WELL-DEFINED POLYGLUTAMATES AS CARRIERS FOR THE TREATMENT OF NEURODEGENERATIVE DISEASES

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CERTIFIES, that the work

“WELL-DEFINED POLYGLUTAMATES AS CARRIERS FOR THE TREATMENT OF NEURODEGENERATIVE DISEASES”

has been developed by Aroa Duro Castaño under her supervision in the Centro de Investigación Príncipe Felipe in Valencia, as a thesis project to obtain a Ph.D degree in Biochemistry and Biomedicine from the University of Valencia, Faculty of Biological Sciences, Biochemistry and Molecular Biology Department.
To all who have accompanied me during this trip up to here.
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Una muy buena amiga dijo una vez, “Una tesis es una vida dentro de la vida”. No sé si puedo definirlo mejor, pero lo cierto es que, al menos en mí, ha representado una parte muy importante. Durante estos años he aprendido muchísimo de los excelentes compañeros de viaje que me han acompañado, y sobre todo, he aprendido mucho de mí misma. Los buenos y malos momentos que debes atravesar (inherentes a la tesis), te enseñan cuáles son tus verdaderas capacidades y lo lejos que puedes llegar si te lo propones. Y no sólo me refiero al ámbito científico, todo ello cala muy hondo también en el ámbito personal. No he conocido estudiante que no se haya quejado durante el desarrollo de su tesis (entre los que me incluyo, por supuesto), pero de justos es reconocer que son más los buenos momentos, las alegrías compartidas, las satisfacciones profesionales y personales… que todo aquello que nos aflige de este pequeño mundillo. Si no, no estaríamos aquí, ¡que tampoco somos tontos! Aunque a veces nos vemos total e inevitablemente inmersos en nuestros experimentos, hasta llegar a perder la perspectiva de las cosas, la carrera científica también nos permite vivir situaciones personales especiales, que jamás habríamos experimentado de no haber elegido este camino. Sin duda, creo que no soy la misma persona que cuando empecé este viaje. Y el sendero hasta aquí no lo hemos recorrido solos. Es por ello, que dedico estas páginas de mi libro a todos aquellos que han contribuido a que hoy esté aquí.

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Segundo, me gustaría agradecer a esa gran familia que siempre hemos sido el I-36. ¡El ambiente de este laboratorio es envidiable! ¡Lo que tenemos aquí tiene mucho valor y hay que preservarlo! Tengo que decir que siempre me he sentido integrada y querida por todos aquellos que han ido pasando durante estos años por aquí. Empezando por los que ya no están aquí, las que fueron mis muy queridas compañeras de
tesis, Coralie, Gaby e Inma. Hemos compartido muchos buenos momentos y nos hemos apoyados las unas a las otras, cual hermanas, cuando venían malas rachas. ¡Echo de menos esas visitas a Häagen-Dazs! Jejeje. Las tres os habéis hecho un hueco grande en mi corazón. Coralie, muchas gracias por todo lo que has hecho por mí. Contigo he vivido preciosos recuerdos que jamás olvidaré. Nuestras noches de confidencias, las salidas a umbracle y nuestras aventuras por Tailandia nos unieron mucho. Gracias por acogerme cuando llegué, ¡no sabes lo que eso significó para mí! Inma, ¿qué decir de ti? Hemos sido un gran equipo, dentro y fuera del laboratorio. Nos entendemos tan bien, que no hacía falta pronunciar las palabras para saber lo que la otra pensaba. Siempre dos cabezas compenetradas. Trabajar contigo, no era un trabajo, ¡era un gustazo! Siempre recordaré los temazos, y esos findes de resaca en el lab cuando no salen los cálculos en las reacciones y ¡el Vivaspin no quiere colaborar! Mi querida Gaby, eres una grandísima persona, de las que apenas quedan y espero que a estas alturas… ¡ya lo veas tú también! Muchas gracias por tu apoyo, por preocuparte siempre de que todos estemos bien, por confiar en mí y por tu gran tesón… ¡eres un gran ejemplo! Y sobre todo, muchas gracias por abrirme un huequito en tu corazón, ha sido muy importante para mí.

Siguiendo con los que ya no están, no puedo dejarme a ¡Matthias! (o Perry, como Inma y yo lo llamábamos). La verdad es que sin ti, mis comienzos hubieran sido diferentes, ¡estoy segura! Gracias por compartir contigo todo lo que sabías, has sido una gran inspiración y una persona digna de admirar. Richard, mucho hemos compartido y reído, sobre todo en ese PGA TEAM junto con Inma, donde aún me acuerdo de cuando íbamos a agitar la reacción con la barquita remando para hacer esa “multi-tone” de PGA jajaja. Ximo, he echado de menos tu optimismo y tu “humor especial” desde que te fuiste, gracias por siempre creer en mí. Julie, siempre recordaré tus bromas, y tu ironía constante jejeje, ¡no descartes que vayamos por Bilbao a verte! Sin olvidarme de Marina, Fabiana, Vanessa, Rut, Mareli, Ant…con las cuales he compartido muchos momentos en el lab. Gracias también a todos esos estudiantes que he tenido el placer de enseñar, como Hannah o Álex (a Luz y Dani os dejo para luego jeje).

Y pasamos ahora a los que aún compartimos nuestros días aquí. ¡Maria Helena! Tú la primera, muchas gracias por ser como eres, por apoyarme siempre, y por ayudarme tanto en lo científico como en lo personal. ¡Eres un sol! Ana y Esther, las bio del lab. Muchas gracias
[Acknowledgements]

por vuestra ayuda incondicional siempre que la he necesitado. La gran Ana, vales muchísimo y espero que lo sepas. ¡Pues todos lo sabemos! La luchadora Esther, siempre recordaré que empecé con Javi, gracias a que tú me hiciste la pregunta que yo nunca me había planteado. ¡David! Eres la alegría del laboratorio, siempre con tus bailecitos y tu electro-latino jajaja. ¡Nunca pierdas eso! Elena, muchas gracias por ir siempre con una sonrisa, ¡no sé cómo llegas a todo sin agobiarte! Tengo que aprender de ti. Vicent, mil gracias por todo lo que has hecho por mí. Te debo un jamón y tres quesos jajaja. Trabajar contigo siempre es un placer, gracias por tu apoyo, tus críticas constructivas, y tu ayuda. Sin ti, hay partes de esta tesis que jamás se habrían desarrollado. Y… ¡pasamos al nuevo grupo de PhD students! Sonia, aunque estás empezando, sé que todo te irá muy bien, porque te lo mereces. Mis compis de office, ¡Amaya y Juanjo! ¡Amaya! Muchas gracias por tus consejos, por devolverme a la realidad cuando entro en mis bucles, por estar siempre pendiente, y por tu optimismo. ¡Eres muy grande, que no se te olvide! ¡Juanjo! Eres un gran compañero, dentro y fuera de aquí, muchas gracias por subirme siempre el ánimo, por estar ahí cuando haces falta y sobre todo por confiar en mí. Has crecido mucho desde que empezaste, ¡sigue así que llegarás alto! Ahora sí, Luz y Dani, mis estudiantes favoritos, que ahora formáis parte de PTS. Muchas gracias por vuestro entusiasmo, por vuestro gran interés y por vuestras ganas por aprender y hacer siempre más. Me alegro muchísimo de que estéis aquí. ¡PTS no puede ir mal con vosotros dentro!

Dejando ya al I-36, pero sin irnos de la ciencia, Salva, muchas gracias por todo lo que siempre haces por mí. Has sido un apoyo constante, una persona de las que no se olvidan. A mis chicos de Máster, Andy, Miriam, Chris y Caro… muchas gracias por todos esos días que compartimos juntos, ¡siempre nos quedarán nuestras fiestas, nuestros viajecitos y nuestros desfases!

A mis amigos de toda la vida, gracias por comprenderme siempre, que sé que no es fácil entender el por qué tienes que trabajar en fin de semana (si no te pagan jajaja) o el hacer horas extra. Gracias por hacerme saber que siempre estás ahí.

Sin lugar a duda, no pudiera haber hecho todo esto sin mi familia. Papá y Mamá, siempre habéis estado aquí de manera incondicional para lo que he necesitado. Muchas gracias por entenderme siempre, y por creer en mí más de lo que lo hace nadie. Por vuestra preocupación constante porque esté bien, por aguantarme en
épocas de estrés cuando nadie más podría y por venir siempre a verme cuando yo no puedo. Si Mahoma no va a la montaña… Alba, ¡muchas gracias por existir! Jajaja. No puedo imaginarme no tenerte, como amiga, como confidente, como hermana del alma… Eres un soplo de aire fresco siempre oportuno cuando se necesita. Gracias por entenderme siempre, por transmitirme tu vitalidad y tu fuerza. Eres un torbellino por donde pasas jejeje.

Y te dejo para el final, Javi. Estoy convencida de que todo habría sido mucho más duro si no hubiéramos hecho este camino juntos. Has conseguido aguantarme todo este tiempo (y sin apenas quejarte jajaja). Eres la única persona que consigue hacerme desconectar del día a día. Estoy enganchada a ti como a la más adictiva de las drogas. Has conseguido que me cambie la perspectiva de las cosas, y que sepa valorar lo que verdaderamente importa en esta vida, que es ser felices. Contigo soy mejor persona y creo que los dos hemos aprendido mucho en esta relación. ¡Ya queda menos para que podamos estar juntos! Yo ya he dado el paso en la tesis, ahora te toca a ti, ¡mucho ánimo en la recta final futuro Doctor!

Fuera de lo personal, tengo que agradecer también a todos aquellos con los que hemos colaborado para llegar aquí. En primer lugar, al proyecto europeo LIVIMODE, y en especial a los grupos de: Vicent Dive en CEA-SAACLAY, por proveernos con las sondas de MMPs, y por permitirme aprender en su laboratorio a hacer los análisis enzimáticos; Karsten Schultz, de EMBL en Frankfurt, por proveernos de sondas de Catepsina y FAP, Hideaki Nagase, del Imperial College de Oxford, por probar nuestros compuestos en sus modelos animales de osteoartritis e inflamación; Boris Turk, del Josef-Stefan Institute en Slovenia, por realizar análisis celulares en su laboratorio y por acogerme durante una estancia de casi dos meses; y Markus Rudin de ETH-Zurich, por acogerme en su laboratorio para realizar las pruebas en modelos animales, de los compuestos de mi tesis. En segundo lugar, me gustaría agradecer también a Miguel Angel Morcillo y su equipo, por realizar análisis celulares en su laboratorio y por acogerme durante una estancia de casi dos meses; y Markus Rudin de ETH-Zurich, por acogerme en su laboratorio para realizar las pruebas en modelos animales, de los compuestos de mi tesis. En segundo lugar, me gustaría agradecer también a Miguel Angel Morcillo y su equipo, por realizar los ensayos de biodistribución por PET, en el CIEMAT de Madrid. Y para terminar, a la empresa Neuropharmatest S.L. de Valencia, a Juan y Javi, por realizar los ensayos en cultivos organotípicos y permitirme aprender sobre ello.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt%</td>
<td>Drug loading in weight percent</td>
</tr>
<tr>
<td>2TP</td>
<td>2-thiopyridine</td>
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<tr>
<td>4HPR</td>
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</tr>
<tr>
<td>α-mPNL</td>
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</tr>
<tr>
<td>aa</td>
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<td>Antibody</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>ATRP</td>
<td>Atom transfer free-radical polymerization</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>AuNP</td>
<td>Gold nanoparticles</td>
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<td>Aβ</td>
<td>Amyloid beta protein</td>
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<td>BACE1</td>
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<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
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<tr>
<td>BDMC</td>
<td>Bisdemethoxycurcumin</td>
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<tr>
<td>Boc</td>
<td>Tertiary-butyloxy carbonyl</td>
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<tr>
<td>BSA</td>
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<tr>
<td>BTA</td>
<td>1,3,5-benzene tricarboxamide</td>
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<tr>
<td>BTB</td>
<td>Blood tumor barrier</td>
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<td>Bz</td>
<td>Benzyl oxy carbonyl</td>
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<tr>
<td>CAA</td>
<td>Cerebral amyloid angiopathy</td>
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<tr>
<td>CAC</td>
<td>Critical aggregation concentration</td>
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<tr>
<td>CD</td>
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</tr>
<tr>
<td>CDCl$_3$</td>
<td>Deuterated chloroform</td>
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<tr>
<td>CDP</td>
<td>Linear cyclodextrin polymer (β-cyclodextrin + PEG)</td>
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<tr>
<td>CED</td>
<td>Convention-enhanced delivery</td>
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<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>COSY</td>
<td>Correlated spectroscopy</td>
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<td>cPEP</td>
<td>Cyclic peptide</td>
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<td>CPT</td>
<td>Camptothecin</td>
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<tr>
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<td>Copper</td>
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<tr>
<td>CuAAC</td>
<td>Copper catalyzed azide/alkyne cycloaddition</td>
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<td>Cys</td>
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<td>DB</td>
<td>Di-block</td>
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<tr>
<td>DCC</td>
<td>N,N’-dicyclohexyl carbodiimide</td>
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<tr>
<td>ddH2O</td>
<td>Deionized water</td>
</tr>
<tr>
<td>DDS</td>
<td>Drug delivery system</td>
</tr>
<tr>
<td>DEPT</td>
<td>Distortionless enhancement by polarization transfer</td>
</tr>
<tr>
<td>DIC</td>
<td>N,N’-Diisopropyl carbodiimide</td>
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</tbody>
</table>
DIEA  N,N-Diisopropylethylamine
DLS  Dynamic light scattering
DMC  Demethoxycurcumin
DMEM  Dulbecco’s modified eagle’s medium
DMF  N,N'-Dimethyl formamide
DMSO  Dimethyl sulfoxide
DMSP  Dimethylsphingosine
DMTMM  4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium
DNA  Deoxyribonucleic acid
DO3A-tBuNH2  tert-butyl 2,2',2''-(10-(2-(2aminoethylamino)-2oxoethyl)-1,4,7-tetrazacyclododecane-1,4,7-triyl)triacetate
DOOA  3,6-dioxa-octanediamine
DOSY  Diffusion-ordered spectroscopy
DOTA  1,4,7-tetrazacyclododecane-1,4,7-10-tetraacetic acid
DOX  Doxorubicin
DP  Degree of polymerization
DPPC  Dipalmitoylphosphatidyl choline
DSPC-Chol  1,2-distearoyl-sn-glycerol-3-phosphatidylcholine and choline
DTPA  Diethylene triamine pentaacetic acid
DTT  1,4-dithiothreitol
DXM  Dexamethasone
EA  Elemental analysis
EC  European commission
EDC  1-Ethyl-3-(-3-dimethylaminopropyl)carbodiimide hydrochloride
EDTA  Ethylenediaminetetraacetic acid
EG  Ethylene glycol
E°  Standard reduction potential
EPI  Epirubicin
EPO  Epoetin beta
EPR  Enhanced permeability and retention
eq  Equivalents
ESF  European science foundation
FA  Fluocinolone acetonide
Fab  Fragment antigen-binding
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Fluorescence correlation spectroscopy</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>FDC</td>
<td>Free drug content</td>
</tr>
<tr>
<td>Fmoc</td>
<td>Fluorenylmethyloxycarbonyl</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>G6S</td>
<td>Glucosamine-6-sulphate</td>
</tr>
<tr>
<td>Ga</td>
<td>Gallium</td>
</tr>
<tr>
<td>GA</td>
<td>Geldanamycin</td>
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<tr>
<td>GAU</td>
<td>Glutamic acid units</td>
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<td>Gd</td>
<td>Gadolinium</td>
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<td>Gem</td>
<td>Gemcitabine</td>
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<td>GFLG</td>
<td>Gly-Phe-Leu-Gly</td>
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<tr>
<td>gHSQC</td>
<td>Gradient heteronuclear single quantum coherence</td>
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<tr>
<td>GI</td>
<td>Gastro-intestinal</td>
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<tr>
<td>GLFG</td>
<td>Gly-Leu-Phe-Gly</td>
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<td>Glycine</td>
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<td>GPC</td>
<td>Gel permeation chromatography</td>
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<td>GSH</td>
<td>Glutathione</td>
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<tr>
<td>HBr</td>
<td>Hydrobromide Acid</td>
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<tr>
<td>HCl</td>
<td>Hydrochloride Acid</td>
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<tr>
<td>HCN-1A</td>
<td>Human cortical neuronal cells</td>
</tr>
<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
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<tr>
<td>HES</td>
<td>Hydroxyethylstarch</td>
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<td>HEWL</td>
<td>Hen egg white lysozyme</td>
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<td>HFIP</td>
<td>1,1,1,3,3,3-Hexafluoroisopropanol</td>
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<td>HGH</td>
<td>Human growth hormone</td>
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<td>HMDS</td>
<td>Hexamethyldisilazane</td>
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<td>HOBr</td>
<td>Hydroxybenzotriazole</td>
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<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
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<tr>
<td>HPMA</td>
<td>N-(2’-Hydroxypropyl)methacrylamide</td>
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<tr>
<td>hrGCSF</td>
<td>Human recombinant granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat-shock protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>------------</td>
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<tr>
<td>HTV</td>
<td>High vacuum techniques</td>
</tr>
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<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
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<tr>
<td>i.m.</td>
<td>Intramuscular</td>
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<td>i.p.</td>
<td>Intraperitoneal</td>
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<td>i.v.</td>
<td>Intravenous</td>
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<tr>
<td>ID</td>
<td>Injected dose</td>
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<td>IHC</td>
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<td>IL</td>
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<td>In</td>
<td>Indium</td>
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<td>K8-ELP</td>
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<td>KDa</td>
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<tr>
<td>keV</td>
<td>Kiloelectron Volts</td>
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<td>LAM</td>
<td>Lamivudine</td>
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<tr>
<td>LE</td>
<td>Linking efficiency</td>
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<td>Leu</td>
<td>Leucine</td>
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<tr>
<td>LiBr</td>
<td>Lithium bromide</td>
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<tr>
<td>LiOH</td>
<td>Lithium hydroxide</td>
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<tr>
<td>LRP1</td>
<td>Low-density lipoprotein receptor-related protein-1</td>
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<tr>
<td>LSGS</td>
<td>Low serum growth supplement</td>
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<tr>
<td>M/I</td>
<td>Monomer/initiator ratio</td>
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<tr>
<td>m/z</td>
<td>Mass/charge</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<td>MAEHP</td>
<td>2-methyl-N-(2’-aminoethyl)-3-hydroxyl-4-pyridinone</td>
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<td>MAO</td>
<td>Monoamine oxidase</td>
</tr>
<tr>
<td>MBq</td>
<td>Mega Becquerel</td>
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<td>MCDK</td>
<td>Madin-Darbycanine kidney</td>
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<tr>
<td>MCR</td>
<td>Mean Count Rate</td>
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<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
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<td>ME</td>
<td>Macrophage elastase</td>
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<td>MFI</td>
<td>Maximum fluorescence intensity</td>
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<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>Mn</td>
<td>Number average molecular weight</td>
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<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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</table>
| MTS          | 3-(4,5-dimethylthiazol-2-yl)-5-(3-
[Abbreviations]

carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

MTT 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide

MTX Methotrexate

MW Weight average molecular weight

MWCO Molecular weight cut off

N$_2$ Nitrogen

NA Normal amine

NAC N-acetyl cysteine

NAM Normal amine mechanism

NaOH Sodium hydroxide

nBu N-Butylamine

NCA N-Carboxyanhydride

NCE New chemical entity

NCS Neocarzinostatin

NGS Normal goat serum

NHS N-Hydroxysuccinimide

NIR Near InfraRed

NIRF Near InfraRed Fluorophores

NMDA N-methyl-D-aspartate

NMR Nuclear Magnetic Resonance

NO Nitric oxide

NOESY Nuclear Overhauser Effect Spectroscopy

NOTA 1,4,7-triazacyclododecane-1,4,7-tetraacetic acid

NPs Nanoparticles

Npt Neopentylamine

NSAIDs Non-steroidal antiinflammatory drugs

NSCLC Non-small cell lung cancer

OBzl(Glu) NCA y-Benzyl L-glutamate N-carboxyanhydride

OG Oregon green cadaverine

PAH Poly(aspartate hydrazide)

PAMAM Poly(amido amine)

PB Phosphate buffer

PBLG poly(γ-benzyl L-glutamate)

PBS Phosphate buffer saline

PC-DCP Phosphocholine-dihexadecyl phosphate cholesterol
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PCS</td>
<td>Photon correlation spectroscopy</td>
</tr>
<tr>
<td>PD</td>
<td>Pharmacodynamics</td>
</tr>
<tr>
<td>PDC</td>
<td>Polymer drug conjugate</td>
</tr>
<tr>
<td>PDEPT</td>
<td>Polymer-directed enzyme prodrug therapy</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PDI</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>pDMAEMA</td>
<td>Poly(dimethylamino)ethylmethacrylate</td>
</tr>
<tr>
<td>PEA</td>
<td>β-phenylethylamine</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEI</td>
<td>Poly(ethylene imine)</td>
</tr>
<tr>
<td>PELT</td>
<td>Polymer-enzyme liposome therapy</td>
</tr>
<tr>
<td>PEO</td>
<td>Poly(ethylene oxide)</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<tr>
<td>PGA</td>
<td>Poly(L-glutamic acid)</td>
</tr>
<tr>
<td>PGE₁</td>
<td>Prostaglandine E₁</td>
</tr>
<tr>
<td>P-gp</td>
<td>Glycoprotein P</td>
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<tr>
<td>PGSE</td>
<td>Pulse-field gradient Spin-Echo</td>
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<td>Phe</td>
<td>Phenylalanine</td>
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<td>PHF</td>
<td>Polycetal</td>
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<tr>
<td>PI-3 kinase</td>
<td>Phosphoinositide 3-kinase</td>
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<td>PK</td>
<td>Pharmacokinetics</td>
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<tr>
<td>PLA</td>
<td>Polylactic acid</td>
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<tr>
<td>PLL</td>
<td>Poly(L-Lysine)</td>
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<td>PLMA</td>
<td>Poly(L-maleic acid)</td>
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<tr>
<td>PMDTA</td>
<td>N,N,N',N',N&quot;-pentamethyldiethylenetriamine</td>
</tr>
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<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
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<tr>
<td>PMS</td>
<td>Phenazine methyl sulfate</td>
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<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
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<tr>
<td>POM</td>
<td>Polyoxometalate</td>
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<td>PPC</td>
<td>Polymer protein conjugate</td>
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<tr>
<td>PPO</td>
<td>Poly(propylene oxide)</td>
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<td>PS</td>
<td>Presenilin</td>
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<tr>
<td>PSS</td>
<td>Polymer standards service</td>
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<td>Pt</td>
<td>Platinate</td>
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<td>PT</td>
<td>Polymer therapeutics</td>
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<tr>
<td>PTX</td>
<td>Paclitaxel</td>
</tr>
<tr>
<td>RAFT</td>
<td>Reversible addition-fragmentation chain-transfer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>-------------------------------------------------</td>
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<tr>
<td>RES</td>
<td>Reticulum endothelium system</td>
</tr>
<tr>
<td>rf</td>
<td>radio-frequency</td>
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<tr>
<td>RFU</td>
<td>Relative fluorescence units</td>
</tr>
<tr>
<td>Rg</td>
<td>Gyration radius</td>
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<tr>
<td>Rh</td>
<td>Hydrodynamic radius</td>
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<tr>
<td>RI</td>
<td>Refractive index</td>
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<tr>
<td>RMT</td>
<td>Receptor mediated transport</td>
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<tr>
<td>RNP</td>
<td>Radionuclide purity</td>
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<td>ROCK</td>
<td>Rho associated kinase</td>
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<td>ROP</td>
<td>Ring opening polymerization</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species.</td>
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<tr>
<td>r.t.</td>
<td>Room temperature</td>
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<tr>
<td>RY</td>
<td>Radiochemical yield</td>
</tr>
<tr>
<td>s.c</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SALP</td>
<td>Sulphate alkyl laminaripentaoside</td>
</tr>
<tr>
<td>SANS</td>
<td>Small angle neutron scattering</td>
</tr>
<tr>
<td>SAS</td>
<td>Small angle scattering</td>
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<tr>
<td>SAXS</td>
<td>Small angle X-ray scattering</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SD</td>
<td>Sprague-Dawley</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>siRNA</td>
<td>Silence interfering ribonucleic acid</td>
</tr>
<tr>
<td>SMA</td>
<td>Copoly(styrene-maleic acid)</td>
</tr>
<tr>
<td>SMANCS</td>
<td>Styrene maleic acid neocarzinostatin</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single photon emission computed tomography</td>
</tr>
<tr>
<td>SPPS</td>
<td>Solid phase peptide synthesis</td>
</tr>
<tr>
<td>TAMRA</td>
<td>Tetramethyl-6-carboxyrhodamine</td>
</tr>
<tr>
<td>TDC</td>
<td>Total drug content</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TfR</td>
<td>Transferrin receptor</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>ThT</td>
<td>Thioflavin T</td>
</tr>
<tr>
<td>TIPS</td>
<td>Triisopropylsilane</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junction</td>
</tr>
</tbody>
</table>

30
[Abbreviations]

TNF  Tumor necrosis factor
tr   Retention time
Tyr  Tyrosine
UV   Ultraviolet
v/v  Volume/volume
VEGF Vascular endothelial growth factor
WHO World health organization
WSTs Water soluble tetrazolium salts
XTT  2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
Y    Yield
ZVD  Zidovudine
Abstract
Alzheimer’s disease (AD) is a neurodegenerative multiple process of the central nervous system, which currently represents the most common cost of Dementia. The already high incidence of AD is predicted to dramatically increase over the years. In fact, the experts claim that it will become a global epidemic by 2050. Consequently, direct and indirect costs related to AD are doomed to dramatically increase. For instance, only in America, AD related burden will overcome the trillion of dollars by 2050. Moreover, available medication (Exelon®, Namenda®, Aricept®, and Razadyne®) produce moderate symptomatic benefits, but do not stop disease progression. Hence, AD, among other neurodegenerative disorders, can be considered an unmet medical need. Neuroprotective drugs, such as, curcuminoids are being taken in high consideration in order to approach these fatal disorders from a protective and preventive point of view. In this context, nanomedicine and, in particular, Polymer Therapeutics (PT) emerge as a powerful alternative to overcome the limitations of low MW drugs including their poor pharmacokinetic and pharmacodynamic profiles and low solubility in aqueous solvents, required for i.v. administration.

Nonetheless, in the PT field, there is a need to develop new and innovative polymer carriers to be used as drug delivery systems and/or imaging agents owing to the fact that there is no universal polymeric system that can be used in the treatment of all diseases. Apart from biodegradability, the development of novel well-defined architectures with higher MW (in order to increase passive targeting provided by the EPR effect), predictable structure and conformation (defined three-dimensional architecture in solution), higher homogeneity, greater drug loading capacity and increased multivalency is considered crucial. To this respect, polypeptides are envisaged to achieve a major impact on a number of different relevant areas including nanomedicine. Thus, new PT based on amino acids are excellent candidates for drug delivery, as they do not suffer from the previously mentioned limitations. Concretely, polyglutamates constitute a versatile platform, which has been effectively used as building blocks in polymer drug conjugates and polymeric micelles for various medical applications ranging from cancer to regenerative medicine. Moreover, it is expected its FDA approval after approval of PGA-paclitaxel conjugate, Opaxio™ for the treatment of various cancers alone or in combination (Opaxio™ has been recently designated as orphan drug in combination with
radiotherapy and temozolomide for the treatment of glioblastoma multiforme). Nevertheless, control on polymer chain length, polydispersities and stereochemistry has been the major challenge in the development of synthetic polypeptides over the past years. Besides, the use of branched polymers is emerging in order to accomplish the previously described requisites. They exhibit special properties when compared to their linear counterparts. As a result of their different architectures, solution conformation, size and shape as well as greater multivalency, different therapeutic outputs could be gained. Due to their compact and globular shapes they are postulated to perform better regarding to overcome biological barriers, a pre-requisite in neurodegenerative disorders treatment as well as diagnostics due to the presence of the blood-brain barrier (BBB), one of the most challenging to surpass.

Therefore, the main aim of this thesis was the design of new versatile polyglutamate-based nanotherapeutics to be used in the treatment and/or diagnosis of devastating neurodegenerative pathologies such as AD.

In order to accomplish our final goal, firstly, we report the development of synthetic pathways to a plethora of functional polyglutamates with well-defined structure, adjustable MW and low polydispersities (Đ <1.2) applying the ring opening polymerization (ROP) of N-Carboxyanhydrides (NCA) with novel initiators. Furthermore, this methodology has been extended to reach a number of architectures based on PGA, including stars, grafts, and hybrid di-block copolymers. In addition, a versatile post-polymerization modification method to introduce a variety of functionalities such as alkyne, azides, reactive disulfides, maleimide groups or protected amines has been developed, yielding a set of orthogonal reactive attachment sites suitable for further bioconjugations. The physico-chemical properties of the obtained polyglutamates have been exhaustively investigated, in terms of size and solution conformation by the use of a battery of complex techniques including DLS, DOSY-NMR, CD, TEM and SANS. Furthermore, we have developed a novel PGA-based family of systems that, according to their physico-chemical characterization, underwent a self-assembly process where it did exist a structure/conformation-concentration dependency encountering at low concentrations “unimers” of 5-10 nm size, whereas bigger structures of around 100-180 nm were formed at high concentrations. After covalent entrapment of these bigger structures by means of click chemistry, the
concentration dependence conformation was clearly eliminated. We have taken profit from that special behavior to develop a strategy in order to reach complex polypeptide based architectures through bottom-up approaches.

Preliminary \textit{in vitro} evaluation in selected cell models in terms of biodegradability, biocompatibility and cellular uptake is presented. Furthermore, after an adequate labeling with fluorescence/NIR probes or/and cation complexing moieties towards the use of MRI and/or PET techniques, the \textit{in vivo} fate (pharmacokinetics and biodistribution) of our polyglutamates is also described. Preliminary results suggest that they were non-toxic entities, validating them as possible carriers for drug delivery.

The covalently entrapped unique architectures have been ultimately used to reach carriers for BBB crossing by means of surface modifications with targeting units and imaging agents. Their BBB crossing properties have being explored \textit{in vivo}, reaching at least 1.2\% of injected dose in the brain. Thus, those results make them optimal candidates to be used in AD treatment.

Among all the biological hallmarks of AD, we are centering our efforts in the amyloid pathway, by the use of curcuminoids and with a neuroprotective approach by combining them with the presence of propargyl moieties within the construct. Their biological output regarding cellular uptake, cell viability, drug release profile and biodistribution has been investigated. Moreover, proof of concept of their activity was achieved \textit{in vitro}, in organotypic hippocampal cultures and is currently being validated \textit{in vivo}.

Finally, the potential of PGA-based conjugates as tissue-specific smart imaging probes is also explored within the frame of the European consortium LIVIMODE. The combination of NIRF enzyme specific smart probes together with the tissue specificity provided by PGA as carrier is explored to be applied in the early detection of disease-related events \textit{in vitro} as well as \textit{in vivo}. This strategy could be used for the development of theranostics towards the early detection and treatment of neurodegenerative disorders.
Aims
[Aims]

The present thesis dissertation is focused on the design of a new versatile polymeric platform to be used as carrier in the development of nanomedicines for the treatment and/or diagnosis of devastating pathologies, such as, neurodegenerative diseases.

Such general objective, encompasses several specific pyramidal tasks that can be summarize as follows:

(i) Design and tune up of a new controlled and versatile polymerization methodology to yield novel biocompatible, biodegradable and multifunctional polymeric carriers based on polyglutamic acid (PGA). An exhaustive carrier characterization will be followed by means of a battery of physico-chemical techniques. Once established, the mentioned polymerization strategy will allow the achievement of the subsequent aims.

(ii) Design, synthesis, exhaustive physico-chemical characterization and biological evaluation in adequate cell and in vivo models of complex PGA-based macromolecular architectures obtained by controlled NCA polymerization techniques and bottom up approaches. This includes to fully understand the solution conformation behavior inherent to such architectures with the purpose of unravel the structure underlying self-assembly processes

(iii) Development of a new and versatile methodology for post-polymerization modification of polyglutamates allowing orthogonal reactive attachment sides, highly suitable for further conjugations of bioactive agents of different nature as well as imaging probes.

(iv) Synthesis, characterization and biological evaluation of PGA-based constructs capable to promote active transport through the Blood-Brain Barrier (BBB) by covalent binding of known targeting vectors.

(v) Synthesis, characterization as well as validation of the novel platforms for its use in targeted drug delivery and/or molecular diagnostic tools in neurodegenerative disorders, such as Alzheimer’s disease. The main goal for this part of the project is to obtain PGA-based combination conjugates using the neuroprotective-neurorescuer propargyl moieties and the neuro-antiinflammatory curcuminoids.

(vi) Development of an in vivo imaging polymeric platform based on protease-activated smart ligands that allow non-invasive
[Aims]

quantitative assessment of target expression in diseased tissue (diagnosis) and monitoring of disease progression (staging). These tools are of high relevance for disease detection, monitoring of disease progression, developing animal models of human disease and evaluating novel therapies. This part of the thesis is included on the frame of a European FP7 Project (LIVIMODE Light-based Functional In Vivo Monitoring of Disease-related Enzymes).

With all that objectives in mind, the long-term goal is the establishment of different versatile building blocks: BBB crossing nanovectors, polymer-based combination conjugates for AD treatment, and smart imaging macromolecular probes. The combination of such building blocks would ideally be the base of a novel theranostic platform for effective monitoring and treatment of neurodegenerative diseases.
Chapter 1

General Introduction
Statistics provided by the World Health Organization (WHO) reveal that every 4 seconds a person dies of cancer, every 11 seconds from AIDS and every 86 seconds of Alzheimer's disease (AD). Thus, for these and other degenerative, chronic or chemo-resistant diseases, an urgent need for better therapies arises.

Currently, the development of new therapies is approached by two different trends. In the first approach, research in genomics and proteomics is enabling the identification of new specific molecular targets. However, in practice it is still nearly impossible to find or synthesize such a "perfect" drug for any given target. This line of research is being conducted mainly through (i) screening of natural products, (ii) the synthesis of compounds of low molecular weight (Mw) via traditional medicinal chemistry or through combinatorial approaches, and (iii) by the identification of natural macromolecules, including antibodies, proteins and oligonucleotides with inherent biological activity. The limited progress made by this strategy is mainly attributed to (i) the use of non-adequate preclinical models, which resulted to be poorly predictive, (ii) the lack of drug specificity in clinical practice, and (iii) the problem of acquired resistance.

The second approach, in many ways complementary to the first one, is focused on the use of drug delivery systems (DDS), in particular in the design of innovative carriers, developed to precisely guide the bioactive agent(s) to the site of action. DDS have been developed to accommodate the transfer of a lead compound from “bench to bedside” which is often limited by the lack of systems or technologies suitable for specific molecular transport. Concretely, the full therapeutic potential of a bioactive agent requires a specific molecular delivery, as the specific localization of therapeutics to the diseased cell promotes the efficient delivery to a precise intracellular compartment and ensures availability within the required timeframe.

In this context, nanoscience and nanotechnology provide the basis for the development of innovative delivery strategies and have enormous potential to improve human health, particularly in serious chronic diseases like cancer or neurodegeneration, and are opening new markets to the pharmaceutical industry. The “nanomedicine” field is distinguishable as it uniquely focuses on medically related, patient-centric nanotechnologies. In the words of the European Science Foundation’s (ESF) Forward Look Nanomedicine “Nanomedicine uses nano-sized tools for diagnosis, prevention and treatment of disease
and to gain increased understanding of the complex underlying pathophysiology of disease. The ultimate goal is improved quality-of-life”. The European Commission’s (EC) Joint Research Centre Report “Nanomedicine; Drivers for development and possible impacts” classifies Polymer Therapeutics as nanomedicines.

With 40 products already in the market and more than 70 in cancer clinical trials, the nanomedicine field has experienced a notably exponential growth within the last decade. This fact is due to its potential to serve as a candidate solution for the urgent requirement of dealing with unsolved pharmaceutical and clinical needs in life-threatening diseases. In the recent years, nanomedicine has gained special attention in different research areas, especially for drug and gene delivery, in diagnostics and molecular imaging as well as in tissue repair and engineering amongst other uses.

1.1. POLYMER THERAPEUTICS.

Polymer therapeutics (PT) can be underlined as one of the most successful first generation of nanomedicines, with 15 products in routine clinical use. Furthermore, 2 of them are within the US Top 10 selling drugs, the polymeric drug glatiramer acetate for the treatment of multiple sclerosis (Copaxone®, Teva Pharm; $3.7 billion), and the polymer conjugate polyethylene glycol (PEG)-filgrastim for the treatment of neutropenia (Neulasta®, Amgen; $3.6 billion).

1.1.1. Concept and Classification.

The term “polymer therapeutics” was firstly introduced by Ruth Duncan to describe hybrid nanoconstructs that combine a bioactive agent with a polymer by either covalent or electrostatic interactions. A bioactive compound is described as a substance able to produce a biological response. The expression “Polymer Therapeutics” is then used to describe polymeric drugs, polymer-drug conjugates, polymer-protein conjugates, polymeric micelles where the drug is attached by covalent bonding, and multicomponent polyplexes (polyelectrolyte complexes) which are being developed as non-viral vectors (Figure 1.1). Polymeric micelles are self-assembling colloidal aggregates of polymeric block copolymers (amphipathic molecules), while polyplexes are polyelectrolyte complexes usually formed by a polycation and an anionic oligonucleotide or plasmid. These nano-sized medicines (5-100 nm in diameter) are considered as
new chemical entities (NCEs) from an industrial standpoint, whose versatility in the synthetic chemistry used, the possibility of addition of biomimetic features, and even the addition of bioresponsive units, make them one of the most versatile nanotechnologies currently available. 

![Figure 1.1. Schematic representation of the Polymer Therapeutics family. Redrawn from Duncan.](image)

Through clinical use of polymer–protein conjugates (Table 1.1), and clinical development of polymer–anticancer drug conjugates (Table 1.2), PT is already well established as a new therapeutic class not only for cancer treatment, but is also expanding its use to treat diseases other than cancer (i.e. PEG–interferon α conjugate known as Pegasys® or Peg-intron® can be used to cure hepatitis). Besides, many recent studies using polymer-drug conjugates have embraced a broad number of pathologies, including tissue regeneration. Furthermore, acquired knowledge based on the development of this first generation of PT has helped to inform on the improved design of second generation products.
Table 1.1. First generation marketed polymer therapeutics. Adapted and updated from refs.\textsuperscript{14,40}

<table>
<thead>
<tr>
<th>Product name</th>
<th>Technology</th>
<th>Indication</th>
<th>Route</th>
<th>Information source</th>
</tr>
</thead>
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<td><strong>Polymer-protein conjugates</strong></td>
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<td>Zinostatin stimalmer®</td>
<td>Styrene maleic anhydride</td>
<td>Cancer-hepatocellular carcinoma</td>
<td>Local via hepatic artery infusion</td>
<td>Yamanouchi\textsuperscript{a} Japan</td>
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<td></td>
<td>neocarzinostatin (SMANCS)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oncaspar®</td>
<td>PEG-asparaginase</td>
<td>Cancer-acute</td>
<td>i.v./i.m.</td>
<td>Enzon\textsuperscript{a}</td>
</tr>
<tr>
<td>Peg-intron®</td>
<td>PEG-Interferon alpha 2b</td>
<td>Hepatitis C</td>
<td>s.c.</td>
<td>Schering-Plough\textsuperscript{a}</td>
</tr>
<tr>
<td>Pegasys®</td>
<td>PEG-Interferon alpha 2a</td>
<td>Hepatitis C</td>
<td>s.c.</td>
<td>Roche\textsuperscript{a}</td>
</tr>
<tr>
<td>Neulasta™</td>
<td>PEG-hrGCSF</td>
<td>Chemotherapy-induced neutropenia</td>
<td>s.c.</td>
<td>Amgen\textsuperscript{a}</td>
</tr>
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<td>Adagen®</td>
<td>PEG-adenosine deaminase</td>
<td>Severe combined immune deficiency syndrome</td>
<td>i.m.</td>
<td>Enzon\textsuperscript{a}</td>
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<td>Somavert®</td>
<td>PEG-HGH antagonist</td>
<td>Acromegalia</td>
<td>s.c.</td>
<td>Pfizer\textsuperscript{a}</td>
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<td>Mircera®</td>
<td>PEG-EPO (polyethylene glycol-epoetin beta)</td>
<td>Treatment of anemia associated with chronic kidney disease</td>
<td>i.v./s.c.</td>
<td>Roche\textsuperscript{a}</td>
</tr>
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<td>Cimzia (certolizumab pegol)</td>
<td>PEG-anti-TNF Fab</td>
<td>Rheumatoid arthritis</td>
<td>s.c.</td>
<td>UCB\textsuperscript{a}</td>
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<td>Krystexxa™ peglotiase</td>
<td>PEG-uricase</td>
<td>Chronic gout</td>
<td>i.v.</td>
<td>Savient Pharmaceuticals</td>
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<td>Product name</td>
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<td>Indication</td>
<td>Route</td>
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<td><strong>Polymer-aptamer conjugate</strong></td>
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<tr>
<td>Macugen®</td>
<td>PEG-aptamer (apatanib)</td>
<td>AMD</td>
<td></td>
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<td><strong>Polymer-drug conjugate</strong></td>
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<td>Movantik™/Moventig®</td>
<td>PEG-naxolol</td>
<td>Opioid-induced constipation</td>
<td>Oral</td>
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<td>(NKTR-118)</td>
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<td><strong>Polymeric drugs</strong></td>
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<tr>
<td>Copaxone®</td>
<td>Glu, Ala, Tyr copolymer</td>
<td>Multiple sclerosis</td>
<td>s.c.</td>
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<tr>
<td>Renagel®</td>
<td>Phosphate binding polymer</td>
<td>End stage renal failure</td>
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<tr>
<td>Welchol®</td>
<td>Cholesterol binding polymer</td>
<td>Type 2 diabetes</td>
<td>Oral</td>
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</table>

*Ala: Alanine, ALL: Acute lymphoblastic leukemia, AMD: Age-related Macular Degeneration, EPO: Epoetin beta; Fab: Fragment antigen-binding, HGH: Human Growth Hormone, hrGCSF: human recombinant Granulocyte-Colony Stimulating Factor, i.m.: intramuscular, i.v.: intravenous, s.c.: subcutaneous, Tyr: Tyrosine, TNF: Tumor Necrosis Factor.

Table 1.2. Examples of polymer therapeutics in clinical development. Adapted and updated from refs<sup>14, 40</sup>
### Polymer-aptamer conjugate

<table>
<thead>
<tr>
<th>Compound</th>
<th>Description</th>
<th>Indication</th>
<th>Route</th>
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<th>Company</th>
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<tr>
<td>ARC1779</td>
<td>PEG-anti-platelet-binding function of von Eillebrand Factor</td>
<td>Thrombotic microangiopathies</td>
<td>i.v.</td>
<td>Phase II</td>
<td>Archemix</td>
</tr>
<tr>
<td>E10030</td>
<td>PEG-anti-PDGF aptamer combination with Lucentis ®</td>
<td>AMD</td>
<td>Local intravitreal</td>
<td>Phase III</td>
<td>Ophthotech</td>
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### Polymeric drugs

<table>
<thead>
<tr>
<th>Compound</th>
<th>Description</th>
<th>Indication</th>
<th>Route</th>
<th>Phase</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMG 223</td>
<td>Phosphate binding polymer</td>
<td>Hyperphosphatemia in CKD patients on hemodialysis</td>
<td>Oral</td>
<td>Phase II</td>
<td>Amgen</td>
</tr>
<tr>
<td>VivaGel®</td>
<td>Lysine-based dendrimer</td>
<td>microbiocide</td>
<td>Topical</td>
<td>Phase III</td>
<td>Starpharma</td>
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### Polymeric Drug-Conjugates

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<th>Compound</th>
<th>Description</th>
<th>Indication</th>
<th>Route</th>
<th>Phase</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT-2103; Xyotax; Opaxio</td>
<td>Poly-glutamic acid (PGA)-paclitaxel</td>
<td>Cancer-NSCLC, ovarian, various other cancers and combinations</td>
<td>i.v.</td>
<td>Phase III</td>
<td>Cell Therapeutics Inc</td>
</tr>
<tr>
<td>Prolindac®</td>
<td>HMPA-copolymer-DACH platinate</td>
<td>Cancer-melanoma, ovarian</td>
<td>i.v.</td>
<td>Phase III</td>
<td>Access Pharmaceuticals</td>
</tr>
<tr>
<td>FCE 28068 (PK1)</td>
<td>HMPA-copolymer-DOX</td>
<td>Breast, lung and colon cancer</td>
<td>i.v.</td>
<td>Phase II</td>
<td>Pfizer</td>
</tr>
<tr>
<td>FCE 28069 (PK2)</td>
<td>HMPA-copolymer-DOX</td>
<td>Hepatocellular carcinoma</td>
<td>i.v.</td>
<td>Phase I/II</td>
<td>Pfizer</td>
</tr>
<tr>
<td>PEG-SN38</td>
<td>Multiarm PEG-camptothecan</td>
<td>Cancer-various</td>
<td>i.v.</td>
<td>Phase II/III</td>
<td>Enzon Inc</td>
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<tr>
<td>CRLX101</td>
<td>CD-PEG-camptothecin</td>
<td>Cancer-various</td>
<td>i.v.</td>
<td>Phase I/II</td>
<td>Cerulean Pharma</td>
</tr>
<tr>
<td>CRLX301</td>
<td>CD-PEG-Docetaxel</td>
<td>Solid tumors</td>
<td>i.v.</td>
<td>Phase I</td>
<td>Cerulean Pharma</td>
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<tr>
<td>DEPT™ Docetaxel</td>
<td>Dendrimer-Docetaxel</td>
<td>Solid tumors</td>
<td>i.v.</td>
<td>Phase I</td>
<td>Starpharma/AstraZeneca</td>
</tr>
<tr>
<td>Code</td>
<td>Compound</td>
<td>Disease</td>
<td>Route</td>
<td>Phase</td>
<td>Company</td>
</tr>
<tr>
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<tr>
<td>NKTR-181</td>
<td>PEG-naloxone</td>
<td>Chronic pain</td>
<td>Oral</td>
<td>Phase III</td>
<td>Nektar</td>
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<tr>
<td>NKTR-171</td>
<td>PEG-Na channel blocker</td>
<td>Neuropathic pain</td>
<td>Oral</td>
<td>Phase I</td>
<td>Nektar</td>
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<tr>
<td>NKTR-102</td>
<td>PEG-irinotecan</td>
<td>Cancer-variouse</td>
<td>i.v.</td>
<td>Phase II/III</td>
<td>Nektar</td>
</tr>
<tr>
<td>XMT-1001</td>
<td>Polyacetal-camptothecin</td>
<td>Cancer-variouse</td>
<td>i.v.</td>
<td>Phase I/II</td>
<td>Mersana</td>
</tr>
<tr>
<td>XMT-1107</td>
<td>Polyacetal-fumagillin</td>
<td>Solid tumors</td>
<td>i.v.</td>
<td>Phase I</td>
<td>Mersana/Teva</td>
</tr>
<tr>
<td><strong>Micelles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SP1049C</td>
<td>Pluronic® formulation of DOX</td>
<td>Cancer-upper GI, NSCLC colorectal</td>
<td>i.v.</td>
<td>Phase III</td>
<td>Supratek Pharma Inc</td>
</tr>
<tr>
<td>NK 105</td>
<td>Paclitaxel block copolymer micelle</td>
<td>Breast cancer</td>
<td>i.v.</td>
<td>Phase III</td>
<td>Nippon Kayaku Co</td>
</tr>
<tr>
<td>NC-6004,</td>
<td>Cisplatin block copolymer micelle</td>
<td>Cancer-variouse</td>
<td>i.v.</td>
<td>Phase I/II</td>
<td>NanoCarrier Co./ Orient Neuropharma</td>
</tr>
<tr>
<td>Nanoplatin™</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NC-4016</td>
<td>Oxaliplatin block copolymer micelle</td>
<td>Solid Tumors</td>
<td>i.v.</td>
<td>Phase I</td>
<td>NanoCarrier Co</td>
</tr>
<tr>
<td>NC-6300 (K-912)</td>
<td>Epirubicin block copolymer micelle</td>
<td>Solid Tumors</td>
<td>i.v.</td>
<td>Phase I</td>
<td>NanoCarrier Co/Kowa</td>
</tr>
</tbody>
</table>

Drug delivery concept from PT point of view, encompasses two important concepts; (i) targeting, what refers to the release of therapeutics at a desired body site (tissue, cell, cellular compartment), and (ii) controlled drug release, to ensure appropriate bioactive agent concentrations within the suitable therapeutic window for a desired duration. As it can be observed from the examples listed in the Tables 1.1 and 1.2, intravenous injection (i.v.) is the preferred administration route for polymer-drug conjugates, owing to allow bioavailability in the bloodstream in a faster way, when compared to other routes, and avoidance of specific biological barriers (i.e. oral, which encounters the problem of poor penetration into the intestinal mucosa).41, 42

Drug conjugation to a polymer chain offers many advantages, including (i) enhanced water solubility (relevant for poorly soluble drugs such as camptothecin (CPT) or platinate (Pt) derivatives or taxol (PTX)); (ii) increased plasma half-life by means of a higher hydrodynamic volume that presumably decreases kidney clearance; (iii) protection against proteolytic enzymes, or unspecific cellular uptakes; (iv) prevention or reduction of aggregation, immunogenicity and antigenicity. Polymer chains can prevent systemic generation of antibodies (complement activation), which is particularly relevant in polymer-protein design; and (v) changed pharmacokinetics (PK) at whole body as well as at cellular and even subcellular level. It is possible to achieve specificity due to passive targeting based on disease related vasculature abnormalities (EPR effect 'enhanced permeability and retention effect') and, therefore, lower systemic toxicity and may even overcome chemoresistant mechanisms (i.e. multidrug resistance (MDR) induced by glycoprotein P (P-gp) overexpression in plasma membrane) and thereby, restricting cellular uptake to endocytic pathway (lysosomotropic intracellular drug delivery).38

The EPR effect phenomenon was firstly described by Matsumura and Maeda46 and is based on the unique pathophysiological features of most solid tumors: extensive angiogenesis, defective capillaries and impaired lymphatic drainage. Hence, after intravenous (i.v.) administration, the ‘leakiness’ of the angiogenic tumor vasculature allows selective extravasation of the conjugate into tumor tissue. Additionally, tumor tissue lacks of an effective lymphatic drainage, which subsequently promotes polymer retention. Combination of these two factors leads to the accumulation of the conjugate in tumor tissue (Figure 1.2). Thus, due to their size, PT (as
other nanomedicines), take advantage from passive targeting provided by EPR effect. Moreover, such effect is also present in inflamed areas, opening the window for the design of nanovectors towards infectious and inflammatory diseases.\textsuperscript{48} EPR-mediated targeting is ultimately driven by circulating plasma concentration of the polymer conjugate.\textsuperscript{37} Nevertheless, the strength of the EPR effect differs on tumor type, tumor region, inflamed area, etc., being highly dependent on tumor vascularization. Hence, poor-vascularized damaged tissues are less susceptible to therapies based on nanosystems that rely solely on such effect,\textsuperscript{49} exposing the need for active targeting strategies in order to approach all possible scenarios.

![Enhanced permeability and retention (EPR) effect and passive targeting](image)

**Figure 1.2.** Enhanced permeability and retention (EPR) effect and passive targeting. Nanocarriers can extravasate into the tumors or inflamed areas through the fenestrations between endothelial cells and accumulate there due to poor lymphatic drainage. Adapted from ref.\textsuperscript{50}

Once accumulated in tumor or target tissue, the conjugates are generally internalized into cells by endocytic mechanisms driven by interactions with proteins and receptors on the cell surface. Drug conjugation to a water-soluble polymer platform restricts cellular uptake to the endocytic pathway and hence, bypasses mechanisms of resistance such as MDR induced by P-gp overexpression in plasma membrane. Indeed, phase I/II clinical trials with 2-N-(Hydroxypropyl methacrylamide) (HPMA) copolymer-Doxorubicin (DOX) (PK1, FCE
Macromolecules are captured by invaginations of cell plasma membrane forming vesicles named endosomes. Such vesicles undergo a complex sequence of fusion events directing the substances to the right compartment. In this process, the endosomal compartment pH drops down from the early to late endosome to values around 5.5. Moreover, at the end of the fusion process, the formed lysosomes host a battery of enzyme machinery utilized to degrade complex molecules such as lipids or proteins. Most of polymer-drug conjugates rely on the lysosomotropic drug delivery pathway, where either proteolytic enzymes or acidic pH triggers drug release. However, the hostile environment of lysosomes must be avoided if the presence of hydrolytic enzymes and acidic pH drives to inactivation/degradation of the bioactive agent. This issue is particularly important in peptide/protein and nucleic acid delivery. To circumvent that, pH-responsive endosomolytic carriers are developed in order to promote endosomal escape upon pH stimuli. Several examples of such systems can be found in literature.

Figure 1.3. Lysosomotropic intracellular drug delivery process followed by Polymer-Drug conjugates.
1.1.2. Rational design of polymer conjugates.

The previous section regarding the use of different endocytic pathways highlights the need for a rational design of nanosystems, given a selected molecular target and a chosen therapeutic agent.

Polymer conjugates can be separated into two groups: polymer-protein and polymer-drug conjugates. The biological purpose or the rational pursued in each case is different and, therefore, so are the parameters to be considered for its construction. While protein conjugation seeks to promote greater stability in serum and decreased immunogenicity, polymer-drug conjugates are designed to influence low Mw drug pharmacokinetics at whole organism and at cellular level, allowing also to cross biological barriers, improving cellular specificity, controlling drug release rate and consequently, decreasing non-specific toxicities and enhancing its therapeutic value.

Thus, all that aspects include the choice of an appropriate polymeric carrier, suitable linker(s) or spacer(s), depending on the bioactive agent(s) to deliver and the molecular target to approach.

1.1.2.1. Polymer-Protein Conjugates.

Many of the limitations of peptide/protein/antibody therapies deal with low stability, short circulation times, poor therapeutic outputs as well as possible immunogenic responses. Polymer-protein conjugates emerge as a potential solution to circumvent these limitations. Although this family started in the early 90s with the development of SMANCS (poly-styrene-co-maleic acid, SMA conjugate of neocarzinostatin NCS), the most popular technique by far to reach polymer-protein conjugates is PEGylation. PEGylation is an FDA (Food and Drug Administration) approved technology based on the use of PEG to build polymer-protein conjugates with various examples already in the market (Table 1.1). PEG conjugated to a protein increases its solubility and plasma stability, reduces immunogenicity and prolongs plasma half-life by avoiding rapid excretion through reticulum endothelium system (RES), overall yielding to a better patient compliance. The PEG-L-asparaginase (Oncaspar®) was the first anticancer conjugate to get approval from the FDA in 1994 (used for the treatment of acute lymphoblastic leukemia), while the PEGylated enzyme PEG-Uricase (Pegloticase®) approved for the treatment of gout in 2010 was the last one to follow (see Tables 1.1 and 1.2). It is important to mention the exponential growth of PEG-
Aptamer conjugates after the approval of Macugen in 2004 for intraocular administration in the treatment of macular degeneration, as well as, Cimzia, an immunoconjugate where site-specific conjugation through bioresponsive linkers was a major achievement.

Regarding their design, site-specific and reproducible protein conjugation is a desired feature. Therefore, the use of semi-telechelic polymers and site-specific conjugation chemistries are preferred to avoid cross-linking reactions and provide a specific point of linkage. In most of polymer-protein conjugates already found in the market or currently in clinical practice, protein release is not desired, therefore non-biodegradable bonds are usually applied (i.e. Oncaspar®, Neulasta™ or PEG-Asys®). On the contrary, if an intracellular transport is pretended, endosomotropic transport is indispensable and biodegradable linkers are essential. Importantly, the polymer carrier to be used should present such endosomolytic properties to enhance cytosolic delivery. As this thesis focuses on the use of polymer-drug conjugates/imaging agents, detailed examples of polymer-protein conjugates are out of the scope and plenty of such constructs can be found in literature.

1.1.2.2. Polymer-Drug Conjugates.

The rational design of a polymer drug conjugate for systemic administration following Ringsdorf’s traditional model is based on a three component design: a water-soluble polymer, a bioresponsive linker and a bioactive agent. Nonetheless, due to polymer multivalency more than one compound can easily be introduced into the polymer main chain allowing the introduction of targeting residues (in order to trigger active targeting-receptor-mediated endocytosis) or more than one drug (polymer-based combination therapy), as well as imaging agents for theranostic purposes, which could enhance conjugate therapeutics value.

The choice of an appropriate polymer carrier is a key step in the design of polymer-drug conjugates. It is currently accepted that an ideal polymer carrier should be characterized by (i) biodegradability or adequate MW, to avoid progressive accumulation in vivo; (ii) low polydispersity, to ensure an acceptable homogeneity of the final conjugates allowing to adjust PK; (iii) longer body circulation time, in order to extend conjugate activity and to promote adequate biodistribution and accumulation in body compartments of interest; and
(iv), multivalency: multiple reactive groups to achieve a satisfactory drug loading.

Traditionally, linear polymers with random-coil conformations have been used to synthesize most PT already in the clinics. These include natural polymers (dextran (α-1,6 polyglucose), dextrin (α-1,4 polyglucose), hyaluronic acid, cyclodextrin); and synthetic polymers, such PEG, HPMA copolymers, polyacetals and poly-L-glutamic acid (PGA). 14, 78

Because of their nature, polymers present specific challenges for pharmaceutical development. A manufactured drug substance should be homogeneous and composed of a single, defined species. By contrast, all synthetic polymers are inherently heterogeneous and, as macromolecules, they can bear special challenges for characterization. Total control of crucial parameters such as chain length, MW, polydispersity (Đ), microstructure, final conformation as well as localization of charge or hydrophobic-hydrophilic balance are key to adequately tailor conjugate body distribution, fate, biological activity and toxicity. 44 79-82 Relative stereochemistry of polymeric chains (tacticity) is been now considered of major importance since it modifies secondary structures and can directly influence polymer degradation profile, which is particularly relevant when using polypeptides as carriers. 73 Surface charge is also important in some biological aspects, including cellular trafficking and biodistribution profile. For instance, neutral or negatively charged surfaces usually avoid non-specific uptakes and positively charged surfaces are prone to tubular reabsorption in the kidneys. All these issues must be considered when chosing the appropriate polymer carrier for the desired application. 83

The average molecular weight is described by the “weight average molecular weight” (MW) and “number average molecular weight” (Mn) and the ratio MW/Mn gives a measure of the polydispersity Đ. For instance, polysaccharides extracted from natural sources are particularly disperse (Đ > 2) and normally have high MW (>200 kDa). New polymer chemistry approaches depending on the polymerization strategy used provide with synthetic polymers with narrow dispersities. For example, PEG has been synthesized by anionic polymerization yielding polymers with Đ ~ 1.01, which can be considered as almost monodisperse.

Besides, design of bioresponsive polymer-drug linkers stable during blood circulation and capable of triggering drug release under
specific conditions is another key feature to achieve an effective intracellular drug release by lysosomotropic delivery.\textsuperscript{38} As previously mentioned, after being internalized by an endocytic mechanisms, polymer-drug conjugates travel through the endosomal pathway until reaching the lysosome, where in presence of proteolytic enzymes and/or acidic pH, degradation of the carrier and/or the biodegradable polymer-drug linkers occurs allowing drug release. Most drugs used in these constructs are hydrophilic/lipophilic, therefore once released, the drugs diffuse out to cytosol to achieve the desired molecular target.\textsuperscript{10}

Different types of linkers are already under use. These include: (i) \textit{Linkers that respond to pH changes}, mainly used for small drugs conjugation as acid-labile spacers, such as acetal, ester bonds, \textit{N-cis-aconityl} acid, or hydrazone linkage.\textsuperscript{84, 85} (ii) \textit{Linkers susceptible to lysosomal enzymes}, (exclusively for small drugs), usually oligopeptides specifically designed to be stable in blood but fast cleaved by lysosomal enzymes (i.e. Cathepsin B or D, and other metalloproteinases), allowing a lysosomotropic drug delivery. Examples of such oligopeptide linkers include GFLG (Gly-Phe-Leu-Gly) and GLFG (Gly-Leu-Phe-Gly).\textsuperscript{86-88} (iii) \textit{Self-inmolative linkers}.\textsuperscript{89} Among then, \textit{coiled coil linkers} represent an example, where the cargo is attached to a polymer backbone via a non-covalent, biologically inspired coiled coil linker. This linker consists of a pair of complementary peptides that are bound around each other in a superhelical fashion to form a tertiary structural motif that is referred as a coiled coil.\textsuperscript{90-92} (iv) \textit{Reductive-sensitive linkers} that trigger drug release upon reductive environments mainly due to gluthatione.\textsuperscript{93} (v) \textit{Drug release by anchimeric-assisted hydrolysis}, where the drug-linker is first released from the polymer by hydrolysis (first prodrug), which triggers the linker (second prodrug) that finally releases the free and active drug. Examples of this include the 1,6-elimination reaction or trimethyl lock lactonization.\textsuperscript{94, 95}

Importantly, the chemistry used in conjugate synthesis must not generate toxicity or immunogenicity in biological systems, and of course, will not modify the active properties of the conjugated element.

\textbf{1.2. FROM THE VERY BEGINNING TILL THE CURRENT STATE OF THE ART.}

Although the first polymer anticancer-drug conjugate entered clinical trials in 1994 and was followed by more than 17 polymer-
anticancer drug conjugates. Their way to become a real product on the market has been slowed down due to some initial setbacks. This included rational design errors that yielded an unspecific drug release for MAG-CPT™ (HPMA copolymer-CPT conjugate) and PNU166945™ (HPMA copolymer-PTX conjugate), commercial issues in the case of FCE 28068 and FCE 28069 and the lack of activity found in early Phase III trials with Opaxio™ due to the use of non-suitable clinical models.

HPMA copolymer-DOX conjugate (PK1, FCE 28068), developed by Kopecek and Duncan was the first anticancer drug conjugate to be clinically evaluated in 1994. After PK1, other six HPMA conjugates have been clinically evaluated with HPMA copolymer-DACH-platinum (AP5306, Prolindac®) being the most advanced in Phase III clinical trials for ovarian cancer treatment. It is important to note, that the only targeted polymer-drug conjugate in clinical trials so far is also an HPMA conjugate (PK2, CF28069, HPMA copolymer-DOX-galactosamine). This conjugate is used for hepatocellular carcinoma treatment, using galactosamine as targeting residue, which recognizes the asialoglycoprotein receptor in the liver (see Tables 1.1 and 1.2). In the case of PGA as platform, Opaxio™ (PGA-paclitaxel (PGA-PTX) conjugate formerly known as Xyotax™, developed by Cell Therapeutics Inc.) is the most clinically advanced in phase III-IV, mainly for ovarian, prostate and esophageal cancer alone or in combination with cisplatin or radiotherapy. In 2012, orphan drug designation was granted to Opaxio™ for the treatment of glioblastoma multiforme in combination of temozolomide and radiotherapy. Unlike HPMA-PTX conjugate, Opaxio™ contains a greater drug loading (37 wt% vs 5 wt%) that confers stability in blood (PTX is linked through an ester bond that should be shielded during blood circulation in order to avoid release triggered by plasma esterases). Important to note, the therapeutic value of Opaxio™ as anticancer agent has been found to be gender-dependent, with increased survival in women but not in men. The accepted hypothesis for this relates to the correlation between estrogen levels and cathepsin B activity, responsible of PTX release as PGA is susceptible to degradation in the presence of this serine protease. For that reason, cathepsin B is now used as a clinical biomarker guiding ongoing trials that only enroll chemotherapy-naïve advanced NSCLC female patients with estradiol levels greater than 25 pg.mL⁻¹. This finding is in consonance with novel strategies for the
near future based on the design of nanopharmacutics for their clinical use in personalized therapy, allowing the selection of patients who will benefit more from therapy and suffer less side-effects. Apart from Opaxio\textsuperscript{TM}, the conjugate CT-2106, PGA-CPT, is also in Phase II trials.

Tables 1.1 and 1.2 summarize current efforts done in the field of PT up to date, including the screening of the first generation of marketed PT (Table 1.1) and those currently under clinical evaluation (Table 1.2). Certain notable advances include PEGylated products NKTR-102 (in advanced Phase III trials for metastatic breast cancer, and Phase II trials in ovarian and colorectal cancers),\textsuperscript{101,102} and NKTR-181, an orally administered conjugate for opioid-induced constipation developed by Nektar Therapeutics;\textsuperscript{103} XMT-1001 (Fleximer1-CPT) and the potent anti-angiogenic conjugate XMT-1107 (Fleximer1-fumagillin) in Phase I developed by Mersana;\textsuperscript{104} and the first conjugate administered as a supramolecular assembled particle of 30 nm, CRLX101 (formerly IT-101) a cyclodextrin-CPT nanoparticle advancing in Phase III developed by Cerulean Pharma Inc.\textsuperscript{105}

Polymer conjugates have been also used in diagnostics by means of a large variety of imaging agents proposed as nanoprobe candidates. Nanosystems are commonly labeled with reported tracer probes for \textit{in vivo} and \textit{ex vivo} monitoring of pharmacokinetic profiles, biodistributions (clearance), targeting cellular and subcellular localizations. The first imaging agents in the clinics were\textsuperscript{125}\textsuperscript{I}-labeled PK1 and PK2 used to monitor conjugate biodistribution in patients.\textsuperscript{97} Since then, several imaging techniques have been approached, encompassing MRI, PET or optical imaging using near infra-red (NIR) fluorescent and luminescent probes.\textsuperscript{106-112} Following these approaches, an intravascular contrast agent, Gadomer\textsuperscript{®} based on a dendrimeric polymer containing 24 gadolinium ions was transferred into clinics, but was soon retired due to poor pharmacokinetics.\textsuperscript{113} Nevertheless, there are plenty of examples of polymeric probes under development in literature, due to several inherent advantages including enhance circulation times and tissue targeting.\textsuperscript{114} Indeed, this thesis deals with the development of smart activatable polymeric probes in Chapter 8. Furthermore, the concept “theranostics” was recently born as a promising and feasible approach to combine therapeutic and diagnostic capabilities within the same polymeric platform offering the possibility of not only diagnosis but also monitoring of disease evolution upon
treatment using only one construct. Many relevant examples of theranostic approaches can be found in literature.\cite{14,77,115-117}

1.3. WHAT’S NEXT? CHALLENGES, NEW TRENDS AND OPPORTUNITIES.

Lessons learned from the development of the first generation of polymer conjugates have facilitated the development of an improved second generation of PT. These improvements encompass the optimization of polymerization techniques and linking chemistry, as well as data gained in efficacy profiles, clinical toxicities and side effects. Current efforts are directed towards practical and cost-effective designs for specific targets with defined routes of administrations and dosage in order to reach personalize medicine as the ultimate goal. Towards this aim, four major research lines are now being explored including: (i) the synthesis of novel well-defined polymeric architectures as carriers, (ii) the search for suitable potent and reliable characterization techniques, (iii) the use of polymer-based combination therapy, and (iv) their application towards new molecular targets. All these strategies are summarized in Figure 1.4 and discussed below.

![Figure 1.4. Current research lines in PT field: novel molecular targets in cancer as well as other disease, polymer-based combination therapy,](image-url)
new architectures and polymeric systems, and an exhaustive physico-
chemical characterization essential to clinical translation following
regulatory indications. Redrawn from ref\textsuperscript{24}.

1.3.1. Use of novel well-defined polymeric carriers and
adequate physico-chemical characterization techniques.

As previously mentioned, there is a growing interest in novel
biodegradable polymeric systems with new well-defined homogeneous
architectures (to enhance passive accumulation by EPR effect), with
low polydispersities, higher possibility for multivalency and greater
drug loading capacity. Their potential advantages include a more
defined chemical composition, tailored surface multivalency, and
creation of a defined three-dimensional architecture in solution.

The use of less heterogeneous systems in combination with
their characterization by means of novel sophisticated physico-chemical
techniques, will ultimately allow a better understanding of structure-
activity relationships, key to define safety and efficacy parameters set
by regulatory demands.\textsuperscript{118}

Non-biodegradable polymers such as HPMA or PEG have been
widely used in PT with some examples already in the clinics.
Nevertheless, such polymeric carriers are limited in terms of MW, in
order to allow renal elimination, and to avoid lysosomal accumulations
that could lead to lysosomal storage diseases. Biodegradable polymers
allow the utilization of higher MW platforms to optimize
pharmacokinetics and higher dose administrations.\textsuperscript{19, 78} These systems
are essential for the treatment of diseases that require chronic
administration, such as tissue repair, and neurodegenerative
disorders,\textsuperscript{119-121} and so the development of better polymeric carriers is
ongoing. Cathepsin B degradable PGA,\textsuperscript{122} dextrins degradable by
amylase,\textsuperscript{120, 123, 124} polyacetals\textsuperscript{125-127} that display pH-dependent
degradation, degradable polysaccharide polysialic acids\textsuperscript{128, 129} and
hydroxyethyl starch (HES)\textsuperscript{130, 131} are interesting options.

As already mentioned, advances in organic and polymer
chemistry, including the use of \textit{click} chemistry\textsuperscript{132} and optimization of
controlled and scalable polymerization techniques (Reversible
Addition-Fragmentation chain Transfer (RAFT) polymerization, Atom
Transfer Radical Polymerization, (ATRP) or Ring Opening
Polymerization (ROP) among others)\textsuperscript{133-136} is allowing to reach novel
and well-defined polymeric carriers from different architectures
including multi-block copolymers and branched systems such as hyperbranched, stars, brush-like, dendrons and dendrimers.\textsuperscript{137, 138} These new polymeric platforms possess inherent characteristics and sometimes, unexpected properties derived from their nature and structures what can be translate into different mechanism to cross biological barriers and cellular trafficking.

The development of complex architectures must however be accompanied by the development of appropriate techniques for their characterization. Sophisticated novel physico-chemical techniques include SANS (small-angle neutron scattering),\textsuperscript{126, 139, 140} SAXS (small-angle X-ray scattering), wide angle X-ray scattering (WAXS), PGSE-NMR (pulse-field gradient spin-echo nuclear magnetic resonance),\textsuperscript{127} and novel electron microscopies, such as Cryo-TEM (Transmission Electron Microscopy).\textsuperscript{141, 142} Their combination is contributing to increase knowledge regarding size, morphology, conformations in solution as well as the establishment of accurate structure-activity relationships. Furthermore, improved bio-physical techniques (mostly imaging techniques) are making possible \textit{in vitro} and \textit{in vivo} monitoring of biological outputs.\textsuperscript{143}

\subsection*{1.3.2. Polymer-based combination therapy.}

The use of polymer–drug conjugates in combination therapy can be also seen as an important opportunity to enhance therapeutic value provided that there is no disease characterized only by single molecular events. The polymeric carrier itself can provide an ideal platform for the simultaneous delivery of a cocktail of drugs simultaneously.\textsuperscript{76} To this respect, four types of polymer-based combination therapy have been proposed (Figure 1.5). These encompass the administration of (i) polymer-drug conjugate + free drug(s) (Type I), (ii) polymer-drug conjugate + polymer-drug conjugate (Type II), (iii) single polymeric carrier bearing the combination of drugs (Type III), and (iv) polymer-directed enzyme prodrug therapy (PDEPT) or polymer-enzyme liposome therapy (PELT).\textsuperscript{76} PDEPT is based on the combination of polymer-drug conjugate together with a polymer carrying the enzyme responsible for the release the drug at the target site, whereas PELT relies on a liposome encapsulated drug delivery system together with a polymeric carrier bearing the enzyme to degrade the liposome for drug release.
Figure 1.5. Polymer-based combination therapy strategies.

The first endocrine-chemotherapy combination reported, HPMA copolymer-aminoglutethimide-DOX,\textsuperscript{144} (HPMA-AGM-DOX) revealed that conjugates containing both drugs showed markedly enhanced cytotoxicity compared with the single counterpart HPMA copolymer–DOX, a conjugate which has already shown clinical activity in breast cancer patients.\textsuperscript{145} Mixtures of polymer conjugates containing only AGM or only DOX did not show a synergistic benefit. Other drug cocktails using HPMA as polymeric platform for cancer treatment, have been used in combination therapy, including the antiangiogenic TNP-470 + alendronates or the two drugs gemcitabine and DOX.\textsuperscript{76}

Combination therapy is currently being pursued for the treatment of many different pathologies, including cancer, infectious diseases and neurological disorders with high expectations and with examples appearing in an exponential manner.\textsuperscript{76, 146} However, many aspects still require refinement, related to the rational design of combination systems (drug selection and drug ratios in the combinations). For instance, the study of release profiles in Type III combination therapies is vitally important as the presence of one drug can hinder or delay the release of the other. This influences drug concentrations at the target site in a given moment and so modifies their synergistic properties.

1.3.3. Approaching novel molecular targets.

Novel sophisticated strategies in cancer therapy are currently under intense development thanks mainly to advances in genomics and proteomics regarding molecular mechanisms in tumorigenesis. Polymer conjugates containing drugs directed against novel anticancer targets are also emerging, including the first anti-angiogenic conjugate, HPMA copolymer–TNP-470.\textsuperscript{147} There are also preliminary attempts to develop therapies that target the apoptotic signaling cascade at molecular level with promising \textit{in vivo} results in ovarian cancer.\textsuperscript{148} Specific inhibition
of kinases and heat-shock proteins are also targeted.\textsuperscript{149, 150} while a combination of chemotherapy and photodynamic therapy (chemoradiotherapy) is also being explored.\textsuperscript{151, 152} A summary of these novel approaches in cancer treatment is shown in Table 1.3.

### Table 1.3. New molecular targets in cancer therapy using Polymer-drug conjugates. Adapted from Sanchis et al.\textsuperscript{39}

<table>
<thead>
<tr>
<th>Molecular Target</th>
<th>Polymer</th>
<th>Drug</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI-3 kinase</td>
<td>HPMA copolymer</td>
<td>Wortmannin</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td>PEG-PAH</td>
<td>Wortmannin</td>
<td>154</td>
</tr>
<tr>
<td>Sphingosine kinase</td>
<td>PGA</td>
<td>DMSP</td>
<td>150</td>
</tr>
<tr>
<td>HSP-90</td>
<td>K8-ELP(1-60)</td>
<td>GA</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td>HPMA copolymer</td>
<td>AH-GDM</td>
<td>149</td>
</tr>
<tr>
<td>Jab1</td>
<td>PEG</td>
<td>Curcumin</td>
<td>156</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>HPMA copolymer</td>
<td>HA14-1</td>
<td>157</td>
</tr>
<tr>
<td>Angiogenesis inhibitors</td>
<td>HPMA copolymer</td>
<td>TNP-470</td>
<td>147, 158, 159</td>
</tr>
<tr>
<td></td>
<td>mPEG-PLA</td>
<td>TNP-470</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>HPMA copolymer</td>
<td>TNP-470</td>
<td>161</td>
</tr>
<tr>
<td></td>
<td>HPMA copolymer</td>
<td>PTX</td>
<td>162</td>
</tr>
<tr>
<td>Aromatase inhibitors</td>
<td>HPMA copolymer</td>
<td>DOX, AGM</td>
<td>144, 163, 164</td>
</tr>
<tr>
<td>DNA alkylating, ROS</td>
<td>HPMA copolymer</td>
<td>Gem, DOX</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>PEG</td>
<td>EPI, NO</td>
<td>166</td>
</tr>
<tr>
<td>Chemoradiotherapy</td>
<td>PGA</td>
<td>4-HPR</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td>HPMA copolymer</td>
<td>Mesochlorin e\textsubscript{e}</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>HPMA copolymer</td>
<td>DOX, Gem</td>
<td>151, 168</td>
</tr>
<tr>
<td></td>
<td>PEG</td>
<td>Psolaren</td>
<td>169</td>
</tr>
</tbody>
</table>

*4HPR: N-(4-hydroxyphenyl) retinamide; AH: Aminohexanoic acid, AH-GDM: 17-(6-aminohexylamino)-17-demethoxygeldanamycin; ALN: Alendronate; DMSP: Dimethylsphingosine; EPI: Epirubicin; GA: Geldanamycin; Gem: Gemcitabine; HSP: heat-shock protein; K8-ELP: Lysine-8-elastin-like polypeptides; NO: Nitric oxide; PAH: Poly(aspartate hydrazide); PI-3 kinase: Phosphoinositide 3-kinase; PLA: Polylactic acid; ROS: Reactive oxygen species.

Advances in drug discovery have expanded the identification of new drugs with relevant activities in different human pathologies, encouraging the development of new polymer therapeutic families targeting many other diseases beyond cancer. Some examples already present in clinics include Cimzia\textsuperscript{®} for rheumatoid arthritis (RA), Macugen\textsuperscript{®} for age-related macular degeneration and Krystexxa\textsuperscript{®} for chronic gout. Diabetes, hypertension, infectious diseases, digestive tract diseases or RA, are also being currently targeted (See Table 1.4). Additionally, PT have emerged as useful tools to promote tissue repair,
applications in wound healing, bone resorption or ischemia/reperfusion injuries are promising strategies. Some of the ongoing examples are extensively reviewed by Sanchis et al.,\textsuperscript{39} and summarized in Table 1.4.

**Table 1.4.** Polymer-drug conjugates in the treatment of diseases other than cancer. Adapted from Sanchis et al.\textsuperscript{39}

<table>
<thead>
<tr>
<th>Disease</th>
<th>Polymer</th>
<th>Drug</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td>PGA</td>
<td>Phloridzin</td>
<td>170, 171</td>
</tr>
<tr>
<td>Hypertension</td>
<td>SMA</td>
<td>AHPP</td>
<td>172</td>
</tr>
<tr>
<td>HIV</td>
<td>PEG</td>
<td>Saquinavir</td>
<td>173</td>
</tr>
<tr>
<td></td>
<td>SALP</td>
<td>ZVD</td>
<td>174</td>
</tr>
<tr>
<td></td>
<td>k-carrageenan</td>
<td>ZVD</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td>Dextrin</td>
<td>ZVD</td>
<td>176</td>
</tr>
<tr>
<td></td>
<td>PHEA</td>
<td>ZVD</td>
<td>177</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>Dextran</td>
<td>LAM</td>
<td>178</td>
</tr>
<tr>
<td>Fungal infection</td>
<td>PEG</td>
<td>Amph B</td>
<td>179, 180</td>
</tr>
<tr>
<td>Leishmaniasis</td>
<td>Arabino-galactan</td>
<td>Amph B</td>
<td>181, 182</td>
</tr>
<tr>
<td></td>
<td>HPMA copolymer</td>
<td>Amph B</td>
<td>183</td>
</tr>
<tr>
<td></td>
<td>HPMA copolymer</td>
<td>NPC1161</td>
<td>184</td>
</tr>
<tr>
<td>Sepsis</td>
<td>PEG</td>
<td>Peptoid 7</td>
<td>185</td>
</tr>
<tr>
<td>Bowel constipation</td>
<td>PEG</td>
<td>Naxolol*</td>
<td>69</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>Dextran</td>
<td>Budesonide</td>
<td>186, 187</td>
</tr>
<tr>
<td>Inflammatory bowel</td>
<td>pDMAEMA</td>
<td>DXM</td>
<td>188</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>HPMA copolymer</td>
<td>DXM</td>
<td>189</td>
</tr>
<tr>
<td></td>
<td>CDP</td>
<td>α-mPNL</td>
<td>190</td>
</tr>
<tr>
<td>Neuroinflammation</td>
<td>PAMAM</td>
<td>NAC</td>
<td>191</td>
</tr>
<tr>
<td>Wound healing</td>
<td>PAMAM</td>
<td>G6S</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>Dextrin</td>
<td>rhEGF</td>
<td>120, 192</td>
</tr>
<tr>
<td>Ischemia/reperfusion injury</td>
<td>PGA</td>
<td>APAF-1 inh.</td>
<td>121, 193-195</td>
</tr>
<tr>
<td></td>
<td>Modified dextran</td>
<td>17β-estradiol</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td>SMA</td>
<td>AHPP</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>PEG</td>
<td>NO</td>
<td>198</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>HPMA copolymer</td>
<td>PGE\textsubscript{1}</td>
<td>189</td>
</tr>
<tr>
<td></td>
<td>HPMA copolymer</td>
<td>ALN</td>
<td>189</td>
</tr>
<tr>
<td></td>
<td>HPMA copolymer</td>
<td>D-Asp8</td>
<td>189</td>
</tr>
<tr>
<td>Rare disease (FAP)</td>
<td>PGA</td>
<td>Doxycycline</td>
<td>199</td>
</tr>
<tr>
<td></td>
<td>PEG</td>
<td>RAGE peptide</td>
<td>200</td>
</tr>
</tbody>
</table>

\*PEG-Naxolol (NKTR-118); α-mPNL: α-methylprednisolone; AHPP: 4-amino-6-hydroxy.pyrazolo[3,4-D]pyrimidine; Amph B: Amphotericin B; APAF-1 inh.: Apoptotic protease activating factor 1 inhibitors; CDP: linear cyclodextrin polymer (β-cyclodextrin + PEG); D-Asp8: D-aspartic acid octapeptide; DXM: Dexamethasone; G6S: Glucosamine-6-sulphate; LAM: Lamivudine; NAC: N-acetyl cysteine; PAMAM: Poly(amidoamine); pDMAEMA: Poly(dimethylamino)ethylmethacrylate; PGE\textsubscript{1}: Prostaglandin E\textsubscript{1};
PT are also expanding to areas of neurodegenerative disorders treatment and diagnosis, and will be addressed in more detail in the following section.

In conclusion, the PT sector is a growing field with many challenges but with many opportunities for improvement. Conjugates for the treatment of diseases other than cancer are still at an early stage of preclinical development, however, these underlie a strong research base for future PT that will hopefully reach clinical use in the next decade.

Overall, a new golden era for PT is just beginning driven by the growing acceptance of PT as clinically important agents, continued search for innovation in big Pharma, and the rapid convergence of interests of many scientific disciplines arising from the increased popularity of nanomedicine. Over the last decade, new hybrid nanotechnologies have emerged, i.e. polymer combination therapies, theranostics, complex polymer conjugates assembled into nanoparticles, new polymer chemistries and compositions trying to capitalize on the unique physico-chemical behavior of nanomaterials. Indeed, investments in nanomedicine is increasing and as a consequence, the number of publications related to PT/polymer-drug conjugates has increased 10-folds since 1996, highlighting the relevance of this relatively new area (Figure 1.6).
1.4. POLYMER THERAPEUTICS FOR THE TREATMENT OF NEURODEGENERATIVE DISORDERS.

Nanomedicine offers an opportunity to overcome problems related to neurodegenerative disorders treatment mainly related with passage through the blood-brain barrier (BBB) (extensively explained in Chapter 6). Most drugs under development and the strategies used to reach the central (CNS) or peripheral (PNS) nervous systems lack of optimal therapeutic efficacy due to poor pharmacokinetics, high toxicities and low BBB penetration. Drug concentrations within the therapeutic window needed to effectively treat most of the CNS disorders are difficult to reach at the desired target site inside brain. In this respect, PT are promising candidates for the treatment and diagnosis of CNS related diseases (including brain tumor) since they hold key characteristics to surpass main limitations from low MW tracers/drugs. Those features include (i) changed pharmacokinetic profiles and enhanced plasma half-life necessary to promote specific tissue/organs accumulations (ii) the possibility to include targeting moieties for specific active targeting strategies in order to increase brain accumulation by selective ligands or monoclonal antibodies; (iii) controlled and sustained drug release of conjugated drugs using cleavable linkers under specific environments; (iv) stability and reduced immunogenicity of the bioactive compound and possess optimal size to allow better penetration (when compared with other nanoconstructs such as liposomes).
FDA approval of Gliadel® wafer (MGI Pharma, Bloomington, USA) implants for brain therapy in malignant gliomas has made real the possibility of using polymeric systems to reach CNS. Nonetheless, implants in deep brain regions are required for an effective drug delivery, and so represent a highly aggressive treatment. Consequently, research is currently focused on the development of non-invasive methods based on different administration routes, all attempting to avoid brain tissue disturbance where risks could frequently be higher than benefits. The main strategies pursued for CNS delivery in the PT field involve systemic administrations with the use of the commonly known “Trojan Horses” to cross the BBB. These strategies are based on the conjugation of targeting ligands of specific receptors present in BBB. Although some systems have already demonstrated some translocation across BBB, the vast majority only achieve about 1% of the administered dose, with 4% the maximum obtained.

Pluronic® block copolymers consisting on hydrophilic poly(ethylenoxide) (PEO) and hydrophobic poly(propylene oxide) (PPO) blocks (PEO-b-PPO-b-PEO) are promising drug delivery systems that can act as polymeric drugs themselves or be used as polymeric micelles for imaging probes/drug delivery. This technology is already in Phase III clinical trials (Pluronic®-DOX, SP1049C, Supratek Pharma) for the treatment of highly resistant tumors) and is being highly explored for CNS delivery due to their inherent ability to interact with hydrophobic surfaces and so improved capability to cross biological barriers. For instance, conjugation of polyclonal antibodies against brain α2-glycoprotein or insulin, allowed Pluronic® bearing drugs or fluorescence probes via receptor mediated transport (RMT) to cross the BBB. This technology has also been applied for peptide/protein delivery to the CNS carrying opioid peptides, horseradish peroxidase or leptin.

The biodegradable PLMA (poly-β-L-malic acid) has been used for the development of PT to treat glioma via i.v. administration achieving specific accumulation in brain tumors and the suppression of intracranial glioma growth by the administration a complex PLMA conjugate. These conjugates combine targeting vectors for the BBB and the blood-tumor barrier (BTB), the endosomal disrupting unit trileucine, and the delivery of the antisense oligonucleotide laminin-411 for tumor inhibition.
PEG based PT are also under investigation for CNS treatment strategies including PEG as carrier itself or as extra unit to provide stealth properties to the final construct. Examples include the PEGylation of several growth factors for neuroprotective purposes, or OX26 antibody conjugation.

The globular structure of PAMAM dendrimers, together with their cationic surfaces, make them suitable systems due to their higher diffusivity in brain parenchyma after convention-enhanced delivery (CED). Plentiful examples of the use of these systems can be found in literature, mainly for glioma treatments and neuroinflammation. For instance, PAMAM-Methotrexate (MTX) dendrimers bearing the targeting antibody Cetuximab (ICM-C225) demonstrated tumor accumulation after CED administration. Additionally, intravitreal administration of PAMAM dendrimers demonstrated novel, intrinsic targeting properties by selectively localizing in activated microglia and astrocytes in the brain and retina. A clinically used steroid, fluocinolone acetonide (FA), has also been conjugated to the hydroxyl-functionalized; G4; PAMAM dendrimer, producing a nanodevice with sustained drug release for up to 90 days. A single systemic dose of hydroxyl terminated; PAMAM-G4 conjugated to N-acetyl cysteine (D-NAC) also produced dramatic improvements in motor function, neuronal counts and myelination, and reduced neuroinflammation. Overall, these PAMAM dendrimer–drug conjugates have shown promising preclinical efficacy in the alleviation of neuroinflammation associated with both ocular and brain diseases. PAMAM dendrimers have been also exploited in combination with Pluronics for small interfering (si)RNA brain delivery by means of polyplexes. For instance, radio-labeled siRNA-PAMAM-G7 dendriplexes incorporated in poloxamer 407 (Pluronic® F127) and chitosan gels were shown to cross the BBB following intranasal administration. Apart from their intrinsic properties to cross barriers, the targeting vectors Angiopep-2 (ANGIO1005, Angiochem Inc., Canada) and Transferring have been efficiently conjugated to PAMAM dendrimers improving their brain accumulation. Nevertheless, PAMAM dendrimers have safety issues for systemic administration that have to be still solved.

The examples listed are just a few and further nanosystems for CNS delivery are currently under development. Despite the fact that those systems are still in early stages, results obtained are encouraging
the use of PT as promising and exciting candidates for CNS drug delivery and molecular diagnostics. Nevertheless, further investigations in respect to the intrinsic barrier crossing properties of new and existing polymeric carriers (prioritizing vesicular and globular shapes) as well as the development of new targeting vectors and in deep exploration of the already discovered is in high demand in order to achieve effective treatments.
1.5. REFERENCES.


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Chapter 2

A controlled and versatile NCA polymerization method for the synthesis of polypeptides
The work presented within this thesis chapter was carried out in tight collaboration with Dr. Inmaculada Conejos Sánchez, who also presented part of it in her thesis dissertation. As a result both of us are co-first authors of the publication in the journal Polymer Chemistry, Volume 4, Issue 11, Pages 3182-3186 (“A controlled and versatile NCA polymerization method for the synthesis of polypeptides”).

2.1. INTRODUCTION AND BACKGROUND.

The development of more defined architectures with higher MW (to enhance passive targeting), predictable structure and conformation, lower heterogeneity, higher drug loading capacity and greater multivalency are main research lines in nanomedicine.

Polypeptides are envisaged to achieve a major impact on a number of different relevant areas such as biomedicine and biotechnology. Acquired knowledge and the increasing interest on amino acids, peptides and proteins is establishing a large panel of these biopolymers whose physical, chemical and biological properties are ruled by their controlled sequences and composition. Polymer therapeutics has helped to establish these polypeptide-based constructs as polymeric nanomedicines for different applications, such as disease treatment and diagnostics among others.

Indeed, routine clinical use of copaxone\textsuperscript{1, 2} and the promising phase III clinical results including the recent designation as orphan drug of the polymer drug conjugate Opaxio\textsuperscript{TM} (polyglutamic acid (PGA) paclitaxel conjugate, formerly Xyotax, PPX, CT-2103)\textsuperscript{3} have underlined the high potential of synthetic polypeptides within nanomedicine, in particular polyglutamic acid (PGA)\textsuperscript{4}.

2.1.1. Polypeptide Design and Synthesis.

Polypeptides belong to a family of macromolecules diverse both in applications and structural features. As a result, there are several strategies for their synthesis and preparation. In general, the methods for the design of polypeptides can be divided in two main different methodologies: the synthetic techniques, and the recombinant DNA techniques.

In the synthetic approaches, stepwise solid-phase polypeptide synthesis (SPPS),\textsuperscript{5, 6} native chemical ligation (NCL)\textsuperscript{7} or ring opening
polymerization of α-amino-\(N\)-carboxyanhydrides (NCAs)\(^{8-10}\) can be included. Those techniques are mainly based on the use of amino acids or their derivatives as monomers and are particularly useful in the design of hybrid architectures that combine sequences of peptidic and non-peptidic nature.

Recombinant DNA techniques can be seen as an alternative genetically encoded approaches for the synthesis of polypeptides. The main advantage of this technique is the inherent accuracy of the methodology, which includes high specificity in the sequence and stereochemistry of the newly synthesized polypeptide. On the other hand, not all polypeptides can be properly expressed in a heterologous host, and non-peptidic moieties cannot be included unless post-expression modification techniques are applied.

### 2.1.1.1. Recombinant DNA Expression of Proteins.

Recombinant DNA expression of proteins is often used for the synthesis of proteins with different purposes.\(^{11}\) One of the most relevant is protein and peptide engineering and design, which is the one that will be reviewed herein.

![Figure 2.1. Recombinant DNA technique for protein synthesis. Redrawn from Rhodes et al.\(^{12}\)](image)

In recombinant techniques (genetically encoded synthesis) three steps can be described. First of all, the creation of a recombinant gene segment that encodes for the protein of interest: a protein target is identified and translated into the corresponding genetic code. Then, the target oligonucleotide is synthesized; secondly, the insertion of this segment into a DNA vector, which is classically a plasmid from
bacteria to produce a recombinant DNA molecule; and finally, transformation of this recombinant DNA molecule into a host cell. Cells that are successfully transformed with the recombinant DNA molecule are grown in culture. This gene produces large amounts of the desired protein, which is later isolated from cells (Figure 2.1). Due to low cost and convenience, bacteria (such as *Escherichia coli* (*E. coli*) or *Bacillus subtilis*) are the most frequently used. It is important to mention that achievement of recombinant genes is the rate-limiting step, in particular if the polypeptidic product of interest is based on a large number of repeats.

This technique presents several advantages over synthetic methods: (i) It produces polypeptides with defined sequence, stereochemistry, and MW based on a genetic template; (ii) Continuous supply of polypeptide can be achieve after optimization (iii) The *in vivo* folding machinery of cells can assist to ensure the correct secondary or tertiary structures and conformations of the peptides.

However, it also present some drawbacks that must be mentioned: (i) Is time and effort consuming, mainly due to synthesis optimization of the gene (especially if large MW are desired); and to optimization of the expression levels in the host cell; (ii) Only short sequences up to 100 nucleotides can be produced by chemical synthesis using an automated solid phase DNA synthesizer. For larger sequences, combination with other techniques is needed (concatenation of oligonucleotides, recursive directional ligation (RDL), and mutagenesis or amplification of existing gene segments using polymerase chain reaction (PCR)); (iii) Multi-domain proteins are much more challenging to express than proteins smaller than 30 kDa; (iv) Incompatibility between protein and bacteria leads to toxicity, reducing protein production; (v) Only the 20 natural L-amino acids can be incorporated using standard cellular components. Although, this problem is slowly being surpassed.

**2.1.1.2. Synthetic Approaches.**

The majority of limitations of genetically encoded synthesis to produce polypeptides can be surpassed with the use of synthetic techniques. Among the advantages of synthetic approaches, the following can be pointed out: (i) Unnatural amino acids can be used to produce a vast number of novel architectures with different properties and structure-function relationships; (ii) It enables combination of
polypeptides with other synthetic polymers such as PEG, (iii) They are an easier and faster methodologies compared with genetically encoded techniques; (iv) Higher yields and large scale synthesis.

Nevertheless, controlled chain length and stereochemistry have represented an issue over the past years.

2.1.1.2.1. Solid-Phase Peptide Synthesis (SPPS).

Stepwise Solid-Phase Peptide Synthesis (SPPS) was first reported by Merrifield in 1963. In his protocol, Merrifield attached the first N-protected amino acid group through an ester bond to a polystyrene resin partially chlorinated. After that, the protecting group was removed yielding a free amino group ready to react with the next N-protected amino acid. Thus, the general principle of SPPS consists on repeating cycles of coupling-wash-deprotection-wash. The peptide is ‘immobilized’ onto the solid-phase and can be retained during a filtration process, whereas liquid-phase reagents and synthesis by-products are flushed away. Finally, the resin can be removed and the peptide isolated (Figure 2.2). The main amino protecting groups usually used are 9-fluorenylmethyloxycarbonyl (FMOC) and t-butyloxycarbonyl (BOC), and each of them requires different resins and amino acid side-chain protection and, consequently, different cleavage/deprotection steps.

The main advantages of this approach can be summarized as: (i) It allows the synthesis of natural peptides as well as incorporation of unnatural amino acids, D-amino acids, and peptide/protein backbone modification; (ii) Total control over peptide composition is achieved; (iii) Clean method due to the possibility to perform wash cycles after each reaction; (iv) Process can be automated using a peptide synthesizer.
Chapter 2

Figure 2.2. Schematic representation of Merrifield solid-phase peptide synthesis (SPPS).

On the other hand, some drawbacks must also be taken into account: (i) Large, complex polypeptides/proteins cannot be prepared, due to a low coupling efficiency as the length of the peptide increases; (ii) High purity is not usually obtained when large polypeptides are synthesized; (iii) It is necessary to use an excess of amino acids and coupling reagents; (iv) solubility of protected peptide segments is sometimes challenging.

Due to all these drawbacks, SPPS is typically restricted to the synthesis of polypeptides of less than 50 amino acid residues in order to not compromise purity.

2.1.1.2.2. Native Chemical Ligation (NCL).

Chemical ligation can be considered as a simple technique based on the chemoselective reaction of two unprotected peptide segments that will react exclusively among them diminishing potential side reactions and generation of undesirable by-products. This methodology was first introduced in 1991 by Schnölzer and co-workers, which consisted on the formation of a non-amide bond at the ligation site (i.e. thioester-linked products). Although introduction of a non-native linker within the protein was well-tolerated in folded proteins, it is always better if possible, the use of peptidic bonds as
linkers in order to mimic natural enzymes/proteins. This problem was solved by Dawson and co-workers\textsuperscript{7, 19} with the introduction of native chemical ligation (NCL) approach (Figure 2.3). It is based on the formation of an unstable covalent bound thioester-linked intermediate that spontaneously rearranges forming the most thermodynamically favored amide bond at the ligation site. This robust approach has been widely used to produce a series of model peptides,\textsuperscript{20} protein inhibitors,\textsuperscript{21} and a vast variety of other proteins.\textsuperscript{22}

![Diagram of Native Chemical Ligation]

**Figure 2.3.** Native chemical ligation. (a) Water, pH = 7; (b) rearrangement of thioester intermediate amide linked product.

The main advantage of this strategy is the absence of complex combinations of protecting groups as unprotected amino acids can be directly linked. However, its use is restricted to link peptide segments, therefore, is necessary to synthesize those segments using other alternative techniques.

### 2.1.1.2.3. Ring-Opening Polymerization (ROP) of α-Amino Acid N-Carboxyanhydrides (NCAs).

The ring-opening polymerization (ROP) of amino acid-N-carboxyanhydrides (NCA) is the most commonly applied polymerization technique to produce polypeptides and polypeptide-based block copolymers on a multigram scale (Scheme 1.1). Although the obtained polymers are less defined than natural peptides, the polymerization method enables access to polypeptidic architectures, which are beyond nature’s possibilities. The ROP of NCAs has already
been used for the synthesis of polypeptides with various applications that range from drug delivery systems, tissue engineering, sensing to catalysis. The first NCA were synthesized by Leuchs in 1906. Since then, a plethora of polypeptides has been created due to the variety of natural and non-natural amino acids and the versatility of the polymerization method, as it has been reviewed in the following excellent literature.

\[ \text{Ring-opening polymerization (ROP) of } \alpha\text{-amino acid } N\text{-carboxyanhydrides (NCAs).} \]

The opportunity to use functional non-natural polymers in combination with the scalable synthesis makes the ROP of NCA a great choice to reach well-defined polypeptides. Polypeptides produced from the NCAs possess the ability (as natural proteins) to form secondary structures e.g., \( \alpha \)-helixes and \( \beta \)-sheets. Moreover, their multiple functionalities, adjustable MWs (1-1000 kDa) and structural homogeneity favor self-assembly into defined supramolecular nanostructures with potential biomedical and pharmaceutical applications.

One of the trends in the development of polymeric based nanomedicines is the building of well-defined, reproducible and homogenous architectures. Although NCA polymerization can never provide perfect control in polypeptide synthesis, the living character of the polymerization allows to get close to it. Synthesis of narrowly distributed polypeptides for their potential use in biomedicine is a desirable goal, which turned out to be rather demanding. Furthermore, the characterization of such synthetic proteins remains a challenge, mainly due to the fact that standard methods for polymer characterization are based on mathematical models for random coiled architectures. In addition, standard protein analytics cannot be applied to samples disperse in sequence as well as MW.

In principle, every NCA can be polymerized by a nucleophile or a base. Both initiation steps lead to a different propagation mechanism. But it has to be kept in mind that, in aprotic solvents, every
nucleophile can also act as a base leading to the coexistence of both mechanisms. This fact can be considered to be one of the main reasons for rather undefined polypeptides. Therefore, the ROP of NCAs can proceed via two mechanisms: normal amine mechanism (NAM, induced by nucleophilic substitution) and activated monomer mechanism (AMM, induced by deprotonation).

As mentioned above, NAM is based on the nucleophile attack of the initiator. The intermediate formed is an unstable carbamic acid that decarboxylates yielding a new free amino group, thus, propagating the polymerization. Therefore, mainly primary amines can be used to polymerize NCAs. A proof of this mechanism was demonstrated independently by Peggion et al.\textsuperscript{30} and Goodman et al.\textsuperscript{31} by confirming the incorporation of the initiator fragment in the final polymer. Scheme 2.2 summarizes initiation and propagation steps.

\begin{equation}
\text{Scheme 2.2. Initiation and propagation steps according to NAM.}
\end{equation}

In contrast, AMM (first proposed by Ballard et al.)\textsuperscript{32, 33} is based on the basic character of the initiator and its ability to subtract the proton of the 3-N of NCA in a preinitiation step leading to the “real initiator”. This real initiator attacks the 5-CO to give a tadpole dimer, followed by reaction with another NCA, creating a new anion with the simultaneous release of CO\textsubscript{2}. This tadpole dimer is attacked by a new anion, to give a tadpole trimer, and so on, followed by the creation of NCA anion at each reaction step (see Scheme 2.3). For that reason this latter mechanism is attributed to tertiary amines or metal alkoxide, stronger basic than nucleophile initiators. In the cases of secondary amine, as well as of alkali halide-initiated polymerizations, it is believed that AMM and NAM coexist.\textsuperscript{34} Whereas NAM is valid for N-unsubstituted NCAs and N-substituted NCAs, AAM mechanism is only
for N-unsubstituted NCAs (due to the preinitiation step). Furthermore it should be noticed, that NCA anion itself can rearrange into an \( \alpha \)-isocyanatocarboxylate (See Scheme 2.5).

\[ \text{Scheme 2.3. Preinitiation, initiation and propagation steps in AMM.} \]

Moreover, in NAM initiation rate is faster than propagation, since primary amines are more nucleophilic than the \( \omega \)-amino groups. A fast initiation is one of the key requirements for a controlled polymerization, leading to MW control and additionally polypeptides with low dispersities due to the fact that every initiator is supposed to start a polymer chain. On the contrary, as AMM proceeds via anion, it is expected that the propagation rate is faster than in NAM leading to higher MWs. Nonetheless, polypeptides with high polydispersity indices (D) are obtained.\textsuperscript{34} Due to this, AMM should be avoided in order to achieve control polymerizations. Since amines can act as both a nucleophile and a base, polymerization will always switch back and forth between “amine” and “activated monomer” mechanisms. Especially, the use of secondary amines is unfavorable, because such compounds are sterically more hindered and have a more basic character. In summary, primary amines with reduced basic character seemed to be the key to a polymerization of NCA following just NAM.

Besides both mentioned polymerization mechanisms, a number of unwanted side reactions exist destroying the living nature of ROP of
NCAs. It is important to take into account all factors hindering the controlled polymerization such as purity of NCAs as well as reaction system (purity of solvents...), presence of water or moisture, CO$_2$ pressure, pH, temperature), presence of salts, cleavage of protecting groups, and undesired termination processes (carbamate mechanisms or reaction with $\alpha$-isocyanatocarboxylates). The carbamate mechanism is based on the nucleophilic attack of the intermediate carbamate to another NCA leading to the formation of urea groups within the polymer and termination of the chain growth reaction itself (see Scheme 2.5). On top of all this, most oligopeptides tend to form secondary structures even at very low degrees of polymerization, most notably $\alpha$-helices and $\beta$-sheets. Both forms differ strongly in solubility and reactivity towards further polymerization. To conclude, AMM should be diminished to yield well-defined homo and block copolymers as control over polymer end groups is essential for the synthesis of multiblock architectures or end group functionalization. Classic NCA polymerization tends to be very problematic, even when initiated by primary amines leading in most cases to reduced control about polymerization process itself. Especially whenever a higher degree of polymerization or complex architectures are desired, the occurring side reactions interfere.

For those reasons, efforts have been devoted to develop new approaches in order to overcome these drawbacks, such as, the use of heavy metal catalysts, high vacuum techniques (HVT), primary amine hydrochloride salts, the combination of low temperature with primary amines, the use of silazane derivatives as initiators or the optimization of reaction conditions (pressure, temperature, etc.). Unfortunately, all methods present limitations:

In 1997 Deming, developed a new class of NCA-initiators based in organickel and cobalt (0) compounds, able to overcome termination reaction and leading to homo and block copolypeptides with narrow $\bar{M}$ ($<1.2$), controlled MW (500-500,000 Da), preserving the original chirality of NCA monomer and useful for a wide range of NCAs. The main drawback is the fact that heavy metal catalysts must be carefully removed to avoid non-specific toxicity in biomedical applications. Besides, some other disadvantages are initiating complex synthesis and requirement of an hydrogen atom at the 3-N of the NCA. Schlaad et al. in 2003 reported the use of primary amine hydrochloride salts as macroinitiators in order to avoid AMM
mechanism. The nucleophilic amine terminus is transferred into a dormant (i.e. protonated) state. Reactivity of primary amine hydrochlorides toward NCAs was first investigated by Knobler et al.\textsuperscript{51} in the 1960s. Main drawbacks are that polymerization time increases due to reduced reactivity of the active sites leading to incomplete monomer conversion and presence of nucleophilic chloride anions which can act as initiators of NCA polymerization as well. Therefore, remaining monomer has to be removed before a second block can be synthesized. In their work Schlaad and co-workers reported $D < 1.1$ and $M_n$ not higher than 22 kDa.\textsuperscript{44}

![Scheme 2.4](image)

**Scheme 2.4.** Hydrochloric salt of a primary amine reaction with NCA.

The use of primary amines and High Vacuum Techniques (HVT) for the living polymerization was also proposed by Hadjichristidis et al. in 2004.\textsuperscript{47} According to the authors, HVT enables the synthesis of well-defined, homo, copolypeptides with complex macromolecular architectures and are also valid for polypeptides hybrids. However, HVTs require complex and expensive experimental setup, complicating synthesis. In addition, the closed system generates a pressure gradient during polymerization, which may influence polymerization kinetics. The use of low temperatures with primary amines was reported by Vayaboury et al in 2004,\textsuperscript{48} decreasing termination reactions. At 0 °C, activation energy barrier for chain propagation becomes lower than that of side reactions. The main disadvantage of this methodology is that reaction times increase about 2-4 times while yields decrease. And finally, the use of silazane derivatives as initiators was carried out by Lu et al. in 2007,\textsuperscript{46,52} leading to low $D$, the expected MWs and almost quantitative yields. The weak point of this approach is that is not general since it cannot be used for polymerization of N-unsubstituted NCA and Hexamethyldisilazanes (HMDS) amines are sensitive to hydrolytic reactions. Scheme 2.5 represents the complexity involved in the chemistry of NCA polymerizations.
Scheme 2.5. NCA polymerization mechanistic aspects from Barz et al.⁴⁴
2.1.2. Polypeptides as Polymer Therapeutics.

The use of amino acids as building blocks offers the opportunity to reach different polymeric systems (homo, or hybrid block copolymers) with diverse macromolecular architectures and a variety of possibilities for many therapeutic applications (Figure 2.4).

![Figure 2.4](image.png)

**Figure 2.4.** Schematic representation of the possibilities offered by the used of amino acids as building blocks.

Some of the inherent characteristics of polypeptides make them excellent candidates for drug delivery, such as presence of functional groups in their sequence that provide specific sites for direct attachments or charged-induced interactions with different biomolecules. Furthermore, polypeptides are susceptible to enzymatic degradation within a safe profile and the generation of non-toxic metabolites. All together stand for remarkable properties to turn them into marketed products. The importance and potential of polypeptides in the development of drug delivery systems have been clearly proven. In fact, it must be noticed that, the most successful first generation polymer therapeutics introduced in Chapter 1, is mainly represented by homopolymers, di-

*block or tri-

*block copolymers formed overall by PGA, poly(L-lysine) (PLL) or poly(L-aspartate).
Figure 2.5. Examples of polypeptide-based Polymer Therapeutics in the clinics.

2.1.2.1. Current Applications of Polypeptides in Drug Delivery.

Polypeptides have demonstrated their potential as drug delivery systems (DDS) with enough examples in literature of their use in Polymer Therapeutics. There are indeed successful products from all categories listed in the introduction highlighting its relevance.

Polymeric drugs. The already mentioned random copolymer Copaxone® (glatiramer acetate) was the first polymeric drug reaching the market for the treatment of multiple sclerosis and it was considered one of the top 10 selling drugs worldwide in the first quarter of 2013 (Figure 2.5). In early 90s, the use of poly(Arg-Gly-Asp) demonstrated to inhibit lung metastasis and migration of B16-BL6 melanoma in mice. Finally, the multivalent lysine-dendrimer VivaGel® is another example of a polymeric drug under clinical development for topical applications as a vaginal virucide to prevent HIV-1 infection.

Polymer-drug conjugates. As stated in the introductory chapter, the initial steps of polymer-drug conjugates were focused on anticancer agent development that were able to favor tumor accumulation, facilitated by the EPR effect and demonstrated tissue penetration. To
this respect, the already mentioned Opaxio\textsuperscript{TM},\textsuperscript{59} represents one of the best examples of the potential of polypeptides as drug delivery systems. Indeed, polyglutamates are highly biocompatible, biodegradable and multifunctional polymers, which have been successfully for various medical applications.\textsuperscript{1,60} In the body, such degradability is triggered by cysteine proteases (particularly cathepsin B), which play a key role in lysosomal degradation of this polymer.\textsuperscript{61} Many worth mentioning examples of PGA use as polymeric carriers can be found in literature. For instance, the also mentioned PGA-camptothecin (CPT)\textsuperscript{62} (formerly CT-2106) is in Phase II for colorectal and ovarian cancers, although the company Cell Therapeutics Inc. (CTI, Seattle, WA, USA) has no immediate plans to conduct any further clinical studies.\textsuperscript{63} Eldar-Boock \textit{et al.} have designed a PGA-PTX conjugate which incorporates an integrin-targeted moiety: a cyclic RGD peptidomimetic which significantly improved tumor accumulation and increased anti-tumor efficacy in an orthotopic murine 4T1 metastatic breast cancer model.\textsuperscript{64} PGA-Dopamine conjugate represents another example. This conjugate has significantly increased the short half-life of dopamine, indispensable in the regulation of angiogenesis, opening up a door for possible treatments of angiogenesis-dependent diseases.\textsuperscript{65} It is also worth mentioning the PGA conjugate of a hydrophobic pro-apoptotic sphingolipid: N,N-dimethylsphingosine (DMSP) linked through an ester bonding. This drug induces apoptosis competing with sphingosine kinase (SpK), a key regulator of tumor angiogenesis.\textsuperscript{66}

When talking about combination therapy approaches for cancer treatment, there are also several examples of PGA use as efficient carrier. Deladriere \textit{et al.} have designed and evaluated a family of PGA-AGM-DOX conjugates with demonstrated activity in an orthotopic 4T1 breast cancer mouse model.\textsuperscript{67} In recent years, several studies have confirmed the therapeutic potential of this application\textsuperscript{68} and, as expected, polypeptides have followed this trend. For instance, Wadhwa et al. have conjugated D-penicillamine and idarubicin to PGA, proving \textit{in vivo} longer residence time in blood and a significant enhancement in survival of athymic mice bearing NCI-H460 tumor xenografts.\textsuperscript{69}

Although pro-apoptotic approaches are related with cancer therapy, anti-apoptotic strategies have also applications in regenerative medicine.\textsuperscript{70} Vicent and coworkers designed a PGA-based Apaf-1 inhibitor conjugate,\textsuperscript{71} which represented the first anti-apoptotic polymeric nanomedicine. This conjugate has efficiently shown to
promote regeneration in the course of inflammation-induced tissue injury. More recently, in the same group, PGA-doxycycline (DOXY) conjugates as fibril disrupters where developed as an approach towards the treatment of the rare amyloidotic disease FAP (Familial Amyloidotic Polyneuropathy). Moreover, PGA can act as an adjuvant itself making appealing its application in vaccinations by the simple antigen presentation within the polymer to generate a directed immune response.

Apart from homo-polyamino acids, engineering of elastin-like peptides (ELPs) has also been explored for drug delivery. ELPs are 5-1500 amino acids in length based on the pentamer sequence Val-Pro-Gly-X-Gly, where X represents any amino acid. Synthesis is achieved through recombinant DNA techniques (Section 2.1.2). Their most significant property relies on a temperature-conformation relationship going from unordered structures to ordered β-turns when surpassing their temperature transition. Exploiting this property and by means of the application of local hyperthermia, these carriers have been targeted to solid tumors. This feature from ELP carriers classifies them as promising candidates for thermally responsive polymer-drug conjugates in cancer treatment. For instance, Geldanamycin has been conjugated to the block polymer lysine 8-elastin-like polypeptides (K8-ELP) showing higher cytotoxicity combining heat and K8-ELP-GA in cell cultures. DOX has been also conjugated to ELPs alone or in combination with cell penetrating peptides. Chilkoti et al. have reviewed multiple alternatives for these systems.

Polymeric micelles. The majority of these micellar systems include a biodegradable polypeptide block, such as poly(aspartic acid) (PAA), PGA or PLL and a variety of hydrophilic polymers, mostly PEG. The first example was reported by Ringsdorf et al. by conjugating cyclophosphamide (CP) sulfide in PEG-PLL copolymer. Kataoka and co-workers are pioneers in this area with several block copolypeptide micelles in clinical trials as anticancer agents, and many similar analogues in preclinical evaluation, i.e., NC-6301 (analogue of NK911 with docetaxel) and NC-4016 [analogue of NC-6004 with oxaliplatin (DACH-platinate)]. Inoue and co-workers developed a PEG-block-poly(aspartic acid)-DOX copolymer forming polymeric micelles that were delivered intravenously to the brain through conventional-enhanced delivery (CED), resulting in prolonged median survival compared with free DOX. With the same polymeric structure
and under the same concept, NC-6300/K-912 releases epirubicin at low pHs and is currently in phase I clinical trials.\(^{85}\)

Once again, under the concept of combination therapy and including pH-response stimuli, PEG-poly(aspartate-hydrazide) (PEG-PAH) block copolymers have been conjugated to DOX and wortmannin, alone or in combination to achieve pH-sensitive polymeric micelles.\(^{86}\) Nevertheless, wortmannin is not a FDA approved drug and its synergistic activity with DOX was not fully demonstrated in this work.

There also many examples of the use of polypeptides to form polyplexes (especially PLL) for gene delivery applications\(^{87}\) as well as their use in protein delivery.\(^{88}\) For the purposes of this thesis dissertation, those systems will not be reviewed.

### 2.1.2.2. Applications in Molecular Imaging and Theraonotics.

As cited in Chapter 1, polymer conjugates have been also proposed as nanoprobe for disease monitoring by means of tracer probe labeling.\(^{89}\), \(^{90}\) Even more interesting is the concept of “theranostics” based on the “find, fight and follow approach” that offers the possibility of an early detection, disease targeting and treatment.\(^{4}\), \(^{91}\) As example Li et al. have carried out biodistribution studies in solid tumor models using PGA-DTPA-Gd by MRI that allowed them to observe localization of the PGA-based probe in the necrotic zone of tumors.\(^{92}\) Recently, they have added a second label for multi-modal imaging by incorporating the near-infrared dye NIR813.\(^{93}\) The authors were able to ratify an increased uptake of PGA-Gd-NIR813 into tumor and a selective accumulation into the necrotic/apoptotic region of the tumor. In addition, polypeptide-based micellar MRI contrast agents are under current development ensuring the validity of these constructs for imaging purposes.\(^{94}\) Furthermore, there are plenty of studies of HPMA polymer in combination with diagnostic agents. For instance, HPMA-DOX conjugate labeled with \(^{131}\)I was considered in Phase I clinical trials.\(^{90}\) In another study, HPMA with Gadolinium was prepared by copolymerization method, including RGD moieties and labeled with \(^{111}\)In as diagnostic agent.\(^{95}\) Recently HPMA labeled with \(^{64}\)Cu and loaded with RGD moieties was evaluated as targeted system of tumor angiogenesis in prostate cancer xenografts by PET (positron emission
tomography) showing increased accumulation when compared with non-targeted control.96

2.2. RESULTS AND DISCUSSION.

As stated in the introduction, PGA can be synthesized by controlled ROP of NCAs,10, 23, 97 Control on polymer chain length and stereochemistry have been one of the major challenges in synthetic approaches and all of the techniques developed up to know to surpass all the problems derived from the inherent characteristics of NCA polymerization have their own limitations. Herein, a versatile and simple methodology for the preparation of well-defined polyglutamate nanocarriers, overcoming those limitations, is described.

2.2.1. Monomer synthesis and stability. Synthesis of γ-benzyl L-glutamate NCA (OBzl(Glu) NCA) (1).

α-Amino acid N-carboxy anhydrides (NCAs) synthesis can be divided into two groups depending on the nature of the amino acid substrate (Scheme 2.6). The first is Leuchs method and is based on the cyclization of N-alkoxycarbonyl amino acid halides to form α-amino acid N-carboxy anhydride. The second is called Fuchs-Farthing method, and involves direct phosgenation of unprotected α-amino acids

\[ \text{Scheme 2.6. N-α-carboxyanhydrides synthesis described by (1) } \text{Leuchs and (2) Fuchs-Farthing.} \]

We adapted the protocol from N.M.B Smeets et al.,98 a variation of Fuchs-Farthing method (Scheme 2.7). In addition, some variations were also implemented such as removal of remaining phosgene or HCl by nitrogen flow prior to precipitation followed by recrystallization and filtration under Schlenk conditions to avoid impurities and enhance storage stability.
Hence, trichloromethyl chloroformate was used instead, which leads to NCA polymerization and will lead to a broad or even multimodal D. Both byproducts are critical in the NCA ring, which can be phosgenated in a second step to form α-isocyanate acid chlorides (Scheme 2.8). Both byproducts are critical in NCA polymerization and will lead to a broad or even multimodal D. Hence, trichloromethyl chloroformate was used instead, which decomposes by temperature yielding phosgene. Due to the need for thermal decomposition higher temperatures than in conventional Fuchs-Farthing method (40-50 °C) are mandatory.

Scheme 2.8. Byproduct formation due to excess of phosgene and HCl.

Reaction mechanism is shown in the Scheme 2.9 and involves the direct phosgenation of unprotected α-amino acids. Cyclization proceeds through the formation of N-chloroformyl amino acid intermediates and loss of a second HCl molecule completes the NCA.

Scheme 2.9. Monomer synthesis mechanism.
The reaction generates 2 equivalents (eq.) of HCl per molecule of NCA capable to initiate NCA self-degradation. To avoid that process, limonene was used to scavenge HCl by addition to its double bonds (Scheme 2.10). Amines or other strong bases cannot be used due to the fact that they would attack the formed NCA and lead to its decomposition. In contrast limonene acts as an almost non-nucleophilic/-basic HCl scavenger. In addition to limonene, a N\textsubscript{2} or Ar stream was applied for 2-4 hours after reaction time to remove the excess of HCl.

**Scheme 2.10.** Mechanism of action of Limonene.

The white solid obtained after precipitation was then recrystallized several times. The presence of HCl impurities can be identified by precipitation of silver chloride when some drops of a solution 1 M of AgNO\textsubscript{3} are added to a solution of monomer. NCA purity is the main criteria to ensure long term storage. This issue was corroborated with stability studies of the NCA under different storage conditions. Results are shown in Table 1.1 where a “-“symbol means that monomer was found already polymerized.

**Table 1.1.** Stability of NCA under different storage conditions.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Storage Time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>r.t.</td>
<td>-</td>
</tr>
<tr>
<td>r.t./Ar</td>
<td>+/-</td>
</tr>
<tr>
<td>4\textdegree C</td>
<td>+</td>
</tr>
<tr>
<td>4\textdegree C/Ar</td>
<td>+</td>
</tr>
<tr>
<td>-23\textdegree C</td>
<td>+</td>
</tr>
<tr>
<td>-23\textdegree C/Ar</td>
<td>+</td>
</tr>
</tbody>
</table>

*r.t.: room temperature. Ar: storage under Argon.*

Thus, monomers were stored at -23\textdegree C under inert atmosphere. It must be mentioned that, prior polymerization, monomer integrity was always checked by (i) its solubility in tetrahydrofurane (THF) (polymerized monomer is only partially soluble whereas monomer is
completely soluble), (ii) its melting point (93.4 °C), (iii) the already mentioned AgNO₃ test, and (iv) its ¹H-NMR spectra in CDCl₃.

¹H-NMR and ¹³C-NMR spectra with corresponding assignments are depicted in Figure 2.6.

![NMR spectra in CDCl₃ of OBzl(Glu) NCA (1). a) ¹H-NMR, b) ¹³C-NMR.](image)

**Figure 2.6.** NMR spectra in CDCl₃ of OBzl(Glu) NCA (1). a) ¹H-NMR, b) ¹³C-NMR.

### 2.2.2. Exploration of NCA polymerization techniques using novel initiators.

#### 2.2.2.1. Exposing the need for novel methods.

As stated in the introduction, the wide range of reaction pathways that can occur during polymerization process, make controlled polymerization of NCAs a challenging task. For that reason, reaction conditions were optimized by using sophisticated and ultra-dried Schlenk equipment, freshly dried and distilled solvents as well as well-dried reagents. Under these conditions, and as a starting point in
order to address the degree of control and weak points of most applied methodologies of NCA polymerization techniques such as the use of primary amines and hydrochloride salts (Schlaad methodology). Several attempts varying monomer/initiator (M/I) ratio were carried out in N,N’-dimethylformamide (DMF) as a solvent, at 40 °C during three days by using n-butylamine and n-butylammonium hydrochloride salt (Scheme 2.11).

Scheme 2.11. General scheme for polymerizations using initiators based in n-butylamine and its derivatives salts.

Several conclusions can be drawn from the obtained results (Table 2.2). In the case of NA initiated polymerizations, degree of polymerization (DP) obtained is, apparently, independent of the M/I above a DP of 100, which make this methodology invalid when high MW are required. In the case of polymerizations initiated by hydrochloride salts, it can be observed that MW obtained increases as the ratio M/I increases, however, is still far lower from the theoretically expected. In both methodologies, $Ɖ$ increased when higher MW were pursued. In conclusion, although both methods are easy to apply without sophisticated equipment or complex synthesis they are invalid whenever DP above 100 is desired. Hence, there is a need for a different approach.
Table 2.2. NCA Polymerization in DMF with n-butylamine and n-butylammonium hydrochloride salt as initiators at 40 °C during 3 days at a monomer concentration of 0.38 mol·L⁻¹.

<table>
<thead>
<tr>
<th>Initiator</th>
<th>DP_{theo}</th>
<th>Y (%)</th>
<th>Mnᵃ (kDa)</th>
<th>DPᵇ</th>
<th>Dᵃ</th>
<th>DPᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Bu-NH₂</td>
<td>100</td>
<td>77</td>
<td>20.3</td>
<td>93</td>
<td>1.25</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>75</td>
<td>22.4</td>
<td>103</td>
<td>1.43</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>1600</td>
<td>83</td>
<td>20.5</td>
<td>94</td>
<td>1.42</td>
<td>60</td>
</tr>
<tr>
<td>n-Bu-NH₃Cl</td>
<td>100</td>
<td>60</td>
<td>4.4</td>
<td>20</td>
<td>1.35</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>72</td>
<td>18.1</td>
<td>83</td>
<td>1.56</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>1600</td>
<td>73</td>
<td>20.7</td>
<td>95</td>
<td>1.52</td>
<td>90</td>
</tr>
</tbody>
</table>

a. Data obtained by SEC in DMF; b. Data obtained by ¹H-NMR. DP= degree of polymerization, DP_{theo}= [M]/[I]= Monomer Concentration/Initiator Concentration, n-Bu-NH₂= n-butylamine, n-Bu-NH₃Cl= n-butylammonium hydrochloride salt, D= dispersity, Mn= apparent, Y= Yield.

DP by ¹H-NMR was calculated by comparison of the integral of the corresponding signals of the n-butyl initiator at low shifts (between 0.8-1.5 ppm), which can be assigned to the seven protons CH₃CH₂CH₂ of the molecule; and the corresponding signal of the CHα of the polypeptide chain (Figure 2.7).

Figure 2.7. Typical ¹H-NMR spectrum of poly(γ-benzyl L-glutamate) (PBLG) (2) in DMF-d7.

From the previous results, it could be concluded that there is a clear need for new approaches to reach living defined polypeptides with
control over chain length. Herein, a novel synthetic approach which uses, for the first time, ammonium salts with non-nucleophilic counter ions such as tetrafluoroborate (BF$_4$) was proposed as an alternative. The BF$_4$ ammonium salts as initiators combine the advantages of primary amine hydrochloride salts repelling AMM mechanism and reducing the reactivity of the amine (<nucleophilic, << basic); and the use of a counterion in the salt without nucleophilic character, correcting the main drawback of the Schlaad methodology. Its inert character is based on the symmetry of the ion leading to an equal charge distribution and its composition of highly electronegative fluorine atoms, which diminish the basicity of the anion. For this reason, a more controlled polymerization is expected with these initiators. However, remaining drawbacks still are that AMM mechanism is only suppressed and the influence of secondary structure remains untouched.

Based on the advantages listed above, a whole study of this hypothesis was planned. Thus, a screening of NCA polymerization of OBzI(Glu) NCA (1) using tetrafluoroborate salts as initiators was designed (Scheme 2.12). Within this screening, several parameters were varied, such as solvents (THF, DMF, dimethyl sulphoxide (DMSO), and dioxane), temperature (0, 25, 40, 60 and 80 °C) and initiators (n-butylammonium (3), neopentylammonium (4), PEG-ammonium (5), as well as functionalized initiators such as propargylammonium (6), (N$_3$-EG(2) ammonium (7)) BF$_4$.  

Scheme 2.12. Synthesis of PBLG (2) through the different ammonium tetrafluoroborate initiators.

2.2.2.2. Initiators.

Apart from initiating the polymerization process, due to the versatility of this polymerization methodology that includes the initiator within the C-terminus polymer backbone, the initiator can be used to:
(i) Introduce C-terminus end functionalities that can serve as linkage points or reactive sites for further conjugations or polymerization processes respectively. This was demonstrated herein with the use of propargylammonium (6), (N$_3$-EG(2) ammonium) (7) BF$_4$ initiators to introduce alkyne and azide moieties, respectively. The presence of an end alkyne or azide functionality allows site-specific conjugation of antibodies, proteins or imaging probes at the same time that side chain multivalency enables the design of advanced theranostics or polymer-based combination nanopharmaceutics, a hot topic in this area due to current clinical needs.$^{68,99}$

(ii) Synthesize hybrid di-block copolymers when macro-initiators consisting on polymeric chains are used. This was also demonstrated by the use of MeO-PEG-NH$_3$BF$_4$ as macroinitiator to lead PEG-PGA hybrid di-block copolymers (8). These hybrid di-block copolymers might offer different properties due to differences in hydrophilicity of the blocks leading to amphiphilic polymers whose properties might be tunable by controlling the blocks size.

(iii) Quantify by proton NMR the DP obtained during polymerization process. To this respect, in this work, n-butylamine was initially used to determine DP. Nevertheless, this initiator was later on substituted by neopentylamine in order to increase signal-to-noise ratio due to the 9 equivalent protons of neopentyl group.

The initiators based on tetrafluoroborate ammonium salts can be easily prepared by reaction of the corresponding amine with HBF$_4$ diethyl ether complex or by ion exchange using ammonium chloride and tetrafluoroborate Meerwein salt (BF$_4^-$-OEt$_3^+$) (Scheme 2.13)$^{100}$ purified by recrystallization and stored without detected decomposition or impurities (Figure 2.8).

Scheme 2.13 | Synthetic route for BF$_4$ salts obtaining.
2.2.2.3. NCA polymerization optimization.

First, variation of solvent conditions was performed using in all cases n-butylammonium BF₄ (3) as initiator of the polymerizations as well as MeO-PEG(2000)ammonium BF₄ (5) at 40 °C during three days. Then, several studies using freshly distilled solvents such as THF, DMSO, dioxane, and DMF were carried out in order to select an appropriate solvent. All solvents have been carefully purified according to literature. From these experiments, it could be clearly concluded that DMF as solvent in polymerizations leads to acceptable yields, controlled MW with narrow Đ as determined by GPC. This is not an unexpected result since in DMF, formation of the salt between the

\[
\text{NH}_3^+ BF_4^-
\]
intermediate of the carbamic acid and the amino groups of the propagating chains can be avoided, which leads to higher than the normal first order kinetics. DMSO was the second choice in the ranking, because performed polymerizations yielded defined systems, but the lower yields and the fact that work up is much more complicated since DMSO is not miscible with apolar organic solvents required in precipitation. Polymers synthesized in dioxane or THF were found to show bi/trimodal distributions as shown by gel permeation chromatography (GPC). Moreover, their MW did not correlate with the expected by $^1$H-NMR.

Following with the optimization process, the effect of temperature was also studied. From the literature mentioned in the introduction, it is already known that lower temperatures suppress some side reactions but elongates reaction times. As in the process of optimization, time was kept as a constant parameter, when reactions were done at lower temperature, lower yields were obtained (20-30%). Higher temperatures (60, and 80 °C) resulted in slightly yellow colored reactions in the case of 60 °C, and strong yellow colored with 80 °C, which can be explained by decomposition of DMF into dimethylamine and cleavage of the protecting group at high temperatures, leading to benzyl alcohol, which is yellow colored. In conclusion, a lack of control is present when high temperatures like 60 °C or above are applied in polymerizations. When low temperatures are applied, very poor yields were obtained (when 3 days of reaction time are kept), which is non-tolerable for the production of polypeptides on large scale.

With DMF established as solvent, and 40 °C as the appropriate temperature for a 3 days reaction, effect of monomer concentration was studied. When higher dilutions were used, less defined polymers (in terms of $\bar{D}$ and expectable MW) were obtained. This fact can be related to secondary structure formation, which depends on polypeptide concentration in solution. Hence, 0.1 g·mL$^{-1}$ monomer concentration was established as optimal according to results from Table 2.3.
Table 2.3. NCA polymerization in DMF with different monomer concentrations.

<table>
<thead>
<tr>
<th>Initiator</th>
<th>[C] M</th>
<th>[C] g·mL⁻¹</th>
<th>DP_theo</th>
<th>Y (%)</th>
<th>Mnᵃ (kDa)</th>
<th>DPᵃ</th>
<th>Dᵃ</th>
<th>DPᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Bu NH₃BF₄</td>
<td>0.1</td>
<td>0.38</td>
<td>200</td>
<td>85</td>
<td>13.9</td>
<td>65</td>
<td>1.28</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.19</td>
<td>200</td>
<td>60</td>
<td>10.6</td>
<td>48</td>
<td>1.27</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>0.025</td>
<td>0.09</td>
<td>200</td>
<td>60</td>
<td>2.43</td>
<td>11</td>
<td>2.71</td>
<td>57</td>
</tr>
</tbody>
</table>

a. Data obtained by SEC in DMF; b. Data obtained by ¹H-NMR.

DP_theo = [M]/[I] = [Monomer]/[Initiator], n-Bu-NH₃BF₄ = n-butylation ammonium BF₄ salt, Mn= apparent, Y= yield.

The versatility of this method was explored by polymerization of OBzI(Glu) NCA (1) with BF₄ salts of neopentyl amine (4), n-butylamine (3), propargylamine (6), azide amine (7) as well as MeO-PEG amine (MW 2000, D₆ 1.03) (5) yielding controlled block copolymers. Some results are summarized in Table 2.4.

Table 2.4. Demonstration of the versatility of the technique by preparation of a variety of functional end groups with different DPs.

<table>
<thead>
<tr>
<th>Initiator</th>
<th>DP_theo</th>
<th>Y(%)</th>
<th>Mn (kDa)ᵃ</th>
<th>Dᵃ</th>
<th>DPᵃ</th>
<th>DPᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Npt-NH₃BF₄</td>
<td>50</td>
<td>73</td>
<td>10.5</td>
<td>1.15</td>
<td>48</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>70</td>
<td>16.4</td>
<td>1.10</td>
<td>75</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>75</td>
<td>22.5</td>
<td>1.13</td>
<td>103</td>
<td>197</td>
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<td></td>
<td>400</td>
<td>85</td>
<td>44.2</td>
<td>1.08</td>
<td>202</td>
<td>405</td>
</tr>
<tr>
<td></td>
<td>1200</td>
<td>80</td>
<td>156.3</td>
<td>1.19</td>
<td>715</td>
<td>*</td>
</tr>
<tr>
<td>n-Bu-NH₃BF₄</td>
<td>50</td>
<td>64</td>
<td>6.8</td>
<td>1.11</td>
<td>31</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>64</td>
<td>13.8</td>
<td>1.09</td>
<td>63</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>63</td>
<td>16.2</td>
<td>1.12</td>
<td>74</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>72</td>
<td>31.5</td>
<td>1.18</td>
<td>144</td>
<td>212</td>
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<td></td>
<td>800</td>
<td>53</td>
<td>48.4</td>
<td>1.19</td>
<td>221</td>
<td>*</td>
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<tr>
<td></td>
<td>1200</td>
<td>51</td>
<td>94.4</td>
<td>1.21</td>
<td>431</td>
<td>*</td>
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<tr>
<td>MeO-PEG(2000)-NH₃BF₄</td>
<td>50</td>
<td>73</td>
<td>6.9</td>
<td>1.17</td>
<td>23</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>81</td>
<td>13.9</td>
<td>1.19</td>
<td>55</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>80</td>
<td>17.7</td>
<td>1.19</td>
<td>72</td>
<td>149</td>
</tr>
<tr>
<td>propargyl-NH₃BF₄</td>
<td>200</td>
<td>84</td>
<td>16.4</td>
<td>1.21</td>
<td>75</td>
<td>*</td>
</tr>
<tr>
<td>N₃-EG(2)-NH₃BF₄</td>
<td>200</td>
<td>77</td>
<td>18.2</td>
<td>1.19</td>
<td>83</td>
<td>*</td>
</tr>
</tbody>
</table>

a. Data obtained by SEC in DMF b. Data obtained by ¹H-NMR.

*Values below the detection limit.
IR was used to monitor monomer conversion with reaction time as a general tool to identify the end of polymerization. DMF strongly absorbs in IR region, therefore suitable peaks to follow the reaction had to be found. Figure 2.9 shows IR spectra of OBzl(Glu) NCA (1) DMF and PBLG (2) in DMF. Peaks corresponding only to monomer were localized at 1857, 1785 and 920 cm\(^{-1}\). Peaks at 1785 and 920 cm\(^{-1}\) were selected due to their higher signal-to-noise ratio.

![Figure 2.9](image)

**Figure 2.9.** Polymerization monitoring through FT-IR. Spectra of PBLG (2) in DMF solution over reaction time.

In all cases, polymers were characterized by \(^1\)H-NMR and GPC analysis (DMF and/or 1,1,1,3,3,3-hexafluoroisopropanol, HFIP). Some examples are given in Figures 2.10 and 2.11. Additionally, Circular Dichroism (CD) was used to confirm secondary structure showing α helix formation in all cases for benzyl protected polymers (Figure 2.11).

![Figure 2.10](image)

**Figure 2.10.** \(^1\)H-NMR spectra of polymerizations carried out with n-butyl BF\(_4\) salts at different [M]/[I] ratio of polymerization in DMF-\(d7\).
For polymerization kinetics, reaction was performed under nitrogen flow and at 40 °C using neopentylammonium BF$_4$ salt as initiator. Each point of kinetics was took from one Schlenk tube, thus, system was not opened while polymerization was running in order to not perturb kinetics. As expected, reaction follows a first order kinetics and $\tilde{D}$ increases as reaction time does when higher chains are generated. Results are summarized in Figure 2.12.

For further confirmation of the advantages of our novel methodology over the use of primary amines, neopentyl ammonium BF$_4$ salt was compared to its analogous amine form (Table 2.5). NCA polymerization with primary amine initiator was performed at 4 °C and 25 °C. However, BF$_4$ salt initiation was carried out only at 25 °C due to
the fact that reaction time was considerable increased at low temperatures (because of the dormant amine species).

Table 2.5. Summary of results from NCA-ROP through NA and BF\textsubscript{4} initiators in DMF, at different temperatures.

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>DP\textsubscript{the}</th>
<th>T °C</th>
<th>Y %</th>
<th>Mn\textsuperscript{a} kDa</th>
<th>D\textsuperscript{a}</th>
<th>DP\textsuperscript{a}</th>
<th>Mn\textsuperscript{b} kDa</th>
<th>D\textsuperscript{b}</th>
<th>DP\textsuperscript{c}</th>
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</thead>
<tbody>
<tr>
<td>Npt-NH\textsubscript{2}</td>
<td>50</td>
<td>4</td>
<td>74</td>
<td>9.4</td>
<td>1.13</td>
<td>43</td>
<td>9.7</td>
<td>1.10</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>64</td>
<td>27.8</td>
<td>1.06</td>
<td>127</td>
<td>22.2</td>
<td>1.11</td>
<td>144</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>68</td>
<td>28.7</td>
<td>1.10</td>
<td>131</td>
<td>23.6</td>
<td>1.14</td>
<td>202</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>90</td>
<td>15.7</td>
<td>1.14</td>
<td>72</td>
<td>29.5</td>
<td>1.10</td>
<td>190</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Npt-NH\textsubscript{2}</td>
<td>50</td>
<td>25</td>
<td>74</td>
<td>9.6</td>
<td>1.21</td>
<td>44</td>
<td>9.3</td>
<td>1.11</td>
<td>47</td>
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<tr>
<td></td>
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<td>80</td>
<td>11.9</td>
<td>1.17</td>
<td>54</td>
<td>11.0</td>
<td>1.14</td>
<td>78</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>79</td>
<td>13.9</td>
<td>1.14</td>
<td>64</td>
<td>12.1</td>
<td>1.16</td>
<td>114</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>78</td>
<td>16.4</td>
<td>1.16</td>
<td>75</td>
<td>12.5</td>
<td>1.16</td>
<td>121</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Npt-BF\textsubscript{4}</td>
<td>50</td>
<td>25</td>
<td>73</td>
<td>10.5</td>
<td>1.15</td>
<td>48</td>
<td>7.9</td>
<td>1.22</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>70</td>
<td>16.4</td>
<td>1.10</td>
<td>75</td>
<td>15.1</td>
<td>1.17</td>
<td>73</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>200</td>
<td>75</td>
<td>22.5</td>
<td>1.13</td>
<td>183</td>
<td>20.4</td>
<td>1.15</td>
<td>197</td>
<td></td>
<td></td>
</tr>
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<td></td>
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<td>85</td>
<td>44.2</td>
<td>1.08</td>
<td>202</td>
<td>35.8</td>
<td>1.16</td>
<td>405</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. Data obtained by SEC in DMF. b Data obtained by SEC in HFIP. c. Data obtained by \textsuperscript{1}H-NMR. I= Initiator, Npt= neopentylamine, Mn= apparent Mn, Y= yield.

HFIP was used as alternative solvent to DMF to ratify an adequate MW, and D data determination. DMF is usually effective enough in disrupting hydrogen bonds, nevertheless, secondary structures cannot be neglected in GPC measurements even at 70 °C (temperature in the GPC).\textsuperscript{103, 104} For that reason, HFIP was proposed expecting absence of secondary structures. However, comparison of polymer MW obtained showed not significant differences for both solvents. Looking at GPC graphs, slight tailing can be observed for PBLGs (2) bearing 50-100 glutamic acid units (GAU), since in this range a small amount of polymers are too short to form helical structures and therefore a mixture of random coils and helical structures is present within the same polymer sample. This can be confirmed by CD spectroscopy and NMR (4.8 ppm, alpha proton) applying GPC conditions. Indeed, this was observed in neopentyl initiator peak which
was divided into two signals (0.83 and 0.79 ppm), none of which comes from the free initiator (0.93 ppm), when low MW polymers were analyzed by NMR. As in nature, low MW PGAs display a more pronounced contribution of random coil structures. However, those effects are absent for higher MW PGAs and therefore are not attributed to undefined polypeptides but to the nature of the polypeptide itself.

After the study, it was concluded that both, the use of NH$_2$ at low temperature or BF$_4$ at 25 °C lead to MW control of the polymers, up to a DP of 200, with low Đ (1.05-1.20) which pointed out the living character of the polymerization process. Nevertheless, and as expected, when higher MW were required, only BF$_4$ achieved the desired DP.

In conclusion, MW of polymers can be precisely controlled up to a DP of ~800 without the use of complex initiators or demanding experimental setup. The derived polymers have low Đ (<1.2) indicating the well-controlled character of the polymerization. The use of these novel initiators provided reproducibility batch-to-batch as well as enabled scalability of the synthesis, from 100 mg to 10 g of starting NCA monomer. Indeed, a spin-off company was grown up from the laboratory, which applies the above described methodology.

### 2.2.3. Optimization of the deprotection protocols of PBLG (2).

Once synthesized and properly characterized, removal of the protecting groups from PBLG (2) is necessary in order to obtain free carboxylic groups for further bioconjugations. Due to the presence of functional groups, glutamic acid based NCAs must be polymerized using protecting groups in order to avoid undesired side reactions and benzylxycarbonyl (Bz) is the most widely used protecting group. There are different protocols for the removal of these protecting groups, such as strong acidic conditions, aqueous basic conditions or catalytic hydrogenation. However, in respect to deprotection of polypeptidic chains there are some issues that must be considered prior deprotection:

1. Harsh acidic conditions can lead to chain cleavage from protonation of side-chain ester groups which react with the amide backbone. TFA/HBr or acetic acid/HBr is known to lead to deprotection in the absence of racemization, however, these conditions are not suitable for sensitive functional end groups derived from functional initiators, e.g. alkyne or biotin moieties as well as PEG based block copolymers (i.e. PEG is rapidly degraded under those conditions).
(ii) Catalytic hydrogenation might lead to reduced yields caused by purification steps need which usually involve the use of resins that can interact within the polypeptide backbone. Furthermore, is ineffective for MW higher that 10 kDa (the helical conformation impedes access of the catalyst)\(^{107}\) and as in the case of acidic conditions, it can be used for functional end groups derived from functional initiators that can be hydrogenated such as alkynes, azides, vinyl moieties, etc.

(iii) Basic conditions might solve the problem of chain cleavage caused by acidic conditions, nevertheless, racemization can occur due to deprotonation of \(\alpha\) protons of the polymeric chains.

Hence, the final PGA homo (9) and di-block polymers (10) must be obtained using a deprotection method that ensures full removal of protecting groups with acceptable yields to be scalable, without damaging the polymer chains and without racemization; an issue, which is often not considered carefully and could induce important changes in nanomedicine behavior in a clinical setting. Changes in secondary structure due to polypeptide racemization will potentially alter immunological properties, degradation profile, and secondary structure. Consequently drug release, the pharmacokinetics profile and biodistribution might be unpredictable upon polypeptide-drug conjugation.

The aim of this study was then to establish an optimal deprotection conditions for all different polymeric architectures based on PBLG. As depicted in Scheme 2.14, three methodologies were tested either for homopolymers or di-block copolymers: acidic or basic media and catalytic hydrogenation.

Scheme 2.14. Deprotection protocols. (i) Acidic method: HBr (2 eq.)/TFA, 5 h, 25 °C; (ii) Catalytic hydrogenation: \(\text{H}_2/\text{Pd(OH)}_2\), 16 h, 25 °C; (iii) Basic method: NaOH aq. (1.5 eq.)/THF (1:8 (v/v)), 16 h, 4 °C. R represents Npt- or nBu-, MeO-PEG(2000)-, propargyl, or azide initiators, \(n\) is the number of repeating units of the glutamic block and \(m\) the ethylene glycol units.
2.2.3.1. Classic conditions. Catalytic hydrogenation vs. Acidic conditions.

To begin with, two methodologies for the removal of benzyl groups for homopolymers synthesis were studied. First, the use of acidic media with HBr/TFA and second, catalytic hydrogenation with the use of \( \text{H}_2 \) atmosphere with \( \text{Pd(OH)}_2/\text{C} \) as catalyst. Both of them lead to complete removal of benzyl groups, however, HBr/TFA was chosen due to simplicity of reaction conditions, higher yields obtained (quantitative versus 40 %) and the easier work up. As mentioned before, catalytic hydrogenation presents the handicap of the removal of Carbon afterwards using specific purification methods (celite columns), which can be the cause of the poor yield obtained.

Standard acidic conditions (HBr/TFA) were optimized for the deprotection of homopolymers. In order to avoid polymer damage, different eq. of HBr as well as different reaction times were evaluated to establish the minimum amounts and reaction time to yield complete deprotection. Classical use of 4 eq. of HBr per carboxylic group in TFA solution were reduced to 2 eq. and complete removal of protecting groups was observed after 5 hours reacting at r.t.. Polymers were obtained in quantitative yields, after purification by precipitation into a large excess of cold diethyl ether leading to a white solid that was recovered after centrifugation. After washing with diethyl ether, the product was then purified by acid-base precipitation (\( \text{NaHCO}_3 /\text{HCl} \) 6 M). Dialysis, ultrafiltration or Sephadex G25 columns were done leading to the sodium salt form. Once purified, polymers were characterized by \(^1\text{H}-\text{NMR}\). In Figure 2.13 it is shown the characteristic \(^1\text{H}-\text{NMR}\) spectrum of \( \alpha \)-polyglutamic acid (PGA) (9). Figure 2.14b shows the typical random coil conformation obtained in CD of deprotected PGAs of different MW ranging from 50-400 GAU.

Importantly, longer reaction times (up to 16 h) are required when undertaking large scale deprotection at acidic conditions to ensure full benzyl group removal and polypeptide stability.
Figure 2.13. $^1$H-NMR spectrum in D$_2$O of nBu initiated PGA deprotected (9a).

Figure 2.14. a) $^1$H-NMR (D$_2$O) analysis of PGAs (9b) with different DP (50, 100, 200 and 400). In red is pointed out the signal of the initiator used (neopentyl) indicating polymer integrity after deprotection. b) CD spectra in ddH$_2$O at 20 °C.

Thus, it was demonstrated that HBr/TFA acidic method lead to well-defined PGA polymer chains with complete removal of benzylic groups without chain cleavage, avoiding racemization processes. Nonetheless, is not a suitable method for deprotection of hybrid di-block copolymers PEG-PBLG, since HBr is a common reagent used for ethers cleavage. To confirm the damage produced to the PEG block, PEG stability studies under acidic and basic conditions were performed obtaining the GPC profiles (Figure 2.15). This study clearly showed that HBr/TFA deprotection is not applicable for PEG-PBLG.
2.2.3.2. Basic conditions optimization.

In respect to the exposed need for a suitable method to deprotect polypeptides bearing sensitive groups to both acidic and catalytic hydrogenation conditions, a basic deprotection protocol was developed.

First of all, and in order to validate the possible use of basic deprotections, different assays using LiOH and NaOH were carried out by subjecting PEG to different conditions summarized in Table 2.6. The resulting polymers were analyzed by GPC and proton NMR to check PEG integrity after exposure time and purification. Within this first study, it was concluded that PEG chains did not resulted damaged with the use of sodium hydroxide solutions. This encouraged us to continue with the optimization of basic conditions.

Kataoka’s protocol for PEG-poly(β-benzyl L-aspartate) deprotection\textsuperscript{108} was proposed as a first approach. Within this method, benzyl groups from aspartate units are easily removed by using a solution of NaOH at 0 °C during 10 minutes. However, when those conditions were applied to the deprotection of PBLG, using mixtures of THF/H\textsubscript{2}O in order to solubilize the products, deprotection did not occur. For that reason, longer reaction times, as well as control over the eq. of NaOH added were studied to achieve our purpose. In a first trial showed in Table 2.6, 2 eq. of NaOH and 5 h at 25 °C were used to deprotect PEG-PBLG (8), resulting in complete deprotection as analyzed by \textsuperscript{1}H-NMR in order to estimate the deprotection efficacy.
Table 2.6. Evaluation of different basic conditions.

<table>
<thead>
<tr>
<th>Method</th>
<th>eq.</th>
<th>t (h)</th>
<th>Bz removal</th>
<th>PEG integrity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PEG integrity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
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<td>LiOH</td>
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<tr>
<td>PEG&lt;sub&gt;2000&lt;/sub&gt;-PGA&lt;sub&gt;71&lt;/sub&gt;</td>
<td>NaOH</td>
<td>4</td>
<td>24</td>
<td>√</td>
<td>X</td>
</tr>
<tr>
<td>MeO-PEG&lt;sub&gt;2000&lt;/sub&gt;</td>
<td>LiOH</td>
<td>4</td>
<td>24</td>
<td>-</td>
<td>X</td>
</tr>
<tr>
<td>MeO-PEG&lt;sub&gt;2000&lt;/sub&gt;</td>
<td>NaOH</td>
<td>4</td>
<td>24</td>
<td>-</td>
<td>X</td>
</tr>
<tr>
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<td>2</td>
<td>7</td>
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<td>X</td>
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<tr>
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<td>7</td>
<td>-</td>
<td>X</td>
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<tr>
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<td>2</td>
<td>5</td>
<td>-</td>
<td>√</td>
</tr>
<tr>
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<td>NaOH</td>
<td>2</td>
<td>5</td>
<td>√</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>H-NMR data, <sup>b</sup>GPC data, eq.: eq. GAU

2.2.3.2.1. Optimization with/for homopolymers.

With 2 eq. of NaOH, at 25 °C and 5 hours as a starting point, conditions were optimized using homopolymers, in order to assess the balance between complete deprotection and avoidance of racemization processes. This basic deprotection study of polyglutamates was carried out varying parameters such as reaction time, NaOH eq. and THF/ddH<sub>2</sub>O ratio.

In all cases, 50 mg of polymer were dissolved in THF at r.t. Then, solution was cooled down and maintained under stirring at 4 °C. Finally NaOH solution was added drop wise. Turbidity was found in all cases once NaOH was added. Solutions were left under vigorous stirring the desire time. The organic solvent was removed under vacuum, and the aqueous phase was purified by ultracentrifugation using a Vivaspin® (MWCO 3000 Da).

The study was monitored by polarimeter measurements to evaluate α coefficient as a measure of the rotation angle of polarized light. Each sample was dissolved in ddH<sub>2</sub>O at 10 mg∙mL<sup>-1</sup> concentration and average data was obtained after 20 measurements. The obtained products were analyzed by <sup>1</sup>H-NMR to prove complete deprotection.

The alpha carbon of the amino acids is a chiral carbon that provides optical activity to the molecule. Amino acids in nature have an L configuration, which is related to rotation of the plane of polarized
light to the left giving negative α values. The rotation degree of polarized light depends on the number of chiral molecules that it encounters through the polarimeter cell. In this study, the synthesized glutamic acid NCA possess an L conformation that it must be retained during polymerization process if polymerization is initiated by a nucleophile, and not a base. This is the case of the described methodology within this work. L configuration must be also retained after deprotection to ensure biocompatibility of the polymeric carriers synthesized.

As the purpose of the study was to find appropriate basic conditions were racemization does not occur, each deprotected polymer was evaluated to determine its specific rotation ([α]Tλ), value that was calculated according to the literature with the formula in Equation 1.1.

\[
[\alpha]_\lambda^T = \frac{\alpha \cdot 100}{L \cdot c}
\]

**Equation 1.1.** Formula of the specific rotation [α] of the polypeptide chain. α= observed rotation, L= polarimeter cell length (dm), c= concentration (g·100·mL\(^{-1}\)) and T= temperature.

As strong basic conditions can damage the stereochemistry of glutamic acid units by deprotection of alpha proton and subsequent reprotonation, the protocol was modified to find softer conditions. For that reason, a whole studied was carried out at 4 °C. In a first approach, THF/ddH\(_2\)O ratio was varied, using a decreased amount of NaOH eq. (1.2) and 24 hours as reaction time (Table 2.7). The best THF/ddH\(_2\)O ratio value found so far, according to α measurements was 8:1.

**Table 2.7.** Optimization of THF/ddH\(_2\)O (v/v) ratio and concentration with 1.2 eq., 24 hours.

<table>
<thead>
<tr>
<th>eq. GAU</th>
<th>eq. NaOH</th>
<th>THF: H(_2)O</th>
<th>(\alpha_{av})</th>
<th>SD</th>
<th>(%_{\text{obs}}^2) coeff</th>
<th>([\alpha]_{25\lambda}^T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBLG (45 GAU)</td>
<td>1 1.2</td>
<td>4:1</td>
<td>-0.146</td>
<td>0.001</td>
<td>-0.727</td>
<td>-1.46</td>
</tr>
<tr>
<td>1 1.2</td>
<td>5.3:1</td>
<td>-0.283</td>
<td>0.001</td>
<td>-0.473</td>
<td>-2.83</td>
<td></td>
</tr>
<tr>
<td>1 1.2</td>
<td>8:1</td>
<td>-0.311</td>
<td>0.001</td>
<td>-0.428</td>
<td>-3.11</td>
<td></td>
</tr>
</tbody>
</table>

Reaction time was still too long in order to prevent racemization, nevertheless, when reaction time was decreased to 16 hours using only 1.2 eq. of NaOH per carboxylic group, complete
deprotection was not achieved. For that reason, the study was repeated using 1.5 eq. and a reaction time of 16 hours. Results validated as optimum THF/ddH₂O ratio the founded before (8:1). Interestingly, when higher ratios of THF/ddH₂O were used, deprotection did not occurred, probably due to limited accessibility of NaOH to the polymer chains (Table 2.8).

**Table 2.8.** Optimization of the THF/ddH₂O ratio and concentration with 1.5 eq., 16 hours.

<table>
<thead>
<tr>
<th>eq. GAU</th>
<th>eq. NaOH</th>
<th>THF:ddH₂O</th>
<th>α&lt;sub&gt;av&lt;/sub&gt;</th>
<th>SD</th>
<th>%s&lt;sup&gt;2&lt;/sup&gt; coeff</th>
<th>[α]&lt;sup&gt;25λ&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBLG (45 GAU)</td>
<td>1</td>
<td>1.5</td>
<td>64:1</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.5</td>
<td>32:1</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.5</td>
<td>16:1</td>
<td>-0.466</td>
<td>0.002</td>
<td>-0.330</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.5</td>
<td>8:1</td>
<td>-0.479</td>
<td>0.002</td>
<td>-0.345</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.5</td>
<td>4:1</td>
<td>-0.237</td>
<td>0.001</td>
<td>-0.598</td>
</tr>
</tbody>
</table>

Once the optimal ratio THF/ddH₂O was established, reaction time was varied by using 1.5 eq. of NaOH. Results summarized in Table 2.9 lead to the conclusion that, although 8 hours were enough time (and minimum needed) to achieve complete PBLG deprotection, the best α value was obtained for 16 hours reaction time. Reduced reaction times did not end up in deprotection.

**Table 2.9.** Time optimization using 8:1 THF/ddH₂O and 1.5 eq. NaOH.

<table>
<thead>
<tr>
<th>eq. GAU</th>
<th>eq. NaOH</th>
<th>t (h)</th>
<th>α&lt;sub&gt;av&lt;/sub&gt;</th>
<th>SD</th>
<th>%s&lt;sup&gt;2&lt;/sup&gt; coeff</th>
<th>[α]&lt;sup&gt;25λ&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBLG (45 GAU)</td>
<td>1</td>
<td>1.5</td>
<td>2</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.5</td>
<td>4</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.5</td>
<td>6</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.5</td>
<td>8</td>
<td>-0.484</td>
<td>0.002</td>
<td>-0.758</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.5</td>
<td>16</td>
<td>-0.615</td>
<td>0.002</td>
<td>-0.287</td>
</tr>
</tbody>
</table>

As conclusion, it can be said that the use of 1.5 eq. NaOH, 16 hours and THF/ddH₂O 8:1 efficiently deprotected PBLG, with the highest negative α value that we could obtained, being around -0.6. To our knowledge, there was no information available on literature to
assess whether those values are enough to undoubtedly claim that racemization processes are not occurring. For that reason, α values of comparable polymers with well-known L configurations were studied:

(i) In one hand, L-polyglutamates obtained by solid phase peptide synthesis (SPPS). (ii) On the other hand, HBr deprotected polyglutamates obtained by our methodology. As mentioned before, acid deprotection has been demonstrated not to affect the stereochemistry of the initial polymer. (iii) A commercial deprotected PGA\(_n\) with different GA units (n= 100, 200 and 300).

Comparison with SPPS polymers will allow us to confirm that racemization processes did not occur either during polymerization, ratifying nucleophilic initiation of the methodology described as above mentioned. Results obtained after measuring α values are summarized in Table 2.10

**Table 2.10.** Observed rotation for SPPS synthesized PGA in comparison with commercial (Com.) PGAs and ROP-NCA synthesized PGA (9).

<table>
<thead>
<tr>
<th></th>
<th>α(_{av})</th>
<th>SD</th>
<th>%s2 coeff.</th>
<th>[α](_{25\lambda})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Com. PGA(_n) (n= 100)</td>
<td>-0.567</td>
<td>0.001</td>
<td>-0.224</td>
<td>-5.67</td>
</tr>
<tr>
<td>Com. PGA(_n) (n= 200)</td>
<td>-0.545</td>
<td>0.001</td>
<td>-0.243</td>
<td>-5.45</td>
</tr>
<tr>
<td>Com. PGA(_n) (n= 300)</td>
<td>-0.485</td>
<td>0.002</td>
<td>-0.315</td>
<td>-4.85</td>
</tr>
<tr>
<td>PGA(_{20}) HBr dep. (SPPS)</td>
<td>-0.555</td>
<td>0.001</td>
<td>-0.210</td>
<td>-5.55</td>
</tr>
<tr>
<td>PGA(_{15}) (SPPS)</td>
<td>-0.512</td>
<td>0.002</td>
<td>-0.357</td>
<td>-5.12</td>
</tr>
<tr>
<td>PGA(_{15}) (SPPS) NaOH 2 M</td>
<td>0.007</td>
<td>0.001</td>
<td>18.470</td>
<td>0.07</td>
</tr>
</tbody>
</table>

As it can be extracted from Table 2.10, α values of SPPS PGA, commercial PGA and PGA HBr deprotected are on the same range that the ones achieved with the optimized basic deprotection methodology. Moreover, damage on stereochemistry and therefore optical activity caused by the use of strong basic conditions was proven by exposing PGA\(_{15}\) to concentrated NaOH (2 M) during 16 hours. This gave a positive α value (0.007) of complete racemization.

Finally, the versatility of the method was validated by studying the deprotection of different MW polymers with the optimized conditions (Table 2.11). In all cases, according to α coefficients, optical activity remained untouched.
Table 2.11. Comparison of observed rotation from PGAs (9) with different DP after deprotection with the optimal basic protocol.

| PBLG<sub>n</sub> | \(\alpha_{av}\) | SD | %\(\delta^2\) coeff. | \(|\alpha|^{25}_{rots}\) |
|-----------------|----------------|----|----------------------|----------------------|
| n= 100          | -0.654         | 0.002 | -0.331               | -6.55               |
| n= 200          | -0.659         | 0.002 | -0.287               | -6.59               |
| n= 400          | -0.664         | 0.002 | -0.285               | -6.64               |

2.2.3.2.2. Optimization with/for hybrid DB PEG-PGA.

Once the conditions were established, the study was then conducted on hybrid di-block copolymers PEG-PBLG (3). Nevertheless, when polymers were evaluated under the same optimized methodology, incomplete reactions took place due to the use of 1.5 eq. of NaOH. For that reason, the procedure was re-optimized to deprotect di-block copolymers. Products were purified as for homopolymers and analyzed by \(^1\)H-NMR to check complete deprotection (Figure 2.16).

![Figure 2.16. \(^1\)H-NMR spectrum in D<sub>2</sub>O of di-block PEG-PGA (10) with the signals assigned.]

Variation of NaOH eq. was first carried out by using 16 hours and the ratio THF/ddH<sub>2</sub>O as fixed parameter. From the results obtained (Table 2.12), it was concluded that although yield was slightly increased, the use of more than 2 eq. NaOH began to cause changes on stereochemistry of the polymer chains as seen by less negative \(\alpha\) values.
Table 2.12. Optimization of NaOH eq. for di-block deprotection using the same conditions of time and THF/ddH₂O ratio (16 h, 8:1).

<table>
<thead>
<tr>
<th>eq. NaOH</th>
<th>α\text{av} (% s² coeff.)</th>
<th>[α]₂₅λ</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-PGAn (50 GAU)</td>
<td>2 -0.551 (0.002) -0.294 -5.51</td>
<td></td>
</tr>
<tr>
<td>3 -0.510 (0.001) -0.292 -5.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 -0.440 (0.005) -0.325 -4.40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Reaction time was varied in order to achieve higher yields (Table 2.13). However, longer reaction times did not improve significantly those yields and α values were on detriment upon increasing reaction time. Therefore, 16 hours was established as the optimal time frame.

Table 2.13. Optimization of reaction time for di-block deprotection using the same conditions of NaOH eq. and THF/ddH₂O ratio (2, 8:1).

<table>
<thead>
<tr>
<th>eq. NaOH</th>
<th>t (h)</th>
<th>α\text{av} (% s² coeff.)</th>
<th>[α]₂₅λ</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-PGA₅₀ GAU</td>
<td>2 16 -0.535 (0.003) -0.468 -5.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 21 -0.517 (0.001) -0.283 -5.17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 24 -0.494 (0.003) -0.648 -4.94</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Summarizing, complete deprotection without deviations in specific rotation ([α]₀λ) value of deprotected PGA was achieved. Moreover, the [α]₀λ values were on the range of PGA 15mers prepared by solid phase peptide synthesis (SPPS) independent from the optimized acidic or basic methodology.

A summary of results obtained for homo and di-block copolymers regarding acid basic deprotection are represented in Figure 2.17.
Figure 2.17. Comparison between in specific rotation ([\(\alpha\)]\(_\mathrm{r}\)) was performed analyzing a PGA\(_{15}\) synthesized by SPPS, PGA\(_{50}\) after acidic (1) and basic deprotection and PEG-PGA\(_{50}\) after basic deprotection as it is reflected in the diagram.

The deprotection protocol enabled acidic as well as basic deprotection of PBLG allowing the synthesis of PEG based block copolymers and the incorporation of sensitive functional groups. As conclusions, it can be said, that, for homopolymers deprotection, HBr/TFA optimized method was preferred since quantitative yields were always obtained without chain cleavage as well as without changes on stereochemistry of \(\alpha\) carbons of the polymer chain. Moreover, for low scale reactions, reaction time is much lower when compared to basic protocol. For large scale reactions, the optimal reaction time was set as 16 hours. In the case of di-block copolymers, the basic protocol was always preferred in order to avoid chain cleavage of PEG block. In addition, this protocol was also used for the deprotection of PBLG bearing functional groups at the chain end sensitive to acidic and hydrogenation conditions.

To draw to a close with this part, it is worth mentioning that, although BF\(_4\) salts have been reported to be safe\(^{100,109}\), BF\(_4\) traces have never been detected in \(^{19}\)F-NMR after removal of protection groups either by acidic or basic methodologies and workup (Figure 2.18).
Figure 2.18. a) $^{19}$F-NMR of PBLG (2) before benzyl deprotection in DMSO-$d_6$, b) $^{19}$F-NMR of PGA (8), after benzyl deprotection in DMSO-$d_6$. Explanation of chemical shifts: B has two isotopes with spin > ½ and because the ion is symmetric one can observe quadrupole splitting rather than quadrupole broadening. For $[(^{10}\text{B})\text{F}_4]^{-10}\text{B}$ (19.58 % abundance) has spin (I) 3 so $(2nI +1)$, n= 1 resonance will be split into seven lines of equal intensity (19.58/7). On top of this (chemical shift probably but not necessarily the same) $[(^{11}\text{B})\text{F}_4]^{11}\text{B}$ (80.42 % abundant) has spin 3/2 so $(2nI +1)$ resonance will be split into 4 lines of equal intensity so about 20 % (80/4) with a different B-F coupling.

2.3. CONCLUSIONS.

Summarizing the above results, a new methodology for NCA polymerization was applied to enable easy, multigram scale synthesis of narrowly distributed PGA. The optimization of the monomer synthesis, as well as its storage was studied in a first place following a modification of the Fuchs-Farthing method. As every impurity with nucleophilic or basic character can initiate the polymerization, careful recrystallization and purification under dry atmosphere is necessary to store and to apply NCAs to reach controlled ring opening polymerization. Once that step was optimized, various polymerization parameters where adjusted such as solvent used, temperature and concentration in order to get well-defined polymers. Moreover, DP could be adjusted precisely over a long range of MWs. In addition, the deprotection protocol enabled acidic as well as basic deprotection of PBLG allowing the synthesis of PEG based block copolymers and the incorporation of sensitive functional groups.
Conformation and polydispersity are relevant parameters in the described methodology. Recently, Huesmann et al. have addressed both topics in poly(L-lysine) synthesis.\textsuperscript{104}

It has to be mentioned that large scale PGA synthesis under GMP conditions are currently being implemented ratifying the feasibility of the described methodology to enable a rapid clinical transfer of our PGA-based nanomedicines. Importantly, these methods are more widely applicable to other synthetic polypeptides or polypeptide-based constructs which ratifies the broad interest of this approach.

Figure 2.19. Schematic representation of NCA polymerization methodology developed within this chapter.

2.4. MATERIALS AND METHODS.

2.4.1. Materials.

All chemicals were reagent grade, obtained from Aldrich and used without further purification, unless otherwise indicated. MeO-PEG(2000)-NH\textsubscript{2} and H-L-Glu(OBzl)-OH were obtained from Iris Biotech. All solvents were of analytical grade and were dried and freshly distilled. Deuterated chloroform-\textit{d}1, DMSO-\textit{d}6, DMF-\textit{d}7 and D\textsubscript{2}O were purchased from Deutero GmbH. Preparative SEC was performed using Sephadex G-25 superfine from GE as well as PD MiniTrap G-10 \textsuperscript{TM} columns containing 2.1 mL of Sephadex\textsuperscript{TM} G-10.
Dialysis was performed in a Millipore ultrafiltration device fitted with a 1, 3, or 10 kDa MWCO (Vivaspin®) regenerated cellulose membrane.

2.4.2. Characterization techniques.

2.4.2.1. NMR spectroscopy.

$^1$H and $^{13}$C-NMR spectra were recorded on a Bruker AC 300 at r.t. and at a frequency of 300 and 75 MHz respectively and analyzed using the MestreNova 6.2 software.

2.4.2.2. DMF GPC.

For SEC measurements in DMF containing $1 \text{ g} \cdot \text{L}^{-1}$ of lithium bromide as an additive, an Agilent 1100 series system was used with a flow rate of $1 \text{ mL} \cdot \text{min}^{-1}$ at $30 ^\circ \text{C}$ as an integrated instrument, including three HEMA-based columns (10$^5$/10$^3$/10$^2$ Å porosity) from MZ-Analysentechnik GmbH, a UV (275 nm) and an RI detector. Calibration was achieved with well-defined PEG/DMF or poly(methyl methacrylate) (PMMA)/DMF standards, provided by Polymer Standards Service (PSS)/Mainz Germany.

2.4.2.3. HFIP GPC.

GPC was performed with HFIP containing $3 \text{ g} \cdot \text{L}^{-1}$ potassium trifluoroacetate as eluent at $40 ^\circ \text{C}$. The columns were packed with modified silica (PFG columns particle size: 7 µm, porosity: 100 & 1000 Å. A refractive index detector (G 1362A RID) was used to detect the polymer. Molecular weights were calculated using a calibration performed with PMMA standards (Polymer Standards Services GmbH) and toluene as internal standard.

2.4.2.4. Circular Dichroism (CD).

CD Spectroscopy was performed with a J-815 CD Spectrometer (JASCO Corporation) using a Peltier thermostated cell holder (PTC-423, JASCO Corporation) with a recirculating cooler (JULABO F250, JASCO Corporation). A nitrogen flow (~2.7 L·min$^{-1}$) was lead through the spectrometer and controlled with a nitrogen flow monitor (Afriso Euro-Index). Samples were dissolved in HFIP for protected and ddH$_2$O for deprotected samples and diluted to a concentration of $0.25 \text{ mg} \cdot \text{mL}^{-1}$. Samples were measured repeatedly (n=3) in a quartz cuvette with d= 0.1 cm at 20 °C. Obtained molar ellipticities were plotted as mean residue ellipticity.
2.4.2.5. Infrared (IR) spectroscopy analysis for polymerization monitoring.

IR spectra were recorded using thermo scientific Nicolet 380 FT-IR spectrometer with a spectral range 7800-350 cm\(^{-1}\), optical resolution (apodized) < 0.9 cm\(^{-1}\) resolution (standard) and peak-to-peak noise < 2.2\(\cdot10^{-5}\) abs. (> 22000:1) (1 minute scan). All samples analyzed were under solution or previously dissolved in DMF. Analysis was carried out at 25 °C.

2.4.2.6. Polarimetry.

Chirality of deprotected polymers was checked using the automatic polarimeter Jasco P1020 measuring 3 times x20 scans each. Solutions were all prepared in ddH\(_2\)O at 10 mg\(\cdot\)mL\(^{-1}\). Analysis were carried out at 25 °C.

2.4.3. Protocols.

2.4.3.1. NCA monomer synthesis. Synthesis of \(\gamma\)-benzyl L-Glutamate N-carboxyanhydride (NCA) (1).

H-L-Glu(OBzl)-OH (17 g, 71.66 mmol, 264 g\(\cdot\)mol\(^{-1}\)) was added to a two-neck 250 mL round bottom flask fitted with a stirrer bar, reflux column, dropping funnel and an argon in and outlet. The apparatus was purged with Ar for 5 min. Afterwards anhydrous (anh.) THF (120 mL) was added and the contents were heated to 60 °C. Limonene (11.6 mL, 71.66 mmol, 1 eq.) was added to the stirring suspension before diphosgene (5.2 mL, 8.5 g, 43 mmol, 0.6 eq.) dissolved in THF (10 mL, anh.) was added via a dropping funnel over a period of 10 min. The reaction was left stirring for 3 hours at 60 °C whilst purging with Ar leading to a clear solution. The reaction mixture was bubbled with Ar to aid the removal of remaining HCl for 2 hours whilst the Ar outlet was directed through an aqueous 1 M sodium NaOH to neutralize the gas. The reaction solvent was reduced to a quarter of the original volume by rotary evaporation and ethyl acetate (32 mL) was added. The contents were added to ice cold hexane (200 mL) to form a white precipitate, which was isolated by vacuum filtration and washed with cold hexane. The solid was recrystallized from toluene (50 mL, anh.) and THF (30 mL, anh.) under inert atmosphere (\(\text{N}_2\) or Ar) by using a 250 mL two neck-flask 250 round bottom flask fitted with a stirrer bar, reflux column and an argon inlet and outlet where crystallization was induced by a drop wise addition of cold hexane (27 mL). Finally the white
crystals were filtered under Ar conditions by using Schlenk techniques, and stored at -20 °C.

To ensure that residual HCl had been successfully remove, NCA (2-4 mg) was dissolved in THF (0.5 mL) and added to a 0.1 mmol silver nitrate solution (1 mL) where the solution remained clear. When Ag⁺ and Cl⁻ ions meet they form the colorless insoluble AgCl salt, which can be easily detected. Another test is checking the solubility in THF. The NCA is soluble in THF, if turbidity is seen in the solution, can be due to the presence of remaining hexane and should disappear by heating the solution, but if precipitation is seen is due to the presence of polymer or starting material (both not soluble in THF).

Yields: 70-80 %. mp: 93.4 °C. ¹H-NMR: δ_H (300 MHz, CDCl₃): 2.00-2.30 (2H, m, CH₂), 2.52-2.60 (2H, m, CH₂), 4.30-4.34 (1H, t, CH), 5.09 (2H, s, OCH₂), 6.40 (1H, s, NH), 7.30 (5H, m, Ph). ¹³C-NMR: δ_C (75 MHz, CDCl₃) 27.5, 30.6, 57.6, 67.8, 129.2, 129.4, 129.5, 135.9, 152.4, 170.2, 173.3.

2.4.3.2. General method for the preparation of Hydrochloride salts.
In a one-neck 100 mL round bottom flask, 10 mL (7.4 g, 101 mmol) n-butylamine were added to 20 mL of dichloromethane. The mixture was cooled in an ice bath and 8.43 mL (101 mmol) of concentrated hydrochloric acid was slowly added. The mixture was concentrated in vacuo until white crystals started to precipitate. The crystals were filtrated and recrystallized from dichloromethane.

Yield: 87 % of white needles. ¹H-NMR: δ_H (300 MHz, DMSO-d₆) 8.13 (3H, s), 2.72 (2H, t), 1.53 (2H, quint), 1.31 (1H, sext), 0.86 (1H, t).

2.4.3.3. General method for the preparation of BF₄ salts.
These salts were easily prepared by reaction of the corresponding amine with tetrafluoroboric acid diethyl ether complex, HBF₄·Et₂O, and posterior purification by recrystallization.

2.4.3.3.1. Synthesis of tetrafluoroborate n-butylammonium salt (3).
Butylamine (200 mg, 2.7 mmol) was dissolved in 1 mL diethyl ether, and 442 mg (2.7 mmol) of HBF₄·Et₂O, was added to the solution leading to the formation of a white solid salt in a quantitative yield. The
product was filtered off and recrystallized two times from ethyl acetate. The product was then dried under high vacuum and stored at -20 °C.

Yield: 50 % of a white solid. \(^\text{\textsuperscript{1}}\)H-NMR \(\delta_{\text{H}}\) (300 MHz, DMSO-\(d_6\)) 7.58 (3H, s), 2.84-2.71 (2H, m), 1.56-1.43 (2H, m), 1.39-1.25 (2H, m), 0.89 (3H, t) \(^\text{\textsuperscript{13}}\)C-NMR \(\delta_{\text{C}}\) (75 MHz, DMSO-\(d_6\)) 38.64, 29.09, 19.08, 13.49. EA: C: 29.61 % (calc.: 29.85 %), H: 7.27 % (calc.: 7.51 %), N: 8.60 % (calc.: 8.70 %).

2.4.3.3.2. Synthesis of tetrafluoroborate neopentylammonium salt (4).

To 5 mL (5.59 g, 36.74 mmol) of HBF\(_4\)∙Et\(_2\)O, 4.31 mL (3.20 g, 36.74 mmol) of neopentylamine were slowly added. The addition resulted in the precipitation of a white solid. The solvent was removed under vacuum and the solid was recrystallized twice from ethyl acetate and washed with cyclohexane. The product was dried under vacuum.

Yield: 42 % of a white solid. \(^\text{\textsuperscript{1}}\)H-NMR \(\delta_{\text{H}}\) (300 MHz, DMSO-\(d_6\)) 7.58 (3H, s), 2.63 (2H, s), 0.93 (9H, s) (Fig. 5S). \(^\text{\textsuperscript{13}}\)C-NMR \(\delta_{\text{C}}\) (75 MHz, DMSO-\(d_6\)) 49.94, 30.21, 26.78 EA: C: 34.35 % (calc.: 34.43 %), H: 7.99 % (calc.: 8.06 %), N: 8.07 % (calc.: 8.00 %).

2.4.3.3.3. Synthesis of tetrafluoroborate PEGammonium salts (5).

MeO-PEG(2000)-amine (600 mg, 0.3 mmol, 1892 g·mol\(^{-1}\)) was dissolve in 3 mL of THF, and 53.4 mg (0.3 mmol, 45 µL) of HBF\(_4\)∙Et\(_2\)O, was added to the solution leading to the formation of a faint yellow salt in a quantitative yield. The solvent was removed in a rotary evaporator. Solvent was evaporated and the solid residue was washed three times with hexane (washes were repeated until pH was not acidic). The product was then dried under high vacuum and stored at -20 °C.

Yield: Quantitative.\(^\text{\textsuperscript{1}}\)H-NMR \(\delta_{\text{H}}\) (300 MHz, DMSO-\(d_6\)) 7.69 (3H, s), 3.78-3.70 (2H, m), 3.52 (139H, d), 3.47-3.39 (6H, m), 3.24 (3H, s), 3.06-2.91 (2H, m)

2.4.3.4. General procedure for NCA polymerization. Synthesis of poly(\(\gamma\)-benzyl L-Glutamate) (PBLG) (2) under \(\text{N}_2\) conditions by using Schlenk techniques.

OBzl(Glu) NCA (0.5 g, 1.9 mmol, 264 g·mol\(^{-1}\)) was added to a Schlenk tube fitted with a stirrer bar, a stopper and purged with 3 cycles
of vacuum/Ar, under Ar flow and dissolved in 5 mL of solvent (freshly purified). Afterwards the initiator was added and the mixture was left stirring at 40 °C in an oil bath for 3 days under Ar/N₂ atmosphere with constant pressure. After 3 days reacting, the solution was poured into 40 mL of cold diethyl ether leading to a white suspension that was centrifuged at 4,000 rpm during 10 minutes. Supernatant was removed and the white solid was then suspended in ddH₂O and freeze-dried.

Yield: 70-90 %. ¹H-NMR δ_H (300 MHz, DMF-d7) 8.58 (1H, s), 7.42 (5H, s), 5.19 (2H, s), 4.21 (1H, s), 2.81 (2H, s), 2.45 (2H, s). ¹³C-NMR δ_C (75 MHz, DMF-d7) 175.94 (s), 172.26 (s), 162.77-162.18 (m), 161.98 (s), 136.76 (s), 128.87 - 127.75 (m), 66.05 (s), 57.13 (s), 35.41-34.17 (m), 32.48 (s), 30.84, 30.30-29.04 (m), 27.28 (s), 25.99 (s).

*Note: the corresponding signals of initiator depend on the initiator used: n-butyl ammonium; neopentyl ammonium or methoxy polyethylene glycol ammonium.

2.4.3.5. NCA polymerization kinetics.

OBzl(Glu) NCA (0.6 g, 2.27 mmol, 264 g·mol⁻¹) was dissolved in 6 mL of previously purified DMF. The content was split into 6 Schlenk tubes fitted with a stirrer bar, a stopper, and under N₂ atmosphere. Afterwards, a solution in DMF of initiator was prepared, and the corresponding amount was added to each tube. The mixtures were left stirring at 40 °C in an oil bath for 4 days under Ar/N₂ atmosphere with constant pressure. Time points of kinetics were collected each 14, 22, 38, 46, 70, and 96 hours, by precipitation of the polymers into diethyl ether tree times, freeze-dry of the sample in ddH₂O and GPC analysis of the MW eluting with DMF/LiBr.

2.4.3.6. Deprotection of PBLG.

2.4.3.6.1. Deprotection of PBLG under catalytic hydrogenation with Pd(OH)₂ Charcoal in DMF.

100 mg (0.0035 mmol, 28251 g·mol⁻¹) of PBLG was dissolved in 15 mL DMF absolute, in a round bottom flask fitted with a septum and a stirring bar. Then Pd(OH)₂ Charcoal was added and the flask was purged with N₂ in order to remove air and afterwards N₂ was removed by purging with H₂. The reaction was left stirring for two days under H₂ atmosphere (with a balloon full of H₂). Purification was carried out pouring the solution into a large excess of diethyl ether. Centrifugation leads to a mixture of a white solid with palladium/charcoal. The solid
was re-dissolved in acidic water and filtered through a celite column in a syringe and a filter. As a result, PGA (9) was obtained as a white powder.

Yield: 40 %. \( ^1 \text{H-NMR } \delta_\text{H} \,(300 \text{ MHz, D}_2\text{O}) \) 4.31-4.26 (1H, m), 2.38-2.14 (2H, m) 2.10-1.80 (2H, m) 2.10-1.80 (2H, m).

### 2.4.3.6.2. Deprotection of PBLG with HBr in trifluoroacetic acid. Optimal conditions.

In a round bottom flask fitted with a glass stopper and a stirrer bar, 100 mg of PBLG (0.0035 mmol) were dissolved in 3 mL TFA. Once dissolved, 2 eq. of HBr (48 % v/v, 1.49 g\( \cdot \)cm\(^{-3}\), 81 g\( \cdot \)mol\(^{-1}\)) per carboxylic group were added drop wise, and the yellow mixture was left stirring for 5 hours. *Note: For big scale deprotection of PBLG (> 600 mg), 16 hours were needed in order to achieve full deprotection. Then, the solution was poured into a large excess of cold diethyl ether leading to a white solid that was recovered after centrifugation (2600 rpm, 4 °C, 10 minutes). The product (9) was washed per triplicate with diethyl ether and dried over high vacuum. After that, the product was then purified by acid-base precipitation (NaHCO\(_3\) /HCl 6 M). Dialysis or ultrafiltration was done leading to the sodium salt form.

Yield: 75-86 %. \( ^1 \text{H-NMR } \delta_\text{H} \,(300 \text{ MHz, D}_2\text{O}) \) 4.31-4.26 (1H, m), 2.38-2.14 (2H, m) 2.10-1.80 (2H, m) 2.10-1.80 (2H, m).

### 2.4.3.6.3. Basic deprotection with NaOH aq./THF mixture. Optimal conditions.

HOMOPOLYMER (9): In a round bottom flask, PBLG (5.1 \( \mu \)mol, 9760 g\( \cdot \)mol\(^{-1}\), 50 GAU) was dissolved in THF (16 mL) at r.t. Then, the solution was cool down up to 4 °C and kept under stirring. In a vial, 1.5 eq. of NaOH per carboxylic group (7.7 \( \mu \)mol, 40 g\( \cdot \)mol\(^{-1}\)) were dissolved in 2 mL of ddH\(_2\)O and then added to the main solution drop wise. Turbidity was found after NaOH addition. The solutions were left under vigorous stirring for 16 h. Afterwards, THF was removed by evaporation. The residue was diluted with ddH\(_2\)O, concentrated and purified by ultrafiltration (Vivaspin®, MWCO 3000 Da). Upper part of the tube was freeze dried and the obtained white solid was analyzed by NMR (D\(_2\)O).

Yield: 40-60 %. \( ^1 \text{H-NMR } \delta_\text{H} \,(300 \text{ MHz, D}_2\text{O}) \) 4.31-4.26 (1H, m), 2.38-2.14 (2H, m) 2.10-1.80 (2H, m) 2.10-1.80 (2H, m).
DI-BLOCK (10): In a 50 mL round bottom flask, protected di-block (0.184 mmol, 12208 g·mol⁻¹, 50 GAU) was dissolved in 16 mL of THF at r.t. Then, the solution was cooled down up to 4 °C and kept under stirring. In a vial, 2 eq. of NaOH per carboxylic group of the polypeptide block (0.369 mmol, 40 g·mol⁻¹) were dissolved in 2 mL of ddH₂O and then added to the main solution drop wise. Turbidity was found after NaOH addition. The solutions were left under vigorous stirring for 16 h. Afterwards, THF was removed under evaporation and the residue was diluted with ddH₂O, concentrated and purified by ultrafiltration (Vivaspin®, MWCO 3000 Da). Upper part of the tube was freeze dried and the obtained white solid was analyzed by NMR (D₂O).

Yield: 40-70 %. ¹H-NMR δ_H (300 MHz, D₂O) 4.16 (1H, m), 3.45 (xH, m), 2.13 (2H, m), 1.76-1.89 (2H, m). i.e. DB₅₀ (δ) 1.76-1.89 (100H, m), 2.13 (100H, m), 3.45 (172H, m), 4.16 (50H, m).
2.5. REFERENCES.


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Chapter 3

Driving towards Well-Defined
Polypeptide-based Architectures
Star-shaped polyglutamic acid as an excellent candidate for biomedical applications
3.1. INTRODUCTION AND BACKGROUND.

As stated in Chapter 1, there is still an urgent need for the development of new and smart polymeric systems. Key desirable characteristics of these new upcoming systems are higher MW and degree of homogeneity, predictable conformations in solution, multivalency and increased drug loading capacity among others.

Recent efforts in the field are devoted towards novel biodegradable polymers as they allow utilization of higher MW platforms to optimize PK, essential for the treatment of diseases that require chronic administration, such as neurological disorders or tissue regeneration.\(^1\) Apart from biodegradability, the development of novel well-defined architectures with higher MW, predictable structure and conformation, higher homogeneity, greater drug loading capacity and increased multivalency is considered crucial. In this context, the use of branched polymers is emerging in order to accomplish the previously described requisites. They exhibit special properties when compared to the linear analogues as a result of their different architectures, solution conformations, sizes and shapes as well as greater multivalency, what could yield to different therapeutic output.\(^2\), \(^3\) Branched polymers include star,\(^4\) hyperbranched and dendritic-like polymers,\(^5\) dendrimers,\(^6\) graft, brush and comb-like polymers\(^7\) as well as polymer networks.\(^8\) (Figure 3.1).

\[\text{Figure 3.1. Examples of branched architectures.}\]
3.1.1. Star polymers definition.

Star polymers are branched polymers consisting on several linear chains attached to a central core. They can be sub-classified depending on the nature of the different branches: if the branches are identical linear chains they are named “Symmetric Stars”, if the branches have different MW or topology they are considered as “Asymmetric Stars”, and as “Miktoarm Stars” if the branches are chemically different. In all cases, these arms can be constituted by one-block or multi-block copolymers.

This special category of polymers has become popular in different research areas (chemistry, physics, biochemistry and engineering) due to the unique mechanical, rheological, as well as biomedical properties that are unreachable for linear polymers.\textsuperscript{9-11}

As general basis, star polymers are characterized by a compact structure, presumably with globular shape, and have large surface areas, increased concentrations of end groups with functionalities, when compared to polymers with similar MW. Moreover, they offer unique rheological properties which make them optimal platforms for drug delivery\textsuperscript{12} among other biological applications.\textsuperscript{13} Furthermore, multi-arm stars as well as hyperbranched polymers show enhanced solubility, lower melt viscosity, different thermal and physical properties in general, in comparison to their corresponding linear structures.\textsuperscript{14} Viscosity and other properties depend more on arm MW than on the total MW of the star polymer.\textsuperscript{15} If compared to dendrimers, star polymers offer the advantages of feature accelerated and tunable methods of synthesis. Apart from bio-applications, these unique materials are being considered of growing interest in other areas as thermoplastics,\textsuperscript{16} nanoelectronics\textsuperscript{17} and many other applications.\textsuperscript{10, 11}

As stated before, star polymers are defined by a smaller size and therefore higher segment density as judged against to linear polymers with the same MW. One of the most appealing properties, apart from their rheological characteristics and thermoplastic character, is their self-assembly behavior. Nonetheless, it must be said that reports up to date suggest that macromolecular architecture is a key parameter for the tuning of micellar behavior and properties, and thus, it must be well-considered for the design of new materials and their potential biological applications, in particular as drug delivery systems.
3.1.2. Synthetic approaches.

The first attempt to synthesize star polymers was in 1948 by Schaefgen and Flory\textsuperscript{18} who, by using the core first approach with multifunctional initiators such as cyclohexanenetetrapropionic or dicyclohexaneneoctacarboxylic acid, polymerized $\varepsilon$-caprolactam to give rise to tetra- and octa-arm star-like polyamides. Later on, Morton \textit{et al.}\textsuperscript{19} used the so-called arm first approach for the synthesis of 4-arm star polystyrene by neutralizing polystyrillithium living chains with tetrachlorosilane. From that period plentiful efforts have been done to build novel star-shaped architectures as well as to understand from a theoretical and experimental point of view,\textsuperscript{20} their unique properties arising from their intrinsic nature. Advances in modern polymer chemistry, especially with the introduction of controlled/living radical polymerizations in the 1990s, made possible the exponential growth of these complex materials. The most important polymerization techniques used comprise anionic, cationic, controlled radical, ring opening, ring-opening metathesis, group transfer, step-growth polycondensation, metal template-assisted, and electrostatic self-assembly and covalent fixation methods. The advances in “click chemistry” represented an important contribution especially when the strategy followed is the arm first approach. Nevertheless, the synthetic methods for the development of star-related architectures are out of the scope of this thesis and detailed information could be obtained from literature.\textsuperscript{10,11}

For the synthesis of star polymers, two major strategies have been widely applied: the core-first approach (divergent approach) and the arm-first approach (convergent approach).\textsuperscript{10}

The core-first approach (or divergent approach) is anchored in the use of a multifunctional initiator as core that initiates the polymerization of several arms on a simultaneous way (Figure 3.2a). In order to achieve control in polymerization for the synthesis of homogenous constructs, it is crucial to have equally reactive initiating sites. Moreover, initiation step must be always faster than propagation. Historically, the major disadvantage of this methodology is the difficulty in the characterization of the polymers obtained as arm MW cannot be directly measured. Nevertheless, advances in characterization techniques are progressively solving the problem and this strategy is the most widely used in the synthesis of star polypeptides.\textsuperscript{21}

On the other hand, the arm-first approach (or convergent approach), consists on the reaction of living macromolecular chains
previously synthesized, with a multifunctional reagent that serves as core (Figure 3.2b). Two main strategies can be followed in this case; either by using a multifunctional molecule that will neutralize linear living chains (use of “multifunctional linking agents”) or can be based on the covalent attachment of telechelic linear polymers to a multifunctional central core (“coupling onto”). The main advantage of the “arm first” approach is that characterization should be easier since the living arms can be characterized in a previous step before linking. Nevertheless, the main disadvantage of this methodology is the steric hindrance component which could limit the number of arms linked. Moreover a large excess of living chains is always a requisite. For that reason, and in order to get star polymers with high purity, purification-fractionation steps are unavoidable.

**Figure 3.2.** Synthetic approaches for the preparation of peptide-based star polymers via (a) core-first or divergent approach; b) arm first or convergent approach; c) core-cross linked stars.
Besides these two widely used approaches, a latest classification takes into account a new synthetic strategy (Figure 3.2c). This approach consists on the reaction of living macroinitiators (MI) (also named macromonomers) with multifunctional molecules acting as cross-linkers giving rise to star-shaped architectures known as core cross-linked star (CCS) polymers.\(^{22}\)

### 3.1.3. Drug delivery applications.

There are plenty of examples of drug encapsulations within these unique architectures due to their inherent nature.\(^{23}\) However, only few examples can be found in literature on their use as polymer-drug conjugates and they are still far to enter any clinical stage. Most of them are based on the use of the well-known HPMA copolymers.\(^{24}\) For instance, in 2000 Wang et al.\(^{25}\) reported the synthesis of star-like HPMA by conjugating semitelechelic HPMA macromolecules to PAMAM dendrimers from generations G2 to G4 as core. DOX was then conjugated to HPMA chains. Jelínková et al.\(^{26}\) compared two different antibody-targeted HPMA copolymers of -GlyPheLeuGly-DOX (star-like vs. linear HPMA). The star-like conjugate consisted on 30 to 40 copolymer chains of HPMA bearing DOX linked to the central antibody molecule via an amide bond between the end of each backbone chain and the lysine ε-amino groups of the antibody. Whereas binding affinity was independent from the polymer architecture, the star-like conjugate exhibited 10-fold higher cytotoxic effect \textit{in vitro} in different cancer cell lines and 6.5-fold higher concentration in blood in biodistribution studies in mice, as compared to their linear counterparts. Both types of anti-Thy-1.2 targeted conjugates cured all mice bearing T-cell lymphoma EL4, however, the star-like conjugates containing anti-CD71/A or B antibodies performed better than classic linear ones in colorectal cancer SW 620.

Then, Etrych et al.\(^{27}\) described the synthesis of a family of new biodegradable star polymer-DOX conjugates based also on a macromolecular core formed by PAMAM dendrimers from which semitelechelic HPMA copolymer DOX conjugates (hydrazone linked) were grafted. They were able to synthesize different MW constructs from 200 to 1000 g.mol\(^{-1}\) with relatively low \(D\) (~1.7). The star conjugates exhibited higher \textit{in vivo} anti-tumor activities when compared to the free DOX or linear polymer conjugates in a EL4T-cell lymphoma mouse model.\(^{28}\) Previously, the same group has developed
star-shaped immunoglobulin-containing HPMA-based conjugates\textsuperscript{29} with hydrazone-DOX, that showed comparable cytostatic activity as for free DOX.HCl in several cancer cell lines, and significantly higher antitumor activity \textit{in vivo} in mice bearing EL4 T-cell lymphoma than immunoglobulin free conjugates.

Another example of hybrid dendritic-star like polymers is the work of Cao \textit{et al.}\textsuperscript{30} who reported the synthesis of a dendrimer-like star polymer based on well-defined poly(L-lactic acid) (PLLA) star polymer with six carboxylic acid-terminated polyester dendrons of 2,2-bis(hydroxymethyl)propionic acid. Amine-functionalized folic acid moieties were effectively conjugated and uptake of those constructs was much higher than for non-targeted ones against mouth epidermal carcinoma (KB) cells (overexpressing folate-receptor).

Kowalczuk \textit{et al.}\textsuperscript{31} described the synthesis of star-shaped cisplatin nanoconjugates (12-14 nm radii) based on a highly branched poly(styrene) core and poly(tert-butyl acrylate) arms. They were able to achieve a high cisplatin loading (45 wt %) and their \textit{in vitro} evaluation showed a sustained drug release, an endocytic mechanism of uptake, and a lower cytotoxic effect when compared to the free drug.

Very recently, Li \textit{et al.}\textsuperscript{32} published nanoparticle systems (~15 nm radii) based on star polymers as theranostic vectors bearing aldehyde groups for the covalent conjugation of DOX and activated esters for the 1-(5-amino-3-aza-2-oxypentyl)-4,7,10-tris(tert-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (DO3A-tBu-NH\textsubscript{2})—a Gadolinium (Gd\textsuperscript{3+}) chelating agent. Among other results, they found that the DOX/Gd-conjugated nanoparticles yielded a similar IC\textsubscript{50} to free DOX for breast cancer cell lines, confirming DOX integrity after nanoparticle conjugation. Moreover, relaxivity of Gd loaded in star-shaped polymers was found to be 3 times higher than conventional organic non-polymeric Gd/DO3A complexes.

Navath \textit{et al.} reported the synthesis and biological evaluation of N-Acetyl cystein (NAC) conjugated to 6, and 8 PEG star-shaped polymers via disulfide bonds for applications in neuroinflammation.\textsuperscript{33} Conjugates diameter sizes were between 21-28 nm and 34-43 nm for 6 arm an 8 arm, respectively. The two synthesized constructs showed a release of NAC of 74 \% in 2 hours when exposed to glutathione (GSH) at intracellular concentrations (2-10 mM) whereas no release was observed with extracellular concentrations of GSH (2 µM). The conjugates showed 2-fold increased antioxidant activity compared to
free drug when they were tested by monitoring cytokine release in lipopolysaccharide (LPS) induced inflammatory response in microglial cells looking at the ROS (reactive oxygen species), NO (free radical nitrile), anti-inflammatory activity and GSH depletion.

There are also some examples of star-polymer-drug conjugates for antifungal applications. Sedlák et al., in 2008 reported the synthesis of β-glucosidase-sensitive star-PEG-Amphotericin B (AmB) conjugates. Amphotericin B is a powerful antifungal drug. By the use of the linker β-D-glucopyranoside (molecular switch sensitive to β-glucosidases), the release of AmB is ensured to occur only in parasitical fungal pathogens that have specific hydrolase β-glucosidases, and not in healthy human tissues where these enzymes are not present. Their preliminary studies showed an efficient targeted delivery at the areas of activity of pathogens. The same group has used the star-shaped PEG platform described before as drug delivery carrier for the antifungal agent nystatin, with similar results.


Various polypeptide-based star polymers have been synthesized over the years. For example, Klok et al. used perylene derivatives with four primary amine groups as initiators to lead 4-arm PBLG and PZLL and Inoue et al. used hexafunctional initiators for the synthesis of 6-arm PBLG star polymers both taking profit of the NCA polymerization techniques. Other examples are provided from the work of Aliferis et al. who used 2-(aminomethyl)-2-methyl-1,3-propanediamine as a trifunctional initiator for the synthesis of P(BLL-b-BLG)3 3-arm star-block co-polypeptides; or the studies of Karatzas et al. in the synthesis of 4-arm (PEO-b-PBLG)4 hybrid star block copolymers using four-arm PEO stars end-functionalized with primary amines as initiators to polymerize OBzl(Glu) NCA among others.

More recent examples include the use of dendritic cores as initiators such as of poly(ethylene imine) (PEI), poly(propylene imine) (PPI), or poly(amidoamine) (PAMAM). The examples listed are all based on the use of amine initiation in NCA polymerization techniques with the inherent limitations of the mentioned methodology, either leading to low MW, or star polymers with very broad D. The use of HVT from Hadjichristidis group can be seen as a suitable solution, however, requirement of complex
equipment is always a disadvantage in order to develop a versatile synthetic procedure.

Despite the fact that there is a growing interest in the development of hybrid and peptide-based star polymers as prospective advanced materials for biological applications in the field of nanomedicine, most of the studies undertaken to date have been focused on the synthesis of new and varied star polymers and their study in terms of self-assembly under a variety of conditions. Only recently, peptide-based star polymers have been considered and explored as drug delivery systems. Sulistio et al. synthesized peptide-based CCS polymers composed entirely of amino acid building blocks. These constructs were composed of PLL arms emerging from a poly(L-cysteine) (PLC) core and could be core-functionalized via reaction with primary amines bearing different functional groups (e.g., pyrene, alkyne). These types of stars were able to entrap hydrophobic drugs, such as the anti-cancer drug pirarubicin, through physical interactions with pyrene moieties of the core. Moreover, due to the presence of disulfide bonds at the core, the stars could also be cleaved by reducing agents such as dithiothreitol, yielding redox-sensitive polymers. Apart from that, the same group developed CCS polymers with PLL arms, PLC cores and peripheral allyl functionalities by using an allylamine initiator, allowing the conjugation of targeting moieties such as folic acid moieties for cancer cell targeting. Their in vitro studies against breast cancer cells showed absence of toxicity and an enhanced cell uptake for those CCS bearing folic acid residues. Xing et al. prepared CCS polymers using MeO-PEG1900-NH$_2$ as a macroinitiator for ROP cysteine and OBzl(Glu) NCA derivatives which resulted in the formation of nanogels where the polymers had 9700 PEG arms and a $M_w$ of $4.2 \times 10^7$ Da. Those nanogels were tested in vitro in terms of cytotoxicity, being non-toxic and biocompatible. The hydrophobic drug indomethacin, was encapsulated and efficiently released using glutathione in physiological media. Byrne et al. have reported the synthesis of multi-arm star polypeptides by NCA polymerization from dendritic initiators based on PPI. Such systems were loaded with rhodamine B and showed and enzyme controlled payload release when incubated with thermolysin. The same group have recently published the glycosylation of those star shaped polymers and found selective binding to lectin ConA as a function of degree of branching and glycosylation.
The examples presented are based on the encapsulation of hydrophobic drugs and not on a chemical conjugation, since there are not yet examples on literature (as far as our knowledge). Nonetheless, they represent good examples to point out the possible use of star-shaped polypeptides in drug delivery applications.

Summing up, branched polymers are outstanding aspirants as carriers due to their unique rheological, mechanical and biomedical properties derived from their structure, inaccessible for linear polymers. There is a wide range of opportunities in the field of PT for these relatively new and interesting architectures. Preliminary results suggest that they are non-toxic entities, validating them as possible carriers for drug delivery among other applications. However, there is still a long way to go in this respect and the true potential of these constructs has not yet been realized. In addition, in vivo studies must be accomplished in order to confirm the absence of an immunogenic response and an adequate fate. Furthermore, many other possible architectures, with the use of natural and unnatural amino acids, are yet to be unexplored, which may increase the versatility and applicability of these nanostructures.

For that reason, one step further was taken in this work and the BF$_4$ methodology for NCA polymerization was used to synthesize well-defined branched polymers, in a divergent method from novel multifunctional initiators. In addition, their exhaustive physicochemical characterization and preliminary in vitro evaluation is also presented as it is considered compulsory data in the process towards their validation as nanocarriers.

### 3.2. RESULTS AND DISCUSSION.

The previously described methodology based on the use of BF$_4$ salts as initiators for NCA polymerization$^{47}$ have been extended to synthesize well-defined 3-arm star polyglutamates (Scheme 3.1).
Scheme 3.1. Schematic representation of the NCA polymerization using star-shaped initiators.

Control in the initiation step that this newly described methodology offers is key to the synthesis of branched polymers with predictable MW and low Đ. The use of a variety of multifunctional amine initiators in their BF\(_4\) salt form to ring-open polymerize OBzI(Glu) NCA (1), results in a fast and homogeneous initiation and efficient propagation to achieve well-defined architectures.

To this aim we synthesized four different multifunctional 3-arm initiators, including one that contained disulfide bonds within the arms. In the presence of a reducing agent (Glutathione GSH, or 1,4-dithiothreitol DTT) the star could then be disassembled into the individual arms. This novel initiator for polypeptide synthesis enables characterization of the individual arms to ensure similar chain length resulting from a living polymerization and efficient initiation.

3.2.1. Initiator synthesis.

In all cases, the synthesis of initiators was carried out starting from the multifunctional core 1,3,5-benzenetricarbonyl trichloride, to which, the mono-Boc protected diamines, N-Boc-ethylendiamine N-Boc-1,6-hexanediame, 1-(t-Butyloxycarbonyl-amino)-3,6-dioxa-8-octaneamine (N-Boc-DOOA), and N-Boc-cysteamine were efficiently coupled in a simple and clean reaction. Briefly, reactions took place under inert conditions (N\(_2\) flow) where 1 eq of 1,3,5-benzenetricarbonyl trichloride in dried THF was reacted with 3.3 eq. of the corresponding Boc protected amine in the presence of N,N’N’’-disopropylethylendiamine for 2 hours. Then, the dried product was obtained pure after several purification steps including washing and recrystallization (See Materials and Methods, M&M). In general, the mono-Boc protected 3-arm initiator precursors were carefully purified.
to avoid di- or mono-functional cores that would lead to unwanted mixtures of 1- and 2-arm star polymers.

In the case of cysteamine based initiator, a previous step for the synthesis of mono-Boc cysteamine (11) from cysteamine dihydrochloride was carried out using a general method taken from Girgenti et al. with slight modifications (Scheme 3.2). Briefly, a suspension of 3 eq. cysteamine dihydrochloride in chloroform was treated with 6 eq. of triethylendiamine and stirred for 20 minutes at r.t. A solution of 1 eq. di-tert-butyl dicarbonate in anh. chloroform was added drop wise and left reacting for 16 hours more. After that, the product was purified according to M&M and characterized by $^1$H-NMR (Figure 3.3).

![Scheme 3.2. Synthesis of mono-Boc cysteamine (11) from cysteamine dihydrochloride.](image)

**Scheme 3.2.** Synthesis of mono-Boc cysteamine (11) from cysteamine dihydrochloride.
Once purified, the identity of the mono-Boc protected initiators (12) was assessed by 1D NMR ($^1$H-NMR, $^{13}$C-NMR), with the aid of DEPT-135 and 2D NMR spectra (gHSQCED and COSY) for assignments. An example of NMR spectra and assignments is given in Figure 3.4 for the initiator 12a. The rest can be found in SI (Appendix III).
Figure 3.4. NMR spectra of 1,3,5-Tri-tert-butyl ((benzenetricarbonyltris(azanediyl)) tris(ethane-2,1-diyl))tricarbamate (12a). a) $^1$H-NMR in DMSO-$d_6$; b) $^{13}$C-NMR in CDCl$_3$.

Then, the BF$_4$ ammonium salt initiators (13) were obtained simply by removal of Boc groups using tetrafluoroboric acid. Once more, identity of the compounds was investigated by $^1$H-NMR, $^{13}$C-NMR, and $^{19}$F-NMR in this case. An example of NMR set is given for the initiator 13a. Other NMR spectra can be found in SI.
3.2.2. Synthesis of 3-arm star shaped polyglutamates through NCA polymerization techniques.

Using the newly synthesized initiators, several polymerizations of OBzl(Glu) NCA (1) were carried out in order to prove the versatility of the BF$_4$ salts based methodology in the synthesis of star-shaped architectures. In all cases, reactions yield 3-arm star shaped polyglutamates (St-PBLG) (14) with different MW ranging from 15.0-
60.0 kDa (Table 3.1, Figure 3.6) and $\bar{D}$ in the range of 1-1.2 independently of the initiator used. This fact suggests that a large spacer between the initiator core and the initiation site is not necessary, despite concerns over steric hindrance.

**Table 3.1.** Variety of initiators used in the polymerization processes and different DPs obtained, demonstrating the versatility of the technique.

<table>
<thead>
<tr>
<th>Star</th>
<th>I</th>
<th>$\text{DP}_{\text{theo}}$</th>
<th>$\text{Mn}^a$ (kDa)</th>
<th>$\text{Mn}^b$ (kDa)</th>
<th>$\text{DP}^a$</th>
<th>$\text{DP}^b$</th>
<th>$\bar{D}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>14a_1</td>
<td>100</td>
<td>21.3</td>
<td>21.0</td>
<td>97</td>
<td>96</td>
<td>1.26</td>
<td></td>
</tr>
<tr>
<td>14a_2</td>
<td>Ethyl based</td>
<td>150</td>
<td>24.1</td>
<td>27.6</td>
<td>110</td>
<td>126</td>
<td>1.22</td>
</tr>
<tr>
<td>14a_3</td>
<td>250</td>
<td>50.3</td>
<td>51.5</td>
<td>229</td>
<td>235</td>
<td>1.09</td>
<td></td>
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<tr>
<td>14a_4</td>
<td>300</td>
<td>59.2</td>
<td>60.9</td>
<td>270</td>
<td>278</td>
<td>1.19</td>
<td></td>
</tr>
<tr>
<td>14b_1</td>
<td>Hexyl based</td>
<td>75</td>
<td>16.4</td>
<td>-</td>
<td>75</td>
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<td>1.25</td>
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<td>23.9</td>
<td>23.7</td>
<td>109</td>
<td>108</td>
<td>1.23</td>
<td></td>
</tr>
<tr>
<td>14b_3</td>
<td>250</td>
<td>51.5</td>
<td>52.6</td>
<td>235</td>
<td>240</td>
<td>1.17</td>
<td></td>
</tr>
<tr>
<td>14c_1</td>
<td>DOOA based</td>
<td>75</td>
<td>15.8</td>
<td>16.9</td>
<td>72</td>
<td>77</td>
<td>1.13</td>
</tr>
<tr>
<td>14c_2</td>
<td>100</td>
<td>22.2</td>
<td>24.1</td>
<td>101</td>
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<td>1.23</td>
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</tr>
<tr>
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<td>31.1</td>
<td>152</td>
<td>142</td>
<td>1.10</td>
<td></td>
</tr>
<tr>
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<td>41.6</td>
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<td></td>
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<tr>
<td>14d_1</td>
<td>Cyst based</td>
<td>200</td>
<td>43.1</td>
<td>-</td>
<td>196</td>
<td>-</td>
<td>1.22</td>
</tr>
</tbody>
</table>

a) Data obtained by SEC in DMF. b) Data obtained by $^1$H-NMR. $I=$ initiator DP$=$ degree of polymerization, $\bar{D}=$ polydispersity.

**Figure 3.6.** a) Selected GPCs from St-PBLG (14a) in DMF (1 % LiBr) at 8 mg·mL$^{-1}$. b) CD of St-PBLG (14) at 20 °C in HFIP at 0.1 mg·mL$^{-1}$. 
Polymers were characterized using a number of physicochemical analytical techniques, yielding the desired MW by GPC and $^1$H-NMR. Data obtained from GPC and $^1$H-NMR was, in general, consistent with the feed ratios for polymer synthesis thus, obtaining the desired MW even up to 60 kDa. This is a highly significant fact and a major benefit offered by the technology we are describing herein, since most star polypeptides described in literature have not achieved high MW with narrow Đ. $^{36-38, 43-45, 49}$ All data obtained so far indicates an excellent control over polymerization using these initiators, however, to fully demonstrate this fact, we endeavored to prove the homogeneity achieved on the arms MW. To do that, we employed the above-mentioned initiator bearing disulfide bonds. The polymer synthesized was efficiently reduced by means of DTT, releasing the individual arms and allowing monitoring by SEC. Remarkably, we were able to almost perfectly disassemble the star polymers to yield linear chains of 1/3 MW of the original star and with low Đ (<1.25) (Figure 3.7).

![Figure 3.7. GPC traces in DMF/LiBr of cysteamine initiated St-PGA (14d_1) before and after treatment with DTT 1 M during 72 hours.](image)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mn (KDa)</th>
<th>Mw (KDa)</th>
<th>Rh</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>14d_1 (Star-SS initiated)</td>
<td>43.1</td>
<td>52.6</td>
<td>5.07</td>
<td>1.22</td>
</tr>
<tr>
<td>14d_1 with DTT</td>
<td>16.4</td>
<td>19.0</td>
<td>2.81</td>
<td>1.16</td>
</tr>
</tbody>
</table>

This evidence, in addition to the expected MW of stars from GPC and $^1$H-NMR fully demonstrate that star polymers synthesized have very similar length arms resulting from fast and efficient initiation and controlled propagation. Knowing that this synthesis of St-PBLG
leads to homogenous material, makes them much more favorable candidates to be used as drug delivery carriers or imaging probes.

**3.2.3. Deprotection of St-PBLG.**

In order to have a full understanding on the quality of the star polymers synthesized, the analysis of the deprotected polymers had to be also performed. Benzyl protecting groups were removed using conventional methods already reported for linear polymers. Nevertheless, it was important to choose the correct deprotection approach depending on the initiator used. For example, to maintain the integrity of the DOOA core, a procedure using NaOH with previously optimized conditions was used. Standard HBr/TFA acidic conditions were used in all other cases. Complete removal of benzyl protecting groups was certified by $^1$H-NMR (7.4 ppm and 5.3 ppm) and the remaining aromatic signal observed, therefore, were assigned to the benzylic core of the star at 8.2 ppm (3 protons). Integration of this signal against those of glutamic acid provided the average number of units per chain and consequently, MW. After deprotection, the expected random coil conformation of polyglutamate chains was observed in all samples when analyzed using Circular Dichroism (CD) (Figure 3.8). Finally, DLS (Dynamic Light Scattering) at 0.2 mg·mL$^{-1}$ showed particle sizes between 4-8 nm in all measurements (Figure 3.9).

![Figure 3.8.](image-url)

**Figure 3.8.** a) $^1$H-NMR of St-PGA (15c) of different MW. The red square is surrounding benzyl core signals. b) CD of St-PGAs (15) in PB at 37 °C showing typical random coil conformation of PGAs.
3.2.4. *In vitro* biological characterization of the synthesized polyglutamate based architectures.

Key features for the validation of the newly synthesized polymers as potential carriers for drug delivery or imaging probes are their biodegradability, their toxicity in cell cultures and the way cells are taken them up. For that purpose, the present chapter is also devoted to the biological characterization of these architectures, where preliminary *in vitro* evaluation in selected cell models is described.

### 3.2.4.1. *In vitro* Biodegradability.

As mentioned before, PGA is a biodegradable polymer carrier. The degradation of poly(L-glutamic acid) by lysosomal enzymes has been extensively studied *in vitro* using purified enzymes and cell lysates.\(^5^0\), \(^5^1\) These studies showed that poly(L-glutamic acid) is more susceptible to lysosomal degradation than poly(aspartic acid) and poly(D-glutamic acid),\(^5^2\) and that cysteine proteases, particularly cathepsin B, play key roles in the lysosomal degradation of poly(L-glutamic acid).\(^5^0\)

To ensure that enzyme-dependent biodegradability of St-PGA had not been compromised by the architecture, all synthesized polymers were incubated in presence of lysosomal enzyme cathepsin B (at pH 6 in acetate buffer and EDTA, under reductive conditions by using DTT in order to favor the enzyme activity). The degradation rate was monitored by GPC under aqueous conditions. As expected, polymers were degraded in presence of cathepsin B but following different kinetics upon the polymerization initiator used (Figure 3.10).
Figure 3.10. Example of degradation profiles by cathepsin B of two St-PGA initiated by a) Ethyl based initiator (15a) and b) Hexyl based initiator (15b). GPC traces in PBS at different time points.

3.2.4.2. Cell viability assays.

Another key feature for the validation of the newly synthesized polymers as potential carriers for drug delivery or imaging probes is their toxicity in cell cultures.

Cell-based assays are often used in order to screen collections of compounds with the purpose of determining possible cytotoxic as well as cell proliferation effects. This kind of assays are also commonly used for receptor binding measuring, trafficking of cellular components, or monitoring organelle function. It is important to know how many viable cells are remaining at the end of the experiment independently on the type of assay used. There are currently a wide variety of methods that can be used to estimate the number of viable eukaryotic cells. Most of them are based on multi-well formats where data are recorded using a plate reader, due to their facile preparation, less cost-effective and the needless of sophisticated equipment, in contrast to methods by flow cytometry and high content imaging. These methods include tetrazolium reduction, resazurin reduction, protease markers, and ATP (Adenosine Triphosphate) detection. The first three methods measure some aspect of general metabolism or an enzymatic activity as a marker of cell viability. All of them are based on the incubation of a specific reagent with a population of viable cells to convert a substrate to a colored or fluorescent product, generating a signal that is proportional to the number of viable cells present. This signal can be detected with a plate reader. The ATP assay is also based on the measurement of a fluorescent signal produced, although in this case no incubation period is needed since the addition of assay reagent immediately ruptures the cells.
Within this project, tetrazolium reduction assays have been applied in order to validate the different polymeric carriers synthesized. The reduction of tetrazolium salts is currently a reliable process and can be directly correlated to the number of viable cells since this process only occurs when mitochondrial reductase enzymes are active. In general, the most commonly tetrazolium compounds used include: MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide), and WSTs (water soluble tetrazolium salts) family that can be classified in two categories due to their mechanism of action. On one hand, MTT which is positively charged and readily penetrates viable eukaryotic cells and; on the other hand MTS, XTT, and WST-1 which are negatively charged and do not readily penetrate cells. For that reason, the latest are usually applied in combination with an intermediate electron acceptor that can penetrate viable cells where is reduced in the cytoplasm or at the cell surface and exit the cells where they can convert the tetrazolium to the soluble formazan product\(^5\) (Figure 3.11). A common electron acceptor used is PMS (phenazine methyl sulfate). One of the advantages of the tetrazolium assays that produce an aqueous soluble formazan is that absorbance can be recorded form the assay plates periodically during early stages of incubation. In contrast to MTT reagent, the more recent developed MTS, XTT and WST series can be reduced by viable cells to generate formazan products that are directly soluble in cell culture medium.\(^5\) Therefore, a liquid handling step during the assay procedure as required for MTT is not further needed making the protocols more convenient and reliable. In contrast to XTT, MTS exhibits a greater concentration range with linearity in the absorbance (comparable to MTT) what makes this reagent optimal for cell viability determination.
Figure 3.11. Intermediate electron acceptor PMS transfers electron from NADH in cytoplasm to reduce MTS in culture medium into an aqueous soluble formazan.

Within this project, combination of MTS/PMS was used to assess cell viability after treatments. In brief, after cell incubations, 10 µL of manufacturer solution of MTS/PMS (20:1) was added to each well, and the cells were incubated for a further 2 hours. Mitochondrial dehydrogenase enzymes of viable cells indirectly converted MTS tetrazolium into a colored formazan product. Optical density of each well was measured at 490 nm.

Two different cell lines were used in order to determine the cytotoxicity of selected polymers from the family synthesized: SHSY5Y human derived neuroblastoma cell line and HUVEC human umbilical vein endothelial cells. Those two cell lines were rationally selected, as one of the main aims of this dissertation was the evaluation of the ability of the synthesized compounds to cross biological barriers, and more concretely, the BBB where endothelial cells plays a major role. HUVEC cell line was an established cell line in the laboratory, and all the parameters such as cell density were optimized for compound testing. However, this fact was not true for SHSY5Y cell line, and therefore, it had to be set by cell growing curves starting from different seeding densities up to 1 week. These curves where obtained by MTS assays to cells incubated in 96-well plates over different periods of time and maintained at 37 °C in an atmosphere of 5 %
carbon dioxide and 95 % air. Experiments were done with 6 replicates and per triplicate.

Figure 3.12. SHSY5Y cell line growing curves obtained by MTS assays. n> 3, mean ± SEM.

According to the results obtained from the growing curves, 12000 cell/well (in 96-well microtitre plates, 35000 cell·cm$^{-2}$) for SHSY5Y, was established as a proper seeding density in order to maintain the cells under exponential growth during the treatments (72 hours). Hence, for cell viability experiments, 72 hours MTS assays against SHSY5Y and HUVEC where then performed. Firstly, different architectures were compared: linear-PGA, PEG-PGA hybrid di-block copolymer, and 3-arm St-PGA (ethyl based initiated). As it can be observed from Figure 3.13a, no toxicity was observed against SHSY5Y cell line up to 3 mg·mL$^{-1}$ (higher concentrations were not tested).

Afterwards, several 3 arm St-PGAs with different MW and core due to the use of different initiators were also evaluated, this time against HUVEC cell line. As it can be seeing from Figure 3.13b, all polymeric structures tested where non-toxic against HUVEC cell line up to 3 mg·mL$^{-1}$ (higher concentrations were not tested).
Figure 3.13. Cell viability assay of a) 3 different PGA architectures against SHSY5Y cell line, 72 hours of treatments. b) Different 3-arm St-PGAs against HUVEC cell line, 72 hours of treatments. n > 3, mean ± SEM.

3.2.4.3. Cellular uptake.

Understanding of cellular internalization mechanisms used by nanopharmaceuticals has become a key player in the field of drug delivery. Nanomedicines mainly use endocytic vesicles or endosomes, which in turn employ a complex mechanism to address the different molecules to specific intracellular locations. It can be said that charge, shape, material composition, and surface functional groups are basic physico-chemical parameters that determine cell entry of nanomedicines by endocytic pathway.\textsuperscript{55}

Confocal microscopy techniques and flow cytometry are routinely used with fluorescence-labeled polymers in order to evaluate their uptake by cells. Live-cell confocal imaging, allows visualizing trafficking between multiple compartments within individual living
cells over time, avoiding any possible artifacts derived from fixation protocols. On the other hand, flow cytometry give us semi-quantitative information about the mechanism of internalization.

3.2.4.3.1. Fluorescence labeling of PGA-based architectures with Oregon Green Cadaverine.

Thus, after evaluation of degradation kinetics and biocompatibility of the systems, different PGA architectures were fluorescently labeled for in vitro evaluation.

For macromolecular therapeutics and nano-sized drug delivery systems, fluorescent labeling is commonly applied to allow intracellular trafficking studies, conjugate cell-specific localization and/or in vivo fate and PK. Probes such as the fluorophore Oregon Green (OG) have been extensively reviewed for cellular studies to determine cell uptake and binding. To this aim, the conjugated probe must fulfill some requirements such as high stability of the probe itself as well as stability of the linkage to ensure adequate carrier monitoring. On the other hand, a minimal percentage of probe loading is desirable in order to avoid data misinterpretation due to changes in polymer conformation resulting from changes in charge and solubility. In order to fulfill all that criteria, less than 1 mol% of OG was conjugated through a non-biodegradable amine bond. In the case of linear PGA\textsubscript{COOH} and St-PGA\textsubscript{COOH}, conjugation was carried out by using diisopropylcarbodiimide (DIC) / Hydroxybenzotriazole (HOBt) as carboxylic acid activators in organic solvents. On the other hand, for OG labeling of PEG-PGA\textsubscript{COONa}, hybrid di-block copolymer, DMTMM·Cl was used. The protocol of OG conjugation was previously established and routinely used with DIC/HOBt in Dr Vicent laboratory, ensuring 80-90% conjugation efficiency of the fluorescence dye. A schematic representation of polymers labeling is depicted in Scheme 3.3.
Scheme 3.3. Synthesis of OG 488 labeled products (21).

The formation of amide bonds by condensation of carboxylic acids with amines in presence of carbodiimides is one of the most common synthetic procedures.\(^{58}\) DIC is a carbodiimide used in peptide synthesis. As a liquid, it is easier to handle than the commonly used N,N′-dicyclohexylcarbodiimide DCC and EDC (N′,N′-dicyclohexyl carbodiimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride respectively) especially since its urea is soluble in most organic solvents, facilitating work up processes. To optimize the couplings, additives such as HOBt are widely used for the generation of active esters able of efficient acylation of amino groups, especially in the case of amino acids and peptides.\(^{59}\) Furthermore, HOBt is well known to act as racemization suppression agent.
The use of different carboxylic acid activators responds to practical and technical reasons, since the hybrid di-block copolymer is obtained directly as sodium salt form after deprotection (see Scheme 3.4), in contrast to linear and St-PGAs. The acid form conversion of PEG-PGA was avoided in order to not compromise PEG block integrity.\textsuperscript{47} In any case, OG labeling was successfully accomplished independently of the selected PGA derivative and methodology. The labeled polymers where always converted into sodium salt form, and purified by Sephadex G25 using commercial PD10 columns. The fractions containing OG labeled polymer where selected after measuring their fluorescence at 485 nm in the plate reader Victor Wallac\textsuperscript{TM}, collected and re-purified by dialysis using Vivaspin\textsuperscript{®} MWCO 3000 Da, in order to avoid any trace of free OG which would lead to interference and different mechanisms of uptake. A typical curve obtained after fluorescence measuring of the fractions from PD10 columns is shown in Figure 3.14. After purification, dye loading was calculated by (i) the fluorescence associated with the polymer containing fractions related to the total fluorescence output coming from all the fractions after PD10 column (Figure 3.14) and by (ii) recording a calibration curve of the labeled polymeric architecture as well as the free OG.
Figure 3.14. Example of a typical curve obtained after fluorescence measuring of 1 mL fractions collected from PD10 columns.

In order to properly quantify the OG content of every conjugate, a calibration curve was recorded. It has to be taken into account that quenching phenomena are obtained at high OG concentrations (Figure 3.15). Hence, it is necessary to be in the proper fluorescence range for an accurate estimation of OG content.

Figure 3.15. OG fluorescence calibration curve in PBS pH 7.4 with increasing concentrations showing quenching phenomena.

Moreover, it is has to be also mentioned that slight changes in fluorescence intensity could be found when using different pH buffers to dissolve the polymeric conjugates as well as free OG. Figure 3.16 represents OG calibration curve obtained at different pHs highlighting the importance of controlled pHs during the OG loading estimations as well as during uptake experiments.
3.2.4.3.2. Cellular uptake study by Flow Cytometry.

The experiments carried out were pursuing different answers:
(i) On one hand, the effect of MW on cellular uptake was studied by using different linear PGA-OG and hybrid DB PEG-PGA-OG of different number of GAU. (ii) On the other hand, the new architectures star-shaped polymers where characterized in terms of cellular uptake for the first time. (iii) Finally, the three different architectures with similar MW were compared to assess the effect of polymeric structure on uptake mechanisms.

Flow cytometry (cell uptake and binding) together with live-cell confocal microscopy analysis (subcellular fate and pathway) in SHSY5Y human derived neuroblastoma cell line, were used to study cellular trafficking of the OG-labeled polymers. Uptake experiments were carried out at different temperatures, 37 °C (total uptake) and 4 °C (binding) in order to determine the presence of energy dependent or non-dependent internalization mechanisms, such as endocytosis or diffusion, respectively. It is worth mentioning that all experiments were done in the presence of cathepsin B inhibitor CA-047 in order to avoid possible degradation of PGA chains along the incubation periods. Results were represented by means of cell associated fluorescence (CAF) over incubation time. CAF represents the percentage of positive cells multiplied by fluorescence intensity and divided by 100, always removing CAF of control cells (without treatments) in order to avoid any artifacts from autofluorescence phenomena.

Results clearly showed energy-dependent mechanisms of internalization (endocytosis) in all the cases due to absence of uptake at 4 °C as observed by flow cytometry. Figure 3.17 shows an example of the uptake profile of a 3 arm St-PGA (250 GA units). Energy dependent
uptake represents the result of subtracting uptake at 4 °C from uptake at 37 °C.

**Figure 3.17.** Uptake study by flow cytometry of St-PGA fluorescently labeled in SHSY5Y cell line as example. n > 3, mean ± SEM.

When different linear PGA homopolymers of different number of GAU (80, 180, and 250) where compared, no significant differences in cellular uptake profiles were observed. In the case of DB, although CAF at 5 hours was comparable to that of PGA homopolymers, its uptake kinetics was slower (Figure 3.18).

**Figure 3.18.** Uptake profile (energy dependent only) of different OG-labeled polymers against SHSY5Y cell line including different MW linear PGAs and one DB PGA. n > 3, mean ± SEM.

Then, linear homopolymer and star structures with greater MW (with potential to increase EPR effect) were compared. Interestingly, St-PGA-OG showed a significant increase (about 3-fold) in CAF at 5 h time point when compared with linear-PGA-OG conjugate of similar MW (~250 GAU). (Figure 3.19).
Figure 3.19. Uptake study against SHSY5Y cell line of St-PGA-OG in comparison with linear PGA of similar MW (around 250 GAU). a) CAF of both polymers over time. b) CAF of both polymers at 5 hour showing significant differences when statistics was performed using one-way ANOVA. $p^*< 0.05$. c) % of positive (+) cells to OG fluorescence, comparison of both polymers at 5 hours showing
statistical differences. $\rho < 0.05$. d) % of positive cells representation comparing both polymers together with the control used (cell autofluorescence). $n > 3$, mean $\pm$ SEM.

This finding might be attributed to the inherent properties of the different architectures. As general basis, star polymers have a more compact structure, presumably with globular shape, and have large surface areas, increased concentrations of functional end groups for polymers with equal molecular weight, and unique rheological properties which make them optimal platforms for drug delivery and imaging among other biological applications.\textsuperscript{10,11}

### 3.2.4.3.3. Cellular uptake by Live-Cell Confocal Imaging.

As mentioned before, live-cell confocal imaging, allows visualizing trafficking between multiple compartments within individual living cells over time. Results obtained by flow cytometry were further confirmed with confocal microscopy experiments at 2 hours post-treatment with OG labeled polymers in SHSY5Y cells following a pulse-chase experiment. Co-localization in lysosomes was observed upon the use of lysosomal marker Lysotracker Red (Figure 3.20).
Figure 3.20. Confocal image of uptake at 2 hours post-treatment of OG-labeled polymers in SHSY5Y cell line following a pulse-chase experiment. Co-localization with lysosomal marker Lysotracker Red was observed.

A co-localization histogram can be built by using Leica Software (Figure 3.21), confirming a high degree of co-localization in lysosomes for all polymers.

Figure 3.21. Representative co-localization histogram of St-PGA-OG as example. In red, fluorescence intensity corresponding to Lysotracker
Red Channel; in green, fluorescence intensity corresponding to OG 488 Channel; in Blue, fluorescence intensity of DAPI channel (for Hoetch).

### 3.3. CONCLUSION.

In summary, herein, a controlled living polymerization approach to obtain polyglutamate-based star shaped polymers have been described based on our recently developed ROP of NCAs with novel non-nucleophilic initiators (Chapter 2). This fact represents a sign of its versatility. Our approach does not require of neither complex and expensive equipment nor low temperatures, but is capable to yield star-shaped polypeptides with low Đ (< 1.2) in a relatively short time and in a variety of MW including high and adjustable molecular mass. Moreover, it has been also described a method that gives very convincing evidence that the arms on stars were of equal MW by using reducible initiators containing disulfide bonds. This has been highlighted as a highly significant result when considering new polymer carriers as drug delivery systems or imaging probes due to the need for homogeneity in these materials.

*In vitro* biocompatibility (MTS assays) was not compromised with the use of different polymeric architectures according to results obtained from the polymers tested. Regarding uptake profiles, all polymers tested (independently of MW or architecture) followed an energy-dependent mechanism of uptake, presumably through endocytic mechanism due to the absence of uptake at 4 °C. MW of the polymers (at least in linear structures) did not significantly affect the degree of internalization. Nonetheless, this degree of internalization was highly increased when star-shaped polymers were used, highlighting the importance of the architecture in the biological output.

### 3.4. MATERIALS AND METHODS.

#### 3.4.1. Materials.

All chemicals were reagent grade, obtained from Aldrich and used without further purification, unless otherwise indicated. H-L-Glu(OBzl)-OH, was obtained from Iris Biotech. Phenazinemethosulfate (PMS) was supplied by Sigma (Sp). Dulbecco’s Modified Eagle’s Medium (DMEM), Leibovitz, Phosphate buffer saline (PBS), Foetal bovine serum (FBS) Medium 200, Low Serum Growth Supplement (LSGS) and Trypsin, were provided from Gibco.
dimethylthiazol-2-yl)-5- (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) was supplied by Promega (Sp).

All solvents were of analytical grade and were dried and freshly distilled. Deuterated chloroform-$d_1$, DMSO-$d_6$, DMF-$d_7$ and $D_2O$ were purchased from Deutero GmbH.

Preparative SEC was performed using Sephadex G-25 superfine from GE as well as PD MiniTrap G-10™ columns containing 2.1 mL of Sephadex™ G-10. Dialysis was performed in a Millipore ultrafiltration device fitted with a 1, 3, or 10 kDa MWCO regenerated cellulose membrane (Vivaspin®).

### 3.4.2. Characterization techniques.

NMR spectroscopy was performed as for section 2.4.2.1 from Chapter 2.

#### 3.4.2.1. GPC in DMF.

For SEC measurements in DMF containing 1g·L$^{-1}$ of lithium bromide as an additive, an Agilent 1100 series system was used with a flow rate of 1 mL·min$^{-1}$ at 70 °C as an integrated instrument, including three HEMA-based columns ($10^5/10^3/10^2$ Å porosity) from MZ-Analysentechnik GmbH, Viscoteck TDA™ 302 triple detector 87 with UV detection coupled, and Calibration was achieved with well-defined poly(methyl methacrylate) (PMMA)/DMF standards, provided by Polymer Standards Service (PSS)/Mainz Germany.

#### 3.4.2.2. Circular Dichroism (CD).

CD Spectroscopy was performed with a J-815 CD Spectrometer (JASCO Corporation) using a Peltierthermostated cell holder (PTC-423, JASCO Corporation) with a recirculating cooler (JULABO F250, JASCO Corporation). A nitrogen flow ($\sim$2.7 L·min$^{-1}$) was lead through the spectrometer and controlled with a nitrogen flow monitor (Afriso Euro-Index). The samples were dissolved in HFIP for protected and PBS pH 7.4 for deprotected samples and diluted to a concentration of 0.1 mg·mL$^{-1}$. Samples were measured repeatedly (n=3) in a quartz cuvette with d= 0.1 cm at 20 °C for HFIP samples and 37 °C for PBS dissolved samples. Obtained molar ellipticities were plotted as mean residue ellipticity.
3.4.2.3. Dynamic Light Scattering (DLS).

DLS measurements were performed at 25 °C using a Malvern ZetasizerNanoZS instrument, equipped with a 532 nm laser at a fixed scattering angle of 173°. Polymer solutions (0.2 mg·mL⁻¹) were prepared in PBS pH 7.4. Solutions were sonicated for 10 min and filtered through a 0.45 μm cellulose membrane filter. Size distribution by volume was measured (diameter, nm) for each polymer per triplicate with n> 3 measurements.

3.4.2.4. UV-VIS.

UV-VIS measurements were performed using JASCO V-630 spectrophotometer at 25 °C with 1.0 cm matched quartz cells and with spectral bandwidth of 0.5 nm.

3.4.2.5. Victor² Wallace™.

Percentage of OG 488 loading in different polymers was determined by fluorescence measurements using Multilabel HTS counter Wallac Victor² 1420 + software Wallac 1420 workstation from Perkin Elmer. Dark 96-Well Multiwell Plates will 100 μL where used for that purpose. Fluorescence was measured using 490 filter for excitation and 535 for emission wavelengths.

3.4.3. Protocols.

Monomer synthesis was performed as for Section 2.4.3.1.

3.4.3.1. Initiators Synthesis.

3.4.3.1.1. Ethyl-based initiator synthesis.

a. Synthesis of 1,3,5-Tri-tert-butyl ((benzenetricarbonyltris (azanediyl)) tris(ethane-2,1-diyl))tricarbamate (12a).

In a two-neck round bottom flask fitted with a stirrer bar, and a N₂ inlet and outlet, 1,3,5-benzenetricarbonyl trichloride (1.88 mmol, 1 eq.) was dissolved in 12 mL of anh. THF. N,N’,N”-diisopropylethylendiamine (DIEA) (6.22 mmol, 3.3 eq.) was added to the reaction mixture followed by drop wise addition of N-Boc-ethylendiamine (6.22 mmol, 3.3 eq.) over a period of 10 minutes. Reaction was then left to proceed for 2 hours. After that time, solvent was completely removed under vacuum. The product was re-dissolved in chloroform and washed (3x) with ddH₂O, and (3x) with acidic water (pH~3). Finally, the organic phase was isolated under vacuum and the
product was recrystallized from THF/Methanol/Hexane yielding a white crystalline solid. The product was then dried under high vacuum and stored at -20 °C.

Yield: 82 %. $\delta_H$ (300 MHz, DMSO) 8.68-8.65 (m, 3H), 8.41 (s, 3H), 6.92-6.88 (m,3H), 3.34-3.31 (m, 6H), 3.16-3.13 (m, 6H), 1.37 (s, 27H). $^{13}$C NMR (75 MHz, CDCl$_3$) 166.80 (C=O), 156.84 (C=O), 134.58 (C$_{Ar}$ quaternary), 128.47 (CH$_{Ar}$), 79.57 (C quaternary), 40.93 (CH$_2$), 40.43 (CH$_2$), 28.45 (CH$_3$).

b. **Synthesis of 1,3,5-(benzenetricarbonyltris(azanediyl)) triethanammonium BF$_4$ salt (13a).**

In a round bottom flask fitted with a stirrer bar and a stopper, compound 12a (1 eq.) was dissolved in dichloromethane. Afterwards, 3.3 eq. of tetrafluoroboric acid diethyl ether complex, HBF$_4$∙Et$_2$O, was added to the solution leading to the instantaneous formation of a white solid. The precipitate was filtered off and recrystallized (3x) from THF/methanol/hexane. The product was then dried under high vacuum and stored at -20 °C.

Yield: 98 %. $\delta_H$ (300 MHz, D$_2$O) 8.32 (s, 3H), 3.72-3.68 (m, 6H) 3.25-3.21 (m, 6H). $^{13}$C NMR (75 MHz, D$_2$O) 169.45 (C=O), 134.38 (C$_{Ar}$ quaternary), 129.36 (C$_{Ar}$), 39.23(CH$_2$), 37.52 (CH$_2$). $^{19}$F-NMR: -150.48 (BF$_4$). MALDI-TOF: 337.1709 [M$^{+1}$].

**3.4.3.1.2. Hexyl-based initiator synthesis.**

a. **Synthesis of 1,3,5-tri-tert-butyl((benzenetricarbonyltris(azanediyl)tris(hexane-1,6-diyl))tricarbamate (12b).**

In a two-neck round bottom flask fitted with a stir bar, and a N$_2$ inlet and outlet, 1,3,5-benzenetricarbonyl trichloride (1.88 mmol, 1 eq.) was dissolved in 12 mL of dried THF. After that, DIEA (6.22 mmol, 3.3 eq.) was added to the reaction mixture followed by the drop wise addition of N-Boc-1,6-hexanediamine (6.22 mmol, 3.3 eq.) over a period of 10 minutes. Reaction was then left to proceed for 2 hours. After that time, solvent was completely removed under vacuum. The product was re-dissolved in chloroform and washed (3x) with ddH$_2$O, and acidic water (3x). Finally, the organic phase was isolated under vacuum and the product was recrystallized from THF/Methanol/Hexane yielding a white crystalline solid. The product was then dried under high vacuum and stored at -20 °C.
Yield: 72 % $\delta_H$ (300 MHz, DMSO) 8.37 (s, 3H), 6.75 (m, 3H), 3.30-3.24 (m, 6H), 2.91-2.87 (m, 6H), 1.55-1.51 (m, 6H), 1.37-1.29 (m, 45H). $\delta_C$ (75 MHz, CDCl$_3$) 166.28 (C=O), 156.49 (C=O), 134.93 (CAr quaternary), 128.68 (CAr), 79.34 (C quaternary), 40.33 (CH$_2$), 39.92 (CH$_2$), 29.89 (CH$_2$), 29.19 (CH$_2$), 28.42 (CH$_3$), 26.19 (CH$_2$), 25.90 (CH$_2$).

b. Synthesis of 1,3,5-(benzenetricarbonyltris(azanediyl))trishexan-1-ammonium BF$_4$ salt (13b).

In a one-necked round bottom flask fitted with a stir bar and a stopper, compound 12b (1 eq.) was dissolved in dichloromethane. Afterwards, 3.3 eq. of HBF$_4$∙Et$_2$O, was added to the solution leading to the formation of a white solid salt in almost quantitative yield. The product was then filtered off and recrystallized (3x) from THF/methanol/hexane. The product was then dried under high vacuum and stored at -20 °C.

Yield: 90 %. $\delta_H$ (300 MHz, D$_2$O) 8.12 (s, 3H), 3.37-3.32 (m, 6H), 2.96-2.91 (m, 6H), 1.61-1.57 (m, 12H), 1.39-1.34 (m, 12H). $\delta_C$ (75 MHz, D$_2$O) 168.92 (C=O), 135.00 (CAr quaternary), 128.49 (CAr), 39.97 (CH$_2$), 39.42 (CH$_2$), 28.10 (CH$_2$), 26.60 (CH$_2$), 25.57 (CH$_2$), 25.23 (CH$_2$).$^{19}$F-NMR-150.41. MALDI-TOF: 505.3563 [M$^+$].

3.4.3.1.3. DOOA-based initiator synthesis.

a. Synthesis of 1,3,5-tri-tert-butyl (((benzenetricarbonyltris(azanediyl))tris(ethane-2,1-diyl))tris(oxy))tris(ethane-2,1-diyl))tris(ethane-2,1-diyl))tricarbamate (12c).

In a two-neck round bottom flask fitted with a stir bar, and a N$_2$ inlet and outlet, 1,3,5-benzenetricarbonyl trichloride (0.75 mmol, 1 eq.) was dissolved in 12 mL of dried THF After that, DIEA (6.22 mmol, 3.3 eq.) was added to the reaction mixture followed by the drop wise addition of N-Boc-DOOA (2.49 mmol, 3.3 eq.) over a period of 10 minutes. Reaction was then left to proceed for 2 hours. After that time, solvent was completely removed under vacuum. The product was re-dissolved in chloroform and washed with ddH$_2$O, and acidic water (3x each). Finally, the organic phase was isolated under vacuum and the product was recrystallized (3x) from THF/Methanol/Hexane yielding a white crystalline solid. The product was then dried under high vacuum and stored at -20 °C.
Yield: 70 %. δ<sub>H</sub> (300 MHz, CDCl<sub>3</sub>) 8.35 (s, 3H), 3.62-3.58 (m, 6H), 3.24-3.20 (m, 6H), 1.34 (s, 27H). δ<sub>C</sub> (75 MHz, CDCl<sub>3</sub>) 165.99 (C=O), 156.19 (C=O), 135.05 (C<sub>Ar</sub> quaternary), 128.55 (C<sub>Ar</sub>), 79.42 (Cquaternary), 79.32 (CH<sub>2</sub>), 77.22 (CH<sub>2</sub>), 70.20 (CH<sub>2</sub>), 69.66 (CH<sub>2</sub>), 40.51 (CH<sub>2</sub>), 40.09 (CH<sub>2</sub>), 28.39 (CH<sub>3</sub>).

b. Synthesis of 1,3,5’-((((benzenetricarbonyltris(azanediyl))tris(ethane-2,1-diyl))tris(oxy))tris(ethane-2,1-diyl))tris(oxy))triethanammonium BF4 salt (13c).

In a one-necked round bottom flask fitted with a stir bar and a stopper, compound 12c (1 eq.) was dissolved in dichloromethane. Afterwards, 3.3 eq. HBF<sub>4</sub>∙Et<sub>2</sub>O, was added to the solution leading to the formation of a white solid salt in almost quantitative yield. The product was then filtered off and recrystallized (3x) from THF/methanol/hexane. The product was then dried under high vacuum and stored at -20 °C.

Yield: 85 %. δ<sub>H</sub> (300 MHz, D<sub>2</sub>O) 8.24 (s, 3H), 3.74-3.67 (m, 30H), 3.61-3.58 (m, 6H), 3.14-3.10 (m, 6H). δ<sub>C</sub> (75 MHz, D<sub>2</sub>O) 168.95 (C=O), 134.86 (C<sub>Ar</sub> quaternary), 128.89 (C<sub>Ar</sub>), 69.56 (CH<sub>2</sub>), 69.49 (CH<sub>2</sub>), 68.78 (CH<sub>2</sub>), 66.37 (CH<sub>2</sub>), 39.56 (CH<sub>2</sub>), 39.08 (CH<sub>2</sub>), 19F NMR: -150.48. MALDI-TOF: 601.3354 [M<sup>+</sup>].

3.4.3.1.4. Cysteamine-based initiator synthesis.

General method from Girgenti et al.<sup>48</sup> with slight modifications. Briefly, a suspension of cysteamine dihydrochloride (15 mmol) in 100 mL of anh. chloroform was treated with triethylamine (30 mmol) and stirred for 20 minutes at r.t.. A solution of di-tert-butyldicarbonate (5 mmol) in another 50 mL of anh. chloroform was added drop wise over a period of 1.5 hours, keeping the reaction at 0 °C. The reaction mixture was warmed to r.t. and stirred for 16 hours more. After that time, 20 mL of ddH<sub>2</sub>O were added and the organic layer was washed with ddH<sub>2</sub>O (3x). A colorless oil was obtained as a pure compound after removal of the solvent under high vacuum.

Yield: 50 %. <sup>1</sup>H-NMR δ<sub>H</sub> (300 MHz, DMSO-d6) 6.97-6.96 (1H, d), 3.23-3.17 (2H, q), 2.81-2.68 (6H, m), 1.50 (1H, s), 1.38 (9H, s). <sup>13</sup>C-NMR δ<sub>C</sub> (75 MHz, DMSO-d6) 155.94 (C=O), 78.21 (C
quaternary), 42.57 (CH₂), 41.40 (CH₂), 39.90 (CH₂), 38.13 (CH₂), 28.67 (CH₃).

b. Synthesis of 1,3,5-tri-tert-butyl ((((benzenetricarbonyltris(azanediyl)tris(ethane-2,1-diyl))tris(disulfanediyl))tris(ethane-2,1-diyl))tricarbamate (12d).

In a two-neck round bottom flask fitted with a stir bar, and a N₂ inlet and outlet, 1,3,5-benzenetricarbonyl trichloride (0.38 mmol, 1 eq.) was dissolved in 12 mL of dried THF. After that, DIEA (1.24 mmol, 3.3 eq.) was added to the reaction mixture followed by the drop wise addition of N-Boc-cysteamine (1.24 mmol, 3.3 eq.) over a period of 10 minutes. Reaction was then left to proceed for 2 hours. After that time, solvent was completely removed under vacuum. The product was redissolved in chloroform and washed with ddH₂O, and acidic water (3x each). Finally, the organic phase was isolated under vacuum and the product was obtained by column chromatography on silica gel using CH₂Cl₂:MeOH (90:10). Rf= 0.5. The product was then dried under high vacuum and stored at -20 °C.

Yield: 70 %. ¹H-NMR δH (300 MHz, Acetone-d6) 8.21 (3H, m), 8.43 (3H, m), 6.17 (3H, s), 3.73-3.67 (6H, m), 3.37-3.30 (6H, m), 2.98-2.94 (6H, m), 2.84-2.79 (6H, m), 1.32 (27H, s). ¹³C-NMR δC (75 MHz, CDCl₃) 165.87 (C=O), 156.21 (C=O), 135.45 (Cₐr quaternary), 129.06 (Cₐr), 78.16 (Cquaternary), 39.87 (CH₂), 39.31 (CH₂), 38.07 (CH₂), 37.43 (CH₂), 28.68 (CH₃).

c. Synthesis of 1,3,5-((((benzenetricarbonyltris(azanediyl))tris(ethane-2,1-diyl))tris(disulfanediyl))triethanaminium BF₄⁻ (13d).

In a one-necked round bottom flask fitted with a stir bar and a stopper, compound 12d (1 eq.) was dissolved in dichloromethane. Afterwards, 3.3 eq. of HBF₄-Et₂O, was added to the solution leading to the formation of a white solid salt in almost quantitative yield. The product was then filtered off and washed (3x) with ether. For further purification, the product is recrystallized from THF/methanol/hexane and then dried under high vacuum and stored at -20 °C.

Yield: 80 %. ¹H-NMR δH (300 MHz, D₂O) 8.08 (3H, s), 3.62-3.57 (6H, m), 3.23-3.18 (6H, m), 2.86-2.84 (12H, m). ¹³C-NMR δC (75 MHz, DMSO-d6) 165.47 (C=O), 134.55 (Cₐr quaternary), 128.06 (Cₐr), 39.77 (CH₂), 39.31 (CH₂), 38.28 (CH₂), 37.65 (CH₂). ¹⁹F-NMR: -148.31.
3.4.3.2. Synthesis of St-PBLG (14).

As described in Section 2.4.3.4. 23

Yield: 70-90 %. $^1$H-NMR $\delta_H$ (300 MHz, DMF-$d_7$) 8.58 (xH, s), 7.42 (5H, s), 5.19 (2H, s), 4.21 (1H, s), 2.81 (2H, s), 2.45 (2H, s). $^{13}$C-NMR $\delta_C$ (75 MHz, DMF-$d_7$) 175.94 (s), 172.26 (s), 162.77-162.18 (m), 161.98 (s), 136.76 (s), 128.87-127.75 (m), 66.05 (s), 57.13 (s), 35.41 - 34.17 (m), 32.48 (s), 30.84, 30.30-29.04 (m), 27.28 (s), 25.99 (s). x: DP obtained/3 arms

3.4.3.3. Deprotection of St-PBLG.

Different methods were followed depending on the initiator used: acid conditions (described in Section 2.4.3.6.2.) were applied when ethyl and hexyl based initiators were used. On the other hand, basic conditions (described in Section 2.4.3.6.3.) were applied for DOOA, and cysteamine based initiator synthesis.

3.4.3.4. Kinetics of polymer degradation in presence of Cathepsin B.

Several PGA-based polymers were degraded in vitro in presence of lysosomal enzyme cathepsin B. To test their degradation kinetic profile by cathepsin B, the different polymer solutions (3 mg mL$^{-1}$) were prepared. Exactly 3 mg were weighed and 700 $\mu$L of acetate buffer 20 mM, pH 6, 100 $\mu$L of EDTA 2 mM, 100 $\mu$L of DTT 5 mM were added. Finally, 6.25 units of Cathepsin B (100 $\mu$L of a solution of 25 units of cathepsin B in 400 $\mu$L of acetate buffer pH 6 20 mM) were added. Cathepsin B needs acidic pH (5-6) to be active as well as reductive environment, therefore a DTT solution was also added together with EDTA in order to complex possible free cations that can inactivate cathepsins (mainly Ca$^{2+}$).

Once solutions were prepared, aliquots of 100 $\mu$L were picked at different time points after homogenization. Meanwhile, samples were kept at 37 °C under stirring. Aliquots at different time points (0, 0.5, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144, 168 hours) were taken, frozen and later analyzed by GPC. To evaluate the mass of the polymers, 100 $\mu$L of 3 mg mL$^{-1}$ polymer solution in PBS was injected in GPC using two TSK Gel columns in series G2500 PWXL and G3000 PWXL with a Viscotek TDA™ 302 triple detector 87 with UV detection coupled. The mobile phase used was PBS 0.1 M, flow 1 mL min$^{-1}$. 

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3.4.3.5. Fluorescence labeling with Oregon Green 488 Cadaverine.

3.4.3.5.1. Protocol to label linear and St-PGA.

In a round two necked bottom flask fitted with a stirrer bar and two septums, 29 mg of St-PGA (0.225 mmol GAU, 1 eq.) was weighted and dissolved in 1.5 mL of dry DMF under N$_2$ flow. Then, 1.12 μL of DIC (0.85 mg, 0.00674 mmol, 0.03 eq.) were added and the reaction was left to proceed for 5 minutes at r.t. Afterwards, HOBt (1 mg, 0.00674 mmol, 0.03 eq.) was added directly. Reaction was then left to proceed for 10 minutes before OG (1 mg, 2.25·10$^{-3}$ mmol, 0.01 eq.) was added. The pH was adjusted to 8 by adding ~100 μL of DIEA. The mixture was left stirring overnight at r.t. and protected from light. Finally, solvent was removed under vacuo and the product was dissolved in 300 μL of water and then adding ~50 μL of NaHCO$_3$ 1 M. The solution was purified by Sephadex PD10 column eluting with ddH$_2$O. OG loading was calculated by fluorescence using a Victor$^2$Wallac™ plate reader with excitation filter of 490 nm and emission filter of 535 nm. A calibration curve with OG was first performed. Yields: 95 %. OG loadings: 0.8 - 0.95 mol GAU.

3.4.3.5.2. Protocol to label DB PEG-PGA.

In a one neck round bottom flask fitted with a stir bar and a stopper, 50 mg of DB PEG-PGA (0.36 mmol GAU, 1 eq.) were suspended in 5 mL of ddH$_2$O. Afterwards 2.1 mg of DMTMM·Cl were added dissolved in 2 mL of ddH$_2$O (7.1·10$^{-3}$ mmol, 0.02 eq.). After 10 minutes OG (1.5 mg, 3.36 10$^{-3}$ mmol 0.01 eq.) was added and the pH was adjusted to 8 by adding some drops of 1 M NaHCO$_3$ solution. Reaction was allowed to proceed 16 hours stirring at r.t. The solution was then concentrated in the freeze-dryer and purified by Sephadex PD10 column eluting with ddH$_2$O. OG was calculated by fluorescence using a Victor$^2$Wallac™ plate reader with excitation filter of 490 nm and emission filter of 535 nm. A calibration curve with OG was first performed. Yields: 95 %. OG loadings: 0.7-0.8 mol GAU.

3.4.3.6. Cell Culture protocols.

HUVEC cells were cultured in Medium 200 supplemented with Low Serum Growth Supplement (LSGS). SHSY5Y cells were cultured in DMEM media supplemented with Foetal Bovine Serum (FBS). Cells were maintained at 37 °C in an atmosphere of 5 % carbon dioxide and
95 % air. Medium was replaced every 2-3 days and underwent passage once weekly when 80 % of cell conflu ence was reached.

3.4.3.7. MTS assay for cell viability.
MTS cell viability assay (72 h incubation). Cells were seeded in sterile 96-well microtitre plates at a cell density of 35000 cell·cm⁻² for SHSY5Y and 1260 cell·cm⁻² for HUVEC. Plates were incubated for 24 hours and compounds (0.2 μm filter sterilized) were then added to give a final concentration of 0-3 mg·mL⁻¹. After 72 h of incubation, MTS/PMS (20:1) (10 μL of manufacturer solution) was added to each well, and the cells were incubated for a further 2 hours. Optical density of each well was measured at 490 nm. Plates were read spectrophotometrically using Victor² Wallac plate reader. The absorbance values were represented as the percentage of cell viability taken as 100 % cell viability of untreated control cells.

3.4.3.8. Cellular uptake by flow cytometry of OG-labeled polymers in SHSY5Y cells.
SHSY5Y cells were seeded in 6-well plates at a density of 35000 cell·cm⁻² (1 mL cell suspension per well) and allowed to adhere for 24 hours. In binding experiments conducted at 4 °C, cells were pre-incubated at this temperature for 30 min prior to start the experiment. For both experiments, 4 °C and 37 °C, the cathepsin B inhibitor CA-074 (0.4 μL from a solution of 5 μM to reach a final concentration of 2 μM) was added 30 min before the addition of the conjugate. Then, 10 μL of OG-labeled polymer (0.01 mg OG·mL⁻¹) were added at different time points from 0 to 300 min meanwhile cells were incubated either at 37 °C or 4 °C for each experiment. Finally, cells were placed on iced in order to stop energy dependent mechanisms and washed twice with cold PBS-BSA 0.1 %. (PBS supplemented with Bovine Serum Albumin (BSA)). Then cells were suspended in 0.5 mL of cold PBS by the use of a scraper. Finally, the cell pellet was placed in flow cytometer tubes. Cell-associated fluorescence was then analyzed using a Becton Dickinson FACS Calibur cytometer (California, USA) equipped with an argon laser (488 nm) and emission filter for 550 nm. Data collection involved 10,000 counts per sample, and data were analyzed using CELLQuest™ version 3.3 software. Data are expressed by plotting the cell-associated fluorescence, which is the result of the % of positive cells by multiplied by the mean fluorescence. CAF= % positive
cells*mean fluorescence/100. Cells incubated without polymer were used to account for the background fluorescence.

Statistical Analysis. Data from the experiments was analyzed using one-way ANOVA. In all cases, we considered differences to be significant when p***<0.001; p**<0.01; p*<0.05; ns: non-significant.

3.4.3.9. Confocal fluorescence microscopy: Live-cell imaging.

For live-cell imaging, SHSY5Y cells were seeded at a density of 35000 cell·cm⁻², on glass bottom culture dishes (1 cm² Petri plate) and allowed to seed for 24 hours at 37 °C. The experiment was performed following a pulse-chase mode at 37 °C. First, the cathepsin B inhibitor CA-074 (0.4 μL from a solution of 5 μM to reach a final concentration of 2 μM) was added 30 min prior the addition of the St-PGA-OG. Then, 10 μL of OG-labeled polymer (0.01 mg OG·mL⁻¹) were added and the cells were incubated for 2 hours at 37°C (pulse). The medium was removed and cells were washed twice with PBS. 1 mL of medium containing 2 μM of the CA-074 inhibitor was then added, and cells were incubated for 4 hours at 37 °C (chase). 30 min before washing the cells with PBS-BSA 0.1 %, the nuclear marker Hoech (1 μL from a solution 5 mM), and the lysosomal marker Lysotracker Red (0.75 μL from a solution of 100 μM) were added in order to identify possible co-localizations and therefore establish an endocytic pathway. Finally, cells were washed with PBS-BSA 0.1 %. Then, the glass was removed and placed on the microscope chamber with fresh media containing 2 μM of CA-074 inhibitor. Samples were analyzed under the microscope. Images were captured with an inverted DM IRE2 microscope equipped with a λ-blue 60 x oil immersion objective and handled with a TCS SP2 system, equipped with an Acoustic Optical Beam Splitter (AOBS). Excitation was performed with an argon laser ((OG 496 nm) and HeNe laser (Lysotracker Red 594 nm), and blue diode (Hoetch 405 nm). Images were captured at an 8-bit grey scale and processed with LCS software (version 2.5.1347a, Leica Germany) containing multicolor, macro and 3D components. Control cells that followed the same incubation time were also analyzed to establish the autofluorescence, as well as cells treated only with Hoech or Lysotracker Red.
3.5. REFERENCES


42 C. Hua, C. M. Dong and Y. Wei, *Biomacromolecules*, 2009, 10, 1140.


Chapter 4

A versatile post-polymerization modification method for polyglutamic acid: Synthesis of orthogonal reactive polyglutamates and their use in "Click Chemistry"
4.1. INTRODUCTION AND BACKGROUND.

As stated in Chapter 1, the choice of an appropriate polymer to serve as drug delivery system/imaging agent is key for an adequate biological output. Besides, the chemistry for the attachment of bioactive molecules must be seriously considered. To this respect, conjugation chemistry of polymers to natural or non-natural agents is of major importance. The incorporation of reactive sites becomes even more demanding whenever orthogonal reactive groups are mandatory for site-specific conjugation of bioactive compounds (i.e. peptides, proteins, antibodies) (Figure 4.1).

There are two main approaches to introduce reactive groups for later bioconjugations, into polymers: the (co)polymerization of reactive monomers\(^1\) and the post-polymerization modification techniques.\(^2\) Recent advances in synthetic polymer chemistry have made possible the construction of complex macromolecules with control over many parameters such as functionality, topology, MW and Đ. While co-polymerization may offer a certain control about distribution of reactive groups among the polymer chain, post-polymerization modification is usually much easier to perform, since the synthesis of monomer bearing sterically bulky, highly aggregating or reactive groups can be avoided. Moreover, post-polymerization modification can be a powerful and attractive approach for the synthesis of functional polymers overcoming the limited functional group tolerance of many controlled polymerization techniques.\(^2,3\)

The concept of post-polymerization modification was developed by Hermann Staudinger in the 1920s who laid down the foundation to use this approach to fabricate functional materials.\(^4\) Since then, the scope of post-polymerization modification reactions has suffered a great expansion, especially in the 1990s due to the re-discovery of highly efficient coupling chemistries that allow quantitative, chemoselective and orthogonal functionalization of reactive polymer precursors.\(^1\)

For all that reasons, a new and versatile post-polymerization modification methodology allowing to introduce a series of functionalities into PGA polymer backbone is described within this chapter. Part of this work was published in the journal Polymer Chemistry, year 2013, volume 4, issue 10, pages 2980 - 2994 (A
versatile post-polymerization modification method for polyglutamic acid: Synthesis of orthogonal reactive polyglutamates and their use in "click chemistry").

![Diagram](image)

**Figure 4.1.** Schematic representation of the versatility of the strategy pursued within this chapter in the development of methods for efficient introduction of orthogonal reactive sites into PGA polymeric chains for further bioconjugations.

### 4.2. RESULTS AND DISCUSSION.

#### 4.2.1. Direct post-polymerization modification of PBLG (2) by aminolysis.

As a starting strategy for the direct post-polymerization modification of PBLG one could think in direct aminolysis reaction. Aminolysis is any type of reaction in which a molecule is split into two parts by reacting with a molecule of ammonia or an amine. In the case of this particular aminolysis reaction, is a nucleophilic acyl substitution where the benzylic esters (protective benzyl groups) of the polymer backbone of PBLG will be substituted by the attack of an amine to the C=O group resulting in the amide formation. As the polymer is entirely composed of amide bonds due to the nature of PBLG, this kind of substitution could be dangerous for the integrity of the polymer backbone. This will depend on the aminolysis reaction conditions as well as the basicity of the amine used. To reduce the risks of backbone damage produce by aminolysis, 2-hydroxypyridine was used as catalyst. The use of 2-hydroxypyridine will presumably speeds up
reaction time, and will allow the use of less harsh reaction conditions. The mechanism of action of this catalyst is not fully elucidated, and there are different theories. One of them claims that an 8-membered-ring transition state is created with the involvement of three molecules which entropically is not very favorable. The other one claims that the protecting group is first substituted by the catalyst and subsequently the catalyst is substituted by the reacting amine. Those mechanisms are depicted in Scheme 4.1.

**Scheme 4.1.** Different possible mechanisms of the aminolysis reaction catalyzed by 2-hydroxypyridine. A) With an 8-membered-ring transition state; B) With an intermediated resulting from the substitution of the benzyl group for the catalyst.

To test the versatility and possible risks of this approach within polyglutamate backbone, we used a Boc end capped PBLG (16) (MW=18177 g·mol⁻¹, D: 1.14). The use of Boc end capped polymers responds to the need of monitoring backbone integrity which could be easily observed by the end cap group signals in \(^1\)H-NMR. For that purpose, first PBLG was end capped by using 5 eq. of trimethylacetylchloride as a capping agent and DIEA as a base to activate the N-terminus of the backbone nucleophilicity. Reaction was carried out in DMF, during 4 hours under nitrogen atmosphere leading to complete conversion of the end group according to the \(^1\)H-NMR spectrum. (Scheme 4.2, Figure 4.2)
Scheme 4.2. Synthesis of end capped PBLG (16).

\[
\begin{align*}
\text{R}_1 \quad \text{N}^+ \quad \text{O} \quad \text{H} \quad \text{N}^+ \quad \text{O} \quad \text{R}_1 \\
\text{O} \quad \text{H} \quad \text{O} \quad \text{H} \quad \text{N}^+ \quad \text{O} \quad \text{R}_1 \\
\text{DMF} \quad \text{DIEA} \quad \text{R.T.} \quad 4\text{h}
\end{align*}
\]

Figure 4.2. $^1$H-NMR in CDCl$_3$ of end capped poly($\gamma$-benzyl L-glutamate) (16) with corresponding assignments. Signal 5 ($\text{CH}_2$) was integrated by 186 protons, since the polymer used was of ~83 GAUs according to GPC results. Signal 5 was compared with signal 1 (corresponding with three $\text{CH}_3$ groups from end capping group) to confirm complete conversion as it can be seen from the integrations.

Once end capping was complete, the polymer backbone was modified by the use of three different mono-Boc protected diamines: N-Boc-DOOA, N-Boc-hexaneamine and N-Boc-ethylenediamine (Scheme 4.3). These amines are not much different in their $pK_b$ values ($pK_b$ N-Boc-DOOA: 9.4; N-Boc-hexaneamine: 10.2 and N-Boc-ethylenediamine: 9.2 according to ref$^7$). However, they differ in their hydrophobic character. The use of mono-Boc protected amines could be interesting to build complex architectures based on polyglutamates such as graft, brush and comb-like polypeptides, due to the creation of different reactive sites, in this case randomly distributed, which can be used for later polymerization processes in order to grow branches.
Scheme 4.3. General reaction scheme of the synthesis of modified PBLG via aminolysis, with different amines Boc protected amines.

Different percentages of modifications with the three different amines were aimed (5, 10, 20 mol% GAU). Reactions were always carried out in DMF as a solvent, under nitrogen atmosphere and at 50 °C during 48 hours. The reagents ratio was optimized as follows. The use of 2 eq. of the desired modification of the amines (X, i.e., 0.1 for 10 % modification), and 1 eq. of catalyst (ratio 1:2X:1X) did not lead to any modification. Then, the use of 1:20X:5X ratio worked for the reaction with N-Boc-DOOA, but not for N-Boc-hexaneamine and N-Boc-ethylenediamine modifications which could be explained due to the higher hydrophobicity of these two latest amines when compared to the N-Boc-DOOA. Finally, the use of the ratio 1:40X:5X, lead to successful results for N-Boc-DOOA as well as N-Boc-ethylenediamine. In the case of N-Boc hexaneamine, poor modifications were obtained in all reactions (never higher than 4 %). In fact, when the polymer backbone integrity was analyzed by GPC as well as by $^1$H-NMR (endcapping integrity), cleavage was observed in the ones modified with N-Boc-hexaneamine (Figure 4.3).

Figure 4.3. GPC trace from the polymers modified with N-Boc-hexaneamine through aminolysis reaction.
The degree of modification can be calculated with the aid of $^1$H-NMR spectra (Figure 4.4). It was calculated by comparison of the signal of the $\alpha$-carbon of the polymer backbone and the signal of the Boc-group. The integral of the proton in CH$\alpha$ corresponds with one proton, while the integral of the Boc-group should correspond to 9 protons for 100%. Moreover, signals corresponding to OCH$_2$C$_6$H$_5$ decrease in comparison with CH$\alpha$ due to aminolysis substitution. Figure 4.4 shows an example of characterization performed and the rest can be found in SI.

As mentioned before, GPC was used to check the integrity of polymer backbone after modification, as well as to ratify that $D$ of polymers after modification remains constant, what suggests an homogeneous substitution during reaction. For modifications with N-Boc-ethylendiamine (17a) as well as for N-Boc-DOOA (17c), results were consistent, the $D$’s were below 1.2 in all the cases (Table 4.1).

**Figure 4.4.** Example of poly(γ-benzyl L-glutamate-co-N-Boc-ethylendiamine glutamate) (17a) characterization. a) $^1$H-NMR spectrum in CDCl$_3$ with corresponding assignments. b) GPC trace in DMF/LiBr.
Table 4.1. Overview of results obtained within aminolysis reactions.

<table>
<thead>
<tr>
<th>N-Boc protected amine</th>
<th>% mod. (cal)</th>
<th>% mod. (real)</th>
<th>Y (%)</th>
<th>Mn(^b) kDa</th>
<th>MW(^b) kDa</th>
<th>D(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Boc-DOOA (17c)</td>
<td>5</td>
<td>4.7</td>
<td>57</td>
<td>20.8</td>
<td>22.6</td>
<td>1.09</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8</td>
<td>76</td>
<td>21.0</td>
<td>23.5</td>
<td>1.12</td>
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<tr>
<td></td>
<td>20</td>
<td>15</td>
<td>76</td>
<td>21.0</td>
<td>22.9</td>
<td>1.09</td>
</tr>
<tr>
<td>N-Boc-ethylendiamine (17a)</td>
<td>5</td>
<td>3.3</td>
<td>86</td>
<td>18.4</td>
<td>20.9</td>
<td>1.13</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10</td>
<td>94</td>
<td>18.8</td>
<td>22.4</td>
<td>1.19</td>
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<tr>
<td></td>
<td>20</td>
<td>21</td>
<td>75</td>
<td>19.6</td>
<td>22.8</td>
<td>1.16</td>
</tr>
<tr>
<td>N-Boc-hexaneamine (17b)</td>
<td>5</td>
<td>3.6</td>
<td>47</td>
<td>Degradation observed</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.4</td>
<td>47</td>
<td>Degradation observed</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>3</td>
<td>42</td>
<td>Degradation observed</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. Data obtained by \(^1\)H-NMR. b. Data obtained by GPC in DMF/LiBr.

CD spectra were also recorded from the samples to examine the conformations of the modified polymers in HFIP 0.1 mg·mL\(^{-1}\) at 20 °C (Figure 4.5). According to the CD spectra of modified polymers with N-Boc-DOOA (17c) and N-Boc-ethylendiamine (17a) with different percentages of modification, the α-helix is retained in both cases.

Figure 4.5. CD spectrum of poly(γ-benzyl L-glutamate-co-N-Boc-ethylendiamine glutamate) (17a) as example at 0.1 mg·mL\(^{-1}\) in HFIP and 20 °C. Similar profiles were obtained for N-Boc-DOOA modified polymers.

Additionally, and as mentioned before, these modifications could be used to produce reactive sites for further polymerizations. The Boc groups can be easily removed with HBF\(_4\)·Et\(_2\)O (Scheme 4.4) without damaging the end cap protecting group, neither the benzylic esters as confirmed by \(^1\)H-NMR (Figure 4.6). Although it might be
interesting to compare the structures produced by polymerization of these different polymers with potentially different degrees of branching due to the different percentages of modifications by aminolysis (reactive sites), the synthesis of graft co-polymers was not on the scope of this project.

Scheme 4.4. Boc-deprotection of N-Boc amine modified PBLGs.

Figure 4.6. $^1$H-NMR spectra in CDCl$_3$ of poly($\gamma$-benzyl L-glutamate-co-ethylendiamine glutamate) (18a), overlapping with the protected polymer (17a). The red circle highlights the disappearance of Boc signal.

As conclusion, it can be stated that, aminolysis reaction is a very straightforward reaction which could be used for direct modification of benzyl protected polyglutamates, of particular interest when reactive sites for posterior polymerizations are required. Nevertheless, the lack of versatility of this reaction (it depends on polymer stability upon reaction conditions, as well as on the amine used for modifications), the need for harsh conditions and a large excess of reagents, and the restriction to organic solvents were polyglutamate chains will be solubilized, make it a non-suitable choice for the introduction of functional reactive groups for orthogonal bioconjugations.
For that reason, there was a need for a versatile methodology which must fulfill some requirements such as: (i) the use of mild reaction conditions in order to keep the polymer backbone, (ii) high versatility, making possible the use of aqueous and organic solvents as well as the introduction of hydrophobic and hydrophilic moieties.

### 4.2.2. Post-polymerization modification of PGA with DMTMM.

Taking into account the points above discussed, in this work we pursued the development of a versatile post-polymerization modification methodology allowing the introduction of different functionalities such as alkyne, azide, reactive disulfides, protected amines and protected alcohols, or PEG moieties into PGA.

The selected strategy to achieve this goal was based on *in situ* activation of carboxyl functionalities of PGA by 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium (DMTMM) chloride (DMTMM-Cl) \((19a)\) in aqueous solution, and DMTMM-BF\(_4\) \((19b)\) salt for organic solvents. DMTMM-Cl \((19a)\) was synthesized according to literature. Briefly, 1 eq of 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT), was reacted with 0.93 eq of N-methylmorpholine (NMM) at r.t. (Scheme 4.5). A white solid appeared within several minutes that can be easily isolated. In the case of the BF\(_4\) form \((19b)\) for organic solvents, the chloride salt was displaced by the BF\(_4\) *in situ* by reacting CDMT and NMM in water, and addition of NaBF\(_4\). Therefore, modifications on the procedure reported by Michielsen and coworkers have been performed to adjust the protocol to PGA. Activation of the carboxylic acids within polymer backbone was carried out by using DMTMM-Cl \((19a)\) for aqueous solutions with PGA as a sodium salt, and DMTMM-BF\(_4\) \((19b)\) for organic solvents such as DMF with PGA as an acid (insoluble in aqueous media). The use of one or the other was based on different criteria such as the solubility of the selected amine, or taken into account practical reasons.

![Scheme 4.5. DMTMM-Cl synthesis](image)
depending on subsequent reaction needs (i.e. if PGA is needed as acid or as salt). Afterwards, the selected amine was added into the reaction mixture leading to the corresponding modified polymer. The reaction is described in Scheme 4.6.

After the required optimization, this strategy has been implemented herein to accomplish direct bioconjugation of contrast agents (i.e. DO3A), imaging probes (Cy5.5, Oregon green), drugs and also PEGylation. PEGylation is well known as the process of covalent attachment of PEG chains to another molecule, normally a drug or a therapeutic protein. The covalent attachment of PEG to a drug or therapeutic protein can "mask" it from the host's immune system (reduced immunogenicity and antigenicity) and increase its hydrodynamic size which prolongs circulation time by reducing its renal clearance. PEGylation can also provide water solubility to hydrophobic drugs and proteins. Therefore, the introduction of PEGylated units into polymer backbone not only allowed us the inclusion of a spacer between the polymer and the corresponding bioactive compound, but also can modify the polymer in vivo fate, biodistribution and therefore, the possible therapeutic application.

\[ \text{Scheme 4.6. Synthesis of reactive PGA derivatives (20) and their use for site-specific conjugation.} \]
The mechanism of action of DMTMM·Cl consists on the formation of an activated ester with the release of 4-methylmorpholine in a first step. This activated ester reacts with the corresponding amine compound. The pH of the reacting mixture is always adjusted to 8 in order to favor the amine form of the compound instead of the protonated one that coexist in equilibrium, with non nucleophilic character. In the case of DMTMM·BF$_4$ salt, the base DIEA was used for the same reasons. In Scheme 4.7, the mechanism of the reaction is depicted for the concrete case of the use of DMTMM·Cl.

**Scheme 4.7.** General scheme of post-polymerization modification of PGA sodium salt with DMTMM·Cl as activator of the carboxylic groups of the polymer backbone.

Once conversion was fully achieved (usually 16 hours), different procedures for purification of the resulting constructs were explored. In the case of reactions with DMTMM·BF$_4$ under organic solvents, a first step of ether precipitation was carried out. Then, polymer was suspended in an aqueous solution and dissolved by conversion into sodium salt form upon addition of sodium bicarbonate. After that, the aqueous solutions were purified using different methods. One of them was based on acid/base precipitation since PGA is insoluble as carboxylic acid but soluble as sodium salt. As all by-products were water soluble, the resulting polymers could be easily purified by precipitation in acidic water (pH~3-4) and re-dissolved with sodium bicarbonate (3x). After freeze-drying pure colorless PGA modified polymers (20) as sodium salt could be collected.

Additionally, ultrafiltration by using a membrane of MWCO 3000 was also explored since polymers with a higher content of mEG(6) (>30 %) cannot be precipitated under the above described conditions. Likewise precipitation, ultrafiltration yielded pure colorless
PGA derivatives after freeze-drying. Finally, size exclusion chromatography (SEC) using Sephadex G25 columns was also investigated as alternative purification method. In all three cases, the product was obtained pure and in comparable yields (>80 %), but, it could be said that ultrafiltration was preferable at large scale whereas Sephadex G25/PD10 columns were the preferred methodology at small scale. Once purified, percentages of modification achieved was determined by $^1$H-NMR.

Table 4.2. Characteristics of the polymers synthesized using DMTMM-Cl in aqueous solutions.

<table>
<thead>
<tr>
<th>PGA-prop (20a)</th>
<th>% Alkyne (calc.)</th>
<th>% Alkyne $^1$H-NMR</th>
<th>Mn kDa</th>
<th>MW kDa</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>20a_1</td>
<td>50</td>
<td>31</td>
<td>19.5</td>
<td>22.0</td>
<td>1.13</td>
</tr>
<tr>
<td>20a_2</td>
<td>30</td>
<td>18</td>
<td>18.8</td>
<td>21.3</td>
<td>1.13</td>
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<tr>
<td>20a_3</td>
<td>20</td>
<td>11</td>
<td>18.5</td>
<td>20.9</td>
<td>1.13</td>
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<tr>
<td>20a_4</td>
<td>10</td>
<td>6</td>
<td>18.2</td>
<td>20.6</td>
<td>1.13</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PGA-EG(2/6/9)N$_3$ (20b-d)</th>
<th>% Azide (calc.)</th>
<th>% Azide $^1$H-NMR</th>
<th>Mn kDa</th>
<th>MW kDa</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>20b_1 (n=2)</td>
<td>60</td>
<td>30</td>
<td>26.2</td>
<td>29.6</td>
<td>1.13</td>
</tr>
<tr>
<td>20b_2 (n=2)</td>
<td>20</td>
<td>15</td>
<td>22.3</td>
<td>25.2</td>
<td>1.13</td>
</tr>
<tr>
<td>20b_3 (n=2)</td>
<td>15</td>
<td>11</td>
<td>21.0</td>
<td>23.7</td>
<td>1.13</td>
</tr>
<tr>
<td>20c_1 (n=6)</td>
<td>50</td>
<td>34</td>
<td>33.6</td>
<td>38.0</td>
<td>1.13</td>
</tr>
<tr>
<td>20c_2 (n=6)</td>
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<td>18</td>
<td>26.2</td>
<td>29.6</td>
<td>1.13</td>
</tr>
<tr>
<td>20c_3 (n=6)</td>
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<td>16</td>
<td>25.3</td>
<td>28.5</td>
<td>1.13</td>
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<tr>
<td>20d_1 (n=9)</td>
<td>50</td>
<td>28</td>
<td>40.7</td>
<td>46.0</td>
<td>1.13</td>
</tr>
<tr>
<td>20d_2 (n=9)</td>
<td>20</td>
<td>16</td>
<td>33.3</td>
<td>37.6</td>
<td>1.13</td>
</tr>
<tr>
<td>20d_3 (n=9)</td>
<td>10</td>
<td>9</td>
<td>29.0</td>
<td>32.8</td>
<td>1.13</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PGA-mEG(6) (20e)</th>
<th>% EG (calc.)</th>
<th>% EG $^1$H-NMR</th>
<th>Mn kDa</th>
<th>MW kDa</th>
<th>D</th>
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<td>49</td>
<td>39.7</td>
<td>45.0</td>
<td>1.13</td>
</tr>
<tr>
<td>20e_4</td>
<td>60</td>
<td>30</td>
<td>31.3</td>
<td>35.3</td>
<td>1.13</td>
</tr>
<tr>
<td>20e_5</td>
<td>20</td>
<td>8</td>
<td>21.5</td>
<td>24.3</td>
<td>1.13</td>
</tr>
</tbody>
</table>
### Table 4.3. Characteristics of the polymers synthesized using DMTMM·BF₄ in organic solutions (DMF).

<table>
<thead>
<tr>
<th>PGA-SS-2TP (20f)</th>
<th>% Cysteamine2TP (calc.)</th>
<th>% Cysteamine2TP ¹H-NMR</th>
<th>Mn kDa</th>
<th>MW kDa</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>20f_1</td>
<td>50</td>
<td>32</td>
<td>23.6</td>
<td>27.2</td>
<td>1.15</td>
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<td>20f_2</td>
<td>35</td>
<td>30</td>
<td>23.4</td>
<td>26.9</td>
<td>1.15</td>
</tr>
<tr>
<td>20f_3</td>
<td>10</td>
<td>9</td>
<td>23.0</td>
<td>26.5</td>
<td>1.15</td>
</tr>
<tr>
<td>20f_4</td>
<td>5</td>
<td>4</td>
<td>23.0</td>
<td>26.3</td>
<td>1.15</td>
</tr>
</tbody>
</table>

*PGA-prop (20a): poly(glutamic acid-co-propargyl glutamate); PGA-EG(2/6/9)N₃ (20b-d): poly(glutamic acid-co-EG(n)N₃ glutamate); PGA-mEG(6) (20e): poly(glutamic acid-co-EG(6)OMe glutamate); PGA-SS-2TP: poly(glutamic acid-co-cysteamine2TP glutamate)

<table>
<thead>
<tr>
<th>PGA-N-Boc-ethylendiamine (20g)</th>
<th>% N-Boc-ethylendiamine (calc.)</th>
<th>% N-Boc-ethylendiamine ¹H-NMR</th>
<th>Mn kDa</th>
<th>MW kDa</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>20g_1</td>
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<td>54</td>
<td>18.1</td>
<td>21.5</td>
<td>1.19</td>
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<td>20</td>
<td>17</td>
<td>14.1</td>
<td>16.7</td>
<td>1.19</td>
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<td>20g_3</td>
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<td>12</td>
<td>13.0</td>
<td>15.5</td>
<td>1.19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PGA-N-Boc-DOOA (20h)</th>
<th>% N-Boc-DOOA (calc.)</th>
<th>% N-Boc-DOOA ¹H-NMR</th>
<th>Mn kDa</th>
<th>MW kDa</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>20h_1</td>
<td>50</td>
<td>62</td>
<td>13.4</td>
<td>16.0</td>
<td>1.19</td>
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<td>20h_2</td>
<td>20</td>
<td>17</td>
<td>12.8</td>
<td>15.2</td>
<td>1.19</td>
</tr>
<tr>
<td>20h_3</td>
<td>10</td>
<td>5</td>
<td>12.5</td>
<td>14.8</td>
<td>1.19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PGA-prop (20a)</th>
<th>% Alkyne (calc.)</th>
<th>% Alkyne ¹H-NMR</th>
<th>Mn kDa</th>
<th>MW kDa</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>20a_5</td>
<td>75</td>
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<td>15.6</td>
<td>18.1</td>
<td>1.16</td>
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<td>20a_6</td>
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<td>30</td>
<td>15.5</td>
<td>18.0</td>
<td>1.16</td>
</tr>
<tr>
<td>20a_7</td>
<td>35</td>
<td>22</td>
<td>15.4</td>
<td>17.9</td>
<td>1.16</td>
</tr>
<tr>
<td>20a_8</td>
<td>20</td>
<td>16</td>
<td>15.3</td>
<td>17.8</td>
<td>1.16</td>
</tr>
<tr>
<td>20a_9</td>
<td>10</td>
<td>9</td>
<td>15.2</td>
<td>17.7</td>
<td>1.16</td>
</tr>
</tbody>
</table>

*PGA-N-Boc-ethylendiamine (20g): poly(glutamic acid-co-N-Boc-ethylendiamine glutamate); PGA-N-Boc-DOOA (20h): poly(glutamic acid-co-N-Boc-DOOA glutamate).
Figure 4.7. $^1$H-NMR spectrum (D$_2$O) of poly(glutamic acid-co-propargyl glutamate) (20a).

Figure 4.8. $^1$H-NMR spectrum (D$_2$O) of poly(glutamic acid-co-EG(n)N$_3$ glutamate) (20b-d).

Figure 4.9. $^1$H-NMR spectrum (D$_2$O) of poly(glutamic acid-co-EG(6)OMe glutamate) (20e).
Figure 4.10. $^1$H-NMR spectrum (D$_2$O) of poly(glutamic acid-co-cysteamine2TP glutamate) (20f).

Figure 4.11. $^1$H-NMR spectrum (D$_2$O) of poly(glutamic acid-co-N-Boc-ethylendiamine glutamate) (20g).

Figure 4.12. $^1$H-NMR spectrum (D$_2$O) of poly(glutamic acid-co-N-Boc-DOOA glutamate) (20h).
In PGA-prop (20a), alkyne content was quantified by integration of the signals from propargyl alkyne (3.81 ppm CH$_2$- and 2.48 ppm acetylenic proton) in relation to signals of PGA (2.40-1.52 ppm PGA side chain and 4.01 ppm α-proton) from $^1$H-NMR spectra in D$_2$O (see Figure 4.7). In the case of PGA-mEG(6) (20e), and PGA-EG(2/6/9)N$_3$ (20b-d), the oligoethylene glycol content was quantified by integration of the corresponding signals of ethylene glycol unit in $^1$H-NMR spectra in D$_2$O, in comparison with the corresponding signals of PGA (see Figure 4.8). The peaks at 3.26, 3.35 and 3.48 ppm correspond to -CH$_2$- protons near to amide group and azide group. The big signal at 3.55 ppm belongs to -CH$_2$- of oligoethylene glycol chain plus one of the triplet corresponding to terminal CH$_2$ protons (Figure 4.9). This latter signal changes in its integration whenever modification is done with EG$_2$ (2 units of ethylene glycol in the inside chain which correspond with 10 protons) or EG$_6$ (which corresponds with 50 protons). In the case of modifications with the N-Boc-ethylendiamine, peaks at 3.21 ppm (-CH$_2$-) and 3.11 ppm (-CH$_2$-) chemical shifts and more precisely the peak of the Boc signal at 1.35 ppm (-CH$_3$)$_3$ were used for quantifications (Figure 4.11). Similarly, in the case of N-Boc-DOOA modifications, the big signal at 3.52 ppm corresponds to the -CH$_2$- of the oligoethylene glycol unit, and peaks at 3.32 and 3.20 ppm were assigned to -CH$_2$- close to amide groups (Figure 4.12). As for N-Boc-ethylendiamine modified products (20g-h), Boc signal at 1.35 ppm was used for quantification of the percentage of substitution. Finally, in modifications with pyridyl dithiol cysteamine (cysteamine2TP) (20f), the ratio of the integrals of pyridine ring 8.4 ppm (CH$_{aromat}$), 7.84 ppm (CH$_{aromat}$), and 7.28 ppm (CH$_{aromat}$) to the integral of α-proton of PGA was used to calculate the percentage of derivatization. Peaks at 3.48 and 2.95 ppm correspond to -CH$_2$- of cysteamine as shown in Figure 4.10.

Thus, in comparison with PGA signals, % of substitution of each polymer could be easily calculated. Characteristics of all PGA derivatives are displayed in Tables 4.2 and 4.3.

Overall, linking efficiencies are comparable or even better than post-polymerization modification of poly(acrylic acid) PAA as reported by Michielsen and coworkers $^{20}$ and are around 60-80 % when low degrees of substitution are desired. Only in some cases of sterically demanding amines as well as for solubility reasons, reduced linking efficiency of 40-50 % at high degrees of substitution were obtained.
4.2.3. Study of the influence of the percentage of modification in polymer size and solution conformation.

Once the protocol for post-polymerization modification reactions was optimized, the influence of different percentages of substitutions was tested in terms of size, solution conformation, and biodegradability of the newly synthesized constructs. Some representative examples will be illustrated in the following paragraphs.

CD spectra in PB 0.1 M (phosphate buffer without saline) at 37 °C and 0.1 mg·mL⁻¹ were recorded in order to assess changes (if they were) in secondary structures of modified polymers (Figure 4.13). PB is used in order to keep constant the pH avoiding problems caused in voltage when measurements are done in PBS buffer, due to salt content. As it can be seen in Figure 4.13, all polymers analyzed showed the typical random coil conformation as for linear PGA without significant changes in the secondary structure.

![Figure 4.13](image1.png)

**Figure 4.13.** CD spectra of a) Different PGA-prop (20a), b) PGA-N-Boc-DOOA (20h), in PB at 37 °C and at [C] of 0.1 mg·mL⁻¹.

The effect of polymer concentration and temperature of the analysis was also checked with some examples as it can be seen in Figure 4.14. From results obtained no big influence due to the temperature of the analysis was found. On the other hand, CD plots changed with the concentration used, being constant at concentrations from 0.05-0.5 mg·mL⁻¹. Above that concentration, a red shift could be observed for the negative band from 196 to 203, this might be due to partial transition of the polymeric backbone random coil to a more organized conformation, however, further studies on this regard should be done in the future to unravel this conformational issue.
Figure 4.14. CD spectra of A) PGA-EG(6)N₃ (20c), in PB at [C] of 0.1 mg·mL⁻¹ and different temperatures, B) PGA-N-Boc-DOOA (20h), in PB at 37 °C and at different concentrations.

In the case of Boc protected amines, Boc groups were removed with a mixture of trifluoroacetic acid/dichloromethane TFA/CH₂Cl₂ (40:60), in order to check the influence on secondary structure of the deprotected amines with the pH. For that purpose, CD spectra were recorded at different pHs using polymers with different percentage of modifications. In Figure 4.15, examples with DOOA modified PGAs are shown. As it can be seen in CD plots, pH influence on secondary structure seems to be slightly higher when amine percentage of modification is greater. However, no major changes in secondary structure are found at physiological pH, what is significantly relevant regarding their potential use for biomedical applications.

Figure 4.15. CD spectra in PBS at [C] of 0.1 mg·mL⁻¹ at different pHs, of a) PGA-DOOA 10 % (20j), b) PGA-DOOA 20 % (20j), both polymers bearing free amine groups.

The solution conformation of several PGA modified polymers was analyzed by Small Angle Neutron Scattering (SANS). From the
curve fitting obtained by means of FISH software, it could be concluded that solution conformation of all constructs corresponded to “capped rods” with two different radius ($R_a$ and $R_b$). The values obtained for different PGAs bearing EG(n) azide groups with similar degree of modification but different EG length showed smaller rods (small $R_a$ and $R_b$) for EG(6) when compared with EG(2) and EG(9). This could be in concordance with the degradation rate obtained, higher for EG(6) (see next section), and its posterior biological output (see Chapter 8). In the case of ethyldiamine and DOOA modified polymers, $R_a$, remained more or less constant independently of degree of modification. Nevertheless, longer rods (higher $R_b$) where obtained when the percentage of functionalization was increased.

**Table 4.4.** Summary of the results obtained from SANS characterization.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% mod</th>
<th>MW kDa</th>
<th>SANS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGA-EG(2) (20b)</td>
<td>32</td>
<td>32.3</td>
<td>1.40</td>
</tr>
<tr>
<td>PGA-EG(6) (20c)</td>
<td>27</td>
<td>36.4</td>
<td>1.00</td>
</tr>
<tr>
<td>PGA-EG(9) (20d)</td>
<td>28</td>
<td>40.7</td>
<td>1.40</td>
</tr>
<tr>
<td>PGA-ethylendiamine (20i)</td>
<td>12</td>
<td>12.7</td>
<td>1.45</td>
</tr>
<tr>
<td>PGA-DOOA (20j)</td>
<td>54</td>
<td>13.4</td>
<td>1.40</td>
</tr>
<tr>
<td>PGA-DOOA (20j)</td>
<td>5</td>
<td>13.0</td>
<td>1.40</td>
</tr>
<tr>
<td>PGA-DOOA (20j)</td>
<td>17</td>
<td>14.1</td>
<td>1.32</td>
</tr>
</tbody>
</table>

It has to be mentioned that the protocol of post-polymerization modification was successfully applied in the introduction of functional moieties in the case of hybrid di-block copolymers and star-shaped polymers as well, highlighting its versatility.

**4.2.4. Click Chemistry model couplings with small molecules.**

The purpose of the introduction of functional moieties into the polymer backbone was their posterior use for bioconjugations in orthogonal reactions. Conjugation strategies can be divided into two categories: chemical\(^{11, 12}\) and biological ligation.\(^{13}\) Among chemical strategies, Copper Catalyzed Azide/Alkyne Cycloaddition (CuAAC) formally known as Cu\(^1\)-mediated Huisgen 1,3-dipolar cycloaddition reaction has been widely applied\(^{14}\) since it is a versatile but easy to perform methodology for site-specific chemical ligation of complex
molecules to polymers. Moreover, this class of reaction is atom efficient, broad in scope, and it provides high yields under relatively mild conditions in aqueous as well as organic media; it presents high tolerance of functional groups (except for those that are self-reactive (e.g. azides and alkynes)); and in addition, reaction can be developed at various types of interfaces, such as solid/liquid, liquid/liquid, or even solid/solid interfaces. Therefore, the characteristics of this reaction completely fits with the definition of Barry Sharpless\(^\text{12}\): “A click reaction must be modular, wide in scope, high yielding, create only inoffensive by-products (that can be removed without chromatography), are stereospecific, simple to perform and that require benign or easily removed solvent.”

Apart from the advantages pointed out, it has to be noticed that the process is thermodynamic and kinetically favorable (50 and 26 kcal/mol, respectively), regiospecific (whereas the classic Huisgen 1,3-dipolar cycloaddition often gives mixtures of regioisomers, the copper-catalyzed reaction allows the synthesis of the 1,4-disubstituted regioisomers specifically), and chemoselective, with a $10^7$ rate enhancement over non-catalyzed reaction.

This type of reactions have been widely used for many applications of post-polymerization modification to couple telechelic polymers with differently functionalized chain ends (yielding di-and triblock copolymers), to prepare graft copolymers (by grafting from or to methods), to conjugate synthetic polymers to biomacromolecules, to cross-link polymers or to achieve complex architectures including dendrimers, hyperbranched polymers and core-cross-linked star polymers.

These newly synthesized PGA derivatives have been used to develop a conjugation protocol for the attachment of either hydrophobic or hydrophilic molecules employing the CuAAC methodology (Scheme 4.8).
Scheme 4.8. Click reactions toward PGA-prop copolymer and PEG-EG(n)N₃ as example.

From the different catalyst known, the system CuSO₄·5 H₂O/sodium ascorbate is widely used when reaction takes places in aqueous solutions, and CuBr/N,N,N',N',N"-pentamethyldiethylenetriamine (PMDTA) when organic solvents are used. The active specie is Copper (I), which is easily oxidized to Copper (II) in aqueous solutions as its potential of reduction predicts:

\[
E^\circ_{O_2/OH} = +0.40 \text{V} \\
E^\circ_{Cu^{2+}/Cu^{+}} = 0.16 \text{V} \\
E^T = E^\circ_{O_2/OH} - E^\circ_{Cu^{2+}/Cu^{+}} = +0.24 \text{V} \quad (\text{favorable reaction when the } E \text{ is positive})
\]

For that reason, systems were always carefully degassed prior to addition of the catalyst and afterwards as well.

In the case of the catalyst CuSO₄·5 H₂O/sodium ascorbate, the active specie Cu (I) is formed “in situ” with the reductive power of ascorbate (known as a potent antioxidant). An excess of reductive agent is always added in order to prevent re-oxidation of the catalyst. In the case of CuBr/PMDTA, the active specie does not need to be formed in situ. PMDTA is a basic, bulky, and flexible, tridentate ligand which stabilizes Cu(I) in solution. Its basic character is required as a base is necessary in the first step to deprotonate the acetylene and generate a Cu acetylide intermediate. When reaction takes place in water, it is the water acting as a base.

The coupling reaction was monitored by FT-IR by disappearance of the asymmetric vibration band. Figure 4.16 displays a representative FT-IR spectrum. Time zero represents the pure azide in absence of the corresponding alkyne reagent. As it can be seen, the
asymmetric vibration band was observed at 2485 cm\(^{-1}\), this band was disappearing with time up to 48 h when it was non-longer detectable. Finally, the polymers were purified according to the above-mentioned methodologies for post-polymerization modification. In all cases colorless polymers could be obtained after freeze drying from solution.

**Figure 4.16.** Representative FT-IR-spectra of a CuAAC reaction between a polymeric azide and a low molecular weight alkyne.

Linking efficiency was determined according to \(^1\)H-NMR spectra of the resulting compound by integration of the peak at 7.8 ppm corresponding to the proton of the triazole ring newly formed. In Table 4.5, optimization of reaction conditions is summarized looking for an acceptable % of coupling and yield in aqueous conditions and in organic solvents. Reaction conditions have been optimized to nearly quantitative linking efficiencies by screening different solvents, temperatures, catalysts and concentrations (see Table 4.5). Besides monitoring the conversion of azides by IR, linking efficiency of the CuAAC reaction was also monitored by \(^1\)H-NMR by correlating the integration of triazole proton signal, which is formed during the CuAAC, with the \(\alpha\)-proton of PGA (see Figure 4.17).

**Figure 4.17.** \(^1\)H-NMR spectrum (D\(_2\)O) of CuAAC reaction product between PGA-prop (20a) and NH\(_2\)-EG(2)N\(_3\). The peak surrounded by a circle associates to the proton of triazole ring.
### Table 4.5. Reaction conditions used during CuAAC.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Solv.</th>
<th>$T$ °C</th>
<th>CuSO$_4$/NaA.</th>
<th>eq.$^a$</th>
<th>LE$^b$ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H$_2$O</td>
<td>25</td>
<td>0.3/0.5</td>
<td>3(20%)</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>H$_2$O</td>
<td>40</td>
<td>0.3/0.5</td>
<td>3(20%)</td>
<td>88</td>
</tr>
<tr>
<td>3</td>
<td>H$_2$O</td>
<td>40</td>
<td>1/5</td>
<td>6(40%)</td>
<td>97</td>
</tr>
<tr>
<td>4</td>
<td>DMF/H$_2$O</td>
<td>40</td>
<td>0.3/0.5</td>
<td>6(40%)</td>
<td>48</td>
</tr>
<tr>
<td>5</td>
<td>DMF/H$_2$O</td>
<td>40</td>
<td>1/5</td>
<td>2(9%)</td>
<td>67</td>
</tr>
<tr>
<td>6</td>
<td>H$_2$O</td>
<td>40</td>
<td>1/5</td>
<td>6(40%)</td>
<td>96</td>
</tr>
<tr>
<td>7</td>
<td>H$_2$O</td>
<td>25</td>
<td>0.3/0.5</td>
<td>3(14%)</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>H$_2$O</td>
<td>40</td>
<td>0.3/0.5</td>
<td>3(14%)</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>DMF/H$_2$O</td>
<td>60</td>
<td>1/5</td>
<td>4(18%)</td>
<td>33</td>
</tr>
<tr>
<td>10</td>
<td>DMF/H$_2$O</td>
<td>60</td>
<td>1/10</td>
<td>2(9%)</td>
<td>55</td>
</tr>
<tr>
<td>11</td>
<td>H$_2$O</td>
<td>25</td>
<td>0.3/0.5</td>
<td>3(14%)</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>H$_2$O</td>
<td>40</td>
<td>0.3/0.5</td>
<td>3(14%)</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>DMF/H$_2$O</td>
<td>40</td>
<td>1/5</td>
<td>2(9%)</td>
<td>67</td>
</tr>
<tr>
<td>14</td>
<td>DMF/H$_2$O</td>
<td>40</td>
<td>1/5</td>
<td>2.4 (11%)</td>
<td>99</td>
</tr>
</tbody>
</table>

a. eq. Equivalents of clickable molecule per polymer. The % corresponds with the % calculated of modified GAU (bearing N$_3$ or alkyne depending on the polymer used) that will react theoretically. b. L.E: Linking efficiency calculated taking into account the previous percentage, and the % achieved.

Summarizing, CuAAC reaction on PGA derivatives was achieved in aqueous as well as organic solutions. The use of
CuSO$_4$/Sodium ascorbate (1/5) as catalyst at 40 °C was the optimal condition for CuAAC in aqueous media yielding to quantitative conversions. The use of DMF/H$_2$O mixtures (4:1) with CuSO$_4$/Sodium ascorbate (1/5) also allowed conjugation of hydrophobic but DMF soluble peptides/drugs. The linking efficiency was always lower when DMF/H$_2$O mixtures were used (55-67 %), but acceptable, reproducible and predictable. This effect was attributed to intra-chain aggregation during conjugation of hydrophobic compounds making it increasingly difficult to reach the conjugation site due to steric hindrance.

**4.2.5. In vitro biological characterization.**

As for star-shaped polymers described in previous chapters, degradation of the modified polymers under presence of lysosomal enzyme cathepsin B was studied. In all cases, polymers were incubated in presence of cathepsin B, at pH 6 in acetate buffer and EDTA, under reductive conditions by using DTT in order to favor enzyme activity. Aliquots were collected over time and analyzed by GPC under aqueous conditions.

On one hand, the influence of % of modification of one concrete moiety was tested by using PGA-prop (20a). Figure 4.18 shows the plots of some examples of degradation profiles from 5, 10 and 20 % of propargylamine modifications of linear PGAs. According to the GPC traces of the degraded samples over time, differences among the polymers where found. Concretely, degradation rate was greater upon polymer modification grade (at least in the case of PGA-prop). This finding is in good agreement with literature where Kopecek and collegues demonstrated that both, the incorporation of hydrophobic co-monomers and modification of the carboxylic groups of glutamic acid side-chains with hydrophobic groups increased the lysosomal degradability of the co-polymers.$^{15}$
The same experiment was performed using star-shaped polymers, bearing 10 and 20% of propargylamine moieties showing similar results as those found for the linear counterparts.

On the other hand, the influence of the introduced moiety size (length) for a fixed percentage of polymer modification (~30%) was studied by using PGA-EG(n)N₃ with different EG number of units (20b-d).

**Figure 4.18.** GPC traces showing kinetics of PGA-prop (20a) degradation upon Cathepsin B incubation.
As it can be observed in Figure 4.19, polymer degradation profile changed depending on EG length. In all cases, percentage of modification was around 30 %, however, for EG(9), no degradation was observed upon incubation with cathepsin B. This result could be attributed to the fact that EG(9) pendant groups might be shielding cathepsin B activity due to steric hindrance and consequently diminished enzyme accessibility. When EG(2) and EG(6), are compared, it can be observed that polymers modified with EG(6) moieties were degraded faster than EG(2) modified polymers. This could be again attributed to steric hindrance issues caused by the use of a shorter spacer which could limit the accessibility of the enzyme to the polymer backbone. More importantly, these findings could be directly correlated with a different biological performance of the modified polymers when used as Near InfraRed (NIR) fluorescence smart probes. As it will be explained in further detail in Chapter 8, the polymeric probe based on the EG(6) spacer showed the best performance in vitro.

Finally, cell viability of a set of polyglutamic acid polymers (star-shaped and linear PGAs) modified by post-polymerization modification techniques with propargylamine residues was tested against SHSY5Y cell line. This time, in order to compare with propargylamine moiety related cytotoxicity, concentrations tested were according to % weight of propargylamine introduced in the polymer chains. As it can be observed in Figure 4.20, no toxicity was found at the concentrations tested 72 hours of treatments.
4.3. CONCLUSIONS.

In this chapter, a versatile methodology for the synthesis of polyglutamic acid (PGA) derivatives bearing orthogonal reactive sites, e.g. azides, alkynes, reactive disulfides, protected amines, etc. in combination with the carboxyl functionalities of PGA has been reported. Additionally, the linkage of water as well as DMF soluble compounds to PGA has been also efficiently carried out. This could be used for site-specific conjugation of a variety of bioactive agents of different nature (i.e. peptides, proteins, drugs). Therefore, the described protocols enable a versatile and controlled approach towards the development of next generation PGA-based polymer therapeutics.

Figure 4.20. Cell viability assay of a) 4 star-shaped PGA-prop (St-prop) against SHSY5Y cell line, 72 hours of treatments. b) 3 linear PGA-propargyl and propargylamine moiety against SHSY5Y cell line, 72 hours of treatments. n > 3, mean ± SEM.
From \textit{in vitro} results, several conclusions can be drawn. As it could be expected, degree of functionalization as well as nature of the motifs added within the polymer backbone do affect the degradation rate of the final construct. Those parameters should be then taken into account when talking about biodegradability and biocompatibility of polymeric systems, since the nature of the final structure will drive the final polymer properties. Upon modification, cell viability (72 h MTS assays) of the derivatized polyglutamates was not compromised up to a concentration of 10 $\mu$M propargyl eq. against SHSY5Y cells.

4.4. MATERIALS AND METHODS.

4.4.1. Materials.

All chemicals were synthesis grade, obtained from Aldrich and used without further purification, unless otherwise indicated. All EG amines were obtained from Iris Biotech GMB. All solvents were of analytical grade. Preparative SEC was performed using Sephadex G-25 superfine from GE as well as PD MiniTrap G-10 ™ columns containing 2.1 mL of Sephadex™ G-10.

4.4.2. Characterization techniques.

NMR spectroscopy, GPC, CD, DLS, and IR were performed using the equipment and techniques according to Sections 2.4.2 and 3.4.2.

4.4.2.1. Small Angle Neutron Scattering (SANS).

Small-angle neutron scattering (SANS) experiments were performed on the D11 difractometer at the Institute Laue-Langevin in Grenoble (France) by our collaborators from the School of Chemistry in Cardiff University (Cardiff, UK). Scattering data are expressed in terms of the scattering vector, Q, which is given by $Q = 4\pi\lambda \cdot \sin(\Theta/2)$ where $\lambda$ is wavelength and $\Theta$ the angle at which neutrons are scattered. The incident neutron wavelengths were variable between 4.5 and 40 Å, giving accessible Q-ranges of 0.0003 to 1 Å$^{-1}$ using four different sample-detector distances. The detector may be placed at any distance between 1.2 and 39 metres from the sample position. Sample solutions were prepared at 10 mg·mL$^{-1}$ on a 1 g scale in D$_2$O (pH 5.5, 0.1 M phosphate buffer) and placed in 2mm path length quartz cells, mounted in a sample changer thermostated at 37 °C (± 0.2). Data were corrected
for transmission intensity, electronic background and normalized against a flat scatter according to standard procedures for the instrument. The obtained scattering profiles $I(Q) \, vs. \, Q$ were analysed according to $I(Q) \propto \Phi \, V_p \, P(Q) \, S(Q) + Binc$ where $\Phi$ is the volume fraction and $V_p$ the particle volume. Binc is the incoherent scattering, generally dominated by the protons present in the sample, which usually depends on the concentration of polymer. The FISH modelling suite was used for the analysis. FISH incorporates parameterized form factors, $P(Q)$ and structure factors, $S(Q)$, to describe the dimensions of the scattering particle and inter-particle interaction.

4.4.3. Protocols.
Degradations under the presence of cathepsin B as well as MTS assays for cell viability were performed according to protocols described in Chapter 3.

4.4.3.1. End capping of PBLG with trimethylacetylchloride.
In a two-neck round bottom flask of 50 mL fitted with a stir bar, an outlet and inlet of $N_2$ and two septums, 2.74 g PBLG (0.151 mmol 18177 g·mol$^{-1}$, 1.14 $\Delta$, 1 eq.) was dissolved in 5 mL of anh. DMF. After that 132 $\mu$L of DIEA, (0.756 mmol, 129.24 g·mol$^{-1}$, 0.742 g·mL$^{-1}$, 5 eq.) were added to the flask, followed by 93 $\mu$L of end capping agent, trimethylacetylchloride (0.756 mmol, 120.58 g·mol$^{-1}$, 0.979 g·mL$^{-1}$, 5 eq.). Reaction was left to proceed for 4 hours. After that time, the solution was precipitated in an excess of cold diethyl ether and filtered off. The resultant polymer was washed with ddH$_2$O. Finally, the polymer was obtained as a dried white solid after lyophilization. Yield: 85 %. $^1$H-NMR $\delta_H$ (300 MHz, CDCl$_3$) 7.28 (5H, m) 5.06 (2H, m), 3.96 (1H, m), 2.54 (2H, m), 2.29 (2H, m), 1.19 (s, 9/83H, s).

4.4.3.2. General procedure for the post-polymerization modification of PBLG by aminolysis.
4.4.3.2.1. Synthesis of poly(γ-benzyl L-glutamate-co-N-Boc-DOOA glutamate).
In a two-neck round bottom flask of 25 mL fitted with a stir bar, an outlet and inlet of $N_2$ and two septums, 200 mg of end capped polymer (0.913 mmol GAU, 1 eq.) were dissolved in 6 mL of anh. DMF. Then 20 eq. of the desired modification of N-Boc-DOOA were added in 2 mL of DMF under nitrogen atmosphere (i.e. 226.8 mg, 0.913
mmol for an amine modification of 5 %). After that, 21.7 mg of 2-hydroxypyridine (0.228 mmol, 5 eq. from the desired percentage of modification) where added in two more mL of anh. DMF. Reaction was then left to proceed under N₂ flow at 50 °C for 48 hours. After that time, 5 mL of THF were added to the flask in order to help with precipitation process. The solution was then precipitated in a large excess of cold diethyl ether, filtered off and washed with ddH₂O. Finally, the polymer was obtained as a white solid after lyophilization. Yields: 60 - 90 % ¹H-NMR δ_H (300 MHz, CDCl₃) 7.29 (5H, m), 4.99 (2H, m), 3.78 (1H, m), 3.61-3.44 (8xH, m), 3.39-3.31 (2xH, m), 3.25 (2xH, s), 2.54 (2H, m), 2.16 (2H, m), 1.38 (9H, s), 1.19 (9/83H, s). x: percentage of modification

4.4.3.2.2. Synthesis of poly(γ-benzyl L-glutamate-co-N-Boc-ethylenediamine glutamate).

In a two-neck round bottom flask of 25 mL fitted with a stir bar, an outlet and inlet of N₂ and two septums, 200 mg of end capped polymer (0.913 mmol GAU, 1 eq.) were dissolved in 6 mL of anh. DMF. Then 40 eq. of the desired modification of N-Boc-ethylenediamine were added in 2 mL of DMF under nitrogen atmosphere (i.e. 292.6 mg, 1.826 mmol for an amine modification of 5 %). After that, 21.7 mg of 2-hydroxypyridine (0.228 mmol, 5 eq. from the desired percentage of modification) where added in two more mL of anh. DMF. Reaction was then left to proceed under N₂ flow at 50 °C for 48 hours. After that time, 5 mL of THF were added to the flask in order to help with the precipitation process. The solution was then precipitated in a large excess of cold diethyl ether, filtered off and washed with ddH₂O. Finally, the polymer was obtained as a white solid after freeze-drying. Yield: 70-90 % ¹H-NMR δ_H (300 MHz, CDCl₃) 7.29 (5H, m), 4.97 (2H, m), 3.86 (1H, m), 3.42 (2xH, m), 3.15 (2xH, m), 2.50 (2H, m), 2.17 (2H, m), 1.34 (9H, s), 1.19 (9/83H, s). x: percentage of modification

4.4.3.3. Boc removal from modified PBLGs.

To a round one neck bottom flask, fitted with a stir bar, 30 mg of N-Boc modified polymer was dissolved in 2 mL of chloroform. After that 10 eq. of HBF₄·Et₂O (0.0625 mmol, 1.18 g·mL⁻¹), per Boc group of polymer, were added dissolved in 1 mL of chloroform. The
reaction was left to proceed for 3 hours. After that time, the polymer was precipitated in a large excess of cold diethyl ether, washed with diethyl ether (3x) and filtered off. A white solid was obtained after freeze-drying.

Yields: 80-90 % ¹H-NMR δ_H (300 MHz, CDCl₃):
a) Poly(γ-benzyl L-glutamate-co-N-Boc-ethylendiamine glutamate) 7.29 (5H, m), 4.97 (2H, m), 3.86 (1H, m), 3.43 (2xH, m), 3.29 (2xH, m), 2.62 (2H, m), 2.24 (2H, m), 1.19 (9/83H, s).
b) Poly(γ-benzyl L-glutamate-co-N-Boc-DOOA glutamate) 7.29 (5H, m), 4.99 (2H, m), 3.78 (1H, m), 3.61-3.44 (8xH, m), 3.39-3.31 (2xH, m), 3.25 (2xH, s), 2.54 (2H, m), 2.16 (2H, m), 1.19 (9/83H, s). x: percentage of modification.

4.4.3.4. Method for post-polymerization modification of PGA using DMTMM.

4.4.3.4.1. DMTMM·Cl for aqueous solutions.

In a one neck round bottom flask fitted with a stir bar and a stopper, 200 mg of PGA (1.55 mol GAU, 1 eq.) were suspended in 10 mL of ddH₂O. Afterwards the eq. for the desired modification of DMTMM·Cl were added dissolved in 5 mL of ddH₂O (i.e. 128.7 mg, 0.465 mmol, 0.3 eq. for 30 % modification). After 10 minutes (0.93 mmol 0.6 eq. for 30 % modification) of the corresponding amine were added and the pH was adjusted to 8 by adding some drops of 1 M NaHCO₃ solution. Reaction was allowed to proceed overnight stirring at r.t. After this, as all by products are soluble in acid aqueous solution, either acid/base precipitation, dialysis (Vivaspin® MWCO 3000 Da), or size exclusion chromatography with Sephadex G25 columns, was done in order to purify the copolymer. A colorless amorphous solid was obtained after freeze-drying.

Yields: 80-90 %. ¹H-NMR δ_H (300 MHz, D₂O):
a) Poly(glutamic acid-co-propargyl glutamate): 4.30-4.02 (1H, m), 3.81 (2xH, s), 2.48 (1xH, s), 2.35-2.02 (2H, m), 2.01-1.65 (2H, m). x: percentage of modification.
b) Poly(glutamic acid-co-EG(6)OMe glutamate): 4.33-4.19 (1H, m), 3.95-3.78 (20xH, m), 3.77-3.49 (2xH, m), 3.34 (3xH, s), 2.41-1.76 (4H, m). x: percentage of modification.
c) Poly(glutamic acid-co-EG(n)N₃ glutamate): 4.28-4.07 (1H, m), 3.65-3.51 (RxH, m), 3.48 (2xH, t), 3.40-3.30 (2xH, m), 3.25 (2xH, d), 2.29 -
2.00 (2H, m), 1.98 -1.65 (2H, m). *R: 8 for EG2, 20 for EG6, 32 for EG9. x: percentage of modification.

d) Poly(glutamic acid-co-cysteamine2TP glutamate): 8.4 (xH, m), 7.84 (2xH, m), 7.28 (xH, m), 4.33 (1H, m), 3.48 (2xH, m), 2.95 (2xH, m), 1.9-2.3 (4H, m). x: percentage of modification.

4.4.3.4.2. DMTMM-BF$_4$ for organic solutions.

In a two-neck round bottom flask fitted with a stir bar and two septums, 200 mg of PGA (acid form) (1.55 mol GAU, 1 eq.) were suspended in 10 mL of anh. DMF under N$_2$ atmosphere. Afterwards the eq. for the desired modification of DMTMM-BF$_4$ were added dissolved in 5 mL of anh. DMF (i.e. 152.5 mg, 0.465 mmol, 0.3 eq. for 30 % modification). After 10 minutes (0.93 mmol 0.6 eq., for 30 % modification) of the corresponding amine were added and the pH was adjusted to 8 by adding DIEA. Reaction was allowed to proceed for 16 hours, stirring at r.t. under N$_2$ atmosphere. After this, the solvent was completely removed. The modified polymer was then suspended in ddH$_2$O and converted into the sodium salt form by addition of NaHCO$_3$ 1 M. As all by products are soluble in acid aqueous solution, either acid/base precipitation, dialysis (Vivaspin® MWCO 3000 Da), or size exclusion chromatography with Sephadex G25 columns, was done in order to purify the copolymer. A colorless amorphous solid was obtained after freeze-drying.

Yields: 80-90 % $^1$H-NMR $\delta$H (300 MHz, D$_2$O):

a) Poly(glutamic acid-co-N-Boc-DOOA glutamate): 4.23 (1H, m), 3.52 (8xH, m), 3.32 (2xH, m), 3.20 (2xH, m), 2.35-2.13 (2H, m), 1.99 (2H, m), 1.36 (9xH, s). x: percentage of modification.

b) Poly(glutamic acid-co-N-Boc-ethylendiamine glutamate): 4.26 (1H, m), 3.21 (2xH, m), 3.11 (2xH, m), 2.21 (2H, m), 1.91 (2H, m), 1.35 (9xH, s). x: percentage of modification.

4.4.3.5. Boc removal from N-Boc-ethylendiamine and N-Boc-DOOA modified PGAs.

To a one neck round bottom flask fitted with a stir, 70 mg of modified PGA were dissolved in 10 mL of a 50/50 v/v CHCl$_3$/TFA. Reaction was allowed to proceed for 5 hours. After that time, solvents were evaporated under vacuum. For purification, polymers were re-dissolved in ddH$_2$O with addition of NaHCO$_3$. Then sodium salts forms
of the polymers were purified using dialysis (Vivaspin® MWCO 3000 Da) or size exclusion chromatography with Sephadex G25. Yield: 80-90 % \(^1\)H-NMR \(\delta_H\) (300 MHz, D\(_2\)O) 4.27 (1H, m), 3.44 (2xH, m), 3.09 (2xH, m), 2.66 (2H, m), 2.39-2.13 (2H, m). (i.e. for poly(glutamic acid-co-ethylendiamine glutamate))

4.4.3.6. Methodology for copper catalyzed alkyne-azide coupling (CuAAC) of PGA derivatives.

a) CuAAC conditions for the coupling of hydrophobic peptides/drugs.

In a two-neck round bottom flask fitted with a stirrer bar and a stopper, 1 eq. of copolymer (PGA-EG(2)N\(_3\), PGA-EG(6)N\(_3\) or PGA-prop in each case) sodium salt was dissolved in ddH\(_2\)O. After that, the corresponding amount for the desired % of substitution of clickable agent was added in dry DMF solution. Then, 5 eq. of sodium ascorbate in ddH\(_2\)O solution were added. Then, the mixture was degassed by performing two freeze-pump-thaw cycles. One eq. of CuSO\(_4\) was weighted under N\(_2\) flow and added in ddH\(_2\)O solution to the reaction mixture. The final complete mixture, containing a proportion DMF/H\(_2\)O of 4:1, was degassed by performing another freeze-pump-thaw cycle and left to react at 40 °C in an oil bath protected from light.

b) CuAAC conditions for the coupling of water-soluble molecules. The synthesis was done according to a) but previously degassing ddH\(_2\)O. \(^1\)H-NMR \(\delta_H\) (300 MHz, D\(_2\)O): 7.8 (s)*, 4.30-4.02 (1H, m), 3.79 (s)*, 3.63-3.46 (m)* 2.46 (s)*, 2.35-2.02 (2H, m), 2.01-1.65 (2H, m) when NH\(_2\)PEG(n)N\(_3\) was coupled to PGA-prop polymers

*Integration of peaks corresponds with the amount of functionalization (PEG, alkyne or azide). When PGA-EG(2/6)N\(_3\) polymers were used, propargyl singlet at 2.46 ppm disappeared. Peak at 7.8 ppm corresponds with the proton of the triazole ring formed in the CuAAC, and was used to determine coupling efficiencies.
4.5. REFERENCES


16 R. K. Heenan, *Rutherford Appleton Laboratory, Didcot, U.K.*
Chapter 5

Synthesis of polyglutamate-based complex architectures through bottom-up approach
5.1. INTRODUCTION AND BACKGROUND.

One of the most appealing properties of some polymeric systems, apart from their rheological characteristics and thermoplastic character, is their self-assembly behavior that can be promoted in solution by the presence of functional moieties along the chain arms or by using selective solvents. Micellar structural parameters such as critical micellar concentration (CMC), aggregation number, core and shell dimensions, overall micelle concentration as well as thermodynamics and kinetics of micellization of complex structures, such as star-block copolymers and miktoarm stars, have been poorly investigated if compared to linear analogues. In general basis, star structures have higher CMC values and consequently, lower aggregation numbers than their linear block copolymers counterparts.

The work of He et al. (He et al., 2009) can serve as an example of this micellar behavior. They synthesized a family of 4-arm star-block copolymers based on polyethylene oxide (PEO) (as inner block) and poly(methyl methacrylate) (PMMA). They found that micellar behavior of the polymers was affected by the pH of aqueous solutions: whereas at high pH values the star-blocks were dissolved adopting an extended conformation, at low pH and low degrees of neutralization large spherical micelles were formed presenting lower $R_h$ (hydrodynamic radius) as degree of neutralization also decreased. In this concrete case, micellization behavior depended basically on the balance between the existing interactions, including electrostatic (due to carboxylic groups), hydrophobic and hydrogen bonding. A second and more clarifying example of the fact that the number of arms can influence micellization character has been reported by Strandman et al. They synthesized two different amphiphilic 4 and 8-arms PMMA-PAA (poly(acrylic acid)) star-block copolymers with PMMA as inner blocks. They found for the 4-arm a morphological transition from spherical multimolecular micelles at pH 5 in salt-free aqueous solutions, to cylindrical micelles upon the addition of salts that were again transformed into spherical micelles with a pH increase up to 12 (swelling of the corona). In contrast, this effect was not occurring for 8-arm stars due to the fact that the higher number of arms resulted in higher repulsion and stretching of the PMMA core, leading only to spherical structures.

Overall, it is well-known that macromolecular architecture is a key parameter for the tuning of micellar behavior and properties, and thus, it
must be well-considered for the design of new materials and their potential biological applications, in particular as drug delivery systems.

Besides, the design of polymer self-assembled DDS has been mainly focused on amphiphilic and ionic systems which take advantage of hydrophobic effect and electrostatic interactions respectively. In the case of amphiphiles, it is relevant to mention that although intermolecular hydrophobic interactions are predominant forces to drive self-assembly processes in water, they lack of the directionality achievable through other non-covalent interactions such as hydrogen bonding or π-π stacking. This leads to amphiphile shape dependent assemblies morphologies, where the balance and length of hydrophobic and hydrophilic domains rules the formation of spherical (micelles, vesicles...), elongated (fibers, worm-like micelles) or planar structures (bilayers). Overwhelming examples of amphiphilic block polymers can be found in literature in which the above described design rules are exploited to design structural but also versatile drug carriers. In fact, polymeric micelles have been extensively studied during the last decades as long circulating vehicles for i.v. administration of drugs.

It has been pointed out the myriad of possibilities that might arise from the combination of polymer and supramolecular chemistry combining robustness, multivalency and adaptability. In this context, the combination of polymer backbones incorporating self-assembling motifs is gaining interest as a novel strategy towards the development of functional materials.

In the context of the present work, introduction of functional motifs along polypeptide backbone can be achieved following different strategies: i) using functional NCA monomers; ii) grafting on the polymer backbone side chains through post-polymerization modifications as described in Chapter 4; iii) end-capping or chemical modification of terminal reactive ω-nitrogen atom and iv) use of functional amine initiators in the ROP of NCAs. Some of those functional initiators have been explored in different fields over the last decades due to their structural simplicity, chemical accessibility, multivalency and deep understanding of their supramolecular self-assembly.
Figure 5.1. Schematic representation of a special motif self-assembly into helical one-dimensional aggregates, which are stabilized by threefold intermolecular H-bonding. (Adapted from Cantekin et al. 35)

For instance, Meijer and coworkers have studied extensively some of those self-assembly triggering motifs to build architectural diverse compounds with interesting self-assembling properties. Particularly relevant are the applications to build collagen like triple helix assemblies,36 one dimensional elongated fibers,37 thermoplastic elastomers based on nanorods,38 among others. Enhancing water solubility of those motifs through peripheral groups might, in some cases, result in the formation of stimuli responsive hydrogels.39-42 Previous antecedents combining such motifs with polymers include the design of folding polymers based on polymethacrylates.43

Regarding its biological applications, plentiful of those motifs been employed as scaffold to connect bioactive units such as cyclic RGD ligands,44 glycans as bacterial lectin inhibitors45 or as anti-inflammatory compounds.46 Self-assembling properties can be modulated in aqueous systems through pH or ionic strength to build tailor made nanorods47-49 and peptide functionalized nanofibers50, 51 with applications in biomedicine.52

Overall, within this chapter, we will describe the synthesis of novel constructs bearing self-assembly triggering motifs as building blocks to promote the design of self-assembled materials to be used as PT.
5.2. RESULTS DISCUSSION.

5.2.1. Use of self-assembly triggering motifs.

Novel PGA-based systems bearing self-assembly triggering motifs were synthesized. When such systems were characterized by SANS, interesting data was found regarding compounds size. SANS experiments have been performed as routine technique in the lab in order to elucidate size and solution conformation of new constructs. When these architectures were analyzed by SANS and after adequate data treatment and fittings (Figure 5.2), gyration radius were found in the range of 70-160 nm, much higher than the ones expected for the “unimeric form” of these systems (between 5-10 nm) (Table 5.1). These experiments were carried out at relatively high concentration (10 mg·mL⁻¹) and therefore, self-assembly could be triggered. SANS fitting analysis correlated these structures with “hard spheres with branches pointing outside”.

![Figure 5.2. SANS data plotting of various PGA-based constructs with self-assembling properties at 10 mg·mL⁻¹ (X).](image)

Moreover, when DLS measurements in PBS buffer pH 7.4 were performed, it was found out that those systems undergo a concentration dependent self-assembly process. At low concentrations “unimers” of 5-10 nm diameter size were identified, whereas bigger structures of around 100-200 nm diameter size were formed at high concentrations.
This phenomenon occurred in all systems bearing self-assembly triggering motifs, independently on the spacer used (ethyl, hexyl or DOOA). Nevertheless it did not occur in linear PGA (Figure 5.3). With increasing concentrations, it could be clearly observed the disappearance of the small structures and progressive appearance of the bigger ones, up to a point where only big structures of 100-200 nm size (diameter) were observed (2 mg·mL⁻¹). By plotting the scattered intensity, Mean Count Rate (MCR) in Kcps obtained against concentration, a value of critical aggregation concentration (CAC) can be obtained with the intersection of the two lineal curves (Figure 5.3). This CAC value not only represents the concentration above which aggregation processes are taking place, but also represents the maximum concentration of free non-aggregated polymer species present in the sample under that specific conditions (temperature, ionic strength, pH).

**Figure 5.3.** Size-concentration dependence analysis by DLS in PBS buffer at pH 7.4. a) Mean Count Rate (MCR) vs. concentration of PGA-based constructs bearing self-assembly triggering motifs, DP 180 as example. b) Mean Count Rate vs. concentration of linear PGA polymer DP 150 as example. c) Example of correlation coefficient curves
obtained for PGA-based constructs bearing self-assembly triggering motifs. d) DLS size by intensity plot at different concentrations from PGA-based constructs bearing self-assembly triggering motifs, showing the increase in the population with high hydrodynamic radius when increasing the concentration.

Table 5.1 summarizes CAC values, hydrodynamic radius ($R_h$) and gyration radius ($R_g$) obtained by DLS and SANS respectively, for several PGA-based constructs bearing self-assembly triggering motifs, with different chain lengths. Similar $R_h$ values were obtained for all of the measured polymers, and in general, higher CAC values were observed with greater chain lengths.

Table 5.1. Summary of CAC values, hydrodynamic radius ($R_h$) and gyration radius ($R_g$) obtained by DLS and SANS for different PGA-based constructs bearing self-assembly triggering motifs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>GAU$^a$</th>
<th>C.A.C.$^b$</th>
<th>$R_h$$^c$ (nm)</th>
<th>$R_g$$^d$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X_1</td>
<td>99</td>
<td>0.20</td>
<td>60.1</td>
<td>160.7</td>
</tr>
<tr>
<td>X_2</td>
<td>231</td>
<td>0.40</td>
<td>62.7</td>
<td>80.8</td>
</tr>
<tr>
<td>X_3</td>
<td>102</td>
<td>0.30</td>
<td>53.0</td>
<td>69.1</td>
</tr>
<tr>
<td>X_4</td>
<td>186</td>
<td>0.30</td>
<td>47.7</td>
<td>91.1</td>
</tr>
<tr>
<td>X_5</td>
<td>234</td>
<td>0.55</td>
<td>123.7</td>
<td>84.5</td>
</tr>
</tbody>
</table>

$^a$ GPC in DMF/LiBr at 8 mg·mL$^{-1}$. $^b$ Critical Aggregation Concentration (CAC) measured by DLS (mean count rate vs. concentration) in PBS at 20 °C. $^c$ DLS data at 2 mg·mL$^{-1}$ in PBS buffer pH 7.4 at 20 °C expressed by intensity mean. $^d$SANS data (ILL, Grenoble, measured at 10 mg·mL$^{-1}$ in PBS buffer pH 7.4 at 20 °C.

Accordingly, a self-assembly process is proposed for these systems to lead bigger structures with hard sphere shapes bearing branching points outside directed (Figure 5.4). It must be noticed, that self-assembly process of these systems represents a reversible and dynamic equilibrium between free non-aggregated species and large assemblies with broad size distributions. Therefore, as it is a dynamic process, several considerations must be taken into account: First of all, kinetic effects might be playing an important role and the observed
metastable nanoconstructs might evolve with time. It has to be considered that the present study has been carried out after stabilization of all samples during 24 hours. Future work on these systems must address such kinetic consideration. Secondly, this process might be sensitive to different stimuli (physical or chemical) that might affect not only equilibrium in terms of CAC but also morphology and molecular arrangement of the assemblies. Lastly, although control and study of all parameters affecting the equilibrium seems a complicated task, it also opens the opportunity to prepare tailor-made DDS with a high potential to cover a wide range of different biological applications.

\[ \text{Figure 5.4. Schematic representation of the self-assembly process} \]

\[ \text{followed by PGA-based constructs bearing self-assembly triggering motifs, studied according to DLS and SANS data interpretation.} \]

\[ \text{5.2.2. Elucidating the structure underlying self-assembly processes.} \]

In order to further elucidate the structures underlying this self-assembly process, further advanced characterization was carried out. We used conventional techniques employed in supramolecular chemistry to unravel the role of the different non-covalent interactions responsible for self-assembly, the molecular packing within assemblies and the morphology of the nanoconstructs. Unfortunately, presence of a large polypeptide backbone hinders a suitable characterization through CD and NMR that might have been useful to study the non-covalent interactions responsible for the self-assembly process.\textsuperscript{57, 58}

On one hand, these architectures were tested following pyrene assay in order to investigate, not only the presence or absence of hydrophobic environments, but also to corroborate CACs determined via DLS analysis. Pyrene fluorescence has been generally used to measure CMC in micellar systems primarily due to the fact that its spectroscopic properties are highly sensitive to changes in the environmental polarity, and secondly, due to its poor water solubility
being prone to accommodate into hydrophobic cavities present specially in micellar aqueous solutions. The five emission bands of pyrene undergo significant changes to their vibrational fine structure intensities when fluorescence measurements are made in polar versus non-polar environment. The first and third bands show the greatest relative variation to one another when the microenvironment changes. Therefore, these two bands are used to determine the fluorescence ratio in the so-called \( I_3 : I_1 \) method. This ratio is dependent on solvent polarity and hydrophobicity of the surrounding environment, making it useful to study aggregation properties. An alternative to this method is the study of the (0,0) bands in pyrene excitation spectra and the comparison of the intensity ratio \( I_{338} / I_{333} \). At low amphiphile concentrations this ratio corresponds to value characteristic of pyrene in water. At high concentrations this value corresponds to the value of pyrene entirely in the hydrophobic environment following an inverse sigmoidal curve to that obtained using the emission bands in \( I_3 : I_1 \) method. For this assay, several polymeric aqueous solutions at different concentrations ranging from 0.004 to 2 mg·mL\(^{-1}\) were mixed with 3 μL of a pyrene stock solution (0.02 mg·mL\(^{-1}\)) in acetone. Samples were then heated at 37 °C for 2 hours and fluorescence measurements were performed 24 hours after. Each excitation spectra is recorded from 300 to 360 nm with an emission wavelength of 390 nm at r.t. Finally, intensity ratio \( I_{338} / I_{333} \) is plotted against polymer concentration in order to determine CAC values. As shown in Figure 5.5, the intensity ratio remains constant along the whole range of concentrations. This result suggests that hydrophobic environments within the assemblies, if present, are not capable of accommodating pyrene molecules within their cavities. According to previous reports, these motifs are prone to stack into one dimensional columnar assemblies in water when hydrophilic peripheral PEG groups are attached to the central core. Such assemblies might be consistent with this observation since such stacked column represents a hydrophobic domain but with a small width to allow pyrene molecules to intercalate.
Figure 5.5. Pyrene fluorescence assay with PGA-based constructs bearing self-assembly triggering motifs of 150 GAU.

In order to unravel the molecular organization of the self-assembly triggering motifs within the assemblies, SANS contrast experiments were performed with polymers bearing a D-labeled version of those motifs in LOQ SANS instrument at ISIS (UK) very recently, and data is still under treatment. Due to the large scales accessible with this technique (1-1000 Å) it is not only possible to obtain overall information on solution conformation but also exhaustive minor local details. In addition, selective deuterium labeling of different domains within polymers allows the differential “visualization” of the multiple domains in different solvents.66-69

For that purpose, a deuterium (D) labeled motifs were synthesized in two steps using fully deuterated reagents. In the first step, Boc protected motifs were synthesized using DMTMM-BF₄ (19b) as carboxylic acid activator. Reaction proceeded for 48 hours in THF under N₂. After purification, identity of this molecule was confirmed by ¹H-NMR and ²H-NMR. Then, Boc was easily removed with HBF₄-Et₂O. Again, ¹H-NMR and ²H-NMR confirmed the product identity (Figure 5.6).
Figure 5.6. $^2$H NMR spectra of the D-labeled motifs. a) N-Boc-protected motifs (22) in H$_2$O + 3 μL Acetone-$d_6$ at 300 MHz. b) Amine free protected motifs (23) in H$_2$O + 3 μL Acetone at 500 MHz.

The D-labeled motif was used to yield PGA-based polymers bearing D-labeled self-assembly triggering motifs (Đ ~1.2, 26.3 kDa, 40 GAU per arm), and this system was studied through SANS contrast experiments both in H$_2$O and D$_2$O solvents.

Qualitatively, the contrast experiment in H$_2$O shows a prominent bump compared to the sample in D$_2$O. Aggregation of the self-assembling motif will result in differences on the scattering length density between the hydrophobic domain and the polymer backbone expressed in our system as a “bump” in I(Q) versus Q plot at high Q values. This feature provides a direct indication of a characteristic ‘short’ dimension in the structure, suggesting the presence of self-assembled domains. This points out the presence of organized domains in contrast to a random distribution of self-assembling moieties along
the nanostructure, in agreement with previous reports in literature. This fact confirms that such moieties are the driving motif for the assembly of these architectures. Further data analysis and mathematical fitting strategies might help to elucidate molecular packing of such motifs within assemblies and help to draw a more realistic picture of the molecular organization of self-assembled PGA-based constructs (Figure 5.7).

![SANS contrast Experiments with D-Labeled core](image)

**Figure 5.7.** SANS contrast experiments with D-labeled core in D$_2$O (outer H molecular organization determination) and H$_2$O (D-labeled core molecular organization determination), at 10 mg·mL$^{-1}$ and 20 °C.

When observed under the microscope using Transmission Electron Microscopy (TEM), PGA-based structures bearing self-assembly triggering motifs, exhibited homogenous globular shaped nanoparticles of about 80-100 nm diameter with relatively low dispersities, further confirming the findings obtained in the first SANS experiment and DLS analysis (Figure 5.8).

![TEM micrographs](image)

**Figure 5.8.** TEM micrographs from PGA-based structures bearing self-assembly triggering motifs of a sample prepared in ddH$_2$O at 1 mg·mL$^{-1}$.
5.2.3. Towards more complex structures through bottom-up strategies.

These interesting findings together with the need of new and more defined architectures with higher MW (to enhance passive targeting by the EPR effect), predictable structure and conformation, lower heterogeneity, higher drug loading capacity and greater possibility for multivalency, in nanomedicine in general, and in this project in particular, encouraged the following strategy. Based on the self-assembling motifs we proposed a strategy for the preparation of new DDS with higher MW in order to get longer circulation times in vivo, a requisite to potentially achieve brain accumulation. Functionalization of such PGA-based constructs via post-polymerization methodologies (See Chapter 4) allowed to introduce cross-linkers. Reversible self-assembly of the amphiphilic block copolymers under specific physical stimuli (concentration, temperature, ionic strength) will be followed by covalent capture (cross-linking) to yield nanoconstructs stable to physical stimuli but prone to disassembly into the parent building-blocks under specific chemical/physiological triggers (depending on the linking chemistry used). The particularly relevant fact in this strategy is the synthetic economy through the use of bottom-up approach and the possibility of introducing several relevant biological molecules such as drugs (or combinations thereof) or imaging agents in a relative easy and orthogonal synthetic manner (See Chapters 6, 7).

More interestingly, after covalent entrapment of the structures, surface modifications can be performed (See Chapter 6) again with drugs, imaging agents or targeting residues in order to guarantee their adequate exposure to the receptors when needed, or even stealth/antifouling motifs. That will lead to carriers with greater circulation times yielding to an enhanced tumor accumulation by EPR effect. The bioresponsive chemistry also implemented will allow nanostructure disassembly under selected triggers (i.e. pH, reductive environment) allowing better tumor penetration and subsequent drug release. These novel constructs will offer the following advantages: (i) Modulable size. (ii) Lower dependence on solution conformation regarding the cargo. (iii) Fully biodegradable, long circulating carriers. (iv) Multivalency suitable for combination therapy, even with chemically incompatible groups due to the possibility of co-assembling different components and, (v) Easy modulation of polymer surface for
stealth/antifouling properties, Z-potential and hydrophobicity, as diffusion across biological barriers is strongly dependent on these parameters.\textsuperscript{73}

\textbf{5.2.3.1. Polymers modification and co-assembly studies.}

As stated before, the purposed strategy will be based on the entrapment of these bigger structures, obtained when the solution concentration used is above CAC values, by introduction of covalent bounds among polymer chains from different “unimers”. For that purpose, orthogonal functional moieties must be firstly introduced within the polymer chains, either in the same polymer (in order to achieve self-assembly), or in different polymer chains (pursuing co-assembly processes). As mentioned before, the idea underlying this strategy is the use of these functional moieties for covalent entrapment of the large structures in order to obtain size concentration independence, precluding the dynamic and reversible equilibrium and therefore obtaining more stable assemblies against changes in the physico-chemical environment such as ionic strength or concentration. This fact is particularly relevant when i.v. administration of the designed DDS are pursued due to the dilution factor that those structures will face after administration, ensuring, in this way, the stability in size of those structures during circulation time and therefore enhancing their blood half-life.\textsuperscript{13}

As a first approach, in order to simplify the study and achieve the required proof of concept, irreversible \textit{click} chemistry was proposed for covalent entrapment due to the easier characterization (when compared to reversible chemistry) (Figure 5.9). Nonetheless, the long term goal of this strategy is the use of biodegradable linkers in order to have nanostructures with adequate 3D features to promote passive targeting by EPR effect after i.v. administration and once accumulated in the target, capable to disassemble under specific physiological triggers, in order to enhance tissue penetration and subsequent drug release.
Figure 5.9. Schematic representation of covalent capture of co-assembled polymers bearing orthogonal functionalities (alkynes and azides) through CuAAC *click* chemistry.

For that purpose, several PGA-based structures bearing self-assembly triggering motifs were modified with alkynes and azides using the optimized post-polymerization techniques either both in the same polymer chain or in different polymers. Those moieties will be used to promote covalent capture by the already optimized CuAAC chemistry but firstly, the co- and self-assembly properties of the new constructs must be studied.

From 5 to 50 % of GAUs of the polymers were modified with propargylamine (24a) and NH$_2$EG(2)N$_3$ (24b) respectively. One polymer was also dually modified with 10 % alkyne and 20 % azide mol GAUs. Those polymers where analyzed by DLS and CAC was calculated. As negative control for the study, linear alkyne modified PGAs (5 and 10 mol% GAUs) were also measured leading to absence of aggregation processes in the concentration range studied. Surprisingly, compounds bearing both moieties (to be used in the covalent capture of self-assembled architectures) did not show aggregation and CAC could not be calculated in the concentration range studied (Figure 5.10). This could be attributed to the relatively high degree of structures modification that could interfere in the assembly process. Although, in the case of the structures bearing above
20 mol% of EG(2)N₃ a CAC could not be determined by this method, a closer inspection to size distribution graphs looks controversial: whereas size distribution by number evidences the presence of star unimers (~2.5 nm radius), size distribution by intensity reveals the presence of large aggregates (> 40 nm radius). These findings point out that size measurements were carried out just in the equilibrium region were both species coexist, although in this particular case probably unimers are the major component.

From these results, a general conclusion can be drawn. As expected, degree of modification, as well as nature of those modifications do have an influence in the aggregation processes. From Figure 5.10, it can be observed that, when hydrophilic moieties are included within star polymer backbone, CAC value of the assemblies increased by increasing the degree of modification and unimers seem to be the major component in those systems. Nevertheless, if hydrophobic moieties are included, CAC values are decreased with degree of modification and in this case, no unimers could be detected under the size measurement conditions used (See Table 5.2). Consequently, a general picture of the self-assembling process on these systems could be drawn. Firstly, the self-assembly equilibrium does take place, however, as its concentration range (CAC) is located close to that used in the measurement conditions, both species involved in the equilibrium can be observed. Secondly, increasing system hydrophobicity shifts the equilibrium towards the aggregated components leading to reduced CAC values and the absence of unimers. Aiming to reach a set of complex and bigger architectures with different inherent properties, such equilibrium was studied in more detail. These studies could be found later in this chapter (looking at ionic strength, [C] and temperature influence).
Figure 5.10. Mean count rate (MCR) vs. increasing concentrations plotting of a) alkyne modified polymers with different degrees of functionalization (24a); b) azide modified polymers with different degrees of functionalization (24b); c) linear PGA alkyne modified as negative control and (20a) d) alkyne + azide modified polymers with a total degree of functionalization of 30 mol% GAUs.

Data obtained by DLS is summarized in Table 5.2. Modified polymers were also analyzed by SANS in order to gain insights into the real behavior of these constructs (data under mathematical treatment).

Table 5.2. Summary of CAC values and hydrodynamic radius (R\(_h\)) obtained by DLS.

<table>
<thead>
<tr>
<th>Compound</th>
<th>GAU(^a)</th>
<th>Mod. GAU(^b)</th>
<th>CAC(^c)</th>
<th>(R_h)(^d) (nm)</th>
<th>(R_h)(^e) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-prop(5) 24a_1</td>
<td>150</td>
<td>5</td>
<td>0.60</td>
<td>44.0</td>
<td>67.2</td>
</tr>
<tr>
<td>X-prop(10) 24a_2</td>
<td>150</td>
<td>10</td>
<td>0.50</td>
<td>38.5</td>
<td>77.3</td>
</tr>
<tr>
<td>X-prop(20) 24a_3</td>
<td>150</td>
<td>20</td>
<td>0.40</td>
<td>37.4</td>
<td>68.6</td>
</tr>
<tr>
<td>X-prop(30) 24a_4</td>
<td>150</td>
<td>30</td>
<td>0.35</td>
<td>49.2</td>
<td>95.5</td>
</tr>
<tr>
<td>X-prop(50) 24a_5</td>
<td>150</td>
<td>50</td>
<td>0.35</td>
<td>45.1</td>
<td>90.6</td>
</tr>
<tr>
<td>X-EG(2)N(_3)(5) 24b_1</td>
<td>150</td>
<td>5</td>
<td>0.50</td>
<td>2.3</td>
<td>69.0</td>
</tr>
<tr>
<td>X-EG(2)N(_3)(10) 24b_2</td>
<td>150</td>
<td>10</td>
<td>0.55</td>
<td>2.7</td>
<td>58.2</td>
</tr>
<tr>
<td>X-EG(2)N(_3)(20) 24b_3</td>
<td>150</td>
<td>20</td>
<td>-*</td>
<td>2.6</td>
<td>75.2</td>
</tr>
<tr>
<td>X-EG(2)N(_3)(30) 24b_4</td>
<td>150</td>
<td>30</td>
<td>-*</td>
<td>2.5</td>
<td>65.8</td>
</tr>
<tr>
<td>X-EG(2)N(_3)(50) 24b_5</td>
<td>150</td>
<td>50</td>
<td>-*</td>
<td>2.6</td>
<td>71.1</td>
</tr>
</tbody>
</table>

* C.A.C. could not be calculated in the concentrations range employed. Aggregation (if occurs) might be found over 2 mg∙mL\(^{-1}\). a. GPC in DMF/LiBr at 8 mg∙mL\(^{-1}\). b. Data obtained by \(^1\)H-NMR in mol%. c. CAC measured by DLS in PBS at 20 °C and size measured by DLS at 2 mg∙mL\(^{-1}\) in PBS at 20 °C by d. Number mean, and e. Intensity mean.
Assemblies’ morphology was also investigated through TEM. As shown in Figure 5.11, this morphology does not vary significantly from the parent compound with the different chemical modifications introduced. In all cases globular aggregates in the range of 100 nm were found. It must be noticed that TEM requires a full drying of samples, which is achieved by solvent evaporation at r.t. Such samples processing is not ideal for soft materials since the change of concentration upon solvent evaporation might result in further aggregation or distortion of the nanostructures present in the native solvated state. Nevertheless, in general these results are in good agreement with those found for the parent compound and also with DLS and SANS data obtained for these series of compounds.

Figure 5.11. TEM micrographs of modified PGA-based structures bearing self-assembly triggering motifs at 1 mg·mL$^{-1}$ in ddH$_2$O; a) X-EG(2)N$_3$(5); b) X-prop(10).

Once self-assembly process of the modified polymers was confirmed, studies to assess co-assembly where done using DLS, by observation of CAC value shift of one of the compounds upon addition of constant amount (always below its CAC) of the second component. Thus four series of solutions were prepared for the CAC determination experiments: X-EG(2)N$_3$(5) (24b_1), X-prop(10) (24a_2), and the same series but with addition of the second component in a concentration below their CAC. Figure 5.12 shows the plots of scattered intensity against variable concentration of one of the components keeping constant the concentration of the counterpart (always bellow their CAC). Although the MCR values are lower for series containing both components, which can be attributed to a smaller size of the aggregates, qualitatively, it can be seen a decrease in CAC value in both cases when the second compound was added to the solution. These findings somehow suggest a synergy in the formation of mixed assemblies.
through co-assembly processes (Figure 5.12) what is in good agreement with previous reports on PEG modified BTA species but also block-copolymer systems. Nonetheless, these results are not yet conclusive, what motivated a deeper study of the co-assembly process through NMR techniques.

**Figure 5.12.** Co-assembly study by DLS. Graphs showing CAC determination for X-EG(2)N₃(5) (24b_1), in the presence of constant concentration of X-prop(10) (24a_2), (a) and vice versa (b).

Further evidences on co-assembly of these architectures were studied through NMR techniques. Pulsed-gradient spin-echo NMR spectroscopy, known as diffusion NMR spectroscopy (or DOSY NMR), allows determining the self-diffusion coefficient of the species present in solution. The diffusion coefficient is related not only to intrinsic properties of the molecules (size, shape, MW, charge, etc.) but also to the surrounding environment such as concentration, solvent, temperature or ionic strength among others. In brief, application of NMR field gradients allows to “label” the spins along the direction of the applied gradient. Upon the use of certain pulse sequences (based on spin-echo) the spectra of the components in a mixture (chemical shifts) can be separated according to their diffusion coefficient, in a similar way to a size-exclusion chromatography. This technique has been applied in different contexts such as MW prediction, effect of shape in polymers, bioconjugation studies, polypeptides and the study of supramolecular self-assembling systems among other examples.

In order to perform the present study, diffusion coefficients of X-EG(2)N₃(5) (24b_1) above (2 mg·mL⁻¹) and below (0.1 mg·mL⁻¹) its CAC was investigated in a first step. Firstly, diffusion coefficients obtained from fitting the intensities to Stejskal-Tanner equation (see M&M) for the chemical shifts associated to both PGA (4.3 ppm) and
ethylene glycol (3.6 ppm) in the concentrated sample were identical, confirming the covalent attachment of the EG-azide residue to the polymer, as expected. Diffusion coefficient values obtained for the diluted (unimer) and concentrated (nano assembly) samples were $3.12 \times 10^{-11} \text{m}^2\text{s}^{-1}$ and $4.54 \times 10^{-12} \text{m}^2\text{s}^{-1}$ respectively. As expected, diffusion coefficient is reduced in one order of magnitude upon self-assembly of the small unimers into the large nanostructures due to the inverse relationship between size or hydrodynamic radius and diffusion coefficient in the Stokes-Einstein equation:

$$R_h = \frac{K_B \cdot T}{6\pi \cdot \eta \cdot D}$$

**Equation 5.1.** Stokes-Einstein equation, where $K_B$ is Boltzman constant ($1.3806488 \times 10^{-23} \text{m}^2\text{Kg}\cdot\text{s}^{-2}\cdot\text{K}^{-1}$), $T$ is temperature (K), $\eta$ is dynamic viscosity ($1.095 \times 10^{-3} \text{Kg}\cdot\text{m}^{-1}\cdot\text{s}^{-1}$ for D$_2$O at 298.15 K and 0.1 MPa), $R_h$ is hydrodynamic radius and $D$ is the diffusion coefficient.

Size values obtained from this first analysis reveals that below CAC, these compounds are characterized by a $R_h$ of 6.4 nm whereas the large nanostructures formed upon self-assembly above their CAC have a size of 44 nm ($R_h$). Similar results were obtained for X-prop(10) at 2 mg\cdot mL$^{-1}$ with a hydrodynamic radius of 46 nm. These findings are in good agreement with DLS data even though both techniques measure size in a different manner.

Then, co-assembly process was tested in a similar way to that described in literature.$^{81, 82}$ A sample containing X-prop(10) above its CAC (2 mg\cdot mL$^{-1}$) in the presence of X-EG(2)N$_3$(5) below its CAC (0.1 mg\cdot mL$^{-1}$) was prepared. X-EG(2)N$_3$ was used at the lower concentration for this study, due to the fact that EG signals are more intense (3.2 ppm, 8H) in comparison with signals from propargyl group in compound X-prop with lower intensity (3.8 ppm, 2H). Besides, DOSY arrayed experiments are difficult to process in diluted samples yielding low quality fittings due to low intensities of the spectra recorded at high gradient strength values. As it can be seen in Figure 5.13, $^1$H NMR spectra shows the signals corresponding to each of the components employed in DOSY NMR analysis. After data treatment, it can be seen that compound X-prop(10) shows the characteristic diffusion coefficient of self-assembled species ($5.03 \times 10^{-12} \text{m}^2\text{s}^{-1}$) expected for the concentration studied. However, compound X-
EG(2)N₃, that, at 0.1 mg·mL⁻¹ should present a larger diffusion coefficient when compared to the self-assembled constructs, reduced its diffusion coefficient in one order of magnitude from (3.12·10⁻¹¹ m²·s⁻¹) to (5.24·10⁻¹² m²·s⁻¹), being virtually equivalent to that found for X-prop(10) component. These results suggest that although X-EG(2)N₃ is below the CAC, it moves along with the self-assembled constructs from the counterpart X-prop(10), and thus, indirectly confirms that these architectures are able to co-assemble.

Figure 5.13. Co-assembly studies through DOSY NMR: a) Schematic representation of the conditions for co-assembly study; b) ¹H NMR in D₂O of a mixture containing 2 mg·mL⁻¹ of compound X-prop(10) (24a_2) and X-EG(2)N₃(5) (24b_1); c) Graphs obtained by fitting the intensities of the arrayed DOSY spectra into Stejskal-Tanner equation and the calculated diffusion coefficients (D).
Moreover, confirmation of co-assembly process was assessed with the help of NOESY experiments (see M&M for further details). As observed in Figure 5.14, a clear NOE correlation was found for propargyl and ethylene glycol signals, a result that confirms the spatial proximity between both groups.

![2D NOESY spectra showing NOE correlation of propargyl and EG protons of a mixture containing 2 mg·mL\(^{-1}\) of each compound: X-prop(10) \((24a_2)\) and X-EG(2)N\(_3\)(5) \((24b_1)\).]

**Figure 5.14.**

5.2.3.2. Assembly size modulation and stimuli-responsiveness to different parameters.

With size-concentration dependence verified, the effect of different stimuli such as temperature and ionic strength were further investigated using a PGA-based construct bearing self-assembly triggering motifs without any modification as a model system. Size dependence on ionic strength of media was investigated after the first evidences found (Table 5.3) when measuring the same sample in ddH\(_2\)O or PBS buffer 0.1 M pH 7.4.

**Table 5.3.** Size determination of PGA-based construct bearing self-assembly triggering motifs by DLS (PBS and ddH\(_2\)O) and DOSY.

<table>
<thead>
<tr>
<th>Compound</th>
<th>(R_h^a) (nm)</th>
<th>(R_h^b) (nm)</th>
<th>(R_h^e) (nm)</th>
<th>(R_h^d) (nm)</th>
<th>(R_h^f) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-PGA</td>
<td>36.4</td>
<td>69.1</td>
<td>123.7</td>
<td>2.7</td>
<td>124.4</td>
</tr>
</tbody>
</table>

*Data obtained of a 2 mg·mL\(^{-1}\) sample from a. DOSY NMR in D\(_2\)O. b. DLS number mean in ddH\(_2\)O. c. DLS intensity mean in ddH\(_2\)O. d. DLS number mean in PBS 7.4. e. DLS intensity mean in PBS 7.4.
From results obtained in Table 5.3, it can be concluded that presence of salt, and therefore, modulation of the ionic strength, highly affects the self-assembly equilibrium by shifting it towards unimer region. In the absence of salts, no unimers could be observed by DLS. This finding must be taken into account when performing covalent capture strategies since presence of unimers should be avoided in order to efficiently entrap the big assemblies. Thereafter, ionic strength was further studied as we decided to investigate the influence of different salts on aggregate size (Figure 5.15). Sodium chloride (NaCl), guanidinium hydrochloride (GuHCl), and sodium Sulphate (Na$_2$SO$_4$) were chosen due to their different nature. As can be observed in Figure 5.15, the scattered intensity is progressively reduced with increasing salt content, being Na$_2$SO$_4$ the most disruptive. Furthermore, $R_h$ (mean number) dependence on salt content reveals disassembly of aggregates just by addition of 50 mM of any salt.$^{84,85}$

![Figure 5.15](image)

**Figure 5.15.** Ionic strength effect on size of PGA-based construct bearing self-assembly triggering motifs at 2 mg·mL$^{-1}$ and at 37 °C represented by the changes suffered in scattered intensity (MCR) (left) and in $R_h$ by number (right) upon addition of increasing amount of different salts.

Size dependence on concentration was studied at 37 °C in the concentration range of 1 to 10 mg·mL$^{-1}$. As it can be observed in Figure 5.16, a sudden increase in size was observed above 5 mg·mL$^{-1}$ from ~70 nm to ~100 nm. Size dependence on temperature was also found when the system was studied by DLS measurements at 10 mg·mL$^{-1}$ in the temperature range between 10 and 60 °C. As it can be observed in Figure 5.16, the highest construct was found at 60 °C.
Figure 5.16. a) Temperature effect on size of PGA-based construct bearing self-assembly triggering motifs at 10 mg·mL⁻¹. b) Size-concentration dependence of PGA-based construct bearing self-assembly triggering motifs at 37 °C.

All these findings point out the fact that size modulation of the constructs is possible through fine tuning parameters such as concentration, temperature and ionic strength during covalent capture reaction conditions. This might open a door towards the preparation of a toolbox of tailor-made nanostructures with diverse sizes based on the same polymeric building block and modulated through physico-chemical parameters. Following this line of reasoning, future work should be carried out to test the feasibility of covalently capture the dynamic and reversible metastable nanostructures under different environmental conditions.

In the present chapter, we focused our strategy in single selected physico-chemical conditions in order to further achieve proof of concept for this hypothesis.

5.2.3.3. Covalent entrapment of co-assembled structures by CuAAC.

With clear evidences highlighting co-assembly of these structures, covalent capture was carried out through CuAAC click chemistry, using the optimized conditions in Chapter 4, and the polymers X-prop(10) and X-EG(2)N₃(5). Those polymers were chosen in order to have an excess of propargyl units to ensure complete conversion, as the reaction will be performed in equimolar ratio of both functionalities. The reaction was carried out in ddH₂O (constructs were
present in aggregated state as seen before), using a concentration to ensure the only presence of big structures within the polymeric mixture (ratio 1:1, 2 mg·mL$^{-1}$). The mixture was firstly sonicated for 5 minutes in order to promote homogenization. Then, CuAAC was performed according to M&M. Complete conversion was achieved after 3 days, according to $^1$H-NMR (triazole signal at 7.8 integrates for 5 mol%) (Figure 5.17).

![Figure 5.17. $^1$H-NMR in D$_2$O showing the comparison of the two different polymers and the clicked system (25a). The red box highlights the signal corresponding to triazole peak at 7.8 ppm.](image)

The clicked system was studied by DLS measurements in comparison with a physical mixture 1:1 of both components separately after sonication. Dilution experiments were performed by diluting both samples up to 32 fold 1 mg·mL$^{-1}$ stock solution. In the case of the physical mixture, two different structures were already found at the first dilution (1:2 ratio). Nevertheless, for the clicked construct (25a), only big structures of about ~ 80-100 nm diameter were encountered, even at 1/32 of the initial concentration (~0.03 mg·mL$^{-1}$) (Figure 5.18). The small decrease in the assemblies found for the clicked system (from 45 to 30 nm in radius) might be due to the low eq. of effective cross-linking groups (in this case azide, 5 mol%) resulting in an incomplete cross-linking of the self-assembled nanostructures. Future work will be carried out working with higher eq. of effective cross-linking groups in order to improve the stability upon dilution.
Figure 5.18. DLS data showing the a) Concentration dependence of the physical mixture of X-prop(10) and X-EG(2)N₃(5) and b) Concentration independence of the clicked structure (25a). c) Comparison of both measured systems in terms of size against concentration. All values correspond to measurements in PBS 7.4 and are represented in number.

Cryo-TEM pictures of the clicked system confirmed the formation of spherical structures with a diameter size ~ 100 nm (Figure 5.19). In contrast to conventional TEM technique, cryo-TEM allows to visualize samples under conditions similar to those found in the native hydrated state.²⁶, ²⁷ Further analysis of the nanoscopic morphologies in these systems should be performed under cryogenic conditions to avoid artifacts arising from the drying process.

Figure 5.19. Cryo-TEM pictures of 25a at 2 mg·mL⁻¹. Dark spots represent spherical structures of 25a.
As expected for the proposed spherical structure, where carboxylic groups would be presumably exposed, Z-potential measured was rather negative (Figure 5.20). Although we cannot discard the possibility of partial protonation of inner carboxylic acids within the assemblies due to pK shift through cooperative intermolecular interactions upon self-assembly, CD data seems to discard the presence of α-helix conformation within the assemblies (attributed to protonation of carboxylates). As shown in Figure 5.20b, CD spectra of PGA-based construct bearing self-assembly triggering motifs (150 GAU) looks controversial. Whereas PGA shows a clear random coil conformation pattern independently of concentration, the PGA-based construct bearing self-assembly triggering motifs shows a transition from random coil below CAC to a different secondary structure (Figure 5.20). Such structure cannot be attributed to random coil nor α-helix pattern, evidencing the presence of a more complex conformation. Further studies need to be performed in order to unravel this phenomenon.

![Figure 5.20. a) Z-Potential Distribution of PGA-based X-Click structures (25a). b) CD spectra of PGA-based construct bearing self-assembly triggering motifs of 150 GAU and linear PGA of 150 GAU both at 0.1 and 1 mg·mL⁻¹, in PB pH 7.4 at 37 °C.](image)

### 5.2.3.4. Alternative covalent capture strategies.

In order to validate the versatility of this approach, other covalent capture strategies were implemented. The also non-reversible thiol-ene click chemistry, and the reductive-reversible disulfide chemistry were optimized in our systems. Thiol-based conjugation reactions are often performed using thiol-ene and thiol-yne click reactions. Thiol-maleimide chemistry represents indeed an interesting
alternative that has been demonstrated to proceed in an efficient manner under reagent free, mild conditions.\(^{93-96}\) On the other hand, di-thiol chemistry have been widely used for instance in cross-linked micelles\(^{97-99}\) and hydrogels\(^{100-104}\) cross-linking by means of disulfide bonding due to its reversibility under reductive conditions. The use of disulfide bonding in our designed strategy will allow us to yield nanoconstructs stable to physical stimuli but prone to dissassembly into the building-blocks under reductive media.

First of all, the modification of such structures with activated di-thiol units \((24c)\) (using cysteamine-2TP) and with maleimide groups \((24d)\) (using \(\text{NH}_2-\text{CH}_2\text{CH}_2\)-maleimide) was performed. Compound identity was determined by \(^1\text{H}-\text{NMR, according to corresponding signals of pyridyl group (in the case of SS-2TP), and those from maleimide group (6.78 ppms) as depicted in Figure 5.21.}\)

![Figure 5.21](image)

**Figure 5.21.** \(^1\text{H}-\text{NMR spectra (D2O) with corresponding assignments of a) X-SS2TP (24c_4); b) X-malei (24d_3).}\)
Their aggregation behavior was also studied by DLS as for the previous compounds, leading to aggregated structures of around 100 nm upon increasing the concentration as shown in Figure 5.22 and Table 5.4.

**Table 5.4.** Summary of CAC values and $R_h$ obtained by DLS for different modified polymers with SS-2TP and maleimide groups.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mod GAU$^a$</th>
<th>CAC$^b$</th>
<th>$R_h^c$ (nm)</th>
<th>$R_h^d$ (nm)</th>
<th>$R_h^e$ (nm)</th>
<th>$R_h^f$ (nm)</th>
<th>$R_h^g$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-SS-2TP(5) 24c_1</td>
<td>5</td>
<td>0.40</td>
<td>2.3</td>
<td>78.5</td>
<td>50.2</td>
<td>145.4</td>
<td>30.2</td>
</tr>
<tr>
<td>X-SS-2TP(10) 24c_2</td>
<td>10</td>
<td>0.30</td>
<td>2.6</td>
<td>94.5</td>
<td>56.5</td>
<td>79.9</td>
<td>29.9</td>
</tr>
<tr>
<td>X-SS-2TP(15) 24c_3</td>
<td>15</td>
<td>0.20</td>
<td>4.1</td>
<td>111.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>X-SS-2TP(30) 24c_4</td>
<td>30</td>
<td>0.30</td>
<td>2.5</td>
<td>51.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>X-malei(5) 24d_1</td>
<td>5</td>
<td>0.40</td>
<td>2.7</td>
<td>133.9</td>
<td>82.3</td>
<td>118.9</td>
<td>38.5</td>
</tr>
<tr>
<td>X-malei(10) 24d_2</td>
<td>10</td>
<td>0.35</td>
<td>2.7</td>
<td>101.3</td>
<td>74.6</td>
<td>135.1</td>
<td>31.9</td>
</tr>
<tr>
<td>X-malei(35) 24d_3</td>
<td>35</td>
<td>0.30</td>
<td>4.2</td>
<td>100.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a. Data obtained by $^1$H-NMR in mol% GAU. b. CAC measured by DLS in PBS at 20 °C. c&d. Size measured by DLS at 2 mg·mL$^{-1}$ in PBS pH 7.4 at 20 °C by c. Number mean, and d. Intensity mean. e&f. Size measured by DLS at 2 mg·mL$^{-1}$ in ddH$_2$O at 20 °C by e. Number mean, and f. Intensity mean. g. DOSY data in D$_2$O.

**Figure 5.22.** Mean count rate vs. concentrations plotting of a) SS-2TP modified polymers (24c) with different % functionalization, b) maleimide modified polymers (24d) with different % functionalization.

As it can be observed from Table 5.4, differences in size values obtained by number mean depending on the salt content (PBS or ddH$_2$O) are remarkable once again, highlighting the previous argument.
of a system under equilibrium processes. In addition, TEM microscopy confirmed the presence of big aggregates (Figure 5.23).

Figure 5.23. TEM micrographs of modified polymers at 1 mg·mL$^{-1}$ in ddH$_2$O; a) X-malei(35) (24d_3); b) X-SS-2TP(30) (24c_4).

Once aggregation process was confirmed, the resulting structures were covalently captured by using both chemical strategies (Scheme 5.1). For this, polymers bearing 30 mol% SS2TP, and 35 mol% maleimide groups were chosen, in order to have an excess of maleimide groups to ensure complete conversion (in the case of thiol-ene chemistry). For di-thiol chemistry, two different attempts were performed using 5 and 30 % modified polymers. All reactions were performed at concentrations of 4 mg mL$^{-1}$ for each compound in ddH$_2$O for di-thiol chemistry and PBS buffer at pH 7.4 for thiol-ene, due to the need of controlling the pH over reaction time in order to guarantee maleimide group stability. After purification by dialysis, the success of the entrapment was ratified by $^1$H-NMR. In the case of di-thiol chemistry confirmation was achieved by disappearance of the aromatic signals corresponding to pyridyl groups while CH$_2$ signals of cysteamine were kept, in the case of di-thiol chemistry. For thiol-ene reactions, the absence of the characteristic maleimide peak around 6.7 as well as the pyridyl signals were indicative of effective couplings. One example of such characterization is depicted in Figure 5.24.
Scheme 5.1. Schematic representation of two strategies for covalent capture of self-assembled structures by click chemistry. a) Disulfide chemistry, b) Thiol-ene chemistry.

Figure 5.24. $^1$H-NMR spectra (D$_2$O) comparison of X-SS-2TP(5) and the clicked compound.

Interestingly, depending on the degree of cross-linking, different structures can be built. While 5 mol% of SS cross-link lead to nanosystems perfectly soluble in aqueous solutions, higher degrees of cross-linking (30 mol%) trigger gelation processes. This finding highlights the versatility of those structures and the possibility to tune the final construct properties depending on desired application. Those gels could be easily used for other therapeutic applications that require
a local administration such as wound healing or spinal cord injury among others.

DLS measurements confirmed the covalent capture leading to stable structures of around 100 nm diameter. TEM microscopy of these two clicked systems showed the presence of better defined spherical structures when compared with non-covalently entrapped analogous as shown in Figure 5.25. Further studies regarding stability upon dilution and stimuli triggered disassembly are been carried out to explore the suitability of this approach.

![Figure 5.25. Examples of TEM micrographs of covalently captured structures at 1 mg·mL\(^{-1}\) in ddH\(_2\)O; a) X-Click through thiol-ene; b) X-Click through di-thiol using 30 % SS groups.](image)

5.2.3.5. Preliminary biological evaluation of the clicked architectures.

As indicated in Chapter 3, and after exhaustive physico-chemical characterization presented in the above paragraphs, some of the key features for the validation of the newly synthesized architectures as potential carriers for drug delivery or imaging probes are their toxicity in cell cultures and cellular trafficking.

First of all, cell viability against SHSY5Y cell line of the three chemically different clicked architectures was studied. All of them resulted non-toxic up to 3 mg·mL\(^{-1}\) when tested at 72 hours of incubation following an MTS protocol for cell viability determination. (Figure 5.26).
Figure 5.26. Cell viability assay of 3 different X-Click architectures against SHSY5Y cell line up to 3 mg·mL⁻¹, 72 hours of treatments (n>3, mean ± SEM).

In parallel, cellular trafficking of CuAAC-clicked polymers (23a) was evaluated in SHSY5Y cells by flow cytometry at 37 °C (total uptake) and 4 °C (binding) in order to identify the role of energy-dependent mechanisms (i.e. endocytosis). For that, the polymer was labeled with Oregon Green (λ = 488, 1 mol% GAU) following the protocol described in Chapter 4 (DIC/HOBt in DMF under inert conditions). As it could be expected, this globular shaped structure was fast internalized, showing around 95 % of positive already at 15 minutes (Figure 5.27a). Furthermore, when this construct was compared with the linear PGA and star PGA, a significant increase in cell-associated fluorescence (CAF) was observed (Figure 5.27b). Not only 23a goes through a faster uptake (according to both CAF and % positive cells) but also, the amount of construct internalized is significantly greater when compared with the other 2 systems (Figure 5.27).
Figure 5.27. a) Uptake kinetics against SHSY5Y cell line of X-Click-OG-labeled polymer at different time points and different temperatures (4 °C for binding, 37 °C for total uptake). b) CAF representing the energy-dependent fraction of uptake, comparing the three architectures over time. n> 3, mean ± SEM.

Confocal pictures were also obtained in order to confirm the results and to better identify subcellular localization (Figure 5.28). A clear co-localization with the lysosomal marker Lysotracker red was observed indicating that 23a would be an ideal carrier for drugs requiring a lysosomotropic delivery.
5.3. CONCLUSIONS.

Herein and based on the use of self-assembling motif, we have developed a strategy for the preparation of DDS incorporating polypeptidic (polyglutamates) chains. One of the most relevant features in this work is the combination of a discotic self-assembling core overwhelmingly exploited in supramolecular chemistry and materials science for the design of liquid crystals, 1D fibers or organo/hydrogels with a polymeric carrier such as poly-L-glutamic acid widely used in PT field. According to DLS, TEM and SANS, the resulting polymer undergoes spontaneous self-assembly in water yielding spherical objects. At first glance these results were surprising considering that the relative contribution of the hydrophobic self-assembling unit (352.26 Da) in the polymers represents only 0.7-1.7 wt% of the whole polymer mass. However, evidences drawn from the present study reveals that such units are able to trigger the organization of anionic polyglutamate backbones into spherical objects and partially neglecting the electrostatic repulsions between anionic side chains. It might be argued that the discrete spherical morphology of the present nanostructures results from the combination of hydrophobic interactions and H-bonding among motifs promoting 1D aggregation (as reported in literature, but hard to demonstrate in our system), and compensated by the electrostatic repulsions between PGA units.

Figure 5.28. Confocal image of the uptake at 2 hours post-treatment of OG-labeled X-Click in SHSY5Y cell line following a pulse-chase experiment. Co-localization with Lysotracker Red was observed (yellow).
Interestingly, chemical modifications within the polypeptidic backbone by post-polymerization modifications can prevent or significantly modify this aggregation behavior depending on the degree of modification as well as the nature of the groups included.

Furthermore, fine tuning of the physico-chemical environment can lead to a wide variety of nanometric architectures of different size and shape which can be covalently entrapped to retain their solution conformation in the biological settings.

Moreover, co-assembly of different building blocks has been effectively demonstrated. This behavior can be employed to construct relatively complex architectures combining drugs, imaging agents and active targeting moieties through bottom-up approaches in order to yield drug delivery systems or imaging probes for different therapeutic needs. Indeed, this possibility will be implemented in the next two chapters by means of surface modifications of covalently entrapped structures where the in vivo biodistribution as well as their potential use in the design of Alzheimer’s disease therapeutics will be addressed.

In addition, current ongoing studies point out that molecular design and surface modification of those entrapped structures have been shown to play a crucial role in the morphologies of the aggregates from spherical particles to 1D self-assembled fibers or organo/hydrogels (data not shown). Undoubtedly, these findings open up the door for multiple libraries of structures. The reported data is only the “tip of the iceberg” for a family of compounds with promising potential to be used in nanomedicine.

5.4. MATERIALS AND METHODS.

5.4.1. Materials.

All chemicals were reagent grade, obtained from Aldrich and used without further purification, unless otherwise indicated. All solvents were of analytical grade and were dried and freshly distilled. Deuterated chloroform-d1, DMSO-d6 and D2O were purchased from Deutero GmbH. Preparative SEC was performed using Sephadex G-25 superfine from GE as well as PD MiniTrap G-10 ™ columns containing 2.1 mL of Sephadex™ G-10. Dialysis was performed in a Millipore ultrafiltration device fitted with a 1, 3, or 10 kDa MWCO regenerated cellulose membrane (Vivaspin®).
5.4.2. Characterization techniques.

GPC, DLS, and SANS equipment used were those described in previous chapters (Chapters 2, 3 and 4).

5.4.2.1. CRYO-TEM.

Samples were vitrified in an FEI's vitrobot: A 60 μL drop of an aqueous suspension of the material was placed on a TEM holey carbon copper grid, excess of water was blotted away at the vitrobot with filter paper and the grid were freeze-plunged in liquid ethane. Samples were then transferred under liquid nitrogen atmosphere to a Gatan TEM cryo-holder equipped with a liquid nitrogen reservoir. After that, samples were transferred to a Tecnai T20 (FEI company) operated at 200 KV. All observations were done at low temperature (100 K).

5.4.2.2. TEM.

Pictures were obtained from a transmission electron microscope FEI Tecnai G2 Spirit (FEI Europe, Eindhoven, Netherlands) using the digital camera Morada (Olympus Soft Image Solutions GmbH, Münster, Germany). Samples were prepared as detailed: a Mesh grid was placed over one sample solution drop for 3 minutes; then the grid was transferred to a drop of uranile acetate (2 % in ddH₂O for one minute. Excess of uranile acetate was dried and the grid was placed in the grid holder and observed under the microscope.

5.4.2.3. NMR Studies.

NMR Spectroscopy. NMR spectra were recorded at 27 °C (300 K) on a Bruker Avance III 500 MHz Bruker spectrometer equipped with a 5 mm TBI broadband probe. Data were processed with the software Mestrenova (Bruker GmbH, Karlsruhe, Germany). Samples were prepared at the desired concentration in D₂O.

Diffusion Experiments. Pulsed field gradient NMR spectroscopy was used to measure translational diffusion by fitting the integrals or intensities of the NMR signals to Stejskal–Tanner\textsuperscript{105, 106} equation: \( I = I_0 \exp[-D\gamma^2g^2\delta^2(\Delta-\delta/3)] \), where \( I \) is the observed intensity, \( I_0 \), the reference intensity (unattenuated signal intensity), \( D \), diffusion coefficient, \( \gamma \), the gyromagnetic ratio of the observed nucleus, \( g \), the gradient strength, \( \delta \), the length of the gradient, and \( \Delta \), the diffusion time. Two-dimensional diffusion-ordered NMR spectroscopy (DOSY) was performed with a stimulated echo sequence using bipolar gradient...
pulses. The lengths of delays were held constant at $\Delta = 100$ ms, and 32 spectra of 64 scans each were acquired with the strength of the diffusion gradient varying between 5 % and 95 %. The lengths of the diffusion gradient and the stimulated echo were optimized for each sample. Typical values were $\delta = 1.5$ ms for the analysis of non-aggregated species and 5 ms to study the nano assemblies.

NOE experiments. Two-dimensional NOE experiments (NOESY) were recorded with a mixing time of 100 ms with 128 scans.

5.4.3. Protocols.

5.4.3.1. Synthetic procedures.

PGA synthesis, post-polymerization modifications and CuAAC chemistry used were done according to protocols described in previous chapters. Concretely: Chapter 2 (PGA synthesis) and Chapter 4 (Post-polymerization modification, and CuAAC chemistry).

5.4.3.1.1. Synthesis of D-labeled Boc-protected self-assembly triggering motif (22).

In a two-necked round bottom flask, fitted with a stir bar and two septums, the deuterated precursor of the motif (19.7 mg, 0.093 mmol, 1 eq.) was suspended in 5 mL THF anh. To the resulting suspension, DMTMM-BF$_4$ (106.84 mg, 0.326 mmol, 3.5 eq.) was added drop wise and stirred at r.t. for 15 minutes. After that, ethylene-d$_4$-diamine-N-tBoc diamine (50 mg, 0.6048 mmol, 6.5 eq.) was added and the reaction mixture was stirred for 48 h. The solvent was removed under reduced pressure, and the product was re-suspended in CHCl$_3$ and extracted with aqueous HCl (0.5 M) (x3), aqueous NaHCO$_3$ (1 M) (x3) and brine (x3). The organic residues were dried over Na$_2$SO$_4$ (anh.) and concentrated in vacuo. The product was purified through flash chromatography (TLC analysis (RF = 0.3, CH$_2$Cl$_2$/MeOH (95:5))). A white solid was obtained after solvent removal under vacuo.

Yield: 67 %. $^1$H-NMR $\delta_H$ (300 MHz, Acetone-$d_6$) 8.64 (3H, m), 6.88 (3H, m) 1.37 (27H, s). $^2$H-NMR $\delta_H$ (300 MHz, Acetone (+3uL Acetone-$d_6$) 9.45 (3H, m), 4.47 (6H, m) 4.28 (6H, m).
5.4.3.1.2. Synthesis of D-labeled self-assembly triggering motif (23).

In a one-neck round bottom flask fitted with a stir bar and a stopper, the product 22 (40 mg, 0.062 mmol, 1 eq.) was dissolved in THF 10 mL, and HBF$_4$∙Et$_2$O (0.08 mL, 0.62 mmol, 10 eq.) was added to the solution. After stirring for 3 days TLC revealed the complete deprotection of Boc-amines (Rf = 0.5 CH$_2$Cl$_2$/MeOH (95:5), UV+ ninhydrine). Purification was performed through sequential and repetitive precipitation in MeOH/Et$_2$O and MeOH/Hexane. Yield: 80 %.

$^2$H-NMR $\delta_H$ (500 MHz, H$_2$O (+3uL Acetone-d6)) 9.23 (3H, m), 4.52 (6H, m) 4.06 (6H, m).

5.4.3.1.3. Thiol-ene click chemistry for covalent entrapment.

In a one neck round bottom flask fitted with a stir bar and a stopper, 50 mg of each of the functionalized sodium salt form of the polymers were dissolved in PBS buffer 7.4 both at a concentration of 4 mg mL$^{-1}$ for co-assembly. Sample was sonicated for 5 minutes. Afterwards, 11.9 mg (0.041 mmol, 0.15 eq.) of TCEP (tris(2-carboxyethyl)phosphine) was added to the reaction mixture. The pH was checked to be around 7.4 and the reaction mixture was left to proceed under vigorous stirring, at r.t., over 16 hours. After that time, the resulting product was dialyzed using a MWCO 3000 Da. $^1$H NMR was used to check reaction success by taking into account the maleimide peak disappearance at 6.77 ppms.

5.4.3.1.4. Di-sulfide chemistry for covalent entrapment.

In a one neck round bottom flask fitted with a stir bar and a stopper, 100 mg of the -SS- functionalized sodium salt form of the polymer was dissolved in ddH$_2$O at a concentration of 4 mg-mL$^{-1}$ for self-assembly. The sample was sonicated for 5 minutes. Afterwards, 11.9 mg (0.041 mmol, 0.15 eq.) of TCEP (tris(2-carboxyethyl)phosphine) was added to the reaction mixture. Reaction mixture was left to proceed under vigorous stirring, at r.t., over 2 hours to promote the removal of the 2TP groups. After that time, the resulting product was dialyzed using a MWCO 3000 Da. $^1$H NMR and DLS measurements were used to check reaction success.
5.4.3.2. CAC determinations by DLS.

The compounds tested were dissolved in PBS 7.4 at different concentrations ranging from 0.004 to 2 mg·mL⁻¹. Linear PGAs were also used as negative controls. Each solution was freshly prepared for the measurements, sonicated for 5 minutes and left 24 hours to stabilize. DLS measurements were done per triplicate with fixed parameters for all samples. Data was expressed as the mean count rate (Kcps) against the concentration. CAC values were obtained from the curves intersections.

5.4.3.3. Pyrene Assay.

For these measurements, dilution series of the conjugates under study were prepared. A stock of 4 mg·mL⁻¹ of each compound was prepared in PBS 7.4 as well as a stock of pyrene of 0.02 mg·mL⁻¹ in acetone. Several dilutions are prepared to cover a wide range of polymer concentrations (from 2-0.004 mg·mL⁻¹) to which 3 µL of the pyrene stock solution in acetone were added. Then, all solutions are placed in vials and are incubated in an oven at 37 °C for 2 hours in order to evaporate the acetone. After storing the samples for 24 hours, measurements were carried out into a 1 cm path length PMMA fluorescence cell reaching a final volume: 1 mL). Each excitation spectra is recorded from 300 to 360 nm with an emission wavelength of 390 nm at r.t. in a Jasco FP-8300 Spectrofluorimeter. The excitation and emission band slits are 5 and 2.5 nm respectively. Finally, data was expressed by plotting the intensity ratio I₃₃₈/I₃₃₃ against the polymer concentration in order to determine the CMC value.

5.4.3.4. Co-assembly studies by DLS.

X-EG(2)N₅(5) and X-prop(10) solutions prepared from CAC determination studies ranging from 0.004 to 2 mg·mL⁻¹ were modified with 1 µL of X-prop(10) and X-EG(2)N₅(5) respectively from stock solutions in order to have a final concentration of the added compound below its CAC. That means 0.1 mg·mL⁻¹ of X-prop(10) in X-EG(2)N₅(5) series, and 0.2 mg·mL⁻¹ of X-EG(2)N₅(5) in X-prop(10) series.
5.4.3.5. Cell viability against SHSY5Y cell line.
Cell assays to assess cell viability were carried out according to the protocol described in Chapter 4.

5.4.3.6. Uptake studies against SHSY5Y cell line by flow cytometry and Live-cell Confocal Imaging.
Cell assays and procedures for cellular uptake were carried out according to the protocol described in Chapter 4.
5.5. REFERENCES


56. *Journal*.


Chapter 6

Design and evaluation of polymeric carriers to enhance BBB crossing
6.1. INTRODUCTION AND BACKGROUND.


Brain disorders, including developmental, psychiatric and neurodegenerative diseases, represent an enormous disease burden, in terms of human suffering and economic cost. More than 600 disorders afflict the nervous system.¹ Neurodegenerative diseases are defined as hereditary and sporadic conditions characterized by progressive nervous system dysfunction. These disorders are often associated with atrophy of the affected central or peripheral structures of the nervous system. They include diseases such as Alzheimer's disease and other dementias, brain cancer, degenerative nerve diseases, encephalitis, epilepsy, genetic brain disorders, head and brain malformations, hydrocephalus, stroke, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease), Huntington's disease, Prion diseases, and others. With progressive human ageing and the overall deterioration of our ecological environment, an increasing number of people is year by year affected by neurological disorders that encompass, in many cases, chronic and incurable states. Only in Europe it has been estimated that 35 % of all disease burden is attributable to brain-related disorders.²

Thus, economic costs of brain disorders are correspondingly large, including not only cost of treatments, but also the lost productivity of patients and their caregivers, for whom it can represent an enormous emotional, practical, and financial burden. Hence, the overall disease burden from these disorders is much greater than would be suggested by mortality number.³ Just in Europe, the cost of brain diseases increased from 386 € billion per year (twice the estimated cost of cancer) in 2004⁴ (including direct costs of treatment and indirect costs) to 798 € billion in 2010.⁵ That value includes direct health care cost (37 %), direct non-medical cost (23 %), and indirect cost (40 %). See Figure 6.1 for disaggregated data. The average cost per inhabitant was 5,550 €.

Overall, the growing incidence and increasing costs caused by this family of diseases together with the lack of effective treatments, points out the need for novel approaches in order to address their enormous burden.
6.1.2. The nervous system.

The nervous system is the part of an animal's body that coordinates its voluntary and involuntary actions and transmits signals between different parts of its body. In most animal species, it encompasses two main components, the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS is formed by the brain and spinal cord, while the PNS consists mainly of nerves, whose axons connect the CNS to every other part of the body. The PNS consist of motor neurons (mediating voluntary movement), the autonomic nervous system (including the sympathetic nervous system), the parasympathetic nervous system (which regulate involuntary functions) and the enteric nervous system, that controls the gastrointestinal system.

Nervous tissue is the main component of the brain and spinal cord in the CNS, and the branching peripheral nerves in the PNS. It is composed of neurons, or nerve cells, which receive and transmit impulses, and neuroglia (glial cells or glia) (see Figure 6.2).

Neurons are nerve impulse-conducting cells considered the functional unit of the nervous system, which form the nerves, brain and spinal column. A typical neuron has a nucleated soma or cell body and the processes (axon and dendrites). They are classically classified
according to polarity in: bipolar neurons (in which the processes extend from opposite ends of the soma), (pseudo)unipolar neurons (in which the process extends from one end of the soma) and multipolar neurons (a neuron in which an axon and several dendrites extend from the soma). Furthermore, there is another classification according to the direction of nerve impulses in: sensory neurons or afferents (that transmit sensory information in the form of an action potential from the PNS to the CNS), motor neurons or efferents (that send nerve impulses away from the CNS to muscles or glands) and interneurons (that send impulses to another neuron).

Figure 6.2. Cells from neuronal tissue redrawn from ref 6.

Glial cells are considered as supporting cells that assist the propagation of the nerve impulse as well as provide nutrients to the neuron. They are smaller than neurons, and vary in structure according to their function encountering astrocytes, microglial cells and oligodendrocytes NG2 glia in CNS; Schwann cells, satellite glial cells and enteric glia in PNS.

The brain is the control center of the body. It controls thoughts, memory, speech, movement and regulates the function of many organs. The normal neuronal-vascular relationship is crucial for a good brain functioning. Indeed, neurons need a constant supply of oxygen and nutrients, thus, are located among 8-20 μm distance from capillaries. Adult brain contains approximately 650 km of capillaries, and it has been estimated that almost every neuron in human brain has its own capillary.
On the contrary, it is also critical to maintain brain homeostasis to keep normal brain function, and neurons are sensitive to many compounds and are subjected to many concentrations balances between outside and inside. Due to the high relevance of CNS functions, the brain is one of the most (if not the most) protected organs in the human body, being guarded by various barriers. Such barriers regulate nervous tissue homeostasis, and control the highly selective and specific uptake/efflux mechanisms, as well as the metabolism of endogenous and exogenous molecules. For that reason, accessibility of blood circulating compounds to the brain is one of the lowest compared to other organs, despite its dense capillary network. Most drug candidates have little effect in serious CNS disorders and many CNS drug development programs are discontinued mainly due to poor BBB permeation as the majority of candidates are either polar, with a MW > 500 Da, are bound to plasma proteins or are pumped out of the brain by means of efflux pumps. Indeed, according to Pardridge, only 2% of small-molecule drugs and almost 0% of biologic drugs do reach the brain, thus limiting the development of efficient treatments for brain diseases, especially in the case of antisense pharmaceuticals, gene medicines, and recombinant proteins.

Current drugs developed by pharmaceutical companies, capable to cross cell membranes and physiological barriers are both, smaller than 500 Da and lipophilic. In spite of all the limitations mentioned above, up to date, the main efforts to design strategies for the treatment of CNS disorders have been focused on drug discovery, rather than on the design of new approaches for brain targeting and active delivery.

**6.1.3. Barriers at the CNS.**

Concretely, three barriers regulate the transport of bioactives to brain parenchyma: (i) the blood brain barrier (BBB), localized in brain capillaries, between blood and interstitial fluid (ISF), representing the barrier between the lumen of cerebral blood vessels and brain parenchyma; (ii) arachnoid epithelial membrane (or meningeal barrier), an epithelial cell layer that covers brain tissue in the ventricles, limiting transfer from cerebrospinal fluid (CSF) to brain tissue. This barrier is the less studied and structurally the most complex of all brain barriers; and (iii) the blood-cerebrospinal fluid barrier (BCSFB), that involves the choroid plexus epithelium in the ventricles, representing the barrier among choroid plexus blood vessels and the CSF (See Figure 6.3).
Figure 6.3. Schematic representation of CNS barriers. a) Neurovascular unit from BBB, As: astrocyte, Peri: pericytes, Endo: endothelial cell and B.M.: basement membrane. b) The meningeal barrier. Blood vessels of dura are fenestrated and provide little barrier function; however, outer cells of arachnoid membrane (Arach) have tight junctions (arrowheads) forming the physical barrier between the CSF-filled subarachnoid space (SAS) and overlaying structures. Blood vessels in arachnoid and on pial surface (PIA) have tight junctions with similar barrier characteristics as BBB. c) Blood-CSF barrier. Choroid plexus blood vessels are fenestrated and form a non-restrictive barrier (small arrows); however, the epithelial cells (Ep) have apical tight junctions (arrowheads) that restrict intercellular passage of molecules. d) Adult ventricular ependymal does not restrict the exchange of molecules at least as large as proteins between CSF and brain. Fetal CSF-brain barrier, a barrier between the CSF and brain parenchyma, has only been shown to be a functional barrier in the early developing. Redrawn from Saunders et al.14
6.1.3.1. Blood-brain barrier (BBB).

6.1.3.1.1. Structure.

In order to preserve brain homeostasis, brain vessels are modified to form the BBB. Since its discovery by Paul Ehrlich in 1885,15 BBB has been considered the most critical and nearly undefeatable cellular barrier in the human beings that protects both brain and spinal cord. The BBB is a highly specialized and selective physical and physiological barrier that maintains such mentioned brain homeostasis by controlling the entry of compounds from blood to brain, and their efflux, as well as protects nervous tissue against potentially harmful xenobiotics and endogenous molecules that could be present in the bloodstream. It acts also as a metabolic barrier due to the high turnover of the intra and extracellular enzymes, what it must be taken into account in drug development.16, 17 The BBB is mainly formed by brain capillary endothelial cells (BCECs),18 although other cell types, such as pericytes, neuronal terminations, and perivascular astrocytes play an important role in BBB structure and function,19-21 forming the neurovascular unit (Figures 6.3 and 6.4). Moreover, the BCECs are closely connected by the presence of intercellular tight junctions (TJs).

Due to their specific functions, BCECs possess specific features when compared to peripheral ECs:22-25 (i) they present remarkably lower number of endocytic vesicles, limiting the transcellular transport; (ii) absence of fenestrations; (iii) high electrical resistance due to the presence of the TJs that restrict the paracellular flux; (iv) higher mitochondrial volume, what reflects a higher metabolism; (v) specialized transport systems and (vi) lack of lymphatic drainage.

The main components responsible for the structural integrity of the BBB are TJs and the adherent junctions (AJs).26 Such structures are closely located forming the junction complex between adjacent BCECs. While AJs are more involved in the initiation and maintenance of BCECs contact, TJs seal them to form a continuous tubular structure.27 TJs are located in the upper part of the apical region of plasma membrane from BCECs and represents the most important seal to prevent paracellular diffusion, also taking part in the regulation of lateral diffusion between apical and basolateral plasma membrane domains, thus, keeping constant plasma membrane lipid and protein polarity.28 Such structures are composed by a complex network of parallel, interconnected, transmembrane and cytoplasmatic families of proteins (junctional adhesion molecules (JAMs),29, 30 occluding,31
claudins \(^{32, 33}\) and cytoplasmic accessory proteins). The high transendothelial electrical resistance (TEER) in brain endothelium (2000 ohm.cm\(^2\) compared to 2-20 ohm.cm\(^2\) in peripheral capillaries)\(^{34}\) is mainly due to the presence of the TJ complex.\(^{35}\) In respect to AJs, they have been reported to be essential in the maintenance of TJ and the junctional complex.\(^{36}\) Their main components are transmembrane glycoproteins from the cadherin superfamily linked to the cytoskeleton.\(^{37, 38}\)

Apart from the already described BCECs, pericytes, astrocytes, neurons, microglia and extracellular base membrane significantly contribute to BBB function and maintenance, although their full role remains still unknown.

Astrocytes, a type of microglia cells, play a key role in BBB creation and maintenance since their end-feet contact directly with BCECs forming a net that acts as a very restrictive barrier.\(^{39, 40}\) They have been described to significantly influence neurovascular structure, maintenance and integrity; participating in the nutrition and metabolic support of neurons.\(^{41}\) The effect of astrocytes in BCECs relies on the secretion of numerous signaling molecules, such as cytokines, neutrophins and many factors.\(^{42, 43}\) Astrocytes are also related with the regulation of expression and polarized location of transporters such as P-glycoprotein (P-gp)\(^{44}\) and GLUT1.\(^{39}\) They have been suggested to play an important role in TJs formation and integrity.\(^{27, 45}\) Indeed, in vitro co-cultures of astrocytes and BCECs achieve high electrical resistances, a strong evidence of their implication.\(^{46, 47}\)

Pericytes are a type of mesenchymal cell that occupy the vascular space, between the capillary wall and astrocytes end-feet. Pericytes have been historically considered as mere scaffolds, but they have important roles in the communication with BCECs by direct physical contact and paracrine signaling pathways.\(^{48}\) They are known to play a regulatory role in vascular tone, stability, repair and angiogenesis both in CNS and non CNS.\(^{41, 49-51}\) Moreover, they have been related to the efflux transporters expression (i.e. ATP-binding cassette (ABC) transporters) as well as to the growth and function of the endothelium.\(^{51}\) Furthermore, it has been demonstrated that pericytes deficiency increases BBB permeability to water and to a range of low and high molecular mass tracers injected i.v.\(^{50}\) In addition, it has been recently reported that the lack of pericytes in mice models lead to neuronal degenerative changes, learning and memory impairment and
neuroinflammatory responses. All that together highlights the relevance of pericytes in the neurovascular unit.

Microglia are the resident immune cells in the CNS derived from monocytes and macrophages, and perform an essential role in the immune response. Whereas in normal conditions, neurons and glial cells interact together to promote brain homeostasis, microglia become activated under brain injury or immunological stimuli and undergo alterations from the so-called resting state to an activated one. This activation starts a coordinated neuroinflammatory response which has been demonstrated to be involved in BBB impairments and consequently neurodegeneration. Nonetheless, the mechanisms by which this occurs are not fully understood yet.

The basement membrane at BBB plays important roles including maintenance of capillary vessel morphology, cell adhesion, and prevention of plasma protein leakage from capillary vessels. It is constructed from extracellular matrix (ECM) such as collagen type IV, laminin, and bronectin, and regulated by matrix metalloproteinases (MMPs) and plasmin.

Finally, neurons are also actively participating in this structure since neuronal terminations arrive to all cells forming the BBB.

Figure 6.4. Blood Brain Barrier structure. Redrawn from ref 63.
6.1.3.1.2. Transport mechanisms across BBB.

In general, nutrients, ions and other molecules cross the BBB following a paracellular diffusion between BCECs cells through the junctional complex or by the transcellular pathway across the cells.

**Paracellular pathway.** The paracellular diffusion is a passive mechanism coordinated by TJs. Small water-soluble molecules as well as lipophilic solutes cross the BBB depending on electrochemical, hydrostatic and osmotic gradients. However, this pathway only plays a minor role in brain targeted drugs and is significantly reduced in BBB, when compared with other organs.\(^{64}\)

**Transcellular pathway.** This pathway can be energy or non-energy dependent and includes all types of transport mechanisms across cells. Among them, it can be classified as passive or active, depending on energy requirement (adenosine triphosphate, ATP).

**Passive transcellular mechanisms:** are based on the transport of molecules by simple diffusion (transcellular diffusion) or facilitated diffusion (carrier-mediated transport) always following the concentration gradient.

**Transcellular diffusion.** Using this pathway, lipid-soluble substances, such as oxygen, CO\(_2\), alcohol or steroid hormones are able to diffuse across both the luminal and the abluminal membrane of the capillary endothelium.\(^{65}\) This route is the most favored among passive mechanisms. Indeed the vast majority of CNS drugs follow this mechanism.\(^{66}\)

**Carrier-mediated transport (CMT),** is a saturable transport process.\(^{67}\) There are two forms of CMT, active transport (which could should be included in active transcellular mechanisms) and facilitated diffusion. For instance, entry of glucose by glucose transporter 1 (GLUT1) is by facilitated diffusion, in the concentration gradient direction. In case of amino acids, there are different and selective energy dependent carrier proteins (active).\(^{68}\) These carriers are normally polarized and localized on both the luminal and abluminal membrane of the BCECs, operating in both directions.\(^{69}\) Overall, there are many selective carrier systems expressed in brain ECs.

**Active transcellular mechanisms:** Based on the use of ATP as energy, these transport mechanisms allow the interchange of molecules against gradient concentration, relying mostly on vesicle formation.
Receptor-mediated transcytosis (RMT). RMT represents the principal mechanism used by brain ECs to uptake essential molecules such as hormones, or high molecular mass proteins such as insulin, leptin, low density lipoproteins, transferrin or insulin-like growth factors (IGF) across BBB. This transport process is based on the receptor-ligand recognition at the luminal membrane of BCECs inducing the formation of coated pits that engulf the ligand leading to an endocytic vesicle. Then, such vesicle suffers the endosomal fusion when dissociation of the receptor from the ligand occurs releasing the content by exocytosis. Finally, free receptor is recycled to cell surface or degraded through lysosomes. Nevertheless, complete crossing is never achieved since some vesicles fuse with lysosomes and their content is degraded.

Currently, efforts done to achieve BBB crossing in CNS drug delivery are mainly directed to the use of this pathway since it is neither size limited nor lipophilicity-dependent. Basically, the strategies are based on association or conjugation to ligands capable to promote transcytosis upon receptor binding, forming the so-called molecular “Trojan horses”. Among such receptors, there are three widely investigated for drug delivery approaches: (i) insulin receptor (IR), (ii) transferrin receptor (TfR) and (iii) low-density lipoprotein receptor related proteins 1 and 2 (LDP-1 and 2).

Adsorptive endocytosis (AE or adsorptive-mediated transcytosis AMT). This mechanism takes place through ionic interactions. Positively charge molecules such as cationized albumin, protamine or histone interact with the negatively charged components of the EC membrane. This approach has been used for the delivery of drugs by means of albumin conjugation for instance, and it has been also explored with antibodies for applications in diagnostics, neuroimaging and brain treatment.

Efflux transport system. The efflux transport is an active process for substance removal from the CNS to the circulation in order to prevent brain accumulation. Present in the BBB and in the BCSFB as well, this mechanism is the reason by which most of drugs that cross BBB, do not reach the necessary concentration for being therapeutically active. One of the best known efflux transporter is P-glycoprotein (P-gp), a member of the ATP-binding cassette (ABC) transporters, localized in the luminal membrane of BCECs. P-gp forms part from the multidrug resistance receptors (MDRs), and actively transport many
anticancer drugs, antibiotics, immune system suppressors or ionic channel modulators out of the brain,\textsuperscript{81, 82} avoiding the accumulation of toxins or drugs in the brain.

On the contrary to P-gp, there are also energy independent efflux transporters such as the organic anion transporter (OAT) and organic anion transporter polypeptide (OATP), that have dual function as efflux and influx transporters depending on compounds concentration on both sides of the BBB.\textsuperscript{83}

\textbf{Figure 6.5.} Schematic representation of transport mechanisms across BBB including diffusion, paracellular transport, carrier-mediated transport, receptor-mediated transport, adsorptive-mediated transport and efflux systems.

\textbf{6.1.4. BBB models.}

In addition to a better understanding of physiological and pathophysiological conditions of the brain and its diseases, methods able to select drug candidates more efficiently in the early preclinical stages are needed. Currently, selection of potential candidates for CNS diseases strongly relies on either \textit{in vitro} or \textit{in silico} estimation of BBB permeability, or/and on \textit{in vivo} measurements of the total brain to total plasma drug concentration ratio.\textsuperscript{84} \textit{In silico}, \textit{in vitro} and \textit{in vivo} methodologies have been the subject of much research and development in recent years.\textsuperscript{46, 47, 52, 85, 86} Several \textit{in silico} and \textit{in vivo} methods are well-established in the development of new drugs. \textit{In vitro} methods are, however, more controversial. It is well-known that no simple \textit{in vitro} model can mimic all BBB functionalities due to its inherent complexity.\textsuperscript{85} Nonetheless, it is nowadays consensual that the
model used must at least hold the most relevant features of BBB needed for the aim of the investigations, that are commonly directed either to CNS drug permeability, elucidation of drug interactions at the BBB, or to perform physiopathological, toxicological and immunological studies. For the first line of research mentioned, an in vitro model suitable for compounds screening in terms of BBB permeability, it should possess features such as low paracellular permeability and express key enzymes and carrier transport systems. The main advantages of in vitro models, in comparison to the in vivo models rely on their simplicity, lower cost and lower amounts of compounds required, their high throughput screening capacity, the possibility to quantify compounds in physiological buffers and the identification of early signs of cell toxicity, apart from the need of lower number of animals, better from an ethical as well as an economical point of view. Nevertheless, in vitro methods cannot substitute in vivo tests. At general basis, in vitro models encompass.

(i) Physico-chemical models. Represent simple methods used to select candidates for CNS diseases based on their physiochemical properties. Immobilized artificial membrane chromatography (IAM), parallel artificial permeability assays (PAMPA) and lipophilicity measurements are the most commonly used, however, they are mainly used for compounds transported by passive diffusion.

(ii) Cell-based models were developed in order to obtain more reliable prediction. Isolated microvessels present already a 3D structure. They are useful to study morphological aspects, expression and activity of transporters and to carry out drug delivery assays. Primary or low passage of immortalized cell lines of cerebral origin provide the closest phenotypic resemblance to that of the in vivo BBB because many of their characteristics are genetically programed. Their main limitations rely on the fact that are not able to retain BBB properties. To overcome such drawbacks co-culture and triple co-culture systems have been implemented through combination of primary or immortalized cell lines of BCECs, with astrocytes, astrocytic cell lines, C6 glioma cell lines, pericytes, mixed glial cells and/or cell conditioned media. Such models represent a more realistic scenario since all elements of the neurovascular unit strongly contribute to the development and maintenance of the BBB phenotype. Nonetheless, they are time-consuming and still do not fully reproduce
in vivo conditions. Cells lines of non-cerebral origin are also used to mimic BBB. Among them, of the most characterized and used cell line is the Madin- Darby Canine Kidney (MDCK) cell line, which is easy to grow, achieves a reproducible TEER value and can be transfected in a stable manner with the MDR1 gene, resulting in the polarized expression of P-gp, for instance. More recently, Lippmann and coworkers have developed a promising model based on human pluripotent stem cells (hPSCs), were neural progenitor cells differentiated to mature neurons and astrocytes that can be used in co-culture, representing a robust BBB model. Traditional techniques for mimicking the BBB are based on static two chamber systems (Transwell™) separated by a cell monolayer grown on a polycarbonate membrane that represents the barrier. These static models have the limitation of low reproducibility of the anatomical and physiological features of the BBB, and consequently, poor correlation with the in vivo results. To address that, microfluidic BBB models to simulate the real environment have been also tested in 2D systems by applying flow-induced shear stress across the monolayer. One step further is the use of dynamic in vitro models with tridimensional architecture (3D models) developed to reproduce more accurately the physiological features of the brain vascular segments, taking into account cellular movements, cell-cell interactions and the blood flow in the prediction of brain permeation. However these systems are not routinely used due to their technical demand and high cost. Extensive information about in vitro models can be found in literature.

Pathological in vitro models of BBB have been also developed to address drug permeability in disease-altered BBB. Among other alternatives involve the use of cells derived from invertebrates such as Drosophila melanogaster and Zebrafish.

Nevertheless, predicting CNS drug efficacy on the basis of an estimated BBB permeability is far from reality since there are many parameter that are not taken into account related with their pharmacokinetics (PK) and pharmacodynamics (PD) once administered. Such parameters include the ratio between drug concentration in brain parenchyma and blood levels as well as the further distribution within the brain compartments with special features. Thus, all those factors need to be addressed directly by in vivo studies. To date the most predictive PK/PD parameter for a desired effect in brain is now recognized to be the free drug concentration in
To this respect, tissue microdialysis is the only technique currently available that allows not only direct *in vivo* measurement of brain ISF concentration of unbound drug *in vivo*, but also the establishment of highly valuable PK/PD relationships.\(^\text{54, 112, 113}\)

### 6.1.5. Delivery strategies to the brain.

As stated before, many drugs with potential therapeutic effects in the treatment of CNS pathologies fail due to poor pharmacokinetics and the limitations to cross BBB. Up to date, the challenge of brain drug delivery has been partly accomplished through several strategies, including invasive or non-invasive approaches. The majority of such techniques rely on the combination of several skills such as biology, biophysics as well as the use of nanotechnology to achieve this specific task. Invasive techniques encloses surgery-needed approaches and BBB disruption whilst non-invasive ones encompass drug modification by medicinal chemistry approaches and the use of nanotechnology (Figure 6.6).

Among non-invasive approaches, medicinal chemistry is a widely used approach to enhance passive diffusion across BBB by increasing the lipid solubility of polar small drug candidates with poor BBB permeability.\(^\text{9, 25, 123}\) Methylations, halogenations and in general polar groups blockage has been traditionally used together with the introduction of lipid moieties.\(^\text{67}\) Nevertheless, this approach encounters several difficulties: (i) when increasing the lipid solubility of the drug, non-specific bindings to plasma proteins and lipids contained in fat tissues are prone to increase too, as well as penetration in all body organs; (ii) structural modifications to the original drug can diminish the original affinity/activity for the target receptor;\(^\text{131-135}\) (iii) chemical modifications usually lead to increase on MW, thus affecting brain penetration.\(^\text{11}\) For that reasons is really difficult to design small drugs able to cross BBB since a well-optimized balance from physicochemical properties and pharmacokinetic profiles must be first accomplished, ending on effective CNS activity, acceptable absorption, distribution, metabolism and excretion/elimination (ADME) properties and favorable PK/PD profiles.
Figure 6.6. Schematic representation of current strategies to deliver drugs to the brain. Invasive techniques encloses surgery-needed approaches and BBB disruption whilst non-invasive techniques encompasses drug modification by medicinal chemistry and alternative approaches. Redrawn from ref\(^{25}\)

The use of *fusion proteins* and *cell-penetrating peptides (CPP)* to achieve BBB crossing included in medicinal chemistry approaches is becoming a popular strategy.\(^{75}\) CPPs are short amphipathic cationic peptides (less than 30 amino acids) that facilitate rapid internalization of exogenous cargo including drugs, proteins, nucleic acids, liposomes or nanoparticles after chemical conjugation.\(^{139, 140}\) Although the mechanism is not fully understood, their first contacts with cell surface are mediated by electrostatic interactions with proteoglycans, and there are some hints that point out to non-specific AMT. Several natural and synthetic peptides are under use for this
purpose encompassing the well-known TAT (transactivator of transcription), Penetratin, the Syn B vectors (derived from the antimicrobial peptide protegrin 1), or Mastoparan/transportan." In spite of their potential in promoting drug delivery, CPPs suffer from some limitations such as: (i) toxic effects on cell membranes and organelles; (ii) poor pharmacokinetics and short half-lives in biological media due to degradation by proteolytic enzymes; (iii) immunogenicity and complement activation.

Microspheres and biodegradable wafers technologies have been also implemented to increase drug retention in the brain by using lipid-based polymeric devices in the first case, and biodegradable polymers in the second to form drug complexes. However, such technology is locally applied to the brain by stereotaxic surgery, what is safer than the previous invasive methods described, but still harmful. Alternative methods include intranasal administration, the use of molecular Trojan horses (TH), genetic engineering, the use of nanotechnology-based systems, their combinations and combination with medicinal chemistry strategies (Figure 6.6).

Intranasal route of administration have been successfully applied in the delivery of some small lipid molecules, nano-sized micelles and liposomes. Nevertheless, there are many physiological variables that influence the administration such as pH, high enzymatic activity of the epithelium, mucosal irritation or even nasal pathologies. Moreover, it is limited by drug’s lipophilicity. Genetic engineering strategies are based on the implantation of living tissue inside brain with the ability to secrete therapeutic molecules. For instance neural stem cells (NCS) have been implanted into hippocampus of AD and Down syndrome mice model in order to modify levels of tau/reelin-positive granules. Recent studies confirm the potential of such approaches, however, they are still limited by the short survival rate of the inserted tissues.

Among the strategies based on nanosystems two main approaches can be followed: the use of CNS delivery systems relying on BCECs uptake and notably adsorptive-mediated endocytosis or the use of targeted delivery systems via targeting BBB receptors (carrier-mediated or receptor-mediated). The first strategy is followed by liposomal formulations (neutral or cationic), colloidal nanoparticles (neutral or cationic) and solid lipid nanoparticles, relying only in their intrinsic characteristics to cross biological barriers. Since this thesis
deals with the use of targeted drug delivery systems, the first approach will not be further commented and examples of those systems are reviewed in the excellent literature.\textsuperscript{9,25}

Special mention will be done to the approach based on the use of TH and some examples in their use in targeted drug delivery will be addressed. TH are vectors able to bind specific receptors while carrying cargo molecules that can be transported across BBB.\textsuperscript{148-150} The general strategy to deliver biologics to the brain by the use of TH follows RMT, and involves the conjugation of a receptor-targeting moiety with the therapeutic of interest.\textsuperscript{151-153} that can be mAbs, recombinant proteins, RNA, DNA, or nanomedicines. The most well studied BBB RMT targets include transferrin receptor (TfR), insulin receptor and the LDL receptors.

TfR mediates iron delivery to the brain by binding the iron-binding protein transferrin (Tf)\textsuperscript{154} and is expressed at high level at the BBB.\textsuperscript{155,156} It has been widely used for BBB delivery.\textsuperscript{151,152,157} For instance, PEGylated liposomes decorated with Tf and CPP polyarginine demonstrated significant brain accumulation at 24 hours (4 % of injected dose, ID) after i.v. administration in rat. Nonetheless, despite its use, Tf is not the ideal ligand as endogenous Tf is present at high concentrations in the bloodstream and will always compete with the injected one.\textsuperscript{158}

Thus, antibodies targeting epitopes distal to Tf-binding site have been developed in order to overcome the competition limitation, and there are plenty of reported examples about them.\textsuperscript{157} For instance, a fusion protein of cTfRMAb, a chimeric mAb that binds to the mouse TfR, was used to deliver TNF$\alpha$ achieving 1.4 % ID in mouse brain\textsuperscript{159} and showed significant neuroprotection in a mouse model of Parkinson Disease (PD).\textsuperscript{160} Erythropoietin and glial-derived neurotrophic factor (GDNF) have been also delivered by this strategy for PD treatment with promising results.\textsuperscript{161,162} One of the most utilized anti-TfR mAb is OX26, with plenty of examples in literature.\textsuperscript{163-166} For example, PEGylated liposomes decorated with OX26 were used to deliver GDNF expression plasmids to treat PD in rat models achieving significant neuroprotection after 3 weeks of once-weekly injections.\textsuperscript{167} Apart from PD, a wide range of other neurological disorders are been targeted with anti-TfR antibodies.\textsuperscript{137,168-170}

Recent approaches employ the iron-mimicking cyclic peptide, CRTIGPSVC, as the RMT-targeting ligand.\textsuperscript{171} Although it binds to the
Tf-binding site, this peptide is showing promising results in the treatment of CNS disorders\textsuperscript{171,172} For that reason, it has been one of the vectors selected within this thesis for BBB targeting.

However, the use of TfR as a target for RMT may be limited due to: (i) TfR expression in vascular beds and parenchyma of other organs, what will lead to widespread distributions; (ii) Full transcytosis of TfR to the brain may be limited and depends on the affinity of the ligand for the receptor\textsuperscript{173,174}

The insulin receptor, IR, (also expressed at the BBB)\textsuperscript{156} responsible for the transport of insulin\textsuperscript{71, 175, 176} to the brain has been also explored. As insulin itself cannot be used as vector (due to its short half-life in serum and the possibility to cause hypoglcemia upon administration of exogenous insulin),\textsuperscript{177} strategies are centered on the use of human IR mAbs (HIRMAb, and HIRMAb fusion proteins) for brain delivery\textsuperscript{178}. Indeed, the termed AGT-181 (ArmaGen Technologies), a HIRMAb fused with α-L-iduronidase (IDUA), and enzyme missing in Hurler’s syndrome is currently in clinical trials, representing the first effort to bring RMT-targeted antibodies to the clinic for the treatment of genetic lysosomal storage diseases\textsuperscript{178-182} This system achieved approximately 2 % of ID in rhesus monkey brain 2 hours after i.v. injection. Apart from AGT-181, HIRMAb has been fused to numerous other therapeutic proteins such as anti Aβ scFv,\textsuperscript{183, 184} GDNF,\textsuperscript{185, 186} TNFR,\textsuperscript{187, 188} erythropoietin\textsuperscript{189} or paraoxonase.\textsuperscript{190, 191} Moreover, Pardridge et al. have successfully constructed a radiolabeled Aβ peptide conjugated to HIRMAb, as a diagnostic probe for AD.\textsuperscript{192-196} Nevertheless, despite its efficiency, this approach is considered risky due to its involvement in glucose homeostasis.

Other receptors exploited are the low-density lipoprotein receptor (LDLR) and low-density lipoprotein receptor-related proteins 1 (LRP1) and 2 (LRP2), that are expressed in BCECs,\textsuperscript{155, 156} and mediate the transport of lipoproteins and a diverse array of other ligands (such as ApoE, HIV-1, TAT protein, lactoferrin, melanotransferrin, receptor associated protein…) across the BBB via RMT.\textsuperscript{197-199} Although antireceptor Abs have not been reported for this family, overwhelming studies have explored de use of LDLR and LRP ligands and peptide ligand mimics as vectors for brain delivery. For instance NPs decorated with ApoE have been shown to cross the BBB in vivo\textsuperscript{200, 201} Angiopep-2 (ANG-2) a 19 amino acid peptide, was reported as a ligand targeting LRP receptor,\textsuperscript{202-204} and has shown
promising results as a delivery vector in the treatment of glioma mainly due to its elevated reported rate of transcytosis. Furthermore, a peptide-paclitaxel conjugate with 3 molecules of paclitaxel linked to ANG-2, termed ANG1005 has been shown to have activity against glioblastoma, and to extend the survival of mice with intracerebral tumors. These systems enters the brain through LRP1 and is not a substrate for the P-gp efflux transporter. ANG1005 is being developed by Angiochem for glioma treatment and is the most advance system for BBB targeting with numerous Phase I clinical trials completed and Phase II trials under way. Besides, DOX and etopoxide conjugated to ANG-2 lead to ANG1007 and ANG1009, respectively with increased brain penetration. Moreover, ANG-2 has been also investigated in the delivery of genes, peptides, proteins, antibodies and enzymes. Furthermore, ANG-2 is becoming popular in targeted nanomedicines to cross BBB with a growing number of examples in literature. For instance, ANG-2 conjugated with PEG-co-poly(3-caprolactone) NPs was developed as a dual targeting drug delivery system for glioma treatment. ANG-PEG-DOX-AuNPs, loaded with DOX through hydrazone, and functionalized with ANG-2 was able to deliver and release DOX in glioma and to increase survival time of glioma-bearing mice. Dual peptide-modified liposomes with ANG-2 and neuropilin-1 receptor (tLyP-1) for brain tumor targeting and penetration were designed and loaded with siRNA and DTX showing good results after systemic administration against mice with U87MG tumors. ANG-2-pluronic F127-conjugated superparamagnetic iron oxide nanoparticles have been recently proposed as nanotheranostic agents for BBB targeting. The examples listed are just a few from the increasing body of literature about ANG-2 uses in nanoconstructs. Due to all this background information, in this thesis, ANG-2 has been also selected as vector for brain delivery.

Apart from those, other less studied receptors are becoming popular as targets for BBB crossing such as diphtheria toxin receptor, or acetylcholine receptors. Glutathione (GSH) is recently been investigated as targeting vector since BBB express transporters for this molecule. For instance, glutathione pegylated liposomal doxorubicin (2B3-101) is being developed as a new treatment for patients with brain cancer and is currently in Phase I/II clinical trials.
6.1.6. *In vivo Imaging.*

Improving the characterization of compounds and their effects in early, and not yet so costly phases is key to shorten drug discovery development process. Moreover, and linked with that, knowledge about a given disease or disease model, and their early diagnosis and characterization is a required step to develop biomarkers and to discover molecular targets for effective treatments. In this context, *in vivo* imaging emerge as a highly necessary battery of techniques to address several crucial questions concerning drug discovery and development. Although, no single imaging modality can answer all possible questions in this complicated domain, to find out the potential of such techniques in a certain disease area is important. For instance, a combination of neuroimaging tests are needed to provide diagnostic support and to elucidate underlying mechanism of pathologies. Hence, encompassing CNS bioimaging to achieve a whole picture includes the use of nuclear medicine techniques such as Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT); and morphological information obtained by Magnetic Resonance Imaging (MRI) and Computed Tomography (CT). A complete review of imaging strategies within pharmaceutical research is beyond the scope of this thesis, however Table 6.1 gives an overview of current imaging modalities considered of interest.
Table 6.1. *In vivo* imaging techniques currently used in the context of biomedical research and/or medical diagnosis. Adapted from ref\textsuperscript{38}

<table>
<thead>
<tr>
<th>Technique</th>
<th>Clinical imaging</th>
<th>Resolution</th>
<th>Animal imaging</th>
<th>Resolution and time scale</th>
<th>Applications</th>
<th>Main characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPECT (low energy $\gamma$-rays)</td>
<td>Yes</td>
<td>6-8 mm; s</td>
<td>yes</td>
<td>1-2 mm min</td>
<td>Functional</td>
<td>Radioisotopes have longer half-lives than those used in PET; sensitivity 10-100 smaller than PET</td>
</tr>
<tr>
<td>PET (high energy $\gamma$-rays)</td>
<td>Yes</td>
<td>4 mm; s</td>
<td>yes</td>
<td>1-2 mm min</td>
<td>Metabolic, functional, molecular</td>
<td>High sensitivity (pM concentrations). Cyclotron needed</td>
</tr>
<tr>
<td>CT</td>
<td>yes</td>
<td>0.5 mm; s</td>
<td>yes</td>
<td>50-100 $\mu$m min</td>
<td>Anatomical, functional</td>
<td>Poor soft tissue contrast</td>
</tr>
<tr>
<td>Ultrasound</td>
<td>yes</td>
<td>300-500 $\mu$m; s</td>
<td>yes</td>
<td>50 $\mu$m min</td>
<td>Anatomical, functional</td>
<td>Difficulties to image through bone or lungs; microbubbles used for contrast enhancement</td>
</tr>
<tr>
<td>MRI</td>
<td>yes</td>
<td>1 mm; s to min</td>
<td>yes</td>
<td>80-100 $\mu$m s to h</td>
<td>Anatomical, functional, molecular</td>
<td>High spatial resolution and soft tissue contrast</td>
</tr>
<tr>
<td>Bioluminescence</td>
<td>no</td>
<td>-</td>
<td>yes</td>
<td>1-10 mm s to min</td>
<td>Molecular</td>
<td>High sensitivity; transgene-based approach, light emission prone to attenuate with increased tissue depth</td>
</tr>
<tr>
<td>Optical Imaging</td>
<td>no</td>
<td>-</td>
<td>yes</td>
<td>1-3 mm s to min</td>
<td>Molecular</td>
<td>Excititation and emission light prone to attenuate with increased tissue depth</td>
</tr>
</tbody>
</table>
6.2. RESULTS AND DISCUSSION.

Within this dissertation, *in vivo* PET and *ex-vivo* gamma counter were used to follow biodistribution of radionuclide labeled polymers. For fluorescently labeled systems, *ex-vivo* fluorescence imaging was applied. PET was used as imaging technique since it allows monitoring via positron emitter-labeled compounds and it has been demonstrated to be more sensitive than MRI or CT. However, high costs and the need for a cyclotron limit its use. Very briefly, the system detects pairs of gamma rays emitted indirectly by a positron-emitting radionuclide (tracer), which is introduced into the body. As the radionuclide decays, positrons are annihilated by electrons, giving rise to gamma rays that are detected simultaneously. One of the most advantageous characteristic of PET is its capability to detect organ accumulation in a high accurate rate, regardless of tissue depth.

In this thesis, positron emitters $^{68}$Ga, $^{111}$In were incorporated into linear, hybrid di-block and star-shaped PGAs through complexation chemistry. In order to accomplish stable complexation of the metal radioisotope, the incorporation of chelating agents into the polymer backbone represented a prerequisite. The most commonly used chelating agents for $^{68}$Ga and $^{111}$In are based on polyamine carboxylic acids such as diethylene triamine pentaacetic acid (DTPA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), or 1,4,7-triazacyclododecane-1,4,7-tetraacetic acid (NOTA). For the biodistribution of radiolabeled PGA-based architectures, DOTA derivative chelating agent with a free amine group suitable for conjugation (DO3A-NH$_2$) was selected as forms stable complexes with several M$_2^+$ and M$_3^+$ ions such as $^{68}$Ga, $^{111}$In, and others.

6.2.1. Technique validation. Linear non-targeted PGA labeling and *in vivo* evaluation using PET.

In a first approach, in collaboration with Dr Inmaculada Conejos Sanchez, (shared research and also presented in her PhD thesis), *in vivo* biodistribution studies of linear homopolymer PGA (100 GAU) was studied using PET as well as *ex-vivo* radioactivity measurement using a gamma counter. For that purpose, PGA-DO3A- $^{68}$Ga was first synthesized and *in vivo* evaluated. DO3AtBu-NH$_2$ derivative was used instead of DOTA for several reasons: a free amino group was desired for a straightforward conjugation to the PGA.
backbone; the free COOH groups of DO3A-NH$_2$ could interfere with further conjugations to the PGA chains; DO3A-NH$_2$ forms stable complexes with the chosen radionuclides as well as DOTA; tBu protecting groups provide solubility in most organic solvents and the tert-butyl ester removal procedures do not need harsh conditions that could damage the polypeptide chain as well as further molecules included afterwards. Therefore, 9.8 mol% GAU of DO3A-tBu-NH$_2$ was effectively conjugated via amide bond to a linear homopolymers PGA$_{100}$ following the strategy depicted in the Scheme 6.1. Quantitative conjugation efficiency was achieved (since 10 mol% GAU was pursued) with a reasonable mass yield of 80 %. Percentage of modification was calculated according to $^1$H-NMR analysis by comparing the corresponding integral of the $\alpha$-protons of PGA (4.24 ppm) with the 27 protons of tBu groups at 1.40 ppm (Figure 6.7).

**Scheme 6.1.** Synthetic route to PGA-DO3A (27a).

**Figure 6.7.** $^1$H-NMR spectrum (D$_2$O) of PGA-DO3A-tBu (26a).

Removal of tBu groups was optimized in order to limit the exposure time in the acidic solutions of the synthesized architectures to avoid damage to the polymeric carriers as well as to the peptidic sequences for BBB targeting planned to be introduced afterwards.
Finally, 3 hours in a mixture TFA/H$_2$O (95:5) lead to successful deprotections without compromising the integrity of the carriers. Figure 6.8 shows the comparison between PGA-DO3A-tBu (26a) and PGA-DO3A (27a) where the signal at 1.40 ppm corresponds to tBu groups.

Figure 6.8. $^1$H-NMR spectra (D$_2$O) of PGA-DO3A-tBu (26a) and PGA-DO3A (27a).

$^{68}$Ga labeling was performed at CIEMAT by our collaborators (Madrid) reaching radiochemical yields of 86.3 % ± 3.4 (n= 5), and radionuclide purity (RNP) was < 3.4·10$^{-4}$ ± 0.4·10$^{-4}$ in all cases.

Animal experiments to test biodistribution and pharmacokinetics (PK) profile of PGA-DO3A-$^{68}$Ga (28a) were carried out at the Unit of Biomedical Applications of Radioisotopes and Pharmacokinetics at CIEMAT (Madrid). PGA-DO3A-$^{68}$Ga, (28a) was injected i.v. to FVB/NJ mice. Animals were monitored by PET only up to 3 hours due to $^{68}$Ga decay (Figure 6.9). Five mice per time point were sacrificed (corresponding to 0.25, 0.5, 1, 2 and 3 h) and different organs were extracted for radioactivity measurement ex vivo (Figure 6.10). According to the results from biodistribution, a renal excretion profile (bladder and kidney signals) with no specific accumulation in any organ was obtained. This was further confirmed in the ex vivo analysis where the higher percentage of the injected dose (ID) corresponded to the kidneys.
Figure 6.9. PGA-DO3A-\textsuperscript{68}Ga (28a) (50.1 µCi) PET images (Sagittal, coronal and transverse images) obtained 18 min after administration during 60 min acquisition (top) and 160 min after administration during 40 min acquisition (bottom).
**Figure 6.10.** PGA-DO3A$^{68}$Ga (28a) biodistribution. Data expressed as normalized % ID per gram of tissue at different time points. n> 4, mean ± SEM.
Representation of the % ID/mL in plasma was used to assess PK parameters (Figure 6.11). The best fit was obtained with a two-compartmental model. From the parameters obtained, the half-life estimation of the conjugate was 0.59 hours.

![Figure 6.11](image)

**Figure 6.11.** % ID/mL in serum against time of Linear PGA-DO3A-^{68}Ga (28a).

### 6.2.2. First Generation. Linear PGA and DB PEG-PGA labeled and targeted polymers synthesis and *in vivo* evaluation by PET.

#### 6.2.2.1. Synthetic strategies to reach BBB-targeted linear PGA and DB PEG-PGA with peptidic residues.

Within this first attempt to reach the BBB, two peptidic sequences widely recognized as potential candidates to achieve BBB active targeting have been chosen. These peptide sequences are the already mentioned ANG-2 and the iron-mimicking cPEP. As initial approach, both peptides were conjugated to linear PGA_{100} and hybrid di-*block* copolymer (DB) PEG_{42}-PGA_{100} through a small linker previously introduced in the polymer side-chains (Scheme 6.2).
Scheme 6.2. Synthetic routes for the introduction of peptide sequences for BBB active targeting. a) Route for ANG conjugation, b) Route for cPEP conjugation.
By post-polymerization modification techniques using DMTMM-Cl as previously described in Chapter 4, reactive disulfides were easily introduced to allow conjugation of ANG-2 via disulfide bond. Cysteamine-2TP was used for that purpose achieving 7 and 10 mol% of sulfhydryl pendant groups for PGA and DB respectively as calculated by $^1$H-NMR after purification by dialysis using Vivaspin® MWCO 3000 Da (Figure 6.12).

Then, tBu groups were easily deprotected by using the already optimized conditions. In order to avoid disulfide bond breakages, TIPS (triisopropylsilane) was used at the ratio TFA:H$_2$O:TIPS (95:2.5:2.5) during 3 hours reaching quantitative yields. It has to be mentioned, that basic conditions were also tried in order to avoid the exposure of the disulfide bridges to acids, however, these conditions did not lead to any successful results due to solubility issues.

![Figure 6.12. $^1$H-NMR (D$_2$O) of PGA-DO3A-SS2TP (30a) as example after tBu deprotection, showing the peaks used for modification estimations (pyridine ring 8.4 ppm (CH$_{aromat}$), 7.84 ppm (CH$_{aromat}$), and 7.28 ppm (CH$_{aromat}$).](image)

After removal of the tBu protecting groups, ANG-2-SH was effectively conjugated via disulfide bonds. The final conjugate was purified by SEC using Sephadex commercial PD10 columns and functionalization was quantified by Nanodrop™ ($\lambda=280$ nm) using the absorbance from the tryptophans encountered in the targeted conjugates (Figure 6.13).
Figure 6.13. Absorbance elugrams by Nanodrop™ of the fractions obtained by SEC (PD10 columns) of a) PGA-DO3A-ANG (31a) and b) DB-ANG (31b).

Peptide loading was calculated according to $^1$H-NMR and further confirmed by amino acid (AA) analysis (Table 6.2), achieving 95 and 90% conjugation efficiency of ANG to PGA and DB respectively. It has to be mentioned that quantification by $^1$H-NMR was never easy due to complexity of the samples. In this case, the estimation was done according to the aromatic signals corresponding to the tyrosine (1) and phenylalanine (3) (Figure 6.14). For that reason, peptide content was always confirmed by AA analysis.

Figure 6.14. $^1$H-NMR spectrum (D$_2$O) of the polymer conjugate DB-DO3A-ANG (31b) as example. The complex spectrum allow to estimate peptide content by using the 19 aromatic protons (3 phenylalanines and 1 tyrosine).
Table 6.2. ANG conjugate characterization summary.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>MW kDa</th>
<th>mol% GAU</th>
<th>mol% GAU linker</th>
<th>mol% DO3A wt%</th>
<th>DO3A wt%</th>
<th>mol% GAU wt%</th>
<th>ANG</th>
<th>1H-NMR</th>
<th>AA analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGA-DO3A-ANG (31a)</td>
<td>32.0</td>
<td>10 mol%</td>
<td>7</td>
<td>15 wt%</td>
<td>15 wt%</td>
<td>4.7 mol%</td>
<td>3.9 mol%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DB-DO3A-ANG (31b)</td>
<td>30.4</td>
<td>11 mol%</td>
<td>10</td>
<td>15 wt%</td>
<td>32 wt%</td>
<td>4.5 mol%</td>
<td>3.5 mol%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Following the same strategy by using DMTMM·Cl, N-Boc-ethylendiamine was conjugated to the polymer backbone in order to introduce free amines for the conjugation of cPEP via amide bonding. After purification by dialysis, percentage of modification was calculated according to the $^1$H-NMR spectra signal at 1.37 ppm corresponding to the Boc group. 9 and 11 mol% GAU were obtained for PGA and DB respectively (conjugation efficiency of 90% and 110%). Both, Boc and tBu groups were then easily deprotected in CH$_2$Cl$_2$/TFA (3:2). The purified polymers were then reacted with previously N-Hydroxysuccinimide (NHS) activated cPEP, in order to form a stable amide bond between the introduced amines and the C-terminus activated acid of cPEP. NHS activation of cPEP was carried out with DCC and posterior addition of NHS as described in M&M. Percentage of NHS activation was determined as 100%, estimated according to CH$_2$ signal belonging to NHS ring at 2.6 ppm (Figure 6.15).

Figure 6.15. $^1$H-NMR spectrum (DMSO-d6) of NHS-cPEP (34).

Once conjugated, cPEP content was estimated by $^1$H-NMR by integrating the broad signal at 0.88 corresponding to the isopropyl
group of valine and the two CH$_3$ of the isoleucine present in the peptide sequence (Figure 6.16).

![Figure 6.16. 1H-NMR spectrum (D$_2$O) of DB-DO3A-cPEP (35b) as example showing peptide content estimation.](image)

Table 6.3 summarizes the results obtained in the performed cPEP conjugations.

**Table 6.3.** cPEP-derivatized conjugate characterization summary.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>MW kDa</th>
<th>mol% GAU</th>
<th>wt% DO3A</th>
<th>mol% GAU</th>
<th>wt% cPEP</th>
<th>mol% GAU</th>
<th>wt% cPEP</th>
<th>1H-NMR</th>
<th>AA analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGA-DO3A-cPEP (35a)</td>
<td>22.0</td>
<td>2 mol%</td>
<td>5 wt%</td>
<td>9</td>
<td>2.75 mol%</td>
<td>12 wt%</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DB-DO3A-cPEP (35b)</td>
<td>24.6</td>
<td>9 mol%</td>
<td>19 wt%</td>
<td>11</td>
<td>3.4 mol%</td>
<td>13 wt%</td>
<td>2.8 mol%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**6.2.2.2. In vivo evaluation of BBB targeted linear PGA and DB PEG-PGA conjugates.**

These four DO3A-bearing targeted systems based on PGA and PEG-PGA carriers were labeled with radionuclides ($^{68}$Ga) and evaluated in preliminary *in vivo* experiments performed at CIEMAT by our collaborators. Two main purposes were pursued with this first attempt: on one hand, comparison between two different architectures (linear homopolymers PGA and hybrid di-*block* copolymer PEG-PGA); on the other hand the experiment was used to screen the best vector (targeting unit) to reach BBB among ANG-2 and iron mimicking cPEP, both conjugated within the polymers backbones. Thus, PGA- and DB-DO3A-$^{68}$Ga-ANG, and PGA and DB-DO3A-$^{68}$Ga-cPEP were evaluated in FVB/NJ mice. A dose between 0.2-1.4 MBq of the DO3A-
$^{68}$Ga-labeled systems (21 ± 4 µg compound/g body weight was injected intravenously. Animals were monitored up to 3 hours by PET (again no longer times were used due to Ga decay), and one mouse was sacrificed per time point (0.5, 1, 2, 3 h) (Figures 6.17 to 6.20). As for PGA-DO3A-$^{68}$Ga, organs were extracted and radioactivity was measured (Figure 6.21).

**Figure 6.17.** PGA-DO3A-$^{68}$Ga-ANG (36a) (31.8 µCi). PET images (Sagittal, coronal and transverse images) obtained immediately after administration during 30 min acquisition time.

**Figure 6.18.** PGA-DO3A-$^{68}$Ga-cPEP (37a) (25.5 µCi). PET images (Sagittal, coronal and transverse images) obtained immediately after administration during 30 min acquisition time.

**Figure 6.19.** DB-DO3A-$^{68}$Ga-ANG (36b) (35.6 µCi). PET images (Sagittal, coronal and transverse images) obtained immediately after administration during 30 min acquisition time.
Figure 6.20. DB-DO3A\(^{68}\)Ga-cPEP (37b) (36.2 µCi). PET images (Sagittal, coronal and transverse images) obtained immediately after administration during 30 min acquisition time.

Although only one animal per time point was used as a first attempt in order to evaluate the targeting units, as expected, biodistribution studies confirmed renal excretion profiles for all the compounds as it can be observed from the intense signals in bladder and kidneys. Also, the graphs represented in Figure 6.21 corroborated this fact. Figure 21 shows the % ID normalized per gram of tissue of the 4 targeted systems in comparison with non-targeted PGA, after organ extraction and radioactivity measurements. Again, it has to be mentioned that only up to 3 hours after injection was evaluated due to the short half-life of \(^{68}\)Ga radioisotope (68 min). No major differences were observed if compared PGA and DB as nanocarriers. As a general trend, both ANG bearing systems show a greater % ID \(\cdot \text{g}^{-1}\) tissue in most of organs, including brain (the target organ of this study). The greatest accumulation in brain for ANG bearing systems was found at 0.5 hours post injection. On the contrary, for cPEP labeled compounds the highest brain signal was found at 1 hour. Unfortunately, only a maximum of 0.02 % of the injected dose reached the brain, and the signals were not sustained on time as clearance was rapidly observed. This signal decrease could be explained by a possible pump-out of the conjugates from the brain by the strong efflux systems. Moreover, as no perfusion was carried out prior to organ extractions, the obtained brain signal could also correspond to the accumulation in the blood vessels surrounding the brain. The amount that reached the brain was under the expectations as in literature BBB crossing efficiency ranges from 1 %, to a maximum of 4 % ID. This fact could be attributed to system conformation in solution which could mask the peptide ligands avoiding their recognition by the specific receptors. As wt. % loading of cPEP was lower in both structures compared to ANG loading, from
this study, it was not easy to select one of the targeted moieties and both were used in further conjugate generation.

**Figure 6.21.** Normalized data (injected dose (ID) per gram of tissue) for each organ extracted from biodistribution study comparing cPEP and ANG-2 targeted system. Non-targeted linear PGA was used as control. Time course experiment.
Summarizing, these first preliminary results let some hints in order to further move in BBB targeting goal:
- Better exposure of the peptide sequences to their target receptors is a requisite to achieve greater brain accumulation.
- Longer body residence time is needed in order to increase the chances of the polymeric systems to accumulate in the target organ.

To overcome these previous limitations detected with the first pilot study, different synthetic strategies were proposed. First, the use of star-shaped polymers. Their presumably global structures let us to hypothesize that higher loading and greater exposure of the peptidic sequences would be achieved with these systems. Second, the use of a different synthetic pathway for the development of di-block copolymers by introducing the targeting units in a semi-telechelic approach. Although this latter approach would lead to a decrease in peptide loading (only one per polymer chain as maximum), a better could be achieved.

Moreover, a different radionuclide was used in order to be able to explore longer time points post-administration. In this case, $^{111}$In was chosen due to the compatibility with the already designed synthetic strategies using DO3A as complexating agent (it also forms stable complexes). The higher half-life of $^{111}$In (2.08 days) allowed to better identify compounds half-life. Nevertheless, in vivo images could not be taken as SPECT instruments were not available for $^{111}$In.
6.2.3.1. Synthetic strategies towards targeted St-PGA and DB2 PGA-PEG radioactive probes.

For the synthesis of star-shaped labeled polymers, exactly the same strategy as for their linear counterparts was followed as depicted in Scheme 6.2. We used a 3-arm star-shaped PGA initiated with ethylenediamine based initiator (see Chapter 3) with MW 16610 Da (110 GAU), and Đ 1.23. One St-DO3A-${}^{68}$Ga labeled carrier as control (28c) and two different targeted carriers St-DO3A-${}^{68}$Ga-ANG (36c), St-DO3A-${}^{68}$Ga-cPEP (37c), were synthesized and evaluated. The physico-chemical characteristics of those polymers are summarized in Table 6.4.

The new hybrid di-blocks, PGA-PEG (DB2) were synthesized by introducing a PEG block through the N-terminus of the PBLG homopolymers after the polymerization process, as shown in Scheme 6.3.
Scheme 6.3. Synthetic route to reach DB2.
Reactions were carried out using a PBLG block of 92 GAU (D 1.12), in HFIP at 40 °C during 72 hours. HFIP was chosen as solvent due to its well-known ability to open secondary structures in order to facilitate the accessibility of the free amine of the N-terminus of the benzyl protected polymer to the activated carboxylic acid on the PEG block. Three different di-blocks were synthesized: a DB2 as control (38a) using methoxyPEG NHS MW 3023 Da; a DB-Fmoc (38b), with protected amine moiety in order to conjugate cPEP in further steps using Fmoc-PEG NHS MW 4847 Da; and a DB-SS4TP (38c) with activated disulfide in order to conjugate ANG by steps using 4TPSS-PEG NHS MW 3219 Da. PEG signal at 3.62 ppm in $^1$H-NMR in CDCl$_3$ was used in all cases to confirm DB2 synthesis. After di-block synthesis and purification, benzyl groups of PGA block were easily deprotected following the optimized basic conditions using NaOH/THF mixtures (39).

DO3A groups were introduced later on by post-polymerization modification techniques using DMTMM·Cl (Scheme 6.4). DO3A content of the polymers was determined as 16 mol% GAU in all cases according to NMR (conjugation efficiency of 80 %). In the case of DB2-DO3A and DB2-DO3A-ANG synthesis, the tBu groups where deprotected in the following step using the mixture TFA:TIPS:H$_2$O (95:2.5:2.5). For DB2-DO3A-cPEP, the peptide conjugation was prior to tBu groups deprotection. Similar peptide conjugation and characterization approaches as those previously described were followed. Conjugate details are summarized in Table 6.4.
Scheme 6.4. Synthetic schemes for a) DB2-DO3A (41a), b) DB2-DO3A-ANG (41c), c) DB2-DO3A-cPEP (41d).

Table 6.4. St-PGA and DB2 conjugates physico-chemical characteristics.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>MW (kDa)</th>
<th>mol% GAU/ wt% DO3A</th>
<th>mol% GAU linker</th>
<th>mol% GAU/ wt% peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>St-DO3A-ANG (31c)</td>
<td>46.4</td>
<td>20 mol%/ 25 wt%</td>
<td>9</td>
<td>4.0 mol%/ 27 wt%</td>
</tr>
<tr>
<td>St-DO3A-cPEP (35c)</td>
<td>39.1</td>
<td>20 mol%/ 30 wt%</td>
<td>8</td>
<td>6.0 mol%/ 20 wt%</td>
</tr>
<tr>
<td>DB2-DO3A-ANG (41c)</td>
<td>23.7</td>
<td>16 mol%/ 28 wt%</td>
<td>-</td>
<td>0.8 eq. polym./ 7 wt%</td>
</tr>
<tr>
<td>DB2-DO3A-cPEP (41d)</td>
<td>20.2</td>
<td>16 mol%/ 26 wt%</td>
<td>-</td>
<td>0.8 eq. polym./ 3 wt%</td>
</tr>
</tbody>
</table>
At the moment, DB2 conjugates are under evaluation at CIEMAT. The following results correspond to the evaluation of star-shaped family.

As for $^{68}$Ga, radioisotope labeling was carried out in CIEMAT using a similar procedure (see M&M). The radio-labeled solution was purified by size exclusion chromatography (SEC) (Figure 6.22).

Figure 6.22. % Activity measured after $^{111}$In labeling and purification by SEC of St-DO3A as example.

6.2.3.2. In vivo evaluation of non-targeted St-PGA. Biodistribution and pharmacokinetics (PK) comparison with linear PGA.

For biodistribution and PK studies, 88 FVJ/B mice (4-5 per time point) were anesthetized with isofluorane and doses between 37 KBq and 2.5 MBq $^{111}$In-eq of the three star constructs were injected i.v. (1-20 µg·g$^{-1}$ body weight). Then, 4-5 mice were sacrificed per time point (0.5, 1, 2, 4, 8 and 24 h). As for $^{68}$Ga labeled polymers, blood and organs were extracted and radioactivity was measured using a gamma counter.

Biodistribution of linear PGA was previously performed using $^{68}$Ga radioisotope, therefore, only short times (up to 3 hours) could be recorded due to radionuclide decay (about 68 min for $^{68}$Ga). If short time points (0.5, 1 and 2 hours) of the % ID·g$^{-1}$ tissue of PGA-DO3A-$^{68}$Ga (28a) and St-DO3A-$^{111}$In (28c) are studied, a general greater accumulation in all organs with the star shaped polymer was observed, when compared with the linear counterpart. (Figure 6.24).
Figure 6.23. St-DO3A-\textsuperscript{111}In (28c) biodistribution. Data expressed as normalized % Injected Dose (ID) per gram of tissue, different time points. n > 5, mean ± SEM.
Figure 6.24. Radioactivity normalized data for each organ in respect to the % injected dose (% ID) per gram of tissue, of St-PGA compared with its linear counterpart. Time course experiment. n > 4, mean ± SEM.

Representation of the % of ID·mL⁻¹ in plasma was also used to assess PK parameters. The best fit was obtained with a two-compartmental model. The two-compartment model is more complex and takes into account the existence of a gap in the body distribution after i.v. administration of a single dose (Figure 6.25). This model considers the body as two interconnected compartments, one known as
central compartment (where the compound rapidly access), and the peripheral compartment, with greater accessibility constrains, precisely comes through the first one. Since clearance occurs mainly from the central compartment, the drug distributed in the peripheral compartment must return to the center for this phenomenon to occur. Most drugs are adapted to a two compartment model, since the one compartment model is too simple considering only one compartment, and assuming that the drug administered is rapidly and uniformly distribute through the whole organism (Figure 6.25).

![Figure 6.25. Schematic representation of the two models for pharmacokinetics estimations. a) One compartment, where $K_a$: absorption constant and $K_c$ clearance constant, t b) Two-compartments, where PC: peripheral compartment, CC: central compartment, $K_{1,2}$: distribution constant to the PC $K_{2,1}$: return constant from the PC.](image)

After a proper adjustment using the mathematical expression of the two-compartments model (Equation 6.1), pharmacokinetic parameters of both compounds were extracted, and compared as shown in Table 6.5. In general basis $\alpha$ mainly depends on the compound distribution (from central to peripheral compartments) and $\beta$ mainly depends on compounds clearance where $\alpha+\beta = K_{1,2} + K_c + K_{2,1}$.

$$C(t) = A. e^{-\alpha t} + B. e^{-\beta t}$$

**Equation 6.1.** Mathematical expression of the fit to two compartments model. $\alpha$ and $\beta$ are two constants that depend solely on the constants of transference among both compartment ($K_{1,2}$ and $K_{2,1}$).

Although the plasmatic profiles were similar for both compounds, differences could be drawn PK parameters obtained for PGA-DO3A-$^{68}$Ga (28a) with St-DO3A-$^{111}$In (28c) were compared. Their biological or terminal half-life estimated as $\text{Ln}2/\beta$, resulted to be 13 times greater for the star polymer, this fact could be in part attributed to the use of different radionuclides for the study. The use of $^{111}$Indium allowed to study and estimate PK parameters of the stars.
providing more reliable results due to the higher semidesintegration period for $^{111}$In (2.1 days) compared to $^{68}$Ga (68 min).

Table 6.5. Pharmacokinetic parameters of PGA and Star-shaped PGA.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>St-DO3A-$^{111}$In (28c)</th>
<th>PGA-DO3A-$^{68}$Ga (28a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Value</td>
<td>Std E</td>
</tr>
<tr>
<td>$\alpha$ (h$^{-1}$)</td>
<td>4.74</td>
<td>0.42</td>
</tr>
<tr>
<td>$\beta$ (h$^{-1}$)</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>AUC (%ID - h - mL$^{-1}$)</td>
<td>5.45</td>
<td>0.76</td>
</tr>
<tr>
<td>$t_{1/2B}$ (h)</td>
<td>12.05</td>
<td>7.96</td>
</tr>
<tr>
<td>$K_e$ (h$^{-1}$)</td>
<td>4.11</td>
<td>0.53</td>
</tr>
<tr>
<td>$K_{12}$ (h$^{-1}$)</td>
<td>0.62</td>
<td>0.27</td>
</tr>
<tr>
<td>$K_{21}$ (h$^{-1}$)</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td>$C_{max}$ (%ID - mL$^{-1}$)</td>
<td>22.37</td>
<td>5.14</td>
</tr>
<tr>
<td>$C_l$ (mL - h$^{-1}$)</td>
<td>18.35</td>
<td>2.55</td>
</tr>
<tr>
<td>$V_{ss}$ (mL)</td>
<td>46.34</td>
<td>44.45</td>
</tr>
</tbody>
</table>

*Value: Estimate, Std E: Standard Error.

In any biologic system, the loss of a radiolabeled compound is due to both physical decay of the radionuclide, and the biological elimination of the radiolabeled compound. The net or effective half-life, time required for the radioactivity in an organism to be reduced to half through the combined effects of the physical decay of the isotope and the biological elimination of the isotope could be calculated by Equation 6.2:

$$\frac{1}{(t_{1/2})_{effective}} = \frac{1}{(t_{1/2})_{physical}} + \frac{1}{(t_{1/2})_{biological}}$$

Equation 6.2. Effective half-life estimation.

As the effective terminal half-life was 12.05±7.96 hours and 0.59±0.29 hours for star and linear PGA, respectively, the biological terminal half-life was then 15.87±9.47 hours and 1.23±0.40 hours for both compounds, respectively.

In the case of the two compartment model a number of volume terms can be also defined. $V_{ss}$, the appropriate volume of distribution when plasma concentrations are measured in steady state conditions, is
the most useful $V_d$. This $V_{ss}$ value was about 9 times higher for the star polymer compared to the linear one, meaning a greater distribution of the carrier.

The Clearance value (Cl) from the central compartment, estimated as the ratio of dose/AUC (area under the plasma concentration-time curve), was also slightly higher for the star-shaped polymer (18.35 vs. 11.77 mL·h$^{-1}$ for linear PGA). The renal clearance value of inulin (a model compound that is excreted only by glomerular filtration) has been established to be around 20 mL·h$^{-1}$ in FVB mice.$^{241}$ This value is really close to the value obtained for the star polymer. Thus it could be claimed that the polymer was cleared out only by glomerular filtration. In the case of linear PGA, the value was slightly smaller. This could be explained by a possible binding to plasma proteins, reducing the glomerular filtration, or by tubular reabsorption.

6.2.3.3. In vivo evaluation of BBB targeted St-PGA. Biodistribution and pharmacokinetics.

Targeted star polymers bearing ANG (36c) and cPEP (37c) were also evaluated. The % ID per gram of tissue at different time points, of the two targeted polymers in comparison with the non-targeted star-shaped carrier is represented in Figure 6.26. According to the graphs, it can be observed that the majority of the ID for all compounds was found in the kidneys (around 40-60 % in peptide bearing compounds, and 20-40 % for the star control), confirming a renal excretion profile. This was in good agreement with the MW of the compounds, below 60000 Da (cut-off of the renal glomeruli). Liver showed around 1-2 % ID for the targeted compounds, and 3-4 % for the non-targeted star. The % ID in the rest of the organs was very low including the brain, where does not reach 0.05 %.
Figure 6.26. Radioactivity normalized data for each organ in respect to the % injected dose (% ID) per gram of tissue for St-PGA compared with its targeted counterparts. Time course experiment. Error bars are not included for clarity reasons. n> 5, mean ± SEM.

PK parameters were calculated as described before for linear PGAs by using the representation of the % ID⋅mL⁻¹ in serum over time.
As for the previous compounds, PK corresponds to a two-compartment model for the three star systems (Figure 6.27).

a) Non-targeted (28c)

![Graph showing PK for non-targeted star system](image)

b) With ANG (36c)

c) With cPEP (37c)

![Graphs showing PK for targeted star systems](image)

**Figure 6.27.** Two-compartment model fittings (PK) of % ID∙mL\(^{-1}\) in serum with time of star-shaped PGAs targeted (b and c) and non-targeted (a). n > 5, mean ± SEM.

Selected PK parameters are summarized in Table 6.6. As it can be observed, half-life in plasma was found to be between 12-16 hours in all the cases. Always higher than that obtained with linear PGA. \(V_{ss}\) of the targeted stars (171.4 mL for cPEP and 177.8 mL for ANG) is much higher than the one of the non-targeted star (46.34 mL). This is indicative of a significantly higher distribution of the targeted stars in the body.

As explained before, from the clearance value of the inulin we could state that the non-targeted star carrier (Cl: 18.35 mL∙h\(^{-1}\)) was excreted by glomerular filtration. In the case of the peptide bearing stars, this clearance values are slightly higher (31.65 and 33.39 mL∙h\(^{-1}\) for cPEP and ANG respectively). Therefore, other mechanisms of renal excretion (i.e. tubular secretion) could be present in the kidneys in this case.
Table 6.6. Pharmacokinetic parameters of St-DO3A-$^{111}$In-cPEP (37c) and St-DO3A-$^{111}$In-ANG (36c).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>St-DO3A-$^{111}$In-cPEP</th>
<th>St-DO3A-$^{111}$In-ANG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Value</td>
<td>Std E</td>
</tr>
<tr>
<td>$\alpha$ (h$^{-1}$)</td>
<td>2.30</td>
<td>0.25</td>
</tr>
<tr>
<td>$\beta$ (h$^{-1}$)</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>AUC (%ID∙h∙mL$^{-1}$)</td>
<td>3.16</td>
<td>0.63</td>
</tr>
<tr>
<td>$t_{1/2\beta}$ (h)</td>
<td>15.79</td>
<td>19.52</td>
</tr>
<tr>
<td>$K_e$ (h$^{-1}$)</td>
<td>1.79</td>
<td>0.42</td>
</tr>
<tr>
<td>$K_{12}$ (h$^{-1}$)</td>
<td>0.49</td>
<td>0.34</td>
</tr>
<tr>
<td>$K_{21}$ (h$^{-1}$)</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>$C_{max}$ (%ID∙mL$^{-1}$)</td>
<td>5.67</td>
<td>0.96</td>
</tr>
<tr>
<td>$Cl$ (mL∙h$^{-1}$)</td>
<td>31.65</td>
<td>6.31</td>
</tr>
<tr>
<td>$V_{ss}$ (mL)</td>
<td>171.30</td>
<td>265.10</td>
</tr>
</tbody>
</table>

6.2.4. Third Generation Constructs. Development and in vivo evaluation by optical imaging.

6.2.4.1. Synthetic route to reach dually labeled and targeted systems through bottom-up approach of self-assembled polymers.

The constructs synthesized through bottom-up approach via covalent capture of self-assembled polypeptides obtained by controlled NCA polymerization and adequate post-polymerization modifications were in vivo evaluated in terms of biodistribution and pharmacokinetics of targeted and non-targeted constructs. From the results obtained in the biodistribution study with star shaped polymers, only ANG-2 derivatives will be evaluated for BBB crossing, as a first approach. For that, cross-linked polymers through CuAAC were dually labeled with DO3A-Gd$^{3+}$ and Cy5.5 for MRI and optical imaging techniques, respectively in order to have a dual probe with a view to future biomedical applications. Moreover, ANG was easily introduced following the same strategy described in Section 6.2.2.1.
Figure 6.28. Schematic representation of the synthetic route followed for surface modification of the covalently captured polymers (25a) to reach the dual probes. a) 1) DMTMM-Cl, 2) DO3AtBu-NH₂ in ddH₂O, r.t. 24 h. b) and f) 1) DMTMM-Cl, 2) Cy5.5 (6S-IDCC) in ddH₂O, r.t. 24 h. c) and g) TFA:TIPS:ddH₂O (95:2.5:2.5), r.t. 3 h. d) and i) GdCl₃⁺ in PBS 0.1 M 7.4, r.t. 5 h. e) 1) DMTMM-Cl, 2) cysteamine-SS2TP in ddH₂O, r.t. 24 h. h) ANG in HEPES buffer 7.4, r.t. 16 h.
Briefly, PGA-based systems with self-assembly triggering motifs of 150 GAU were modified by post-polymerization techniques with propargylamine units (11 mol%), and NH₂EG(2)N₃ units (7 mol%), respectively. Those polymers were mixed to allow self-assembly at 2 mg·mL⁻¹ in ddH₂O and covalent capture through CuAAC as described in Chapter 5. After an adequate characterization to achieve compound identity by ¹H-NMR (triazole peak at 7.8 ppm) and DLS measurements, the clicked polymer was used in further labeling strategies. DMTMM·Cl was employed in order to activate the carboxylic acids to allow the introduction of DO3AtBu-NH₂ in the first place, followed by Cy5.5 in the synthesis of the non-targeted system. DO3A modified units were quantified as described for the synthesis of radiolabeled polymers by ¹H-NMR. On the other hand, Cy5.5 content estimation was carried out by fluorescence (prior calibration curve of Cy5.5 dye in PBS buffer was obtained (Figure 6.29)).

![Figure 6.29. Cy5.5 calibration curve in PBS at pH 7.4.](image)

For the non-targeted construct, tBu protecting groups from DO3A were easily removed at this point, using the mixture TFA:TIPS:H₂O (95:2.5:2.5). In the case of the targeted polymer, cysteamine-2TP units were introduced again by post-polymerization modification in aqueous media prior to the introduction of Cy5.5. Quantification was determined as 10 mol% of GAU by ¹H-NMR. Then, the tBu protecting groups from DO3A were removed, and ANG was conjugated following previous strategies by means of disulfide bonding. Finally, Gd³⁺ was complexed to DO3A bearing constructs using 1:1 eq. (DO3A:GdCl₃) ratio. The reaction took place in PBS 0.1 M at pH 8 (GdCl₃ precipitation was observed at lower pHs) and monitored by titration using 4-(2-pyridylazo)resorcinol. This titrating
agent turns from yellow to orange in the presence of free Gd\(^{3+}\). After 5 hours reaction time, no free Gd\(^{3+}\) was detected. The reaction was purified by dialysis and absence of free Gd\(^{3+}\) was again tested. Conjugates physico-chemical characteristics are summarized in Table 6.7.

**Table 6.7.** Conjugate physico-chemical characteristics for *in vivo* biodistribution by fluorescence.

<table>
<thead>
<tr>
<th>Compound</th>
<th>mol% GAU</th>
<th>wt% DO3A</th>
<th>wt% Gd</th>
<th>mol% GAU/ wt% Gd</th>
<th>mol% GAU/ wt% Cy5.5</th>
<th>mol% GAU/ wt% ANG</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-Click-DO3A-Gd-Cy5.5 (45)</td>
<td>10.0 mol%</td>
<td>20.3 wt%</td>
<td>12.0</td>
<td>0.5 mol%</td>
<td>3.1 wt%</td>
<td>-</td>
</tr>
<tr>
<td>X-Click-DO3A-Gd-Cy5.5-ANG (50)</td>
<td>10 mol%</td>
<td>17.6 wt%</td>
<td>10.4</td>
<td>0.5 mol%</td>
<td>2.7 wt%</td>
<td>13.8 wt%</td>
</tr>
</tbody>
</table>

The Z-potential of the clicked architectures before, and after surface modifications was recorded in ddH\(_2\)O at 20 °C and the results are depicted in Figure 6.30. As it can be observed, surface modifications with DO3A-tBu (26e) and cysteamine-2TP (46), significantly decrease de negative Z-potential obtained for the clicked structure with all the carboxylic groups unmodified and presumably exposed at the surface (25a).

**Figure 6.30.** Z-potential obtained at 20 °C from clicked structures at 1 mg·mL\(^{-1}\) in ddH\(_2\)O, before and after the subsequent surface modifications.
The introduction of the negatively charged Cy5.5 within the structure resulted in an increase on Z-potential obtained (47). Finally, when ANG-2 peptidic sequences where conjugated (49), Z-potential dramatically decrease to almost neutral, probably due to a shielding effect provided by the 19 aa sequences.

Furthermore, size of the systems was estimated by TEM to be in the range of 70-100 nm diameter (Figure 6.31).

![TEM micrographs of a) X-Click-DO3A-Gd-Cy5.5 (45), and b) X-Click-DO3A-Gd-Cy5.5-ANG (50).](image)

In order to compare the results obtained from fluorescence techniques with PET, a new construct based on X-Click systems was also synthesized following the procedures already described through this chapter. In this case, X-Click PGA was successively modified with DO3A, cysteamine-2TP, and ANG peptide. Conjugate characteristics are shown in Table 6.8. This construct is still under evaluation in CIEMAT, however a good correlation with the results already obtained is expected.

**Table 6.8. X-Click-DO3A-ANG (31d) conjugate physico-chemical details.**

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>mol% GAU wt% DO3A</th>
<th>mol% GAU linker</th>
<th>mol% GAU wt% pep&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-Click-DO3A-ANG (31d)</td>
<td>30 mol%</td>
<td>20</td>
<td>2.75 mol%</td>
</tr>
<tr>
<td></td>
<td>35.3 wt%</td>
<td></td>
<td>16.5 wt%</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data obtained by 1H-NMR
6.2.4.2. *In vivo* evaluation of BBB targeted clicked systems by optical imaging.

Once synthesized and characterized, these complex architectures were evaluated *in vivo*. Biodistribution experiments were carried out using C57Bl/6 mice and fluorescence techniques taking profit from Cy5.5 dye on the polymeric carriers. Targeted and non-targeted architectures were administered i.v. through the tail vein to isofluorane anesthetized mice, at a dose of 4.15 mg·Kg\(^{-1}\) Cy5.5 eq. Two animals were then sacrificed at different time points (1, 3, 7, 14 and 24 hours). Prior to sacrificed, mice were first anesthetized with a lethal anesthesia cocktail, blood was extracted from the cava vein, and perfusion with saline was carried out in order to accurately determine the amount of compound in the brain. Then, organs were extracted and their fluorescence was measured using the red filter in MAESTROM™. For fluorescence quantification, normalized data was obtained by taking always the same pixel area for all organs expressed as average signal (counts·s\(^{-1}\)). A calibration curve of the compounds in the same MAESTROM™ was carried out in order to estimate the fluorescence corresponding to the injected dose. Biodistribution data obtained from non-targeted and targeted polymer is depicted in Figures 6.32 - 6.34.

When both compounds were compared, no major differences in biodistribution were encountered as it can be observed in Figure 6.33. Renal excretion profiles could be observed in both cases. However, the targeted compound was found to accumulate in a higher extend in organs such as liver and kidney. Notably, when the biodistribution data from these bigger architectures was compared with that from the non-clicked stars (Figure 6.26), a greater accumulation in the lungs at early time points was observed. This fact was in good agreement with the nature of the architectures used, since sizes above 100 nm tend to accumulate in lungs. Hence, this family of architectures could have a potential use in order to target lung diseases such as lung cancer. Nevertheless, these carriers also demonstrated to be safe as not weight loss in the animals was observed (see further details in Chapter 7). Besides, lung accumulation was significantly diminished over time, validating them as possible carriers.
a) Non-targeted

X-Click-DO3A-Gd-Cy5.5

% ID

Kidney, Stomach, Liver, Heart, Spleen, Brain, Lung, Blood

1 hour, 3 hours, 7 hours, 14 hours, 24 hours
Figure 6.32. % ID normalized by pixel area of non-targeted (a) and targeted (b) Cy5.5 labeled clicked architectures.
Important to note, the ANG bearing compound offered greater brain accumulation at early time points when compared to the non-targeted counterpart. Nonetheless, similar accumulation was found for both compounds at late time points such as 24 hours. Remarkably, the amount found in the brain in both cases was between 1-1.5 % ID, which is 20-30 times greater than the one obtained for non-clicked stars (0.05 % ID). As mentioned before and according to literature, the normal % ID for those systems who are able to reach the brain is usually between 1-2 % ID, with the maximum obtained with 4 %.  

**Figure 6.33.** Biodistribution by optical imaging at different time points of targeted and non-targeted clicked architectures. Time course experiment. Error bars are not included for clarity reasons.
Figure 6.34. Organ fluorescence images obtained at MAESTRO™ from a) non-targeted, b) targeted clicked compounds.
6.3. CONCLUSIONS.

The BBB represents a huge challenge for the transport of bioactive compounds to the brain. Herein, we aimed to overcome such limitation for the treatment of the vast majority of neurodegenerative diseases. To achieve that, and after being evaluated in vitro, different polymeric carriers with increasing complexity (linear, di-block copolymer, stars and PGA-based clicked systems) were purposed as candidates for BBB crossing. Firstly, their in vivo biocompatibility, safety, non-specific accumulation in any organ, and adequate excretion profile were evaluated, confirming their suitability as nanovectors after targeting moieties conjugation. Secondly, the biodistribution of targeted polymeric derivatives (with peptidic targeting ligands Angiopep2 and the iron mimicking cyclic peptide) were studied paying particular attention to brain accumulation in order to validate them as possible carriers through BBB.

Different imaging techniques were used (PET and fluorescence optical imaging), being PET the most adequate for brain detection due to its sensitivity as the % ID able to cross BBB is usually very low (< 4% ID).

Non-targeted polymeric constructs from linear homopolymers, DBs PEG-PGA or simple stars did not crossed the BBB. When targeted systems were explored, preliminary data with ANG bearing carriers showed greater % ID in the brain than cPEP. Star derivatives seemed to perform better than the linear counterparts although brain concentration was not maintain in any case. DB systems gave in all cases lower percentages probably due to conformational issues, where PEG blocks could presumably form an outer shell hiding the peptide molecules attached in the polypeptide block.

These preliminary results pointed out the need for new architectures with higher MW (to increase blood residence time in order to promote higher brain accumulation), and capable to better expose the peptide ligands to the corresponding receptors. Therefore, a more complex 3rd generation systems by means of ANG-clicked structures was selected. Such constructs, after adequate labeling and ANG conjugation, were evaluated using fluorescence imaging techniques and presented a much greater accumulation in brain (up to 1.5 % ID). Those results encourage us in the use of the clicked nanovectors for neurodegenerative applications. Moreover, taking into
account that data was obtained with fluorescence imaging, which usually provides underestimated values, further studies using PET are currently being conducted to elucidate brain accumulation of those structures in a more realistic way.

6.4. MATERIALS AND METHODS.

6.4.1. Materials.

All chemicals were reagent grade, obtained from Aldrich and used without further purification, unless otherwise indicated. All solvents were of analytical grade and were dried and freshly distilled. Deuterated chloroform-$d_1$, DMSO-$d_6$, and D$_2$O were purchased from Deutero GmbH. PGA-based architectures: PGA, St-PGA, DB PEG-PGA X-Click, were synthesized according to protocols described in Chapters 2, 3, 4 and 5. DO3AtBu was purchased from Chematec. Cy5.5 (6S-IDCC) was obtained from Mivenion. Angiopep2-cystein Ac-FFYGGSRGKRNNFKTEYYC and the iron mimicking cPEP Ac-CRTIGPSVC (disulfide bridge) sequences were obtained from Selleck Chemicals LLC. Preparative SEC was performed using Sephadex G-25 superfine from GE as well as PD MiniTrap G-10™ columns contenting 2.1 mL of Sephadex™ G-10. Dialysis was performed in a Millipore ultrafiltration device fitted with a 3, or 5 kDa MWCO regenerated cellulose membrane (Vivaspin®).

Animals, FVB/NJ albino mice (CIEMAT Laboratory Animals Facility, Madrid) were housed on a 12-hour light and 12-hour dark cycle with free access to food and water. All animal protocols were approved by the Institutional Animal Care and Use Committee at CIEMAT (Madrid).

C57Bl/6 mice (Animal Imaging Center, Zurich, Switzerland) were kept at standard housing conditions: light/dark cycle of 12 hours, temperature of 20-24 °C, relative humidity minimum 40 %, with free access to food and water. All experiments were performed in accordance with the Swiss Federal Act on Animal Protection.

6.4.2. Characterization techniques.

6.4.2.1. Nanodrop™.

The collected fractions from ANG purifications through Sephadex G25 pre-packed commercial columns (PD10), were analyzed by UV-VIS recording the absorbance at 280 nm from each eluted
sample. For that, 2 µL of each fraction were placed in Nanodrop™ ND-1000 UV-Vis Spectrophotometer and the absorbance at 280 nm was measured. Data was plotted in order to be able to identify those desired fractions containing only the polymeric conjugate.

### 6.4.2.2. Maestro™

*Ex vivo* Near-infrared imaging; 2D-*in vivo* imaging was performed using Maestro 500 imaging system (Cambridge Research Inc, Woburn, USA). For Cy5.5 measurements, a band pass filter from 615 nm-665 nm and a highpass filter over 690 nm were used for excitation and emission light, respectively. The fluorescence was detected by a CCD camera. A series of images was acquired at different wavelengths and then subjected to spectral un-mixing as described by Gao et al.\(^{243}\) This enabled the un-mixing of the Alexa680 fluorescence pattern from tissue autofluorescence.

### 6.4.3. Protocols.

#### 6.4.3.1. Radionuclide labeling.

In order to introduce radionuclides within the polymer backbone of the different architectures, modification of those with groups serving as complexating agents for radionuclides is a prerequisite. Within this thesis dissertation, DO3A-NH\(_2\) was chosen. The synthetic strategy for the DO3A is depicted in Scheme 6.1.

##### 6.4.3.1.1. Conjugation of DO3AtBu-NH\(_2\) for radionuclide complexation.

Due to the good solubility either in aqueous or organic solvents of DO3AtBu-NH\(_2\), conjugations were possible in both media. For linear and star PGA in carboxylic acid forms, conjugations were carried out in organic solvents (DMF) by using the standard DIC/HOBt protocol. In the case of DBs PEG-PGA or PGA-PEG and clicked structures as sodium salt forms (either due to the purification steps done before or to incompatibility of acid precipitation methods to obtain the acidic forms), the conjugations were done in aqueous media using the previously described DMTMM-Cl protocol of post-polymerization modification (Chapter 4).

Briefly, for acid forms of PGAs:

In a two-neck round bottom flask fitted with a stir bar and two septums, 300 mg (of PGA, 2.32 mmol GAU, 1 eq.) was dissolved in 20
mL of anh. DMF under nitrogen flow. Then, 53 μL of DIC (44 mg, 0.35 mmol, 0.15 eq.) were added and reaction was left to proceed for 5 minutes at r.t. Afterwards, HOBt (47 mg, 0.35 mmol, 0.15 eq.) was added directly. Reaction was then left to proceed for 10 minutes before DO3AtBu-NH₂ (141 mg, 0.23 mmol, 0.1 eq.) was added for 10 % modification. *Note: amounts of carboxylic acid activators and DOTAtBu-NH₂ vary depending on the desired percentage of modification. The pH was adjusted to 8 by adding ~100 μL of DIEA. The mixture was left stirring for 48 hours at r.t. and protected from light. Finally, solvent was partially removed under vacuo, precipitated into a large excess of cold acetone, filtered off and washed three times with cold acetone. A pale yellow solid was obtained after drying. The percentage of modified GAU was calculated according to the tBu groups’ signal at 1.4 ppm in comparison with the alpha proton of PGA backbones in ¹H-NMR spectra.

For sodium salt forms:

In a one-neck round bottom flask, 0.72 mmol GAU of PGA-based structure (PEG-PGA, PGA-PEG or X-Click) was dissolved in 5 mL ddH₂O. DMTMM ∙ Cl (i.e. 0.144 mmol for 20 % modification) was then added, in another 2 mL of ddH₂O, to the reaction mixture and left stirring for 10 minutes. After that, DO3AtBu-NH₂ (i.e. 0.144 mmol for 20 % modification) was added in 3 mL more of ddH₂O. pH of the reaction mixture was adjusted to 8 by adding sodium bicarbonate 1 M. The contents were left stirring for 24 hours at r.t. After that time, the products were purified via dialysis using Vivaspin® MWCO 3000. Pale yellow solids were obtained after freeze drying. Percentage of DO3AtBu-NH₂ introduced was estimated by ¹H-NMR according to the signal at 1.41 ppm corresponding to tBu groups. Yields: 70-90 %. Conjugation efficiency: 70-80 %.

6.4.3.1.2. Deprotection of DO3A tBu-NH₂.

For radionuclide complexation, the protecting tBu groups from carbonyl moieties must be previously removed. Two different protocols were used depending on the compound nature. For constructs without any sensitive group to TFA conditions, the first protocol was applied. The use of TIPS in the second protocol was introduced in order to prevent disulfide bonds breakage during deprotection conditions. Thus,

Protocol 1. The construct was dissolved in CH₂Cl₂/TFA (3/2, v/v) mixture and left under vigorous stirring for 16 hours at r.t. After
that time, the solution was precipitated by pouring into a large excess of cold diethyl ether. Pale yellow solid was obtained after filtering, washing with diethyl ether and drying under vacuum. Complete deprotection was achieved as confirmed by $^1$H-NMR. Yields: 80-90 %.

Protocol 2. The construct was dissolved in TFA/H$_2$O/TIPS (95/2.5/2.5, v/v) mixture and left stirring at r.t. during 3 hours. After that, the contents were precipitated into a large excess of cold diethyl ether. A pale yellow solid was collected, washed with diethyl ether and dried over vacuum. Complete deprotection was confirmed by $^1$H-NMR analysis. Yields: 80-90 %

6.4.3.1.3. $^{68}$Ga and $^{111}$In complexation.

As a first step, $^{68}$Ga and $^{111}$In radionuclides on each case from commercial solutions, were eluted, purified and concentrated. Afterwards, the obtained radionuclide was transferred into a microwave tube and the pH was adjusted to 3.5-4 by adding HEPES buffer and HCl 2 M (to avoid $^{68}$Ga hydroxylation during complexation). Immediately after, the DO3A labeled system was added in 500 μL of aqueous solution 10 mg∙mL$^{-1}$. The reaction mixture was heated at 90 °C for 5 minutes by using a laboratory microwave with monomodal radiation (Discover Benchmate, CEM). The reaction was stopped after 5 min at r.t. by the addition of 50 μL of 50 mM EDTA. Then, purification was performed with molecular exclusion chromatography cartridge (Bio Gel P-6 Biorad) using PBS pH 7 as eluent. The elution profile was determined by fractionating, 0.77 mL per fraction, and measuring each with a dose calibrator (VDC 405, Veenstra). Radiochemical yield (RY) was calculated as percentage of the activity in each fraction eluted from the molecular exclusion cartridge of the total activity purified and corrected for the decay.

6.4.3.2. Fluorescence labeling with Cy5.5.

For Cy5.5 labeling, the protocol described in Chapter 4 based on the post-polymerization modification using DMTMM·Cl in aqueous solutions was applied. Briefly, in a one-neck round bottom flask, PGA-based polymer was dissolved in ddH$_2$O (1 eq. GAU). Then, the carboxylic groups were activated using DMTMM·Cl (i.e. 0.02 eq. for 2 % modification). Reaction was allowed to proceed for 10 minutes. After that time, Cy5.5 (i.e. 0.02 eq. for 2 % modification) was added in ddH$_2$O. The pH was adjusted to 8 by adding sodium bicarbonate 1 M.
Reaction was left to proceed for 24 hours, protected from light. For purification, the products were submitted to both Sephadex G25 and dialysis using Vivaspin® MWCO 5000.

Cy5.5 content estimation was carried out by fluorescence ($\lambda_{em}$: 595 nm, $\lambda_{ex}$: 680 nm) after the building of an appropriate calibration curve of Cy5.5 dye in PBS buffer.

Yields: 60-70 %. Conjugation efficiency 70-90 %.

6.4.3.3. DO3A-Gd$^{3+}$ labeling for MRI.

In a one-neck round bottom flask, the corresponding DO3A bearing polymer as sodium salt form (1 eq. of modified DO3A GAU units) was dissolved in PBS 0.1 M pH 7.4. Then, GdCl$_3$ (1 eq.) dissolved in ddH$_2$O was dropped into the main solution. During this process, pH was monitored and remained constant to 8. The degree of Gd (III) complexation was determined by titrating aliquots during reaction process using 4-(2-pyridylazo) resorcinol which turns from yellow to orange in the presence of free Gd). No free Gd was detected after 5 hours reaction time. The reaction was then stopped and purified by dialysis using Vivaspin® MWCO 5000. Absence of free Gd was again confirmed by using the titrating method described before with the dialyzed contents.

6.4.3.4. Second Generation DB PGA-PEG synthesis.

The synthetic route to reach DB PGA-PEG (DB2) is depicted in Scheme 6.3.

6.4.3.4.1. PBLG-PEG di-block synthesis.

The new hybrid di-blocks, PGA-PEG (DB2) where synthesized by introducing a PEG block through N-terminus of PBLG homopolymers after the polymerization process. Briefly, PBLG block (100 mg, 0.005 mmol, 1 eq., 20200 g·mol$^{-1}$ Ø 1.12), was dissolved in 10 mL of HFIP. The contents were heated up to 40 °C. Then, 1.3 eq. of corresponding PEG block (19.5 mg methoxyPEGNHS MW 3023 for DB2 control; 31.2 mg of FMOCPEGNHS MW 4847 for NH$_2$ functionalized DB2; or 20.7 mg of 4TPSS-PEGNHS MW 3219, for SS functionalized DB2), were added dissolved in 2 mL more of HFIP. Reaction mixture was allowed to proceed for 72 hours at 40 °C. Then, the products were purified by concentration and precipitation into a
large excess of diethyl ether. After drying, PEG signal at 3.62 ppm in $^1$H-NMR in CDCl$_3$ was used in all cases to confirm DB2 synthesis.

### 6.4.3.4.2. Benzyl removal from PBLG-PEG.

Benzyl groups of PBLG block were easily deprotected following the optimized basic conditions with NaOH/THF mixtures in M&M from Chapter 2. Products were obtained as white powders after lyophilization. Complete deprotection and PEG integrity was confirmed by $^1$H-NMR in D$_2$O.

#### 6.4.3.5. Peptide sequences conjugation.

Two different strategies for peptide sequences conjugation were performed: on one hand, conjugation of the targeting groups into the polymer backbone, through a previously introduced linker for orthogonal bioconjugations (for linear, star, X-Click and DB PGAs); on the other hand, in the case of DB2, a semitelechelic conjugation at the end of the end-functionalized PEG block. Both strategies will be described for ANG and iron mimicking cPEP conjugations.

#### 6.4.3.5.1. Angiopep2-SH conjugation.

- **a. Conjugation to the polymer backbone.**
  
  **a1. Linker/spacer conjugation:** 2TP activated cysteamine coupling. The spacer was introduced following the protocol described for post-polymerization modification with cysteamine-2TP using DMTMM-CI previously described in Chapter 4. Briefly for 10 % GAU modification: in a one-neck round bottom flask fitted with a stirrer bar and a stopper, 0.4 mmol GAU of PGA construct (linear, star, DB or X-Click) was dissolved in 10 mL of ddH$_2$O. Then, DMTMM-CI (0.04 mmol, 0.1 eq., for 10 % modification) was added in 2 more mL of ddH$_2$O. Reaction was left to proceed for 10 minutes. After that time, cysteamine-2TP (0.08 mmol, 0.2 eq., for 10 % modification) was added to the reaction mixture and the pH was adjusted to 8 by the use of NaHCO$_3$ 1 M solution. Reaction was allowed to proceed for 16 hours, and purified by dialysis using Vivaspin® MWCO 3000. The cysteamine-2TP content was determined according to $^1$H-NMR as explained in Chapter 4.

  Yields: 80-95 %. Cysteamine-2TP loading: 7 mol% GAU for linear PGA; 10 % for DB PEG-PGA; 9 % for St-PGA; 10 % for X-Click.
a2. DO3AtBu deprotection. After linker introduction, the tBu groups from DO3A were removed since no side reactions will occur anymore within the carboxylic groups from DO3A. This step was carried out according to the conditions described previously within this chapter, using TFA/H$_2$O/TIPS. Yields: Quantitative

a3. ANG conjugation within the polymeric platform by disulfide bonding. ANG was conjugated following the same protocol for all constructs and using always the same eq. of peptide than the modifications obtained with reactive disulfides. Hence, briefly, in the case of 10 % SS-2TP modified St-PGA: in a one-neck round bottom flask fitted with a stir bar and a stopper, 45 mg of St-PGA-DO3A-Cys2TP (0.18 mmol GAU, 1 eq.) were dissolved in 4 mL of ammonium acetate 150 mM pH 5. In parallel, ANG was dissolved in 3 mL of HEPES buffer 10 nM pH 7.4. The two solutions were mixed up and the pH was checked to be 6. Reaction was allowed to proceed under stirring for 16 hours. Then, the product was concentrated and purified through a PD-10 column. Fractions from the SEC purification were analyzed by absorbance measurements in Nanodrop™ to determine the conjugate fractions. ANG content was firstly estimated by $^1$H-NMR and amino acid analysis performed at the University of Barcelona (Unitat de Tècniques Separatives I Síntesi de Pèptids Centres Científics I Tecnològics). Yields: 60-70 %. Peptide contents:

Table 6.9. ANG loadings summary.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>mol% GAU/ wt% peptide</th>
<th>$^1$H-NMR</th>
<th>AA analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGA-DO3A-ANG</td>
<td>4.7 mol% / 36 wt%</td>
<td>3.9 mol% / 32 wt%</td>
<td></td>
</tr>
<tr>
<td>DB-DO3A-ANG</td>
<td>4.5 mol% / 32 wt%</td>
<td>3.5 mol% / 27 wt%</td>
<td></td>
</tr>
<tr>
<td>St-DO3A-ANG</td>
<td>4.0 mol% / 27 wt%</td>
<td>5.1 mol% / 33 wt%</td>
<td></td>
</tr>
<tr>
<td>X-Click-DO3A-ANG</td>
<td>2.75 mol% / 16 wt%</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>X-Click-DO3A-Gd-Cy5.5-ANG</td>
<td>1.5 mol% / 13.8 wt%</td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>
b. Telechelic Conjugation to DB2-SS4TP

After DO3AtBu-NH2 conjugation and tBu groups’ removal by protocols described before, ANG was conjugated to PEG-SS4TP block chain end. The procedure followed was analogous to the one described in the previous paragraph but using different eq. (1 eq. of PGA-PEG-SS4TP chains to 2 eq. of ANG). Yield: 75 %. Conjugation efficiency: 80 % according to 1H-NMR (0.8 eq. per polymer chain); 30 % according to AA analysis (0.3 eq. per polymer chain).

6.4.3.5.2. Cyclic peptide conjugation. 

a. Conjugation to the polymeric backbone.

a1. Linker/spacer conjugation. N-Boc-ethylendiamine coupling. The protocol used for the N-Boc-ethylendiamine coupling was post-polymerization modification using DMTMM·Cl previously described in Chapter 4. Briefly, for 10 % modification, 0.403 mmol GAU of polymer (1 eq.) was dissolved in 10 mL of ddH2O. Then, 0.04 mmol of DMTMM·Cl (11.14 mg, 0.1 eq.) was added and the reaction was left to proceed for 10 minutes. After that time, N-Boc-ethylendiamine (12.8 mg, 0.08 mmol, 0.2 eq.) and the mixture was left reacting for 16 hours. For purification, dialysis was performed using Vivaspin® MWCO 3000. Percentage of modification was calculated according to the 1H-NMR spectra. Yields: 80-90 %. N-Boc-ethylendiamine loading: 9 mol% GAU for linear PGA; 10 % for DB PEG-PGA; 8 % for star-PGA.

a2. Boc and tBu groups simultaneous deprotection. Both, Boc and tBu groups were then easily deprotected in CH2Cl2:TFA (3:2) for 16 hours following the protocol described previously in this chapter for tBu from DO3A deprotection. Yield: Quantitative.

a3. COOH activation of Iron mimicking cPEP C-terminus. In a two-necked round bottom flask fitted with a stir bar and two septums, 30 mg of cPEP (0.032 mmol, 1 eq.) were dissolved under inert atmosphere in anh. DMSO (0.5 mL). Then, 8.7 mg DCC (0.042 mmol, 1.3 eq.) were added to the reaction mixture. After 5 minutes, 4.8 mg of N-Hydroxysuccinimide (NHS) (0.042 mmol, 1.3 eq.) were added and the reaction was left to proceed for 16 hours at r.t. and under nitrogen flow. After that time, the contents were precipitated into a large excess of cold diethyl ether, filtered off, and dried. Yield: 95 %. Percentage of NHS activation was determined as 100 %, estimated according to the CH2 signal belonging to the NHS ring at 2.6 ppm.
a4. cPEP conjugation through amide bond. As for ANG conjugation, the eq. of peptide used for the conjugation were the same as the percentage of linker incorporation achieved for each case. Thus, briefly, in the case of linear PGA-DO3A-ethylendiamine (9 % linker): in a one-neck round bottom flask fitted with a stir bar and a stopper, 22.5 mg of polymer (0.14 mmol, 1 eq.) were dissolved in 4 mL of PBS 0.1 M pH 7.4. After that, 13 mg of activated cPEP (0.0126 mmol, 0.09 eq.) were added to the reaction mixture. Reaction was left to proceed under vigorous stirring for 5 hours. Then, the product was purified by SEC using a commercial PD-10 column. Peptide content was estimated by $^1$H-NMR in D$_2$O and quantified by amino acid analysis at the University of Barcelona (Unitat de Tècniques Separatives i Síntesi de Pèptids Centres Científics i Tecnològics). Yields: 75-80 %. Peptide Loading:

Table 6.10. Iron mimicking cPEP loadings summary.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>mol% GAU/ wt% peptide</th>
<th>1H-NMR</th>
<th>AA analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGA-DO3A-cPEP</td>
<td>2.75 mol%/ 12 wt%</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>DB-DO3A-cPEP</td>
<td>3.4 mol%/ 13 wt%</td>
<td>2.8 mol%/ 11 wt%</td>
<td></td>
</tr>
<tr>
<td>St-DO3A-cPEP</td>
<td>6.0 mol%/ 20 wt%</td>
<td>7.4 mol%/ 23 wt%</td>
<td></td>
</tr>
</tbody>
</table>

b. Telechelic conjugation.

After DO3AtBu-NH$_2$ conjugation by the protocol previously described in this chapter, cPEP was conjugated to the PEG-NHFMOOC block chain end. Firstly, the Fmoc group was removed. In a round bottom flask fitted with a stir bar and a stopper, PGA-DO3AtBu-PEG-NHFMOOC was dissolved in a mixture of piperidine 20 % in DMF for 3 hours. The product was then precipitated into a large excess of cold diethyl ether, filtered off, and dried over vacuum. Yield: Quantitative. Complete deprotection was achieved according to $^1$H-NMR in D$_2$O.

After that, the procedure for the conjugation of NHS activated cPEP followed was analogous to the one described for the backbone conjugations but using different eq. (1 eq. of PGA-PEG-SS4TP chains to 2 eq. of cPEP). Yield: 70 %. Conjugation efficiency: 80 % according to $^1$H-NMR (0.8 eq. per polymer chain); 50 % according to AA analysis (0.5 eq. per polymer chain).
6.4.3.6. Z-Potential measurements.

Z-potential measurements were performed at 20 °C using a Malvern ZetasizerNanoZS instrument, equipped with a 532 nm laser using Disposable folded capillary cells, provided by Malvern Instruments Ltd. (Worcerstershire, UK). Polymer solutions (0.1 mg/mL) were prepared in ddH$_2$O. The solutions were filtered through a 0.45 µm cellulose membrane filter. Z-potential was measured for each sample per triplicate with n> 3 measurements.

6.4.3.7. Biodistribution by PET imaging and ex-vivo radioactivity measurement.

*PET imaging was only possible with $^{68}$Ga-labeled polymers since SPECT equipment was not available for $^{111}$In-labeled systems. PET imaging of $^{68}$Ga-labeled polymers was complemented with ex-vivo radioactivity measurements in a gamma counter, which was the only option for $^{111}$In-labeled systems.

$^{68}$Ga-labeled polymers. Animals’ weight was 25 ± 4 g. For pharmacokinetics and biodistribution study, 50 mice were used. Mice were anesthetized with 1.5 % isofluorane, and a dose between 0.2 and 1.4 MBq per mouse (21 ± 4 µg compound/g body weight) was injected i.v. through the tail vein. Blood samples were obtained at different time points (0.25, 0.5, 1, 2 and 3 hours) post injection by terminal bleeding via cardiac puncture following isofluorane anesthesia. Organs (lungs, heart, spleen, kidneys, liver and brain) and some tissues (muscle, fat) were isolated, rinsed with normal saline, weighted, and radioactivity of each sample was measured using Cobra II auto-gamma counter per triplicate. Blood samples were centrifuged (3000 rpm, 10 min, 25 °C) and plasma supernatant was collected. % ID and % ID·g$^{-1}$ were calculated by comparison with standards taken from the injected solution for each animal. Data was expressed as the mean ± SD.

$^{111}$In-labeled polymers. Animals’ weight was 22 ± 4 g. For pharmacokinetics and biodistribution study, 88 mice were used (4-5 for each time point). Mice were anesthetized with 2 % isofluorane, and a dose between 37 KBq and 2.5 MBq of compound per mouse (1-20 µg compound/g body weight) was injected i.v. through the tail vein. Blood samples were obtained at different time points (0.5, 1, 2, 4, 8 and 24 hours) post injection by terminal bleeding via cardiac puncture following isofluorane anesthesia. Organs (lungs, heart, spleen, kidneys, liver and brain) and some tissues (muscle, fat) were isolated, rinsed
with normal saline, weighted, and radioactivity of each sample was measured using Cobra II auto-gamma counter per triplicate. Blood samples were centrifuged (3000 rpm, 10 min, 25 °C) and plasma supernatant was collected. % ID and % ID·g⁻¹ were calculated by comparison with standards taken from the injected solution for each animal. Data was expressed as the mean ± SD.

6.4.3.8. Pharmacokinetics of radiolabeled systems.

Plasma concentration versus time data of radioactivity were analysed by a two-compartment model with bolus input and first-order elimination rate. The model is described by the following equation:

\[ C(t) = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} \]

Apparent terminal half-life (t1/2) is calculated as Ln2/β and the plasma clearance (Cl) for each compound is estimated as the ratio of dose/AUC (area under the plasma concentration-time curve). Initial estimates of PK parameters were computed by WinNonLin (ver. 5.2, Pharsight Corporation, Mountain View, CA) using curve stripping. PK parameters were A, B, α and β. From these parameters, several derived PK parameters were computed: AUC (A/α + B/β), Cl (D/AUC), Vss, Cmax (A+B) and apparent terminal half-life. As plasma concentrations often span a wide range, it is useful to employ a weighting procedure for the raw data that allows one to fit low concentrations and high concentrations simultaneously. We used weighting by the deviation standard of the concentration. The Nelder-Mead simplex algorithm, which is implemented in WinNonlin, was used as fitting algorithm.

6.4.3.9. Biodistribution by Optical Imaging.

Biodistribution experiments were carried out using C57Bl/6 mice. Animals weight was 23 ± 4 g. Mice were anesthetized with 1.5 % isofluorane. Targeted and non-targeted architectures were administered i.v. through vein tail via cannula, at a dose of 4.15 mg·Kg⁻¹ Cy5.5 eq. Blood samples were extracted from cava vein at different time points (1, 3, 7, 14 and 24 hours) from anesthetized mice with a lethal anesthesia cocktail (i.e. for 20 g mice 200 µL from the solution containing ketamine 100 mg·Kg⁻¹, xylasin 100 mg·Kg⁻¹, acepromacide 2 mg·Kg⁻¹). Perfusion with 10 mL of saline was then performed. Organs were extracted (brain, liver, kidney, heart, stomach, spleen, lung) immediately after and their fluorescence was measured using the red filter in MAESTRO™. For fluorescence quantification, normalized
data was obtained by taking always the same pixel area for all organs expressed as average signal (counts.s$^{-1}$). See section 6.4.2.2. A calibration curve of the compounds in the same MAESTRO™ was carried out in order to estimate the fluorescence corresponding to the injected dose.
6.5. REFERENCES.


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Chapter 7

Polymer therapeutics for the treatment of neurodegenerative disorders. Alzheimer’s disease as example
7.1. INTRODUCTION AND BACKGROUND.

7.1.1. Alzheimer’s Disease.


Alzheimer's disease (AD) is a neurodegenerative process of the central nervous system, characterized by loss of short term memory and attention, subsequently affecting other cognitive skills such as language, abstract thinking, critical judgment and recognition of places or people. This progressive loss of cognitive abilities is associated with a decline in a person’s capability to be independent and care for one’s own needs. Such devastating disease represents the most unmet medical need in neurological disorders.¹

![Figure 7.1. Human brain under normal conditions compared to advance Alzheimer’s.](image)

Globally and in developed countries in particular, dementias are a growing burden, since main types of dementia are associated with aging and the average population age is increasing worldwide. Indeed, when AD was identified by the German physician Alois Alzheimer in 1906, this disorder was considered a rare disease. Unfortunately nowadays, AD is the most common cause of dementia in elderly people, accounting for about 70 % of all cases in Europe and North America. On the contrary, in Russia and Japan vascular dementia is more frequent than AD and in Africa AD has a low incidence. However, when considered globally, it represents 50-70 % of all forms of dementia worldwide.

AD is one of the largest global public health challenges to be faced. Currently, over 35 million people worldwide is living with the disease and this number is expected to double by 2030 reaching up to
115 million in 2050, more than triple.\textsuperscript{2} Besides the cost of medication and therapy, patients with AD require special medical care. The global economic cost is around US$ 604 billion. It is expected that due to life expectancy and the new lifestyles, the costs of dementia will grow exponentially by 2050 (when the baby boom generation will reach 65 years old). Approximately 10\% of the population in developed countries, with over 65 years of age, and 50\% above 85 years old are diagnosed with AD, representing the 7\textsuperscript{th} leading cause of death in countries such as the United States.

![AD global forecast 2013-2050](image)

**Figure 7.2.** AD global forecast from 2013-2015 represented in millions of people suffering from AD worldwide.\textsuperscript{2}

Only in Spain, this dementia is currently suffered by more than 1.5 million people and the cost of the disease is over 37,000 million euros. Besides, with an aging population, deaths in Spain derived from AD have doubled from 2000 to 2011, year in which 11,907 deaths were registered, according to National Statistics Institute (INE). Diseases of the nervous system are the 4\textsuperscript{th} most common cause of death in Spain (20,254, 5.2\%), behind vascular diseases (30.5\%) cancer (28.2\%) and respiratory pathologies (10.9\%).\textsuperscript{5}

![Main causes of Death in Spain](image)

**Figure 7.3.** Main causes of Death in Spain. Vascular diseases occupy the top position (30.5\%) followed by tumors (28.2\%), respiratory
diseases (28.2 %) and nervous system affections including dementias (5.2 %).³

The therapeutic armory for AD has increased over the last years, and new disease-modifying drugs are coming out. However, current available drugs only induce moderate symptomatic benefits but do not target neuronal impairment.

7.1.1.2. Disease hallmarks and progression.

In general, AD is a multiple and entangled process, caused by complex interactions among multiple genetic, epigenetic and environmental factors. Regarding genetics, mutations in three genes (amyloid protein precursor (APP), presenilin (PS)-1 and PS-2) cause early-onset autosomal dominant AD (< 60 years old), which represents less than 1 % of AD cases.⁴ On the other hand, apolipoprotein (apo) E4 has been genetically linked to a late-onset familial and sporadic AD (> 60 years old) which accounts for most AD cases.⁴

Although there are several clear disease hallmarks, the specific cause of Alzheimer's remains unknown. Based on clinical observation and autopsy, the disease has been characterized by three cardinal changes in the brain:

(i) The appearance of extracellular deposits, named senile cerebral plaques laden with β-amyloid peptide (Aβ) and dystrophic neurites in neocortical terminal fields.

![Figure 7.4. Representation of disease neurons with extracellular Aβ deposits in Alzheimer’s disease vs. healthy neurons from healthy patients. Adapted from ref⁵](image)
(ii) The formation of abnormal intracellular filaments (neurofibrillary tangles) made up of a highly phosphorylated form of microtubule-associated protein tau in the perikaryia of certain neurons in medial temporal-lobe structures, accompanied by neurophil threads in axons and nerve terminals.

![Figure 7.5. Representation of healthy neurons where microtubule-associated protein tau is placed as structural scaffold for microtubule stabilization, vs. disease neurons where hyperphosphorilated state of tau protein leads to microtubule disintegration and the formation of neurofibrillary tangles within the misfolded protein. Adapted from ref.6](image)

(iii) The loss of vulnerable neurons, mainly pyramidal, cholinergic, noradrenergic and serotonergic neurons. The death of cholinergic neurons leads to a deficit in acetylcholine (Ach), a major transmitter involved in memory.

Loss of white matter, congophilic (amyloid) angiopathy, inflammation, and oxidative damage are also present hallmarks of the disease.

Generally, neurodegeneration begins in the entorhinal cortex with pyramidal cell loss (the most abundant neurons in the cortex), neurofibrillary tangles and neurophil threads, and then spreads in an anatomically defined pattern to other brain regions: particularly the hippocampus and parietal and temporal regions of neocortex. Senile plaques first appear in the frontal cortex, and then spread over the entire cortical region, whereas hyperphosphorylated tau and insoluble tangles initially appear in the limbic system (entorhinal cortex, hippocampus, dentate gyrus) and then progress to the neocortex.

Symptoms can vary in severity and chronology but in any case they reflect the gradual expansion of degenerative damages within the
brain. Furthermore, the rate of progression is also highly variable. People with Alzheimer's live an average of eight years, but some people may survive up to 20 years. According to recent studies, changes in brain may start even 20 years or more before symptoms appear. At early stages, before symptoms can be detected with current tests, plaques and tangles begin to form in brain areas involved in learning and memory, thinking and planning. Hence, when people is usually diagnosed with dementia, they are at least in mild-to-moderate Alzheimer’s stages.

In mild to moderate stages, brain regions important in memory, thinking and planning develop more plaques and tangles leading to serious problems with memory or thinking that already interfere with work or social life. In moderate stages, areas involved in speaking and understanding speech are also damaged. Furthermore, as AD progresses, individuals may experience changes in personality and behavior and they find problems in recognizing friends or family.

In advanced AD, most of the cortex is seriously damaged. The brain shrinks dramatically due to widespread cell death. Individuals lose their ability to communicate, to recognize family and loved ones and to care for themselves. Figure 7.6 shows disease progression from healthy to advanced AD brains.

![Figure 7.6. AD progression in brain cross sections from healthy brain without any physiological damage to mild-to-moderate AD brain where typical cortical and hippocampus shrinkage and ventricles enlargement](image)
Many hypothesis have been postulated to explain AD pathogenesis, encompassing Aβ cascade, tau, cholinergic, oxidative stress and metal hypothesis as the main ones. Nevertheless, no single hypothesis can completely explain the causes of AD. Moreover, whether these changes are causes or consequences of AD is still not fully elucidated.

In amyloid cascade hypothesis, the accumulation of soluble Aβ into toxic oligomers and amyloid plaques is thought to promote the pathogenic cascade leading to tau hyperphosphorylation, intracellular neurofibrillary tangles, synaptic dysfunction, neuronal death and, ultimately, loss of cognitive functions. Aβ are the hydrolytic products from APP cleavage by β-secretase (BACE1) and posterior cleavage by γ-secretase. Under non-amyloidogenic conditions, such cleavage is performed first by α-secretase followed by γ-secretase leading to non-amyloidogenic substrates (see Figure 7.7). The main species generated in the amyloidogenic cleavage are Aβ40 and Aβ42. Whereas Aβ40 is the most abundant, Aβ42 is more prone to aggregation. The imbalance among Aβ production and clearance plays a central role in AD pathogenesis. Apart from senile plaques Aβ also deposits in the cerebral vascular wall leading to cerebral amyloid angiopathy (CAA). Thus, according to this cascade, bioactive agents with the ability to reduce Aβ production, inhibit Aβ aggregation or enhance Aβ clearance are good candidates for AD therapies.
Figure 7.7. APP cleavage pathways. Non-amyloidogenic pathway involves APP extracellular cleavage by α-secretase in a first stage generating sAPPα and the fragment C83, which is subsequently cleaved by γ-secretase enzyme leading to non-amyloidogenic peptide p3 that is also released to the extracellular space. On the contrary, the amyloidogenic pathway is initiated by β-secretase cleavage of APP leading to sAPPβ and C99 peptide this time. C99 is then cleaved by γ-secretase leading to Aβ peptides prone to aggregate forming first dimers and small oligomers and later on fibrils and plaques. Redrawn from ref.13

Tau hypothesis is the second most accepted hypothesis, according to which tau hyperphosphorylation triggers the formation of neurofibrillary tangles inside neurons, main cause in AD onset.14 Once tau is aggregated, such agglomerates can be transported from one nerve cell to another along the neural network of axons that connect them. In consort with this hypothesis, tau protein has become a target for AD strategies by inhibition of its phosphorylation or aggregation, reduction of its levels, tau immunization or microtubule stabilization.15

Cholinergic neurons are affected even in very early stages of AD, pointing out (in the cholinergic hypothesis) that this condition contributes to the severity of the cognitive and behavioral deficits.16 Indeed, most of the current drugs for AD symptomatic treatment (donepezil, galantamine, rivastigmine) were born with this hypothesis.17

According to the oxidative hypothesis, oxidative stress generated during aging causes serious damage to cell function and viability18 and is intimately related to AD onset. Consequently,
antioxidants such as flavonoids and polyphenols are under study for AD treatments.\(^\text{19}\)

Linked to the oxidative hypothesis, metal hypothesis includes the role of metal ions in AD development, by postulating that metals such as Cu\(^{2+}\), Zn\(^{2+}\), Fe\(^{3+}\) bind to Aβ causing the production of reactive oxygen species.\(^\text{20}\) Hence, metal chelators have been suggested as therapeutic strategy in AD.\(^\text{21,22}\)

Activated microglia and reactive astrocytes have been found to be located on the surroundings of senile plaques. Furthermore, their biochemical markers are overexpressed in AD brains.\(^\text{23}\) Recent studies suggest that microglia may have contradictory roles along AD onset and progression. Firstly, phagocytic microglia engulf and remove Aβ. Nevertheless, when activated chronically (as in AD), microglia releases chemokines and a cascade of damaging cytokines (interleukines (IL) IL-1, IL-6, and TNFα).\(^\text{24}\) Furthermore, reactive astroglia also release acute-phase reactants such as α\(_1\)-antichymotrypsin, α\(_2\)-macroglobulin, and C-reactive protein that can aggravate as well as ameliorate AD. A crosstalk between systemic and central innate immune system by the release of inflammatory mediators leading to the migration of T cells and immune cells into the brain has also been observed in AD.\(^\text{25}\) Thus, microglia activation leads (indirectly) to BBB disruption.\(^\text{26,27}\) Overall, the contradictory roles of microglia makes more difficult the identification of effective treatments. Non-steroidal anti-inflammatory drugs (NSAIDs) have been reported to decrease the risk of suffering from AD, and slow disease progression.\(^\text{28}\)

Finally, mitochondrial dysfunction is also found in AD brains.\(^\text{29}\) Indeed, the exposure to Aβ triggers the inhibition of key mitochondrial enzymes in the brain.\(^\text{30,31}\) Cytochrome c oxidase is specifically impaired\(^\text{32}\) and subsequently, electron transport, ATP production, oxygen consumption, and mitochondrial membrane potential can be damaged. Furthermore, the increase of radical formation cause oxidative stress, release of cytochrome c and consequently apoptosis.\(^\text{33}\)

### 7.1.1.3. Current treatments and approaches.

Drug development for AD has been a slow and difficult process. Only five drugs are approved for the treatment of AD including four Acetylcholinesterase (AChE) inhibitors (tacrine, donepezil, rivastigmine, galantamine) and an N-methyl-D-aspartate (NMDA) receptor AD antagonist (memantine).\(^\text{34,35}\) Tacrine was
approved by the FDA in 1993, donepezil in 1996, rivastigmine in 1998, galantamine in 2001, and memantine in 2003. Since 2003, no new treatments have been approved mainly due to many failures in AD drug development with both small molecules and immunotherapies due to poor effectiveness or unacceptable toxicities.\textsuperscript{36–40} Indeed, 72\% of the agents failed in phase I, 92\% in phase II and 98\% in phase III, highlighting the high attrition rate for AD treatment. Importantly, from 2002 to 2012, 244 compounds were tested in the 413 AD trials performed, from which, the largest number are focused in addressing symptomatic effect (improve cognition), followed by disease-modifying small molecules and disease-modifying immunotherapies as reviewed recently by Cummings et al.\textsuperscript{37}

Drug strategies under clinical development are in consonance with the different hypothesis described before, as can be seen in Table 7.1. An extensive summary of clinical trials and late-stage drug development for AD up to 2014 can be found in literature.\textsuperscript{41}

**Table 7.1.** Mechanism of action of current AD drugs under clinical development (as of February 2004, from ref.\textsuperscript{37})

<table>
<thead>
<tr>
<th>Mechanism of action</th>
<th>Phase 1</th>
<th>Phase 2</th>
<th>Phase 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptomatic for cognition</td>
<td>5</td>
<td>10</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Symptomatic for behavior</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Disease-modifying small molecule (amyloid-related)</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Disease-modifying small molecule (tau-related)</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Disease-modifying small molecule (neuroprotector)</td>
<td>2</td>
<td>19</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>Disease-modifying immunotherapy</td>
<td>4</td>
<td>8</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>Therapeutic device</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Stem cells</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>22</strong></td>
<td><strong>50</strong></td>
<td><strong>23</strong></td>
<td><strong>95</strong></td>
</tr>
</tbody>
</table>

Overall, there is still a long way to go in order to achieve effective and safe treatments for such chronic neurodegenerative disease. Past failures and the dichotomies of symptomatic versus disease-modifying, early stages versus late stages treatment, and amyloid versus non-amyloid hypothesis, suggest that there is still much to be understood. Moreover, the fact that disease mechanisms are not fully elucidated, the multiple present hallmarks with unclear origin
(what is cause and what is consequence?), BBB handicap, limitations in diagnosis to identify early and treatable stages, the many difficulties found in clinical trials that require long-term treatments (especially for preventive approaches), and the need for multi-target and personalized therapy are hindering the appearance of novel promising candidates for AD treatment. To this respect, nanomedicine is envisaged as possible solution for efficient diagnosis and treatment approaches.

7.1.1.4. Nanomedicine as treatment for AD.

As mentioned before, some of the main challenges of AD treatment rely on the fact that (i) BBB impedes drug delivery into the CNS, and (ii) the lack of understanding on the exact pathogenesis of AD. Among the already described strategies for CNS delivery in Chapter 6, non-invasive approaches are preferred in AD therapy to allow better patient compliance and to avoid any safety concerns, as well as the relatively high costs of the invasive techniques. Among non-invasive approaches, nanomedicine is gaining increasing interest. Current nanopharmaceuticals for AD encompass Aβ targeting, metal ions binding, cholinesterase inhibition, neuroprotection and estrogen replacement therapy.

Aβ targeted nanotherapeutic strategies include the modulation of Aβ production, inhibition of Aβ aggregation, and enhancement of Aβ clearance. Modulation of Aβ production can be achieved by targeting the enzymes responsible for the APP cleavage (β-secretase BACE1 and γ-secretase). However, only BACE1 represents a good target for clinical applications provided that γ-secretase is related to many other processes and its inhibition has demonstrated to lead to unwanted side effects.42-44 To this respect, Alvarez-Erviti et al. developed modified exosomes as carriers for brain-targeting delivery of BACE1 siRNA with promising results (Table 7.1).45 Regarding the inhibition of Aβ aggregation, Li et al. synthesized polyoxometalate (POM)-peptide particles from POM and Aβ15-20 inhibitor of aggregation together with Congo Red to monitor the inhibition process.46 On the other hand, the polyphenolic curcumin (further analyzed later on in this chapter) has been also used against Aβ aggregation47, 48 in several nanosystems including PEG-PLA nanoparticles from Cheng et al.49 and PLGA nanoparticles from Mathew et al (See Table 7.1).50 Song et al. have recently developed a nanostructure based on apolipoproteinE3-reconstituted HDL. Such system demonstrated to accelerate Aβ
degradation, decreased Aβ deposition, while attenuating microgliosis, thus, ameliorating neurologic changes and rescue memory deficits in AD mice model.\textsuperscript{51} Immunotherapy has been also effectively applied to inhibit Aβ deposition. For instance, Canovi \textit{et al.} built a nanoliposome loaded with Aβ-MAb (anti Aβ monoclonal antibody) with high affinity for Aβ\textsubscript{42} monomers and fibrils.\textsuperscript{52}

Normal metal ion distribution in the CNS, (i.e. cooper, iron or zinc) is altered by several fold concentration in AD conditions.\textsuperscript{53-55} Furthermore, senile plaques are enriched with these metals what indicates their involvement in Aβ aggregation and Aβ-induced oxidative stress.\textsuperscript{56, 57} Hence, metal chelators are postulated as candidates to disrupt and prevent Aβ plaques. For instance, Liu et al, conjugated the iron chelator 2-methyl-N-(2’-aminoethyl)-3-hydroxyl-4-pyridinone (MAEHP) to functionalized polystyrene nanoparticles leading to Nano-N2YP a potential inhibitor of Aβ aggregation and neurotoxicity, demonstrated \textit{in vitro}.\textsuperscript{58}

The AChE inhibitor rivastigmine has been delivered using liposomal formulations in order to increase its limited plasma half-life (< 1.5 hours).\textsuperscript{59, 60}

Regarding neuroprotection, PEG-PEI-Rho associated kinase (ROCK-II) siRNA polyplex has demonstrated to decrease Aβ production, protect from neurodegeneration and promote axonal growth.\textsuperscript{61-63}

Finally, Mittal et al. constructed estradiol-loaded tween 80 (T-80)-coated PLGA nanoparticles capable to achieve significantly increased levels of estradiol in brain after oral administration, showing also good results in the prevention of the expression of Aβ\textsubscript{1-42} immunoreactivity.\textsuperscript{64}

Nonetheless, most of the studies are still at very early stages of development (even \textit{in vitro} for some of them, see Table 7.2), thus, further research must be done to evaluate their real potential.
<table>
<thead>
<tr>
<th>Nanocarrier</th>
<th>Targeting Moiety</th>
<th>Drug</th>
<th>Size (nm)</th>
<th>Target</th>
<th>Stage</th>
<th>Model</th>
<th>Outcome</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exosome</td>
<td>Rabies viral glycoprotein peptide</td>
<td>BACE1 siRNA</td>
<td>~88</td>
<td>BACE1 mRNA</td>
<td>In vivo</td>
<td>C57BL/6 mice</td>
<td>High efficiency in BACE1 silencing</td>
<td>45</td>
</tr>
<tr>
<td>Polyoxometalate nanospheres</td>
<td>Aβ_{15-20} peptide</td>
<td>-</td>
<td>~80</td>
<td>-</td>
<td>In vitro</td>
<td>PC12 cells and mice CSF</td>
<td>Inhibition on amyloid aggregation in CSF and rescue Aβ-induced cytotoxicity in PC12 cells</td>
<td>46</td>
</tr>
<tr>
<td>PEG-PLA nanoparticles</td>
<td>-</td>
<td>Curcumin</td>
<td>&lt;80</td>
<td>-</td>
<td>In vitro &amp; in vivo</td>
<td>MDCK and Tg2576 mice</td>
<td>Higher BBB permeability of curcumin and improvements in working and cue memory following oral admin.</td>
<td>49</td>
</tr>
<tr>
<td>PLGA nanoparticles</td>
<td>Tat-1 peptide</td>
<td>Curcumin</td>
<td>150-200</td>
<td>Neurons</td>
<td>In vitro</td>
<td>Amyloid protein and LAG cell line</td>
<td>Dissolved amyloid aggregates and non-cytotoxic</td>
<td>50</td>
</tr>
<tr>
<td>AuNP</td>
<td>Cys-PEP</td>
<td>-</td>
<td>~10</td>
<td>Aβ aggreg.</td>
<td>In vitro</td>
<td>Aβ solutions</td>
<td>Binds to Aβ and dissolved them via weak microwave fields.</td>
<td>66</td>
</tr>
<tr>
<td>AuNP</td>
<td>THR and CLPFFD peptide</td>
<td>-</td>
<td>~13</td>
<td>Aβ aggreg. and TfR</td>
<td>In vitro &amp; in vivo</td>
<td>Aβ solutions, male SD rats</td>
<td>Increased BBB permeability, dissolved Aβ aggregates</td>
<td>67</td>
</tr>
<tr>
<td>Reconstituted HDL</td>
<td>ApoE3</td>
<td>-</td>
<td>27.94 ± 8.9</td>
<td>BBB and Aβ</td>
<td>In vitro &amp; in vivo</td>
<td>Primary glial cells, Chang liver cells and SAMP8 mice</td>
<td>Accelerated degradation of Aβ, decreased Aβ deposition, attenuate microgliosis, ameliorated neurologic changes and rescue memory deficits in AD mice model</td>
<td>51</td>
</tr>
<tr>
<td>Carrier/Conjugates</td>
<td>Drug/Delivery</td>
<td>Binding/Inhibition</td>
<td>Target Cells/Animal Models</td>
<td>Cytotoxicity/Delivery</td>
<td></td>
<td></td>
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<td>--------------------</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>DSPC-Chol liposome</td>
<td>Aβ-MAb</td>
<td>124-134</td>
<td>In vitro Postmorten AD brain tissue</td>
<td>High binding affinity to Aβ monomers and fibrils</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG-PLA nanoparticles</td>
<td>TGN and QSH</td>
<td>~100 Aβ and Aβ_{42}</td>
<td>In vitro &amp; in vivo bEnd.3 and PC12 cells, adult male nude and ICR mice</td>
<td>Little cytotoxicity; better targeted delivery than control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polystyrene nanoparticles</td>
<td>N2PY (iron chelator)</td>
<td>~240 Iron</td>
<td>In vitro HCN-1A</td>
<td>Inhibition of Aβ aggregation and neurotoxicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPPC and cholesterol liposome</td>
<td>-</td>
<td>~3400 Rivastigmine</td>
<td>In vitro &amp; in vivo MDCK and Balb-C male mice</td>
<td>Highest AChE inhibition on the animal model</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC-DCP-Chol liposome</td>
<td>-</td>
<td>~500 Rivastigmine</td>
<td>In vivo Male Wistar rats</td>
<td>Decreased BACE1, AChE and IL1B gene expression of AICl3-treated rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG-PEI</td>
<td>-</td>
<td>ROCK-II-siRNA 104.17 ± 1.4 ROCK-II</td>
<td>In vitro C17.2 neural stem cells</td>
<td>Suppressed ROCK-II mRNA expression with high transfection efficiency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLGA nanoparticles</td>
<td>Tween 80 Estradiol 138.8 ± 4.3</td>
<td>BBB</td>
<td>In vivo Ovariectomized SD rats</td>
<td>Active drug effect and higher brain estradiol levels, 24 h post admin.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Aβ-MAb: Aβ monoclonal antibody; AChE: Acetylcholinesterase; ApoE3: Apolipoprotein E3; AuNP: Gold nanoparticles; BACE1: β-siteAPP-cleaving enzyme 1; DPPC: Dipalmitoylphosphotidyl choline; DSPC-Chol: 1,2-distearoyl-sn-glycerol-3-phosphatidylcholine and choline; HCN-1A: Human cortical neuronal cells; HDL: High density lipoprotein; MDCK: Madin-Darby canine kidney; PC-DCP-Chol: Phosphocholine-dihexadecyl phosphate cholesterol; R.: Reference; ROCK-II: rho-associated kinase II; SD: Sprague-Dawley.
7.2. RESULTS AND DISCUSSION.

7.2.1. Towards neuroprotective therapies for AD.

As stated in Chapter 1, PT possess all the necessary requirements for diagnosis and treatment of CNS disorders and brain tumors. As also mentioned, moderate current treatments to AD offer only symptomatic benefit, but do not aim to delay or stop disease progression. Therefore, scientific efforts have been focused on the understanding of key molecules and pathways in physiological processes, as well as drug discovery for AD treatment. Within our group, to achieve polymeric nanomedicines of controlled architecture bearing drugs with neuroprotective or neuronal rescuer action is one of our main lines of research for the treatment of AD. This PhD thesis is part of such strategy.

One example of neuroprotective agents are the propargylamine-bearing drugs Selegiline®, Rasagiline®, Ladostigil® and M30®, which are described as MAO-B (monoamine oxidase-B) inhibitors. In brain, MAO-B is localized in glial cells, and MAO-B levels increase with neuronal cell death. MAO is an important enzyme to metabolize in vivo endogenous and diet-derived biogenic amines via oxidative deamination. Major substrates are noradrenaline, adrenaline, dopamine, β-phenylethylamine (PEA) and serotonin. It has been recently established that their protective effect does not only result from MAO-B inhibition, as it is found in other propargylamine-containing molecules that do not inhibit this enzyme, such as the s-optical isomer of Rasagiline®. It seems that this effect is directly related to the propargylamine moiety. Indeed, propargylamine moiety itself has been also evaluated confirming its neuroprotective-neurorescuer character.

![Chemical structure of propargyl-derivatives compounds.](image)

**Figure 7.8.** Chemical structure of propargyl-derivatives compounds. The anti-Parkinsonian drugs, Selegiline® and Rasagiline®, and the multifunctional propargylamine-derivatives, Ladostigil® and M30®. The propargylamine moiety is circled.
On the other hand, as already stated, neuroinflammation is implicated in the pathogenesis of many neurodegenerative disorders, including AD. Hence, for AD therapeutics, there is a rational to develop drugs that attenuate inflammatory cascades that contribute to neurodegeneration and amyloid production or accumulation. To this respect, curcumin and curcuminoids are compounds known to inhibit inflammation while reducing plaque deposition in AD models.\(^{74-77}\)

Curcumin (diferuloylmethane) is a polyphenolic compound derived from turmeric spice from curcuma longa.\(^{78}\) Naturally occurring curcuminoids are a mixture of curcumin (77%), demethoxycurcumin (DMC) (17%), and bisdemethoxycurcumin (BDMC) (3%).\(^{79}\) (See Figure 7.9).

![Curcumin, Demethoxycurcumin, and Bisdemethoxycurcumin](image)

**Figure 7.9.** Structure of the different curcuminoids present in curcuma turmeric spice from curcuma longa.

Indeed, the lower prevalence of AD incidence in India, is generally attributed to the turmeric spice consumption as part of curry, and it is assumed that people who eat curcuma regularly, have lower incidence of AD.\(^{80,81}\)

Curcumin antagonizes many steps in the inflammatory cascade, including activator protein-1 transcription, activation of nuclear factor-kB, iNOS, and JNK.\(^{82-84}\) It also exerts a potent antioxidant activity for NO-related radical generation.\(^{85}\) In contrast to non-steroidal anti-inflammatory drugs (NSAIDs) who have adverse side effects including gastrointestinal ulceration and liver or kidney toxicity, curcumin seems to be relatively safe, even in clinical trials for prevention of relapse of ulcerative colitis.\(^{86,87}\) Many therapeutic applications have been already attributed to curcuminoids such as the suppression of mutagenesis, chemoprevention for a wide variety of cancers, inhibition of atherosclerosis or of viral and bacterial growth.\(^{88-93}\) Furthermore,
Curcumin has been shown to mediate anti-proliferative effects through suppression of cyclin D1 and anti-apoptotic gene products,\textsuperscript{94-96} induce cytochrome C release, activate caspases\textsuperscript{97, 98} and p53\textsuperscript{99} and have anti-angiogenic effects through the down-regulation of vascular endothelial growth factor (VEGF).\textsuperscript{94, 100} On the basis of the results from these studies, curcumin is currently in clinical trials for the treatment of various cancers and for AD among many other pathologies.\textsuperscript{101} Some of the biological properties of curcuminoids are summarized in Figure 7.10.

![Figure 7.10](image)

**Figure 7.10.** Established immuno-protective effects of curcumin, highlighting the great potential and ability to act as antioxidant, anti-inflammatory, and anticancer drug. Redrawn from ref.\textsuperscript{102}

Curcuminoids are described to selectively bind to Aβ plaques being able to prevent fibril formation and to disrupt already formed fibrils, thus, acting in the aggregation process.\textsuperscript{103, 104} They also reduced phosphorylated tau protein burden.\textsuperscript{105} Moreover, the curcuminoid family has been recently identified as AChE inhibitors as well as β-secretase inhibitors,\textsuperscript{106, 107} overall, covering most of the pathogenic hypothesis postulated for AD onset and development. Furthermore, curcumin-releasing mechanically adaptive intracortical implants have recently demonstrated that curcumin improve the proximal neuronal density and blood-brain barrier stability.\textsuperscript{108}

Hence, although it has been reported that curcumin/curcuminoid administration attenuates cognitive deficits,
neuroinflammation and plaque pathology in AD models, its poor solubility in water, poor oral absorption in both humans and animals, and the low systemic bioavailability have limited its clinical benefits.

As a maximum goal for this part of the project we aim to obtain combination conjugates for systemic administration with synergistic effect using the neuroprotective-neurorescuer propargyl moieties and the neuro-antiinflammatory curcuminoids, looking for a new therapeutic strategy in AD.

To this aim, the already explored complex PGA structures were chosen as carriers due to their good in vivo performance, in terms of biodistribution and brain accumulation. Hence, the synthetic strategy was based on a bottom-up approach by taking profit of the self and co-assembly properties of the previously described star-shaped polyglutamates. One possibility could be the conjugation of curcuminoids to the 3-arm star shaped polymeric aggregates obtained from the bottom-up approach after covalent entrapment in order to build combination therapy constructs with curcuminoids presumably exposed at the surface (Figure 7.11). An alternative approach would be the conjugation of propargyl residues to a 3-arm star shaped architecture and the linkage of curcuminoids + azide moieties to another 3-arm star polymer. Those structures will presumably co-assemble when mixed at fixed concentrations above their CAC value, and covalent entrapping will follow to obtain a final construct with defined structure independent from the concentration. This later strategy is only valid if the drug is stable under click reaction conditions and do not contain functional groups that might interfere (which is the case). However, a whole study of the new system including the drug had be done before in order to optimize the conditions for the covalent capture of the new architectures. For targeting moieties and/or imaging agents the second approach would not be suitable as these residues should be included at the end of the strategy in order to secure binding and receptor recognition (in the case of targeting moieties) or to avoid quenching effects (in the case of imaging agents).

According to the reported data from propargylamine residues, it seems that release from the drugs depicted in Figure 7.8, is not a request for activity, since the methylated residue itself is able to exhibit neuroprotective-neurorescuer properties. Thus, herein we propose the direct conjugation of these propargylamine residues within the
polymeric architectures selected by post-polymerization modification techniques by using non-biodegradable covalent linkages. On the other hand, conjugation of the curcuminoid will be carried out through a biodegradable ester linkage so that the selected curcuminoid could be released to trigger its biological activity. Among curcuminoids, BDMC was chosen due to its higher stability in physiological conditions (pH 7.4) when compared to curcumin. Similar biological outputs have been described for all curcuminoids.\textsuperscript{112}
Figure 7.11. Schematic representation of the two main strategies in order to obtain curcuminoid conjugated to complex PGA-based architectures based on bottom-up approaches. In approach a), curcuminoids are introduced by surface conjugation whereas in approach b) curcuminoid are conjugated prior to covalent capture of co-assembled structures.
7.2.2. Design of combination therapy through bottom-up approaches.

First of all, in order to optimize the conjugation chemistry of BDMC into PGAs, as well as to study their self-assembly behavior, several PGA-based systems bearing self-assembly triggering motifs, were modified with BDMC. After many attempts using different carboxylic acid activators, DMTMM·BF₄ was chosen with the aid of DMAP as catalyst yielding conjugation efficiencies between 30-40%. It has to be mentioned that the activation of BDMC alcohols by tosylation did not improve conjugation efficiency.

Table 7.3. BDMC-conjugates details.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>TDC wt% (Abs 415 nm)</th>
<th>FDC wt% of TDC (Abs 415 nm, HPLC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-BDMC (0.5 wt%) (51a)</td>
<td>0.5</td>
<td>&gt;1</td>
</tr>
<tr>
<td>X-BDMC (1 wt%) (51b)</td>
<td>1.0</td>
<td>&gt;1</td>
</tr>
<tr>
<td>X-BDMC (2.5 wt%) (51c)</td>
<td>2.5</td>
<td>&gt;1</td>
</tr>
</tbody>
</table>

*TDC: total drug content; FDC: free drug content.

Self-assembly of the BDMC-conjugates necessary to follow the bottom-up approach was confirmed by DLS measurements as shown in Figure 7.12.

Figure 7.12. Mean Count Rate (Kcps) at increasing concentrations obtained by DLS for X-BDMC conjugates showing self-assembly. CAC= 0.6 for 51b and CAC=0.7 for 51c.

As stated before, two strategies towards the design of polymer-based combination constructs bearing propargyl amine moieties + curcuminoids were followed. For that aim, a bottom-up approach based on the synthesis and covalent capture of PGA-based systems was used.
Taking into account that propargyl amine will be used as one of the drugs for these nanostructures, covalent capture through CuAAC was chosen. In order to always have free propargyl groups, double eq. were used when compared to the number of azides introduced. The first strategy consisted on the synthesis of two different modified polymers with propargyl amine moieties (10 mol% GAU) and NH$_2$EG(2)N$_3$ (5 mol% GAU), their covalent capture, and posterior conjugation of BDMC to the already entrapped structure. In the second strategy, BDMC was conjugated into one of the two modified unimers, (concretely into the azide modified one due to its less hydrophobicity), prior to covalent capture by CuAAC. Nonetheless, this latter strategy did not succeed due to poor stability of BDMC upon the CuAAC conditions. Results obtained in some of the experimental attempts are summarized in Table 7.4, where X-BDMC-Click refers to BDMC conjugation prior click chemistry and X-Click-BDMC, stands for BDMC conjugation after the covalent capture step.

**Table 7.4.** Physico-chemical characteristics of BDMC-conjugates through bottom-up approach.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>TDC wt% (Abs 415 nm)</th>
<th>FDC wt% of TDC (Abs 415 nm, HPLC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-EG(2)N$_3$-BDMC (52)</td>
<td>1.25</td>
<td>&gt;1</td>
</tr>
<tr>
<td>X-BDMC-Click (53)</td>
<td>0.11</td>
<td>&gt;1</td>
</tr>
<tr>
<td>X-Click-BDMC (54a)</td>
<td>2.00</td>
<td>&gt;1</td>
</tr>
<tr>
<td>X-Click-BDMC (54b)</td>
<td>4.00</td>
<td>&gt;1</td>
</tr>
</tbody>
</table>

7.2.3. *In vitro* evaluation.

7.2.3.1. Cell Viability.

Firstly, cytotoxicity of BDMC bearing polymers was explored up to 15 μM drug-eq. According to previous studies found in literature, a curcuminoid concentration range of 0.1-1 μM should be enough to induce a therapeutic benefit by diminishing oxidative stress. Moreover, the IC50 value for Aβ aggregation and lipid peroxidation of curcuminoids is also found in that concentration rage, indicating that such a dose should be enough in order to produce antioxidant and anti-inflammatory effects. As it can be observed from Figure 7.13, non-significant toxicities up to 10 μM drug-eq. were found. The compound X-Click-BDMC (54b) with 4 wt% of BDMC was selected for further investigations (100 % cell viability at 10 μM).
Figure 7.13. Cell viability of BDMC derivatives against SHSY5Y cell line. 72 hours MTS assay. n> 3, mean ± SEM.

7.2.3.2. Drug release profiles.
Since a pH degradable linker (ester) was used for the conjugation of BDMC, the kinetics of drug release under hydrolytic conditions was consequently studied. Samples of X-Click-BDMC 4 wt%, (selected from cell viability experiments) were incubated at 37 ºC at different pHs including 5.0 (lysosome), 6.5 (endosome) and 7.4 (blood) up to 96 hours. A sustained and controlled drug release profile was obtained after HPLC analysis. About 20 % of the conjugated drug was released within 2 days at pH 5.0 whereas pH 6.5 and 7.4 showed a much slower release profile.

Figure 7.14. Drug release profiles at different pH (5.0, 6.5 and 7.4) of X-Click-BDMC (4 wt%). Time course experiments were done per triplicate. n> 3, mean ± SEM.
7.2.3.3. Prevention of fibril formation in vitro.

In order to achieve proof of concept, activity of the compounds was checked in a first attempt using an accepted model based on the use of Hen Egg White Lysozyme (HEWL) for protein amyloid formation. HEWL is a monomeric protein composed of 129 amino acids with helix rich conformation, and it represents one of the best known model proteins to study protein aggregation.\textsuperscript{115, 116} It has been demonstrated that under acidic pH this protein undergo amyloid aggregation (Figure 7.15b). Hence, activity of several BDMC bearing conjugates, as inhibitors of fibril formation was checked by Thioflavin T (ThT) fluorescence measuring, which is in correlation with fibril formation. ThT is a benzothiazole salt used as a dye to visualize and quantify the presence or fibrillation of misfolded protein aggregates, or amyloid, both in vitro and in vivo (i.e. plaques composed of amyloid beta found in the brains of Alzheimer's disease patients). ThT Assay measures changes of fluorescence intensity of ThT upon binding to amyloid fibrils (Figure 7.15a). The enhanced fluorescence can be observed by fluorescence microscopy or by fluorescent spectroscopy. The spectroscopic assay is normally used to monitor fibrillization over time.

![Figure 7.15. a) Schematic representation of ThT fluorescence changes upon protein fibrillization. b) Pictures of HEWL unimers and HEWL fibrils upon heating at 60 °C and vigorous stirring during 24 h, pH 2.0.](image)

Then, several BDMC bearing compounds and free BDMC, for comparison, at two different concentrations (10 and 50 μM BDMC-eq.) were incubated for 24 hours with HEWL (2 mg·mL\textsuperscript{-1} solution) at 60 °C under vigorous magnetic stirring, and at low pH in order to favor amyloid aggregation. PBS solutions and the polymeric carrier were
used as positive controls. It is worth mentioning that, no fibrillation was found neither when HEWL was incubated at r.t. nor when no magnetic stirring was used. Aliquots of the fibril samples were taken at different time points and mixed with ThT aliquots for 5 minutes. Finally, fluorescence was measured in a Victor™ Wallace ($\lambda_{\text{exc}}$ 450 nm and $\lambda_{\text{em}}$ 510 nm) and background fluorescence from curcuminoid subtracted. (Figure 7.16). By this assay, it could be concluded that the polymer conjugates exhibits a fibril inhibitor behavior slightly better (although no significantly different) than free BDMC. It was also clear that, activity of the conjugates was mainly due to the presence of curcuminoid and not to the PGA chains. The use of higher concentrations (50 μM drug-eq.) did not improved the results obtained when compared with lower concentrations (10 μM drug-eq.). 10 μM BDMC-eq was selected then, as the concentration to move forward.

a)
Figure 7.16. ThT fluorescence intensity changes upon time in HEWL samples incubated with different BDMC conjugates at a) 10 µM BDMC-eq. and b) 50 µM BDMC-eq. n> 3, mean ± SEM.

These results were further confirmed by TEM, as it can be observed in Figure 7.17.

Figure 7.17. TEM pictures obtained from HEWL incubated samples within the different BDMC polyglutamate derivatives at 10 µM BDMC-eq. (c-e) in comparison with a) Control PBS and b) Free BDMC 10 µM.
7.2.3.4. Effects of X-Click-BDMC on Aβ induced neurotoxicity in hippocampal organotypic cultures.

These studies were performed in collaboration with the company Neuropharmatest S.L. who developed the organotypic cultures. The neuroprotective effect of the curcuminoid bearing polymeric structure was evaluated in organotypic cultures from entorhinal cortex-hypocampus. In order to study neuroprotection, the experimental design involved pretreatments with the conjugate prior to an Amyloid-β peptide (Aβ1-42) triggered injury. This ex vivo model has been previously validated to determine neurotoxicity and constitutes an effective manner to identify the neuroprotective effect of molecules with real therapeutic potential against AD. The organotypic cultures of slices containing both entorhinal cortex and hippocampus are an excellent ex vivo model to monitor the structure and physiology of these regions of the limbic system. They preserve the principal circuits of hippocampus, including its main excitatory input coming from the entorhinal cortex. Besides, they can be maintained for long periods of time, optimal to evaluate pharmacological activity on neurons or glial cells of the different treatments upon time. Hippocampus and entorhinal cortex are among the most affected regions in AD, accumulating a high density of extracellular deposits of Aβ peptide, and are partially responsible of the progressive memory loss and cognitive impairment observed in this neurological disorders.

Previous work has provided strong evidence that the synthetic peptide Aβ1-42 is able to induce neural injury in this type of organotypic culture. Hence, the aim was to analyze this cell damage and its putative prevention by a pretreatment with the X-Click-BDMC 4 wt% using propidium iodide (PI) staining. PI is a polar compound impermeable to intact cell membranes, but capable to penetrate damaged cells and to bind to nuclear DNA, providing a bright red fluorescence. This labeling, allow us the quantification of the density of degenerated cells in a given region. In our case, the region of interest (ROI) was the CA1 region of hippocampus (cornus ammonis 1), where several studies have found neurodegenerative effects induced by Aβ peptides.

Viability of the organotypic cultures in the presence of X-Click-BDMC and absence of Aβ peptides was firstly investigated (48 hours incubation). Slices were stained with PI, fixed and finally analyzed by
confocal microscopy according to M&M. Our polymer conjugate, up to 0.2 \( \mu M \) BDMC-equiv, did not induce significant changes in IP positive nuclei density when compared to control cultures (0.005 \( \mu M \) \( F(4,18) = 11.096, \rho = 1 \); 0.05 \( \mu M \) \( F(4,18) = 11.096, \rho = 1 \); 0.2 \( \mu M \) \( F(4,18) = 11.096, \rho = 0.41 \)). At 0.5 \( \mu M \) drug-equiv. concentration an increase in cell death was observed (\( F(4,18) = 11.096, \rho < 0.0001 \)) (See Figure 7.18).

![Figure 7.18](image)

**Figure 7.18.** Changes in density (nuclei/1000 \( \mu m^2 \)) of PI stained nuclei in pyramidal layer of CA1 region of hippocampal organotypic cultures comparing control cultures treated with vehicle and cultures treated with different concentrations of X-Click-BDMC (0.005, 0.05, 0.2 and 0.5 \( \mu M \) drug-equiv.). Asterisk indicate statistically significant differences after ANOVA analyses followed Bonferroni’s post hoc tests. \( n > 3 \), mean \( \pm \) SEM.

The concentrations of 0.05 and 0.2 \( \mu M \) drug-equiv. were then selected in order to have the maximum tolerated concentration to provide neuroprotective effects in \( A\beta_{1-42} \) treated cultures. In this case, organotypic slices were pretreated with the polymer conjugate 48 hours before \( A\beta \) cell death induction. Thereafter, cultured slices were treated with a second dose of conjugate and \( A\beta_{1-42} \) (1 \( \mu M \) final concentration). 48 hours later, cell death was quantified after staining with PI, fixation according to M&M and analysis by confocal microscopy. In this case, pretreatment with 0.2 \( \mu M \) BDMC-equiv. induced a significant increase in cell death (\( F(5,19) = 9.574, \rho = 0.006 \)) but not in the case of 0.05 \( \mu M \) BDMC-equiv. Cultures treated with \( A\beta_{1-42} \) increased cell death when compared to controls (vehicle (\( F(5,19) = 9.574, \rho = 0.0001 \)), and 0.05 \( \mu M \) drug-equiv. of polymer conjugate (\( F(5,19) = 9.574, \rho = 0.006 \)) as shown in
Figure 7.19. Pretreatment of cultures with 0.05 µM polymer conjugate ($F(5,19)= 9.574, \rho= 0.005$) or 0.2 µM ($F(5,19)= 9.574, \rho= 0.026$) before Aβ$_{1-42}$ addition induced a significant decrease in the density of PI labeled nuclei when compared with cultures treated only with Aβ$_{1-42}$ peptide. (Figure 7.19 and 7.20).

**Figure 7.19.** Changes in density (nuclei/1000 µm$^2$) of PI stained nuclei in pyramidal layer of CA1 region of hippocampal organotypic cultures comparing control cultures treated with vehicle (No polymer/No Aβ), cultures pretreated with different concentrations of polymer conjugate (0.05 µM drug-eq. (Polymer 0.05/No Aβ ) and 0.2 µM drug-eq. (Polymer 0.2/No Aβ), exposed only to Aβ$_{1-42}$ peptide (No polymer/Aβ) or exposed to Aβ$_{1-42}$ and pretreated with different concentrations of polymer conjugate (0.05 µM drug-eq. (Polymer 0.05/Aβ ) and 0.2 µM drug eq. (Polymer 0.2/Aβ)). Blue asterisk in bars indicate statistically significant differences from control group and red asterisk indicate statistically significant differences from cultures exposed only to Aβ$_{1-42}$ peptide (No polymer/Aβ), after ANOVA analyses followed by Bonferroni’s post hoc tests. n> 3, mean ± SEM.
Figure 7.20. Confocal microscopic analysis of PI staining in the pyramidal layer of the CA1 region of hippocampal organotypic cultures. A: Control cultures treated with vehicle (No polymer/ No Aβ) showing the presence of some scarce nuclei faintly labeled with PI. B: Cultures exposed only to Aβ₁₋₄₂ (No polymer/Aβ) showing abundant PI stained nuclei. C & D: Cultures exposed to Aβ₁₋₄₂ peptide and pretreated with 0.05 µM drug-eq. (Polymer 0.05/Aβ) and 0.2 µM drug-eq. (Polymer 0.2/Aβ). Density of PI is sensibly decreased in these conditions. All the microphotographs in this figure are single confocal planes. Scale bar: 25 μm.

Overall, the construct bearing BDMC tested in organotypic cultures shows no toxicity after 48 hours of treatment at the different concentrations tested, except for 0.5 µM concentration. When repeated doses were applied (in the case of the pretreatment experiment), the 0.2 µM concentration resulted toxic for the non Aβ₁₋₄₂ peptide treated cultures, however, this concentration was effective for Aβ₁₋₄₂ toxicity prevention. Pretreatment with polymer conjugate at either 0.05 or 0.2 µM of drug-eq. significantly reduced cell death in Aβ₁₋₄₂ peptide treated cultures. As 0.05 µM concentration resulted enough to produce significant neuroprotective effects against Aβ₁₋₄₂ neurotoxicity without being toxic, this concentration was selected to move forwards. Further experiments are ongoing in order to identify the possible mechanisms of neuroprotection followed by our constructs.
7.2.4. X-Click-DO3A-Gd-Cy5.5-BDMC-ANG as the selected theranostic agent. From synthesis to in vivo biodistribution studies.

Taking into account the results obtained in the biodistribution experiments with the clicked architectures as well as the potential of the BDMC conjugates to treat AD pathology, the design of a final construct combining all those features was planned as the next step. Thus, synthesis of a new architecture including targeting units (ANG), labeling agents (Cy5.5 and DO3A-Gd) and therapeutic agents (propargyl moieties and BDMC), based on a bottom-up approach through covalent capture of self-assembled structures was carried out. In this way, a targeted architecture with potential theranostic applications was built up.

Synthetic wise, first of all, the X-Click-BDMC conjugate was obtained as previously described by achieving 2.1 mol% of BDMC loading according to UV-Vis quantification at 415 nm with a free drug content < 1 wt% of the total drug. DO3A-NH$_2$ cysteamine-2TP and Cy5.5 were subsequently introduced following previously described protocols for synthesis and purifications (see Chapter 6). Finally ANG-2-SH was effectively conjugated via disulfide bonds. The final conjugate was purified by SEC using Sephadex commercial PD10 columns. The fractions eluted where monitored by absorbance at 280 nm in Nanodrop® in order to identify those corresponding to the targeted conjugates. Peptide loading was calculated according to $^1$H-NMR and UV-Vis via calibration curve at 280 nm achieving 75 % conjugation efficiency. Gd$^{3+}$ was complexed in the last step yielding to compound 59 (Figure 7.21, Table 7.5.).
**Figure 7.21.** Schematic representation of the synthetic route followed in the synthesis of targeted dually labeled X-Click-BDMC (59) for theranostic purposes. a) 1) DMTMM∙Cl, 2) DO3AtBu-NH$_2$ in ddH$_2$O, r.t. 24 h. b) 1) DMTMM∙Cl, 2) Cysteamine-SS2TP in ddH$_2$O, r.t. 24 h. c) 1) DMTMM∙Cl, 2) Cy5.5 (6S-IDCC) in ddH$_2$O, r.t. 24 h. d) ANG in HEPES buffer 7.4, r.t. 16 h and e) GdCl$_3^+$ in PBS 0.1M 7.4, r.t. 5 h.

**Table 7.5.** Physico-chemical characteristics of Conjugate 59.

<table>
<thead>
<tr>
<th>Compound</th>
<th>mol% GAU/wt% DO3A</th>
<th>wt% Gd</th>
<th>mol% GAU/wt% Cy5.5</th>
<th>mol% GAU/wt% ANG</th>
<th>mol% GAU/wt% BDMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-Click-DO3A-Gd-Cy5.5-BDMC-ANG (59)</td>
<td>10/ 16.3</td>
<td>9.6</td>
<td>0.95/ 4.7</td>
<td>1.5/ 12.6</td>
<td>2.1/ 2.5</td>
</tr>
</tbody>
</table>
Conjugate size was investigated by TEM microscopy as shown in Figure 7.22. Bigger sizes than for the previous clicked structures were found (100-200 nm) as it could be expected from the high degree of surface modification.

![TEM micrographs of X-Click-DO3A-Gd-Cy5.5-BDMC-ANG (59).](image)

As for the other clicked structures evaluated, biodistribution experiments were carried out using C57Bl/6 mice and optical imaging techniques taking profit from the presence of Cy5.5 dye. The conjugate was administered i.v. through tail vein to isofluorane anesthetized mice, at a dose of 4.15 mg·Kg⁻¹ Cy5.5 eq. Two animals were then sacrificed at different time points (1, 3, 7, 14 and 24 h). Again, before sacrificed, mice were first anesthetized with a lethal anesthesia cocktail, blood was extracted from the cava vein, and perfusion with 10 mL of saline was carried out in order to accurately determine the amount of compound in brain. Organs were then extracted and their fluorescence was measured using the red filter in MAESTRO™. For fluorescence quantification, normalized data was obtained by taking always the same pixel area for all organs and it was expressed as average signal (counts·s⁻¹). % ID was calculated by using a prior calibration curve of the compound. Data obtained has been summarized in Figures 7.23 and 7.24.
Figure 7.23. % ID normalized by pixel area of X-Click-DO3A-Gd-Cy5.5-BDMC-ANG.
The biodistribution profile was comparable to those obtained for the other clicked architectures (45 and 50, both without BDMC). Renal excretion profiles were again observed, and lung accumulation at the early time points highlights the bigger size of the structures. Although no signs of toxicity were observed, further nanotoxicological evaluation is being carried out in order to fully demonstrate the safety of these constructs (biochemical analysis, pathological analysis, etc.).

![Biodistribution at different time points of X-Click-DO3A-Gd-Cy5.5-BDMC-Ang.](image)

**Figure 7.24.** Biodistribution at different time points of X-Click-DO3A-Gd-Cy5.5-BDMC-Ang.

In the brain, ANG bearing compound offered higher accumulation at the early time points, and slow clearance at longer time points. Once more, the amount found in brain for these clicked architectures is 20-25 times greater than the one obtained for the unclicked stars (0.05 % of the ID), being around 1.2 % ID in this case.
Figure 7.25 shows the organ fluorescence obtained at the different time points in the evaluation of compound 59.

![Figure 7.25. C57B1/6 mouse organ fluorescence images obtained at MAESTRO™ upon treatment with X-Click-DO3A-Gd-Cy5.5-BDMC-Ang.](image)

7.2.5. In vivo activity.

7.2.5.1. Initial Aβ plaque burden establishment.

After biodistribution results, a preliminary study to achieve proof of concept of pharmacological activity in vivo was designed. For that purpose, the mouse strain ArcAbeta was used as Alzheimer’s mouse in vivo model. As our idea is to tackle the disease from a neuroprotective point of view, young animals (from 8-11 months) were chosen. Since this mouse model starts to accumulate plaque burden at around 6-9 months of age, excessive and irreversible amounts of Aβ plaques will not be present. All experiments carried out with the ArcAbeta model were performed at Prof. Rudin’s group (ETH-Zurich) through a MINECO-funded research stay.

As AD mouse models usually present a significant variability among the number and stage of plaques, a first experiment was designed in order to assess plaque burden on each specific animal. For that purpose, the well-known Aβ plaques binding tracer AOI987 was used.\(^\text{121}\) Hintersteiner et al. established that the difference of the normalized signal intensities \(I_{\text{rel}}\) between transgenic and non-transgenic mice was highest for a dosage of 0.1 mg·Kg\(^{-1}\) at 3-4 hours after injection, and dye was completely washed out 24 hours after i.v.
Thus, animals were first head saved and fluorescence before and 3 hours after the injection of 0.1 mg·Kg$^{-1}$ of AOI987 was measured at Maestro™. Differences among both measurements provide an idea about plaque burden before starting the treatments. Figure 7.26 shows the results.

![Figure 7.26](image)

**Figure 7.26.** Graphic representation of the difference of fluorescence intensities (average signal in scale counts) registered before and after AOI987 injection for each ArcAbeta animal related to plaque burden. Measurements were register in the spectral section between 660-800 nm. Yellow bars belong to the animals that were treated afterwards with saline, and green bars show the animal group that was treated with compound (59).

In theory, this previous gained data could be used as internal standards for each mouse in order to reduce the high variability inherent from the animal model. This could be done by measuring plaque burden using the same method at the end of the experiment.

### 7.2.5.2. Animal treatments.

Firstly, *in vivo* safety is a go/no go step for any tested compound in order to proceed with its validation. Therefore, a pilot study with 59 was designed with a dose schedule selected based on PK studies. In this first experiment, animal weight was monitored and animal brains were analyzed looking for signs of pharmacological activity. Three different groups of animals were chosen: wild type animals as control (x2), ArcAbeta animals used as non-treated controls injected with saline (x7) and ArcAbeta animals treated with compound 59 at a comparable dose as that used in the biodistribution studies (2
mg·Kg$^{-1}$ BDMC eq.) (x7). Although the selected dose could be considered low by comparing with different curcuminoid treatments found in literature, taking into account that 1 % reaches the brain (Figure 7.24), it would be enough to trigger therapeutic effects.

Animals were injected six times within two weeks without showing signs of toxicity as it is depicted in Figure 7.27 where no weight loss of the treated animals was observed.

![Animal weight as toxicity control](image)

**Figure 7.27.** Experimental design from the treatments performed in ArcAbeta model together with the animal weight registration as a proof of treatment safety.

### 7.2.5.3. Final plaque burden establishment.

As previously done for initial plaque burden, the AOI987 was planned to be injected i.v. through vein tail in order to determine final plaque burden after treatments. Nonetheless, the experiment could not be completed. Animals treated showed a huge signal in the head in the spectral range of AOI987 fluorescence due to Cy5.5 signal coming from compound 59, even before AOI987 injection. To check if this signal was due to compound circulation and not by brain accumulation, blood was collected and also measured at Maestro™ just before animal
killing. Levels in blood were comparable those observed at 24 hours in the biodistribution study (same dose used in both experiments). Thus, confirming a real accumulation of the compound in the brain region.

Figure 7.28. Blood levels from compound (59) measured by fluorescence registered in the spectral range of 660-800 nm wavelengths, of ArcAbeta treated animals, 24 hours after experimental design (day 15). “Biod” refers to the equivalent amount found at 24 hours post-administration in the biodistribution study. The different numbers refer to the individual treated mouse.

Animals were killed 24 hours after the last injection and brains were cryo-preserved for histological analysis in order to alternatively analyze performance.

Further evidence of brain accumulation was obtained when fixed cryo-sections of 10 μm were scanned using the Slide Scanner Pannoramic 250 (3D Histech) (Figure 7.29). Nevertheless, although there is a clear evidence of compound 59 presence in brain after reperfusion, further studies must be done in order to confirm whether these spots are located inside the brain or just attached to brain vasculature. In the worst scenario, as curcuminoids are described to cross the BBB, accumulation in brain vasculature will allow to increase compound 59 concentration in brain areas where BDMC can be released and enter the brain to perform its biological output.
As AOI987 tracer could not provide the expected output from the \textit{in vivo} activity due to the masking problem arose by the significant accumulation of 59 in mice heads, histological analysis was carried out. To that aim, brain slices from samples of treated and untreated ArcAbeta mice, as well as control wild types were processed. 10 µm thickness slices were fixed using paraformaldehyde 4% and subsequently incubated with the monoclonal antibodies mouse anti-Aβ, as well as rat anti-mouse F4/80 antibody for microglia activation detection. Staining with the secondary antibodies mouse Alexa 488 and Rat Alexa 594 allowed the identification of small and few amyloid plaques and microglia activation mainly in the surroundings of such deposits. An example of amyloid plaque detection and microglia activation after staining optimizations can be found in Figure 7.30.
Figure 7.30. Example of staining of half-brain cross-sections from untreated ArcAbeta mice. In green, Aβ deposits detected using 1:1000 dilution of primary mouse monoclonal antibody Beta Amyloid, 1-16 (6E10) stained with secondary mouse Alexa 488. In red, microglia activation detected using 1:500 dilution of rat anti mouse monoclonal antibody F4/80, stained with secondary rat anti-mouse Alexa 594. In yellow, merge.

Although the analysis of these brains is still ongoing including histological analysis as well as TEM micrographs, unfortunately up to now, no determinant proof of pharmacological activity can be drawn.

On one hand, ArcAbeta model itself encompasses a high variation in plaque development including variation in the number of plaques as well as variation in the age where process starts. Furthermore, variations according to the gender were also found. In our specific case, most of the male mice did not present any detectable plaque by immunohistochemistry (regardless treatment) whereas females presented a highly variable number of plaques. At the experiment design, we aimed to overcome such variations by using each animal as its own control with the aid of the AOI987 tracer. However, that was not possible due to the high signal found in mice heads after treatments. With toxicity issues already addressed, next steps will include the design of a proper in vivo activity study. Such experiment will be based on the long-term treatment of a reasonable number of young Alzheimer’s bearing mice for neuroprotection with all of them having exactly the same age and same gender (females) in
order to diminish variations. Long-term experiments will provide a more realistic scenario of the disease and more valuable conclusions regarding the potential of polymer therapeutics could be drawn from there.

7.3. CONCLUSIONS.

In this work, PT, concretely, polymer-drug conjugates for the treatment of AD have been synthesized and properly characterized. According to previous results obtained in Chapter 6 in terms of biodistribution profiles, the bigger covalently captured structures were preferred in order to promote brain accumulation, necessary to achieve effective treatments for CNS disorders. Curcuminoids, concretely BDMC was chosen as a model drug due to its already demonstrated activity and potential for the treatment of AD. In combination with propargylamine moieties (in clinical studies in AD patients), BDMC was conjugated to the clicked structures via ester bonding. In vitro performance of the developed constructs was evaluated in terms of cell viability, drug release profiles, and in vitro activity as inhibitors of fibril formation. Furthermore, when tested in organotypic cultures, pretreatment with either 1 or 5 µM BDMC-eq. significantly reduced cell death in Aβ1-42 peptide treated cultures. In vitro results, together with the biodistribution of BDMC-bearing clicked structure by optical imaging highlighted the potential of these nanomedicines for AD treatments. Furthermore, compound in vivo safety was demonstrated, and a great accumulation in brain surroundings was encountered upon treatment. Considering the preliminary results obtained, further ex vivo investigation considering other pathological hallmarks of the disease where BDMC could be effective are being explored and a proper in vivo activity experiment is planned in order to unravel the full potential of these systems as nanotherapeutics for neurological disorders.
7.4. MATERIALS AND METHODS.

7.4.1. Materials.

All chemicals were reagent grade, obtained from Aldrich and used without further purification, unless otherwise indicated. All solvents were of analytical grade and were dried and freshly distilled. Deuterated chloroform-$d_1$, DMSO-$d_6$, and D$_2$O were purchased from Deutero GmbH. X-Click architectures were synthesized according to conditions described in Chapter 5. DO3AtBu was purchased from Chematec. Cy5.5 (6S-IDCC) was obtained from Mivenion. Angiopep2-cystein Ac-FFYGGSRGKRNFKTEEEYC was obtained from Selleck Chemicals LLC. Bisdemethoxycurcumin was obtained from TCI chemicals. Glucose was obtained from MERCK, L-glutamin in GBSS was purchased from Sigma-Aldrich, Optimem-1 was obtained from GIBCO, Aβ$_{1-42}$ was purchased from TOCRIS. CellMaskOrange® was obtained from Molecular-Probes Life Technologies. Beta amyloid 1-16 (6E10) Monoclonal Antibody was purchased from Covance. Rat anti mouse F4/80 was purchased from AbD Serotec. Anti-Fluorescein/Oregon Green Goat polyclonal IgG Alexa Fluor 488 were purchased from Invitrogen. Preparative SEC was performed using Sephadex G-25 superfine from GE as well as PD MiniTrap G-10™ columns containing 2.1 mL of Sephadex™ G-10. Dialysis was performed in a Millipore ultrafiltration device fitted with a 3, or 5 kDa MWCO regenerated cellulose membrane (Vivaspin®).

Animals: Transgenic ArcAbeta mice and wild type littermates (C57Bl/6 mice) of either gender (Division of Psychiatry Research, University of Zurich) were used. Animals were kept at standard housing conditions: light/dark cycle of 12 hours, temperature of 20-24 °C, relative humidity minimum 40 %, with free access to food and water. All experiments were performed in accordance with the Swiss Federal Act on Animal Protection.

7.4.2. Characterization techniques.

The equipment used for characterization within this chapter is already described in previous ones. See Chapter 2 for NMR and GPC; Chapter 3 for Victor™ Wallace; Chapter 5 for TEM microscopy; Chapter 6 for Nanodrop ® and Maestro™.
7.4.3. Protocols.

7.4.3.1. Bisdemethoxycurcumin (BDMC) conjugation to PGA-based polymers.

Conjugation of BDMC was carried out using DMTMM-BF$_4$ protocol described in Chapter 4 with slight modifications. Briefly, in a two-necked round bottom flask, fitted with a stirrer bar and two septums, the corresponding polymer was dissolved in 10 mL of anh. DMF under nitrogen atmosphere. After that, 1.5 eq. of DMTMM-BF$_4$ of the desired percentage of GAU modification was added in 5 mL more of anh. DMF. Reaction was left to proceed for 10 minutes. Then, 1.5 eq. of the desired percentage of GAU modification were added to the reaction mixture, followed by a catalytic amount of DMAP. The pH was checked to be around 7. Reaction was then left to proceed for 72 hours. For purification, the mixture was poured into a large excess of diethyl ether. After isolation, the yellowish solid was converted into sodium salt form by careful addition of NaHCO$_3$ 1 M. Then, the aqueous solution was washed with diethyl ether till no yellowish coloration was found in the organic phase. Finally, the product in aqueous phase was purified by dialysis using Vivaspin® MWCO 5000, and freeze-dried. BDMC contain was determined by UV-VIS at 415 using a calibration curve with free BDMC. FDC was estimated by HPLC following the method: eluent A was ddH$_2$O and eluent B was acetonitrile. Samples were analyzed using the following gradient: from 40 % B to 80 % B over 20 min using Lichrospher 100 RP 18, 5.0 µm (dimension: length x ID)= 125 x 4.0 mm). BDMC retention time (tr) 5.98 minutes. Experiments were done per triplicate. % of free drug was established by performing a calibration curve with BDMC dissolved in the mixture ddH$_2$O/Acetonitrile (50/50) and injected under the same HPLC conditions.

7.4.3.2. Synthesis of X-Click-DO3A-Gd-Cy5.5-BDMC-ANG (59).

Starting from X-Click-BDMC (2.1 mol% GAU loading of BDMC) sodium salt, all the methods developed for the synthesis of 59 are described in M&M of Chapter 6. Firstly, DO3A-NH$_2$ was conjugated following the already described DMTMM-Cl protocol for reaction conditions. The product was purified by dialysis using Vivaspin® MWCO 5000 and DO3A-NH$_2$ content was estimated as 10 % GAU according to $^1$H-NMR and further confirmed after Gd
complexation. After that, the isolated product was then modified with cysteamine-2TP following the same procedure for conjugation and purification as for DO3A-NH₂. Cysteamine-2TP content was estimated as 10 % GAU by ¹H-NMR in D₂O according to the corresponding aromatic signals of 2TP group. Next, Cy5.5-NH₂ was then conjugated to the already synthesized construct using the same DMTMM·Cl protocol for reaction conditions. Purification was also performed by dialysis using Vivaspin® MWCO 5000, and the Cy5.5 content was determined by fluorescence at λ_{exc}: 595 nm, λ_{emi}: 680 nm as 0.95 mol% of GAU. ANG-2-SH was effectively conjugated via disulfide bonds in HEPES buffer at pH 7.4 over 16 hours. The final conjugate was purified by SEC using Sephadex commercial PD10 columns. The fractions eluted where monitored by absorbance at 280 nm in Nanodrop® in order to identify those corresponding to the targeted conjugates. Peptide loading was calculated according to ¹H-NMR and UV-VIS via calibration curve at 280 nm achieving 1.5 mol% of GAU (75 % conjugation efficiency). Finally, Gd³⁺ was complexed in the last step by following the protocol of Chapter 6, in PBS 0.1 M yielding to compound 59.

7.4.3.3. Cytotoxicity assessment of BDMC-conjugates.

SHSY5Y cell line was seeded in sterile 96-well microtitre plates at a cell density of 35000 cell·cm⁻². Plates were incubated for 24 hours and BDMC bearing compounds (0.2 μm filter sterilized) were then added to give a final concentration range of 0-15 μM eq. BDMC. After 72 hours of incubation, MTS/PMS (20:1) (10 μL of manufacturer solution) was added to each well, and the cells were incubated for a further 2 hours. Optical density of each well was measured at 490 nm. Plates were read spectrophotometrically using a Victor™ Wallac plate reader. The absorbance values were represented as percentage of cell viability taken as 100 % cell viability of untreated control cells.

7.4.3.4. Drug Release Kinetics.

Drug release kinetics of X-Click-BDMC 4 % were performed at three different pHs (5.0, 6.5 and 7.4). In all cases, as BDMC might precipitate in the aqueous solution upon degradation, a stock solution was prepared, and from that one, aliquots of 100 μL containing the same mg of polymers were prepared at the beginning of the experiment. Hence, the whole aliquot will be taken for HPLC analysis, avoiding the
loss of precipitated product. Briefly, X-Click-BDMC 4 wt% was dissolved at 4 mg·mL\(^{-1}\) in PBS buffers at different pH and subsequently aliquoted. Samples were incubated at 37 °C and freeze-dried at different time points, re-suspended in 100 µL of the mixture ddH\(_2\)O/Acetonitrile (50/50) and injected in HPLC following the method: eluent A was ddH\(_2\)O and eluent B was acetonitrile. Samples were analyzed using the following gradient: from 40 % B to 80 % B over 20 min using Lichrospher 100 RP 18, 5.0 µm (dimension: length x ID= 125 x 4.0 mm). BDMC retention time (tr) 5.98 minutes. Experiments were done per triplicate. % of drug released was established by performing a calibration curve with BDMC dissolved in the mixture ddH\(_2\)O/Acetonitrile (50/50) and injected under the same conditions as for the degradation samples.

7.4.3.5. Prevention of HEWL Lysozyme fibril formation. Thioflavin T fluorescence assay.

HEWL fibrils were formed using 2 mg·mL\(^{-1}\) Hen Egg White Lysozyme in acidic buffer 12 Mm HCl containing 140 nM NaCl and 2.7 mM KCl (pH 2). Samples were magnetically stirred at 60 °C up to 24 hours for fibril formation. Kinetics of fibril formation was followed from time zero by ThT-based titration method. Typically, a 100 µM solution of ThT was prepared by dissolving ThT powder in PBS pH 7.4. Aliquots of 20 µL of HEWL solution were collected at time intervals and mixed with 100 µL of ThT solution in a dark 96-well plate. Samples were left to stabilize for 5 minutes and fluorescence was measured at 590 nm in the plate reader Victor\(^{TM}\) Wallace. For compounds evaluation in terms of prevention of fibril formation, those fibrils were incubated with 10 or 50 µM BDMC eq. of the compounds dissolved in a minimum volume of PBS buffer 7.4. In control samples, the same amount of PBS buffer without compound was added. Free BDMC was added dissolved in a minimum amount of ethanol (maximum ethanol percentage 1 % in the final volume).

7.4.3.6. Prevention of neurotoxicity induced by A\(_{\beta}\)\(_{1-42}\) in organotypic cultures.

7.4.3.6.1. Organotypic hippocampal slice cultures.

Mice pups (B6.Cg-Tg(Thy1-YFP)HJrs/J) were used to obtain transverse hippocampal organotypic slice cultures with a McIlwain\(^{TM}\) Tissue Chopper (350 µM thick).\(^{117}\) Briefly, brains were placed into
Petri dishes filled with cold (4 °C) sterile dissecting medium (1 % glucose (0.5 g·mL\(^{-1}\)), 0.2 % penicillin/streptomycin, 0.5 % L-glutamin in GBSS. The overlying pia was gently removed and coronal cuts were made to remove portions of the rostral and caudal poles, leaving the frontoparietal region intact. The right and left cortices were cut simultaneously in the coronal plane at a thickness of 350 µM using McIwain\textsuperscript{TM} Tissue Chopper. Slices obtained were transferred into dissecting medium and separated gently by agitation. Slices containing hippocampus and entorhinal cortex were placed on moistened translucent membranes of tissue culture inserts (0.4 µM, Millicel-CM, Millipore, Bedford, MA. USA) and immersed in 1 mL of Serum-OPTIMEM culture medium (25 % heat inactivated horse serum, 25 % HBSS, 50 % Optimem-1 supplemented with 10 µL·mL\(^{-1}\) glucose. Three slices were cultured in the same insert and six inserts were placed together in 6-well plates. To ensure that slices from control and treated groups were cultured under identical conditions, three inserts from each place were designated as control group and the other three as treated groups. Cultures were stored in a humid atmosphere at 37 °C in 5 % CO\(_2\) for 16 days (HERAcell\textsuperscript{®} 150i, Thermo Scientific) and medium was changed three times per week, always replacing half of it (0.5 mL out of 1).

7.4.3.6.2. Organotypic culture viability assay upon X-Click-BDMC treatments.

The polymer conjugate was dissolved in ddH\(_2\)O and added to the culture media at different concentrations at day 16\textsuperscript{th} of organotypic culturing. Compounds were incubated within the cultures for 48 hours. After that, tissue cultures were stained with PI (10 µM) for 10 minutes, washed with PBS (x3) and fixed with paraformaldehyde (PFA) 4 %. Tissue samples were again washed with PBS (x3), let to dry, and mounted using DAKO-glycergel media containing DAPI. Cell damage was assessed by fluorescent image analysis of PI uptake. Cultures were observed with an upright confocal microscope (Leica TSE). Images were captured and analyzed using FIJI Image Software. After capture of images, the density of PI nuclei was analyzed in the pyramidal layer of CA1. For each experimental group, mean ± SEM was determined and the resulting values analyzed by one-way ANOVA with the number of slices as the “n”. Significant effects were further analyzed by
Bonferroni *post-hoc* test, using the IBM SPSS statistics software (version 22).

7.4.3.6.3. Aβ1-42 neurotoxicity induction and prevention by using X-Click-BDMC.

For the establishment of Aβ1-42 toxicity induction, 1 mg of Aβ1-42 peptide was dissolved in ddH2O to prepare a stock solution of Aβ1-42 (0.5 mM) that was stored at -20 °C. Prior to use, Aβ1-42 was aggregated at 37 °C for 72 hours. In order to establish neurotoxicity inducement, on the 16th day of the organotypic culture, Aβ1-42 peptide was added by replacing half of the media, obtaining a final concentration of 1 µM. In the case of pretreated cultures, (controls with and without Aβ1-42), the polymer conjugated dissolved in ddH2O was added at different concentrations (0.05 and 0.2 µM) at 14th (prevention) and 16th (maintenance) days. After that, tissue cultures were stained with PI (10 µM) for 10 minutes, washed with PBS (x3) and fixed with paraformaldehyde (PFA) 4 %. Tissue samples were again washed with PBS (x3), let to dry, and mounted using DAKO-glycergel media containing DAPI. Cell damage and statistical analysis were carried out as for the previous epigraph.

7.4.3.7. *In vivo* Biodistribution.

*In vivo* biodistribution studies were performed as for Chapter 6, section 6.4.3.7.

7.4.3.8. Plaque burden determination using AOI987 tracer.

Transgenic ArcAbeta mice and wild type littermates (C57Bl/6 mice) were shaved in the head for the study. Then, mice were anesthetized using 1.5 % of isofluorane and fluorescence was recorded as background prior to tracer injection at Maestro™ at the spectral range 660-800 nm. For this, mice were transferred to a pre-warmed (37 °C) platform in the small animal imaging system (CriMaestro 500), with maintained anesthesia using an anesthesia mask. Then, AOI987 tracer was injected i.v. (0.1 mg·Kg⁻¹). Three hours after injection, animals were again anesthetized using 1.5 % isofluorane and fluorescence was again recorded. For fluorescence quantification, normalized data was obtained by taking always the same pixel area for all of the organs expressed as average signal (counts·s⁻¹). (See section 6.4.2.2)
7.4.3.9. In vivo preliminary activity study.

Transgenic ArcAbeta mice and wild type littermates as controls (C57Bl/6 mice) were used. Animals weight was 27.5 ± 5.5 g. Mice were anesthetized with 1.5 % isoflurane. Compound 59 was administered i.v. through the vein tail via cannula, at a dose of 2 mg·Kg⁻¹ BDMC eq. six times during two weeks (see Figure 7.27). ArcAbeta control animals were injected with equivalent volumes of saline. 24 hours after the last injection, animals were sacrificed. Blood samples were extracted from the cava vein of the anesthetized mice with a lethal anesthesia cocktail (i.e. for 20 g mice 200 µL from the solution containing ketamin 100 mg·Kg⁻¹, Xylasin 100 mg·Kg⁻¹, Acepromacide 2 mg·Kg⁻¹). Blood fluorescence was registered at Maestro™ following previous described protocols in Chapter 6. Perfusion with 10 mL of saline was then performed. Brains were extracted immediately after and frozen for histological analysis according to Section 7.4.3.10.1.

7.4.3.10. Histology.
7.4.3.10.1. Tissue freezing and conservation.

Immediately after animal sacrifice, perfused brains were extracted and frozen embedded in OCT (Tissue-Teck®; Sakura Finetek USA) at -40 °C using 2-methylbutane and liquid nitrogen. Cryostat NX70 (Thermo Scientific) was employed for brain sectioning. Coronal frontal sections of 10 µM were cut from previously frozen in OCT brains. When cut, sections were transferred to a microscope slide by touching the slide to the tissue and preserved at -80 °C till used for histology processes.

7.4.3.10.2. Brain sections fixing and immunohistochemistry protocols.

a) Brain fixing and staining for Compound 59 accumulation determination.

Brain sections were left drying for 16 hours prior fixation. A border surrounding brain slices was made using a lipid pen. Then, the microscope slide containing brain sections were placed into a wet chamber and fixed with paraformaldehyde 4 % over 10 minutes. After that, samples were washed three times with PBS 1X for 10 minutes 3 times. Next, samples were stained with a dilution 1:50 in PBS 1X, from the commercial recommendation (1.5X) of CellMaskOrange™ a
specific membrane marker, for 30 minutes protected from light. Afterwards, samples were again washed with PBS 1X for 10 minutes 3 times. Finally, samples were mounted with DAKO glycergel mounting medium and covered with cover slips.

b) Brain fixing and staining for plaque burden determination combined with macrophage activation markers.

Brain sections were left drying for 16 hours prior fixation. A border surrounding brain slices was made using a lipid pen. Then, the microscope slide containing brain sections were placed into a wet chamber and fixed with paraformaldehyde 4 % over 10 minutes. After that, samples were washed three times with PBS 1X for 5 minutes. Next, samples were incubated with a mixture of two antibodies in PBS containing 1:1000 of primary mouse monoclonal antibody Beta Amyloid, 1-16 (6E10), 1:500 rat anti mouse monoclonal antibody F4/80, 1:100 Normal Goat Serum (NGS) and 1:100 Triton. Samples were left slowly shaking overnight at 4 °C. After that tissues were washed with PBS 1X 3 times for 10 minutes, and the stained with the secondary antibodies for each primary one at dilutions 1:1000 for each antibody (mouse 488 and rat 594) with 1:100 NGS and 1:100 triton. Samples were left at r.t. slowly stirring for 1 hour. Then, slides were washed with PBS 1X for 10 minutes three times. Finally, samples were let dry, mounted using DAKO glycergel mounting medium and covered with cover slips.
7.5. REFERENCES.


Chapter 8

Light based *in vivo* imaging of disease related-enzymes with polyglutamate-based molecular probes. A possible neuroimaging approach in preclinical models.
8.1. INTRODUCTION AND BACKGROUND.

8.1.1. Exposing the need for early diagnosis.

This additional chapter has been developed on the frame of a European Project based on the development of molecular diagnosis probes that could be redirected in the frame of this thesis towards neuroimaging, possibility currently being explored with some of the developed probes.

LiVIMODE (Light-based Functional In Vivo Monitoring of Disease-related Enzymes) was a European research consortium within FP7 with scientists from universities, research organizations, and a global healthcare leader, Sanofi-Aventis.

Structural tissue changes are commonly late indicators of a pathological cascade. Understanding the molecular processes in living cells during the formation and progression of diseases such as cancer, rheumatoid arthritis and osteoarthritis is crucial for the development of new diagnostic tools and treatments. Non-invasive visualization of unique molecular processes behind human pathology (or models) would provide highly specific and potentially early indicators of ongoing diseases. These molecular processes are, i.e., up regulation or activation of specific disease related factors, in particular enzymes such as proteases like metalloproteases (MMPs) or cathepsins.

In this context, LiVIMODE main aim was the development of novel powerful imaging agents, which were intended for the direct visualization and investigation of disease-related molecular processes in living cells and in diagnostic imaging. The efforts were mainly focused on highly sensitive optical imaging methods, which allow such direct visualization of specific molecular events (disease-related enzymes activity) in the living cell. The overall objective of LiVIMODE was thus to develop an in vivo imaging platform based on protease-activated smart ligands that allow non-invasive quantitative assessment of target expression in diseased tissue (diagnosis) and monitoring of disease progression (staging). These tools are of high relevance for disease detection, monitoring of disease progression, developing animal models of human disease and evaluating novel therapies.

Imaging of specific molecular events puts high demands on the sensitivity of the imaging technique, therefore the main methods of interest for this task are nuclear and optical imaging methods, in
particular those in the near-infrared spectral domain, capable of sensing nano- and femtomolar concentrations of analytes in vivo. Hence, optical imaging was proposed within this consortium, which is optimally suited for this task as it provides excellent sensitivity and allows visualizing of molecular events via fluorescence signal. Moreover, significant progress has been recently made in the area of optical imaging technology, enabling a three-dimensional quantitative mapping of fluorophore distribution within a turbid medium (tissue). Non-invasive quantitative imaging of disease-specific molecular markers has the potential to overcome the limitations of the currently available imaging technologies such as X-ray-CT, MRI. Conventional imaging methods, such as angiography, CT, MRI, and radionuclide imaging, rely on contrast agents (i.e. iodine, gadolinium, and radioisotopes) that are “always on”. Although these indicators have proven clinically useful, they lack of sensitivity due to inadequate target-to-background signal ratio. A unique aspect of optical imaging is that fluorescence probes can be designed to be activatable, that is, only “turned on” under certain conditions. This feature of “turning on” only at the target has been termed as activatable or “smart”.

However, while optical imaging technology has rapidly evolved over the last years, the availability of smart imaging agents that change their fluorescence properties upon disease related events remains a challenge. To date, activatable protease agents for optical imaging have already been successfully applied to visualize primary tumors and metastases, inflammatory processes in active atherosclerotic plaques, degenerative joint diseases and pulmonary inflammation among other applications. Nonetheless, the most critical gap in molecular imaging approaches is the availability of target-specific and tissue-specific imaging probes. Nevertheless this first generation of activatable imaging agents tend to suffer from insufficient selectivity for the targeted disease-related molecular event, and from suboptimal pharmacokinetic properties. Thus, the purpose of this project was to overcome this gap by providing target specificity as well as tissue specificity.

Among disease-related factors, proteases are a class of enzymes usually highly up-regulated. The combination of their high disease-specific expression levels with their ability to cleave substrates at high turnover rate make them attractive targets for imaging probes. On the frame of this project, targets for the smart probes were selected from
proteolytic enzymes with the aim of enabling the monitoring of critical processes occurring in tumor progression, inflammation and degenerative joint diseases. In all these cases, symptomatic complaints of patients are often made at a late-stage and effective treatments may no longer be available. For that reason, it is crucial to be able to monitor disease progression (and treatment) at the early stages in order to decide suitable treatment options, dosage schedules or even drug combinations. Molecular mechanisms that underlay those diseases are different, however, it has been clearly shown that proteases play key roles in early and progressive stages of all of them.\textsuperscript{19-22} Proteases cleave biologically important molecules with a high turnover rate, in a specific manner and affect the disease progression by changing cellular environments. Moreover, many of them are elevated and localized in the lesions making them the perfect targets for this technology, where non-invasiveness is pursued. Hence, herein, selective chemical probes cleavable by locally activatable proteases (smart probes) leading to signal changes for diagnosis were pursued.

As mentioned at the beginning of the chapter, although the target-diseases are those listed before, this approach could be used for the development of smart-activatable probes for the early diagnose of neurodegenerative diseases as well (particularly MMPs 2, 9, 12 and 13, as well as Cathepsin B for AD diagnosis).\textsuperscript{23-31}

### 8.1.2. Proteases of interest.

#### 8.1.2.1. Monitoring tumor microenvironment, angiogenesis and hypoxia.

Recent approaches for cancer therapy target tumor microenvironment in contrast to classical chemotherapeutic agents that target cancer cells. That communication within the tumor microenvironment is mainly mediated by proteases. During cancer progression, some proteases have been found to be overexpressed disturbing the normal balance between proteases and their endogenous inhibitors and leading to the activation of downstream effector molecules. The following examples of proteases are well studied and it is well known that they are overexpressed in cells or interstitium of the tumor microenvironment, as well as their role in tumor development or treatment. Therefore, that makes them ideally suited for activation of smart probes.
Tumor-associated fibroblasts (TAFS) are involved in carcinoma cell invasion and increase the tumor interstitial fluid pressure, decreasing delivery of anti-cancer drugs.\textsuperscript{32-34} FAP fibroblast activation protein, is his inhibitor and is highly expressed in tumor-associated fibroblasts.\textsuperscript{35, 36} Therefore, a probe detecting FAP protease activity could be used to detect the presence of tumor-associated fibroblasts.\textsuperscript{37} Some metalloproteases have a great potential as diagnostic biomarkers and are highly overexpressed in various tumors,\textsuperscript{21, 38-40} as well as cysteine cathepsins that contribute to tumor angiogenesis, inflammation, metastasis spread and tumor cell apoptosis.\textsuperscript{41-44} Cysteine proteases caspases play a major role in cancer cell apoptosis.\textsuperscript{45-48} Table 8.1 summarizes the opportunities to develop smart activatable probes based on the activity of the mentioned proteases.

**Table 8.1. Proteases of interest for cancer and joint diseases.**

<table>
<thead>
<tr>
<th>Protease</th>
<th>Protease Class</th>
<th>Cancer</th>
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<tr>
<td>ADAMTS-4</td>
<td>Metallo</td>
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<td>+</td>
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<tr>
<td>ADAMTS-5</td>
<td>Metallo</td>
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<td>Metallo</td>
<td>+/?</td>
<td>+/-</td>
</tr>
</tbody>
</table>

*ADAMTS: A disintegrin and metalloproteinase with thrombospondin motifs, CPA: Carboxypeptidase A CDP: Carboxypeptidase D, FAP: fibroblast
activation protein, MMP: Matrix metalloproteinase OA: Osteoarthritis, TAFI: Thrombin-activatable fibrinolysis inhibitor

8.1.2.2. Osteoarthritis: diagnosis, treatment and challenges.

Osteoarthritis is the most prevalent age-related degenerative joint disease characterized mainly by a gradual loss of articular cartilage and deformation of the bone;\textsuperscript{49, 50} and currently there is no effective disease-modifying treatment except surgery. There are many causes of cartilage loss and many etiological factors to its progression.\textsuperscript{51-54} The primary cause is the elevated level of proteases,\textsuperscript{19, 20, 55} therefore, it is attractive to consider selective protease inhibitors as potential therapeutics. The main gap in this field is the lack of early-stage disease diagnostic tools, key for the success of any current therapy. Therefore, the development of non-invasive imaging methods capable to detect molecular changes is required.

8.1.3. Smart Imaging Agents.

Activity based imaging agents (smart probes) are commonly designed as FRET (fluorescence resonance energy transfer) pairs, photoactivatable fluorescent or bioluminescent probes generating a fluorescent or luminescent signal upon enzymatic hydrolysis of a chemical linker or a peptide bond.\textsuperscript{56} Near-infrared (NIR) fluorescence (650-900 nm) detection is the preferred range because it avoids the background fluorescence interference of natural biomolecules, providing a high contrast between target and background tissues. Modern NIR fluorescent \textit{in vivo} imaging is usually performed with internally quenched FRET probes with an emission band in the near infrared. Indeed, protease probes have commonly been based on peptidic substrates coupled to FRET pairs.\textsuperscript{57} An alternative to this approach is the development of high densely loaded fluorophores into a polymer backbone in such a way that self-quenching phenomena does occur. In this case, polymer degradation under specific biological stimuli (including protease activities) will trigger the fluorescence signal.\textsuperscript{3} Figure 8.1 shows both strategies. Up to date, both strategies suffer from different limitations: FRET pair based small probes, exhibit limited cell permeability \textit{in vivo}, poor tissue specificity and non-specific cleavage by related proteases; High-densely loaded polymeric probes offer good tissue selectivity but poor and limited protease selectivity (limited to polymer degradation).
Thus, there is an exposed need for a second generation of smart activatable imaging agents. In order to design a successful activatable smart probe, several considerations must be taken into account. It has to be: (i) highly selective for the disease related enzyme, that (ii) accumulate at the target site, (iii) while being efficiently cleared from the remaining part of the body. Those considerations could be defined as tasks for this project, where:

a) Target specificity was achieved by the use of modern medicinal chemistry based on reverse designs using chemically optimized selective protease inhibitors with high affinity for the desired protease that can be indeed transformed into not only selective but also catalytically efficient smart agents. This task was accomplished by our pharmaceutical collaborators from Sanofi-Aventis, EMBL and CEA-Saclays.

b) Tissue specificity. The second task was to provide a biocompatible polymeric scaffold for the low molecular weight smart activatable probes to enhance the tissue and molecular selectivity as well as the pharmacokinetic properties. The selective enrichment in the tissue of interest should either occur passively through enhanced
permeability and retention effect or by specific tissue-targeting residues. This second task was the one developed in our lab using polyglutamic acid based carriers.

Therefore, our contribution and main aim within LIVIMODE was to enhance the tissue and molecular selectivity of the low molecular weight smart activatable probes synthesized by other partners. This was achieved by means of polymer therapeutics: (i) Seeking selective enrichment in the tumor/joint environment through EPR effect and by specific tissue-targeting residues (RGDs, for tumor targeting, Rothenfluh peptides for cartilage targeting, alendronates for bone targeting...). For specific proteases, this resulted in higher signal intensity in tumors/joints and improve specificity versus protease activity expressed in other locations. (ii) Improving pharmacokinetics of probes (optimized half-life, tissue distribution) and by, (iii) Optimizing signal intensity by targeting retention of cleaved probe.

8.2. RESULTS AND DISCUSSION.

In this study highly specific turnover-based reporters, were combined with polymeric carriers for an efficient tissue selective-delivery of activatable smart probes. The potential of PGA as nanocarrier systems for optical imaging of disease related enzymes is presented here in terms of in vitro assays, in cell cultures and in some cases, in terms of in vivo proof of concept. In the frame of this additional chapter, only the most relevant results from the LIVIMODE project are discussed.

8.2.1. Smart probes.

In order to achieve the previously described goal, linear PGA was used as polymeric platform due to its intrinsic properties already described in the introductory chapter. On one hand, several functionalities (alkynes, azides, or maleimides) for orthogonal couplings were introduced within the developed smart probes in their synthetic strategies carried out by our partners. On the other hand, the corresponding complementary moieties were included within the PGA backbones in order to accomplish those named orthogonal reactions. These functionalities were easily introduced following the post-polymerization modification methodology described in Chapter 4.
8.2.1.1. MMP-based probes.

As introduced before, MMP family is involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes such as inflammatory disorders, regulation of tumor microenvironment and diseases such as atheroma, arthritis, cancer and tissue ulceration. MMP-12 also known as macrophage elastase (ME), is almost exclusively produced by macrophages and is essential for macrophage migration. MMP-12 is considered an important target due to its involvement in diseases such as cancer, asthma, allergy, pulmonary (emphysema) and skin inflammation, as well as in arthritic. Biological studies of Gelatinase B (or MMP-9) relate such proteolytic enzyme primarily with cancer and later on with vascular and inflammatory diseases. These facts have generated interest in targeting MMP-9 in acute lethal conditions, such as bacterial meningitis, sepsis and endotoxin shock, and in acute aggravations of chronic diseases. Finally, MMP-13 or collagenase 3 was firstly encountered and described in human breast cancer. Later, it was correlated with OA, after finding it in chondrocytes and as an mRNA in synovial membrane in OA and rheumatoid arthritis (RA).

Due to their high relevance under pathophysiological conditions, efforts have been devoted in the development of selective probes for such enzymes. A detailed description of the probes developed for MMP detection so far can be found in the very recent review by Knapinska and Fields. The main handicaps in the development of MMP probes is that their recognition sequence is usually broad and shared among MMPs. For instance, the commercial probe MMPsense, with the sequence Gly-Gly-Pro-Arg-Gln~IleThr-Ala-Gly, detects the activity of MMP-2, -7, and -9 in vivo by fluorescent molecular tomography.

8.2.1.1.1. MMP-12 conjugates.

As a first approach, and in order to optimize the chemistry and characterization of the final constructs, “in vitro” smart probes were developed. This family of activatable probes was based on the classical design using a peptidic sequence (substrate for MMP-12) combined with a FRET reporter pair (dye and quencher) and a chemical tag, for their conjugation within the polymer backbone (Scheme 8.1). The
FRET pair of these so-called “in vitro” probes consisted on the highly fluorescent 7-methoxycoumarin group that is efficiently quenched by energy transfer to the 2,4-dinitrophenyl group.

Scheme 8.1. Generic chemical structure of MMP-12 in vitro probes.

Within the optimization process for probe conjugation different parameters were taken into account in a first study: linker design (spacer) and peptide loading. For that purpose, three different spacer length were used: -CH\_2-, in the case of alkyne-functionalized polymers with propargyl amine; -EG(2)-, in the case of azide-functionalized polymers with NH\_2-EG(2)-N\_3, and -EG(6)- in the case of azide-functionalized polymers with NH\_2-EG(6)-N\_3. In all cases, probe conjugation was carried out with the optimized conditions for CuAAC (Chapter 4), in DMF/ddH\_2O mixtures (4:1), under nitrogen atmosphere and at 60 °C during 72 hours (Scheme 8.2).

Scheme 8.2. Conjugation scheme of the activatable probe to functionalized PGAs.

Purifications were done either by SEC using PD10 columns or via dialysis MWCO 3,000. Probe loading was estimated according to absorbance of the fractions at \(\lambda = 332\) nm (extinction coefficient of the free probe \(\varepsilon = 21470\) M\(^{-1}\)·cm\(^{-1}\)) and confirmed by \(^1\)H-NMR. The absence of free probe within the polymeric construct that could lead to interferences and/or artefacts in the in vitro testing was confirmed by mass spectrometry using MALDI-TOF (absence of the corresponding peaks from the free probes); and by diffusion NMR (DOSY), where the
diffusion coefficient of only one specie was observed. The final conjugates were also characterized by CD, where the random coil conformation of linear PGA was conserved after conjugations. Examples of the characterization techniques used in general for the PGA-probe conjugates are depicted in Figure 8.2.

![Figure 8.2](image)

**Figure 8.2.** Physico-chemical characterization carried out for each PGA-probe conjugate. PGA-MMP-12 conjugate as example. a) UV-VIS spectra; b) DOSY-NMR; c) ^1^H-NMR; d) CD.

Table 8.2 summarizes the main characteristics of the PGA-MMP-12 *in vitro* conjugated probes.

**Table 8.2.** PGA-MMP-12 *in vitro* conjugate probes characterization.

<table>
<thead>
<tr>
<th>Conj.</th>
<th>Linker mol%</th>
<th>Spacer</th>
<th>mol% probe</th>
<th>wt% probe</th>
<th>MW kDa</th>
<th>GAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP.1</td>
<td>11 % Alkyne</td>
<td>CH(_2)</td>
<td>1.5</td>
<td>13.0</td>
<td>24.3</td>
<td>138</td>
</tr>
<tr>
<td>SP.2</td>
<td>16 % Azide</td>
<td>EG(6)</td>
<td>1.5</td>
<td>10.0</td>
<td>30.7</td>
<td>138</td>
</tr>
<tr>
<td>SP.3</td>
<td>30 % Azide</td>
<td>EG(2)</td>
<td>3.2</td>
<td>22.5</td>
<td>28.9</td>
<td>138</td>
</tr>
</tbody>
</table>
Once the polymeric probes were synthesized and properly characterized, enzymatic assays against different MMPs were performed in order to establish their selectivity and specificity against MMP-12 probe after probe conjugation. For that purpose, kinetic studies during 2 hours were carried out in multi-well plates where the fluorescence release over time of each probe due to enzyme cleavage was recorded. The obtained degradation curve of the substrate was used to calculate the $K_{\text{cat}}/K_m$ ratio, as a representative measurement of the degradation rate of the substrate upon enzyme addition. Table 8.3 summarizes the results obtained for the three different MMP-12 in vitro probes against the MMPhs 9, 12, 8, 13 and 2 (h stands for human origin).

**Table 8.3.** Catalytic efficiency by means of $K_{\text{cat}}/K_m$ constants ratio.

<table>
<thead>
<tr>
<th>Free probe</th>
<th>$K_{\text{cat}}/K_m$ Values ($M^{-1} s^{-1}$) with $[\text{Enzyme}] = 10$ nM</th>
<th>MMP9h</th>
<th>MMP12h</th>
<th>MMP8h</th>
<th>MMP13h</th>
<th>MMP2h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>116345</td>
<td>185000</td>
<td>269</td>
<td>91474</td>
<td>14534</td>
</tr>
<tr>
<td>SP.1</td>
<td></td>
<td>85246</td>
<td>190000</td>
<td>500</td>
<td>51365</td>
<td>43754</td>
</tr>
<tr>
<td>SP.2</td>
<td></td>
<td>93664</td>
<td>550000</td>
<td>500</td>
<td>154740</td>
<td>71257</td>
</tr>
<tr>
<td>SP.3</td>
<td></td>
<td>1352</td>
<td>67255</td>
<td>245</td>
<td>37680</td>
<td>1354</td>
</tr>
</tbody>
</table>

Figure 8.3 shows the representation of the catalytic efficiency obtained for each polymeric probe in comparison with the free substrate. From the results obtained, several conclusions can be drawn. To begin with, enzyme selectivity for MMP-12 was kept in all cases, however, differences among the enzymatic profiles of the conjugates were observed. In the case of SP.1 conjugate, with the shorter spacer, the selectivity for MMP-12 was kept and slightly increased, whereas for SP.2 conjugate (the longer spacer) this selectivity was dramatically increased. On the other hand, regarding probe loading, SP.3 conjugate (with the highest loading, 3.2 mol%) shows a worse performance when compared with conjugate SP.2 (1.5 mol% probe loading).
Figure 8.3. Catalytic efficiency ($K_{\text{cat}}/K_m$, M$^{-1}$s$^{-1}$) displayed by MMPs in cleaving various probes.

These preliminary results clearly support the overall concept that smart activatable probes can be designed bearing linkers and conjugated to carrier scaffolds without losing catalytic turnover by the target enzyme. In particular, results obtained with SP.2 are very promising as not only selectivity for the specific enzyme was kept but also could be increased depending on the length of the polymer-probe spacer used, as well as the percentage of probe loading gained. All the above results lead to suspect that these probes may adopt a folded conformation in solution placing in close proximity the quencher and the fluorophore groups, a property that may influence the recognition and the efficacy of the corresponding MMP in cleaving these probes. These results were in good agreement with those obtained at CEA-Saclays with free MMP-12 probes, where different spacers among fluorophore and quencher, the use of different fluorophore or quenchers, and the positions of those within the probe structure demonstrated to dramatically affect their selectivity and specificity against MMP-12.

With these preliminary results using in vitro probes one step further in the development of MMP-12 selective probes was performed. For that purpose, in vivo NIR dyes and quencher were used. In the concrete case of MMP-12 developed probes from CEA, Cy5.5 (6S-IDCC) was used as dye, and quenched by the use of QSY21. The chemical tag alkyne was selected in this case, in order to be able to take profit from the optimized chemistry in the previous study.
Scheme 8.3. Chemical structure of the MMP-12 selective probe used for conjugation to PGA.

As a first approach, PGA-EG(6)N₃ was selected for probe conjugation due to the antecedents observed on the spacer influence with *in vitro* MMP-12 probes. Conjugation and conjugate purification were performed following the same protocol as that used for the previous probes. Probe quantification (Table 8.4) was carried out this time by ¹H-NMR as well as by fluorescence output of Cy5.5 λₜₐₜ = 595 nm, λₑₚₑ = 680 nm.

**Table 8.4.** PGA-MMP-12 *in vivo* conjugate probe characterization.

<table>
<thead>
<tr>
<th>Conj.</th>
<th>mol% linker</th>
<th>Spacer</th>
<th>mol% probe</th>
<th>wt% probe</th>
<th>MW kDa</th>
<th>GAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP.4</td>
<td>16</td>
<td>EG(6)</td>
<td>1.6</td>
<td>21.0</td>
<td>38.6</td>
<td>155</td>
</tr>
</tbody>
</table>

After adequate characterization, the polymeric probe was tested *in vitro* on a panel of MMPs, in PBS buffer. The kinetic constants obtained are summarized in Table 8.5 as well as, the related enzymatic curves are plotted in Figure 8.4.

**Table 8.5.** Catalytic efficiency by means of $K_{cat}/K_m$ constants ratio. In MMP12, “h” stands for human and “m” stands for mouse. $K_{cat}/K_m$ Values (M⁻¹·s⁻¹) with [Enzyme]= 10 nM

<table>
<thead>
<tr>
<th>MMP</th>
<th>1h</th>
<th>2h</th>
<th>3h</th>
<th>7h</th>
<th>8h</th>
<th>9h</th>
<th>12h</th>
<th>13h</th>
<th>14h</th>
<th>12m</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free</td>
<td>29696</td>
<td>62500</td>
<td>440</td>
<td>47593</td>
<td>2794</td>
<td>1043</td>
<td>50453</td>
<td>71893</td>
<td>300</td>
<td>650000</td>
</tr>
<tr>
<td>SP.4</td>
<td>260</td>
<td>61383</td>
<td>660</td>
<td>41193</td>
<td>500</td>
<td>2300</td>
<td>561733</td>
<td>41083</td>
<td>6633</td>
<td>600000</td>
</tr>
</tbody>
</table>
As it can be clearly observed in Figure 8.4, selectivity and efficacy were conserved when the probe (MMP12 substrate) is coupled to PGA chains. Moreover, substrate processing ratio was even higher when conjugated to the polymer ($K_{cat}/K_m$ values from $5 \cdot 10^5$ mol$^{-1}$s$^{-1}$ for the free substrate to $5.8 \cdot 10^5$ mol$^{-1}$s$^{-1}$ when conjugated into PGA backbone).

Furthermore, enzymatic degradation on other proteases (ADAMTS-4, ADAMTS-5, Tumor necrosis factor (TNF)-converting enzyme (TACE), Angiotensin-I converting Enzyme (ACE), and Neural endopeptidase (NEP)) have also been performed with this conjugate. The polymeric probe resulted to be highly selective for MMP12h, when
compared with the rest of proteases as summarized in Table 8.6 and Figure 8.5.

**Table 8.6.** Catalytic efficiency of SP.4 towards other proteases in PBS buffer.

<table>
<thead>
<tr>
<th>Protein</th>
<th>( K_{cat}/K_m ) Values (M(^{-1}\cdot\text{s}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAMTS-4</td>
<td>500</td>
</tr>
<tr>
<td>ADAMTS-5</td>
<td>500</td>
</tr>
<tr>
<td>TACE</td>
<td>500</td>
</tr>
<tr>
<td>ACE</td>
<td>500</td>
</tr>
<tr>
<td>NEP</td>
<td>13230</td>
</tr>
<tr>
<td>MMP12h</td>
<td>561733</td>
</tr>
</tbody>
</table>

**Figure 8.5.** Representation of the catalytic efficiency of PGA conjugate SP.4 towards other proteases.

No degradation was observed when the conjugate was exposed to diluted mouse plasma and PBS buffer over time as shown in Figure 8.6.

**Figure 8.6.** Diluted plasma and buffer stability of the SP.4 conjugate. Any probe degradation was observed.
After this first validation of the *in vivo* PGA-MMP12 conjugate probe SP.4, the influence of plasma proteins on the enzyme processing turnover was also studied. As the previous results strongly suggested that this series of probes adopt particular conformations in solution that may influence their ability to be cleaved by MMPs, one could expect that the presence of plasma proteins may also affect these conformations. Hence, the same experiments were performed in diluted mouse plasma (50 times). As it can be seen in Figure 8.7, there was no impact on MMP-12 mouse catalytic efficiency in cleaving the substrate under these conditions, but strikingly, MMP12 human was found to be much less active. These results illustrated how difficult could be to anticipate the effect on the substrate conjugation onto the polymer.

![Kinetic profiles of substrate cleavage upon MMPs addition in diluted mouse plasma at 2 nM MMP concentration.](image)

![Kcat/Km values obtained from the enzymatic curves in diluted mouse plasma at 10 nM MMPs.](image)

**Figure 8.7.** a) Kinetic profiles of substrate cleavage upon MMPs addition in diluted mouse plasma at 2 nM MMP concentration. b) $K_{\text{cat}}/K_{\text{m}}$ values obtained from the enzymatic curves in diluted mouse plasma at 10 nM MMPs.
As it could be expected, the presence of plasma proteins makes a difference in enzymes turnover. Although the polymeric probe exhibits a slightly slower processing by MMP12m in mouse diluted plasma when compared with the same processing in buffer, the selectivity and efficacy of MMP-12m in the substrate cleavage was retained validating it for *in vivo* applications.

Indeed, *in vivo* validation of the probe was performed in CEA-Saclays with a breast cancer orthotopic model based on the tumor cell line 4T1. Briefly, 4T1 cell line was injected in the mammary fat pad of female Balb/C mice to induce a well-vascularized tumor of 100 to 200 mm³ in 15 days. The polymeric probe **SP.4** and the parent free probe were i.v. injected in mice and the *in vivo* tumor fluorescence was followed during 24 hours by IVIS®. Probe biodistribution was assessed by the *ex vivo* imaging of the different organs at 24 h post-injection showing renal filtration and a urinary excretion for both probes.

*In vivo* fluorescence of tumor after injection of the polymeric probe **SP.4** or the free probe exhibits different kinetic profiles. For the non-grafted probe, tumor fluorescence signal shows a maximum at 60 min post-injection, followed by a rapid decrease. For **SP.4** probe, the signal increases during the first hour post-injection and remains stable up to 24 h, demonstrating the high impact of the polymer conjugation significantly enhancing tumor accumulation and therefore specific signaling (data not shown, obtained at Vincent Dive Lab).

Finally, regarding linker design, a family of conjugates with different spacer length, of the same MMP-12 *in vivo* probe were developed (Table 8.7) in order to get further understanding of the spacer influence on their performance. This study is still ongoing.

**Table 8.7.** Physico-chemical characteristics of PGA-MMP-12 *in vivo* conjugate probes (with different spacers).

<table>
<thead>
<tr>
<th>Conj.</th>
<th>mol% linker</th>
<th>Spacer</th>
<th>mol% probe</th>
<th>wt% probe</th>
<th>MW kDa</th>
<th>GAU</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SP.5</strong></td>
<td>32</td>
<td>EG(2)</td>
<td>1.9</td>
<td>22.6</td>
<td>43.4</td>
<td>155</td>
</tr>
<tr>
<td><strong>SP.6</strong></td>
<td>27</td>
<td>EG(6)</td>
<td>1.9</td>
<td>20.8</td>
<td>47.1</td>
<td>155</td>
</tr>
<tr>
<td><strong>SP.7</strong></td>
<td>28</td>
<td>EG(9)</td>
<td>1.7</td>
<td>17.6</td>
<td>50.1</td>
<td>155</td>
</tr>
</tbody>
</table>
8.2.1.1.2. MMP-9 conjugates.

As for MMP-12 selective probes, an optimization process with the so-called *in vitro* probes was first carried out. For that purpose, two MMP-9 *in vitro* probes were provided from CEA-Saclays in order to perform the bioconjugations with the linear polymers (Scheme 8.4).

Scheme 8.4. Schematic chemical structure of MMP-9 *in vitro* probes.

On one hand, and in order to further confirm the need of a spacer and the influence of the probe loading in the final construct processing by the enzyme, a conjugate with direct attachment of the probe (NH$_2$ functionalized), and high loading (5.8 mol%) was synthesized **SP.8** (Table 8.8) and *in vitro* evaluated (Figure 8.8). The synthesized polymeric probe displayed a worse enzymatic processing when compared with the free substrate. The selectivity for MMP-9 was kept, whereas the efficacy in the turnover was dramatically decreased. These results confirmed the need of a longer spacer between probe and polymer (**SP.9**) and the fact that high probe loadings do not improve the probe performance *in vitro*. However, conjugation to the polymer, independently of the spacer length and probe loading, decreased probe selectivity. In both conjugates, probe selectivity was lost upon conjugation to PGA. For **SP.8** conjugate (direct conjugation), catalytic efficiency decreased by only a factor of 2.5, but the conjugate was less selective than the free substrate. With **SP.9** conjugate (EG(6) spacer), the catalytic efficiency showed a 20-fold decreased, compared to the parent free substrate.

Table 8.8. Physico-chemical characterization of PGA-MMP-9 *in vitro* conjugate probes (with different spacers and loadings).

<table>
<thead>
<tr>
<th>Conj.</th>
<th>mol% linker</th>
<th>Spacer</th>
<th>mol% probe</th>
<th>wt% probe</th>
<th>MW kDa</th>
<th>GAU</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SP.8</strong></td>
<td>-</td>
<td>-</td>
<td>5.8</td>
<td>35.9</td>
<td>26.0</td>
<td>110</td>
</tr>
<tr>
<td><strong>SP.9</strong></td>
<td>16</td>
<td>EG(6)</td>
<td>1.0</td>
<td>7.0</td>
<td>33.6</td>
<td>152</td>
</tr>
</tbody>
</table>
Figure 8.8. Catalytic efficiency ($K_{cat}/K_m$, $M^{-1} \cdot s^{-1}$) displayed by MMPs in cleaving various MMP-9 selective probes at 200 nM probe concentration.

The *in vivo* analogous of the probe (NIRF, Cy5.5. and QSY21 as quencher, Scheme 8.5) was then developed and conjugated into the PGA backbone through, once again, CuAAC chemistry. Interestingly, the free *in vivo* probe (same sequence as the *in vitro* probe, but different dye/quencher pair), exhibited really poor specificity for MMP-9h when tested in buffer. Such specificity was slightly increased when the same probe was tested in diluted plasma. Hence, it was decided to draft it onto a polymeric backbone to check whether this specificity could be increased.

![Chemical structure of MMP-9 in vivo probes](image)

Scheme 8.5. Chemical structure of MMP-9 *in vivo* probes.

Following previous guidelines with MMP-12 probes and MMP-9 *in vitro* probes, a low probe loading (SP.10) was aimed (Table 8.9).
Table 8.9. Physico-chemical characteristics of PGA-MMP-9 *in vivo* conjugate probe.

<table>
<thead>
<tr>
<th>Conj.</th>
<th>mol% linker</th>
<th>Spacer</th>
<th>mol% probe</th>
<th>wt% probe</th>
<th>MW kDa</th>
<th>GAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP.10</td>
<td>27</td>
<td>EG(6)</td>
<td>2.2</td>
<td>25.2</td>
<td>57.9</td>
<td>184</td>
</tr>
</tbody>
</table>

The conjugated MMP-9 substrate was then tested *in vitro* in PBS solution in a first step, against a panel of MMPs, showing a decrease in the catalytic efficiency but an improvement on selectivity upon conjugation (Figure 8.9).

![Figure 8.9](image.png)

**Figure 8.9.** a) Kinetic profiles of substrate cleavage upon MMPs (4 nM) addition in PBS at 200 nM of probe concentration. b) $K_{cat}/K_m$ values obtained from the enzymatic curves in PBS.

Besides, when the conjugated probe was tested in mouse diluted plasma, selectivity against MMP-9h was found considerably increased, when compared with the parent free substrate.
Figure 8.10. a) Kinetic profiles of substrate cleavage upon MMPs (4 nM) addition in diluted mouse plasma at 200 nM of probe concentration. b) $K_{cat}/K_m$ values obtained from the enzymatic curves in diluted mouse plasma.

Overall, the conjugate **SP.10** was found to be highly selective for MMP-9h in diluted mouse plasma what validates it for *in vivo* use. Further studies with this conjugate are on the pipeline of this project, including *in vivo* biodistributions and as well as efficacy and selectivity determinations *in vivo*.

Additionally, and in order to further study the probe loading effect without the influence of other parameters, the same polymeric backbone bearing azide groups with EG(6) spacer were used in order to graft different probe loadings and check its effect on protease selectivity. Table 8.10 summarizes the wt% achieved in all cases.
Table 8.10. Physico-chemical characterization of different PGA-MMP-9 \textit{in vivo} conjugate-probes.

<table>
<thead>
<tr>
<th>Conj.</th>
<th>mol% linker</th>
<th>Spacer</th>
<th>mol% probe</th>
<th>wt% probe</th>
<th>MW kDa</th>
<th>GAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP.11</td>
<td>27</td>
<td>EG(6)</td>
<td>0.6</td>
<td>9.2</td>
<td>39.8</td>
<td>155</td>
</tr>
<tr>
<td>SP.12</td>
<td>27</td>
<td>EG(6)</td>
<td>1.3</td>
<td>16.8</td>
<td>43.7</td>
<td>155</td>
</tr>
<tr>
<td>SP.13</td>
<td>27</td>
<td>EG(6)</td>
<td>4.2</td>
<td>39.5</td>
<td>60.1</td>
<td>155</td>
</tr>
</tbody>
</table>

As usual, probe cleavage and fluorescence release was monitored over time for all conjugated probes when exposed to MMP9h (Figure 8.11).

![Figure 8.11](image)

**Figure 8.11.** Kinetic profiles of substrate cleavage upon MMP9h (6.6 nM) addition in PBS at 200 nM of probe concentration.

According to these results, it could be concluded that, with MMP-9, higher probe loading (SP.13) induces auto-quenching phenomenon, as the fluorescence signal does not increase in the expected factor (at least a factor 3 is expected between SP.12 and SP13). Lower probe loading (SP.11 and SP.12) resulted in greater catalytic efficiency.

8.2.1.1.3. MMP-13 conjugates.

After all the general studies carried out on MMP-9 probes as well as MMP-12 probes, MMP-13 probe was directly designed for \textit{in vivo} applications using the FRET pair Cy5.5 and QSY21 and following the already optimized physico-chemical characteristics regarding spacer
as well as probe loading. The general chemical formula of this MMP-13 selective probe was similar to the one of MMP-9 in vivo selective probes (Scheme 8.5), but changing the R substituent. The probe was clicked into PGA polymer backbone via CuAAC as described before and its characteristics are summarized in Table 8.11.

**Table 8.11.** Physico-chemical characterization of PGA-MMP-13 in vivo conjugate probe.

<table>
<thead>
<tr>
<th>Conj.</th>
<th>mol% linker</th>
<th>Spacer</th>
<th>%mol probe</th>
<th>wt% probe</th>
<th>MW kDa</th>
<th>GAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP.14</td>
<td>16</td>
<td>EG(6)</td>
<td>1.5</td>
<td>20.7</td>
<td>39.0</td>
<td>155</td>
</tr>
</tbody>
</table>

Once purified and characterized, the polymeric MMP-13 selective probe was tested against several MMPs in PBS buffer. Probe selectivity and catalytic efficiency was slightly increase upon conjugation (from $k_{cat}/K_m$ $4.6 \cdot 10^5$ M$^{-1}$s$^{-1}$ of free substrate to $6.5 \cdot 10^5$ M$^{-1}$s$^{-1}$ in conjugated probe) (Figure 8.12).

**Figure 8.12.** $K_{cat}/K_m$ values obtained from the enzymatic curves in PBS buffer obtained at 400 nM of probe concentration.

After that, the probe was also validated in mouse diluted plasma in order to be used for in vivo monitoring of disease related enzymes. Although catalytic efficiency was decreased, selectivity against MMP13 enzyme was proven to be guaranteed in plasma, allowing us to proceed for in vivo studies (Figure 8.13).
The polymeric probe (SP.14) potential for *in vivo* monitoring of OA progression due to MMP-13 specificity was then tested in Oxford, at Hideaki Nagase laboratory. Briefly, an OA model was first developed for this project, based on the destabilization of the medial meniscus (DMM) (Figure 8.14).
Figure 8.14. OA model developed within Hideaki Nagase lab in Oxford University.\textsuperscript{75}

This model was well established and used to test two smart probes (free, and conjugated) against MMP13. The probes were tested both i.v. and six weeks after the surgery and fluorescence was recorded over time (48 h) in the DMM knee, the DMM contralateral (from the injured animal but without surgery), and sham knee (from control animals with surgery but without damage) (Figure 8.15). Whereas free substrate was hardly able to be distinguished between the different knees, the polymeric probe was able to discriminate among DMM and sham operated animals 6 weeks after surgery. Moreover, PGA-probe was able to distinguish between DMM operated and contralateral knees also six weeks after surgery.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{free-probe-6-weeks-after-surgery.png}
\caption{Free probe, 6 weeks after surgery.}
\end{figure}
Figure 8.15. Fluorescence intensity due to probe activation in vivo after i.v. injection of the probes over time. a) Free MMP-13, 6 weeks after surgery, n= 3. Paired t test: DMM vs. DMM contralateral, ρ < 0.001; DMM vs. Sham, ρ= 0.29 (n.s.). b) PGA-MMP-13, 6 weeks after surgery, n= 3. Paired t test: DMM vs. DMM contralateral, ρ= 0.0053; DMM vs. Sham, ρ= 0.0008. c) PGA-MMP-13 vs. free MMP-13 probe in DMM damaged knee. n= 3, ρ= 0.0086.

Fluorescence images taken 48 hours after i.v. administration of SP.14 probe in operated animals (Figure 8.16) highlight the results obtained.
Figure 8.16. Fluorescence images raw data, taken 48 hours after PGA-probe SP.14 i.v. injection. Probe was injected 6 weeks after surgery.

When time course experiments over the progression of the DMM damaged knee were performed, differences among the conjugated and non-conjugated probes were also found. The unconjugated MMP-13 selective probe was able to distinguish among knees only when injected 8 weeks after surgery. On the contrary, the PGA-MMP-13 conjugate probe was able to discriminate already at six weeks post-surgery (Figure 8.17). This is crucial due to the main objective pursued within the development of these activatable smart probes was based on early detection.

a)
b)

**Figure 8.17.** Maximum fluorescence intensity (MFI) of probes activation over course of DMM. a) Free activatable probe. Data from 8 h after i.v. delivery of the probe. n= 5 * p<0.05. b) PGA-probe SP.14. Data from 2 days after i.v. delivery of the probe. n= 5 * p<0.05.

One critical gap to address within smart probes development is their specificity for the desired enzyme/protease. In order to answer that question, it is necessary to use specific enzyme inhibitors (MMP-13 in this case) and to look for specific signal attenuation upon inhibitor addition. For that purpose, in another study, an MMP-13i inhibitor was administered (per os) p.o. 1 day before and 4 hours before imaging, always at 6 weeks post-surgery, and the fluorescence due to probe activation was measured (Figure 8.18). The PGA-probe signal was effectively attenuated to fluorescence levels below those obtained for DMM contralateral and Sham knees, confirming the presumed specificity of the polymeric probe.
Figure 8.18. Maximum fluorescence intensity (MFI) registered for PGA-probe over time at 6 weeks post-surgery and influence of the specific inhibitor for MMP-13. Inhibitor was administered p.o. 1 day before and 4 hours before imaging. n= 5.

One step further in the development and validation of this smart probe, as well as one of the aims of this project was to check their ability to monitor the progress on the disease when talking about the evaluation of novel therapies. For this purpose, SP.14 probe was used in order to follow the treatments of injured animals with different doses of the mentioned MMP-13 inhibitor p.o., daily. Importantly, this inhibitor was never administrated simultaneously to probe injection. The polymeric probe was able to show a decrease on the MMP-13 levels (and presumably, a decrease on the inflammation in the OA injured knee) in DMM knees after the treatments with 0.3 mg·Kg⁻¹ of inhibitor. When a dose of inhibitor 10 times higher was administered, the fluorescence levels found were close to those found in the DMM contralateral, already at six weeks post-surgery. Results suggested that 30 mg·Kg⁻¹ inhibitor dose was required to restore OA damage in vivo.
Thus, from these results, it could be concluded that this smart probe could be a potential tool in monitoring specific MMP-13 activity and disease progression in osteoarthritis, being the first of its class with such remarkable behavior.

### 8.2.1.2. Cathepsin-B based probes.

As mentioned in the introduction, Cathepsins are good candidates to be used as targets for activatable probes development. Among cathepsins, cathepsin B is of major relevance for cancer diagnosis and therapy as abnormal regulation of cathepsin B and its overexpression has a direct correlation with increased invasive and metastatic potential in many cancers\(^{43, 77}\). Several cathepsins (B, S and L)-sensitive activity-based probes\(^{78}\) have been designed as suicide substrates, which label the enzyme covalently and inactivate it upon binding.\(^{79-81}\) Several fluorogenic peptide substrates,\(^{82}\) for optical imaging of cathepsin B activity \textit{in vitro} and substrate-based NIRF polymeric probe\(^{83, 84}\) for non-invasive optical imaging of cathepsin B activity \textit{in vivo} have already been synthesized. Nonetheless, such probes might be less suited for \textit{in vivo} applications due to their peptidic character.\(^{3, 85, 86}\)

With the same purposes, Cathepsin B probes were developed, by collaborators from EMBL (Germany). Those activatable probes were synthesized through a strategy known as “reverse design”. The recently developed “reverse design” concept is based on transferring the selective profile of an optimized cathepsin inhibitor into a selective
probe by replacing the warhead of such an inhibitor by a cleavable peptide bond and subsequently attaching appropriate reporter groups for optical imaging (Scheme 8.6). \(^\text{15}\)

**Scheme 8.6.** General chemical formula of Cathepsin B probes. For *in vitro* probes, FRET pair coumarin 343/TAMRA. For *in vivo* probes, FRET pair Cy5.5/BHQ-3.

In a similar way as for MMP probes, *in vitro* probes were first designed in order to perform optimization processes as well as to confirm probe selectivity and efficacy after conjugation to PGA. The *in vitro* probe included the FRET pair coumarin 343/TAMRA (Tetramethyl-6-Carboxyrhodamine), and alkyne as the chemical tag for conjugation. Following CuAAC conditions, the probe was successfully conjugated to azide functionalized PGA polymers using the already optimized EG(6) spacer between probe and polymeric backbone. The synthesized conjugate details are summarized in Table 8.12.

**Table 8.12.** Cathepsin B selective *in vitro* polymeric probes details.

<table>
<thead>
<tr>
<th>Conj.</th>
<th>mol% linker</th>
<th>Spacer</th>
<th>mol% probe</th>
<th>wt% probe</th>
<th>MW kDa</th>
<th>GAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP.15</td>
<td>16</td>
<td>EG(6)</td>
<td>3.5</td>
<td>22.6</td>
<td>39.3</td>
<td>152</td>
</tr>
</tbody>
</table>

After adequate characterization of the Cathepsin B selective *in vitro* polymeric probe (SP.15), its selectivity and efficacy was tested against different recombinant cathepsins.
Figure 8.20. Cleavage of cathepsin B reporter *in vitro* probe at [Probe]= 50 ng·mL⁻¹ and [Enzyme]= 25 nM, *in vitro*. Change in fluorescence emission intensity at $\lambda_{em}= 695$ nm of probe after addition of cathepsins B, K, L, S and V (excitation at $\lambda_{ex}= 670$ nm).

Figure 8.20 noticeably shows how the selectivity of the polymeric probe to be processed by Cathepsin B was retained upon conjugation, thus, validating the strategy of conjugation also for the activatable probes synthesized through reverse design.

Afterwards, selective Cathepsin B probes for *in vivo* applications were also developed. The *in vivo* probes were based on the NIRF Cy5.5/BHQ-3 (Black Hole Quencher®-3), containing also alkyne moieties for bioconjugations. CuAAC chemistry was once again used for probe grafting into the polymeric backbone and the synthesized conjugate details are given in Table 8.13.
Table 8.13. Cathepsin B \textit{in vivo} polymeric probe (SP.16) details.

<table>
<thead>
<tr>
<th>Conjugate (Conj.)</th>
<th>Linker mol%</th>
<th>Spacer</th>
<th>probe mol%</th>
<th>probe wt%</th>
<th>MW (kDa)</th>
<th>GAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP.16</td>
<td>16</td>
<td>EG(6)</td>
<td>1.2</td>
<td>14.4</td>
<td>35.0</td>
<td>152</td>
</tr>
</tbody>
</table>

The selectivity and efficacy of cleavage of the fluorogenic substrate after coupling to the polymer was again verified against recombinant cathepsins \textit{in vitro} and it confirmed that the selectivity was preserved after conjugation (Figure 8.21).

![Figure 8.21. Cleavage of cathepsin B \textit{in vivo} probes at [Probes] = 10 μM and [Enzyme] = 25 nM, \textit{in vitro}. Change in fluorescence emission intensity at $\lambda_{em}$ = 695 nm of probes after addition of cathepsins B, K, L, S and V (excitation at $\lambda_{ex}$ = 670 nm). (a) Free Probe; (b) PGA-Probe (SP.16).](image)

Conjugation of the probe to polymers slightly decreased the turnover efficiency (2.6 fold, Figure 8.22) presumably owing to constrained substrate accessibility after conjugation. Nevertheless, when the kinetics of the PGA-probe was compared with an analogous dendrimeric polyglycerol-based conjugate of the same probe, the linear PGA construct exhibited a much better performing in terms of turnover processing.

With the $K_{cat}/K_{m}$ values exceeding 10,000 M$^{-1}$.s$^{-1}$ the conjugate seemed to be the ideal candidates for monitoring of cathepsin B activity \textit{in vivo}.
Figure 8.22. Probe cleavage rate (V: velocity) against probe concentration (S: substrate) by cathepsin B. Free probe, PGA-probe and a dendrimeric version of the conjugate are compared. Solid lines were generated using nonlinear regression analysis. The table shows the kinetic parameters of cleavage by cathepsin B.

Additionally, mass spectrometry analysis from the probes after being processed by Cathepsin B revealed the same cleavage site for both probes (free and PGA-conjugate) as shown in Figure 8.23.
Figure 8.23. MALDI-TOF mass spectra of PGA-Probe before (blue) and after (red), Free Probe before (green) and after (yellow) cleavage by cathepsin B. The quencher-bearing fragment with m/z 732.4 generated by cathepsin B cleavage is encircled.

Although Cathepsin B can be located extracellularly, is mostly present intracellularly in the lysosomal compartments. This fact make the PGA conjugate even better system for Cathepsin B targeting due to its capacity to follow lysosomal pathways through endocytosis. With a
view to cancer applications, NIFR microscopy was also performed in cultured 4T1-breast cancer cells, confirming intracellular probes localization as shown in Figure 8.24. Moreover the polymer conjugated probe exhibited a substantially higher signal as compared to the non-conjugated probe and the commercially available imaging agent for monitoring cathepsin activity Prosense 680.

![Figure 8.24. NIFR microscopy study of cellular uptake of probes by cultured 4T1 cells.](image)

It is well-known that both tumor and immune cells secrete large amounts of cathepsin B.\(^{88}\) Taking into account that fact, the evaluation of the probes in co-culture of breast carcinoma MDA-MB-231 cells with THP-1 cells differentiated into macrophages seemed to be the next step towards probe validation. The free probe, PGA-probe (SP.16) and the dendrimeric analogue dPG-probe conjugate were incubated in the co-cultures, and a fluorescence signal increase was observed, being the highest associated with our PGA-probe conjugate. These signals were effectively inhibited by the broad spectrum cathepsin inhibitor E-64 and a more cathepsin B selective CA-074 in all the cases, suggesting that the probes were specifically cleaved by cathepsin B also in cell culture (Figure 8.25).
Figure 8.25. Fluorescence intensity for cathepsin B probes in co-cultures. Cathepsin inhibitors E-64 and CA-074 could successfully block the fluorescent signal, while all three probes could detect cathepsin B activity in co-culture supernatants.

Overall, the in vivo probe SP.16 can be considered as a good candidate for in vivo monitoring of Cathepsin B, and further in vivo studies on 4T1 tumor models are currently ongoing to demonstrate that.

On top of that, Cathepsin B has been also reported to be overexpressed in inflammation sites, and its activity is related to inflammation processes.\textsuperscript{89, 90} That fact, make it also good aspirant to be used in inflammatory diseases. As proof of concept, the probe was tested in a rapid model of inflammation developed at Hideaki Nagase’s group. The probes were directly injected into the site of inflammation (paw) at 10 μM probe eq., and fluorescence signal was quantified over time (Figure 8.26).
When data was normalized, similar signal was found for both probes at early time points, however, the polymeric probe exhibited a much greater signal after 24 hours post-injection when compared with the free probe, meaning that the conjugated probe is longer retained in the inflamed paw (Figure 8.26). These encouraging results convinced us to further proceed with the development of this class of polymeric probes.
8.2.1.3. Other probe conjugates.

Within the LIVIMODE project, other enzyme selective probes were also synthesized, conjugated into PGA polymeric carriers, and evaluated. This is the case of cathepsin S selective probes as well as FAP selective probes. As stated in the introduction, both enzymes represent good target candidates for the development of smart probes in order to monitor disease progression and several activatable probes have been already developed using them as target enzyme.\textsuperscript{37, 91}

In the case of Cathepsin S selective probes provided from EMBL, different conjugation strategies were explored. Even though CuAAC was a very efficient coupling method, it entailed exhaustive purifications in order to remove copper below the allowed limits for its use in biological applications. For that reason, acid/base chemistry and thiol-ene maleimide couplings were implemented for the conjugation of the cathepsin S probes whose chemical tag was based on primary amines or maleimide groups (Scheme 8.7).
Scheme 8.7. General chemical formula of Cathepsin S probes. For \textit{in vitro} probes, FRET pair coumarin 343/7-methoxycoumarin. For \textit{in vivo} probes, FRET pair Cy5.5/BHQ-3.

Linear PGA polymer was used for the direct conjugation of the Cathepsin S probe bearing amine as chemical tag on one hand leading to the conjugate \textbf{SP.17}. As already seen for other probes, the introduction of a spacer improved the probe performance \textit{in vitro} against different enzymes. For that reason, an EG(4) spacer was attached to the Cathepsin S \textit{in vitro} probe using FMOC-NH-EG(4)-COOH. Then, the derivatized probe was conjugated to PGA via acid/base chemistry to lead the conjugate \textbf{SP.18}. On the other hand, two probes bearing maleimide groups as chemical tags (\textit{in vitro} and \textit{in vivo} probes) were effectively conjugated to cysteamine-2TP modified PGAs (see Chapter 4) using thiol-ene chemistry, yielding the smart polymeric probes \textbf{SP.19} and \textbf{SP.20}.

Table 8.14. Physico-chemical characteristics of Cathepsin S polymeric probe conjugates.

<table>
<thead>
<tr>
<th>Conj.</th>
<th>mol% linker</th>
<th>Spacer</th>
<th>mol% probe</th>
<th>wt% probe</th>
<th>MW kDa</th>
<th>GAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textbf{SP.17}</td>
<td>-</td>
<td>-</td>
<td>1.0 (\textit{in vitro})</td>
<td>6.8</td>
<td>17.8</td>
<td>110</td>
</tr>
<tr>
<td>\textbf{SP.18}</td>
<td>-</td>
<td>EG(4)</td>
<td>1.5 (\textit{in vitro})</td>
<td>9.7</td>
<td>18.8</td>
<td>110</td>
</tr>
<tr>
<td>\textbf{SP.19}</td>
<td>5</td>
<td>(CH\textsubscript{2})\textsubscript{2}</td>
<td>1.1 (\textit{in vitro})</td>
<td>8.8</td>
<td>14.6</td>
<td>85</td>
</tr>
<tr>
<td>\textbf{SP.20}</td>
<td>5</td>
<td>(CH\textsubscript{2})\textsubscript{2}</td>
<td>0.8 (\textit{in vivo})</td>
<td>13.0</td>
<td>26.0</td>
<td>155</td>
</tr>
</tbody>
</table>

The polymeric \textit{in vivo} probe \textbf{SP.20} deserves a special mention as it was found to be highly selective for Cathepsin S, when tested \textit{in vitro} (data not shown).
The same studies as those explained for SP.16 (Cathepsin B selective probe) were performed with Cathepsin S probes, such as cellular uptake in 4T1 tumor cell line in comparison with free probe, mass spectrometry in order to determine the cleavage site, co-culture experiments for Cathepsin activations in presence and absence of specific inhibitors, etc. (data not shown). All of them showed similar results as those obtained for the Cathepsin B polymeric probe, validating again the use of PGA polymers as nanocarriers for in vivo monitoring of disease related enzymes.

Finally, FAP probes from EMBL were also conjugated. In this case, the main objectives were a) to study the influence of probe lipidation by using lipidated (SP.22) and non-lipidated probes (SP.21) and b) to address the influence of polymer size on the final construct in vitro/in vivo performance (SP.23 and SP.24). FAP probes conjugates synthesized are summarized in Table 8.15. Their in vitro behavior in terms of protein specificity and catalytic efficiency are currently under study.

![Scheme 8.8. General chemical formula of FAP probes. For in vitro probes, FRET pair coumarin 343/7-methoxycoumarin. For in vivo probes, FRET pair Cy5.5/BHQ-3. *Note, for SP.22, the in vitro probe used contained an additional lipid moiety.](image)

Table 8.15. FAP activatable polymeric probes family details.

<table>
<thead>
<tr>
<th>Conj.</th>
<th>mol% linker</th>
<th>Spacer</th>
<th>mol% probe</th>
<th>wt% probe</th>
<th>MW kDa</th>
<th>GAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP.21</td>
<td>16 azide</td>
<td>EG(6)</td>
<td>1.4 (in vitro)</td>
<td>11.7</td>
<td>35.4</td>
<td>152</td>
</tr>
<tr>
<td>SP.22</td>
<td>16 azide</td>
<td>EG(6)</td>
<td>1.4 (in vitro)</td>
<td>14.3</td>
<td>36.3</td>
<td>152</td>
</tr>
<tr>
<td>SP.23</td>
<td>16 azide</td>
<td>EG(6)</td>
<td>4.1 (in vivo)</td>
<td>38.4</td>
<td>49.5</td>
<td>152</td>
</tr>
<tr>
<td>SP.24</td>
<td>27 azide</td>
<td>EG(6)</td>
<td>4.2 (in vivo)</td>
<td>35.2</td>
<td>66.7</td>
<td>184</td>
</tr>
</tbody>
</table>
8.2.2. Targeting strategies.

As stated in the introductory part, targeting strategies were also included in the scope of the LIVIMODE project with the aim of enhancing tissue specificity of the synthesized probes.

8.2.2.1. OA targeting.

With the purpose of improving cartilage targeting in the selected polymeric probes such as the MMP-13 selective SP.14 several strategies for OA targeting were pursued. First of all, the use of the cartilage-binding sequence developed by Rothenfluh et al. was explored. Hence, in order to validate this peptide as cartilage vector, the conjugation of two peptidic sequences to PGA used as carrier was performed. One of them was the oligopeptide WYRGRL, specific to cartilage binding. The other was the scrambled sequence YRLGRW (Tyr-Arg-Leu-Gly-Arg-Trp), to be used as negative control.

The starting peptidic products were provided of a FMOC protecting group in the N-terminus and have the C terminus as free acids. Since PGA is a polyacid, the easiest and fastest way to conjugate these oligopeptides to its backbone, taking into account the peptidic sequences, was by direct attachment of the NH$_2$ at the N-terminus (after removal of the protecting group) to the carboxylic groups of the polymer backbone. For that purpose, as the carboxylic group of the peptide is free, the synthesis was designed in order to avoid any undesired crosslinking. For that reason, the activation of the carboxylic groups of the backbone was done in a previous step by using 5"N-hydroxysuccinimide to yield PGA-NHS activated. The FMOC group was removed from the peptides with piperidine (20 %) in methanol; and on one hand, the direct conjugation between the free amine of the N-terminus of the peptides and the activated carboxylic groups of the polymer backbone was performed.

On the other hand, the introduction of an EG(4) spacer was also designed prior to the conjugation of PGA. Due to its cationic character, the presence of Arg in the peptide sequences, could lead to electrostatic interaction between the carboxylic groups of PGA, which could have an influence in the final conformation of the conjugate, and subsequently, in the targeting moieties exposure. In order to diminish the effect of this interaction, the introduction of the EG spacer was proposed. The peptide was PEGylated with FMOC-EG(4)COOH. Pursuing that aim,
and taking into account that the peptide sequence has the C-terminus as free carboxylic acid, the activation of the carboxylic group of the FMOC-EG(4)-COOH was done previously and separately with N-Hydroxysuccinimide. The FMOC group of the PEGylated peptides was then removed by using piperidine (20 %) in methanol and the product was conjugated to PGA-NHS activated via peptidic bond. As those peptides contain both arginine amino acids in their sequences, in order to avoid secondary reactions with the less reactive secondary amine from arginine, the reaction was performed this time at 4 °C.

The four conjugates (PGA-cartilage binding sequence, PGA-random sequence, PGA-EG(4)-cartilage binding sequence, PGA-EG(4)-random sequence) and a linear PGA were then label with Cy5.5 taking profit from the NHS activation. Below the structures of the 5 conjugates synthesized are shown, and their main characteristics are summarized in Table 8.16.

Scheme 8.9. Chemical formula from cartilage targeted polymeric platform (CT).
Table 8.16. Cartilage targeted polymer platform (CT).

<table>
<thead>
<tr>
<th>Polymer</th>
<th>MW kDa</th>
<th>mol% dye</th>
<th>wt% dye</th>
<th>mol% pep</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGA-Cy$_{5.5}$(CT.1)</td>
<td>23.6</td>
<td>0.70</td>
<td>3.2</td>
<td>0</td>
</tr>
<tr>
<td>PGA-Cart-Cy$_{5.5}$ (CT.2)</td>
<td>24.2</td>
<td>0.80</td>
<td>3.6</td>
<td>3.92</td>
</tr>
<tr>
<td>PGA-Scram-Cy$_{5.5}$(CT.3)</td>
<td>24.2</td>
<td>0.70</td>
<td>3.2</td>
<td>5.00</td>
</tr>
<tr>
<td>PGA-EG(4)-Cart-Cy$_{5.5}$ (CT.4)</td>
<td>24.3</td>
<td>0.86</td>
<td>3.9</td>
<td>3.43</td>
</tr>
<tr>
<td>PGA-EG(4)-Scram-Cy$_{5.5}$(CT.5)</td>
<td>24.5</td>
<td>0.83</td>
<td>3.7</td>
<td>4.80</td>
</tr>
</tbody>
</table>

This family of conjugates bearing targeting and non-targeting units was then tested in vivo in order to determine targeting efficiency. Their half-life was first determined after intra-articular injection in the knees (Figure 8.28) showing no major differences among the different polymeric construct. Half-life in the range of 3.8-6.9 in all cases (Table 8.17).

![Figure 8.28. Half-life determination of different PGA-targeted polymers. n= 4, 2 animals, 2 knees, no significant differences between knees were found.](image)

Table 8.17. Fitted half-life after intra-articular injection of cartilage targeted polymers polymer platform (CT).

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Fitted Half-life (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT_1</td>
<td>5.19</td>
</tr>
<tr>
<td>CT_2</td>
<td>3.84</td>
</tr>
<tr>
<td>CT_4</td>
<td>6.93</td>
</tr>
<tr>
<td>CT_5</td>
<td>4.93</td>
</tr>
</tbody>
</table>

Knees were extracted, frozen and sectioned and their fluorescence was analyzed at 640 nm. From the pictures of Figure 8.29
it could be concluded that all the polymers do reach the chondrocytes in the cartilage and seem to be either inside, or just around them. The results suggest that the use of these targeting moieties were not improving the passive targeting of the polymeric itself given by the EPR effect.

**Figure 8.29.** Knee cryosections from the intra-articular injections at 24 hours post-injection. Pictures taken at 20X magnification. The red channel is tuned to Cy5.5, whereas the green channel is using the autofluorescence of the tissue.

After those preliminary results, the use of Rothenfluh peptides as cartilage binding sequences was discarded. Then further detailed *in vivo* characterization was performed with PGA-Cy5.5 (CT_1) in order to validate its use for OA *in vivo* monitoring. Biodistribution after i.v. administration of CT_1 was studied by fluorescence imaging techniques (Figure 8.30a and b). As expected for a PGA-based probe, it followed a renal excretion profile. However, surprisingly, a high accumulation in the knees was found after 24 hours post administration. This fact was further confirmed with histology. Knees were frozen and sectioned. Cryo-sections were then observed at the microscope (Figure 8.30c) at 40X, showing CT_1 probe accumulation in the knee. These findings all together suggest that PGA-based probes can be considered
highly suitable for their use in OA disease progress monitoring. Moreover, PGA nanocarriers combined with the previously developed MMP-13 highly selective probes offers the perfect recipe for this aim.

**Figure 8.30.** a) PGA-Cy5.5 (CT_1) biodistribution after i.v. administration at 24 hours post-injection obtained *ex-vivo*. b) *Ex-vivo* fluorescence example of the injected probe 24 hours post injection. c)
Knee cryo-section at 40X magnification. Red channel was used for Cy5.5 whereas the green channel for tissue autofluorescence.

Apart from the use of Rothenfluh peptides for cartilage binding, other targeting strategies were also proposed and pursued. Alendronates (ALN) for bond targeting were also included in PGA-based nanocarriers as well as RGD cyclic peptides for cancer targeting. Nevertheless, those families of compounds are not yet tested, thus, their results will not be discussed herein.

8.3. CONCLUSIONS.

As enlightened in the introduction the most critical gap for the monitoring of early events related to early stages of disease lies in the lack of target-specific and tissue-specific imaging probes. Within this project, we pursued to overcome this gap with a novel approach aiming towards highly specific smart (activatable) imaging agents. This approach was exemplified on a selected set of disease-relevant protease targets, with consequences for improved monitoring of life-threatening or chronic diseases such as cancer or degenerative joint disease, respectively. This will impact disease management at multiple levels: early diagnosis and disease staging, development and characterization of novel disease-relevant animal models that are critical for therapy development, and finally the evaluation of novel therapeutic drug candidates. Once validated these imaging strategies will constitute valuable biomarkers for demonstrating proof-of-mechanism and eventually proof-of-efficacy for novel drug candidates.

To this end, linear scaffolds of biocompatible polymers (PGA) were synthesized and derivatized with chemical tags complementary to those used for smart probes and several corresponding conjugates were generated. Overall the results of these studies show that a selective enrichment of dye-labeled polymers was possible and that such probes could be attached to these scaffolds via several conjugation methods while retaining and even increasing their ability to be cleaved by selected proteases.

Preliminary in vivo studies of selected conjugates in those adequate in vivo models carried out by our partners clearly point out that these smart polymeric probes are highly suitable as early-detection molecular diagnostic tools with a high signal to noise ratio and a slow
clearance rate. In particular with MMP-13 probe, the data suggested that this macromolecular probe could be qualified as an excellent candidate for theranostic applications in combination with adequate drugs. Furthermore, at the preclinical level, our approach offers a significant potential for multimodal imaging due to easy conjugation to PET or MRI tracers and would further assist in conceptualizing clinical studies.

8.4. MATERIALS AND METHODS.

8.4.1. Materials.
All chemicals were reagent grade and used without further purification, unless otherwise indicated. All EG amines were obtained from Iris Biotech GMB. All solvents were of analytical grade. Preparative SEC was performed using Sephadex G-25 superfine from GE as well as PD MiniTrap G-10 ™ columns containing 2.1 mL of Sephadex™ G-10.

8.4.2. Characterization techniques.
NMR spectroscopy, GPC, CD, DLS, DOSY-NMR and UV were performed using the equipment and techniques according to previous chapters.

8.4.3. Protocols.
8.4.3.1. General procedure for click conjugation of the probes through CuAAC.
In a two-necked round bottom flask fitted with a stirrer bar and a stopper, 1 eq. of the respective copolymer PGA-EG(2/6)N₃ or PGA-prop sodium salt was dissolved in ddH₂O. After that, the corresponding amount for the desired % of substitution of clickable agent (smart probe) was added in dry DMF solution. Then, 5 eq. of sodium ascorbate in ddH₂O were added. After that, the mixture was degassed by performing two freeze-pump-thaw cycles. Afterwards, 1 eq. of CuSO₄ was added in aqueous solution to the reaction mixture. The final complete mixture, containing a proportion DMF/H₂O of 4:1, was degassed by performing another freeze-pump-thaw cycle and left to react at 40 °C in an oil bath for 3 days protected from the light.

For purification, the solvent was completely removed under vacuum. The colored solid was re-dissolved and purified by PD10
column collecting fractions of 0.5 mL. Two clear fractions were found corresponding with the conjugated and unconjugated probe. Additionally, dialysis using Vivaspin® MWCO 3000 was performed to those fractions belonging to the polymer-probe conjugate. The % of probe conjugated was determined using different methods depending on the probe conjugated. Basically, probe content was determined by $^1$H-NMR. Besides, probe content was estimated by spectroscopic techniques. For those in vitro probes, a calibration curve using the maximum of absorbance of the probes ($\lambda$: 332 for MMPs in vitro probes, $\lambda$: 455 for Cathepsin and FAP in vitro probes) was used. In vivo probes content based on Cy5.5 was quantified according to the fluorescence either of the eluted fractions from SEC, or via calibration curve using Cy5.5. Each fraction was diluted in a 1/200 proportion in a 96 dark well-plate. Fluorescence of each fraction was measured using the filters of Cy5.5. $\lambda_{\text{exc}}=595$ nm, $\lambda_{\text{em}}=680$ nm.

SP.1. Yield: 80 % Conjugation efficiency (CE) 50 %; mol probe to GAU 1.5 % (2.07 eq. per polymer chain), 13 wt%, MW conjugate: 24.3 kDa.

SP.2. Yield: 85 % CE 50 %; mol probe to GAU 1.5 % (2.07 eq. per polymer chain), 10 wt%, MW= 30.7 kDa.

SP.3. Yield: 64 % CE 36 %; mol probe to GAU 3.2 % (3.52 eq. per polymer chain), 22.5 wt%, MW= 28.9 kDa.

SP.4. Yield: 90 % CE 80 %; mol probe to GAU 1.6 % (2.43 eq. per polymer chain), 21 wt%, MW= 38.6 kDa.

SP.5. Yield: 90 % CE 86 %; mol probe to GAU 1.9 % (2.9 eq. per polymer chain), 22.6 wt%, MW= 43.4 kDa.

SP.6. Yield: 85 % CE 86 %; mol probe to GAU 1.9 % (2.9 eq. per polymer chain), 20.8 wt%, MW= 47.1 kDa.

SP.7. Yield: 85 % CE 78 %; mol probe to GAU 1.7 % (2.6 eq. per polymer chain), 17.6 wt%, MW= 50.1 kDa.

SP.9. Yield: 40 % CE 50 %; mol probe to GAU 1 % (1.5 eq. per polymer chain), 7 wt%, MW= 33.6 kDa.

SP.10. Yield: 80 % CE 65 %; mol probe to GAU 2.2 % (4 eq. per polymer chain), 25.21 wt%, MW= 57.9 kDa.

SP.11. Yield: 70 % CE 60 %; mol probe to GAU 0.6 % (1 eq. per polymer chain), 9.2 wt%, MW= 39.8 kDa.

SP.12. Yield: 75 % CE 65 %; mol probe to GAU 1.3 % (2 eq. per polymer chain), 16.8 wt%, MW= 43.7 kDa.
8.4.3.2. Direct attachment of the amino functionalized in vitro smart probe to PGA.

The standard DIC/HOBt procedure with slight modifications was applied in this case. Briefly, in a two-necked round bottom flask fitted with a stirrer bar and two septums, PGA was dissolved in anh. DMF under N₂ flow. After that, DIC (1.5 eq. from the desired percentage of modification) was added to the mixture. After 10 minutes, 1.5 eq. from the desired percentage of modification were added as solid form. Reaction was left to proceed for 10 minutes more. After that time, the corresponding amount of probe was added. The pH was adjusted to 8 and the reaction was allowed to proceed for 16 hours protected from light. Finally, the solvent was removed under vacuum and the product was dissolved in ddH₂O (by adding 100 μL of NaHCO₃ 1M to convert it into the sodium salt form of PGA). The product was purified by SEC using PD10 commercial G25 columns followed by dialysis (Vivaspin®, MWCO 3000). Upper part of the tube was freeze-dried and the obtained yellowish solid was analyzed by NMR (D₂O). Probe content was quantified by UV-VIS at λ: 332 nm in the case of MMP probes, and λ: 455 nm for Cathepsin S probes.

SP.13. Yield: 80 % CE 84 %; mol probe to GAU 4.2 % (6.5 eq. per polymer chain), 39.5 wt%, MW= 60.1 kDa.
SP.14. Yield: 60 % CE 75 %; mol probe to GAU 1.5 % (2.3 eq. per polymer chain), 20.74 wt%, MW= 39.0 kDa.
SP.15. Yield: 70 % CE 70 %; mol probe to GAU 3.5 % (5.32 eq. per polymer chain), 22.6 wt%, MW= 39.4 kDa.
SP.16. Yield: 75 % CE 60 %; mol probe to GAU 1.2 % (1.8 eq. per polymer chain) 14.4 wt%, MW= 35.0 kDa.
SP.21. Yield: 90 % CE 62 %; mol probe to GAU 1.36 % (2.01 eq. per polymer chain), 11.75 wt%, MW= 35.4 kDa.
SP.22. Yield: 62 % CE 64 %; mol probe to GAU 1.4 % (2.13 eq. per polymer chain), 14.29 wt%, MW= 36.3 kDa.
SP.23. Yield: 86 % CE 83 %; mol probe to GAU 4.2 % (6.2 eq. per polymer chain) 38.4 wt%, MW= 49.5 kDa.
SP24. Yield: 97 % CE 84 %; mol probe to GAU 4.2 % (7.7 eq. per polymer chain) 35.2 wt%, MW= 66.7 kDa.
8.4.3.3. EG spacer introduction prior to direct conjugation of the amino functionalized in vitro smart probe to PGA.

In order to introduce an EG spacer between the polymer and the probe, FMOC-EG(4)-COOH was attached to the amine functionalized probe prior to polymer conjugation. The carboxylic group of FMOC-EG(4)-COOH was activated with DIC/HOBt, in anh. DMF. Afterwards, the probe HHY-29 was added and the pH was adjusted to 8 by adding DIEA. The reaction was left to proceed for 16 hours stirring under nitrogen atmosphere. After that time, the solvent was evaporated and the product was passed through a C18 column eluting with methanol. The yellowish fraction was collected. The product was directly deprotected with 2 mL Et$_2$NH/DMF (1/4) during 45 minutes. The solvent was then evaporated and the product was directly used for the conjugation to PGA, by using the above protocol based on DIC/HOBt as activator of the carboxylic groups for probe conjugation and purification.

8.4.3.4. General procedure for the conjugation to maleimide probes.

In a round bottom flask fitted with a stirrer bar and a stopper, 1 eq. of the PGAcoCysteamine2TP PGA sodium salt was dissolved in 300 mM HEPES buffer pH 7.4. Next, the corresponding amount for the desired % of substitution of maleimide compound was dissolved in DMF and added to the polymer solution (buffer/DMF ratio 5.5/1). Finally, 10 eq. of TCEP dissolved in HEPES buffer were added, and the reaction mixture was left stirring for 16 hours at r.t. protected from light. Upon reaction, the mixture was purified using a PD10 column collecting fractions of 0.5 mL. One part of each fraction was diluted with ddH$_2$O water and their fluorescence (for in vivo probe bearing Cy5.5, $\lambda_{exc}$= 595 nm, $\lambda_{emi}$= 680 nm) or absorbance (for in vitro probe at $\lambda$: 455 nm) was measured in order to identify the conjugate and quantify the linking efficiency. Finally, the conjugate containing fractions were combined and freeze dried.
SP.19. Yield: 70 % CE 40 %; mol probe to GAU 1.1 % (1 eq. per polymer chain) 8.8 wt%, MW= 14.6 kDa.
SP20. Yield: 75 % CE 50 %; mol probe to GAU 0.8 % (7.7 eq. per polymer chain) 13.0 wt%, MW= 26.0 kDa.

8.4.3.5. Synthesis of targeted polymers for cartilage binding.

8.4.3.5.1. PGA activation with 5'-N-hydroxysuccinimide (NHS).
In a two-necked round bottom flask fitted with a stirrer bar and two septums, 190 mg (1.4 mmol GAU, 1 eq.) of PGA as acid form was dissolved in 10 mL of anh. DMF. Afterwards, 88 mg (0.7 mmol, 126.2 g·mol\(^{-1}\), 0.5 eq.) of DIC were added. The reaction was left stirring at r.t. for 5 minutes. After that time, a catalytic amount of 4-Dimethylaminopyridine (DMAP) was added and 81 mg (0.7 mmol, 0.5 eq.). The pH was checked to be 8. The mixture was left stirring for 16 hours under \(\text{N}_2\) atmosphere. The solvent was removed and the polymer was precipitated by adding 40 mL of a mixture CHCl\(_3\)/Acetone: (4/1) and centrifuged during 5 minutes at 8.000 r.p.m. After removal of the supernatant, the white solid was washed three times with 30 mL of methanol, and dried under vacuum. Yield: 90 %, 44 % NHS activation according to \(^1\)H-NMR (signal at 2.4 ppm in DMSO-\(d_6\)).

8.4.3.5.2. EG(4) spacer introduction.
As the peptidic sequences (cartilage binding and scramble sequence) have both the C- terminus deprotected, it was necessary to previously activate the FMOC-EG(4)-COOH using NHS protocol for the introduction of an EG spacer.

a. NHS activation of FMOC-EG(4)-COOH.
In a two-necked round bottom flask, fitted with stirrer bar and two septums, Fmoc-EG(4)-COOH (100 mg, 0.205 mmol, 1 eq.) was dissolved in 5 mL of anh. \(\text{CH}_2\text{Cl}_2\) under nitrogen atmosphere. Next, DCC (85 mg, 0.410 mmol, 2 eq.) dissolved in 5 mL more of anh. \(\text{CH}_2\text{Cl}_2\), was added and the mixture was left reacting for 10 minutes. After that time, NHS (47.2 mg, 0.410 mmol, 2 eq.) was added as a solid. The reaction was left stirring for 16 hours. After that time, the crude was filtered off and the solvent was removed under vacuo. Yield: 80 %. The degree of NHS activation was determined as 90 % according to \(^1\)H-NMR as well as using the indirect method TNBS assay\(^{93}\) for
estimation of free amino groups when the NHS activated sample is reacted with an equimolar amount of Glygly-NH₂.

**b. Peptides derivatization with EG(4) spacer.**

Fmoc-EG(4)-NHS was reacted with the free amino groups of the cartilage binding and scramble peptidic sequences. For that purpose, 1 eq. of Fmoc-EG(4)-NHS was dissolved in 5 mL of anh. CH₂Cl₂ under nitrogen atmosphere and 0.8 eq. of the corresponding peptide was added afterwards in 5 more mL of anh. CH₂Cl₂. The pH was adjusted to 8 by adding 50 μL of DIEA. The reaction was left stirring under nitrogen flow for 16 hours. Then, the solvent was completely removed. In a direct step, the reaction crude was Fmoc deprotected by using 4 mL of a mixture of piperidine/methanol (20/80, v/v). Reaction was left to proceed for 1 hour and the solvent was completely removed. The Fmoc deprotected product was then purified using a C18 column in methanol collecting fractions of 1.5 mL. The corresponding fraction were put together, and solvent was evaporated yielding to a white solid. Yields: 60 % for cartilage binding derivatized sequence, and 65 % for scramble sequence. Reaction success was checked by ¹H-NMR were EG(4) signals were present, and the corresponding aromatic Fmoc signals were not. Apart from that, UV-VIS was performed and peptide content was determined as 100 % according to the Absorbance of tryptophan amino acid from the peptides (ε: 5.579 M⁻¹·cm⁻¹ at λ: 278 nm).

**8.4.3.5.3. Peptides conjugation to activated PGA-NHS and Cy5.5 labeling.**

In a two-necked round bottom flask fitted with a stirred bar and two septums, 40 mg (0.233 mmol, 1 eq.) of NHS activated PGA was dissolved in 10 mL of anh. DMF under nitrogen atmosphere. The system was cooled down to 4 °C by using an ice bath. Then 0.012 mmol of peptide (0.05 eq. for 5 % modification with derivatized or not derivatized, cartilage binding sequence, or scramble sequence) was added together with a catalytic amount of DMAP. The pH was adjusted to 8 with the addition of 50 μL of DIEA and the reaction was left to proceed for 24 hours. After that time, 0.0023 mmol of Cy5.5-NH₂ (0.01 eq.) was added and the mixture was left reacting for 24 hours more. After that time, the products were purified by SEC using commercial PD10 columns eluting with ddH₂O. Peptide content was estimated by
UV-VIS using tryptophan absorbance at 278 nm. Cy5.5 percentage of modification was determined by fluorescence using $\lambda_{\text{exc}}= 595$ nm, $\lambda_{\text{emi}}= 680$ nm. Yields: 50-70 % Peptide and Cy5.5 content is summarized in Table 8.16

8.4.3.6. In vitro testing of MMP probes.

In vitro testing of MMP probes was performed at CEA-Saclays, in Paris by Vicent Dive group with the assistance in some of them of this PhD aspirant. Briefly, enzymatic assays were carried out in 50 mM Tris-HCl buffer, pH 6.8, 10 mM CaCl$_2$, at 25 °C. The assays were performed in black 96-well plates (non-binding surface plates; Corning Costar Catalog No. 3651). Progress curves were monitored by recording the increase in fluorescence resulting from substrate cleavage at the corresponding wavelengths depending on the probe tested. The conditions of a typical experiment were 100 mL of buffer and 1-10 nM of MMP (R & R Systems). The reaction was then initiated by addition of 0.1-0.5 µM of probe. Data were collected for 1 hour. The $K_{\text{cat}}/K_m$ values were determined from first-order full-time reaction curves ($S << K_m$; $S= 0.5$ µM) and fitted with the integrated Michaelis-Menten equation by nonlinear regression: $P= S_0(1-\exp(-kt))$, where $P$ is product concentration; $S_0$ is substrate concentration (probe) at $t$ (time)$= 0$; $\exp$ is exponential; $k$ is ($K_{\text{cat}}/K_m$) + $E$; and $E$ stands for enzyme concentration. The kinetic parameters $K_m$ and $K_{\text{cat}}$ were estimated according to the direct linear plot method.

8.4.3.7. In vitro testing of Cathepsin B probes.

In vitro testing of Cathepsin B probes was performed at Josef Stefan Institute in Ljubljana, Slovenia by Boris Turk group.

8.4.3.7.1. Cleavage Site Determination of Cathepsin B probes.

Cleavage site in all three probes was determined using MALDI-TOF/TOF UltraFlextreme III mass spectrometer (Bruker, Germany) controlled by the FlexControl 3.3 software package. All three probes were mixed with cathepsin B in 1:100 molar ratio in 50 mM MES buffer pH 6.0 with 1 mM DTT and incubated until no further increase of fluorescence was observed. Samples were then desalted and concentrated using C18 tip before 1 µL of the sample was applied to a MALDI target plate as a 1:1 (v/v) mixture of sample and matrix.
(matrix: 20 mg·mL⁻¹ DHB, dissolved in 30 % acetonitrile and 0.1 % TFA). UV laser beam (nitrogen laser, λ= 337 nm) was used for ionization, ions were accelerated in positive ion mode to a kinetic energy of 25 kV. External calibration of mass spectra was performed using Peptide Calibration Standard II (Bruker, Germany).

8.4.3.7.2. Kinetics of Cathepsins probe hydrolysis.
Kinetics of cathepsin B probes (conjugated and unconjugated), hydrolysis by cathepsin B was measured in accordance with standard procedures. Accordingly, probe (1-30 µM final concentration) was prepared in 0.1 mM PBS buffer pH 6.0 with 1 mM DTT. Cathepsin B was then added and fluorescence increase was measured continuously using Tecan M1000 (Tecan) fluorescence plate reader. The K_M and K_cat/K_m values were determined by nonlinear regression analysis and GraphPad Prism software (GraphPad Software, Inc.) using the experimentally determined reaction velocities at given substrate concentrations.

8.4.3.7.3. Cleavage of Cathepsin B selective probe and SP.16 (PGA-Probe) in co-cultures of MDA-MB-231 and THP-1 cells.
THP-1 cells were seeded (~10⁶ cells·mL⁻¹) on a 150 mm plate and differentiated with 30 ng·mL⁻¹ PMA for 48 hours in RPMI media. Medium was then exchanged and cells were grown for additional 24 hours. MDA-MB-231 cells were grown in DMEM. For the co-culture, both plates with cells were washed with sterile PBS twice, MDA-MB-231 cells were detached using Hank's based enzyme free cell dissociation solution and suspended in 10 mL PBS pH 6.0 with 0.5 mM DTT. Cells were then added to THP-1 cells for 2 hours in a 2:1 ratio. Next, cells were removed by centrifugation and protein supernatants containing secreted extracellular proteins were concentrated on a MWCO 3.000 (Milipore) filter device to a final concentration of 1-2 mg·mL⁻¹. Before the addition of probes, cathepsin B activity in the supernatant was verified with zRR-AMC (Bachem) substrate. For the test 100 µg of supernatant was pipeted into 96 well plate and probes were added to a final 5 µM concentration. In control experiment, E-64 (Peptide Institute) or CA-074 (Peptanova) inhibitors were used at 10 µM final concentration, which is sufficient to completely block any cathepsin activity in cellular experiments. In order to evaluate the
total fluorescence of the probes in the supernatant, complete hydrolysis was performed by addition of 100 nM recombinant cat B. Fluorescence was then measured in 2 minute intervals over 2 hours using TECAN M1000 at excitation and emission wavelengths of 670 nm, and 695 nm, respectively. All experiments were performed in triplicate.

8.4.3.8. *In vivo* testing of the polymeric probes and targeting strategies.

*In vivo* testing of Cathepsin B conjugate in inflamed paw mouse model, *in vivo* testing of MMP-13 probe conjugate in DMM OA mouse model and *in vivo* testing of targeted polymeric probes were performed at Imperial College London, UK by Hideaki Nagase’s group. Such results were presented with the only purpose of highlighting the potential of our polymeric probes provided the good performance *in vivo*, and the experimental part will not be further discussed herein.
8.5. REFERENCES.


General Discussion
The growing incidence and increasing costs caused by neurodegenerative disorders, together with the lack of effective treatments, point out the need for novel approaches in order to address their enormous burden. Most of the current therapies mainly rely on palliative healing without stopping disease progression. Moreover, Central Nervous System (CNS) drug discovery and development is a challenging task due to the presence of the most impenetrable biological barrier in the human body- the blood brain barrier (BBB).

Invasive and non-invasive strategies are under study in order to bypass the BBB and achieve effective drug concentrations at the brain parenchyma within the therapeutic window. In this thesis, we combined non-invasive approaches based on the use of targeted nanosystems for effective brain accumulation, together with molecular Trojan horses in order to promote BBB entry.

Polymer therapeutics (PT) have been proposed as the ideal technological platform towards the design of valuable treatments as well as diagnostic tools for CNS related diseases. With several products in the market, and a growing number under clinical development, PT hold key characteristics to surpass the main limitations suffered from low Mw tracers or drugs, and even biologicals (peptides, proteins, antibodies, oligonucleotides). Those features include: (i) improved pharmacokinetic profile and enhanced plasma half-life; (ii) the possibility to include targeting moieties for specific active targeting; (iii) carrier multivalency allowing tunable drug(s) loading capacity and combination approaches, including theranostics; (iv) controlled and sustained drug release of conjugated drugs upon bioresponsive linkers under specific environments; (v) PT provide stability and reduced immunogenicity to the bioactive compound and possess optimal size to allow better penetration.

It is currently accepted that future challenges and opportunities to move this platform technology forward are based on (i) new molecular targets, (ii) polymer-based combination therapy, (iii) control on polymeric platforms and their conformational behavior in solution, and (iv) exhaustive physico-chemical characterization, essential to transform a promising conjugate into a candidate for clinical evaluation, following regulatory indications as exposed in Chapter 1. Additionally, limitations with existing polymeric systems such as their lack of biodegradability, biocompatibility and multifunctionality inevitably lead to side effects and poor patient compliance. New PT
based on amino acids are excellent candidates for drug delivery, as they do not suffer from these limitations. In particular, polyglutamates (PGA) constitute a versatile platform which has been effectively used as building blocks in polymer drug conjugates and polymeric micelles for various medical applications ranging from cancer to tissue regeneration.\(^{18, 19}\) Moreover, it is expected its FDA approval after approval of PGA-paclitaxel conjugate, Opaxio\textsuperscript{TM} for the treatment of various cancers alone or in combination (in 2012 Opaxio\textsuperscript{TM} was designated as orphan drug in combination with radiotherapy and temozolomide for the treatment of glioblastoma multiforme).\(^{20-24}\)

Besides, there is a growing interest in the development of smart polymeric systems, where new and more defined architectures with higher MW (to enhance passive targeting by the EPR effect), predictable structure and conformation, lower heterogeneity, higher drug loading capacity and greater possibility for multivalency are key desirable parameters. Furthermore, as mentioned above, the use of adequate physico-chemical techniques in order to exhaustively characterize new complex systems is a requisite.

With all that in mind, this thesis dissertation aimed to achieve new well-defined and exhaustively characterized structures, based on polyglutamates, to be used as carriers for the treatment and/or diagnosis of neurodegenerative disorders (concretely Alzheimer’s Disease (AD)), by means of BBB bypassing using active targeting strategies with molecular Trojan horses.

In order to accomplish the already exposed goals of this highly ambitious project and with the aim of “building the house from the foundation”, the initial efforts were devoted to the development of a new, easy and versatile methodology for the synthesis of polymeric platforms based on PGA. Among the techniques for the synthesis of polypeptides,\(^{25-31}\) the ring opening polymerization (ROP) of N-carboxyanhydrides (NCA) represents the most commonly applied polymerization technique to produce polypeptides and polypeptide-based block copolymers on a multigram scale.\(^{32-34}\) The possibility to use non-natural amino acids combined with the use of functional and macromolecular initiators makes it a powerful approach to yield functional polypeptide based homo- and multi-\textit{block} copolymers, with different architectures that could ultimately lead to different and unexpected properties for the desired biological applications. Unfortunately, this polymerization method suffers from different
inherent mechanistic limitations\textsuperscript{34} making the synthesis of well-defined systems, a challenging task. Indeed, it requires the use of ultra-pure NCA monomers and initiators, ultra-dried solvents, and the avoidance of risk factors such as the presence of moisture, traces of \( \text{CO}_2 \) or any impurity that could lead to undesired reactions, precise control over pressure and temperature...among other factors. Even then, secondary and undesired terminating processes can occur due to the coexistence of two main mechanisms within the polymerization process. Several attempts have been carried out by different research groups in order to minimize or even avoid these secondary reactions.\textsuperscript{35-40} Although great advances have been achieved, all these methods have their own limitations. For example, high vacuum techniques (HVT) require complex and expensive experimental setup, hexamethyldisilazane (HMDS) amines are sensitive to hydrolysis, while heavy metal catalysts must be carefully removed to avoid non-specific toxicity in biomedical applications.

Overall those methods, the Schlaad methodology\textsuperscript{38} caught our attention (Scheme 2.4). This method is based on the use of primary amine hydrochloride salts as initiators in order to avoid the undesired activated monomer mechanism (AMM). The principle of this strategy is based on the so-called dormant species caused by the equilibrium among primary amine and the ammonium salt that minimize the monomer deprotonation. Nonetheless, undesired reactions still occur due to the presence of chloride anions, which are well-known to lead to initiations. Hence, in order to overcome those limitations, we proposed a novel approach using, for the first time, ammonium salts as initiators but with non-nucleophilic tetrafluoroborate counterions what will offer the advantages of the Schlaad method without its main drawback. This anion is less hydrophilic and thus, better soluble in a broad range of organic solvents and it has lower nucleophilicity than the related nitrate or halide salts.\textsuperscript{41-43} Indeed, the \( \text{BF}_4^- \) is considered to be inert during the polymerization due to equal charge distribution provided by its tetrahedral symmetry, and the presence of highly electronegative fluorine atoms, which diminishes the nucleophilicity of the anion avoiding, consequently, the undesired monomer deprotonation and guaranteeing the living character of the polymerization. This hypothesis was indeed experimentally demonstrated with the optimization of a new methodology leading to well-defined polypeptides and polypeptide-based block copolymers with controlled and adjustable MW (up to
degree of polymerization 800), low polydispersity (D) (<1.2), quantitative yields, with preserved end group integrity and avoidance of racemization at the chiral centers (Table 2.4). Furthermore, the synthesis could be performed without the need of complex experimental setup, in large scale, with good batch to batch reproducibility and the lack of toxic impurities, what is crucial for further use in the field of nanomedicine. Indeed, this methodology has been the base of a spin-off company within the laboratory, what demonstrates its industrial feasibility.44

Herein, the versatility of the reported method was firstly explored for the synthesis of homopolymers using n-butyl and neopentilamine tetrafluoroborate salts as initiators. Then, the method was extended to the design of end-functionalized polymers derived from the use of functionalized initiators, such as, alkyne and azide bearing initiators what opened the door for site-specific conjugation of bioactive molecules such as antibodies, proteins or imaging probes. The synthesis of hybrid di-block copolymers was also demonstrated by the use of polyethylene glycol (PEG) based initiators (Table 2.4).45

Once the polymerization process was successfully achieved, the following step was the optimization of the benzyl protecting group removal. An ideal method should be able to reach complete PBLG deprotection in the absence of racemization, in order to avoid changes in the secondary structure of the synthesized polypeptides. These changes could induce dramatic modifications in the biological behavior of a designed nanomedicine, such as, altered immunological properties, degradation profiles, and ultimately, a modified and probably unpredictable pharmacokinetics and biodistribution. All that together, point out the relevance of the selected deprotection methodology. While the use of TFA/HBr or acetic acid/HBr was known to produce complete deprotection in the absence of racemization,46 these conditions were not suitable for sensitive functional end groups derived from functional initiators as well as PEG based block copolymers, both acid-sensitive. Thus, an alternative basic methodology based on the use of a NaOH suspension in THF was also optimized for homopolymers as well as PEG based di-block co-polymers in order to achieve benzyl group removal without stereochemical changes (Figure 2.17).45

Regarding the stated need for the design of novel architectures to be used as carriers in certain drug delivery strategies, branched polymers represent outstanding aspirants due to their unique
rheological, mechanical and biomedical properties derived from their structures, inaccessible for linear polymers. Hence, we decided to explore the possibilities of the newly described BF$_4$ methodology in the synthesis of star-shaped polyglutamates, taking profit from the living character and the control over the initiation step provided by our method. Thus, a simple yet powerful methodology for the synthesis of 3-arm star-shaped polyglutamic acid with well-defined structures, precise MW and low D ($<$1.2) was applied, following a divergent method from novel multifunctional initiators. Four different 3-arm initiators were synthesized and characterized including one with reducible disulfide bonds within the arms. The relevance of this latter initiator relies on the possibility of star disassembling into the individual arms. This fact enables the characterization of the individual arms what demonstrated the homogeneity of our systems, as expected from a living polymerization and efficient initiation$^{47}$ (Figure 3.7, Table 3.1).

As mentioned before, multivalency, inherent to PGA, is a desired characteristic in order to get high loading capacities. This can be further improved with the introduction of orthogonal functionalities within the polymer backbone to allow site-specific bioconjugations. Pursuing that aim, a versatile post-polymerization modification technique was implemented.$^{48}$ This technology encompasses the use of 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium (DMTMM) salts for carboxylic acid activations, necessary for acid/base chemistry with functionalized amines. In this way, various reactive functional groups were easily introduced under mild reaction conditions (needed to keep the polymer backbone integrity), such as, alkynes, azides, reactive disulfides or protected amines (Scheme 4.6). This methodology was very versatile as could be used in aqueous as well as in organic solvents by changing the salt counteranion from chloride to BF$_4$, respectively. This versatility allowed us to introduce hydrophilic as well as hydrophobic molecules within the polymer backbone (Tables 4.2 and 4.3). Hence, the described post-polymerization modification method represents a powerful and attractive approach for the synthesis of functional polymers, overcoming the limited functional group tolerance of many controlled polymerization techniques.$^{48}$

Importantly, these newly synthesized polyglutamates should be biologically evaluated, in vitro and, if promising (safety/benefit) in vivo by means of adequate cell and animal models in order to demonstrate
their potential therapeutic applications. First, their biocompatibility, biodegradability and cell trafficking were considered key aspects to be studied.

Polymer degradation profiles in the presence of the lysosomal enzyme cathepsin B were first performed. The results obtained from several star-shaped polyglutamates as well as with the functionalized polymers yielded to the conclusion that, the architecture and the degree and type of functionalization do have an important influence on the degradation rate. For instance, the hexyl-based star-shaped PGA presented a slower degradation rate when compared with its analogous ethyl-based initiated polymer (Figure 3.10). Furthermore and looking at linear polymers, the degree of functionalization as well as the nature of the chains added within the polymer backbone do affect the degradation rate of the final construct as seen in the case of the different PEGylation grades (Figure 4.19).

Their in vitro biocompatibility (MTS assays) against different cell models was apparently not compromised with the use of different architectures nor with the degree of modifications introduced at least up to the concentrations tested (3 mg/mL) (Figures 3.13 and 4.20).

In the cell uptake studies, different PGA architectures (linear, hybrid di-block PEG-PGA, and star) were prior labeled with the fluorescence tag Oregon Green (λ: 488 nm) to allow in vitro evaluation. Those studies revealed energy-dependent mechanisms, independently of the MW or the architecture, since absence of uptake at 4 °C was found for all the polymers tested. When linear PGAs of different MWs were tested, no changes in the internalization profile and cell associated fluorescence (CAF) intensity were found, meaning that, at least for linear polymers, their MW did not affect the degree of cell internalization. Interestingly, cell uptake was significantly increased (in about 3 fold) when star-shaped polymers where used, highlighting the importance of the architecture in the biological output (Figure 3.19). This fact could be ascribed to the inherent properties of star-shaped polymer, which has in general, more compact structure with globular shape and large surface areas among other interesting features, making them relevant candidates to be used in drug delivery and molecular imaging among other biological applications..

Then we went through the synthesis of PGA-based systems with self-assembly properties. When exhaustive physico-chemical characterization of such systems was carried out through a battery of
techniques including DLS, DOSY and NOESY-NMR, CD, transmission electron microscopy (TEM), fluorescence techniques and small angle neutron scattering (SANS), interesting data regarding solution conformation aspects were found. According to the findings, those systems undergo self-assembly processes showing a clear structure/conformation-concentration dependency encountering “unimers” of 5-10 nm size at low concentrations, whereas supramolecular structures of around 100-180 nm were mainly seen at high concentrations. This phenomenon occur in all those systems, nevertheless it did not occur in linear PGA. Critical Aggregation Concentration (CAC) values could be obtained by DLS measurements.\(^{51-54}\) A self-assembly process leading to organized bigger structures with hard sphere shapes bearing branching points directed to the outside was suggested with the aid of TEM imaging and SANS fittings among other findings (Figures 5.2 and 5.8). Indeed, SANS contrast experiments confirmed the presence of organized domains along the nanostructure, fact in good agreement with previous reports in literature.\(^{55-58}\) These findings suggested that such motifs are the driving force for the assembly of these architectures. The stimuli-responsiveness of such systems under different physico-chemical environments (temperature, ionic strength, or concentration), as well as the influence of the degree and nature of the polymer functionalization on the dynamics of the process were studied. Summarizing, it could be said that the carrier size was clearly dependent on the environmental conditions. Importantly, the aggregation process could be tuned and even prevented depending on the polymer functionalization. Apparently, hydrophilic residues above 30 mol% loading prevented system aggregation. This could be a useful tool in the design of highly controlled polymeric constructs.

Considering all this, we proposed a strategy for the preparation of novel drug delivery systems (DDS) with higher MW that would allow longer circulation times \textit{in vivo}, adequate functionalities to enhance BBB crossing and subsequently accumulate therapeutics as well as imaging probes in the brain in a concentration capable to diminish AD burden.

The developed strategy was based on the covalent entrapment of the supramolecular architectures with the aid of \textit{click} chemistry reactions, in order to yield nanoconstructs stable to physical stimuli (concentration, temperature, ionic strength). For that purpose,
functionalization of these PGA-based systems via post-polymerization methodologies was used to introduce cross-linkers. Co-assembly studies were carried out in order to verify that those construct do intercalate, necessary for cross-linking. After that, the covalent capture of the self-assembled constructs was carried out, firstly by using CuAAC click chemistry, but later extended to the copper free thiol-end and reversible disulfide chemistries. This latter strategy would allow to build architectures with sizes >100 nm suitable for long circulating times, but prone to disassemble under reductive media.

The preliminary biological evaluation of those structures against selected cells revealed their absence of toxicity and exhibited a significantly enhanced cell internalization rate when compared with the linear and star under the conditions tested, as shown by flow cytometry (Figures 5.26 and 5.27).

Once the main architectures established, the selection of the transcytosis-trigger residues to be used towards BBB crossing had to be done. ANG-2\textsuperscript{60-66} and CRTIGPCSVC (cPEP)\textsuperscript{67, 68} were chosen as vectors on a first approach. For that, polymer-conjugates from both peptides by means of different linking chemistries (amide bonds for cPEP whereas disulfide chemistry for ANG) using the first family of synthesized polymeric platforms (homopolymers, di-block PEG-PGA, and star polymers) were developed (Scheme 6.2). Such polymeric family was previously labeled to allow in vivo biodistribution studies through non-invasive imaging by PET, with DO3A-\textsuperscript{68}Ga in the case of homo and di-block copolymers, and DO3A-\textsuperscript{111}In for star-shaped polymers. In vivo studies were chosen to evaluate BBB crossing capability since they represent the most suitable and reliable manner in contrast to reported cell-based BBB models.

Hence, biodistribution studies were carried out in FVB/NJ mice. After i.v. administration, animals were monitored and sacrificed at different time points and radioactivity quantified in each organ, as well as in blood and plasma. Plasma levels allowed the extraction of pharmacokinetic parameters, including polymer plasma half-life. A renal excretion profile and a non-specific organ accumulation were observed for all carriers, showing their suitability to be used in DDS (Figures 6.21 and 6.26). Regarding their pharmacokinetics, branched polymers in general (with and without targeting units) presented a greater plasma half-life (from 12 to 16 hours) when compared to their linear counterparts (0.6 hours) (Table 6.5). Consequently, an enhanced
brain accumulation was observed for the star-shaped polymers in comparison with the linear or di-block copolymers, although it was still considered not significant (< 1% injected dose (ID)). Several explanations could be drawn to this respect, being among them, the need for a better exposing of the targeting ligands allowing better receptor recognition and the need for greater circulation times to further promote brain accumulation. The covalently captured structures, resulted from the self-assembly process of the novel PGA-based systems previously described, offered a higher MW (to improve plasma half-life) and a greater and less hindered surface area (to enable a better exposition of the targeting vectors), therefore were considered potential candidates.

Hence, by means of a bottom-up approach, the surface modification of such nanostructures was carried out. Cy5.5 probe was conjugated in order to allow biodistribution monitoring by fluorescence optical imaging (NIR) on one hand. On the other hand, based on the previous data obtained, ANG-2 was chosen as BBB vector and surface conjugated via disulfide bonds. The synthesized constructs (with and without targeting unit) were in vivo analyzed in terms of biodistribution in C57BI/6 mice (Animal Imaging Center, Zurich, Switzerland). After its i.v. administration through the tail vein, animals were sacrificed at different time points and fluorescence in blood and the extracted organs (after organ reperfusion) was recorded using MAESTRO™. In this case, the nanostructures tested also exhibited renal excretion profiles without unexpected toxicities from unspecific accumulations (Figure 6.32). Remarkably, the % ID obtained in brain in all cases was significantly higher than with the previous carriers, with values up to 1.5 % ID considered similar to those in literature for systems that “cross” the BBB.16

Up to this point it could be said that, one of the major challenge in this thesis was already accomplished, as we were able to design biodegradable, multivalent and well-controlled drug delivery carriers capable to surpass the BBB.

With the optimized covalently captured constructs capable to promote brain accumulation, therapeutics for AD treatment and/or diagnostics were pursued always taken into account the advantages to implement combination therapies. Curcuminoids, in particular bisdemethoxycurcumin (BDMC), was selected as a model drug with far demonstrated activities and a great potential for the treatment of AD. In
combination with propargylamine moieties, BDMC was conjugated to the clicked structures via ester bonding. Then, the BDMC-decorated structures were in vitro evaluated in terms of cell viability, drug release profiles at different pHs, and in vitro activity as inhibitors of fibril formation. In order to achieve proof of concept, the activity of the compounds was firstly checked using an accepted model based on the use of Hen Egg White Lysozyme (HEWL) for protein amyloid formation. Several BDMC bearing compounds and free BDMC for comparison at two different concentrations (10 and 50 μM drug-eq.) were incubated for 24 hours with HEWL and their inhibitory potential was measured by Thioflavin T (ThT) fluorescence at different time points (Figure 7.16). These results were further confirmed by TEM (Figure 7.17).

In addition, the neuroprotective effect of the curcuminoid bearing polymeric structures was evaluated in organotypic cultures from entorhinal cortex-hypocampus. In order to study neuroprotection, the experimental design included pretreatments with the polymer conjugate prior to Amyloid-β peptide (Aβ_{1-42})-induced neurotoxicity. In these conditions, pretreatment with either 1 or 5 μM BDMC-eq. significantly reduced cell death in Aβ_{1-42} peptide treated cultures (Figures 7.19 and 7.20). 1 μM BDMC-eq. was selected as the concentration to move forward and further experiments are ongoing in order to identify possible mechanisms of neuroprotection.

As for the other clicked structures evaluated, biodistribution of BDMC bearing compounds dually labeled with DO3A-Gd^{3+} and Cy5.5 was carried out using C57Bl/6 mice and optical imaging techniques. The biodistribution profile was comparable to those obtained for the other clicked architectures analyzed (targeted and non-targeted both without BDMC). Renal excretion profiles were again observed, significant brain accumulations (around 1.2 % ID), and lung accumulation at the early time points highlights the bigger size of the structures (Figure 7.23). Finally, looking for a definitive proof of pharmacological activity, a preliminary in vivo experiment in a young AD mouse model (ArcABeta 8-11 months) was performed. Multiple doses during a 2-week treatment were administered (Figure 7.27). This model resulted highly variable and up to now no conclusive data regarding activity could be drawn. A more controlled in vivo experiment is ongoing. Importantly, from this first in vivo experiment the safety of our carriers was fully demonstrated.
So far, the data obtained from the organotypic cultures together with the \textit{in vivo} biodistribution data and safety profiles with BDMC-bearing clicked architectures highlights the promising potential for the use of these novel nanomedicines in AD treatments. Those preliminary results encourage us for further \textit{in vitro} investigation considering other pathological hallmarks of the disease where BDMC could be effective, as well as proper \textit{in vivo} activity experiments are planned in order to unravel the full potential of these systems as nanotherapeutics for neurological disorders.

Finally, in the frame of a FP7-European project, LIVIMODE, a PGA-based \textit{in vivo} imaging platform based on protease-activatable smart ligands to allow non-invasive quantitative assessment of target expression in diseased tissue (diagnosis) and monitoring of disease progression (staging) was developed. Such probes were based on a NIRF pair and cleavable sequences by specific enzymes. Upon enzyme cleavage, quencher release led to fluorescence detection. By means of optimized conjugation to PGA-based carriers, tissue specificity was provided to the enzyme specific activatable smart probes synthesized by our collaborators. Several NIRF probes where conjugated applying different linking chemistries to PGA derivatives. To this respect, conjugates from MMP-9, 12 and 13, cathepsins B and S, as well as FAP selective probes were generated and evaluated. Overall, the results from the polymeric probes in terms of enzymatic studies showed that a selective enrichment of dye-labeled polymers was possible and that such probes retained and even enhanced their ability to be cleaved by the specific proteases in a selective manner. \textit{In vivo} studies corroborated this fact.

For instance, the MMP-12 as well as the cathepsin-B generated probes exhibited a really good performance \textit{in vitro}, with great potential to be used in cancer detection. Remarkably, with MMP-13 selective polymeric probe, its potential use in the early detection of osteoarthritis was demonstrated with outstanding results in a mouse model of osteoarthritis based on the destabilization of the medial meniscus (DMM). Active targeting strategies were additionally investigated. Regarding OA targeting, the use of the well-known Rothenfluh peptides did not improve conjugate accumulation, which was found to accumulate “per se” in the damaged knees from the DMM OA model after i.v. injection.
As a conclusion it could be said that the results obtained so far, clearly point out that these smart polymeric probes are highly suitable as early-detection molecular diagnostic tools with a high signal to noise ratio and a slow clearance rate. In particular, with MMP-13 probe the data suggested that this macromolecular probe could be qualified as an excellent candidate for theranostic applications in combination with adequate drugs. Furthermore, at the preclinical level our approach offers a significant potential for multimodal imaging due to easy conjugation to PET or MRI tracers apart from OI and would further assist in conceptualizing clinical studies. Finally, it is important to note that, the therapeutic indication of these molecular diagnosis probes could be easily redirected towards neuroimaging with the developed constructs within this thesis, possibility currently being explored with some of the developed probes (MMP12, MMP9 or cathepsin B).

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Final Conclusions
A novel versatile methodology for N-Carboxyanhydride Ring opening polymerization (NCA ROP) was applied to enable easy, multigram scale synthesis of well-defined narrowly distributed polyglutamates (PGA). Ammonium salts with non-nucleophilic tetrafluoroborate counteranions have been used, for the first time, as initiators, allowing to reach, without the use of complex and expensive techniques, polymers with MW (up to 800 units), low Đ (< 1.2), controlled chain end functionality, adequate stereoselectivity and absence of traces of toxic impurities, a requisite for future biomedical applications.

The living character of this technology, and the fact that the initiator is always introduced within the C-terminus of the polymer backbone, allow the synthesis of different polyglutamate-based systems depending on the initiator used, ranging from homopolymers, to di-block copolymers (peptidic or hybrid), as well as C-terminus functionalized polymers for further site-specific bioconjugations.

The deprotection protocol enabled acidic as well as basic deprotection of PBLG, allowing the synthesis of hybrid PEG based block copolymers and the incorporation of sensitive functional groups, always maintaining the correct stereochemistry.

The versatility of this technology has been far demonstrated with its use to reach polyglutamate-based star-shaped polymers with low Đ (< 1.2) in a relatively short time and in a variety of MW including high and adjustable molecular mass. Moreover, reducible initiators containing disulfide bonds gave convincing evidence of the living character of the methodology with comparable MW in all arms.

A useful methodology for the synthesis of PGA derivatives bearing orthogonal reactive sites, e.g. azides, alkynes, reactive disulfides, protected amines, etc. in combination of the carboxyl functionalities of PGA has been reported. The use of DMTMM and its wide range of possibilities allowed the linkage of water as well as DMF soluble compounds to PGA, which could be used for site-specific conjugation of a variety of bioactive agents of different nature (i.e. peptides, proteins, drugs).

Novel PGA-based structures have been designed, bearing self-assembling motifs that has been demonstrated to induce the
organization of anionic polyglutamate backbones. According to DLS, TEM and SANS, the resulting polymers undergo spontaneous self-assembly in water yielding spherical objects. Chemical modifications within the polypeptidic backbone as well as the tuning of physico-chemical conditions can prevent or significantly modify this aggregation behavior leading to a vast range of nanometric architectures of different size and shape.

- Moreover, co-assembly of different building blocks also takes place, allowing to open a wide field for the construction of relatively complex architectures. These structures can be covalently entrapped to retain their solution conformation in the biological settings using different reversible or irreversible linking chemistries.

- In vitro biocompatibility (MTS assays) was not compromised with the use of the different polymeric architectures as well as degree of functionalization. The degree of functionalization as well as the nature of the chains added within the polymer backbone significantly influenced the degradation rate of the final construct.

- Energy-dependent mechanisms of internalization were observed for all polymers (independently of the MW or the architecture), presumably through endocytic mechanism due to the absence of uptake at 4 °C and lysosomal co-localization with fluorescence markers. This degree of internalization was highly increased when star-shaped polymers where used as unimers and even more with the complex covalently entrapped architectures. All this together highlights the importance of the architecture in the biological output.

- Different PGA-based architectures with and without targeting units for BBB crossing (c-PEP and ANG-2) were in vivo evaluated in terms of biodistribution and pharmacokinetic profiles by means of different imaging techniques, such as PET and fluorescence optical imaging. Linear, di-block copolymers and star-shaped unimers did not reach significant brain accumulation in comparison with already existing systems. Nonetheless, the newly described architectures resulting from self-assembled PGAs, decorated with imaging agents and targeting units (ANG-2), by surface modifications of covalently
entrapped structures, showed interesting \textit{in vivo} biodistributions with relevant brain accumulations (up to \(\sim 1.5\%\) ID).

- The established bottom-up synthetic approach has been effectively used to synthesize bisdemethoxycurcumin (BDMC) and propargyl bearing combination conjugates with potential application as a neurorescuer-neuroprotective therapy in Alzheimer’s disease. \textit{In vitro} proof of activity has been achieved in terms of inhibition of fibril formation, and as neuroprotective agent against \(\text{A}\beta_{1-42}\) peptide induced neurotoxicity in organotypic cultures. Conjugate \textit{in vivo} safety was demonstrated in an AD mouse model after a repeated dose schedule. Proof of pharmacological activity \textit{in vivo} is ongoing.

- Finally, the potential of PGA-based conjugates as tissue-specific smart probes has been fully demonstrated. The combination of NIRF enzyme specific smart probes together with the tissue specificity provided by PGA as carrier allowed the early detection of disease-related events \textit{in vitro} as well as \textit{in vivo}. \textit{In vivo} data of selected candidates (i.e. MMP13) has pointed out the suitability of these polymeric probes in theranostic applications including neuroimaging approaches.
APPENDIX I. THESIS PROJECT, OBJECTIVES, MAIN METHODOLOGY, RESULTS AND CONCLUSIONS IN SPANISH.

1. INTRODUCCIÓN Y MARCO TEMÁTICO DE LA TESIS.

El creciente aumento tanto en la incidencia como en los costes de las enfermedades neurodegenerativas, junto con la falta de tratamientos efectivos, pone de manifiesto la necesidad de nuevas aproximaciones para hacer frente a la enorme carga que dichas enfermedades conllevan. La mayoría de los tratamientos actuales se basan en el tratamiento de los síntomas de la enfermedad, pero no en el tratamiento del origen para parar su progresión. Además, el descubrimiento y desarrollo de nuevos fármacos enfocados a enfermedades del sistema nervioso central se ha convertido en una ardua y desafiante tarea debido a que dichos fármacos deben ser capaces de superar la barrera biológica más infranqueable del cuerpo humano: la barrera hematoencefálica (BHE). Actualmente, se llevan a cabo técnicas tanto invasivas como no invasivas para permitir la entrada de fármacos a través de la BHE, de manera que se alcancen concentraciones de fármaco en el parénquima cerebral que se encuentren dentro de la ventana terapéutica para ser efectivos. En la presente tesis, se han combinado varias estrategias no invasivas basadas por un lado, en el uso de nanosistemas para mejorar la acumulación en cerebro junto con el uso de “caballos de Troya” moleculares para promover la entrada a través de la BHE mediante transporte mediado por receptores. Para ello, se propuso el uso de Polímeros Terapéuticos (PT) como nanosistemas para el tratamiento y/o diagnosis de enfermedades neurodegenerativas. Con varios productos ya en el mercado y un número creciente de ejemplos en desarrollo clínico, los PT exhiben características clave para superar las principales limitaciones del uso de fármacos o sondas de bajo peso molecular. Se considera que los PT poseen todos los requisitos para ser considerados como excelentes candidatos para su uso como plataforma para el tratamiento y diagnosis de enfermedades relacionadas con el sistema nervioso central (SNC). Entre dichas características se encuentran: (i) perfiles farmacocinéticos mejorados y tiempos de vida media en plasma prolongados; (ii) la posibilidad de incluir vectores dirigentes específicos para trasporte activo; (iii) multivalencia, lo que permite
modular la carga de fármaco(s) conjugados y el diseño de polímeros de combinación, incluyendo sondas para imagen, (iv) liberación controlada y sostenida de los fármacos conjugados a través de enlaces biodegradables bajo condiciones específicas; (iv) proporcionan estabilidad y reducen la inmunogenicidad del compuesto bioactivo conjugado, además de poseer un tamaño óptimo para permitir una buena penetración. Actualmente se acepta que los futuros retos y oportunidades para el avance de esta plataforma tecnológica se basan en (i) nuevas dianas moleculares, (ii) la terapia de combinación, (iii) control en las estructuras poliméricas y su conformación en solución con la ayuda de caracterización físico-química exhaustiva, esencial para transformar un conjugado prometedor en un candidato para la evaluación clínica, siguiendo las indicaciones reglamentarias

Existe aún la necesidad dentro del campo de PT de desarrollar nuevos polímeros para su uso en sistemas de transporte de fármacos o agentes de imagen (Capítulo 1). Nuevos PT basados en aminoácidos se postulan como excelentes candidatos para dicho fin, ya que no sufren de las limitaciones clásicas de los sistemas actuales (biodegradabilidad, multifuncionalidad y biocompatibilidad). Concretamente, los poliglutamatos (PGA) constituyen una plataforma versátil que se ha utilizado efectivamente como bloques de construcción en los conjugados polímero-fármaco y micelas poliméricas para diversas aplicaciones médicas que van desde el cáncer a procesos isquémicos. Por otra parte, se espera su aprobación por la FDA después de la aprobación del conjugado PGA-paclitaxel, Opaxio™ para el tratamiento de varios tipos de cáncer solos o en combinación (Opaxio™ ha sido designado recientemente como fármaco huérfano en combinación con radioterapia y temozolomida para el tratamiento de glioblastoma multiforme).

Además, hay un creciente interés en el desarrollo de nuevos sistemas poliméricos biodegradables, con arquitecturas y conformaciones más definidas y predecibles, pesos moleculares superiores (para mejorar el transporte pasivo mediante el efecto EPR), menor heterogeneidad, mayor capacidad de carga de fármaco y mayor posibilidad de multifuncionalidad. Asimismo, el uso de técnicas físico-químicas adecuadas, a fin de caracterizar correcta y exhaustivamente nuevos sistemas complejos, es un requisito.

Con todo esto en mente, la tesis tiene como objetivo lograr nuevas estructuras basadas en poliglutamatos bien definidos, para ser
utilizados como sistemas para el tratamiento y/o diagnóstico de enfermedades neurodegenerativas (concretamente Alzheimer (AD)), con el fin de atravesar la BHE, utilizando estrategias de transporte activo mediante el uso de caballos de Troya moleculares.

2. OBJETIVOS DE LA INVESTIGACIÓN.

El objetivo global de la presente tesis se centra en el diseño y desarrollo de una nueva plataforma polimérica basada en el uso de polímeros biocompatibles y biodegradables de diversas arquitecturas para su aplicación en biomedicina, y en concreto, en el tratamiento y/o diagnóstico de patologías tan devastadoras como las enfermedades neurodegenerativas. Dicho objetivo general, engloba los siguientes objetivos específicos que pueden ser considerados de índole piramidal:

(i) Diseño y puesta a punto de una metodología versátil de polimerización controlada para la obtención de nuevos portadores poliméricos biodegradables, biocompatibles, y con alto grado de multifuncionalidades, basados en el ácido poliglutámico (PGA). Los portadores sintetizados se caracterizarán de forma exhaustiva mediante una batería de técnicas físico-químicas. Una vez establecida, dicha metodología se usará para conseguir los siguientes objetivos.

(ii) Diseño, síntesis y caracterización exhaustiva de arquitecturas macromoleculares complejas basadas en poliglutamatos, obtenidos a través de técnicas de polimerización NCA previamente optimizadas. Se llevará a cabo una caracterización físico-química completa, así como su evaluación biológica (in vitro e in vivo) para validar esta nueva plataforma como portadores poliméricos.

(iii) Desarrollo de una metodología versátil para la modificación post-polimerización de los poliglutamatos sintetizados anteriormente, permitiendo alcanzar funcionalizaciones ortogonales altamente adecuadas para posteriores bioconjugaciones tanto de agentes bioactivos como de sondas de imagen.

(iv) Obtención de sistemas poliméricos basados en PGA para el transporte a través de la barrera hematoencefálica (BHE) como pre-requisito para el tratamiento de enfermedades del sistema nervioso central.

(v) Desarrollo de una nueva plataforma de polímeros para su uso como sistemas de transporte de fármacos o sondas para diagnóstico de enfermedades como el Alzheimer.

(vi) Obtención de una plataforma polimérica para su uso en imagen in vivo, basada en el uso de “sondas inteligentes”, activadas tras
actividad proteolítica. Todo ello dentro del marco del proyecto europeo del FP7 LIVIMODE.

Así pues, durante este proyecto se espera construir y evaluar las diferentes piezas indispensables para un diseño final basado en el concepto de “teranóstico” (del inglés, therapy + diagnostic), en el cual se combinen en una sola entidad, todos los bloques desarrollados en la tesis: nanovektores para el cruce de la BHE, polímeros-conjugado de combinación para el tratamiento de AD, y el uso de sondas inteligentes.

3. METODOLOGÍA

3.1. Materiales e instrumentación.

3.1.1. Materiales.

Todos los reactivos usados durante la tesis fueron de grado analítico o superior, y se usaron sin purificación adicional, a no ser que se indique en el correspondiente apartado. Los disolventes usados también eran de grado analítico o superior, y se secaron y destilaron previamente a su uso. De manera general, las reacciones llevadas a cabo en disolventes orgánicos se realizaron bajo atmósfera inerte de nitrógeno o argón.

Los métodos más utilizados de purificación de los polímeros-conjugados se basaron en el uso de diálisis (usando Vivaspin® con membranas de 1, 3, 5 o 10 kDa), o cromatografías por exclusión de tamaño, usando resina Sephadex G-25, o las columnas comerciales pre-empaquetadas PD10.

Los animales de experimentación usados tanto en CIEMAT, Madrid (FVB/NJ) como en Animal Imaging Center, Zúrich (C57Bl/6 y ArcAbeta), se mantuvieron en las condiciones y acorde a los protocolos de los respectivos comités éticos de experimentación animal. En ambos casos, con ciclos de 12 horas de luz y 12 de oscuridad, acceso libre a comida y agua, temperatura alrededor de 20-24 °C y humedad relativa de un 40 %.

3.1.2. Instrumentación.

Espectroscopía por Resonancia Magnética Nuclear (RMN). Los espectros de protón y carbono mono y bidimensionales, así como los experimentos DOSY y NOESY, se adquirieron en un Bruker AC 300, y Bruker Avance III 500 (dependiendo del caso) a temperatura ambiente y se analizaron usando el Software MestreNova 6.2.
Cromatografía por permeación en gel. Los análisis se realizaron en un Triple Detector Array (TDA™) de Viskotek (TDA3 302) complementado con un índice de refracción (RI), detector de difracción de la luz, detector de viscosidad y un detector UV modelo 2501. Las columnas empleadas fueron dos TSK-Gel en serie (Styragel HR3 y HR4, tamaño partícula 6 μm, 300 x 7.8 mm). Como fases móviles se utilizaron, por un lado, DMF 0.1 % LiBr, con el sistema equilibrado a 70 °C; y por otro, HFIP (3 g·L⁻¹ trifluoroacetato potásico) a 40 °C y columnas de sílica modificada (PFG, tamaño partícula 7 μm, porosidad 100 & 1000 Å). Los pesos moleculares se calcularon usando calibración interna realizada con estándares de PMMA y tolueno como patrón interno. Para el tratamiento de datos, se utilizó en ambos casos el software OmniSec 4.1.

Espectroscopía de infrarrojo (FTIR). Los espectros de IR se obtuvieron mediante espectrómetro FT-IR Thermo Scientific Nicolet 380 en un rango espectral de 7800-350 cm⁻¹, resolución óptica (apodizada) < 0.9 cm⁻¹, resolución estándar. Todas las muestras se analizaron disueltas en DMF, y a 25 °C.

Espectroscopia por Dicroísmo circular. Se realizó en un espectrómetro J-815 CD Spectrometer (JASCO Corporation) equipado con un soporte de celda termostatizado (PTC-423, JASCO Corporation), un refrigerante (JULABO F250, JASCO Corporation) y flujo controlado de nitrógeno (~2.7 L.min⁻¹) (Afriso Euro-Index). Las muestras se midieron en HFIP en el caso de polímeros bencil protegidos y en agua desionizada o PBS 7.4 para polímeros en la forma sal sódica. Las medidas se realizaron en cubetas de cuarzo, de d= 0.1 cm y por triplicado.

Dispersión de luz dinámica. Las medidas de tamaño de partícula así como los experimentos de CAC se realizaron en un dispositivo Malvern Zetasizer Nano ZS equipado con un láser (532 nm) a un ángulo de dispersión fijo de 90°. Las muestras se midieron en tampón PBS o ddH₂O a diferentes concentraciones, generalmente a 25 °C, y por triplicado.

Espectroscopía ultravioleta-visible (UV-VIS). Los espectros de UV se recogieron en un espectrofotómetro Jasco V-630 UV/Vis a 25 °C con celdas de cuarzo de 1 cm y ancho de banda de 0.5 nm.

Polarimetria. La quiralidad de los polímeros desprotegidos se evaluó con un polarímetro Jasco P-1020, midiendo cada muestra por triplicado (20 scans por medida) en una celda de vidrio cilíndrica (φ 3.5x100
Las muestras se prepararon en ddH2O a 10 mg·ml⁻¹ y se midieron a 25 °C. Los datos se analizaron con el software Spectra Manager. Victor² Wallace™ para medidas de absorbancia o fluorescencia. Para determinar absorbancias o fluorescencias en fracciones de purificación o ensayos celulares se usó un equipo Victor² Wallac 1420 Multilabel HTS Counter Perkin Elmer (Northwoolk, CT, EEUU) utilizando placas de 96 pocillos y las correspondientes longitudes de onda (emisión/excitación) propias de cada compuesto.

**Microscopia de transmisión electrónica (TEM).** Las fotos se adquirieron en un microscopio de transmisión electrónica FEI Tecnai G2 Spirit (FEI Europe, Eindhoven, Netherlands) usando una cámara digital Morada (Olympus Soft Image Solutions GmbH, Münster, Germany). Para su preparación, las muestras se adsorbieron en rejillas de cobre recubiertas por una película de carbón de 200 mallas. Tras ello, se realizó una tinción de contraste negativo con una disolución de acetato de uranilo al 2 %.

**Cryo-TEM.** Para adquirir las imágenes en un Cryo-TEM, Se vitrificaron las muestras en un vitrobot FEI. Posteriormente dichas muestras se transfirieron bajo nitrógeno líquido a un soporte de Cryo-TEM (Gatan) equipado con un reservorio de nitrógeno líquido. Tras ello, la muestra se trasfirió a un equipo Tecnai T20 (FEI company) y se adquirieron las fotos a 100 K.

**Nanodrop™** Para determinar la cantidad de péptidos en las conjugaciones se usó un Nanodrop™ ND-1000 UV-Vis Spectrophotometer. Brevemente, las fracciones resultantes de la purificación por cromatografía por exclusión de tamaño se analizaron en el Nanodrop™ a una longitud de onda de 280 nm (triptófano).

**Dispersión de neutrones de ángulo pequeño (SANS).** Los experimentos de SANS se llevaron a cabo en un instrumento D11 en el Institute Laue-Langevin (Grenoble, Francia) por nuestros colaboradores de la School of Chemistry in Cardiff University (Cardiff, UK). Las muestras en disolución fueron preparadas con una concentración de conjugado de 10 mg·mL⁻¹ en PBS deuterado 0.1 M y se colocaron en células de cuarzo de 2 mm de camino óptico, manteniendo una temperatura de 37 °C. Para el análisis de datos se usó el modelo FISH.

**Microscopía confocal de fluorescencia en célula viva.** Los estudios de internalización celular por microscopía confocal se hicieron en el Servicio de Microscopía Confocal del CIPF (Valencia). Las imágenes se adquirieron con un microscopio (invertido) láser confocal Leica,
modelo TCS SP2 AOBS (Leica Microsystems Heidelberg GMBH, Mannheim, Alemania) usando un objetivo de inmersión de 63X Plan-Apochromat-Lambda Blue 1.4 N.A. Se usó longitud de onda de 488 nm para el Oregon Green (OG), utilizando para ello un láser de argón usando aperturas de canal de 503 a 604 nm. Todas las imágenes se adquirieron bajo las mismas condiciones y se analizaron mediante el software de Leica “Leica Lite” versión 2.61.

**Maestro**\(^{\text{TM}}\). Las imágenes de los órganos *ex-vivo* en 2D se obtuvieron usando un Maestro 500 (Cambridge Research Inc, Woburn, USA). La fluorescencia se detectó con una cámara CCD. Las imágenes adquiridas a diferentes longitudes de onda se sometieron a un “spectral un-mixing” para discriminar la autofluorescencia del tejido. Las medidas se realizaron en Zúrich, Suiza, en el Animal Imaging Center.

Los ensayos por tomografía de emisión de positrones (**PET**). Para el estudio de biodistribución mediante PET, se utilizó un aparato Argus PET/CT (SEDECAL, Madrid) para la monitorización *in vivo* y se cuantificó la radioactividad *ex vivo* en un contador de centelleo gamma Cobra II auto-gamma. Los ensayos se realizaron en la unidad de Aplicaciones Biomédicas y Farmacocinéticas del CIEMAT, Madrid.

3.2. Métodos más relevantes.

3.2.1. Protocolos de síntesis.

**Síntesis de monómero NCA del ácido glutámico.** H-L-Glu(OBzl)-OH (17 g, 71.66 mmol) se añadió a un matraz de 250 mL de dos bocas equipado con un agitador magnético, una columna de reflujo, un embudo de adición compensada y una entrada y salida de Ar. Se purgó el sistema por 5 minutos. Después, se añadieron 120 mL de THF anhidro y se calentó la mezcla a 60 °C. Tras ello, se añadió limoneno (11.6 mL, 71.66 mmol, 1 eq.) y posteriormente difosgeno gota a gota (5.2 mL, 8.5 g, 43 mmol, 0.6 eq.), disuelto en 10 mL más de THF. Se dejó la mezcla reaccionar durante 3 horas a 60 °C hasta que se convirtió en una solución clara. Posteriormente se burbujeó la disolución con argón para eliminar el exceso de HCl. Tras ello, se concentró la mezcla hasta un cuarto del volumen original y se añadieron 32 mL de acetato de etilo. El contenido se precipitó en hexano frío dando lugar a un precipitado blanco. Finalmente, el sólido se recristalizó en THF/tolueno con la adición lenta de hexano bajo atmósfera inerte. Finalmente, se filtró el sólido y se almacenó a -20 °C. La pureza del monómero se
analizó por RMN, determinando el punto de fusión, y determinando la ausencia de HCl con el test de nitrato de plata. Rendimiento 70-80 %.

**Método general para la síntesis de las sales de BF₄ como iniciadores.**

De manera general, se prepararon las sales de las diferentes aminas (n-butil amina, neopentil amina y MeO-PEG2000-NH₂) partiendo de la correspondiente amina, disolviendo ésta en THF o dietil éter y usando el ácido HBF₄·Et₂O. Tras ello, se produce la aparición de un precipitado blanco, que es filtrado y recristalizado tres veces en acetato de etilo. Tras secarse a vacío, su identidad se analizó por RMN (fluor, carbono y protón) y se usó como iniciador NCA. Rendimientos cuantitativos.

**Método general para la preparación de iniciadores multifuncionales con forma de estrella.**

La síntesis de los iniciadores de forma estrella se llevó a cabo en dos pasos, de manera general, para el iniciador basado en etilendiamina: En un matraz de fondo redondo de dos bocas provisto de un agitador magnético y entrada y salida de nitrógeno, se disolvió el compuesto tricloruro de 1,3,5-bencenotricarbonilo (1.88 mmol, 1 eq.) en 12 mL de THF anhidro. Posteriormente, se añadieron 3.3 eq. (6.22 mmol) de DIEA, seguido de la adición gota a gota de N-Boc-etilendiamina (6.22 mmol, 3.3 eq). La mezcla se dejó reaccionar durante 2 horas. Después de ello, el disolvente se evaporó completamente, el producto se disolvió en CHCl₃ y se lavó con agua desionizada (3x) y con agua ácida a pH 3 (3x). El producto aislado se recristalizó 3 veces de THF/metanol/hexano dando lugar a un sólido blanco. En un segundo paso, se desprotegió el grupo Boc y se formó la sal directamente mediante el uso de 3.3 eq. de HBF₄·Et₂O, en diclorometano. Se produjo la formación instantánea de un precipitado blanco que se filtró y recristalizó 3 veces de THF/metanol/hexano. La identidad del producto se estableció por RMN. Rendimiento: cuantitativo.

**Procedimiento general para la polimerización NCA.**

Brevemente, 0.5 g, (1.9 mmol) del monómero NCA se añadieron a un tubo Schlenk equipado con un agitador magnético y un tapón. Tras purgar tres veces con ciclos de vacío/argón, se añadieron 5 mL de DMF (recién destilado). Posteriormente, se añadió el correspondiente iniciador y la mezcla se dejó reaccionar a 4 °C, 25 o 40 °C (dependiendo de la síntesis) durante 3 días, bajo atmósfera de nitrógeno y presión constante. Después de 3 días, la disolución se precipitó en un exceso de dietil éter frío dando lugar a un precipitado blanco. Rendimiento: 70-90 %. Los productos se caracterizaron por RMN y GPC.
Procedimientos óptimos de desprotección de los grupos bencilo en los polímeros.

a) Desprotección ácida para homopolímeros. En un matraz de fondo redondo equipado con un agitador magnético y un tapón, se disolvieron 100 mg el polímero PBLG (linear o estrella) (0.0035 mmol UAG, unidades de ácido glutámico) en 3 mL de TFA. Una vez disuelto, 2 eq. de HBr (48 % v/v, 1.49 g·cm\(^{-3}\)) por grupo carboxílico se añadieron gota a gota. La mezcla de reacción se dejó agitando 5 horas. *Nota, para cantidades de 600 mg, se necesitaron 16 horas de reacción. Tras ello, la disolución se precipitó en un exceso de dietil éter frío, dando lugar a un sólido blanco. El producto se convirtió en la forma sal sódica añadiendo bicarbonato sódico, y se desaló por sucesivas precipitaciones ácido-base, o usando G25 o dializando. Rendimientos: 75-86%. El producto se caracterizó por RMN.

b) Desprotección básica para di-bloques híbridos PEG-PBLG. En un matraz de fondo redondo, se disolvieron 50 mg del di-bloque protegido (0.184 mmol UAG) en 16 mL de THF a temperatura ambiente. Entonces, la disolución se enfrió a 4 °C y se añadieron 2 eq. por ácido carboxílico de NaOH (0.369 mmol). Tras ello, la disolución se dejó agitando a 4 °C durante 16 horas. Transcurrido ese tiempo, se evaporó el THF y el producto se diluyó en agua desionizada para su purificación por diálisis (Vivaspin® MWCO 3000 Da). Tras liofilizarse, se obtuvo un sólido blanco que se caracterizó por RMN.

Acoplamientos peptídicos usando reacciones ácido-base. Enlaces amida.

a) Método general para la modificación post-polimerización de PGAs usando DMTMM·Cl.

Protocolo 1. DMTMM·Cl para soluciones acuosas. En un matraz de fondo redondo equipado con un agitador y un tapón se disolvieron 200 mg de PGA forma sal (1.55 mmol UAG, 1 eq.) en 10 mL de agua desionizada. Tras ello, se añadió la correspondiente cantidad de DMTMM·Cl para el % de modificación deseado (para un 30 % de modificación, 0.3 eq.). Tras 10 minutos, se añadió el doble de eq. que de DMTMM·Cl de la correspondiente amina a introducir (para un 30 % de modificación 0.6 eq.). Se ajustó el pH a 8, y la mezcla se deja reaccionar durante 16 horas a temperatura ambiente. Tras ello, se realizaron purificaciones estándar usando diálisis, o G25, o precipitación ácido-base. El producto se caracterizó por RMN. Rendimientos: 80-90 %. Eficacias de conjugación 80-100 %.
Protocolo 2. DMTMM-BF$_4$ para soluciones orgánicas. En un matraz de fondo redondo de dos bocas equipado con un agitador magnético y entrada y salida de nitrógeno se disolvieron 200 mg de PGA forma ácida (1.55 mmol, 1 eq.) en 10 mL de DMF anhidro. Tras ello, se añadió la correspondiente cantidad de DMTMM-BF$_4$ para el % de modificación deseado (para un 30% de modificación, 0.3 eq.). Tras 10 minutos de reacción, se añadió el doble de eq. que de DMTMM-BF$_4$ de la correspondiente amina a introducir (para un 30 % de modificación 0.6 eq.). Se ajustó el pH a 8, y la mezcla se deja reaccionar durante 16 horas a temperatura ambiente. Tras ello, se realizaron purificaciones estándar usando diálisis, o G25, o precipitación ácido-base. El producto se caracterizó por RMN. Rendimientos: 80-90 %. Eficacias de conjugación 90-100 %

b) **Método general mediante DIC/HOBt en soluciones orgánicas.** En un matraz de dos bocas provisto de un agitador magnético y una entrada y salida de nitrógeno, se pesó PGA forma ácida (0.225 mmol UAG, 1 eq.) y se disolvió en DMF anhidro bajo atmósfera de nitrógeno. A continuación, se añadió DIC (i.e. 0.15 eq. para una modificación de un 10 %) y se dejó reaccionar durante 5 minutos a temperatura ambiente. Tras ello, se adicionó HOBt (0.15 eq. para una modificación del 10 %). La reacción se dejó proceder durante 10 minutos más y se añadió a continuación 0.10 eq. de la amina correspondiente. Se ajustó el pH a 8 mediante la adición de DIEA. La mezcla se dejó reaccionar durante 48 horas. Finalmente, el disolvente se eliminó a vacío, y el producto se convirtió en la forma sal mediante la adición de bicarbonato sódico. Tras ello, se procedió a su purificación mediante los métodos estándar de diálisis, G25 o precipitación ácido-base. Rendimientos 80-90 %. Eficacias de conjugación 75-100 %.

**Reacciones Click.**

a) **CuAAC.** En un matraz de dos bocas equipado con un agitador magnético se disolvió 1 eq. de copolímero (modificado con azida o propargilamina) en agua desionizada. Tras ello, se añadió la correspondiente cantidad para el % de modificación deseado del agente a clicar en DMF. Después, se añadieron 5 eq. de ascorbato sódico en disolución acuosa, y la mezcla se desgasificó mediante dos ciclos de congelación, vacío y descongelación. Se añadió posteriormente 1 eq. de CuSO$_4$ en disolución acuosa y la mezcla final, conteniendo una proporción de DMF/H$_2$O de 4:1, se desgasificó de nuevo aplicando otro ciclo. La reacción se dejó proceder durante 72 horas protegida de la luz.
Los productos se purificaron usando las técnicas estándar de diálisis, G25 o precipitación ácido-base. Para el acoplamiento de moléculas solubles en agua, se usó el mismo protocolo pero usando sólo agua desionizada y desgasificada como disolvente. La eficacia de la reacción se valoró por RMN. Dicha reacción se utilizó tanto para la conjugación de sondas inteligentes (ver capítulo 8) como para el atrapamiento covalente de las estructuras auto-ensambladas (ver capítulo 6).

b) Tiol-eno.

Protocolo 1. En un matraz de fondo redondo equipado con un agitador magnético y un tapón se pesaron 50 mg de PGA modificados con tioles activados con el grupo 2TP y se disolvieron en tampón (PBS 7.4). Tras ello, se añadió el polímero modificado con grupos maleimida en una mezcla equimolecular y disuelto también en PBS 7.4 de manera que ambos estuvieran a concentración 4 mg·mL⁻¹. La mezcla se sonicó y seguidamente, se añadió el agente reductor TCEP (0.15 eq.), se comprobó el pH y la reacción se dejó agitando durante 16 horas. Tras ello, el producto se purificó por diálisis usando una membrana de 3.000 Da. Este protocolo se utilizó para el atrapamiento covalente de estructuras auto-ensambladas.

Protocolo 2. En un matraz de fondo redondo equipado con un agitador magnético y un tapón, se pesaron 50 mg de PGA modificados con tioles activados con el grupo 2TP, se disolvieron en tampón (HEPES 7.4). Tras ello, se añadieron los eq. correspondientes al deseado % de modificación del compuesto con grupo maleimida en DMF dando lugar a una mezcla (tampón/DMF ratio 5.5/1). Finalmente, se añadieron 10 eq. de TCEP disueltos en HEPES y la reacción se dejó bajo agitación durante 16 horas a temperatura ambiente y protegida de la luz. El producto se purificó utilizando uno de los métodos estándar de diálisis, G25 o precipitación ácido-base. Este protocolo se utilizó para la conjugación de sondas inteligentes con grupos maleimida.

c) Química de bisulfuros.

Protocolo 1. En un matraz de fondo redondo equipado con un agitador magnético y un tapón, 100 mg del polímero funcionalizado con tioles activados con el grupo 2TP, se disolvieron en tampón PBS 7.4 a una concentración de 2 mg·mL⁻¹. Se sonicó la muestra durante 5 minutos, tras los cuales se añadieron 0.15 eq. de TCEP a la mezcla de reacción. Se comprobó el pH y se dejó la muestra reaccionar bajo agitación vigorosa, a temperatura ambiente durante 2 horas. Transcurrido ese tiempo, el producto se dializó usando Vivaspin®
MWCO 3000. Este protocolo se usó para el atrapamiento covalente de forma reversible de polímeros auto-ensamblados.

Protocolo 2. Brevemente, en un matraz de fondo redondo se disolvió el polímero modificado con SS-2TP en tampón acetato de amonio 150 mM pH 5. En paralelo, se disolvió el compuesto bioactivo con grupos SH (mismos eq. que unidades de SS-2TP), en tampón HEPES a pH 7.4. Se mezclaron ambas disoluciones, y la mezcla se dejó reaccionar a pH 6 durante 16 horas. Tras ello, el producto se purificó usando columnas PD10. Este protocolo se usó para la conjugación del péptido dirigente ANG entre otros. Eficacia de conjugación 50-70 %.

Protocolos para el marcaje con sondas de imagen para estudios in vitro/in vivo.

a)  Marcaje con Oregon Green (OG) Cadaverina 488 para estudios de internalización celular. El marcaje con OG se realizó siguiendo el protocolo de modificación con DIC/HOBt (en el caso de PGAs en forma ácida: linear y estrella) y siguiendo el protocolo de modificación con DMTMM-Cl para el caso de PGAs en forma sal, como es el caso de los di-bloques PEG-PGA. El contenido de OG se determinó por fluorescencia usando el lector de placas Victor² Wallac™ con filtro de excitación de 490 nm de longitud de onda y filtro de emisión de 535 nm a través de una curva de calibrado previa. Rendimientos: 80-95 %. Eficacias de conjugación 80-90 %

b)  Marcaje con Cy5.5 para estudios de biodistribución por imagen óptica de fluorescencia in vivo. El marcaje con Cy5.5 se llevó a cabo siguiendo los mismos procesos y en los mismos casos que para OG. El contenido de Cy5.5 se determinó mediante fluorescencia usando el lector de placas Victor² Wallac™ (λ_em: 595 nm, λ_ex: 680 nm) después de construir una curva de calibrado apropiada. Rendimiento: 60-70 %. Eficacias de conjugacion 70-90 %

c)  Marcaje con sondas radioactivas para biodistribuciones con PET. DO3A/68Ga y DO3A/111In. Para la introducción de núcleos radioactivos en la cadena lateral de los polímeros de diferente arquitectura, primero se procedió a la conjugación del agente complejante DO3A-tBu-NH₂. Dicho agente complejante se conjugó a las diferentes arquitecturas de PGA usando, el protocolo estándar de DIC/HOBt para aquellos en forma ácida, solubles en disolventes orgánicos, y el de DMTMM-Cl, para aquellos en forma sal, solubles en soluciones acuosas. Posteriormente se procedió a la desprotección de los grupos ter-butóxido del agente complejante mediante el uso de dos protocolos diferentes, dependiendo de la naturaleza del compuesto: (i)
Para polímeros con grupos no sensibles a TFA, se desprotegió usando una mezcla de CH<sub>2</sub>Cl<sub>2</sub>/TFA (3/2, v/v) durante 16 horas a temperatura ambiente. (ii) Para polímeros con grupos sensibles a TFA, el polímero se disolvió en una mezcla de TFA/H<sub>2</sub>O/TIPS (95/2.5/2.5, v/v) y se agitó a temperatura ambiente durante tres horas. En ambos casos, tras el tiempo de reacción, la disolución se precipitó en un exceso de éter frío dando lugar a un sólido amarillo pálido. El éxito de la desprotección se analizó por RMN. Rendimientos de 80-90 %.

Una vez desprotegido el agente complejante, se llevó a cabo el marcaje con ⁶⁸Ga o ¹¹¹In en CIEMAT, Madrid. Brevemente, la complejación se llevó a cabo en un tubo de síntesis por microondas, y en tampón HEPES a pH 3.5-4. La mezcla de radionúclido y polímero marcado con agente complejante se calentó a 90 °C durante 5 minutos usando un microondas de laboratorio con radiación monomodal (Discover Benchmate, CEM). Tras ello, se enfrió el contenido con nitrógeno líquido, se purificó la reacción usando cromatografía por exclusión de tamaño (Bio Gel P-6 Biorad) con PBS pH 7 como eluyente. La actividad de las fracciones recogidas se midió en un activímetro (VDC 405 Veenstra).

d) Marcaje para MRI con DO3A-Gd³⁺. El grupo DO3A se conjugó a los polímeros de manera idéntica a la anteriormente explicada. Tras ello, se procedió a la complejación de gadolinio. Brevemente, en un matraz de fondo redondo, se disolvió el correspondiente polímero con unidades DO3A en PBS 0.1 M a pH 7.4. Tras ello, una cantidad equimolar de GdCl<sub>3</sub>, se añadió gota a gota disuelto en agua desionizada. Durante el proceso, el pH se monitorizó en todo momento. El grado de complejación se determinó mediante titración con 4-(2-piridilazo)resorcinol. A 5 horas de reacción no se detectó Gd libre y se procedió a la purificación del producto mediante diálisis usando Vivaspin® MWCO 5000.

**Protocolos para la conjugación de vectores dirigentes capaces de atravesar la BHE.**

Con respecto al diseño de polímeros para el cruce de la BHE, se llevaron a cabo dos estrategias básicas: la conjugación de dichos residuos dirigentes a la cadena lateral del polímero (es el caso de los lineales, estrella, PGA clicados y DB PEG-PGAs), o de forma semi-telequólica, en uno de los extremos (caso de DB2 PGA-PEG). Ambas estrategias se usaron para conjugar Angiopep2-SH (ANG-SH) y cPEP.
[Appendix I]

a) **Conjugación de ANG-SH.** Para la conjugación mediante bisulfuros de ANG-SH a la cadena lateral de los polímeros, primero se modificó el correspondiente polímero con cisteamina-2TP, siguiendo el protocolo anteriormente descrito con DMTMM-Cl. Tras ello, se llevó a cabo la conjugación con ANG según lo descrito en el protocolo 2 de la química de bisulfuros. Las fracciones de péptido se determinaron por absorbancia a 280 nm en Nanodrop™. Finalmente, la cantidad de péptido se estimó por RMN, y se confirmó mediante análisis de aminoácidos, llevado a cabo en la Universidad de Barcelona (Unitat de Tècniques Separatives I Síntesi de Pèptids Centres Científics I Tecnològics). Para la conjugación telequelítica del péptido, se llevó a cabo el mismo protocolo anterior pero usando PGA-PEG-SS4TP como polímero, y un exceso de ANG (2 eq.). Rendimientos 60-75 %.

b) **Conjugación del péptido ciclico (cPEP).** Para la conjugación a la cadena lateral de los polímeros, mediante enlace amida a través del extremo C-terminal del péptido, primero se modificó el correspondiente polímero con N-Boc-etilendiamina, siguiendo el protocolo anteriormente descrito con DMTMM-Cl. A continuación, se desprotegieron los grupos Boc, sometiendo al polímero a una disolución de CHCl₂:CH₃OH (3:2) durante 16 horas. El polímero con grupos amina libres se purificó por precipitación en acetona fría. Por otro lado, se llevó a cabo la activación del grupo ácido carboxílico del extremo c-terminal del péptido con DCC/NHS. Brevemente, en un matraz de dos bocas provisto de un agitador y una entrada y salida de nitrógeno, se disolvieron 30 mg del cPEP (0.032 mmol, 1 eq.) bajo atmósfera inerte, en DMSO anhidro. Tras ello, se añadieron 8.7 mg de DCC (0.043 mmol, 1.3 eq.) a la mezcla de reacción. 5 minutos después, se adicionó NHS (4.8 mg, 0.042 mmol, 1.3 eq.) y la reacción se dejó proceder durante 16 horas a temperatura ambiente y bajo atmósfera de nitrógeno. Transcurrido el tiempo, la mezcla se precipitó en un exceso de dietil éter frío, se filtró y secó. El porcentaje de activación se determinó del 100 % de acuerdo a RMN. Finalmente, se llevó a cabo la conjugación de cPEP a la cadena lateral de los polímeros mediante enlace amida. Para ello, se disolvieron en PBS 0.1 M pH 7.4, el correspondiente polímero, y el mismo número de eq. de cPEP activado que grupos amina del polímero. La mezcla se dejó reaccionar durante 5 horas, tras las cuales, se procedió a su purificación usando PD10. El contenido del péptido se estimó por RMN, y se corroboró mediante análisis de aminoácidos llevado a cabo en la Universidad de Barcelona.
En el caso de la conjugación telequélca del péptido, el procedimiento llevado a cabo fue el mismo, pero usando en este caso, DB2 PGA-PEG-NH₂ y un exceso de cPEP activado (2 eq.). Rendimientos: 75-80 %.

**Protocolo para la conjugación de Bisdemetoxicurcumina (BDMC) a PGA.** La conjugación del fármaco BDMC se llevó a cabo a través de enlaces éster, usando el protocolo de DMTMM-BF₄ pero con modificaciones. Brevemente, en un matraz de dos bocas provisto de un agitador magnético y una entrada y salida de nitrógeno, se disolvió el correspondiente polímero en 10 mL de DMF anhidro. Tras ello, se añadieron 1.5 eq. de DMTMM-BF₄ del tanto por ciento de modificación deseado, en 5 mL más de DMF. La reacción se dejó proceder durante 10 minutos, tras los cuales se añadió BDMC (mismos eq. que de DMTMM-BF₄), y una cantidad catalítica de DMAP. Se comprobó que el pH de la reacción rondase 7, y se dejó agitando a temperatura ambiente y protegida de la luz durante 72 horas. Para la purificación, la mezcla se precipitó en un exceso de dietil éter frío, el producto se transformó en su forma sal, en agua desionizada y se lavó con éter sucesivas veces. Finalmente, se desaló por diálisis usando Vivaspin® MWCO 5000. El contenido de BDMC se estimó por UV-VIS a 415 nm, tras realizar una curva de calibración apropiada. La cantidad de fármaco libre se estimó inferior al 1 % de la cantidad de fármaco total mediante HPLC, usando un método de gradientes donde: eluyente A: agua, eluyente B: acetonitrilo. El gradiente usado fue de 40 % B a 80 % B durante 20 min. Tiempo de retención de BDMC: 5.98

**Protocolos para la conjugación de sondas inteligentes (activadas tras la acción de proteasas específicas) a PGA.** Las sondas se conjugaron siguiendo un protocolo u otro en función del grupo funcional introducido para ese fin. Así pues, sondas con grupos alquino/azida se conjugaron a polímeros modificados con grupos azida/alquino respectivamente siguiendo el protocolo de CuAAC descrito anteriormente. Por otro lado, sondas con grupos maleimida se conjugaron a polímeros modificados con grupos SS-2TP mediante el protocolo descrito anteriormente para reacciones tiol-eno en mezclas tampón HEPES/DMF. Finalmente, sondas con grupos amina, se conjugaron de manera directa (o tras la derivatización con un espaciador de etilenglicol), a través del protocolo de DIC/HOBt. La derivatización de las sondas con el espaciador FMOC-EG(4)-COOH se llevó a cabo mediante la conjugación directa de dicho espaciador a la sonda-NH₂ usando el protocolo estándar de DIC/HOBt. Se purificó el
compuesto usando una columna de fase reversa C18 en metanol. Tras ello, se procedió a la desprotección del grupo FMOC, usando 2 mL Et₂NH/DMF (1/4) durante 45 minutos. El disolvente se evaporó y el producto se usó directamente para su conjugación.

El contenido de sonda en el polímero se determinó mediante absorbancia o fluorescencia a sus correspondientes longitudes de onda. **Protocolos utilizados en la caracterización de los compuestos auto-ensamblados.**

a) Determinación de la CAC mediante DLS. Para ello, se disolvieron los polímeros basados en PGA en PBS 7.4 a diferentes concentraciones dentro de un rango de 0.004-2 mg·mL⁻¹. Se usó PGA lineal como control negativo. Cada solución se preparó fresca para las medidas, se sonó durante 5 minutos y se dejó 24 horas para su estabilización. Las medidas de DLS se hicieron por triplicado con parámetros fijados para todas las muestras. Los datos se expresaron en Kcps vs concentración, donde la intersección entre las curvas nos da el valor de CAC. Los estudios de co-ensamblaje de diferentes polímeros se llevaron a cabo de igual forma, pero añadiendo a cada serie de concentraciones, una cantidad por debajo de su CAC del polímero con quien se quiere averiguar el co-ensamblaje.

b) Prueba del pireno. Para dichas medidas, se prepararon una serie de disoluciones de cada polímero en un rango de concentración de 0.004-2 mg·mL⁻¹. Se preparó además una disolución stock de pireno de 0.02 mg·mL⁻¹ en acetona, de la cual se añadieron 3 µL a cada una de las concentraciones de la gama de polímeros. Tras ello, las disoluciones se incubaron a 37 °C durante 2 horas y se midieron 24 horas después en un espectrofluorímetro Jasco FP-8300. Las medidas se llevaron a cabo a 1 cm de longitud de paso y cada espectro de excitación se adquirió desde 300 a 360 nm con emisión a 390 nm. Finalmente, se expresaron los datos gráficamente representando el cociente de intensidades I₃₃₈/I₃₃₃ vs concentración para determinar valores CMC.

3.2.2. Ensayos In vitro.

**Degradaciones con Catepsina B.** Se evaluó la biodegradabilidad de varios poliglutamatos en presencia de la enzima lisosomal catepsina B. Brevemente, se prepararon disoluciones de 3 mg·mL⁻¹ de polímero en tampón acetato (para 3 mg de polímero, 700 µL de tampón acetato 20 mM, pH 6, 100 µL de EDTA 2 mM, 100 µL de DTT 5 mM). Finalmente, se añadieron 6.25 unidades de Catepsina B disueltas en 100
µL de tampón acetato pH 6 20 mM). Las mezclas se mantuvieron a 37 °C bajo agitación, y se tomaron alícuotas a diferentes tiempos (0, 0.5, 1, 2, 4, 8, 24, 48 y 72 h). La pérdida de masa molecular del polímero durante la degradación se analizó por GPC en tampón PBS 7.4.

Cultivos celulares. Las células HUVEC se cultivaron en Medium 200 suplementado con LSGS, mientras que las células SHSY5Y se cultivaron en DMEM suplementado con FBS. Las células se mantuvieron a 37 °C en atmósfera de 5 % de dióxido de carbono y 95 % aire. El medio se cambió cada 2-3 días y se pasaron al menos una vez por semana cuando se alcanzó el 80 % de confluencia.

Ensayos MTS de viabilidad celular. Para los ensayos MTS, las células se cultivaron en placas estériles de 96 pocillos a una densidad celular de 35000 cell·cm⁻² para SHSY5Y y 1260 cell·cm⁻² en el caso de HUVEC. Las placas se incubaron durante 24 horas y transcurrido ese tiempo se añadieron los compuestos (previamente filtrados para su esterilización con un filtro de 0.2 µm). Transcurridas 72 horas de incubación, se añadió 10 µL de la mezcla, MTS/PMS (20:1) a cada pocillo, y se dejó incubar durante 2 horas más. Finalmente, se midió la densidad óptica de cada pocillo a 490 nm usando el lector de placas Victor² Wallac. Los valores de absorbancia de las células control (sin tratamientos) se tomaron como el 100 % de viabilidad.

Internalización de polímeros marcados con OG mediante FACS. Para ello, se sembraron células SHSY5Y en placas de 6 pocillos a una densidad de células de 35000 cell·cm⁻² y se incubaron por 24 horas. El experimento se realizó transcurridas dichas 24 horas tanto a 4 °C como a 37 °C. Para el experimento a 4 °C, las células se pre-incubaron a esa temperatura durante 30 minutos antes de empezar el experimento. En ambos casos, 30 minutos antes de añadir el compuesto a estudiar, se añadió 0.4 µL de una solución 5 µM de inhibidor de catepsina B CA-074, para alcanzar una concentración final de 2 µM. A continuación, se añadieron 0.01 mg·mL⁻¹ eq. de OG de los polímeros marcados con OG en 10 µL a diferentes tiempos (0 a 300 min) mientras que las células se mantenían en incubación tanto 37 °C como a 4 °C. Finalmente, las placas se pusieron en hielo, se lavaron las células dos veces con PBS-BSA 0.1 %, se suspendieron en 0.5 mL de PBS y el pellet se analizó en el citómetro de flujo Becton Dickinson FACS Calibur cytometer (California, USA) equipado con un láser de argón (488 nm) y filtro de emisión de 550 nm.
Internalización celular por microscopía confocal de fluorescencia.

Técnica en célula viva. Se incubaron las células en las mismas condiciones que para el análisis por citometría de flujo, sin embargo, la siembra se realizó sobre un cristal para poder recoger posteriormente las muestras. El experimento se llevó a cabo siguiendo un modo de pulso-carga a 37 °C. Primero, se añadió el inhibidor de catépsina B (misma cantidad que para citometría) 30 minutos antes de la adición del compuesto marcado con OG. De la misma forma, se añadieron 0.01 mg·mL⁻¹ eq. de OG de los polímeros marcados con OG en 10 μL y se incubaron las células durante 2 horas a 37 °C (pulso). Tras ello, se aspiró el medio de las células y éstas se lavaron con PBS conteniendo 2 μM de inhibidor de catépsina B. Posteriormente, las células se incubaron durante 4 horas a 37 °C (carga). 30 minutos antes de proceder al lavado de las células con PBS-BSA 0.1%, se añadió el marcador nuclear (Hoetch, 1 μL de una disolución 5 mM), y lisosomal (Lysotracker Red, 0.75 μL de una disolución 100 μM). Finalmente, se lavaron las células con PBS-BSA al 0.1 %, y el cristal se acopló a una cámara de microscopio con nuevo medio, conteniendo de nuevo 2 μM de CA-074. Se analizaron las muestras en el microscopio del Servicio de microscopía confocal del CIPF excitando con los láseres de argón (OG 496 nm) HeNe (Lysotracker Red 594 nm), y diodo azul (Hoetch 405 nm).

Ensavo de Tioflavina T para la detección de fibrilación de la lisozima HEWL. Las fibrillas de HEWL se formaron usando una disolución de 2 mg·mL⁻¹ de Hen Egg White Lysozyme en un tampón ácido 12 mM de HCl conteniendo 140 mM NaCl y 2.7 mM KCl (pH 2). Las muestras se agitaron magnéticamente a 60 °C, durante 24 horas para la formación de fibrillas. La cinética de fibrilación se siguió desde el tiempo cero mediante un método de titración basado en la fluorescencia de la Tioflavina T. Tipicamente, se preparó una disolución de 100 μM de Tioflavina T en PBS pH 7.4. Se tomaron alícuotas de 20 μL de la disolución de HEWL a diversos intervalos de tiempo a las cuales se les adicionó 100 μL de disolución de Tioflavina T. Las muestras se dejaron estabilizar durante 5 minutos y posteriormente se midió su fluorescencia en el lector Victor² Wallace™. La evaluación de los compuestos de BDMC en la prevención de la fibrilación se llevó a cabo de la misma forma descrita, solo que con la adición del compuesto a la disolución fibrilante de HEWL.
3.2.3. Ensayos in vivo.

**Biodistribución mediante imagen por PET.** De manera general, tanto para compuestos marcados con $^{68}$Ga como con $^{111}$In, los animales FVJ/N se anestesiaron con isofluorano (1.5 %) y se inyectó el compuesto marcado de forma intravenosa a través de la vena de la cola. Se obtuvieron muestras de sangre a diferentes tiempos tras la inyección, mediante punción cardíaca tras ser anestesiados con isofluorano. Finalmente, se extrajeron los órganos (pulmones, corazón, bazo, riñones, hígado y cerebro) y algunos tejidos (músculo y grasa), se lavaron con suero salino, se pesaron y se midió su radioactividad usando un contador Cobra II auto-gamma por triplicado. Las muestras de sangre se centrifugaron (3000 rpm, 10 min, 25 °C) y se recogió el plasma sobrenadante. Se midió también la radioactividad de plasma y sangre para los estudios de farmacocinética. Se calcularon el % de dosis inyectada (DI) y % DI·g$^{-1}$ mediante comparación con estándares tomados de cada disolución inyectada a los animales. Los datos se expresaron como media ± DS.

**Biodistribución mediante Imagen Óptica por fluorescencia.** La biodistribución se llevó a cabo en animales C57Bl/6. Los animales se anestesiaron con 1.5 % de isofluorano y una dosis de 4.15 mg·Kg$^{-1}$ eq. de Cy5.5 se inyectó a través de la vena de la cola. Se extrajeron muestras de sangre a diferentes tiempos (1, 3, 7, 14 and 24 h) tras anestesiar a los animales con un coctel letal de anestesia (i.e. para un animal de 20 g, 200 µL de una disolución de ketamina 100 mg·Kg$^{-1}$, xylasina 100 mg·Kg$^{-1}$, Acepromacida 2 mg·Kg$^{-1}$). Tras ello, se perfundieron los animales con 10 mL de suero salino, se extrajeron los órganos (cerebro, hígado, riñón, corazón, estómago, bazo y pulmones) inmediatamente después. Se midió la fluorescencia tanto de la sangre como de los órganos en MAESTRO™. Finalmente, para la cuantificación de la fluorescencia, se obtuvieron datos normalizados tomando siempre la misma región de área en pixeles para todos los órganos, expresado en cuentas por segundo. Se realizó una curva de calibrado de los compuestos para poder estimar el % DI.

**Determinación de placas Aβ usando el trazador AOI987.** El nivel de placa Aβ en ratones transgénicos ArcAbeta y wild type se determinó, antes de comenzar con los tratamientos, mediante el uso del trazador AOI987. Para ello, se anestesió a los animales con isofluorano 1.5 %, se afeitó la cabeza de los ratones y se determinó el nivel de autofluorescencia (como línea base) de la cabeza del animal. Para medir
la fluorescencia in vivo, se transfirió al animal a una plataforma pre-calentada dentro del sistema de imagen (CriMaestro 500), con anestesia constante usando una máscara de anestesia. Tras ello, se les inyectó la sonda AOI987 (0.1 mg·Kg⁻¹). Se volvió a medir la fluorescencia de la cabeza del animal tres horas post-inyección, en el Maestro™ en el rango espectral de 660-800 nm. Para la cuantificación de la fluorescencia se procedió del mismo modo que para los estudios de biodistribución, tomando siempre la misma área en píxeles.

Tratamientos para el estudio de actividad en un modelo murino de Alzheimer. Se usaron animales transgénicos ArcAbeta y sus compañeros de jaula wild type como controles (ratones de la cepa C57Bl/6). Los animales pesaron entre 27.5 ± 5.5 g. Para la inyección de los tratamientos, los animales se anestesitaron con 1.5 % de isofluorano, y el compuesto se administró de manera intravenosa a través de la vena de la cola a una dosis de 2 mg·Kg⁻¹ BDMC eq. seis veces, durante un período de 2 semanas. Los animales ArcAbeta control se sometieron a las mismas condiciones de anestesia pero fueron inyectados con suero salino. 24 horas tras la última inyección, se sacrificaron los ratones. Previamente se procedió a la extracción de sangre de la vena cava (tras anestesiar al animal con un cóctel letal de anestésicos conteniendo ketamina 100 mg·Kg⁻¹, Xylasín 100 mg·Kg⁻¹, Acepromacide 2 mg·Kg⁻¹). La fluorescencia de la sangre se registró en el Maestro™ siguiendo protocolos previamente descritos. El animal se perfundió con 10 mL suero salino y se extrajeron los cerebros inmediatamente después. Dichos cerebros se congelaron inmediatamente después de ser embebidos en OCT (Tissue-Tec®; Sakura Finetek USA) a -40 °C usando 2-metilbutano y nitrógeno líquido. Para realizar los cortes histológicos de los cerebros se usó un criostato Cryostat NX70. Se realizaron cortes frontales coronales de 10 µM que se guardaron a -80 °C para posteriores estudios histológicos.
4. RESULTADOS

4.1. Desarrollo de una nueva metodología versátil para la síntesis controlada de polipéptidos. (Capítulo 2)\textsuperscript{30,31}

Se ha expuesto anteriormente la necesidad de nuevos sistemas poliméricos con estructuras definidas, altos pesos moleculares, multifuncionales y con bajo grado de heterogeneidad. Para ello, y centrándonos en la síntesis de poliglutamatos, dadas las ventajas inherentes de estos polímeros, en el presente proyecto, se ha desarrollado y puesto a punto una metodología nueva basada en la polimerización ROP-NCA\textsuperscript{32-34}. Dicha metodología ha permitido la síntesis de polipéptidos bien definidos (homo o polímeros multibloque) con pesos moleculares controlados (hasta un grado de polimerización de 800), bajas polidispersidades (menores a 1.2), rendimientos cuantitativos, con preservación de la integridad del extremo amino, y ausencia de racemización de los centros quirales. Todo ello, con alta reproducibilidad lote a lote, en síntesis escalables, con ausencia de trazas de impurezas (crucial para aplicaciones biológicas) y mediante un método que no requiere del uso de equipamiento caro o complejo.

En dicha metodología, se ha optimizado tanto, la síntesis del monómero NCA del ácido glutámico, como el proceso de polimerización en sí y el método de desprotección de los grupos bencilo. Todo el proceso ha sido patentado por el laboratorio de Polímeros Terapéuticos dirigido por la Dra. Vicent\textsuperscript{31} cuya patente ha dado lugar a la creación de una compañía Spin-Off.

Así pues, la metodología de polimerización se basa en el uso, por primera vez, de sales de amonio con contraiones no nucleofilicos como iniciadores, en concreto, sales de BF\textsubscript{4}. En primera instancia, se llevó a cabo la optimización y escalado de la síntesis del monómero, y el ajuste de parámetros como la temperatura, tiempo de reacción, el disolvente, la concentración y el ratio [monómero/iniciador]. Tras ello, la versatilidad del método se demostró mediante la síntesis de diferentes familias de homopolímeros (usando sales de neopentil y n-butil aminas como iniciadores); después, el método se extendió para la síntesis de polímeros funcionalizados en su extremo c-terminal mediante el uso de iniciadores funcionalizados con grupos alquino y azida, lo que abre la puerta a posteriores conjugaciones específicas de moléculas bioactivas, como es el caso de anticuerpos o proteínas, o de
agentes de imagen. También se demostró la capacidad del método para la obtención de polímeros di-bloque híbridos, mediante el uso de macroniniciadores basados en PEG.

Una vez optimizada la polimerización, se llevó a cabo el estudio de un método adecuado de desprotección de los grupos protectores bencilo. El método óptimo debe ser capaz de dar lugar a desprotecciones completas, con ausencia de procesos de racemización que darían lugar a cambios en la estructura secundaria del polipéptido. La relevancia de ello radica en el hecho de que dichos cambios pueden dar lugar a modificaciones drásticas en el comportamiento biológico de los portadores sintetizados, como alteraciones en las propiedades inmunológicas, perfiles de degradación, y en definitiva, alteraciones en los perfiles farmacocinéticos y de biodistribución. Teniendo ello en cuenta, se pensó en el uso de TFA/HBr como método de desprotección, ya que se ha reportado su eficacia, en ausencia de procesos de racemización. Sin embargo, dichas condiciones no son apropiadas cuando se tienen grupos funcionales susceptibles a condiciones ácidas drásticas, como es el caso del bloque PEG, en polímeros di-bloque. Por ello, se desarrolló y optimizó un nuevo método de desprotección basado en el uso de condiciones básicas (NaOH/THF). Dicho método nos permite la desprotección total de los grupos bencilo en polímeros di-bloque, sin cambios estereoquímicos, tras comprobarse al analizar el grado de rotación de luz polarizada.

Los compuestos se caracterizaron mediante el uso de diferentes técnicas físico-químicas. Por ejemplo, la identidad de cada compuesto se determinó por GPC así como por RMN y la estructura secundaria por dicroismo circular confirmando la existencia de la hélice α, en el caso de polipéptidos protegidos, y random coil, en el caso de los desprotegidos.

4.2. Síntesis de poliglutamatos con forma de estrella como excelentes candidatos en aplicaciones biomédicas. (Capítulo 3).

Los polímeros ramificados se consideran excelentes aspirantes para diversas aplicaciones en biomedicina debido a sus interesantes y exclusivas propiedades reológicas, mecánicas y biomédicas, derivadas de sus estructuras y que son inaccesibles para polímeros lineales. Por ello, la obtención de polímeros con arquitectura ramificada se está convirtiendo en un área de creciente interés para el desarrollo de nuevos
portadores poliméricos para diferentes aplicaciones dentro de los campos de biomedicina y biotecnología, entre otros.

Con todo ello en mente, en este proyecto se decidió explorar las posibilidades de la nueva metodología de polimerización anteriormente optimizada, en la síntesis de poliglutamatos con forma estrella. Todo ello, se postuló posible debido al carácter “living” de la polimerización, y el control preciso que proporciona sobre la etapa de iniciación, crucial para el uso de iniciadores multifuncionales. Así pues, mediante un método simple pero poderoso, se llevó a cabo la síntesis de poliglutamatos de forma estrella, con tres ramas, estructuras bien definidas, pesos moleculares controlados y polidispersidades por debajo de 1.2. Para ello, se aplicó un método divergente basado en el uso de nuevos iniciadores multifuncionales BF₄. Entre los iniciadores usados, uno de ellos presenta enlaces bisulfuro reducibles. La relevancia del uso de dicho iniciador radica en el hecho de que nos permitió reducir el polímero y estudiar los brazos por separado, para confirmar, que efectivamente, todos poseían pesos moleculares aproximados, verificando la eficacia de la polimerización.

4.3. Desarrollo de una nueva metodología versátil para la modificación post-polimerización de poliglutamatos. (Capítulo 4).³⁷

Tal y cómo se ha mencionado anteriormente, la multifuncionalidad es necesaria para aumentar la capacidad de carga de un polímero, y es por tanto, una característica deseable de los poliglutamatos. Esta multifuncionalidad inherente en PGA, se puede mejorar con la introducción de funcionalidades diferentes a los grupos ácidos, en la cadena lateral del polímero, lo que permitirá bioconjugaciones específicas de forma ortogonal.

Persiguiendo dicho objetivo, se implementó una metodología para la modificación post-polimerización de las cadenas laterales de PGA. Esta metodología se basa en el uso de sales de DMTMM como activadores de los grupos carboxílicos, necesaria para dar lugar a enlaces amida mediante química ácido-base. De esta forma, se introdujeron varias funcionalidades entre las que se encuentran alquinos, azidas, bisulfuros activados o aminas protegidas, mediante condiciones de reacción suaves (necesarias para mantener la integridad de la cadena de PGA) y con la versatilidad de un método que permite llevar a cabo reacciones tanto en medio acuoso como en disolventes orgánicos, simplemente cambiando el contraión de la sal de cloruro a
BF₄. Dicha versatilidad permite introducir tanto moléculas hidrofílicas como hidrofóbicas en las cadenas de PGA. Por lo tanto, el método descrito permite introducir todo tipo de funcionalidades que, por ejemplo, no podrían introducirse mediante procesos de polimerización (usando monómeros funcionalizados), debido a las incompatibilidades de ciertos grupos funcionales.

4.4. Caracterización In vitro de los polímeros sintetizados hasta el momento. Validación de los polímeros como portadores de fármacos o sondas de imagen. (Capítulos 3-4).

Por otro lado, la validación de los portadores poliméricos mediante su caracterización in vitro es un requisito a cumplir de cara a su posterior uso en aplicaciones biológicas. La biocompatibilidad, biodegradabilidad y los mecanismos de internalización de los mismos son aspectos clave a estudiar. De acuerdo con ello, se llevó a cabo el estudio in vitro de diferentes vectores poliméricos con estructura lineal, di-bloque PEG-PGA, y polímeros estrella. Así pues, se llevó a cabo la degradación en presencia de la enzima lisosomal catépsina B, de varios polímeros lineales y estrella, con y sin funcionalizaciones (alquinos y azidas). El polímero estrella iniciado con iniciador basado en hexilamina, exhibió una velocidad de degradación menor al iniciado con iniciador basado en etilamina, para un mismo peso molecular. En el caso de los polímeros funcionalizados, se demostró cómo el grado y naturaleza de la funcionalización tienen una clara influencia en la degradación enzimática del polímero.

Puesto que el destino final del uso de dichos compuestos se prevé para su uso en enfermedades neurodegenerativas, se evaluó la citotoxicidad de los compuestos en modelos celulares de células endoteliales HUVEC (human umbilical vein endotelial cells) y SHSY5Y, de glioblastoma. Los polímeros testados (lineal, di-bloque, estrella y funcionalizados) resultaron no tóxicos a las concentraciones testadas (hasta 3 mg·mL⁻¹).

Para los estudios de internalización celular, se marcaron con sondas fluorescentes (OG) diferentes poliglutamatatos de diversas estructuras (lineal, PEG-PGA y estrella). Dicho estudios realizados por citometría de flujo y microscopía confocal revelaron mecanismos de internalización dependentes de energía, independientemente del peso molecular del polímero, ya que no se encontró internalización a 4 °C, en los polímeros testados. En cuanto al peso molecular, se probaron
diferentes polímeros lineales de varios pesos moleculares y se observó que no existían diferencias significativas en los perfiles de internalización. Sin embargo, el grado de internalización encontrado de los polímeros estrella fue 4 veces superior al lineal (para un polímero de peso molecular similar). Todo ello pone de manifiesto la importancia de la arquitectura en cuanto al comportamiento biológico de un portador polimérico. Ese hecho puede atribuirse a propiedades inherentes de los polímeros ramificados que, de manera general, son más compactos, con formas globulares y amplias superficies entre otras características que los convierten en candidatos relevantes para su uso como vehículos para el transporte y liberación de fármacos, así como sondas para imagen, entre otras aplicaciones.\textsuperscript{38, 39}

4.5. Estudio exhaustivo de los procesos de auto-ensamblaje de nuevos polímeros basados en PGA. (Capítulo 5).\textsuperscript{40}

Se sintetizaron nuevos polímeros de PGA con motivos que inducen el autoensamblaje. Cuando se llevó a cabo la caracterización físico-química exhaustiva de dichos sistemas a través de una batería de técnicas físico-químicas (DLS, DOSY y NOESY, CD, TEM, técnicas de fluorescencia y SANS) se encontraron resultados interesantes. De acuerdo con dichos resultados, se encontró que estos polímeros basados en PGA, sufren procesos de auto-ensamblaje dependientes de la concentración, de manera tal que, a bajas temperaturas se encuentran formando unímeros de 5-10 nm de tamaño y estructuras supramoleculares de alrededor de 100-180 nm a altas concentraciones (de acuerdo con DLS). Dicho fenómeno ocurre en todos los sistemas que incluyen esos motivos inductores de ensamblaje, pero no en PGA lineal. Se usó DLS para obtener los valores de concentración crítica de agregación (CAC).\textsuperscript{41-44} Los experimentos de contraste de SANS confirmaron la presencia de dominios organizados en la nano-estructura ensamblada, lo cual está de acuerdo con lo descrito en literatura.\textsuperscript{45-48} Además, los estudios de TEM y SANS revelaron que las nanoestructuras toman forma de esferas con las ramas de los polímeros dirigidas hacia el exterior. Se investigó la influencia de diferentes estímulos físico-químicos (temperatura, concentración o fuerza iónica) y de diferentes grados y naturalezas de funcionalización en el equilibrio dinámico de agregación de las nano-estructuras. Como conclusión de todos ellos, se puede decir que el tamaño adoptado por estos sistemas depende claramente de las condiciones ambientales así como de la
funcionalización. De hecho, el proceso de agregación se puede modular e incluso prevenir dependiendo de la funcionalización. Aparentemente, la presencia de residuos hidrofílicos por encima de un 30 % evitan dicha agregación, lo cual puede servir como herramienta para el diseño de nuevos portadores poliméricos.

Con todo ello en mente, se propuso el desarrollo de nuevos sistemas para el transporte de fármacos, de alto peso molecular para prolongar la circulación in vivo, lo que es un requisito para de manera potencial, alcanzar acumulación en cerebro. Esta estrategia está basada en el atrapamiento covalente de las estructuras supramoleculares descritas, con la ayuda de reacciones de click, dando lugar a nano-construcciones estables a estímulos físicos. Para ello, se funcionalizaron dichos polímeros siguiendo la metodología de post-polimerización optimizada, de manera que se introdujeron puntos de anclaje. Tras confirmarse el co-ensamblaje de los polímeros modificados, se llevó a cabo el atrapamiento covalente mediante química CuAAC, en primer lugar y posteriormente se extendió a las químicas libres de cobre tiol-eno y bisulfuro. El uso de bisulfuros para atrapar covalentemente estas estructuras permite construir sistemas de gran tamaño, adecuados para aumentar los tiempos de circulación, pero capaces de desensamblarse bajo condiciones reductoras.

La evaluación biológica preliminar de las estructuras atrapadas reveló que no son entidades toxicas (MTS) en las condiciones estudiadas y que son internalizadas más rápido y en mayor extensión que sus análogos unímeros, como se demostró mediante citometría de flujo.

4.6. Diseño, síntesis y evaluación de portadores poliméricos capaces de atravesar la BHE (Capítulo 6).40

Tras la síntesis y validación de los diferentes portadores poliméricos descritos en los capítulos anteriores, se procedió a la obtención de sistemas capaces de cruzar la BHE, basando nuestras estrategias en el transporte activo mediante transcitosis. Para ello, se pensó en primera instancia en la utilización de los péptidos Angiopep-2 (ANG)49-55 y CRTIGPCSVC (cPEP)56, 57 como vectores dirigentes. Se procedió a la evaluación de la plataforma polimérica de homopolímeros, di-bloques PEG-PGA y polímeros estrella, en cuanto a su capacidad como portadores para el cruce de la BHE. Para ello, se sintetizaron conjugados de ambos péptidos con las diferentes
estructuras mediante el uso de diferentes químicas de bioconjugación (enlace bisulfuro para ANG-2 y amida para cPEP) y se marcaron con DO3A-\(^{68}\)Ga (en el caso de homo y di-bloques) y DO3A-\(^{111}\)In (para el caso de polímeros estrella) para su evaluación in vivo mediante técnicas de imagen no invasiva (PET, en este caso). Los estudios in vivo son los más adecuados para evaluar la capacidad de transporte activo a través de la BHE, ofreciendo unos resultados más realistas y fiables. Así pues, se llevaron a cabo estudios de biodistribución en FVB/NJ. En todos los casos, se inyectaron los compuestos vía intravenosa, a la concentración necesaria para un nivel de señal óptimo. Una vez realizada la monitorización in vivo, los animales se sacrificaron y se evaluó y cuantificó la radioactividad en cada órgano, así como en sangre y plasma. La cantidad en plasma nos permitió calcular parámetros farmacocinéticos, incluyendo el tiempo de vida medio de los compuestos. De los estudios de biodistribución se pudo concluir que todos los polímeros testados resultaron ser biocompatibles y no se acumularon de forma específica en ningún órgano. Se observaron perfiles de eliminación renal en todos los casos. En cuanto a la farmacocinética, los polímeros ramificados en general (con vectores dirigentes y sin ellos), presentaron tiempos de vida media de entre 12-16 horas, mucho más superiores a los obtenidos con el PGA lineal (0.59 horas). Sin embargo, aunque los polímeros estrella mejoraron la acumulación en cerebro (si los comparamos con lineales o di-bloque), dicha acumulación encontrada resultó no ser significativa. Se pueden establecer diversas justificaciones frente a este hecho, entre ellas, una falta de exposición de los ligandos para su reconocimiento, o la necesidad de tiempos de circulación en sangre superiores, para fomentar la acumulación en cerebro.

Debido a los resultados obtenidos, quedó patente la necesidad de usar nuevas estructuras, de tamaño superior para prolongar los tiempos de vida media, a la vez que con superficies de conjugación mayores y menos impedidas, que faciliten la exposición de los péptidos dirigentes. Las estructuras atrapadas covalentemente, resultado de los procesos de auto-ensamblaje descritos en el apartado anterior, se presentan como posibles candidatos puesto que reúnen los requisitos anteriormente expuestos. Así pues, mediante una estrategia bottom-up, se procedió a la modificación en la superficie de dichas estructuras clicadas, con la sonda Cy5.5, en este caso para imagen óptica de fluorescencia (NIR) y con péptidos dirigentes. De entre los estudios
anteriores, se decidió continuar con el péptido ANG-2, puesto que los polímeros conjugados del mismo demostraron una mayor acumulación en cerebro, cuando se compararon con sus análogos de cPEP. Los compuestos sintetizados (con y sin vector dirigente) se analizaron \textit{in vivo}, en términos de biodistribución en ratones C57Bl/6 (Animal Imaging Center, Zürich, Switzerland). Tras su inyección intravenosa a través de la vena de la cola, se procedió al sacrificio de los animales a diferentes tiempos. La fluorescencia de la sangre y de diferentes órganos extraídos tras perfusión, se midió usando un MAESTRO\textsuperscript{TM}. En este caso, las nano-estructuras testadas también exhibieron perfiles de eliminación renal, sin la presencia de toxicidades específicas. Sin embargo, la cantidad obtenida en cerebro en estos casos, sí fue significativa, llegando a un máximo de un 1.5 % de la dosis inyectada para el compuesto con vectores dirigentes. Dicho resultado se encuentra dentro de la normalidad para un sistema donde se afirma que “cruza” la barrera hematoencefálica.\textsuperscript{58}

4.7. Diseño, síntesis y evaluación de portadores poliméricos para el tratamiento de enfermedades neurodegenerativas. (Capítulo 7).

El siguiente paso fue la síntesis y caracterización exhaustiva de conjugados polímero-fármaco para el tratamiento del Alzheimer. Se decidió usar las estructuras poliméricas atrapadas covalentemente como portadores, debido a los prometedores resultados previos obtenidos en las biodistribuciones. En el diseño de tratamientos eficaces para enfermedades del sistema nervioso central, la acumulación en cerebro es un requisito.

Para ello, se seleccionó el curcuminoide, bisdemetoxicurcumina (BDMC) como fármaco modelo debido a su gran potencial para el tratamiento de la enfermedad del Alzheimer. En combinación con residuos de propargilamina, BDMC se conjugó a las estructuras clicadas a través de enlaces éster. Dichas estructuras se evaluaron \textit{in vitro} en términos de viabilidad celular, perfiles de liberación de fármaco a diferentes pHs, y actividad \textit{in vitro} como inhibidores de la formación de fibrillas. A fin de lograr prueba de concepto, la actividad de los compuestos se comprobó mediante un modelo aceptado basado en el uso lisozima de clara de huevo de gallina (HEWL) con capacidad de formar fibras. Varios conjugados de BDMC junto con el fármaco libre se testaron a dos concentraciones diferentes.
(10 y 50 µM) mediante la incubación, durante 24 horas, con HEWL y su poder inhibidor se midió mediante el ensayo de fluorescencia de Tioflavina T, a diferentes tiempos. Dichos resultados se confirmaron adicionalmente mediante microscopía electrónica de transmisión (TEM) donde se observó una prevención significativa de la formación de fibras. De manera adicional, se estudió el carácter neuroprotector de un sistema clicado con BDMC en cultivos organotípicos de hipocampo y entorrinal. Para ello, se pre-trataron dichos cultivos con el compuesto, antes de inducir neurotoxicidad usando el péptido amiloide Aβ1-42. En estas condiciones, los pre-tratamientos con el compuesto, en concentraciones de 1 y 5 µM en equivalentes de BDMC produjeron una reducción significativa de la muerte celular en cultivos tratados con el péptido Aβ1-42. Dado que la concentración de 1 µM resultó suficiente para producir un efecto neuroprotector significativo sin llegar a ser tóxica, se seleccionó dicha concentración y actualmente se están llevando a cabo experimentos para elucidar los posibles mecanismos por los cuales estos sistemas producen efectos neuroprotectores.

De igual forma que para las otras estructuras clicadas, se realizaron estudios de biodistribución con nanosistemas cargados con BDMC, doblemente etiquetados con las sondas DO3A-Gd³⁺ y Cy5.5 y con unidades dirigentes (Angiopep2) para el cruce de la BHE. Dichos estudios se realizaron en ratones C57Bl/6 mediante técnicas de imagen óptica de fluorescencia, usando la sonda de Cy5.5 de los portadores. Los perfiles de biodistribución obtenidos fueron comparables a los de las otras estructuras clicadas (con Angiopep2 y sin Angiopep2), con perfiles de excreción renal, acumulación significativa en cerebro (sobre 1.2 % ID), y acumulación en pulmón a tiempos cortos, debido al mayor tamaño de estas estructuras. En resumen, los resultados obtenidos in vitro, junto con los estudios de biodistribución de estos compuestos mediante imagen óptica, ponen de manifiesto el potencial y prometedor uso de estas nanomedicinas para el tratamiento de Alzheimer. Además, cuando se administró el compuesto de manera recurrente durante dos semanas en animales ArcAbeta (modelo de la enfermedad), dicho compuesto no resultó tóxico y se encontró una gran acumulación en la cabeza. Estos resultados preliminares nos alientan para investigar más a fondo dichos compuestos, tanto in vitro (considerando nuevas dianas de la enfermedad donde el fármaco BDMC puede tener un impacto favorable), e in vivo, con modelos y diseños experimentales apropiados.
4.8. Diseño, síntesis y evaluación de sondas poliméricas inteligentes para la detección temprana de eventos moleculares relacionados con la enfermedad. (Capítulo 8).

Finalmente, y dentro del marco de un proyecto europeo con el acrónimo de LIVIMODE, se desarrolló una plataforma para la imagen in vivo, basada en el uso de sondas inteligentes activables mediante proteasas específicas. Dicha plataforma se desarrolló con el fin de permitir la cuantificación, de forma no invasiva, de la expresión de la proteasa diana en tejidos enfermos (diagnosis), así como la monitorización del progreso de la enfermedad (estado). Dichas sondas están basadas en la combinación de un par NIRF (Near Infrared FRET) y secuencias específicas de ciertas proteasas. Tras el procesamiento por la enzima en cuestión, se produce la liberación del “quencher” dando lugar a la emisión de fluorescencia del fluoróforo. A través de la conjugación a los portadores poliméricos basados en PGA, se proporcionó especificidad tisular a las sondas sintetizadas por nuestros colaboradores. Se conjugaron varias sondas específicas (de MMP-9, 12 y 13; catepsina B y S; FAP) a derivados de PGA aplicando diferentes químicas de bioconjugación. En resumen, los test enzimáticos realizados a las sondas poliméricas demostraron que dichas sondas mantienen e incluso mejoran su selectividad y especificidad por la proteasa en cuestión.

Los resultados preliminares in vivo, de algunos de los candidatos, en modelos in vivo seleccionados de nuestros colaboradores, claramente pusieron de manifiesto el potencial de dichas sondas poliméricas. Por ejemplo, en el caso de la sonda polimérica para MMP-13, se obtuvieron muy buenos resultados con respecto a su capacidad para la detección temprana de osteoartritis, en un modelo animal basado en la desestabilización del menisco medio. Por otro lado, las sondas poliméricas generadas para la detección de catepsina B demostraron un gran potencial en los estudios in vitro para su uso en la detección temprana de cáncer.

Además, dentro del proyecto se exploró el uso de vectores dirigentes para mejorar la especificidad tisular de las sondas generadas. Con respecto al uso de los conocidos péptidos de Rothenfluh para acumulación en cartílago, no mejoró la acumulación que el portador polimérico basado en PGA demostró “per se” en la rodilla lesionada del modelo murino de OA tras ser administrado i.v.
Todos los resultados anteriores ponen de manifiesto el potencial de los derivados de PGA para su uso en diagnóstico. Además, estas sondas macromoleculares representan excelentes candidatos para aplicaciones en “theranostics”.

5. CONCLUSIONES.

Durante el desarrollo del presente trabajo, se ha conseguido alcanzar una serie de objetivos que lo convierten en un trabajo novedoso y relevante en las áreas de síntesis de polímeros, estudios de auto-ensamblajes así como en áreas relacionadas con sus aplicaciones biológicas. Así pues, se ha alcanzado:

- La puesta a punto de una nueva metodología versátil de polimerización que permite la síntesis de poliglutamatos bien definidos, con pesos moleculares controlados y bajas polidispersidades mediante una nueva aproximación vía ROP-NCA. Además, el uso de diferentes iniciadores permite alcanzar poliglutamatos multifuncionales de un solo bloque o varios, con diferentes composiciones y estructuras. De hecho, la reproducibilidad de esta nueva tecnología, así como la posibilidad de realizar escalado, ha dado lugar a la generación de una compañía Spin-Off (Polypeptide Therapeutic Solutions S.L.). Asimismo, esta sencilla pero poderosa metodología se ha extendido a la síntesis de polímeros estrella, con numerosas aplicaciones potenciales dentro del campo de la biomedicina. Como colofón a la parte de desarrollo de técnicas sintéticas, se ha implementado una nueva metodología para la introducción de diversos grupos funcionales en las cadenas laterales de los poliglutamatos vía modificación post-polimerización. Dichas funcionalidades pueden ser usadas en posteriores bioconjugaciones dando lugar a síntesis controladas y ortogonales.

- El desarrollo, estudio y caracterización de nuevas estructuras resultado de procesos de auto-ensamblaje de poliglutamatos, inducido por el uso de motivos que promueven dicho ensamblaje. La estructura y conformación de dichas construcciones ha demostrado ser altamente dependiente de factores y estímulos físico-químicos, así como de la naturaleza de los polímeros auto-ensamblados, lo que permite una modulación de las mismas. Estos descubrimientos abren la puerta al desarrollo potencial de multitud de portadores poliméricos con características modulables en función de la aplicabilidad de los mismos.
El diseño, síntesis y evaluación de una plataforma de portadores poliméricos con diferentes arquitecturas para su uso como vectores para el trasporte activo a través de la BHE. Se ha desarrollado una familia de compuestos (con y sin vectores dirigentes hacia la BHE) para alcanzar un cruce significativo a través de la BHE con el fin último de ser usados en el tratamiento de enfermedades neurodegenerativas. Dentro de ellos, la tercera generación de arquitecturas, sintetizada a partir de estrategias ‘bottom-up’ usando las estructuras auto-ensambladas, ha demostrado ser lo suficientemente eficaz como para considerarse candidatos para el ya descrito fin.

El diseño, síntesis y evaluación preliminar de conjugados-polímero fármaco con actividad neuroprotectora-neurorescatadora para ser usados en el tratamiento de enfermedades neurodegenerativas, en concreto, Alzheimer. Los primeros resultados corroboran el potencial de dichos conjugados, sin embargo, es necesaria una investigación en mayor profundidad para verificar su eficacia.

El diseño, síntesis y evaluación preliminar de sondas poliméricas para la detección de eventos moleculares que representan biomarcadores para la detección temprana de ciertas enfermedades. Aunque se ha puesto como ejemplo el diagnóstico de cáncer u osteoartritis, el potencial de dichas sondas es extensible al diagnóstico de enfermedades neurodegenerativas.

6. REFERENCIAS.


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[Appendix I]

### APPENDIX II. TABLE OF COMPOUNDS

<table>
<thead>
<tr>
<th>REF</th>
<th>Chemical Structure</th>
<th>Name (Abbreviation)</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td><img src="#" alt="Image" /></td>
<td>$\gamma$-benzyl L-glutamate N-Carboxyanhydride (OBzl(Glu) NCA)</td>
</tr>
<tr>
<td>2</td>
<td><img src="#" alt="Image" /></td>
<td>Poly($\gamma$-benzyl L-glutamate) (PBLG) from different initiators</td>
</tr>
<tr>
<td>3</td>
<td><img src="#" alt="Image" /></td>
<td>Tetrafluoroborate n-butylammonium salt (nBu-NH$_3$BF$_4$)</td>
</tr>
<tr>
<td>4</td>
<td><img src="#" alt="Image" /></td>
<td>Tetrafluoroborate neopentylammonium salt (Npt-NH$_3$BF$_4$)</td>
</tr>
<tr>
<td>5</td>
<td><img src="#" alt="Image" /></td>
<td>Tetrafluoroborate PEGammonium salt (MeO-PEG-NH$_3$BF$_4$)</td>
</tr>
<tr>
<td>6</td>
<td><img src="#" alt="Image" /></td>
<td>Tetrafluoroborate propargylammonium salt (Propargyl-NH$_3$BF$_4$)</td>
</tr>
<tr>
<td>7</td>
<td><img src="#" alt="Image" /></td>
<td>N$_3$-EG(2)-NH$_3$BF$_4$</td>
</tr>
<tr>
<td>8</td>
<td><img src="#" alt="Image" /></td>
<td>Hybrid Di-block polyethylene glycol-co-poly($\gamma$-benzyl L-glutamate) (PEG-PBLG)</td>
</tr>
<tr>
<td>9</td>
<td><img src="#" alt="Image" /></td>
<td>$\alpha$-polyglutamic acid (PGA) from different initiators</td>
</tr>
<tr>
<td>10</td>
<td><img src="#" alt="Image" /></td>
<td>Hybrid Di-block polyethylene glycol-co-polyglutamic acid (PEG-PGA or DB)</td>
</tr>
<tr>
<td>11</td>
<td><img src="#" alt="Image" /></td>
<td>Mono-Boc cysteamine</td>
</tr>
</tbody>
</table>
1,3,5-benzene tricarboxamide (BTA) based Boc protected NCA initiators

Star-shaped PBLG (St-PBLG) from different initiators

Star-shaped PGA (St-PGA) from different initiators
16 End capped PBLG

17

17a: Poly(γ-benzyl L-glutamate-co-N-Boc-ethylenediamine glutamate)
17b: Poly(γ-benzyl L-glutamate-co-N-Boc-hexaneamine glutamate)
17c: Poly(γ-benzyl L-glutamate-co-N-Boc-DOOA glutamate)

18

18a: Poly(γ-benzyl L-glutamate-co-ethylenediamine glutamate)
18b: Poly(γ-benzyl L-glutamate-co-hexaneamine glutamate)
18c: Poly(γ-benzyl L-glutamate-co-DOOA glutamate)

19

4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium (DMTMM)

(19a) Cl or BF₄(19b)
20a: Poly(glutamic acid-co-propargyl glutamate)
20b-d: Poly(glutamic acid-co-EG(n)N₃ glutamate)
20e: Poly(glutamic acid-co-EG(6)OMe glutamate)
20f: Poly(glutamic acid-co-cysteamine2TP glutamate)
20g: Poly(glutamic acid-co-N-Boc-ethylendiamine glutamate)
20h: Poly(glutamic acid-co-N-Boc-DOOA glutamate)
20i: Poly(glutamic acid-co-ethylendiamine glutamate)
20j: Poly(glutamic acid-co-DOOA glutamate)

21: Poly(glutamic acid-co-Oregon Green glutamate)
(linear-PGA-OG, St-PGA-OG or PEG-PGA-OG)

22: D-labeled motif Boc Protected

23: D-labeled motif
24

24a: X-prop
24b: X-EG(2)N$_3$
24c: X-SS-2TP
24d: X-malei

25

X covalently entrapped (X-Click)
25a: X-Click through CuAAC
25b: X-Click through disulfide bonding
25c: X-Click through thiol–ene click chemistry

26

DO3A-tBu labeled polymers
26a: PGA-DO3A-tBu
26b: PEG-PGA-DO3A-tBu (DB-DO3A-tBu)
26c: St-PGA-DO3A-tBu
26d: PGA-PEG-DO3A-tBu (DB2.0-DO3A-tBu)
26e: X-Click-DO3A-tBu
DO3A labeled polymers

27a: PGA-DO3A
27b: PEG-PGA-DO3A (DB-DO3A)
27c: St-PGA-DO3A
27d: PGA-PEG-DO3A (DB2.0-DO3A)
27e: X-Click-DO3A

Radionuclide or MRI labeled polymers

28a: PGA-DO3A-^{68}Ga
28b: PEG-PGA-^{68}Ga (DB-DO3A-^{68}Ga)
28c: St-PGA-DO3A-^{111}In
28d: PGA-PEG-DO3A-^{111}In (DB2.0-DO3A-^{111}In)
28e: X-Click-DO3A-^{111}In
28f: X-Click-DO3A-Gd^{3+}

29a: PGA-DO3A-tBu-SS-2TP
29b: PEG-PGA-DO3A-tBu-SS-2TP (DB-DO3A-tBu-SS-2TP)
29c: St-PGA-DO3A-tBu-SS-2TP
29d: X-Click-DO3A-tBu-SS-2TP
30a: PGA-DO3A-SS-2TP  
30b: PEG-PGA-DO3A-SS-2TP  
(DB-DO3A-SS-2TP)  
30c: St-PGA-DO3A-SS-2TP  
30d: X-Click-DO3A-SS-2TP

31a: PGA-DO3A-ANG  
31b: PEG-PGA-DO3A-ANG  
(DB-DO3A-ANG)  
31c: St-PGA-DO3A-ANG  
31d: X-Click-DO3A-ANG

32a: PGA-DO3A-tBu-N-Boc-ethylendiamine  
32b: PEG-PGA-DO3A-tBu-N-Boc-ethylendiamine  
(DB-DO3A-tBu-N-Boc-ethylendiamine)  
32c: St-PGA-DO3A-tBu-N-Boc-ethylendiamine
Appendix II

33: PGA-DO3A-ethylenediamine
33a: PGA-DO3A-ethylenediamine
33b: PEG-PGA-DO3A-ethylenediamine
33c: St-PGA-DO3A-ethylenediamine

34: AC-CRTIGPSVC-NHS (disulfide bridge within)

35: PGA-DO3A-cPEP
35a: PGA-DO3A-cPEP
35b: PEG-PGA-DO3A-cPEP (DB-DO3A-cPEP)
35c: St-PGA-DO3A-cPEP

36: PGA-DO3A-\(^{68}\)Ga-ANG
36a: PGA-DO3A-\(^{68}\)Ga-ANG
36b: PEG-PGA-DO3A-\(^{68}\)Ga-ANG (DB-DO3A-\(^{68}\)Ga-ANG)
36c: St-PGA-DO3A-\(^{111}\)In-ANG
36d: X-Click-DO3A-\(^{111}\)In-ANG

CPEP NHS
37a: PGA-DO3A-$^{68}$Ga-cPEP
37b: PEG-PGA-DO3A-$^{68}$Ga-cPEP
(DB-DO3A-$^{68}$Ga-cPEP)
37c: St-PGA-DO3A-$^{111}$In-cPEP

38a: PBLG-PEG-OMe
38b: PBLG-PEG-FMOC
37c: PBLG-PEG-SS-4TP

39a: PGA-PEG-OMe (DB2)
39b: PGA-PEG-FMOC (DB2-FMOC)
39c: PGA-PEG-SS-4TP (DB2-SS-4TP)

40a: PGA-PEG-DO3A-tBu (DB2-DO3A-tBu)
40b: PGA-PEG-SS-4TP-DO3A-tBu (DB2-SS-4TP DO3A-tBu)
40c: PGA-PEG-FMOC-DO3A-tBu (DB2-FMOC DO3A-tBu)
40d: PGA-PEG-DO3A-tBu-cPEP (DB2 DO3A-tBu-cPEP)
### Appendix II

<table>
<thead>
<tr>
<th>Equation</th>
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<tbody>
<tr>
<td><strong>41a</strong></td>
<td>PGA-PEG-DO3A (DB2-DO3A)</td>
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<tr>
<td><strong>41b</strong></td>
<td>PGA-PEG-SS-4TP-DO3A (DB2-SS-4TP DO3A)</td>
</tr>
<tr>
<td><strong>41c</strong></td>
<td>PGA-PEG-DO3A-ANG (DB2-DO3A-ANG)</td>
</tr>
<tr>
<td><strong>41d</strong></td>
<td>PGA-PEG-DO3A-cPEP (DB2 DO3A-cPEP)</td>
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![Chemical Structures]

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<tr>
<th>Equation</th>
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<tr>
<td><strong>42a</strong></td>
<td>PGA-PEG-DO3A-(^{111})In (DB2-DO3A-(^{111})In)</td>
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<td><strong>42b</strong></td>
<td>PGA-PEG-DO3A-(^{111})In-ANG (DB2-DO3A-(^{111})In-ANG)</td>
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<td><strong>42c</strong></td>
<td>PGA-PEG-DO3A-(^{111})In-cPEP (DB2 DO3A-(^{111})In-cPEP)</td>
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<th>Description</th>
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<tr>
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<td>X-Click-DO3A-tBu-Cy5.5</td>
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<td>X-Click-DO3A-Gd(^{3+})-Cy5.5</td>
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<td>X-Click-DO3A-tBu-SS-2TP</td>
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<td>X-BDMC-Click (capture after BDMC linking)</td>
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<td>59</td>
<td>X-Click-BDMC-DO3A-Gd³⁺-Cy5.5-ANG</td>
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### [Appendix II]

**SP**

![Chemical structure of SP](image)

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<th>Spacer</th>
<th>Linking chemistry (X-Y)</th>
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<td>CH$_2$</td>
<td>CuAAC</td>
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<td>SP.2</td>
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<td>EG(6)</td>
<td>CuAAC</td>
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<tr>
<td>SP.3</td>
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<td>EG(2)</td>
<td>CuAAC</td>
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<tr>
<td>SP.4</td>
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<tr>
<td>SP.5</td>
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<td>COOH/NH$_2$</td>
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<td>SP.12</td>
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<td>Thiol-ene</td>
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</table>
[Appendix II]

CT

CT.1

CT.2 for pep = WYRGRL
CT.3 for pep = YRLGRW

CT.4 for pep = WYRGRL
CT.5 for pep = YRLGRW

CT
APPENDIX III. SUPPLEMENTARY INFORMATION.
CHAPTER 2.

Figure S1. a) $^1$H-NMR example in DMSO-$d_6$ of Npt-NH$_3$BF$_4$ initiator (4). b) $^{19}$F-NMR example in DMSO-$d_6$ of Npt-NH$_3$BF$_4$ initiator (4).
Figure S2. a) $^1$H-NMR example in DMSO-$d_6$ of MeO-PEG(2000)-NH$_3$BF$_4$ initiator (5). b) $^{19}$F-NMR example in DMSO-$d_6$ of MeO-PEG(2000)-NH$_3$BF$_4$ initiator (5).
 CHAPTER 3.

Figure S3. NMR spectra of 1,3,5-tri-tert-butyl((benzenetricarbonyltris(azanediyl)tris(hexane-1,6-diyl))tricarbamate (12b). a) $^1$H-NMR in DMSO-$d_6$; b) $^{13}$C-NMR in CDCl$_3$. 
Figure S4. NMR spectra in CDCl₃ of 1,3,5-tri-tert-butyl (((((((benzenetricarbonyltris(azanediyl)) tris(ethane-2,1-diyl))tris(oxy))tris(ethane-2,1-diyl))tris(oxy))tris(ethane-2,1-diyl))tricarbamate (12c). a) ¹H-NMR; b) ¹³C-NMR.
Figure S5. $^1$H-NMR spectrum of 1,3,5-tri-tert-butyl (((benzenetricarbonyltris(azanediyl))tris(ethane-2,1-diyl))tris(disulfanediyl)) tris(ethane-2,1-diy1))tricarbamate (12d) in acetone-$d_6$. 

a)
Figure S6. NMR spectra in D$_2$O of 1,3,5-(benzenetricarbonyltris(azanediyl))trishexan-1-ammonium BF$_4$ salt (13b). a) $^1$H-NMR; b) $^{13}$C-NMR; c) $^{19}$F-NMR
[Appendix III]

a)

b)
Figure S7. NMR spectra in D$_2$O of 1,3,5-
((((benzenetricarbonyltris(azanediyl))tris(ethane-2,1-
diyli))tris(oxy))tris(ethane-2,1-diyli))tris(oxy))trialkanammonium BF$_4$
 salt (13c). a) $^1$H-NMR; b) $^{13}$C-NMR; c) $^{19}$F-NMR.

Figure S8. NMR spectra in D$_2$O of 1,3,5-
benzenetricarbonyltris(azanediyl))tris(ethane-2,1-
diyli))tris(disulfanediyl))trialkanaminium BF$_4$
 salt (13c). a) $^1$H-NMR; b) $^{13}$C-NMR; c) $^{19}$F-NMR.
CHAPTER 4.

Figure S9. Poly(γ-benzyl L-glutamate-co-N-Boc-DOOA glutamate) (17c) characterization. $^1$H-NMR spectrum in CDCl$_3$ with the corresponding assignments. GPC trace in DMF/LiBr is shown in the rectangle.

Figure S10. Poly(γ-benzyl L-glutamate-co-N-Boc-hexaneamine glutamate) (17b) characterization. $^1$H-NMR spectrum in CDCl$_3$ with the corresponding assignments. GPC trace in DMF/LiBr is shown in the rectangle.