Solid-phase extraction based on ground methacrylate monolith modified with gold nanoparticles for isolation of proteins

María Vergara-Barberán*, María Jesús Lerma-García, Ernesto Francisco Simó-Alfonso, José Manuel Herrero-Martínez*

Department of Analytical Chemistry, University of Valencia, C. Doctor Moliner 50, E-46100 Burjassot, Valencia, Spain

***Corresponding authors:** Dr. José Manuel Herrero-Martínez

Tel.: +34963544062

Fax: +34963544436

e-mail: jmherrer@uv.es

María Vergara-Barberán

Tel.: +34963544062

Fax: +34963544436

e-mail: maria.vergara@uv.es

Abstract

In this study, a novel polymeric material functionalized with gold nanoparticles (AuNPs) was prepared as solid-phase extraction (SPE) sorbent for isolation of proteins. The sorbent was synthesized from a powdered poly(glycidyl-co-ethylene dimethacrylate) monolith, and modified with ammonia, followed by immobilization of AuNPs on the pore surface of the material. To evaluate the performance of this SPE support, proteins were selected as test solutes, being the extraction conditions and other parameters (loading capacity and regenerative ability of sorbent) established. The results indicated that this sorbent could be employed to selectively capture proteins according to their pI, on the basis of the strong affinity of these biomacromolecules towards to AuNPs surface. The applicability of this sorbent was demonstrated by isolating protein species of interest (bovine serum albumin and cytochrome c and lectins in European mistletoe leaves), followed by SDS-PAGE analysis.

Keywords: gold nanoparticles; glycidyl methacrylate monolith; proteins; solid-phase extraction

1. Introduction

Porous polymer monoliths are a category of materials developed during the last two decades, which have attracted much interest [1]. Their simple in situ preparation, high permeability, stability along wide pH-ranges, and versatile surface chemistries have made these materials to be a competitive alternative to the conventional packed chromatographic columns [2]. Due to their singular porous structure, polymer monoliths provide rapid conventional mass transport with very low resistance to flow. As a result, these stationary phases have been used in HPLC [3] and capillary electroseparation techniques [4]. Recently, the application of polymeric monoliths has undergone a rapid growing in the field of sample pretreatment [5, 6]. Thus, the performance of extraction efficiency of organic monoliths could be modified by tailoring its pore structure and surface chemistry.

Glycidyl methacrylate (GMA) material has been used as "reactive support" to provide monoliths with different chromatographic properties (ion exchange, hydrophobic/hydrophilic, chiral, etc.) [7-10]. GMA-based monoliths have been also used for holding silver and gold nanostructures to generate a good surface enhanced Raman spectroscopy (SERS) performance [11-13]. In addition, GMA-based materials in powder have been demonstrated to be helpful in ultrasensitive SERS detection [12, 13] as an "entrapment support" to immobilize biomacromolecules [14]. The powder material could be easily processed into a thin packed column and contains active epoxy groups that can be susceptible of undergoing functionalization.

Gold nanoparticles (AuNPs) have unique properties such as special stability, quantum and surface effect, and great biocompatibility [15]. Since AuNPs have high surface-area-to-volume ratios, easy chemical modification and strong affinity for thiol-

containing ligands, they have already been used for extracting and enriching analytes from complex matrices [16-19]. Thus, Tseng *et al.* [17] used AuNPs to selectively extract thiol-containing compounds as a result of the specific formation of Au–S bonds. In a similar way, they have also developed methods for determining aminothiols in human urine and protein-bound aminothiols in human plasma [18, 19]. Also, the AuNPs have been suspended in the BGE [20] or attached [21] on the inner wall of capillary in electrodriven separation techniques. In spite of its excellent properties, the combination of AuNPs with organic monoliths has been slightly explored. Thus, Svec's research group [22-24] has studied the attachment of AuNPs on the pore surface of reactive polymer-based monolithic supports. In particular, a GMA-based monolithic capillary column was functionalized with cysteamine to afford a pore surface with thiol groups, to which the AuNPs were attached. These capillary monolithic columns have proved to be useful for the capture and separation of cysteine-containing peptides [23] and as platforms to facilitate further variations in surface functionalities [22, 24]. However, the extension of this novel hybrid material as extraction phase has been scarcely reported [13].

SPE is a powerful tool to preconcentrate and purify analytes of interest from a great variety of sample matrices. In particular, this technique is among the widely employed alternatives for protein separation and preconcentration in protein analysis schemes and proteomics techniques [25]. However, a limited choice of sorbents for protein species is available at present, consequently, the development of novel sorbent materials with satisfactory extraction efficiency and selectivity for protein species is highly desirable.

The aim of this study was the development of a novel SPE sorbent based on the modification of a polymer monolith with AuNPs. For this purpose, a GMA-based

monolith was first synthesized, ground and subsequently amino groups were introduced onto the surface of the material by reacting epoxy groups with ammonia. Then, the AuNPs were immobilized onto the amino-functionalized GMA powder material surface on the basis of the strong interaction between the amino group and AuNPs [26, 27]. The prepared materials were filled into an empty plastic SPE column tube and its extraction efficiency was evaluated by using proteins as test solutes. The conditions for the extraction of proteins (bovine serum albumin and cytochrome c) were optimized. Loading capacity and regenerative ability of the SPE monolithic material was also evaluated. The ability of the developed sorbent to isolate proteins in several samples was also evaluated.

2. Experimental

2.1. Chemicals and reagents

Glycidyl methacrylate (GMA), ethylene dimethacrylate (EDMA), 3-(trimethoxysilyl) propyl methacrylate, acetonitrile (ACN) and methanol (MeOH) were purchased from Scharlab (Barcelona, Spain). Azobisisobutyronitrile (AIBN) was from Fluka (Buchs, Switzerland). AuNP suspension (particle size, 20 nm, stabilized with sodium citrate) and Coomassie Blue were from Alfa Aesar (Landcashire, United Kingdom). Monosodium and disodium phosphate (NaH_2PO_4 and Na_2HPO_4, respectively) and orthophosphoric (H_3PO_4) acid were from Merck (Darmstadt, Germany). Ammonium persulfate (APS), acrylamide, bisacrylamide, bovine serum albumin (BSA), cytochrome c (cyt c), gold (III) chloride trihydrate, hydrochloric acid (HCl), 2-mercaptoethanol, nitric acid (HNO_3), lactose, sodium bromide, sodium

dodecyl sulfate (SDS), tetramethylethylenediamine (TEMED), tris(hydroxymethyl)-aminomethane (Tris) and trisodium citrate were obtained from Sigma-Aldrich (St. Louis, MO, USA). A molecular-weight-size protein standard (6.5 to 200 kDa) was also provided by Sigma-Aldrich. Deionized water (Barnstead deionizer, Sybron, Boston, Mass., U.S.A.) was used in all procedures.

Stock solutions of BSA and cyt c (1 mg mL^{-1}) were prepared by dissolving appropriate amounts of each protein in deionized water, and working standard solutions were obtained by dilution of the stock solutions. Phosphate buffer solutions (PBS) of 20 mM at several pH values were prepared by mixing appropriate amounts of Na_2HPO_4, NaH_2PO_4 and H_3PO_4 according to the required pH.

2.2. Instrumentation

SEM/backscattered electron (BSE) images and energy dispersive X-ray (EDAX) analysis were obtained with a Philips XL 330 ESEM integrated with backscattered electron detector and a QUANTAX 400 energy dispersive spectrometer (Bruker, Germany). For these measurements, the monoliths were coated with a very thin-layer of conductive carbon instead of the more usual Au/Pd coating.

Elemental analysis of synthesized material was performed using an EA 1110 CHNS elemental analyzer (CE Instruments, Milan, Italy). The determination of Au in synthetized materials and Bradford protein assay were carried out by measuring in UV-vis with an 8453 diode-array UV-vis spectrophotometer (Agilent Technologies, Waldbronn, Germany). For Bradford's assay [~~26~~ 28], a calibration curve up to 1 mg mL^{-1} of BSA and cyt c was prepared in the elution solvent (see section 3.2). SDS-PAGE

experiments were performed using a vertical minigel Hoefer SE260 Mighty Small system (Hoefer, MA, USA).

2.3. Preparation and functionalization of GMA-based monolith

The GMA-co-EDMA monolithic material was based on a previous work [10]. Briefly, a polymerization mixture was prepared in a 10 mL glass vial by weighing GMA (20 wt%), EDMA (5 wt%), and a binary porogenic solvent mixture containing cyclohexanol (70 wt%) and 1-dodecanol (5 wt%). AIBN (1 wt% with respect to the monomers) was added as initiator. This mixture was sonicated for 5 min and then purged with nitrogen to remove oxygen for 10 more min. The polymerization was carried out in an oven at 60ºC for 24 h. Next, the polymeric material was washed with methanol to remove the porogenic solvents and possible unreacted monomers. Then, the monolithic bulk material was ground with a mortar and sieved with a steel sieve with sizes ≤ 100 µm. The synthesized powder porous material was treated with aqueous 4.5 M ammonia in a round bottomed-flask at 60ºC (water bath) for 2 h under continuous stirring. Upon completion of the reaction, the material was washed with ultra-pure water to remove the excess of ammonia until the eluent was neutral. The poly(GMA-co-EDMA) powdered material functionalized with amino group was obtained for further use.

2.4. Functionalization of amino modified GMA-co-EDMA powder material with AuNPs

The amino modified powder material (400 mg) was placed in a Falcon tube, and then 20 mL AuNPs solution were added, and the mixture was allowed to react under

168 stirring for 10 h. A GMA-co-EDMA powder attached with AuNPs (pink colour) was
169 obtained. Then, the material was washed with deionized water (containing 38.8 mM
170 sodium citrate at pH 6.6) in order to remove the AuNPs, which had not been attached on
171 the amino-modified material. The AuNP-modified material was characterized by SEM
172 and the Au content was evaluated using both UV-vis and EDAX. For UV-vis detection,
173 the dried methacrylate material with AuNPs was first calcinated at 550 °C for 1 h. Then,
174 the remaining product (which contained the gold material), was treated with freshly
175 prepared *aqua regia*, which was subsequently left to evaporate nitric acid vapor and
176 cool down to room temperature. The solution was properly diluted with HCl 1 M and
177 was subjected to UV-vis analysis. The colorimetric determination of Au was based on
178 the formation of the bromoaurate $[Au(Br)_4]^-$ ion [29,30], which is obtained through the
179 reaction of chloroauric acid with potassium bromide and measured at 380 nm. A stock
180 solution of 200 μg mL^{-1} of Au(III) was prepared from its chloride salt. A calibration
181 curve (1-20 μg mL^{-1}) was obtained by appropriate dilution of the stock solution in
182 presence of an excess of NaBr (4 %).
183
184 *2.5. SPE protocol*
185
186 Fifty milligrams of AuNP-modified polymer was packed between two frits
187 (1/16', 20 μm, Análisis Vínicos, Tomelloso, Spain) into 1 mL empty propylene
188 disposable SPE cartridge (Análisis Vínicos). Activation of the sorbent was done with
189 ACN (200 μL) and then equilibrated with water (500 μL). 200 μL of protein standard
190 was loaded on the SPE material at a flow rate of 0.1 mL min^{-1}. After loading the protein
191 standard, the material was washed with water (500 μL) at 0.7 mL min^{-1}, collecting the
192 washing step solution. Retained analytes were eluted with 30 mM PBS (500 μL) and the

eluted proteins were also collected. Next, the sorbent surface was regenerated with water at 80 ºC during 2h. During the SPE process, the proteins left in the effluent (loading solution) passing through the microcolumn were quantified by Bradford assay (measurement of absorbance at 595 nm), in order to evaluate the retention efficiency of proteins on the AuNP-modified material. Afterwards, the proteins were eluted from the microcolumn and quantified in the same way. The same procedure was applied to prepare a blank sorbent constituted by the GMA-co-EDMA polymer (50 mg).

2.6. Extraction of mistletoe proteins

European mistletoe (*Viscum Album L.*) grown on *Pinus halepensis* was collected and the green parts were wased with water to eliminate bacterial and surface contamination from human hands. Then, the green parts were sliced and transferred to a mortar and frozen in liquid nitrogen. The frozen material was ground to a fine powder in a pre-cooled mortar and pestle. Then, the protein extraction protocol was carried out according to Olsner *et al.* [31]. Briefly, 10 g of this powder was mixed with 100 mL of 10 mM Tris-HCl at pH 8.3, which contained 100 mM lactose to avoid the bind of lectins to carbohydrate components in the homogenate. Then, the suspension was mildly shaken at 4ºC overnight and centrifuged at 10000 x g for 10 min. Then, 500 µL of supernatant adjusted at pH 6 was passed through the SPE sorbent. As described above, the retained proteins were eluted with 30 mM PBS at pH 12 and an aliquot of 20 µL was loaded into the gel.

2.7. SDS-PAGE analysis

SDS-PAGE of proteins was carried out with 40% acrylamide/bis solution, and standard discontinuous buffer systems as described by Laemmli [~~29~~ 32]. The protein bands were visualized with staining by using Coomassie Blue. Molecular sizes of the model proteins were analyzed by comparison with an SDS-PAGE standard marker.

3. Results and discussion

3.1. Preparation and characterization of the AuNP-modified monolith

A generic poly(GMA-*co*-EDMA) monolith was first synthesized [10], ground into fine powder and then functionalized with ammonia to prepare the amino modified column (Fig. 1). Elemental analysis of this bulk material indicated the presence of 1.54 wt% nitrogen (1.10 mmol /g monolith), which is in agreement with previous studies related to the preparation of other amino-based monoliths [7, 33]. Then, a citrate-stabilized AuNP colloidal dispersion of 20 nm was used to functionalize the resulting amino until the ~~monolith~~ polymer surface was fully covered with Au (Fig. 1). It was visually confirmed by a change of color in the powdered monolith from white to pink. In addition, SEM micrographs of this ~~monolith~~ material were taken with a BSE detector, giving a satisfactory surface coverage with AuNPs (white spots randomly distributed) (Fig. 2B). The coverage of AuNPs was estimated by both UV-vis spectroscopy (see Section 2.4) and EDAX. Thus, UV-vis spectroscopy analysis showed the presence of 0.5 wt% Au, being EDAX analysis consistent with this value (0.42 wt% Au). Next, this material was used as SPE sorbent for enrichment of proteins.

3.2. Retention of proteins using AuNP-modified GMA material as SPE sorbent

The sample loading flow rate (0.1-0.5 mL min^{-1}) was first studied. This variable has not only a significant effect on the recovery of proteins, but also determines the time of the whole analytical process. The experimental results indicated that low loading flow rates (0.1 mL min^{-1}) allowed higher retention efficiencies of proteins on the AuNP-modified ~~monolithic~~ polymeric material. Lower flow-rates than 0.1 mL min^{-1} were not tested since they require longer processing times. Next, the effect of the elution flow rate on the recovery was also investigated, being the best elution flow rate 0.1 mL min^{-1} for protein species, which was employed for further studies.

The adsorption of proteins onto solid surfaces is a complex process in which several driving forces could be involved, such as hydrophobic, electrostatic, and hydrogen bonding interactions. In general, hydrophobic and electrostatic interactions tend to rule the entire process [34-36]. A preliminary study have showed that within an appropriate pH range, the surfaces of AuNPs could effectively retain proteins, thus providing a potential medium for isolation of these species of interest from several sample matrices [37]. For this reason, the effect of sample pH (2.5-11) on the loading SPE step was first studied (Fig. 3). Solutions (200 µL) containing 200 µg mL^{-1} of BSA (pI 4.7) or cyt c (pI 10.5) were selected as test solutes to perform the sorption studies. As shown in Fig. 3A, for BSA, the highest retention in the loading step was achieved at pH 5.0. This result is in agreement with the findings reported in literature, where the maximum adsorption from aqueous protein solutions onto the metal surface was usually observed at the pI [38-40]. The adsorption of proteins to AuNPs could be explained taking into account both electrostatic interactions and ligand displacement reactions [41, 42]. The electrostatic interactions are related to the attraction between the positive

surface residues of the protein and the negative charge from the citrate-coated Au surfaces. The displacement phenomena implies that citrate is displaced by the protein upon adsorption with amino acids (functional groups) such as lysine (amine), histidine (imidazole), or cysteine (thiol) interacting directly with the gold surface [43]. At pH close to the pI, the electrostatic interactions between the protein molecule and the sorbent are minimized, being the hydrophobic interactions (displacement mechanism) predominant. When the pH was modified to other values different from the optimal one, a less favorable adsorption or retention was obtained owing to electrostatic interactions (repulsion) between protein molecules-support and/or between adjacent adsorbed protein molecules onto Au surface. On the other hand, several authors [44-46] have reported that structural changes (displacement by the protein in its native structure or denaturation of the protein on the surface) can occur as the result of adsorption on the surface while the protein displaces the citrate stabilizer. In this way, the protein unfolds near the surface, exposing hydrophobic residues and presenting specific functional groups which interact by dispersive and van der Waals forces with the Au surface. Taking into account all above considerations, the washing step was done at pH close to pI (buffer at pH 5.0), giving small losses of BSA (< 5%).

Concerning to the elution step, at higher pH values (pH ≥ 11.0) recoveries higher than 94.8% were yielded. As mentioned above, at this pH, the desorption of this protein was facilitated owing to electrostatic interactions. Additionally, a generic GMA-based polymer (parent ~~monolith~~ material) was used as sorbent in the optimal conditions found for the material modified with AuNPs. Low retention (15 %) was obtained in the parent ~~monolith~~ polymer after loading step, which confirmed the selectivity of the AuNP-modified GMA material.

A similar study was done with cyt c. As shown in Fig. 3B, the maximum adsorption of this protein was achieved at a loading pH solution close to its pI (10.7). A similar explanation than that previously described for BSA can be extended for cyt c [~~44~~ 47]. This may be the result of electrostatic repulsions between the negatively charged protein molecules (pH > pI) and negatively charged AuNPs or even between adjacent proteins retained onto the AuNP surface, giving a decrease in the interaction between protein and the sorbent. Accordingly, the washing and elution steps were adequately adjusted to pH conditions to achieve the highest extraction efficiency. In particular, washing step was performed at pH 11, whereas the elution step was done at pH 12. Also, a comparison in terms of retention was also performed with the parent ~~monolith~~ material. Again a high selectivity of the developed procedure was proven since a retention *ca*. 27 % of cyt c was observed in the parent polymer.

In SPE, the breakthrough volume is another important parameter, which could affect the extraction efficiency. For this purpose, different sample volumes (100-2000 µL) of the standard protein solutions were passed through to the SPE material by keeping constant the amount of protein (1000 µg). Fig. 4 shows the breakthrough curves obtained for BSA and cyt c using the developed sorbent. As shown in this figure, high recoveries (89.5-97.8%) up to 1000 µL were achieved for BSA; however, a considerable decrease in the recovery values of cyt c was observed at volumes ~~< 500~~ >200 µL. For this reason, a volume of 200 µL was chosen for further SPE studies.

The loading capacity of SPE sorbent (50 mg) was also evaluated by passing different amounts of BSA. The maximum loading capacity of the polymer monolith modified with AuNPs was 16.6 mg per g sorbent. Typical capacity values for porous media are 1–10% of the bed weight [48, 49], meaning that 10 mg of analyte could be potentially retained (under the proper condition) per g of sorbent material.

In order to ensure a satisfactory loading capacity of the SPE sorbent, its reusability was also evaluated. Svec *et al.* [23] have described that rinsing with water at elevated temperatures (80ºC) allowed a satisfactory regeneration of AuNP-modified columns after treatment with different ligands. On the basis on this study, the regeneration of the AuNP-modified ~~monolith~~ material was carried out by washing it with water at 80ºC for 2 h. After this treatment, the loading capacity was again checked, giving *ca*. 87% of the original capacity. Further, SEM/BSE micrograph of the regenerated AuNP-modified sorbent was performed, where AuNPs were attached onto the ~~monolith~~ polymer surface (data not shown) Also, UV-vis and EDAX measurements of Au content were conducted, with values *ca*. 0.42 wt%, which confirmed the feasibility of the rinsing protocol. Following this procedure, the sorbent can be reused for 20 times without significant efficiency losses (or with satisfactory recoveries comprised between 81.3-97.6%).

The proposed SPE sorbent described here could be favorably compared with recent reports related to the use of functionalized polymeric films for protein purification [50, 51]. Thus, the loading capacity was higher than that reported using membranes-based devices modified with poly(acrylic acid) (PAA) brushes [52]. However, the derivatization of PAA films with metal-ion complexes developed by these authors in several works [50, 51, 53] allowed an increase in the binding capacity of the platform (5.8 μg/cm^2 for BSA [52] and *ca*. 90 mg/mL for His-tagged ubiquitin [53, 54] and its specificity to protein with accessible histidine groups. From these results, a 12 cm × 12 cm membrane area would be needed to retain the same amount of protein (830 μg BSA) previously found for 50 mg sorbent per cartridge, being this SPE device adequate to process small amounts of sample. In spite of the satisfactory binding capacities of these films, the membrane-modification process to achieve these platforms

342 is laborious (several reaction steps). Moreover, brush synthesis is complicated and
343 controlling the polymer-chain density, which undoubtedly affects the amount of protein
344 binding, is challenging [55]. In our case, the preparation of sorbent (synthesis and
345 modification protocol) involved few simple steps.

346 Another aspect to be considered in these films is that they should be thin enough
347 to prevent clogging and maintain flow. In our case, the resulting polymeric sorbent
348 showed some residual permeable porous monolithic structure, which minimizes the
349 probability of sorbent clogging.

350 Other strength of the proposed sorbent is its large reusability (see data above). In
351 this regard, in some of the related works of polymeric films, these devices were reused
352 at least 4 [52] or 6 times [56].

353

354 *3.3. Application to protein isolation*

355

356 The feasibility of the present extraction method for the isolation of proteins
357 according to their pI was demonstrated by isolating cyt c from BSA. A 200 μg mL^{-1} of
358 BSA sample solution prepared at pH 5 and spiked with 100 μg mL^{-1} of cyt c was
359 employed. The extraction process was conducted as detailed above and the different
360 fractions were subjected to SDS-PAGE as illustrated in Fig. 5. As observed, BSA was
361 effectively isolated from cyt c, which is not retained onto the sorbent at pH 5. The SDS-
362 PAGE showed the cyt c band around 12 kDa in the loading fraction (lane 3); whereas
363 the presence of proteins was not observed in the washing step solution (lane 4). On the
364 other hand, BSA band at 66 kDa was observed in the elution fraction after pass through
365 the column buffer solution at pH 11 (lane 5).

366

3.4. Application to lectins isolation from European mistletoe leaves

To demonstrate the application of this sorbent in complex real samples, the isolation of lectins from an European mistletoe sample was done. The extracts from this plant material have been used for a long time as therapeutic agents, mainly for oncological applications. In particular, the cytotoxic and immune stimulant properties of this plant material have been attributed to lectins [57,58]. These glycoproteins are constitute by two polypeptide chains, the cytotoxic A-chain (Mr ≈ 29 kDa) and the carbohydrate-binding B-chain (Mr ≈ 32 kDa) , linked by a disulfide bond, and they can classified as mistletoe lectins (ML): ML-1, ML-2, and ML-3, depending on sugar-binding specificities [59-61]. In particular, most of the European MLs belong to ML-1 group, being it well characterized. Thus, the chains of ML-1 were distributed over a pI range comprised between 6.6 and 7.0 [62-64]

The isolation of lectins from complex samples is often complicated and requires many purification steps (ammonium precipitation, ionic and affinity chromatography, etc) [65]. These chromatographic methods can be time-consuming and expensive, leading to a low yield of the entire process. In addition, the affinity chromatography maintenance is a hardy task when plant crude extracts are loaded into columns since such samples contain pigments, oily components, and other complex substances that could damage the column and worsen the purification [66]. These awkward purification protocols can be considerably simplified by using the sorbent developed here. For this purpose, the extraction process detailed in section 2.6 was followed and then SDS-PAGE analysis of the collected fractions was carried out. Fig. 6 shows the SDS-PAGE analysis of the mistletoe extract without (lane 2) and with pretreatment (lane 3) using this sorbent under non-reducing conditions. Lane 2 did not yield a clearly visible band

392 at the position of the ML proteins, whereas the lane 3 gave two major bands
393 corresponding to M_r = 60,000 and 58,000 Da. These two prominent bands were
394 identified as ML-1 and ML-3, respectively, which was in agreement with previous
395 studies [67]. Also, SDS-PAGE analysis was performed under reducing conditions
396 (presence of 2-mercaptoethanol) (Fig. 6, lane 4), where two major bands originated
397 from the heterodimeric lectins were evidenced, which corresponded to M_r = 28,000 and
398 34,000 Da, being ascribed to the corresponding A- and B- chains of ML-1 and ML-3,
399 respectively [67]. In addition, a misty band at M_r = 60,000 Da was found, which was
400 caused by uncompleted reaction of 2-mercaptoethanol.
401
402 **4. Conclusions**
403
404 A novel material based on a powdered GMA-based polymeric monolith
405 modified with AuNPs has been developed and used as a sorbent in SPE for proteins
406 separation. Several parameters of the SPE procedure have been optimized and protein
407 recoveries have been evaluated by using Bradford assay. By simply adjusting the pH of
408 the loading and washing solution close to pI values of proteins of interest, the
409 biomacromolecules were readily adsorbed onto the AuNPs surface, and could
410 afterwards be eluted by a proper variation of the pH of the eluent. Satisfactory results in
411 terms of recovery efficiencies (95-98 %), loading capacity (16.6 mg per g sorbent) and
412 reusability (at least 20 times) of this sorbent have been also obtained. The feasibility of
413 this methodology was also successfully demonstrated by the isolation of cyt c from
414 BSA followed by SDS-PAGE analysis. Moreover, the practical applicability of the
415 sorbent was demonstrated by an efficient isolation of lectins from mistletoe samples.
416 The results obtained here showed that the developed sorbent could be useful for the

417 selective separation and/or preconcentration of protein species of interest (according to
418 pI) in complex real samples.
419

Acknowledgements

This work was supported by project CTQ2014-52765-R (MINECO of Spain and FEDER). M. V-B thanks the MEC for an FPU grant for PhD studies. The authors also thank Dr. Salomé Laredo-Ortíz and Dr. Enrique Navarro-Raga from the Atomic spectroscopy and Microscopy sections of the SCSIE (University of Valencia), respectively, for their help in elemental analysis and SEM/EDAX measurements.

References

[1] F. Svec, Y. Lv, Advances and recent trends in the field of monolithic columns for chromatography, Anal. Chem. 87 (2015) 250-273.

[2] N. Smith, Z. Jiang, Developments in the use and fabrication of organic monolithic phases for use with high-performance liquid chromatography and capillary electrochromatography, J. Chromatogr. A 1184 (2008) 416-440.

[3] K. Liu, P. Aggarwal, J.S. Lawson, H.D. Tolley, M.L. Lee, Organic monoliths for high-performance reversed-phase liquid chromatography, J. Sep. Sci. 36 (2013) 2767-2781.

[4] J. Ou, Z. Liu, H. Wang, H. Lin, J. Dong, H. Zou, Recent development of hybrid organic-silica monolithic columns in CEC and capillary LC, Electrophoresis 36 (2015) 62-75.

[5] D.R. Bunch, S. Wang, Applications of monolithic solid-phase extraction in chromatography-based clinical chemistry assays, Anal. Bioanal. Chem. 405 (2013) 3021-3033.

[6] A. Namera, T. Saito, Advances in monolithic materials for sample preparation in drug and pharmaceutical analysis, Trends Anal. Chem. 45 (2013) 182-196.

[7] D. Sykora, F. Svec, J.M.J. Frechet, Separation of oligonucleotides on novel monolithic columns with ion-exchange functional surfaces, J. Chromatogr. A 852 (1999) 297-304.

[8] B. Preinerstorfer, W. Lindner, M. Laemmerhofer, Polymethacrylate-type monoliths functionalized with chiral amino phosphonic acid-derived strong cation exchange moieties for enantioselective nonaqueous capillary

electrochromatography and investigation of the chemical composition of the monolithic polymer, Electrophoresis 26 (2005) 2005-2018.

[9] J. Dong, R. Ou, X. Dong, R. Wu, M. Ye, H. Zou, Preparation and evaluation of rigid porous polyacrylamide-based strong cation-exchange monolithic columns for capillary electrochromatography, J. Sep. Sci. 30 (2007) 2986-2992.

[10] E.J. Carrasco-Correa, G. Ramis-Ramos, J.M. Herrero-Martínez, Methacrylate monolithic columns functionalized with epinephrine for capillary electrochromatography applications, J. Chromatogr. A 1298 (2013) 61- 67.

[11] J. Liu, I. White, D.L. DeVoe, Nanoparticle-functionalized porous polymer monolith detection elements for surface-enhanced Raman scattering, Anal. Chem. 83 (2011) 2119-2124.

[12] Q. Li, Y. Du, H. Tang, X. Wang, G. Chen, J. Iqbal, W. Wang, W. Zhang, Ultra sensitive surface-enhanced Raman scattering detection based on monolithic column as a new type substrate, J. Raman Spectrosc. 43 (2012) 1392-1396.

[13] X. Wang, Y. Du, H. Zhang, Y. Xu, Y. Pan, T. Wu, H. Hu, Fast enrichment and ultrasensitive in-situ detection of pesticide residues on oranges with surface-enhanced Raman spectroscopy based on Au nanoparticles decorated glycidyl methacrylate ethylene dimethacrylate material, Food Control 46 (2014) 108-114.

[14] K. Benčinaa, M. Benčinab, A.Podgornika, A. Štrancara, Influence of the methacrylate monolith structure on genomic DNA mechanical degradation, enzymes activity and clogging, J. Chromatogr. A 1160 (2007) 176-183.

[15] Y-C. Yeh, B. Creran, V.M. Rotello, Gold nanoparticles: preparation, properties, and applications in bionanotechnology, Nanoscale 4 (2012) 1871-1880.

475 [16] D. Sykora, V. Kasicka, I. Miksik, P. Rezanka, K. Zaruba, P. Matejka, V. Kral,
476 Application of gold nanoparticles in separation sciences, J. Sep. Sci. 33 (2010)
477 372-387.
478 [17] C.C. Shen, W.L. Tseng, M.M. Hsieh, Selective enrichment of aminothiols using
479 polysorbate 20-capped gold nanoparticles followed by capillary electrophoresis
480 with laser-induced fluorescence, J. Chromatogr. A 1216 (2009) 288-293.
481 [18] M.D. Li, T.L. Cheng, W.L. Tseng, Nonionic surfactant-capped gold
482 nanoparticles for selective enrichment of aminothiols prior to CE with UV
483 absorption detection, Electrophoresis 30 (2009) 388-395.
484 [19] C.W. Chang, W.L. Tseng, Gold nanoparticle extraction followed by capillary
485 electrophoresis to determine the total, free, and protein-bound aminothiols in
486 plasma, Anal. Chem. 82 (2010) 2696-2702.
487 [20] Y.W. Lin, M.J. Huang, H.T. Chang, Analysis of double-stranded DNA by
488 microchip capillary electrophoresis using polymer solutions containing gold
489 nanoparticles, J. Chromatogr. A 1014 (2003) 47-55.
490 [21] L. Yang, E. Guihen, J.D. Holmes, M. Loughran, G.P. O'Sullivan, J.D. Glennon,
491 Gold nanoparticle-modified etched capillaries for open-tubular capillary
492 electrochromatography, Anal. Chem. 77 (2005) 1840-1846.
493 [22] Q. Cao, Y. Xu, F. Liu, F. Svec, J.M.J. Fréchet, Polymer monoliths with
494 exchangeable chemistries: use of gold nanoparticles as intermediate ligands for
495 capillary columns with varying surface functionalities, Anal Chem. 82 (2010)
496 7416-7421.
497 [23] Y. Xu, Q. Cao, F. Svec, J.M.J. Fréchet, Porous polymer monolithic column with
498 surface-bound gold nanoparticles for the capture and separation of cysteine-
499 containing peptides, Anal. Chem. 82 (2010) 3352-3358.

500 [24] Y. Lv, A. Maya, J.M.J. Fréchet, F. Svec, Preparation of porous polymer
501 monoliths featuring enhanced surface coverage with gold nanoparticles, J.
502 Chromatogr. A 1261 (2012) 121-128.
503 [25] W.Z. Chen, J. Shen, X.F. Yin, Y.N. Yu, Optimization of microfabricated
504 nanoliter-scale solid-phase extraction device for detection of gel-separated
505 proteins in low abundance by matrix-assisted laser desorption/ionization mass
506 spectrometry, Rapid Commun. Mass Spectrom. 21 (2007) 35-43.
507 [26] H. Joshi, P.S. Shirude, V. Bansal, K.N. Ganesh, M. Sastry, Isothermal titration
508 calorimetry studies on the binding of amino acids to gold nanoparticles, Phys.
509 Chem. B 108 (2004) 11535-11540.
510 [27] D. Aureau, Y. Varin, K. Roodenko, O. Seitz, O. Pluchery, Y.J. Chabal,
511 Controlled deposition of gold nanoparticles on well-defined organic monolayer
512 grafted on silicon surfaces, J. Phys. Chem. C 114 (2010) 14180–14186.
513 [28] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram
514 quantities of protein utilizing the principle of protein-dye binding, Anal.
515 Biochem. 72 (1976) 248-54.
516 [29] W.A.E. McBryde, J.H Yoe, Colorimetric determination of gold as bromoaurate,
517 Anal. Chem. 20 (1948) 1094-1099.
518 30] V. Gitis, R.C. Haught, R.M. Clark, J. Gun, O. Lev, Nanoscale probes for the
519 evaluation of the integrity of ultrafiltration membranes, J. Membr. Sci. 276
520 (2006) 199-207.
521 [31] S. Olsnes, F. Stirpe, K. Sandvig, A. Pihl, Isolation and characterization of
522 Viscumin, a toxic lectin from Viscum *album L.* (Mistletoe), J. Biol. Chem. 257
523 (1982) 13263-13270.

[32] U.K. Laemmli. Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680-685.

[33] E.J. Carrasco-Correa, G. Ramis-Ramos, J.M. Herrero-Martínez, Evaluation of 2,3-epoxypropyl groups and functionalization yield in glycidyl methacrylate monoliths using gas chromatography, J. Chromatogr. A 1379 (2015) 100-105.

[34] J.J. Gray, The interaction of proteins with solid surfaces, Curr. Opin. Struct. Biol. 14 (2004) 110-115.

[35] S.H. Lacerda, J.J. Park, C. Meuse, D. Pristinski, M.L. Becker, A. Karim, J.F. Douglas, Interaction of gold nanoparticles with common human blood proteins, ACS Nano 26 (2010) 365-379.

[36] J.E. Gagner, M.D. Lopez, J.S. Dordick, R.W. Siegel, Effect of gold nanoparticle morphology on adsorbed protein structure and function, Biomaterials 32 (2011) 7241-7252.

[37] Z. Du, Y.L. Yu, X.W. Chen, J.H. Wang, The isolation of basic proteins by solid-phase extraction with multiwalled carbon nanotubes, Chem. Eur. J. 13 (2007) 9679-9685.

[38] J.Y. Yoon, J.H. Kim, W.S. Kim, Interpretation of protein adsorption phenomena onto functional microspheres, Colloids Surf. B 12 (1998) 15-22.

[39] J.Y. Yoon, J.H. Kim, W.S. Kim, The relationship of interaction forces in the protein adsorption onto polymeric microspheres, Colloids Surf. A 153 (1999) 413-419.

[40] S. Thobhani, S. Attree, R. Boyd, N. Kumarswami, J. Noble, M. Szymanski, R.A. Porter, Bioconjugation and characterisation of gold colloid-labelled proteins, J. Immunol. Methods 356 (2010) 60-69.

548 [41] S.H. Brewer, W.R. Glomm, M.C. Johnson, M.K. Knag, S. Franzen, Probing BSA
549 binding to citrate-coated gold nanoparticles and surfaces, Langmuir 21 (2005)
550 9303-9307.
551 [42] W. R. Glomm, Ø. Halskau, A.M.D. Hanneseth, S. Volden, Adsorption behavior of
552 acidic and basic proteins onto citrate-coated Au surfaces, correlated to their
553 native fold, stability, and pI, J. Phys. Chem. B 111 (2007) 14329-14345.
554 [43] M. Brust, M. Walker, D. Bethell, D.J Schiffrin, R. Whyman, Synthesis of thiol-
555 derivatised gold nanoparticles in a two-phase liquid-liquid system, J. Chem. Soc.
556 Chem. Commun. 70 (1994) 801-802.
557 [44] K.D. Dobson, P.A. Connor, A.J. McQuillan, Monitoring hydrous metal oxide
558 surface charge and adsorption by STIRS, Langmuir 13 (1997) 2614-2616.
559 [45] S.H. Guo, S.J. Tsai, H.C. Kan, D.H. Tsai, M.R. Zachariah, R.J. Phaneuf, The effect
560 of an active substrate on nanoparticle-enhanced fluorescence, Adv. Mater. 20
561 (2008) 1424-1428.
562 [46] H. Tsai, M. Davila-Morris, F.W. DelRio, S. Guha, M.R. Zachariah, V.A. Hackley,
563 Quantitative determination of competitive molecular adsorption on gold
564 nanoparticles using Attenuated Total Reflectance-Fourier Transform Infrared
565 Spectroscopy, Langmuir 27 (2011) 9302-9313.
566 [47] K. Rezwan, A.R. Studart, J. Voros, L.J.J. Gauckler, Change of ζ potential of
567 biocompatible colloidal oxide particles upon adsorption of bovine serum
568 albumin and lysozyme, Phys. Chem. 109 (2005) 14469-14474.
569 [48] http://www.waters.com/waters/en_US/SPE-Method-Development/nav.htm?cid=
570 10083845&locale=en_US
571 [49]http://www.sigmaaldrich.com/analytical-chromatography/sample-preparation/spe/
572 tube-configuration-guide.html

[50] P. Jain, G.L. Baker, M.L. Bruening, Applications of polymer brushes in protein analysis and purification, Annu. Rev. Anal. Chem. 2 (2009) 387-408.

[51] J. Dong, M.L. Bruening, Functionalizing microporous membranes for protein purification and protein digestion, Annu. Rev. Anal. Chem. 8 (2015) 81-100.

[52] J. Dai, Z. Bao, L.Sun, S.U. Hong, G.L. Baker,M.L. Bruening, High-capacity binding of proteins by poly(acrylic acid) brushes and their derivatives, Langmuir 22 (2006) 4274-4281.

[53] W. Ning, M.L. Bruening, Rapid protein digestion and purification with membranes attached to pipet tips, Anal. Chem. 87 (2015) 11984-11989.

[54] P. Jain, M.K. Vyas, J.H. Geiger, G.L. Baker, M.L. Bruening, Protein purification with polymeric affinity membranes containing functionalized poly(acid) brushes, Biomacromolecules 11 (2010) 1019-1026.

[55] 55: Yang et al. J. Mater. Chem. 2011

[56] S. Bhattacharjee, J. Dong, Y. Ma, S. Hovde, J.H. Geiger, G.L. Baker, M.L. Bruening, Formation of high-capacity protein-adsorbing membranes through simple adsorption of poly(acrylic acid)-containing films at low pH, Langmuir 28 (2012) 6885-6892.

[57] H. Bantel, I.H Engels, W. Voelter, K. Schulze-Osthoff, S. Wesselborg, Mistletoe lectin activates Caspase-8/FLICE independently of death receptor signaling and enhances anticancer drug-induced apoptosis, Cancer Res. 59 (1999) 2083-2090.

[58] C.N. Baxevanis, I.F. Voutsas, M.H. Soler, A.D. Gritzapis, O.E. Tsitsilonis, S. Stoeva, W. Voelter, P. Arsenis, M. Papamichail, Mistletoe lectin I-induced effects on human cytotoxic lymphocytes. I. Synergism with Il-2 in the induction of enhanced lak cytotoxicity, Immunopharmacol. Immunotox. 20 (1998) 355-372.

[59] H. Franz, P. Ziska, A. Kindt, Isolation and properties of three lectins from mistletoe (Viscum *album L.*), Biochem. J. 195 (1981) 481-484.

[60] R. Samtleben, M. Kiefer, P. Luther, Characterization of the different lectins from Viscum *album* (Mistletoe) and their structural relationships with the agglutinins from *Abrus precatorius* and *Ricinus communis*. Lectins: Biol. Biochem. Clin. Biochem. 4 (1985) 617-626.

[61] R. Eifler, K. Pfüller, W. Göckeritz, U. Pfüller, Improved procedures for isolation of mistletoe lectins and their subunits: lectin pattern of the European mistletoe. Lectins: Biol. Biochem. Clin. Biochem. 9 (1993) 144–151.

[63] P. Lutter, H.E. Meyer, M. Langer, K. Witthohn, W. Dormeyer, A. Sickmann, M. Blüggel, Investigation of charge variants of rViscumin by two-dimensional gel electrophoresis and mass spectrometry, Electrophoresis 22 (2001) 2888-2897.

[64] P. Luther, H. Theise, B. Chatterjee, D. Karduck, G. Uhlenbruck, The lectin from Viscum *Album L*. isolation, characterization, properties and structure, Int. J. Biochem. 11 (1980) 429-435.

[65] K.S. Nascimento, A.I. Cunha, K.S. Nascimento, B.S. Cavada, A.M. Azevedo, M.R. Aires-Barros, An overview of lectins purification strategies, J. Mol. Recognit.25 (2012) 527-541.

[66] M.A. de Santana, A.M. Santos, M.E. Oliveira, J.S. de Oliveira, E.H. Baba, M.M. Santoro, M.H. de Andrade. A novel and efficient and low-cost methodology for purification of Macrotyloma axillare (Leguminosae) seed lectin, Int. J. Biol. Macromol. 43 (2008) 352-358.

[67] R. Wacker, S. Stoeva, C.Betzel, W. Voelter, Complete structure determination of N-acetyl-Dgalactosamine-binding mistletoe lectin-3 from *Viscum album L. album,* J. Peptide Sci. 11 (2005) 289-302.

Figures

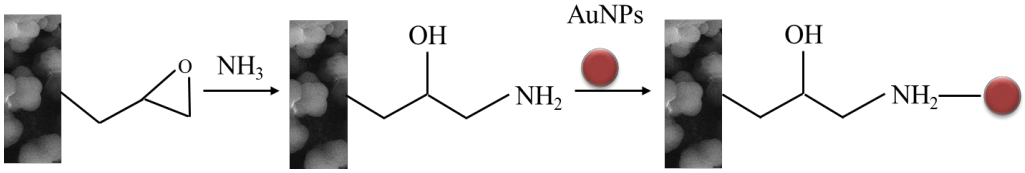


Fig. 1. Preparation of poly(GMA-co-EDMA) monolith and its modifications with ammonia and immobilization of AuNPs.

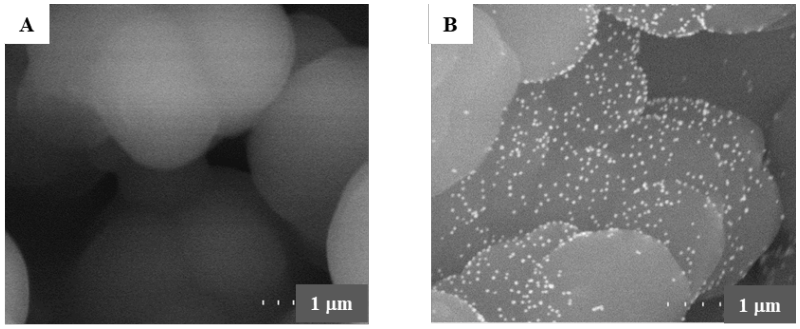


Fig. 2. SEM/BSE micrograph of GMA-co-EDMA powder material without AuNPs (A) and modified with AuNPs (B).

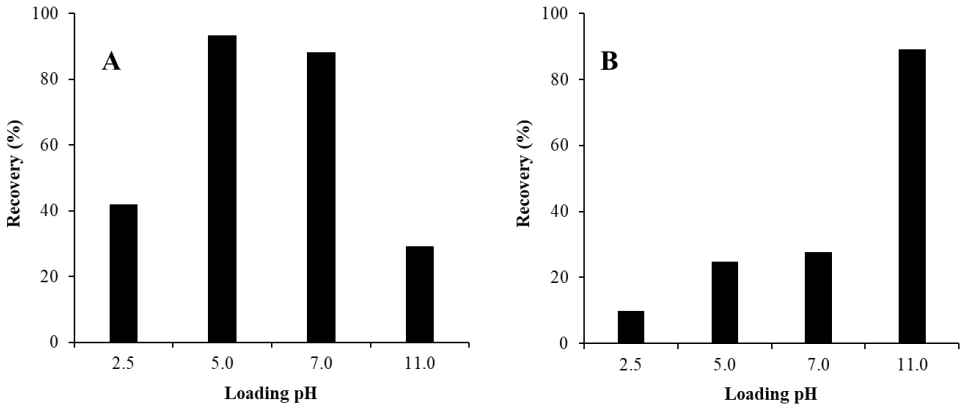
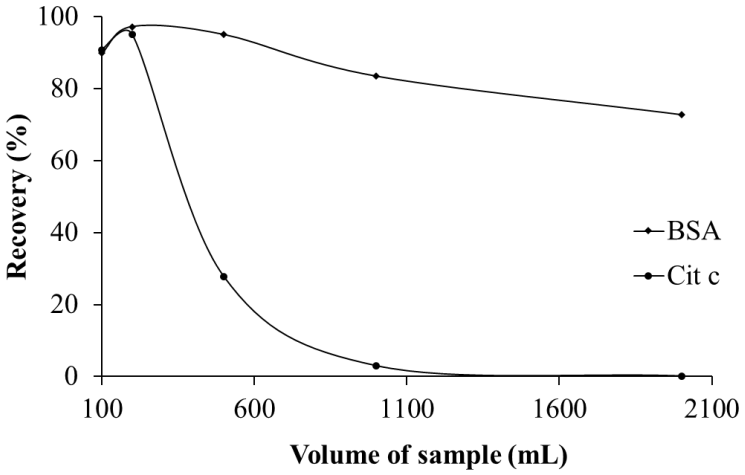
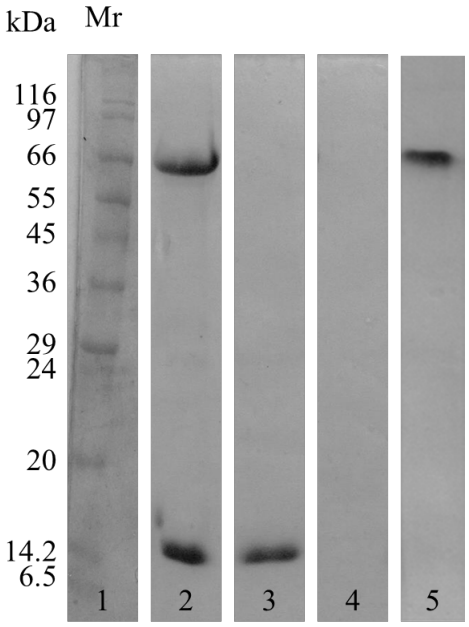


Fig. 3. Effect of loading pH solution on elution step of BSA (part A) and cyt c (part B).



635

636 **Fig. 4.** Effect of sample volume on the recoveries of BSA and cyt c in the GMA-co-
637 EDMA material modified with AuNPs.



638

639 **Fig. 5.** SDS-PAGE of the isolated cyt c from BSA. Identification: lane 1, molecular
640 weight standards (Mr in kDa); lane 2, a 200 μg mL^{-1} of BSA sample solution spiked
641 with 100 μg mL^{-1} of cyt c without pre-treatment; lane 3, the loading step solution
642 passing through the GMA-co-EDMA material modified with AuNPs; lane 4, the
643 washing step solution; lane 4: the elution step solution with the recovered BSA from the
644 sample solution.

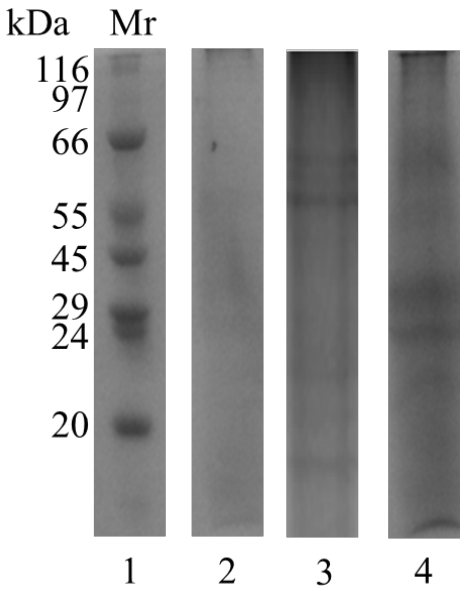


Fig. 6. SDS-PAGE analysis demonstrating isolation of ML lectins from an European mistletoe extract. Lane 1: molecular weight marker, lane 2: mistletoe extract without pretreatment with this sorbent using non-reducing conditions; lane 3: mistletoe extract after pretreatment with this sorbent using non-reducing conditions; lane 4: mistletoe extract after pretreatment with this sorbent using reducing conditions.