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Highlights

- Polymeric AuNP@monoliths were investigated for protein analysis in SPE-CE-MS.
- TTR, which is a biomarker of a rare neurodegenerative disease, was analyzed.
- Protein retention on the Au surface depended on the protein pI.
- The LODs with AuNP@monolith were similar compared to IA sorbent.
- The applicability to serum samples was evaluated.

Polymeric monolithic microcartridges with gold nanoparticles for the analysis of protein biomarkers by on-line solid-phase extraction capillary electrophoresis-mass spectrometry

Laura Pont[1], Gemma Marin[1], María Vergara-Barberán[2], Leonardo G. Gagliardi[3], Victoria Sanz-Nebot[1], José M. Herrero-Martínez*[2], Fernando Benavente*[1]

[1]Department of Chemical Engineering and Analytical Chemistry, Institute for Research on Nutrition and Food Safety (INSA·UB), University of Barcelona, C/Martí i Franquès 1-11, 08028 Barcelona, Spain

[2]Department of Analytical Chemistry, University of Valencia, C/Doctor Moliner 50, 46100 Burjassot, Spain

[3]Laboratorio de Investigación y Desarrollo de Métodos Analíticos, LIDMA, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, CIC-PBA CONICET, C/ 47 esq. 115, B1900AJL La Plata, Argentina

*Corresponding authors: fbenavente@ub.edu (F. Benavente, PhD)

Tel: (+34) 934039116, Fax: (+34) 934021233

and jmherrer@uv.es (J. M. Herrero-Martínez, PhD)

Tel: (+34) 963544062, Fax: (+34) 963544436

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ABSTRACT

In this study, polymeric monoliths with gold nanoparticles (AuNP@monolith) were investigated as microcartridges for the analysis of protein biomarkers by on-line solid-phase extraction capillary electrophoresis-mass spectrometry (SPE-CE-MS). "*Plug-and-play*" microcartridges (7 mm) were prepared from a glycidyl methacrylate (GMA)-based monolithic

capillary column (5 cm x 250 µm i.d.), which was modified with ammonia and subsequently functionalized with gold nanoparticles (AuNPs). The performance of these novel microcartridges was evaluated with human transthyretin (TTR), which is a protein related to different types of familial amyloidotic polyneuropathies (FAP). Protein retention depended on the isoelectric point of the protein (TTR pI~5.4) and elution was achieved with a basic phosphate solution. Under the optimized conditions, limits of detection (LODs) for TTR by AuNP@monolith-SPE-CE-MS were 50 times lower than by CE-MS (5 vs 250 $mg \cdot L^{-1}$, with an ion trap (IT) mass spectrometer). The sensitivity enhancement was similar compared to SPE-CE-MS using immunoaffinity (IA) microcartridges with intact antibodies against TTR. Linearity, repeatability in migration times and peak areas, reusability, reproducibility and application to serum samples were also evaluated.

1. Introduction

One of the issues that affects the limits of detection (LODs) of high-performance separation techniques at the microscale is the reduced volume of sample that must be analyzed to obtain optimum separations [1–6]. This is the case of capillary electrophoresis-mass spectrometry (CE-MS) that has been combined with different electrophoretic and chromatographic strategies to enhance sensitivity [1–6], such as those based on on-line solid-phase extraction (SPE) [4–6]. Today, the on-line coupling of SPE to CE-MS (SPE-CE-MS) is widely accepted as a highly versatile and efficient sensitivity enhancement approach [5,6]. The typical SPE-CE-MS configuration without valves is very simple [6], with the microcartridge connected in series close to the inlet of the separation capillary. The sorbent contained in the microcartridge allows retaining the target analyte from a large sample volume. Then, the retained analyte is subsequently cleaned-up and preconcentrated in a smaller volume of eluent before the electrophoretic separation and MS detection.

The main limitation to broad the applicability of SPE-CE-MS is that the modified capillaries must be lab-made because they are not commercially available [6]. The most widely used microcartridges are packed with sorbent particles, which are typically retained between one or two frits to avoid particle leaking during the analyses [7]. Nevertheless, frits may promote column back-pressure, electroosmotic flow (EOF) disturbance, bubble formation and current instability or breakdowns. Fritless particle-packed microcartridges have been also described, by simply using to prevent bleeding, magnetic particles or sorbents with particle size slightly greater than the inner diameter of the separation capillary [6]. However, the construction of particle-packed microcartridges continues to be perceived as challenging for non-experienced users, and several other alternatives have been proposed based on sorbent membranes, fibers, coatings or monoliths [6].

Fritless monolithic microcartridges are probably the best choice to simplify and systematize the reproducible construction of particle-free microcartridges [8–17]. A monolithic sorbent is a continuous unitary porous structure

without inter-particle voids that can be synthesized inside the separation capillary ("in situ") [8–12] or as a capillary column of a certain length ("ex situ") to cut later several "plug-and-play" microcartridges [13–17]. At best, the monolithic sorbent fills completely the microcartridge lumen [8–16]. Furthermore, the monolith physical structure (*e.g.* porosity and pore size) and the number and type of active surface groups can be further tailored to maximize extraction selectivity and recovery without compromising fluidic and electric performance in SPE-CE. In this sense, the use of gold nanoparticles (AuNPs) in combination with polymeric monoliths (AuNP@monolith) has been explored in separation and sample pretreatment due to their high-surface-area-to-volume ratios, easy chemical modification and strong affinity for thiol-containing compounds [18–23]. Indeed, the application of AuNP@monoliths for the analysis of cysteine-containing peptides, proteins and other compounds [18–23] or as a platform to facilitate further variations in surface functionalities [19,24–27] has been reported. However, despite the greater potential of these hybrid monoliths compared to other monolithic sorbents from the point of view of ease of preparation, high extraction capacity and versatility of functionalization, their integration as microcartridges for SPE-CE-MS has not been explored yet

In this study, polymeric AuNP@monoliths were investigated for the first time as microcartridges in SPE-CE-MS. For this purpose, "plug-and-play" microcartridges were prepared from a glycidyl methacrylate (GMA)-based monolithic capillary column, which was modified with ammonia and subsequently functionalized with AuNPs [22,23]. The performance of the microcartridges was evaluated with human transthyretin (TTR), which is a protein related to different types of familial amyloidotic polyneuropathies (FAP) [28–30]. Protein loading and elution conditions were optimized to obtain the best protein recoveries and the method was validated using an ion trap (IT) mass spectrometer in terms of linearity, limits of detection (LODs), repeatability in migration times and peak areas, microcartridge lifetime and reproducibility. The sensitivity enhancement by AuNP@monolith-SPE-CE-MS was discussed compared to CE-MS and immunoaffinity SPE-CE-MS (IA-SPE-CE-MS). The method was further demonstrated using an accurate mass and high-resolution time-of-flight (TOF) mass spectrometer before the analysis of serum samples.

2. Experimental section

2.1. Materials and reagents

All the chemicals used in the preparation of background electrolytes (BGEs) and solutions were of analytical reagent grade or better. 3-(trimethoxysilyl) propyl methacrylate (98%), glycidyl methacrylate (GMA, ≥97%), ethylene glycol dimethacrylate (EDMA, ≥98%) and azobisisobutyronitrile (AIBN, 98%) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetic acid (HAc) (glacial), ammonium hydroxide (25%), formic acid (HFor) (99.0%), potassium dihydrogenphosphate (≥99.0%), sodium hydrogenphosphate (≥99.0%), ammonium dihydrogenphosphate (≥99.0%), phenol (≥99.5%), and human transthyretin (TTR) (≥95.0%) were acquired from Merck (Darmstadt, Germany). Acetonitrile (LC-MS), methanol (99.9% (v/v)) and ethanol (96% (v/v)) were supplied by Panreac AppliChem (Barcelona, Spain). Ammonium acetate (NH_4Ac) (≥99.9%) and Tween® 20 were provided by Sigma-Aldrich. Propan-2-ol (LC-MS) and AuNP dispersion (particle size, 20 nm, stabilized with sodium citrate) were supplied by Fluka (Buchs, Switzerland) and Alfa Aesar (Lancashire, United Kingdom), respectively. LC-MS grade water (Fisher Scientific, Loughborough, UK) was used in experiments involving MS detection. For the rest of experiments, water with a conductivity value lower than 0.05 $\mu S \cdot cm^{-1}$ was obtained using a Milli-Q water purification system (Millipore, Molsheim, France). Fused silica capillaries were supplied by Polymicro Technologies (Phoenix, AZ, USA).

2.2. Electrolyte solutions, sheath liquid, protein standard and blood sample

All solutions were degassed for 10 min by sonication and filtered through a 0.20 µm nylon filter (MSI, Westboro, MA, USA) before use. The BGEs for the CE-MS and AuNP@monolith-SPE-CE-MS studies contained 1 M HAc (pH 2.3) (acidic BGE) or 10 mM NH_4Ac, pH 5.0 (pI BGE). The sheath liquid solution was a mixture of 60:40 (v/v) propan-2-ol/water with 0.05% (v/v) or 0.25 % (v/v) of HFor for the acidic or the pI BGEs, respectively.

An aqueous standard solution (1,000 μg·mL^{-1}) of TTR was prepared and stored in a freezer at -20ºC until its use. Excipients of low molecular mass were removed by passage through 10,000 relative molecular mass (M_r) cut-off cellulose acetate filters (Amicon Ultra-0.5, Millipore) [28]. The sample was centrifuged at 25ºC for (10 min at 11,000 x g) and the residue was washed three times with an appropriate volume of water or BGE in the same way. The final residue was recovered by inverting the upper reservoir in a vial and spinning once more at a reduced centrifugal force (2 min at 300 x g). Enough water or BGE were added to adjust the concentration of TTR to 1,000 μg·mL^{-1}.

Fresh blood from a healthy donor (male, 43-years old) was obtained by venepuncture and serum was prepared as described in our previous work [28]. The assay was approved by the Ethics Committee of the UB and written informed consent was obtained from the donor. Serum aliquots were stored in a freezer at -20ºC when not in use.

2.3. Apparatus

pH measurements were made with a Crison 2002 potentiometer and a Crison electrode 52-03 (Crison Instruments, Barcelona, Spain). Agitation was performed with a Vortex Genius 3 (Ika®, Staufen, Germany). Centrifugal filtration was carried out in a cooled Rotanta 460 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany).

A KD Scientific 100 series infusion pump (Holliston, MA, USA) was used to modify the GMA-based monolithic capillary column with ammonia and AuNPs, as well as for delivery of the sheath liquid in CE-MS and AuNP@monolith-SPE-CE-MS experiments.

2.4. CE-MS

All CE-MS experiments were performed in a 7100 CE coupled with an orthogonal G1603A sheath-flow interface to a LC/MSD Ion Trap (IT) SL or a 6220 oa-Time-of-flight (TOF) LC/MS mass spectrometer (Agilent

Technologies, Waldbronn, Germany). LCMSD Trap software (Bruker Daltonik, Bremen, Germany) or ChemStation and MassHunter softwares (Agilent Technologies) were used for the CE-IT-MS and CE-TOF-MS instrument control, data acquisition and processing, respectively. The mass spectrometers were operated in ESI+ mode as described in some of our previous works [28–31]. With the IT mass spectrometer, full scan mass spectra were acquired from 700 to 2,200 m/z. ESI, capillary exit, skimmer, octopole 1, octopole 2, octopole radiofrequency, lens 1 and lens 2 voltages were set at 4000 V, 260 V, 30 V, 3.0 V, 2.5 V, 225 V, -2.8 V and -80 V respectively, with the trap drive at 95 (arbitrary units) [31]. With the TOF mass spectrometer, full scan mass spectra were acquired from 100 to 3,200 m/z in the high-resolution mode (4 GHz). Capillary, fragmentor, skimmer and OCT 1 RF voltages were set at 4,000 V, 325 V, 80 V and 300 V, respectively [28–30]. In both mass spectrometers, nebulizer gas (N_2) pressure was 7 psi, drying gas (N_2) flow rate was 4 L·min^{-1} and drying gas temperature was set at 300ºC. The sheath liquid was delivered at a flow rate of 3.3 µL·min^{-1} [28–31].

CE-MS separations were performed at 25°C in a 72 cm total length (L_T) × 75 µm internal diameter (i.d.) × 365 µm outer diameter (o.d.) capillary [28,29]. All capillary rinses were performed flushing at 930 mbar. For new capillaries or between workdays, the capillary was activated flushing with 1 M NaOH (20 or 5 min, respectively), water (20 or 5 min), and BGE (20 or 10 min) (both procedures were performed off-line to avoid the unnecessary contamination of the MS system). Between analyses the capillary was conditioned flushing with water (2 min) and BGE (2 min). Samples were hydrodynamically injected at 50 mbar for 10 s (54 nL, i.e. 1.7% of the capillary, estimated using the Hagen–Poiseuille equation [32]), and a separation voltage of +25 kV (normal polarity, cathode in the outlet) was applied. With the pI BGE (pH 5.0), 100 mbar of pressure were also applied during the electrophoretic separation. The autosampler was kept at 10°C using an external water bath (Minichiller 300, Peter Huber Kältemaschinenbau AG, Offenburg, Germany). For short or overnight storage, the capillary was flushed with water (2 min).

2.4. Preparation of AuNP@monolith microcartridges for SPE-CE-MS

The GMA-*co*-EDMA monolith was prepared as described in a previous work [22]. Briefly, the polymerization mixture was prepared in a 10 mL glass vial by weighing the monomers GMA (20% (m/m)) and EDMA (5% (m/m)), and a binary porogenic solvent mixture of cyclohexanol (70 % (m/m)) and 1-dodecanol (5% (m/m)). AIBN was added as initiator (1% (m/m) with respect to the monomers). The final mixture was sonicated for 5 min and then purged 10 min with nitrogen to remove oxygen.

In order to ensure the covalent attachment of the polymer monolith, the inner wall of a fused silica capillary (1 m L_T x 250 µm i.d. × 365 µm o.d.) was modified with 3-(trimethoxysilyl)propyl methacrylate as described elsewhere [33]. Then, a 5 cm fragment of the silanized capillary was cut and the polymerization mixture was introduced slowly by hand to avoid bubble formation, using a plastic syringe with an appropriate connector. The capillary column was sealed at both ends with two rubber septa and it was polymerized in an oven at 60 ºC for 24 h. After the polymerization reaction was completed, the generic polymer was washed with methanol (30 min) to remove the porogenic solvents and the unreacted monomers. Next, a 4.5 M ammonium hydroxide solution was pumped through the GMA-based monolith at 60ºC (using a column oven) at a flow rate of 100 $\mu L \cdot h^{-1}$ for 2 h. The capillary column was then flushed with water at room temperature until neutral pH. Afterwards, citrate-stabilized AuNPs colloidal dispersion was pumped through the monolithic capillary at room temperature at a flow rate of 200 $\mu L \cdot h^{-1}$, until the entire monolith length turned deep red and a pink solution was observed coming out from the capillary outlet. Finally, the column was thoroughly washed with water (30 min). The surface coverage of monolith with AuNPs was also confirmed by SEM (see Fig. S1A), and its Au content was estimated to be *ca*. 14 % m/m by energy dispersive X-ray (EDAX) (see Fig. S1B).

The AuNP@monolith capillary column was cleanly cut into 7 mm pieces to prepare the fritless monolithic microcartridges. The microcartridge was connected using two plastic sleeves at 7.5 cm from the inlet of a 72 cm L_T × 75 µm i.d. × 365 µm o.d. capillary, which was previously activated as in CE-MS. The junction was tight enough to avoid adhesive sealing, hence the microcartridge was “plug-and-play” and completely replaceable. A plastic syringe with an appropriate connector was used to check the system for abnormal flow restriction, taking as a reference the flow through a CE-MS capillary of the same dimensions.

For AuNP@monolith-SPE-CE-MS under the optimized conditions, the capillary with the microcartridge was first conditioned flushing at 930 mbar with water (2 min) and BGE (2 min). Next, sample dissolved in the pI BGE (pH 5.0) was introduced at 930 mbar for 5 min (30 µL [32]). Then, a flush with pI BGE (1 min) allowed removing non retained molecules and filling the capillary before the elution. All these steps were performed with the nebulizer gas and the ESI capillary voltage switched off to prevent the entrance of contaminants into the MS. Then, both were switched on and a small volume of eluent was injected at 50 mbar for 20 s (108 nL [32], 30 mM $(NH_4)H_2PO_4$ adjusted to pH 9.0 with ammonium hydroxide). The separation was conducted applying +25 kV and assisting with 100 mbar. Between consecutive runs, to avoid carry-over, the capillary was flushed with eluent (1 min) and water (1 min). Separations were performed at 25ºC with the autosampler at 10ºC. The rest of experimental conditions were as in CE-MS.

2.5 Quality parameters

TTR migration times and peak areas were obtained from the extracted ion electropherograms (EIEs) of the most abundantly detected molecular ions (i.e. +7 and +8 molecular ions of monomeric (MO) TTR with the IT mass spectrometer). In AuNP@monolith-SPE-CE-MS, repeatability (n=3) was evaluated as the relative standard deviation (%RSD) of migration times and peak areas for a 10 µg·mL^{-1} TTR standard. Linearity range was studied in the concentration range between 5 and 50 µg mL^{-1}. LODs were estimated by analyzing low-concentration TTR standards (close to the LOD level, as determined from the approach based on S/N=3). The microcartridge lifetime was evaluated by repeatedly analyzing a 10 µg·mL^{-1} TTR standard.

2.6 Analysis of serum samples

Serum samples were pretreated off-line before the analysis of TTR to deplete other high-abundance proteins. This should prevent microcartridge saturation and protein adsorption on the capillary inner surface. The applied off-line sample pretreatment method performed good for the analysis of TTR by IA-SPE-CE-MS [30]. Eight mg of NaCl were added at 2ºC to 100 µL of human serum followed by 100 µL of 5% (v/v) phenol dropwise to precipitate the proteins.

The supernatant was collected after centrifugation at 25 ºC (10 min at 11,000 x g) and then diluted 1:1 (v/v) with the pI BGE (pH 5.0) before its analysis by SPE-CE-MS.

3. Results and discussion

3.1. CE-MS

In a previous investigation, a CE-TOF-MS method was developed for the analysis of TTR using an acidic BGE with 1 M HAc (pH 2.3) and a sheath liquid with 60:40 (v/v) propan-2-ol/water with 0.05% (v/v) of HFor [28,29]. The acidic BGE allowed the best sensitivity for the analysis of TTR because of the higher ionization efficiency of proteins in positive ESI mode at very low pH values. In this particular case, the acidic BGE also promoted disruption of the native tetrameric structure of TTR, hence monomeric (MO) TTR proteoforms were only detected. The same method was applied here using a conventional IT mass spectrometer. This mass spectrometer is not the most ideal choice to analyze proteins due to the limited mass accuracy, mass resolution, scanning speed and m/z scanning ranges. However, it performs reasonably well to obtain average molecular mass values of proteins of considerable complexity, such as we have demonstrated for transferrin or recombinant human erythropoietin [31]. Therefore, it is very convenient for method optimization in CE-MS and SPE-CE-MS without resorting to a more appropriate, but less available, mass spectrometer (e.g. an accurate mass high-resolution TOF mass spectrometer). Figures 1A and 1B (parts i)) show the total ion electropherogram (TIE) and the mass spectrum obtained by CE-IT-MS for a 500 $\mu g \cdot mL^{-1}$ standard solution of TTR using the acidic BGE (pH 2.3), respectively. TTR appears in the electropherogram as a sharp single peak, and the mass spectrum shows the typical cluster of multiply charged ions of MO TTR (with m/z of the most abundant molecular ion corresponding to the MO with charge +9). Indeed, as hinted before, the performance of the IT mass spectrometer was not enough to obtain information about the different MO TTR proteoforms, but only an average M_r for the target protein (i.e. $M_{r\ exp}$ =13,880) which is close to the most abundant MO TTR proteoform (i.e. TTR showing a mixed disulfide with the amino acid cysteine at position 10 of the sequence, TTR-Cys, $M_{r\ theo}$ = 13,880.4022) [28,29].

As an alternative to the acidic BGE (pH 2.3), a BGE with 10 mM NH_4Ac, pH 5.0 (pI BGE) was also investigated to analyze TTR by CE-IT-MS. At this pH value, TTR was close to its pI (5.4), and its retention onto the AuNP surface was expected to be maximum, as indicated before. This is what we observed when a similar methacrylate monolith modified with AuNPs was ground and used as a sorbent for the isolation of bovine serum albumin, cytochrome c and mistletoe leave lectins by off-line SPE [22]. Figures 1A and B (parts ii)) show the TIE and the mass spectrum obtained for a 500 $\mu g \cdot mL^{-1}$ standard solution of TTR using the pI BGE (pH 5.0), respectively. As can be observed in Figure 1A ii), TTR was now detected at a shorter migration time. The cathodic EOF at pH 5.0 was higher than at pH 2.3, but this was mainly due to the application of 100 mbar of pressure during the electrophoretic separation at 25 kV. Without applying a positive pressure between 25 and 100 mbar, analyses were precluded due to the frequent current interruptions and the poor reproducibility. Since total analysis time was shorter by applying 100 mbar, this pressure was selected for further analyses. Furthermore, in order to achieve an appropriate sensitivity (30% increase of TTR peak intensity), it was necessary to increase the HFor content in the 60:40 (v/v) propan-2-ol/water sheath liquid until 0.25 % (v/v). Under these conditions, the mass spectrum of TTR (Figure 1B ii)) shows that the cluster of multiply charged ions of MO TTR was shifted to higher m/z values (with m/z of the most abundant molecular ion corresponding to the MO with charge +8). Besides, as expected, sensitivity was better with the BGE of lower pH (see the TTR peak intensity in both TIEs of Figure 1A for comparison), hence LODs for TTR were 50 and 250 $\mu g \cdot mL^{-1}$, respectively. Repeatability of migration time and peak area with the pI BGE (pH 5.0) was good (i.e. 2.3% and 6.0%, n=3, respectively) and similar to the values with the acidic BGE (pH 2.3) (i.e. 3.4% and 4.8%, n=3, respectively).

3.2 AuNP@monolith-SPE-CE-MS

Protein adsorption onto AuNP surfaces is a complex phenomenon, which is mainly driven by electrostatic and hydrophobic interactions. Electrostatic interactions can be explained by the presence of the positively charged basic groups of the proteins and the negatively charged citrate-stabilized AuNPs. Hydrophobic interactions are mainly due to the free amino, imidazole and thiol groups in the side chains of the amino acid residues, which displace citrate from

the Au surface [34–36]. Different studies have shown that Au surfaces can effectively retain proteins within an appropriate pH range. In a previous investigation [22], we observed that protein retention was specially favored at a pH close to the protein pI, where the hydrophobic interactions are supposed to be predominant.

Despite these considerations, and taking into account the CE-MS results showed in the previous section, we decided to perform the first SPE-CE-MS experiments using the acidic BGE (pH 2.3) for the separation and loading TTR standard solutions in water or in the pI BGE (pH 5.0). However, results were not satisfactory, and no protein was detected. This was probably due to elution of TTR during the washing step with the acidic BGE (pH 2.3). The washing step allows removing impurities and poorly retained TTR after sample loading and it is mandatory to fill the capillary before the elution, separation and detection. In order to improve protein recoveries, different BGEs with 10 mM NH_4Ac and pH values close to the TTR pI were investigated (i.e. pH 4.2, 5.0 and 5.8). In all cases, TTR standard solutions were loaded in the corresponding BGE, as no TTR was detected when the protein was loaded in water. Elution was performed at basic pH, in accordance to our experience with the ground methacrylate monolith modified with AuNPs [22]. However, the solution providing the best results in that study (20 mM sodium phosphate, pH 12.0) was avoided, because it is well-known that the presence of sodium cations and phosphate anions decreases ionization efficiency in MS. As an alternative, based on our previous research with immobilized metal affinity SPE-CE-MS [37], a small plug (i.e. 20s at 50 mbar) of 30 mM $(NH_4)H_2PO_4$, pH 9.0 was used. Figure 2 shows the TIEs obtained for a 20 $\mu g \cdot mL^{-1}$ standard solution of TTR using each BGE and these elution conditions. As can be observed, the most intense TTR peak was detected at pH 5.0 (i.e. pI BGE), hence this BGE was selected for the rest of experiments. At these conditions, TTR was detected as a single peak (Figure 2B), as in CE-MS (Figure 1A ii)). However, the peak was slightly broader and migration time longer, due to the combined effect of the differences on pH and composition of the eluent and the pI BGE and the backpressure promoted by the microcartridge. With regard to the sample loading, the sample loading time was studied by introducing a 20 $\mu g \cdot mL^{-1}$ standard solution of TTR at 930 mbar from 2 to 20 min. As can be seen in Figure 3A, the maximum amount of TTR was detected with a 5 min loading time (30 µL of sample, see Section 2.4). When the protein solution was loaded for a longer time, analyte breakthrough caused a significant

decrease of peak area. Consequently, in order to reduce the total analysis time while achieving the highest recoveries, a sample loading time of 5 min was selected for the rest of experiments.

Once selected the pI BGE (pH 5.0) for sample loading, washing and separation and the sample loading time, different volatile eluents covering acidic and basic pH values were tested as an alternative to the 30 mM $(NH_4)H_2PO_4$, pH 9.0 solution. However, acidic eluents (i.e. 1 M HAc, pH 2.3 and 1 M HFor, pH 1.9) and some basic eluents (i.e. a mixture of 200 mM NH_3 and 200 mM glycine, pH 9.5) gave very irreproducible results, or did not elute the protein (i.e. 30 mM NH_4HCO_3, pH 7.8 and 1 M NH_3, pH 11.6). Addition of 20% (v/v) ACN or MeOH to the 30 mM $(NH_4)H_2PO_4$, pH 9.0 solution was either satisfactory, and TTR peak decreased. Overall, the results showed that elution was mediated by the basic pH, but also by the presence of phosphate anions, which may help to displace the protein from the AuNP surface. Figure 3B further shows that the maximum amount of TTR was eluted when the plug of optimized eluent was injected 20 s at 50 mbar.

Under the optimized conditions, repeatability was similar to CE-MS studies, with %RSDs (n=3, 10 $\mu g \cdot mL^{-1}$ of TTR) of 3.6 and 11.4% for migration time and peak area, respectively. The microcartridges could be reused until 10 analyses without significant changes on this performance. Fig. S2 shows a plot of peak area of the detected TTR after 10 repeated analysis of a 10 $\mu g \cdot mL^{-1}$ TTR standard. The microcartridge was discarded when the peak area of TTR in the EIE decreased more than 25% compared to the mean value of the first three analyses. Reproducibility between microcartridges was acceptable, TTR migration times were very similar but differences on peak areas of the detected TTR until 20% could be observed. The method was linear (r^2>0.998) between 5 and 25 $\mu g \cdot mL^{-1}$ (Figure 3C) and the LOD was slightly lower than 5 $\mu g \cdot mL^{-1}$. This LOD was 50 times lower compared to CE-MS, hence the sensitivity enhancement was similar compared to IA-SPE-CE-MS using microcartridges with intact antibodies against TTR [30].

Before the analysis of serum samples, the SPE-CE-MS method optimized with the conventional IT mass spectrometer was transferred to an accurate mass high-resolution TOF mass spectrometer, which allowed resolving molecular ions from the different MO TTR proteoforms [28–30]. Figures 4A and 4B show a comparison between the EIEs and mass spectra obtained for the SPE-CE-MS analysis of a 10 $\mu g \cdot mL^{-1}$ standard solution of TTR with the IT and

TOF mass spectrometers, respectively. In addition to the slight differences on TTR migration times and peak shapes in the EIEs due to the particularities of the instrumental set-ups (Figures 4A and 4B i)), the TOF mass spectrometer was able to scan with higher sensitivity, mass accuracy and resolution in a wider m/z range (Figure 4B ii)). As a result, the inset in Figure 4B ii) shows that five proteoforms of the MO TTR could be detected in the deconvoluted mass spectrum. The proteoforms corresponded to TTR-Cys ($M_{r\ theo}$ = 13,880.4022), phosphorylated or sulfonated TTR (TTR-Phosphorylated, $M_{r\ theo}$ = 13,841.2439 or TTR-Sulfonated, $M_{r\ theo}$ = 13,841.3283, respectively, mass accuracy was not enough to differentiate this small mass difference [28–30]), dehydroxylated or conjugated cysteine sulfinic acid TTR (TTR-Dehydroxylated or TTR-Sulfinic, $M_{r\ theo}$ = 13,793.2628, MS/MS would be necessary to differentiate between these isobaric proteoforms [28–30]), free TTR (Free-TTR, $M_{r\ theo}$ =13,761.2640), and the isoform resulting from the single amino acid substitution of a cysteine by a glycine at position 10 (TTR-(10) C-G, $M_{r\ theo}$ = 13,715.1713 Da). The LOD for these 5 proteoforms was around 1 $\mu g \cdot mL^{-1}$, similar to the value obtained with the same TOF mass spectrometer by IA-SPE-CE-MS in our previous work [30].

For the analysis of TTR in serum samples, the off-line sample pretreatment based on precipitation of the most abundant proteins with 5% (v/v) of phenol that we applied in a previous work before IA-SPE-CE-MS was investigated [30]. However, TTR was not detected, current was unstable, and reproducibility was low. This was probably due to retention of other proteins with similar pI to TTR that remained in the extract after sample clean-up. Results were also unsatisfactory when phenol precipitation was repeated twice, or when changes in the amount of salt or phenol were made. More selective clean-up methods (e.g. immunoprecipitation) were not investigated to avoid unnecessarily complicating the sample pretreatment. It may therefore be concluded that the limited selectivity of the AuNPs was especially critical at the microscale and precluded the analysis of TTR in serum samples when off-line sample pretreatment was kept simple.

4. Conclusions

The study proofs that polymeric AuNP@monolith materials can be used to prepare "plug-and-play" microcartridges for SPE-CE-MS with enough active surface area to achieve appropriate recoveries and preconcentration factors for protein biomarkers. Despite the particular benefits of incorporating AuNPs into polymer monoliths, the selectivity of the hybrid material, which is based on the protein pI, is compromised when complex biological matrices are analyzed. This investigation could be regarded as a starting point to design in the future AuNP@monoliths with AuNPs further modified with more selective ligands (i.e. antibodies, antibody fragments or aptamers) to find applicability as affinity-based sorbents in the targeted analysis of peptide and protein biomarkers in biological samples by SPE-CE-MS. Otherwise, AuNP@monolith-SPE-CE-MS could be also explored to analyze other type of small molecules (e.g. amino- or thiol-containing compounds) in samples with a lower protein content or, in general, with a less complex matrix (e.g. pharmaceutical products, drugs in urine samples or contaminants in environmental water samples).

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The authors declare no conflicts of interest.

Figure legends

Figure 1. CE-IT-MS for a 500 μg·mL^{-1} TTR standard. **(A)** Total ion electropherogram (TIE) and **(B)** mass spectrum with i) 1 M HAc pH 2.3 (acidic BGE) and ii) 10 mM NH_4Ac pH 5.0 (pI BGE). (With the pI BGE a pressure of 100 mbar is applied during the separation).

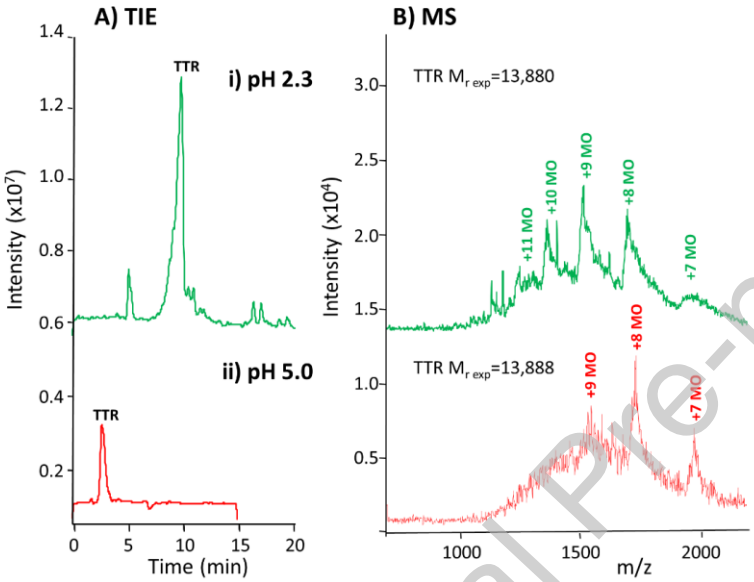

Figure 1

Figure 2. Total ion electropherogram (TIE) by AuNP@monolith-SPE-CE-IT-MS for a 20 µg·mL^{-1} TTR standard with a 10 mM NH_4Ac BGE at pH **(A)** 4.2, **(B)** 5.0 and **(B)** 5.8. (In all cases a pressure of 100 mbar is applied during the separation).

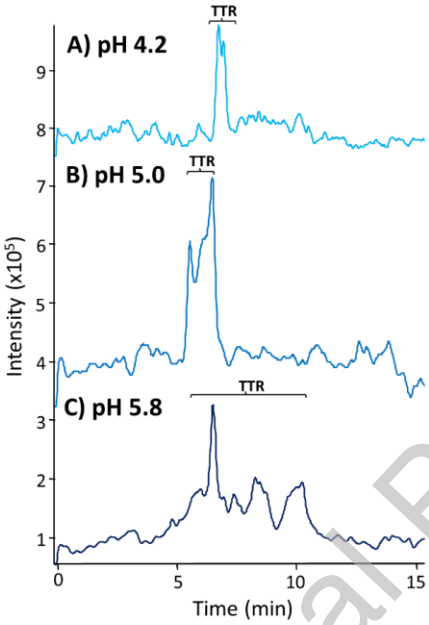

Figure 2

Figure 3. Plot of peak area of the detected TTR vs **(A)** sample loading time at 930 mbar (20 µg·mL^{-1} TTR standard, elution 20 s at 50 mbar), **(B)** elution time at 50 mbar (20 µg·mL^{-1} TTR standard, loading 5 min at 930 mbar) and **(C)**

concentration of the loaded TTR standard solution (using the optimized loading and elution times indicated in **(A)** and **(B)** with an asterisk. Regression parameters in the linear range are shown). In all AuNP@monolith-SPE-CE-IT-MS experiments, 10 mM NH_4Ac pH 5.0 (pI BGE) is used for the loading, washing and separation and 30 mM $(NH_4)H_2PO_4$, pH 9.0 for the elution . A pressure of 100 mbar is applied during the separation.

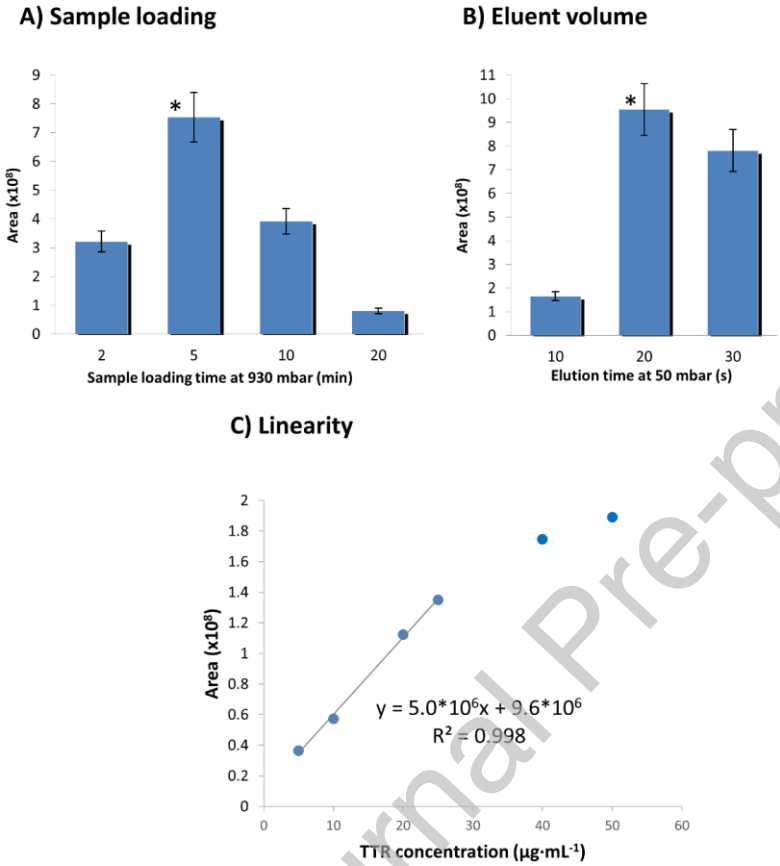

Figure 4. i) Extracted ion electropherogram (EIE) and ii) mass spectrum for a 10 μg·mL^{-1} TTR standard by **(A)** AuNP@monolith-SPE-CE-IT-MS and **(B)** AuNP@monolith-SPE-CE-TOF-MS under the optimized conditions. The deconvoluted mass spectrum is shown for the TOF mass spectrum.

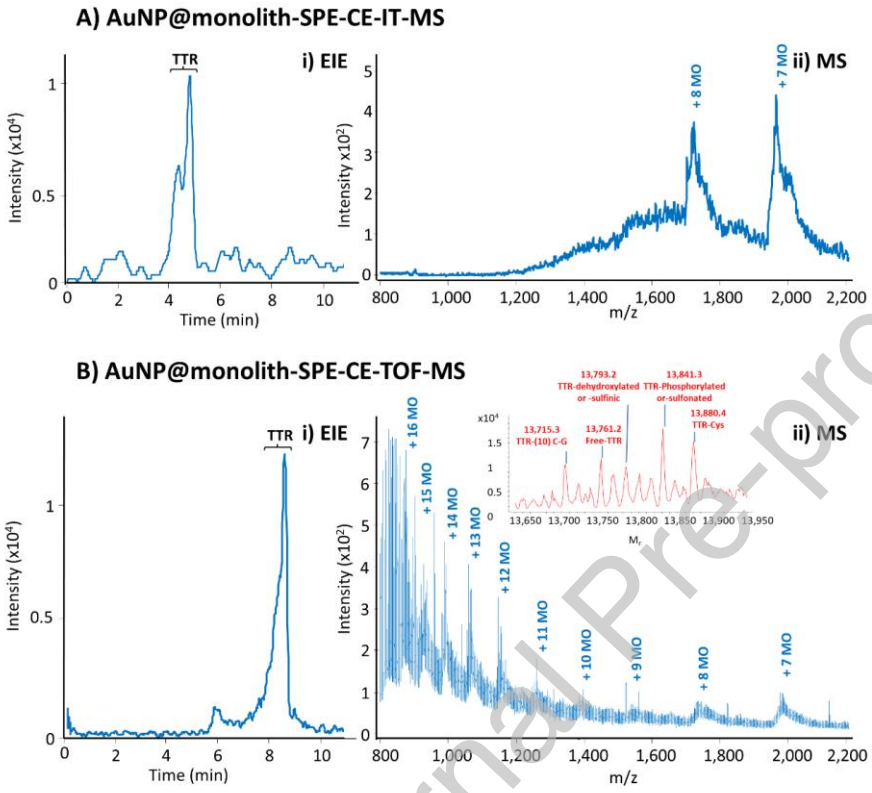

Figure 4

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CRediT authorship contribution statement

Laura Pont: Methodology, Validation, Investigation, Writing original draft, Writing – review & editing. **Gemma Marin:** Methodology, Validation, Investigation, Writing – review & editing. **María Vergara-Barberán**: Methodology, Validation, Investigation, Writing – review & editing. **Leonardo G. Gagliardi**: Conceptualization, Writing – review & editing. **Victoria Sanz-Nebot**: Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **José M. Herrero-Martínez**: Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition, **Fernando Benavente**: Conceptualization, Writing original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.