Use of polymeric sorbents modified with gold and silver nanoparticles for solid-phase extraction of proteins followed by MALDI-TOF analysis

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Abstract

Four different kinds of sorbents for solid-phase extraction (SPE) and preconcentration of proteins from complex samples were developed. All are based on the use of a poly(glycidyl-coethylene dimethacrylate) host monolith that was chemically functionalized using two different ligands (ammonia and cysteamine). Gold nanoparticles (AuNPs) or silver NPs were then assembled to the amino or thiol groups. The resulting materials are shown to be viable stationary phases for use in SPE cartridges. The sorbents are shown to selectively retain bovine serum albumin, and the thiol-modified sorbents containing AuNPs and AgNPs provided the highest recoveries (>90%) and satisfactory loading capacities (29.3 and 17.6 $\mu g \cdot mg^{-1}$ of sorbent, respectively). The applicability of these nanosorbents was demonstrated by preconcentrating viscotoxins from mistletoe extracts. The enriched fractions were subjected to MALDI-TOF analysis to underpin their selectivity.

Keywords Metal nanoparticles; Sorption; Preconcentration; poly(glycidyl-co-ethylene dimethacrylate); BSA; SPE; Stationary phase; MALDI-TOF; viscotoxins, mistletoe

Introduction

Protein/peptide purification and isolation constitute important steps in almost all branches of biosciences and biotechnologies. For this reason, a considerable interest in developing selective and efficient methods to isolate target proteins from complex matrices has attracted much attention [1]. Besides, it is very important that these methodologies present high loading capacities, which will lead to lower detection limits in protein analysis [2]. In this sense, solid-phase extraction (SPE) is the most popular sample treatment procedure employed in protein separation and preconcentration techniques [2]. Thus, SPE is characterized by its high efficiency, low running cost, simplicity, easy automation, and avoidance of the use of toxic organic solvents [2]. However, the selection of an appropriate sorbent is a critical factor to achieve high extraction efficiency, since there is a limited number of sorbents for protein species available at present. Thus, interest has been focused in the development of SPE sorbents for protein isolation using novel materials such as metal-organic frameworks [3] and polymer monoliths [4]. Particularly, these latter sorbents represent an alternative class of stationary phases to traditional packed columns. In this sense, organic polymeric monoliths have many advantages when they are used in sample treatment. For instance, its porous structure can aid to increase the loading capacity of the extraction and offer a good permeability, which allows the use of larger flow rate, thus reducing analysis time [4]. Another important point of monolithic materials is that their extraction efficiency can be changed by tailoring its pore structure and by introducing chemical modifications in the pore surface, which may produce monoliths with enhanced selectivity properties. In this regard, the modification of monoliths via attachment of metallic nanoparticles (NPs) onto the monolithic surface has focused much attention [5-7]. Thus, the most common way to immobilize metallic NPs to polymeric structures is

by incorporating amine or thiol functionalities onto their pore surface [5, 7, 8]. However, up to date, most of these publications have been applied in chromatographic area, being few reports focused on sample treatment [7, 8]. Thus, Alwael *et al.* [7] have described the immobilization of AuNPs onto the amine modified monoliths in pipette tips using ethylenediamine to extract glycoproteins. Vergara *et al.* [8] have immobilized AuNPs onto ground monolithic powder (previously modified with ammonia) to isolate proteins from complex samples. This ground monolithic powder has been also used in ultrasensitive SERS detection [9] as an "entrapment support" to immobilize biomacromolecules [10].

On the other hand, AgNPs have demonstrated to possess distinctive properties compared to other metallic NPs such as their antimicrobial [11] and optical properties [12]. For instance, Banerjee et *al*. [13] investigated changes in their surface plasmon resonance band in presence of different protein concentrations to study the type of interaction (hydrophobic and/or electrostatic) in the binding of proteins to AgNP surface. However, few applications [14] describing its use as preconcentrating supports to retain proteins have been reported. Thus, Shrivas *et al.* [14] have used modified AgNPs to preconcentrate hydrophobic peptides and proteins from biological samples previous MALDI analysis.

This work focuses on the study of different SPE sorbents especially designed for protein isolation from complex samples. For this purpose, polymeric sorbents were prepared from a ground poly(glycidyl-co-ethylene dimethacrylate) based polymer and then treated with different ligands (ammonia or cysteamine). The resulting materials (containing amine or thiol groups, respectively) were functionalized with AuNPs or AgNPs. These SPE sorbents were evaluated in terms of extraction parameters to retain proteins (using bovine serum albumin (BSA) as probe) and other performance features

(loading capacity, reusability and limit of detection) were established. In addition, the ability of these materials to specifically retain small basic and cysteine-rich proteins (thionins) in European mistletoe extract was demonstrated.

Experimental

Chemicals and reagents

Glycidyl methacrylate (GMA), ethylene dimethacrylate (EDMA), acetonitrile (ACN) and methanol (MeOH) were purchased from Scharlab (Barcelona, Spain, http://www.scharlab.com). Azobisisobutyronitrile (AIBN) was purchased from Fluka (Buchs, Switzerland, http://www.sigmaaldrich.com). AuNP and AgNP suspensions (both with a particle size of 20 nm and stabilized with sodium citrate) and Coomassie Blue were from Alfa Aesar (Landcashire, United Kingdom, http://www.alfa.com). Monosodium and disodium phosphate (NaH_2PO_4 and Na_2HPO_4, respectively) and orthophosphoric (H_3PO_4) acid were from Merck (Darmstadt, Germany, http://www.merckgroup.com/en/index.html). BSA, α-cyano-4-hydroxycinnamic acid (HCCA), gold (III) chloride trihydrate, hydrochloric acid (HCl), 2-mercaptoethanol, nitric acid (HNO_3), potassium bromide, tris(hydroxymethyl)-aminomethane (Tris) and trisodium citrate were obtained from Sigma-Aldrich (St. Louis, MO, USA, http://www.sigmaaldrich.com). A molecular-weight-size protein standard (6.5 to 200 kDa) was also provided by Sigma-Aldrich. Deionized water (Barnstead deionizer, Sybron, Boston, Mass., USA, http://www.barnstead-water.com) was used in all procedures.

A stock solution of BSA (1 mg mL^{-1}) was prepared with deionized water, and working standard solutions were obtained by dilution of the stock solution. Phosphate

buffer solutions of 20 mM at several pH values were prepared by dissolving appropriate amounts of Na_2HPO_4, NaH_2PO_4 and H_3PO_4 according to the required pH.

Instrumentation

Scanning electron microscopy /backscattered electron (SEM/BSE) micrographs were obtained with a Hitachi S4800 (Ibaraki, Japan, www.hitachi.com) provided with a field emission gun, and an EMIP 3.0 image acquisition system. Previous to the SEM measurements, the polymeric sorbents were coated with a very thin-layer of conductive carbon.

An EA 1110 CHNS elemental analyzer (CE Instruments, Milan, Italy, http://www.ceinstruments.co.uk) was used for elemental analysis of synthesized materials. An 8453 diode-array UV-vis spectrophotometer (Agilent Technologies, Waldbronn, Germany, http://www.agilent.com/) was used for Bradford assay and for gold determination. An ICP-MS (Agilent model 7900) was employed to determine silver content in the sorbents. Raman spectra of materials were recorded with an XploRA One Raman microscope (Horiba Scientific, Villeneuve d'Ascq, France, http://www.horiba.com/) from 150 to 3500 cm^{-1} using 532 nm as excitation wavelength with a laser power of 90 μW. A 5800 MALDI-TOF/TOF-MS (AB SCIEX, Foster City, CA, https://sciex.com/) was used to determine viscotoxin masses.

Preparation and modification of GMA-based polymer

The GMA-co-EDMA monolithic material was obtained as described in previous works [8, 15]. Briefly, the polymerization mixture composed by GMA (20 wt%), EDMA (5 wt%), cyclohexanol (70 wt%), 1-dodecanol (5 wt%) and AIBN (1 wt% with respect to

145 the monomers) was sonicated for 10 min to obtain a clear solution and, and then, purged
146 with nitrogen for 10 min. The polymerization was carried out in an oven at 60ºC for 24
147 h. A rinse with methanol was employed to remove the unreacted monomers and the
148 pore-forming solvents a wash with methanol was carried out. The bulk material was
149 ground with a mortar and sieved with a steel sieve (with sizes ≤ 100 μm). The resultant
150 GMA powder was chemically modified with ammonia (procedure A) and with
151 cysteamine (procedure B). The procedure A was carried out according to Vergara-
152 Barberán *et al*. [8]. Briefly, the material was treated with aqueous 4.5 M ammonia at
153 60ºC for 2 h. The functionalized GMA-based material with amino groups ("GMA-
154 NH_2") was washed with water until neutral pH and kept at room temperature for further
155 use. Regarding to procedure B, the parent polymer was allowed to react with 2.5 M
156 cysteamine aqueous solution during 2 h at room temperature and finally a washing step
157 was conducted with water until neutral pH [5,6]. The resulting modified thiol-GMA
158 material was referred to as "GMA-SH". Fig. 1 shows the reaction of GMA-based
159 material with both ligands.
160
161 **Functionalization of amino- or thiol-modified GMA material with AuNPs or**
162 **AgNPs**

163 To obtain the four types of sorbents used in this work, 400 mg of GMA-NH_2 or GMA-
164 SH were treated with AuNP or AgNP dispersion. In order to detect the saturation of the
165 monolithic material with metallic NPs, the UV-vis spectra of the supernatants were
166 recorded before and after the addition of AuNPs or AgNPs at several times until the NP
167 surface plasmon resonance band appeared in the UV-vis spectrum. In the case of GMA-
168 NH_2 material, a total volume of 20 mL of metallic NPs was enough to saturate the
169 material, while GMA-SH was saturated after adding a total of 100 mL of metallic NP

suspension. In both cases, the color of the GMA-co-EDMA powders indicated that metallic NPs are attached (pink for AuNPs and yellowish for AgNPs). After attachment of NPs, the materials were washed with 38.8 mM sodium citrate at pH 6.6 in order to remove the non-immobilized AuNPs and AgNPs.

These four materials (designated as GMA-NH_2-Au, GMA-SH-Au, GMA-NH_2-Ag and GMA-SH-Ag) were characterized by SEM. Moreover, the Au and Ag contents were evaluated using UV-vis and ICP-MS, respectively. For these measurements, all materials were previously calcined at 550 °C for 1 h. In the case of sorbents containing AuNPs, the remaining product (which contained Au) was dissolved in *aqua regia,* properly diluted with HCl 1 M and then subjected to UV-vis analysis [16]. On the other hand, the materials with AgNPs were dissolved in concentrated HNO_3, adequately diluted with water and analyzed by ICP-MS.

SPE protocols

SPE cartridges were prepared according to the literature [8]. For this purpose, 50 mg of AuNP- or AgNP-modified polymer sorbents was placed into 1 mL empty propylene disposable SPE cartridge (Análisis Vínicos, Tomelloso, Spain) using two frits (1/16', 20 µm, Análisis Vínicos). In all cases, the SPE sorbents were activated with 200 µL ACN and equilibrated with 500 µL H_2O. Then, 200 µL BSA (200 mg L^{-1} in phosphate buffer, adjusted to a proper pH) was loaded on the SPE material at a flow of 0.1 mL min^{-1}. The washing step was carried out with phosphate buffer (under the same pH conditions as the loading step) at 0.7 mL min^{-1}. The elution step was done with 200 µL of 30 mM phosphate buffer (pH 12.0) at 0.1 mL min^{-1}. In all steps, the fractions were collected and subjected to Bradford assay [17] in order to evaluate the retention efficiency of proteins on these AuNP- and AgNP-modified materials. The regeneration of sorbents was

195 accomplished with water at 80 ºC during 2h [6]. The same procedure was applied to
196 prepare a blank sorbent (GMA-based polymer) (50 mg).

197 To establish the capability of our SPE protocol to preconcentrate proteins, the limit of
198 detection (LOD) of BSA was evaluated. For this purpose, BSA solutions of several
199 concentrations (0.1-100 nM) were prepared in deionized water and subjected to the
200 extraction process. The resulting eluates were then mixed with the matrix solution (0.5
201 g/L HCCA in 70% aqueous acetonitrile and 0.1% TFA), and were deposited onto an
202 ABSCiex MALDI plate and analyzed in a 5800 MALDI TOF/TOF instrument
203 (ABSciex) in the positive ion mode (3000 shots every position). The MS and MS/MS
204 information was sent to be identified by the MASCOT software (v 2.3.02; Matrix
205 Science) via the Protein Pilot (ABSciex).

206 **Extraction of mistletoe viscotoxins and MALDI-TOF analysis**

207 European mistletoe (*Viscum Album L.*) grown on *Pinus halepensis* was collected and the
208 green parts were separated. To eliminate bacterial and surface contamination these parts
209 were rinsed with water, and subsequently sliced, transferred to a mortar and ground into
210 powder using liquid nitrogen. Then, the viscotoxin extraction protocol was adapted
211 from Hussain *et al.* [18]. Briefly, 5 g of this powder was treated with 20 mL of 10 mM
212 phosphate buffer at pH 7.4, mildly shaken at 4ºC overnight and centrifuged at 10000 × g
213 for 10 min. For viscotoxin isolation, 500 µL of the supernatant were directly subjected
214 to the SPE procedure. As mentioned before, the protein elution was made with 30 mM
215 phosphate buffer at pH 12. In this case of mistletoe extract these fractions were
216 subjected to both, Bradford assay and MALDI-TOF measurements.

217 MALDI samples were prepared by mixing equal volumes of the extract and the
218 matrix solution before spotting on the MALDI plate followed by MALDI-TOF
219 analysis.

Results and discussion

Choice of materials

As already mentioned in Introduction, organic polymer monoliths exhibit several benefits such as their simple in situ preparation and large permeability due to their large through pores. In particular, GMA-based polymers are chemically and mechanically stable materials containing reactive functionalities (epoxy groups) susceptible of undergoing functionalization, thus allowing the attachment of organic material or even metallic NPs such as gold [5, 6, 8]. Taking into account these good features, and the strong affinity of both gold and silver surfaces for thiol-containing compounds, the development of novel SPE sorbents in sample treatment for selective extraction and preconcentration of proteins is highly desirable to translate the benefits of this fruitful combination.

Studies with GMA-based materials modified with AuNPs

The use of SPE sorbents based on AuNPs immobilized onto amino-modified GMA materials has been previously implemented in our research group [8]. In fact, this sorbent provided advantageous features in terms of extraction efficiency (easy preparation, satisfactory loading capacity (16.6 mg/g sorbent) and reusability); however, its gold content was low (0.5 wt%). In order to increase this content, and consequently the loading capacity of material, another ligand (cysteamine) was considered. This ligand has been previously used by Svec and co-workers [6] to enrich the surface of GMA-based monoliths with thiol groups for posterior attachment of AuNPs. Thus, the ground GMA modified with cysteamine (GMA-SH) was obtained and then subjected to elemental analysis, giving 1.31 wt% sulfur (0.4 mmol thiol groups /g monolith). This

value was lower than those found by Svec's group (1.05 mmol/g) [5, 6]. These differences can be attributed to the format employed in the synthesis of monoliths. In our case, bulk monoliths (prepared in glass vials) were carried out, while the reported data of the functionalized monoliths were made in capillaries. Evidence on the different properties (e.g. number of accessible reactive and unfunctionalized groups) of monoliths prepared in rather different formats has been previously reported [19]; where the accessible number of epoxy groups was lower for the bulk polymers than for the corresponding capillary monolith.

After functionalization with AuNPs, the GMA-SH-Au material led to a 2.90 wt% Au, which is higher than that previously obtained for GMA-NH_2-Au sorbent. It can be explained by the high amount of thiol groups present in the sorbent surface, which bind strongly to AuNPs. Moreover, SEM micrographs of the GMA-SH-Au materials, taken with a BSE detector, show the presence of large number of AuNPs compared with the GMA-NH_2-Au (Fig. S1). Raman spectroscopy was also employed to verify the binding of AuNPs onto the surface of the modified GMA-based polymers (Fig. S2). Thus, the characteristic stretching modes of primary amine groups (appeared at 3100-3300 and ~1560 cm^{-1}) and thiol groups (appeared at 2510 cm^{-1}) were evidenced for GMA-NH_2 and GMA-SH, respectively. After attachment of AuNPs onto the surface of these polymers, the characteristic stretching modes of amine and thiol groups diminished or even disappeared. It indicates that the AuNPs were assembled to GMA-based materials.

Then, the loading capacity of the GMA-SH-Au sorbent was evaluated. For this purpose, different amounts of BSA (200-5000 μg mL^{-1}) were passed through this SPE device by keeping constant the working conditions of SPE procedure detailed in our previous work [8]. The maximum loading capacity obtained for GMA-SH-Au material was 29.3

mg/g sorbent, which was higher than that found previously for GMA-NH_2-Au material. The reusability of this sorbent was also evaluated. For this purpose, the regeneration protocol described in SPE protocol Section was applied. Thus, the BSA adsorption capacity decreased only 5.3% after 20 cycles.

Studies with GMA-based materials modified with AgNPs

In order to extend the application of SPE supports for protein analysis, sorbents based on the immobilization of AgNPs were also prepared using the parent materials described in the previous section (GMA-NH_2 and GMA-SH). After attachment of AgNPs, the silver content in both materials (GMA-NH_2-Ag and GMA-SH-Ag) was measured by ICP-MS giving 0.29 and 2.7 wt% Ag, respectively. These results were in agreement with SEM micrographs of both sorbents (Fig. S1). Also, Raman spectra of parent and AgNP-modified polymers were done (data not shown), giving similar results to those found for AuNP-based materials (see Fig. S2), thus confirming the binding of AgNPs onto the surface of amino- or thiol-modified polymers. As mentioned above, these differences in silver content can be explained by the higher affinity of these NPs or other metallic nanostructures (such as AuNPs) towards to thiol groups than the amino functionalities. In fact, several authors have suggested that the amino-Ag bond (resulting from a mixed mechanism of electrostatic and donor-acceptor interactions) is weaker than the thiol-Ag bond, which has a strongly covalent behavior [20, 22].

In any case, the retention of proteins onto these Ag modified sorbents was next considered. Both, loading sample and elution pHs were next optimized (see supplementary material text and Figs. S3 and S4). The best results were obtained at pH values of 7 and 12 for loading sample and elution steps, respectively. Once the working SPE conditions were optimized, the breakthrough volume was evaluated for

295 both Ag modified sorbents using BSA as probe. For this purpose, different sample
296 volumes (25-2000 μL) of the standard protein solutions were passed through to the SPE
297 material by keeping constant the amount of protein (0.1 mg). Experimental results
298 showed that for both AgNP-based sorbents excellent recovery values (> 95%) were
299 achieved up to 200 μL of sample volume. Then, the loading capacities of GMA-NH_2-
300 Ag and GMA-SH-Ag were established, giving values of 9.7 and 17.6 mg of BSA per g
301 sorbent, respectively. These values were in strong correlation with the content of Ag
302 found for each sorbent (see values above). The reusability of both sorbents was also
303 tested using the protocol previously described [8]. Thus, the GMA-NH_2-Ag and GMA-
304 SH-Ag materials can be reused until 9 and 12 times, respectively, without significant
305 efficiency losses (satisfactory recovery comprised between 80.2-92.4%). During these
306 cycle experiments, the Ag content (measured by ICP-MS) was evaluated, and no
307 significant changes in Ag concentration were found (data not shown), which
308 demonstrated the viability of the regeneration protocol.

309 A comparison in terms of extraction performance using the sorbents was next
310 considered. Under their respective SPE optimum conditions, the recoveries values of
311 BSA were higher than 98.2% for all sorbents investigated, whereas for the loading
312 capacity, the GMA-SH-Au sorbent shows the highest value (29.3 mg/g). This fact can
313 be explained taking into account the large Au content present in this material and the
314 large affinity of this metallic NP towards to thiol functionalities present in GMA
315 support, as we discussed above.

316 Prior to evaluate the applicability of the current approach to real samples,
317 experiments were conducted to establish the limit of detection (LOD) of standard BSA
318 spiked in water using the GMA-SH-Au sorbent, achieving a LOD value for BSA in
319 water of 0.1 nM.

Then, the SPE sorbent described here was compared with reports related to the use of other nanomaterials for protein/peptide preconcentration prior MALDI-MS analysis. Thus, the LOD assayed in this study was similar [12, 29, 30] or even better [14, 24-28, 31] than those described in studies given in Table 2. Besides, our SPE support method offers an increased surface area-to-volume ratio, without losses of material and analyte compared to some of these microextraction techniques (such as NP-LLME or dSPE) [14, 23-26], where analyte loss or desorption from the surface of NPs occurred during washing or centrifugation steps. Moreover, our SPE protocol simplifies the handling of more samples simultaneously and speeds the preconcentration process of proteins. In particular, this method allowed a sample throughput of 10 samples h^{-1}, whereas a rate of 1-2 samples h^{-1} could be achieved with other NP-based protocols. Other strength of the proposed sorbents is its satisfactory reusability (see data above). In this regard, in the related studies of NP-based materials (see Table 2), this important parameter was not evaluated or this information was missing. This suitable reusability combined with the possibility of manufacturing several SPE cartridges (ca. 7) from the bulk NPs-modified material (see Experimental section) undoubtedly makes this protocol economically attractive.

Application to the isolation of viscotoxins from European mistletoe

The designed sorbent materials were employed to selective isolate and preconcentrate viscotoxins from European mistletoe samples. These small basic and cysteine-rich polypeptides (with molecular weights *ca*. 5 kDa), belonging to the thionin family, consist of a 45-50 amino acids residues with 3-4 internal disulfide bonds. They display cytotoxic activity against different types of tumor cells [32]. Their biological activity can be related to plant defense since its high expression gives enhanced resistance to

pathogens [33]. According to the amino acid sequence, disulphide bridge arrangement and distribution in plant tissues, seven isoforms of viscotoxins, namely A1, A2, A3, B, B2, 1-PS and C1 have been reported [18, 34]. From these thionins, viscotoxin A3 is the most cytotoxic whereas viscotoxin B is the less potent [35]. The pI of the viscotoxins is comprised in a range from 8.9 to 9.3.

The extraction and isolation of the viscotoxins is generally a tedious task that implies several purification steps (cation-exchange columns, fractionation by preparative HPLC, etc) [36]. To avoid these time-consuming processes, the sorbents previously developed to isolate mistletoe viscotoxins were used. The experimental procedure is detailed in Extraction of mistletoe viscotoxins Section, and subsequently the collected fractions were subjected to MALDI-TOF analysis. Fig. 2 shows the MALDI-TOF MS spectra of mistletoe extract before SPE (A), the wash fraction (B) and the eluted viscotoxins using the GMA-SH-Au sorbent (C). The trace A represents the entire range of peptides (up to *m/z* 6000) found in Mistletoe extract, whereas in the washing fraction (trace B), any signal around 4800 (molecular masses of viscotoxins) is observed. Trace C shows the eluted fraction (including an expanded mass region around 4800 *m/z*), being the identified viscotoxins listed in Table 1. Protein information resource software (MASCOT) was used for the determination of the exact mass of peptides. These peptides were also detected in the eluates obtained from the other sorbents of this study (data not shown).

As shown in Fig 4C, the most abundant viscotoxin found was B in contrast to that reported by Hussain *et al.* [18, 37], where the isoforms predominant were A2 and A3. These differences in isoform contents can be explained by taking into account several factors such as the *V. album* subspecies considered and seasonal changes. In our case, the Mistletoe leaves were from the subspecie *Viscum album austriacum,* which

were found over *Pinus nigra,* where the viscotoxin B showed a large content [38]. Besides, the content of this viscotoxin remained unchanged during the seasonal course of leaves of *V. album*, whereas viscotoxins A1, A2, and A3 showed degradation [38].

Conclusions

In this work, several SPE sorbents based on a methacrylate polymer modified with both AuNPs and AgNPs have been used for protein isolation. The ability of two ligands (ammonia and cysteamine) to prepare parent supports to be subsequently functionalized with these NPs was evaluated. The results show that the materials containing thiol functionalities afford a large coverage of AuNPs or AgNPs onto the polymer surface due to the large affinity of thiol moieties and these NPs. After SPE protocol optimization, the sorbents show large recovery efficiencies, loading capacities (up to 29.3 mg/g sorbent) and satisfactory reusabilities. Compared to the existing NP-based methods for sample preparation in MALDI-TOF analysis, our SPE protocol is simpler, more cost-effective, and shorter in terms of processing time; however, it requires more sorbent amount (50 mg) than these (micro)extraction techniques. To demonstrate the practical applicability of these materials, the GMA-SH-Au sorbent, which provided the highest loading capacity and excellent LOD, was chosen and successfully applied to selectively isolate viscotoxins from European mistletoe extracts. The sorbents used in this work open an alternative avenue to facilitate satisfactory separation of peptides/proteins from complex samples using very simple extraction methodologies.

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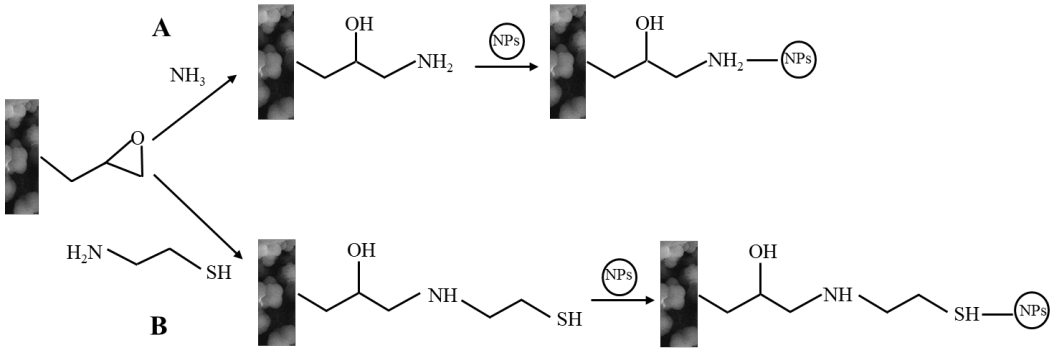
518

519 **Figures**

520



521

522 **Fig. 1** Scheme of functionalization of poly(GMA-co-EDMA) material with ammonia
523 (A) and cysteamine (B), and subsequently immobilization with metallic nanoparticles
524 (AuNPs or AgNPs).

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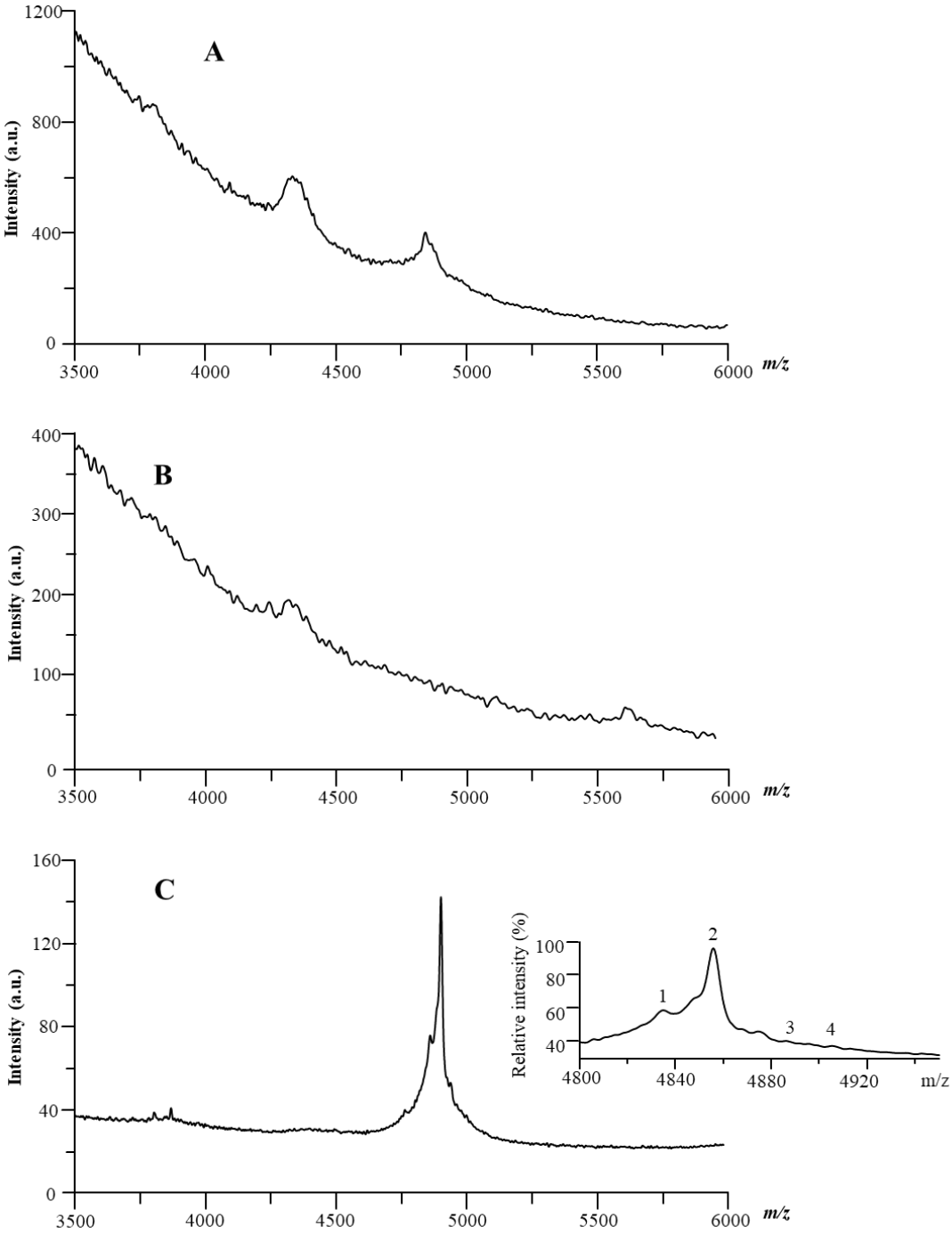

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527

528 **Fig. 2** MALDI-TOF MS spectra of mistletoe extract. Mass spectra of extract before SPE

529 procedure (A), wash fraction after SPE procedure (B) and the elution of the retained

530 viscotoxins (C) using the GMA-SH-Au sorbent.

531

532 **Table 1** Identified viscotoxins from the SPE elution fractions of mistletoe extracts and
533 their theoretical mass reported in literature.

534

Viscotoxin	Observed mass (Da)	Theoretical mass (Da) [24, 45]
A2	4835.8	4834.42
A3	4835.8	4835.53
B	4857.7	4857.45
A1	4889.9	4889.53
1-PS	4904.3	4904.02

535

536 **Table 2** An overview on reported nanomaterial-based methods for preconcentration of proteins and peptides using MALDI-TOF analysis.
537

Material	Analyte	Matrix	Extraction technique	LOD (nM)	Ref.
ZipTip C_{18}	Cytochrome c, myoglobin and BSA	Blood serum	SPE	0.5-1	[23]
AgNPs	Myoglobin, ubiquitin BSA and gramicidin D	Urine and plasma	NP-LLME	130-160	[14]
PdNPs	Insulin, ubiquitin Lysozyme and gramidicin D	Water and urine	NP-LLME	17-37	[24]
Diamond NPs	Cytochrome c, myoglobin and BSA	Blood serum	dSPE	0.1	[23]
ZnSNPs	Ubiquitin and insulin	Aqueous solution, Oyster mushroom and biological samples	dSPE	85-91	[25]
MWCNT/CdSNPs	Cytochrome c and lysozyme	Milk and urine	dSPE	1-7	[26]
Bidentate AgNPs	Cytochrome c and insulin	Milk and human urine	SDME	1-80	[27]
Fe_3O_4 NPs-TiO_2	Angiotensin I, insulin, cytochrome c and trypsinogen	Aqueous solution	MSPE	50	[28]
Fe_3O_4 NPs (coated with carboxylate groups)	Angiotensin I, insulin, myoglobin and cytochrome C digest	Aqueous solution	MSPE	0.1-10	[29]

538