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Boronate affinity sorbents based on thiol-functionalized polysiloxane-polymethacrylate composite materials in syringe format for selective extraction of glycopeptides

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ABSTRACT

In this work, two novel boronate affinity monolithic materials able to extract glycopeptides within a polypropylene syringe are described and compared. The first material was synthesized from glycidyl methacrylate (GMA)-based monoliths modified with poly-3-mercaptopropyl-methylsiloxane (PMPMS) followed by attachment of 4-vinylphenylboronic acid (VPBA) via thiol-ene click reaction. The second material was prepared by using gold nanoparticle (AuNP)-modified monoliths as substrate followed by subsequent attachment of PMPMS and VPBA. The resulting materials were used as sorbents for solid-phase extraction (SPE) to selectively preconcentrate glycopeptides from horseradish peroxidase (HRP) digests. The material that gave the superior performance was that prepared with AuNPs due to the presence of abundant boronic acid groups, being its practical applicability also examined. The hybrid material exhibited a satisfactory efficiency of glycopeptide enrichment (identifying 24 glycopeptides from a total of 27) in mixture of tryptic digests of HRP and bovine serum albumin (BSA) (1:100, w/w). The sorbent shows low sensitivity (0.5 fmol/μL), good adsorption capacity (25 mg g^{-1}) and suitable reusability (over 10 times). Moreover, the hybrid monolith was successfully applied to the selective enrichment of glycopeptides from human serum digests, without any pretreatment, in which 85 glycopeptides were identified by nano-LC-MS/MS, suggesting a great potential for application in glycoproteome field.

1. Introduction

Glycoproteins play an essential role in diverse biological and clinical events, such as inter and intracellular transport, signal transduction, immune response, and molecular recognition [1–4]. In addition, they have been associated with many diseases, being used as biomarkers and therapeutic targets in clinical diagnostics [5]. However, the low abundance of glycoproteins in biological samples, jointly with the complexity of these matrices, make that the direct identification of these compounds remains challenging [3,4,6]. Consequently, a sample treatment is required before proceeding with their determination. Several methods have been described for glycoproteins enrichment such as lectin affinity chromatography [7,8], hydrophilic interaction chromatography [9], hydrazide chemistry [10], or boronate affinity chromatography [3–6]. In particular, boronate-based supports present particular affinity properties towards solutes (e.g. glycoproteins) containing cis-diol groups, where the interaction based on a specific covalent binding can be easily tailored by adjusting the pH of medium [3,5]. Additionally, these boronic-based supports can avoid irreversible glycan variations during extraction process [11]. Indeed, various boronate affinity materials have been synthesized, such as boronic acid-functionalized magnetic nanoparticles (NPs) [12], mesoporous silica particles [13], molecularly imprinted polymers (MIPs) [14,15] and monolithic supports [2–4,6].

The well-known features of monolithic materials, particularly polymer monoliths (viz. simple and cost-effective fabrication, high stability over a wide pH range, low back-pressure and fast separation of macromolecules) [16] combined with boronic ligands represent an attractive support for sample pretreatment methodologies as evidenced by several reviews [11,17]. Thus, these materials have been prepared using hydrophilic boronic acid ligands as functional monomers such as 3-acrylamidophenylboronic acid [18], 4-vinylphenylboronic acid (VPBA) [19], 2,4-difluoro-3-formyl-phenylboronic acid [20] and 3-(acrylamido)phenylboronic acid [5], among others. Nevertheless, due to the small surface area of the resulting polymer monoliths, glycoprotein binding

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capacity needs to be enhanced. Also, organic/inorganic hybrid boronate affinity monoliths [4,21,22] have been adopted to extract these target analytes. Although a slight improvement in surface area is produced in these hybrid monoliths, the nonspecific adsorption of non-glycoproteins on such monoliths is delicate, which decreases the selectivity. Therefore, the development of boronate affinity monolithic sorbents with large surface areas and high selectivity is strongly required to efficiently accomplish the selective capture of glycopeptides.

In the last years, the incorporation of nanomaterials into the monoliths has been considered a valuable strategy to increase the effective surface area of polymer monoliths as well as to tailor their selectivity [23,24]. Several nanomaterials such as gold nanoparticles (AuNPs) [1,3,25] and graphene oxide (GO) [3,26] have been used to prepare boronate affinity-based materials for (micro)extraction purposes. Most of these works have been applied in-capillary format; however, their scale-up to other usual (micro)extraction formats (with enhanced loading capacity) has been scarcely investigated [25]. In this respect, AuNPs have attracted much interest among scientists of monolith field [25,27–29], since AuNP-modified monoliths could be considered perfect candidates as host matrices for selective glycopeptides enrichment [1,2]. For example, Wu *et al.* [1] reported the modification of a GMA-based monolith with AuNPs (using cysteamine) to attach 4-mercaptophenylboronic acid (MPBA) and 2-mercaptoethylamine. The resulting hybrid monolithic capillaries were applied to enrich horseradish peroxidase (HRP) and glycoproteins from human plasma. The same authors [2] used the same AuNP-modified monolithic platform to attach cysteine and PNGase F for glycopeptide enrichment and on-line deglycosilation, respectively. Despite these contributions, it is still necessary to improve the immobilized amount of boronic acid groups of supports, which would consequently enhance its binding capacity. Additionally, this information is commonly missing in most works reported on this topic. Therefore, it is essential to develop novel materials with high coverage density of boronate ligands.

In this work, with the aim of obtaining hybrid boronate affinity materials with a large surface area and enhanced surface coverage of boronate functionalities, poly-3-mercaptopropyl-methylsiloxane (PMPMS) was attached to the surface of the epoxide groups of a poly (GMA-*co*-EDMA) monoliths. Although this polymer has been used to generate large thiol-covered monolith surfaces, which has been posterior functionalized (by thiol-ene click reaction) with chiral selectors in miniaturized separation techniques [30,31], its use to develop boronate affinity sorbents has not been yet explored. Besides, the development of these novel hybrid monolithic sorbents in miniaturized devices (such as syringe) has been proposed for the first time. In order to prepare the boronate affinity sorbents, two distinct approaches were adopted: i) functionalization of the poly(GMA-*co*-EDMA) monolith with the PMPMS polymer followed by thiol-ene click attachment of VPBA, and ii) synthesis of an AuNP-modified poly(GMA-*co*-EDMA) monolith to be used as host material for binding PMPMS polymer followed by attachment of VPBA ligand by click thiol-ene chemistry (Fig. 1). The resulting materials obtained by these approaches were morphologically characterized. Also, their potential as solid-phase extraction (SPE) sorbents to selectively preconcentrate glycopeptides from horseradish peroxidase (HRP) digests as model protein was evaluated. Then, the best material was investigated in terms of adsorption capacity, sensitivity, selectivity, reusability and reproducibility. Furthermore, the practical applicability of selected material was evaluated by enriching glycopeptides from a biological sample such as human serum.

2. Experimental

2.1. Chemicals and reagents

Glycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EDMA), cyclohexanol, 1-dodecanol, 2,2-dimethoxy-2-phenyl-acetophenone (DMPA), cystamine dihydrochloride, poly-3-

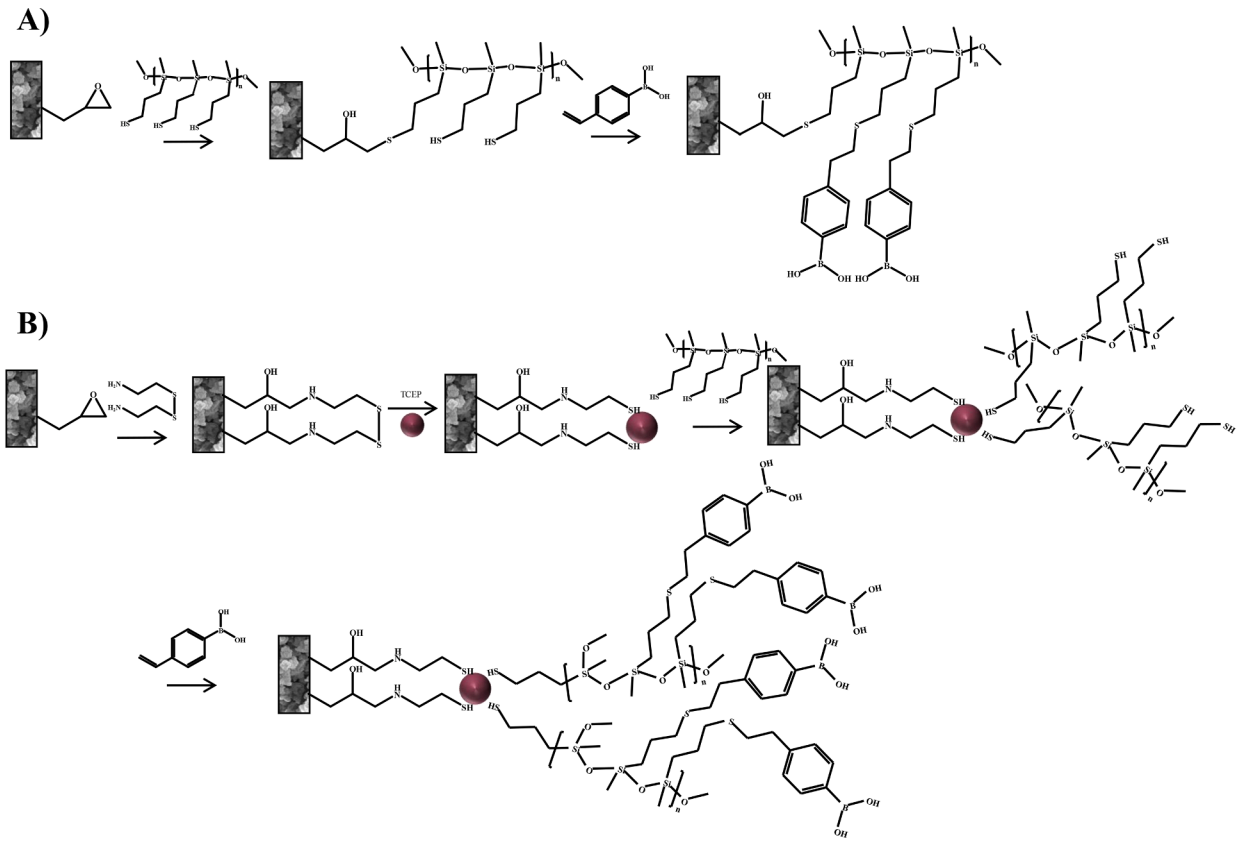

Fig. 1. Scheme of synthesis of (A) GMA-PMPMS-VPBA and (B) GMA-SH@AuNP@PMPMS-VPBA monolithic materials.

mercaptopropyl methylsiloxane (PMPMS), 4-vinylphenylboronic acid (VPBA), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), benzophenone (BP), α-cyano-4-hydroxycinnamic acid (HCCA), trifluoroacetic acid (TFA), dithiothreitol (DTT), ammonium bicarbonate, trypsin, iodoacetamide, sodium hydroxide (NaOH), formic acid (FA), horseradish peroxidase (HRP) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Milwaukee, WI, USA). PNGase F Glycan cleavage kit was acquired from Thermo Fisher Scientific (Bremen, Germany). Azobisisobutyronitrile (AIBN) was purchased from Fluka (Buchs, Switzerland). Gold nanoparticles (AuNPs) suspension stabilized with sodium citrate (particle size, 20 nm, $6.54{\cdot}10^{11}$ particles mL^{-1}) and Coomassie Blue were from Alfa Aesar (Lancashire, UK). Acetonitrile (ACN), ethanol (EtOH), acetone and methanol (MeOH) were purchased from Scharlab (Barcelona, Spain). Deionized water was prepared in Crystal B30 EDI Adrona deionizer (Riga, Latvia) was used in all procedures.

Polypropylene syringes of 1 mL (used as support for monolithic phases) were purchased from Henke Sass Wolf (Keltenstraβe4, Tuttlingen, Germany).

2.2. Instrumentation

Photografting of polypropylene wall surface and photopolymerization of monoliths were carried out using a UV radiation chamber CL-1000 (UVP, Upland, Canada). A syringe pump (model 101, KD Scientific, Holliston, MA, USA) was used to perform functionalization (or modification) of monolithic beds. The morphology of resulting materials was characterized by a scattering electron microscope (S-4800, Hitachi, Ibaraki, Japan) equipped with a backscattered electron detector and a X-ray microanalysis system (EDAX Genesis 400). High-resolution transmission electron microscopy (TEM) analysis of sorbents was performed in a JEOL microscope (JEM 2100F, Freising, Germany) operated at 200 kV. The determination of gold and boron content in the materials was also done by using an inductive coupling plasma equipment connected to mass spectrometer (ICP-MS) (model 7900, Agilent Technologies, Waldbronn, Germany). For these latter measurements, all materials were previously calcined at 550 °C for 1 h, and the resulting pellets were dissolved in aqua regia, properly diluted with water and analyzed by ICP-MS.

An EA 1110 CHNS elemental analyzer (CE Instruments, Milan, Italy) was used for elemental analysis of synthesized materials. Fourier-transform infrared (FTIR) spectroscopy was also conducted in characterization studies. For this purpose, a FTIR spectrometer model Tensor 27 from Bruker (Bremen, Germany) was used. Measurements were recorded using a DLaTGS detector and a Dura Sample IR II attenuated total reflection (ATR) accessory from Smiths Detection Inc. (Warrington, UK) equipped with a nine-reflection diamond/ZnSe DuraDisk plate was used. Spectra were collected in the region compressed between 4000 and 550 cm^{-1} at a resolution of 4 cm^{-1} with 15 scans. Background spectrum was acquired under the same measurement conditions.

An 8453 diode-array UV–vis spectrophotometer (Agilent Technologies, Waldbronn, Germany) was used to obtain HRP content by Bradford assay.

A 5800 MALDI-TOF/TOF-MS (AB SCIEX, Foster City, CA, USA) was used to establish peptide masses.

Peptides were also analyzed by using an Eksigent 425 nano-LC system (Dublin, CA) which was connected to a TripleTOF 6600plus mass spectrometer (AB Sciex).

2.3. Synthesis of GMA-based monolith within the syringe

The inner surface of the syringe was firstly modified by photografting with BP and EDMA [32] in order to enable a covalent attachment of monolithic bed to the wall. The parent monolith consisted of a poly (GMA-*co*-EDMA) material, which was prepared in the vinylized syringe (50 μL of the polymerization mixture, 20 mg of monolith after polymerization) by UV-initiated radical polymerization. The composition of polymerization mixture was taken from a previous work [32]: 40 wt% monomers (32 wt% GMA and 8 wt% EDMA) and 60 wt% porogens (56 wt% cyclohexanol and 4 wt% 1-dodecanol), in the presence of 0.1 wt % DMPA (out of the total weight of the monomers). This mixture was next sonicated for 10 min and purged with nitrogen for 10 more min and then filled into the pretreated syringe. Polymerization was carried out with UV light for 2 h at 1 J cm^{-2}. To remove possible unreacted monomers and rests of porogenic solvents, the monolith was then washed with MeOH and the obtained material was dried in the oven at 60 °C overnight

2.4. Synthesis of the different boronate affinity monolithic materials

Using the parent monolith previously synthesized, two different materials to be tested as SPE sorbents were prepared as illustrated in Fig. 1. The first monolithic material (route A) was obtained by passing through the parent monolith a 10% (v/v) PMPMS solution in ACN at 5 μL min^{-1} using a syringe pump at 60 °C for 2 h. Then, a VPBA solution (40 mg mL^{-1} of VPBA in ACN and also containing 1% AIBN) was used to fill the syringe and its end was sealed with rubber stoppers and kept in an oven at 60 °C for 24 h. The resulting material (GMA-PMPMS-VPBA) was washed with ACN and dried in an oven at 60 °C overnight.

To prepare the second material (route B), the GMA-based monolith was chemically modified with cystamine (to provide thiol moieties) and further attached with AuNPs following a procedure previously described [32,33]. Briefly, a 1.0 mol L^{-1} cystamine dihydrochloride solution (prepared in 2.0 mol L^{-1} aqueous NaOH) was flushed through the bare monolith at 60 °C at a flow rate of 5 μL min^{-1} for 2 h and then washed until neutral pH. Subsequently, to achieve the reduction of disufide bonds, a TCEP solution (0.25 mol L^{-1} in an equal concentration NaOH solution) was pumped through the monolith at room temperature at the same flow rate for 2 h. After washing with water until neutral pH, the resulting thiol-modified monolith was treated with a commercial AuNPs dispersion at 0.3 mL min^{-1} as previously described [34]. The resulting hybrid monoliths were identified as GMA-SH@AuNP. After that, a solution of 10% PMPMS (v/v) in acetone passed through the hybrid monolith at room temperature for 2 h, and then washed with acetone. Afterwards, a solution of VPBA (with the same composition as indicated above) was used to fill the PMPMS-modified material and subjected to the same click *thiol-ene* reaction conditions as above. Finally, the resulting material (namely GMA-SH@AuNP@PMPMS-VPBA) was washed with ACN and dried in an oven at 60 °C overnight.

2.5. HRP and human serum digestion

The standard glycoprotein was dissolved in 1 mL of 50 mM NH_4HCO_3 (pH 7.8) and denatured at 100 °C for 15 min and then allowed to cool at room temperature. Next, the resulting solution was reduced with 1.5 mg mL^{-1} DTT (in 50 mM NH_4HCO_3 7.8) at 56 °C for 1 h and alkylated with 10 mg mL^{-1} iodoacetamide (in the same buffer solution) at room temperature for 45 min. To obtain the glycopeptides, the solution was digested with 0.02 μg $μL^{-1}$ trypsin (in 25 mM NH_4HCO_3 pH 7.8) at 37 °C overnight. The digestion was stopped with FA up to a final concentration of 10%.

10 μL of human serum were diluted with 400 μL of 50 mM NH_4HCO_3 (pH 7.8) and centrifuged at 12000 rpm (ca. 15,000 g) for 5 min. Supernatant was collected and heated at 100 °C for 10 min. Next, the sample was incubated with 100 μL de 30 mg mL^{-1} DTT (in 50 mM NH_4HCO_3 7.8) at 60 °C for 20 min. The cooled solution was alkylated by 10 mg mL^{-1} iodoacetamide (in the same buffer solution) for 30 min at room temperature in the dark. Finally, trypsin (40 μg) was added and digestion was left at 37 °C overnight.

2.6. microSPE protocol

The syringe with the different boronate affinity monolithic sorbents was firstly activated with 500 μL ACN and equilibrated with 500 μL of loading buffer (50 mM NH_4HCO_3 pH 7.8). Then, 500 μL HRP digest (prepared in the loading buffer) were passed through the syringe at 0.1 mL min^{-1} flow rate. The washing step was carried out with the same loading solution and the retained glycopeptides were eluted with 100 μL of ACN/H_2O (50:50, v/v) containing 0.5% TFA prior to the subsequent MS analysis.

2.7. MALDI-TOF analysis

The resulting eluates (1 μL) were spotted onto a MALDI plate, and after the droplets were air-dried at room temperature. 1 μL of matrix (10 mg mL^{-1} HCCA in ACN/H_2O (1:1, v/v) containing 0.1% TFA) was added and allowed to air-dry at room temperature for MALDI-TOF/TOF MS analysis. Peptide mass maps were acquired in in the positive ion mode (1500 shots every position) in a *m/z* range of 800–6000. The MS and MS/MS information was sent to be identified by the MASCOT software (v 2.3.02; Matrix Science) via the Protein Pilot (ABSciex).

2.8. Glycopeptides enrichment from human serum

To enrich glycopeptides from the tryptic digest of human serum, the adopted microSPE protocol was the same that described in Section 2.6. Once the retained glycopeptides were eluted, this solution was evaporated to dryness, and the residue was dissolved in 50 μL of ammonium bicarbonate. Then, the resulting solution was deglycosilated using the PNGase F Glycan Cleavage Kit following the supplier instructions. Finally, the deglycosilated peptides were subjected to nano-LC–MS/MS.

2.9. Nano-LC-MS/MS analysis

Briefly, 2 μL of deglycosilated peptides was loaded onto a trap column (NanoLC Column, 3 μm, C18-CL, 350 μm × 0.5 mm, Eksigent) and desalted using TFA 0.1% as mobile phase at a flow of 5 μL min^{-1} during 5 min. The peptides were then loaded onto an analytical column (3C18-CL-120, 3 μm, 120 Å, 75 μm × 15 cm, Eksigent) equilibrated in 5% ACN and 0.1% FA. The elution was done using a linear gradient of 7–40% B in A for 20 min at a flow rate of 300 nL min^{-1}, where A is deionized water with 0.1% FA and B is ACN with 0.1% FA. Analysis of peptides by MS was carried out in a data-dependent mode. Survey MS1 scans were acquired from 350 to 1400 *m/z* for 250 ms. The quadrupole resolution was set to 'LOW' for MS^2 experiments, which were acquired from 100 to 1500 *m/z* for 25 ms in 'high sensitivity' mode. Main conditions in the MS analysis were ions from 2+ to 4+ with a minimum intensity of 250 cps. Up to 100 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 15 s.

The analysis of the obtained spectra was done using ProteinPilot v5.0 search engine. Protein-Pilot default parameters were used to generate a peak list directly from the nano-LC-MS/MS instrument. The Paragon algorithm [35] of ProteinPilot was used to search the SwissProt database (200601, 562,246 proteins) and Uniprot Trematoda database (200604, 362615). Search criteria included fixed and variable modifications as carbamidomethylation (C) and oxidation (M), respectively. Only peptides with N-!P-S/T were identified as N-linked glycopeptides.

2.10. Quality control and quality assurance (QC/QA)

The application of QA/QC procedures has been carried out in order to provide accurate and reproducible results.

Adsorption capacity of boronate affinity monoliths for glycopeptides was adapted according to the protocols described in literature [36,37]. Briefly, 500 μL of HRP digest at 1 mg mL^{-1} were passed through microSPE devices containing different sorbent amounts (from 10 to 40 mg). After enrichment, the eluted fraction was analyzed by MALDI-TOF-MS. The adsorption capacity was calculated by the amount of HRP tryptic digest (500 μg) to the amount of sorbent.

An estimation of the limit of detection (LOD) was obtained by analyzing digested HRP at low-concentrations (close to the LOD level, as determined from the approach based on S/N = 3).

To evaluate the selectivity of boronate affinity sorbents for glycopeptides, a mixture of HRP and BSA tryptic digests (at a mass ratio of 1:100, w/w) was enriched by the affinity-based monolith and analyzed by MALDI-TOF-MS.

The reusability of the sorbent in syringe devices was evaluated by repeatedly analyzing digested HRP at a concentration of 0.1 mg mL^{-1}.

3. Results and discussion

3.1. Preparation and characterization of boronate affinity monolithic materials

Fig. 1 shows the preparation routes of the different boronate affinity monoliths synthesized in this work. Firstly, poly (GMA-*co*-EDMA) monolith described in previous works of our research group [32,34,38] was selected as starting host material for the preparation of polymeric phases due to the presence of reactive epoxy groups in its structure, which facilitates the modification with different functional groups [1]. Then, two distinct strategies were pursued to increase the density of reactive boronate groups on the surface of the modified monoliths. For this purpose, in the route A, PMPMS was selected as polythiol reagent to modify epoxy groups by nucleophilic substitution following the reaction conditions described elsewhere [30]. The attached PMPMS significantly enhanced the hydrophilicity of parent polymer due to its abundant thiol groups. Then, the available unreacted thiol groups present on the PMMPS-modified monolith surface reacted with VPBA ligands via thiol-ene click chemistry to produce the material named as GMA-PMPMS-VPBA. Alternatively, in the second strategy (route B), AuNPs were immobilized onto the GMA-based monolith surface (previously treated with cystamine [27,28,32]), which provides a smart way not only to increase the surface area for enhancing the active sites, but also to perform a facile surface modification [27,28,32,34,38]. Then, the hybrid monolith was used as support for immobilizing PMPMS and further bonding of VPBA to prepare GMA-SH@AuNP@PMMPS-VPBA composites.

The resulting materials were firstly characterized by SEM to get information of their morphology. The morphology of the GMA-PMPMS-VPBA (see Fig. 2A) resembled the typical microglobular structure of polymethacrylate monoliths, with large-through pores, which is beneficial for flow-through purposes. EDAX analysis of this material gave silicon and boron contents of 2.48 and 2.78 wt%, respectively (see Table S1), thus demonstrating the incorporation of the PMPMPS and VMNPs to the polymeric matrix. Next, SEM/BSE image of the cystamine-modified monolith obtained after AuNP immobilization (Fig. 2B) showed a remarkable AuNPs coverage. This surface coverage was also estimated by EDAX, giving the presence of *ca.* 22 wt% Au (see Table S1), which provided enough sites for further functionalization. Due to the high affinity between gold and the thiol groups of PMPMS, this reagent was used to functionalize AuNP-modified monolith and subsequently to attach VPBA. SEM micrographs of this material (GMA-SH@AuNP@PMMPS-VPBA) were also taken (Fig. 2C). As observed, AuNPs were also evidenced on monolith surface, although most of them were covered by the layer of bonded polythiol, and posterior attachment of VPBA ligand. Although there were no clear morphological differences between both materials (Fig. 2B and C), EDAX analysis of this latter material (see Table S1) showed the presence of silicon (2.02 wt%) and boron (3.04 wt%) demonstrating the successful attachment of PMPMS and VPBA onto AuNPs. Additionally, TEM measurements of these latter materials was carried out (Fig. S1). From these measurements, AuNPs showed diameters of ca. 17 and 19 nm, respectively. The increase in

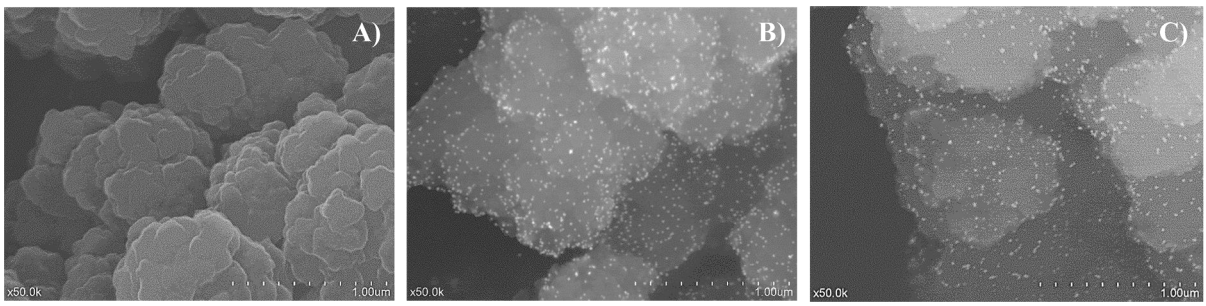

Fig. 2. SEM micrographs of (A) GMA-PMPMS-VPBA, (B) AuNP-modified monolith and (C) GMA-SH@AuNP@PMPMS-VPBA monolithic materials. Images taken at 80,000× magnification.

AuNP diameter in GMA-SH@AuNP@PMMPS-VPBA corroborated the presence of the bonded ligands (PMMPS and VPBA) onto the AuNP surface.

As mentioned in the Introduction, the immobilized amount of boronate groups played an important role in the extraction performance of glycopeptides. Thus, the determination of bulk boron content in both materials was also done by ICP-MS (as described in Section 2.2), giving 0.10 and 0.45 wt% for the GMA-PMPMS-VPBA monolith and the GMA-SH@AuNP@PMMPS-VPBA, respectively. The high immobilization amount observed for the composite might be attributed to the large number of AuNPs available, which provided enhanced reactive thiol coverage (via PMPMS) sites for the attachment of VPBA.

The successful preparation of both monoliths was also confirmed by FT-IR (see Fig. S2). The GMA-based monolith (parent monolith) presented an adsorption band due to the –C–H stretching of epoxy groups at around 3000 cm^{-1} (Fig. S2A). This band decreased in both boronic affinity-based materials (Fig. S2B and C), which proved the epoxide ring-opening reactions with the different ligands (see Fig. 1). Moreover, the band at 1361 cm^{-1}, which correspond to the B–O stretching vibration bond in the phenylboronic acid moieties was observed in the two synthesized materials [5,6], which demonstrated the successful immobilization of VPBA onto the resulting hybrid monoliths. A broad absorption band at 3450 (or 3500) cm^{-1}, which was attributed to O–H stretching arising from the diol moieties present in VPBA, was also observed in these materials, which confirmed VPBA binding to the polymer surface.

3.2. Glycopeptide enrichment performance of synthesized monolithic materials

In order to evaluate the enrichment performance of boronate affinity sorbents, a standard glycoprotein (HRP) tryptic digest was used as a model sample. According to previous or studies [39,40], NH_4HCO_3 (50 mM, pH 7.8) and ACN/H_2O/TFA (50/50, v/v) containing 0.5% TFA were used as loading and elution buffers, respectively.

The MALDI-TOF mass spectra of the HRP tryptic digest without enrichment and with enrichment using the synthesized materials are presented in Fig. 3. Without enrichment, 10 peaks attributed to glycopeptides or their fragments were identified (see Fig. 3A). However, after enrichment, as shown in Fig. 3B and C, 17 and 27 glycopeptides were identified with enhanced MS intensities using the GMA-PMPMS-VPBA and GMA-SH@AuNP@PMPMS-VPBA materials, respectively. The detailed information of identified glycopeptides is shown in Table S2. The superior performance found in this last material was attributed to high immobilized amount of boronic acid groups in this composite, and consequently, large capability for capturing target molecules with cis-diol moieties (in this case, glycopeptides) according to the recognition mechanism depicted in Fig. S3. On the basis of these results, this composite was preferentially chosen as sorbent for extraction of glycopeptides, and several analytical parameters of this material were evaluated as follows.

3.3. Adsorption capacity, detection sensitivity, enrichment selectivity, reproducibility and reusability of hybrid material

The adsorption capacity of the GMA-SH@AuNP@PMPMS-VPBA composite was evaluated by enriching the glycopeptides from a fixed amount of HRP digest using different amounts of sorbent. As shown in Fig. S4, the signal intensities of 6 high-abundant glycopeptides firstly increased reaching a maximum value when 20 mg of sorbent were used. With higher sorbent amounts, signal intensities have not been significantly improved. Therefore, the adsorption capacity of the proposed sorbent is calculated to be 25 mg g^{-1}. This adsorption capacity value is comparable or even higher than those obtained by using other hydrophilic materials [37,41].

The detection sensitivity of the aforementioned hybrid monolith was investigated by using different concentrations of HRP tryptic digest. When the concentration was reduced to 0.5 fmol/μL, 3 glycopeptide peaks with S/N ratios over 6 can still be detected (see Fig. S5). Therefore, the limit of detection (LOD) of the composite was 0.5 fmol/μL. This result suggests that the prepared material has a good sensitivity towards glycopeptides. This value was comparable to others previously reported using HILIC-based monoliths (without boronic functionalities) [41–44] and magnetic materials [45,46], thus indicating that the prepared hybrid monolith had great sensitivity toward glycopeptides.

To evaluate the enrichment selectivity of the hybrid monolith for glycopeptides, a mixture of tryptic digests of HRP and BSA at a mass ratio of 1:100 (w/w) was used as the testing sample. As shown in Fig. S6A for the direct analysis of this mixture, only two glycopeptide peaks could be observed. However, after enrichment with the proposed material, 24 glycopeptide peaks (from the 27 peaks detected when only HRP digest was enriched) were identified (see Fig. S6B). These results proved that the GMA-SH@AuNP@PMPMS-VPBA had high selectivity for glycopeptide enrichment.

To investigate the material reusability performance, the sorbent (within device) was repeatedly used to enrich HRP. Similar mass spectra were obtained after using the same material for 10 consecutive uses, which demonstrated its good stability. Furthermore, the device-to-device reproducibility for glycopeptide enrichment was also evaluated. The number of identified glycopeptides from HRP digests enriched by three in syringe devices was the same with similar signal intensities, thus indicating the good preparation and enrichment reproducibility of GMA-SH@AuNP@PMPMS-VPBA monolithic material.

3.4. N-glycopeptides enrichment from tryptic digest of human serum

To demonstrate the enrichment performance of the proposed material, a real complex biological sample such as human serum (without any pretreatment) was used. For this purpose, as previously stated, the human serum sample (10 μL) was digested with trypsin and passed through the microSPE device. After washing and elution steps, the resulting eluate was deglycosilated with PNGase F and further analyzed by nano-LC–MS/MS. As a result, a total of 85 N-glycosylated peptides

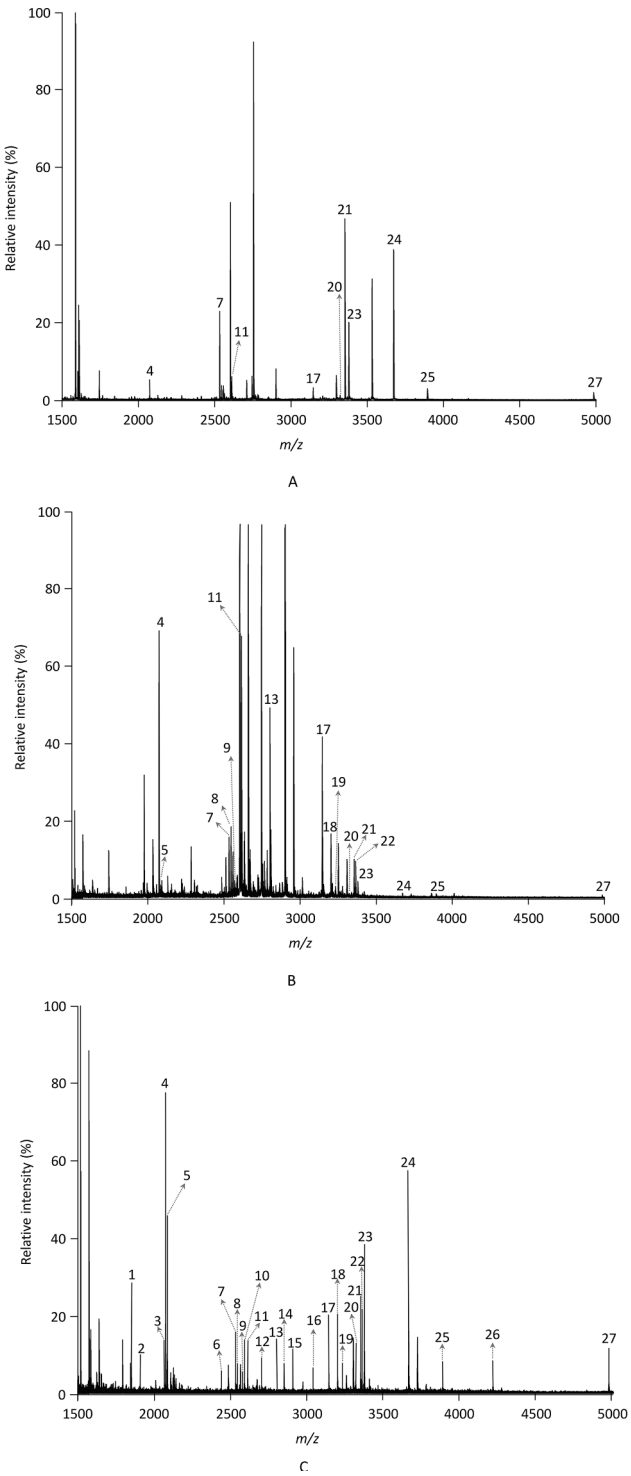

Fig. 3. MALDI-TOF mass spectra of HRP tryptic digests: (A) without enrichment and after enrichment using (B) GMA-PMPMS-VPBA and (C) GMA-SH@AuNP@PMPMS-VPBA materials. Glycopeptide peak identification as in Table S2.

derived from 42 N-glycosylated proteins were identified without removing any high-abundant protein (see Table S3 for detailed information). The enrichment and identification performance of this boronate affinity hybrid monolith was higher than the identification results reported in some papers using hydrophilic monoliths [41,43,44]. However, the resulting identification performance was lower than that obtained using hybrid monoliths with immobilized AuNPs and modified with cysteine [2], although in this work, a previous removal of high-abundant proteins was carried out in combination with a long pre-treatment (70 min).

4. Conclusions

This study presents the preparation of novel boronate affinity monolithic materials in a syringe format for the capture and pre-concentration of glycopeptides. For this purpose, two materials were synthesized using polythiol-functionalized monoliths as host substrates to produce sorbents with high boronic functionalities for glycopeptides enrichment. Thus, the first material was prepared from a generic monolith (GMA) modified with PMPMS and VPBA, whereas the second one used an AuNP-modified monolith as parent support. This last material showed higher efficiency to trap glycopeptides, which can be attributable to the increased binding sites of the resulting material. The hybrid material showed good performances in selective enrichment of glycopeptides, such as satisfactory adsorption capacity, high sensitivity, selectivity and good reusability. Also, the proposed methodology is of facile operation and it was feasibly applied to enrich glycopeptides from complex biological samples such as human serum, without any pre-treatment. Thus, the work described here not only offered the description and application of novel boronate affinity materials, but also opened its application to large-scale glycoproteome analysis.

CRediT authorship contribution statement

O. Mompó-Roselló: Investigation, Writing - original draft, Conceptualization. **M. Vergara-Barberán:** Investigation, Writing - original draft, Conceptualization. **M.J. Lerma-García:** Investigation. **E. F. Simó-Alfonso:** Resources, Funding acquisition. **J.M. Herrero-Martínez:** Resources, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2021.106018.

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