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Preparation of monolithic polymer-magnetite nanoparticle composites into poly(ethylene-co-tetrafluoroethylene) tubes for micro-bore HPLC separation and extraction of phosphorylated compounds

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Abstract

This paper describes the fabrication of a novel microbore monolithic column modified with magnetite nanoparticles (MNPs) prepared in a poly(ethylene-co-tetrafluoroethylene) (EFTE) tubing, and its application as stationary phase for the chromatographic separation of phosphorylated compounds. In order to obtain the composite column, a two-step procedure was performed. The formation of a glycidyl methacrylate-based monolith inside the activated ETFE tube was firstly carried out. Then, two incorporation approaches of MNPs in monoliths were investigated. The generic polymer was modified with 3-aminopropyltrimethoxysilane (APTMS) to be subsequently attached to MNP surfaces. Alternatively, APTMS-coated MNPs were firstly prepared and subsequently used for attachment onto the monolith surface through reaction of epoxy groups present in the generic monolith. This last strategy gave a reproducible layer of MNPs coated onto the polymer monolith as well as robust and permeable chromatographic columns. The retention behaviour of this MNP-based composite monolithic column was studied by using small phosphorylated compounds (adenosine phosphates). It was found that the retention of model analytes was ruled by partitioning and adsorption HILIC mechanisms. The columns also exhibited satisfactory performance in the separation of these target compounds, showing good chromatographic behaviour after two months of continued use. These composite monolithic columns were also successfully applied to the extraction of a tryptic digest of β-casein.

Keywords: monolithic column, magnetite nanoparticles, hybrid monoliths; adenosine phosphates, EFTE tubing, HILIC

1. Introduction

Polymer monolithic columns have been an alternative to the HPLC particle-packed columns for the last thirty years due to their ease of *in-situ* preparation, good permeability, wide pH stability, and readily available surface chemistries [1,2]. Despite these advantages, polymer monoliths have smaller specific surface area and a limited separation performance of small solutes compared with particulate and monolithic silica-based stationary phases. To circumvent these shortcomings, one smart way lies in the development of hybrid or composite materials resulting from synergetic combination of the monolithic phases and nanoparticles (NPs) (*viz.*, large surface/volume ratio, easy functionalization and flexible interaction chemistries). Indeed, hybrid monolithic phases with increased selectivity, sorbent capacity and chromatographic performance have been reported [3-5]. In this sense, a number of nanostructures such as carbon nanotubes [6,7], gold NPs [8,9] and iron oxide NPs [10] have been used in recent years in combination with polymer monoliths to develop novel hybrid monolithic phases for (electro)chromatographic separation purposes. In particular, magnetic iron oxide NPs (MNPs) in its several forms have also been widely used for multiple applications, such as biomedical [11,12] and chemistry technologies [13,14], including analytical field [15]. Apart from their magnetic properties, other advantageous features are their easy chemical modification and strong affinity for phosphorus-containing molecules [16]. Thus, surface modification with hydrophilic ligands (*e.g.* silane coupling reagents) and/or biocompatible polymers (*viz*. chitosan, poly(ethylene glycol)) on the surface of MNPs has been commonly reported [12,17] in order to prevent their agglomeration and thus achieving colloidally stable composites with enhanced and differentiated properties.

In the specific case of MNP-based hybrid monoliths, a common synthesis approach involves the copolymerization of MNPs with suitable monomers. In a previous work, our research group described a polymethacrylate based monolithic column containing vinylized iron oxide MNPs as stationary phase for capillary electrochromatography [10]. The column efficiency in the resulting hybrid monoliths was greatly enhanced by embedding of these MNPs. Despite these good results, in this approach, the MNPs were mostly encapsulated within the polymer matrix, being only few of them accessible to interact with analytes.

To overcome this limitation, an approach based on direct attachment of MNPs to pore surface of monolith has been described. Thus, Krenkova *et al.* immobilized citrate

stabilized Fe_3O_4 NPs via multivalent electrostatic attachment onto a quaternary ammonium modified methacrylate monolith in a capillary [18] or pipette-tip format [19]. Since the MNPs were attached to the monolith only by electrostatic interaction, they may easily leach out from the polymer during application. Up to now, to the best of our knowledge, no study focused on the attachment of MNPs to polymer monoliths by covalent bonding has been reported, which should be an effective way to solve the limitation mentioned above. On the other hand, these composite materials have demonstrated to be useful for efficient phosphopeptide enrichment in a batch mode. However, the application of composites with covalently attached MNPs as stationary phases in conventional HPLC has not been explored to date.

In this work, a novel composite MNP-polymeric monolith as stationary phase for microbore HPLC was developed in poly(ethylene-co-tetrafluoroethylene) (ETFE) tubing, and its applicability was evaluated. For this purpose, two incorporation routes were explored. In the first strategy, a glycidyl methacrylate-based monolith was modified by 3-aminopropyl trimethoxysilane (APTMS) followed by MNP binding. In the second approach, amine-modified MNPs were attached onto pore surface of monolith. This second strategy allowed developing the best connectivity between the MNPs and organic polymer. The resulting composites were characterized and were used as stationary phases for the separation of a mixture of adenosine phosphates in order to investigate their retention on iron oxide NP surfaces. In addition, the reproducibility of these hybrid monolithic columns was evaluated. Moreover, the applicability of these columns was satisfactorily tested by the enrichment of a tryptic digest of β-casein.

2. Materials and methods

2.1. Chemicals and reagents

Divinylbenzene (DVB), ethyleneglycol dimetacrylate (EDMA), glycidyl methacrylate (GMA), lauroyl peroxide (LPO), tetrahydrofuran (THF), formic acid (FA) and trifluoroacetic acid (TFA) were from Sigma-Aldrich (Steinheim, Germany). Benzophenone (BP), (3-aminopropyl)trimethoxysilane (APTMS) and 1-decanol were supplied by Alfa Aesar (Karlsruhe, Germany). HPLC-grade acetonitrile (ACN), methanol (MeOH), ethanol (EtOH) and dimethylsulfoxide (DMSO) were from Merck (Darmstadt, Germany). MNPs were synthesized with $FeCl_2 \cdot 4H_2O$ and $FeCl_3 \cdot 6H_2O$ from Panreac (Barcelona, Spain) and ammonia from Scharlab. Adenosine (AD), adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP),

β-casein from bovine milk and trypsin from bovine pancreas were provided by Sigma-Aldrich.

Ultra-pure water was obtained with a Puranity TU6 water purification system from VWR (Bedford, MA, USA) provided with a 0.2 μm filter. Anhydrous-N_2 was supplied by Abelló Linde (Valencia, Spain). ETFE tubing of 1/16'' (1.6 mm o.d. × 0.75 mm i.d.) was used for in-situ fabrication of composite monoliths and was supplied by Vici Jour (Schenkon, Switzerland).

Stock solutions (2000 $\mu g \cdot mL^{-1}$) of AD and adenosine phosphates were weekly prepared in ACN-water (1:1, v/v) and kept at 4ºC. A test mixture (used as working standard solution) composed of AD (75 $\mu g \cdot mL^{-1}$), AMP, ADP and ATP (150 $\mu g\ mL^{-1}$ each one) was prepared daily by diluting the stock standard solutions with ACN-water (1:1, v/v).

2.2. Instrumentation

Photografting of the ETFE tubing was done using a CL1000 UV Crosslinker (UVP, Upland, CA, USA) at 254 nm. A KD Scientific syringe pump (Model 100, New Hope, PA, USA) was used for the introduction of the reagents into the housing supports (ETFE tubing). A JP Selecta drying oven (Barcelona, Spain) was employed to carry out the polymerization step.

The morphology of the materials was characterized using a scanning electron microscope (S-4800, Hitachi, Ibaraki, Japan) equipped with an X-ray microanalysis system (EDAX Genesis 400). High-resolution transmission electron microscopy (TEM) analysis of MNPs was performed in a JEOL microscope (JEM 2100F, Freising, Germany) operated at 200 kV. Elemental analysis of nanostructure materials was performed using an EA 1110 CHNS elemental analyzer (CE Instruments, Milan, Italy).

An HP1100 HPLC pump (Agilent Technologies, Waldbronn, Bermany) connected to a Rheodyne 7725 manual six-port sample injection valve (Rohnert Park, CA) (with a 3 mL loop) and a column oven (Análisis Vínicos, Ciudad Real, Spain) was used to perform functionalization experiments of monolithic beds with MNPs. Chromatographic measurements were performed using an Agilent 1260 Infinity instrument (Agilent Technologies) equipped with a quaternary gradient pump, a solvent degasser system, an autosampler and a diode-array detector. The chromatographic system was controlled by an OpenLAB CDS LC ChemStation from Agilent (B.04.03)

MALDI-TOF MS experiments were performed using a 5800 MALDI-TOF/TOF mass spectrometer (AB Sciex, CA, USA). The spectra of phosphopeptides before and after

selective enrichment were collected using DHB as a matrix. A 10 mg mL^{-1} DHB solution was prepared in 50% aqueous ACN containing 0.1% TFA. Before analysis, the samples and matrix were mixed in a 1:1 ratio. Mass spectrometric measurements were carried out in the reflector positive mode with a scan range of 1,000-4,000 m/z. 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).

Peptide mixtures were also analyzed by using an Eksigent 425 nano-LC system (Dublin, CA) which was connected to a TripleTOF 5600 mass spectrometer (AB Sciex). Briefly, 5 µL of sample 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 2 µL min^{-1} during 10 min. The peptides were then loaded onto an analytical column (3C18-CL-120, 3 µm, 120 Ă, 75 µm × 15cm, Eksigent) equilibrated in 5% ACN and 0.1% FA. The elution was done using a linear gradient of 7 to 35% B in A for 30 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 350-1250 m/z for 250 ms. The quadrupole resolution was set to 'UNIT' for MS2 experiments, which were acquired 100-1500 m/z for 50 ms in 'high sensitivity' mode. Main conditions in the MS analysis were ions from 1+ to 5+ with a minimum intensity of 70 cps. Up to 50 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 15 s. The system sensitivity was controlled by analyzing 500 ng of K562 trypsin digestion (Sciex), and under these conditions 2077 proteins were identified (FDR <1%) in 45 minutes gradient.

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 MS instrument. The Paragon algorithm [20] of ProteinPilot was used to search the Uniprot_Aves (Nov 2018) database with the following parameters: trypsin specificity, cysteine alkylation, without taxonomy restriction and phosphorylated emphasis.

2.3. Synthesis, functionalization and characterization of MNPs

MNPs were prepared using the co-precipitation method as described elsewhere by Yang *et al.* [21]. To obtain the amino functionalized MNPs (namely NH_2-MNPs), a protocol described by Ahmadi *et al.* was adopted [22]. Briefly, the bare MNPs were added to a 2.7 wt% APTMS solution prepared in MeOH-water-ammonia (82.3:14.6:0.4, v/v/v) at

room temperature for 12 h under continuous stirring. The resulting NH_2-MNPs were washed several times with EtOH and deionized water, and then were dried at 60°C in an oven.

The morphology of the synthesized MNPs and NH_2-MNPs was performed by TEM (Figure S1). From these measurements, MNPs and NH_2-MNPs showed diameters of *ca*. 10 and 13 nm, respectively. The increase in diameter after silanization suggested the presence of the bonded ligand (aminosilane) onto the NP surface. Additionally, elemental analysis of NH_2-MNPs was done, giving a content of 0.76 wt% of nitrogen, which corroborates the presence of aminosilane in MNPs.

2.4. Preparation of polymeric monolithic columns within ETFE tubing

The inner surface of the ETFE tubing was modified prior to the *in-situ* polymerization step to provide covalent anchoring points for the polymer. A two-step photo-grafting procedure was carried out as previously reported [23]. First, the ETFE tube was filled with a deoxygenated solution of BP (5 wt%) in MeOH. The filled column was irradiated at 0.9 J cm^{-2} for 30 min with a distance from the lamps of 2.5 cm. Next, the BP-modified tube was flushed with a deoxygenated solution of EDMA (15 wt%) in MeOH and irradiated using the same conditions as above. The modified ETFE tubing was rinsed with MeOH and dried under nitrogen.

The selected polymerization mixture consisted of 33.33 wt% monomers (40 wt% GMA and 60 wt% DVB), and 66.67 wt% porogenic solvents (91 wt% 1-decanol and 9 wt% THF) in the presence of 1.0 wt% LPO as initiator (relative to the monomers) [24]. It was weighted in a vial, sonicated for 4 min and deoxygenated with nitrogen for 10 min. Then, the activated EFTE tubing was placed inside an external polypropylene mold for posterior filling with the polymerization mixture and subsequent thermal polymerization (70ºC for 4 h) as previously described [24]. After polymerization, the column was cut to the desired length (usually 10 cm), fitted with end fittings, and connected to an HPLC pump to be rinsed with MeOH to remove the possible unreacted monomers and the porogenic solvents.

2.5. Immobilization of MNPs onto polymeric monoliths in ETFE tubing

The incorporation of MNPs to GMA-based monoliths was accomplished by two approaches: a) modification of the parent monolith with APMTS and posterior

attachment of bare MNPs, and b) functionalization of MNPs with APTMS and subsequent immobilization of NH_2-MNPs onto the surface of monolith.

The first strategy (procedure A) was adapted from a previous work [25]. Thus, the GMA-based monolith in ETFE tubing was modified by pumping through a solution of APTMS in acetone at 60°C (column oven) at 0.05 mL min^{-1} using an HPLC pump for 2 h. Upon completion of the reaction, the column was washed with ethanol and dried. Then, a dispersion of MNPs (0.1%) in EtOH:water (1:1, v/v) was pumped through the APTMS-modified monolithic column with a syringe pump at 0.05 mL min^{-1} for 8 h. Regarding to the other approach (procedure B), the parent monolith was modified with a dispersion of NH_2-MNPs (25 μg mL^{-1}) in DMSO, which was used to fill the 3 mL loop and it was pumped through the monolithic bed at 60ºC (placed in an oven) at a flow rate of 0.05 mL min^{-1}. This process was repeated until a completely brown-coloured column was obtained and a brown solution was observed coming out of the column outlet. The column was then rinsed thoroughly with MeOH and with the mobile phase before being attached to the chromatographic system. Figure 1 depicts a scheme of the preparation of composite monolithic columns in ETFE tubing using both procedures.

2.6. Chromatographic calculations and conditions

The dead time was taken at the time of the first significant baseline disturbance [26]. However, it was confirmed by injecting an unretained solute (a solution of 100 μg mL^{-1} of KBr or AD). The dwell time was obtained by replacing the column with a short piece of connecting tubing and running a blank gradient at 260 nm where acetone was increased from 0 to 0.1% in 20 min [26].

The following retention models were used to examine the retention of target compounds in isocratic studies. For partitioning, the linear solvent strength model was adopted [26]:

$$\log k = \log k_w - S\varphi \qquad (1)$$

where k and k_w are the retention factor in the mobile phase and an eluent composed of pure water, respectively, φ is the volumetric fraction of the organic modifier, and S is the empirical slope that gives a measurement of the elution strength of the mobile phase. Concerning adsorption as the retention mechanism, it was based on confined surface adsorption as used in normal-phase chromatography [27,28] and is given by:

$$\log k = \log k_0 - n \log \varphi \qquad (2)$$

where k_0 is the extrapolated intercept (retention factor in pure weak eluent), and *n* is the ratio of cross-sectional areas occupied by analyte molecules and by water molecules.

Peak capacity (P_C) was experimentally determined by using [29]:

$$P_C = 1 + \frac{t_G}{1.679 \times w_{1/2}} \qquad (3)$$

where t_G (min) is the total gradient time of the chromatogram and $w_{1/2}$ is average peak width at half height (min) of different solutes across the elution window. The global resolution (R_G) was also measured as the geometric mean of the resolution between the consecutive peak pairs.

The chromatographic separation of the probe analytes (AD and adenosine phosphates) was carried out under the following conditions. For isocratic studies, mobile phases, at 0.3 mL min^{-1}, were prepared with 5 mM phosphate buffer (pH 6.5) and variable percentages of ACN. For gradient elution experiments, a 5 mM phosphate buffer (pH 6.5) (solvent A) and 100% ACN (solvent B) were used. The gradient program, at 0.5 mL min^{-1}, was as follows: from 0 to 0.5 min, the percentage of B was changed to 90%, and this composition was kept for 1 min. Later, the concentration of B was changed to 86%B in 0.25 min and kept for 1 min. From 2.75 to 4 min, the composition was changed to 70%B, and kept for 2 min more. Finally, the composition returned to initial conditions (100%B) in 1 minute and it was kept for 4 min more. The injection volume was 1 μL, and detection wavelength was set at 230 nm.

2.7. Tryptic digestion of β-casein and phosphopeptide enrichment

The trypsin digest of β-casein was prepared as described in the literature [19]. Briefly, β-casein (2.5 mg mL^{-1}) was dissolved in 10 mM ammonium bicarbonate solution, pH 8.2. Then, the protein solution was digested with trypsin at 37ºC for 20 h (substrate-to-enzyme ratio, 50 : 1, w/w). The proteolysis was stopped by addition of 10% acetic acid.

The enrichment of phosphopeptides using the MNP-modified monolithic column was adapted from previous works [19, 30]. The column was flushed with a mixture of ACN and 0.1% TFA (4:1) for 10 min at a flow rate of 8 μL min^{-1} using a syringe pump. Protein digest was then pumped through the column at 6 μL min^{-1} for 20 min. To wash out the non-phosphorylated peptides, the column was flushed with water for 10 min at 8 μL min^{-1}. Phosphopeptides were eluted using 10 % ammonium hydroxide solution (50 μL) pumped at 8 μL min^{-1}. The fractions were analyzed MALDI-TOF MS as well as nano-LC-ESI-MS/MS.

3. Results and discussion

3.1. Surface attachment of MNPs to polymeric monoliths

As it was mentioned in the Introduction, two strategies were assayed to obtain the hybrid MNP-monolithic columns. As shown in Fig. 1, for the approach A, the amine groups of APTMS initially reacted with the epoxy groups of GMA-based monolith. Elemental analysis showed that resulting aminosilane-modified monoliths provided a nitrogen content of 1.51 wt%, thus confirming the successful modification. Then, a dispersion of MNPs in EtOH:water (1:1, v/v) at 0.1 wt.% was prepared, and pumped through the monolithic column. However, a non-homogeneous distribution of these MNPs along the column length was obtained, remaining mainly at the head of the monolithic column in the housing ETFE tube. These results suggested that agglomeration phenomena of the MNPs were present, which translated into clog the monolith inlet during loading and a concomitant undesirable increase in backpressure of system. Next, dispersions with lower contents of MNPs (up to 0.001 wt%) were tried; however, similar results were found.

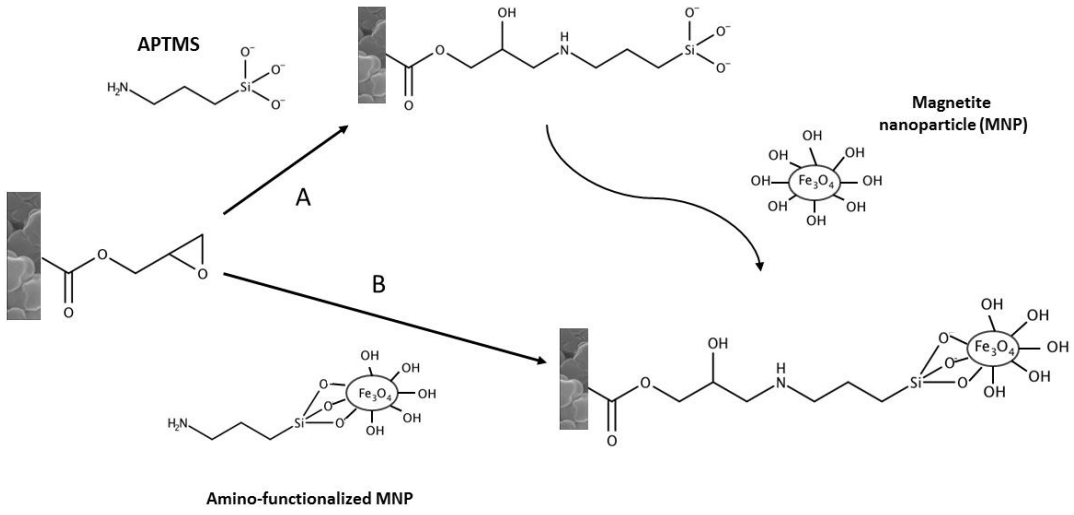

Figure 1. Schemes of modification of GMA-based monoliths in ETFE tubing with: (A) APTMS followed by attachment of bare MNPs and (B) direct immobilization of amino-MNPs.

Alternatively, citrate-coated MNPs were prepared (in the presence of 1.5% w/v citrate ions) as described elsewhere [18,19] to prevent rapid aggregation and poor colloidal stability, and subjected to the protocol A. Unfortunately, the coverage of polymeric monolith was also limited. A possible explanation could be related to the excess citrate

present in the MNP surface, which can hinder the accessibility or direct attachment of the hydroxyl groups on the iron oxide NP surface with silane reagent. Consequently, it was necessary to explore another alternative for the immobilization of the MNPs to the surface monolith.

Thus, the second approach tested involved the covalent attachment of amino-modified MNPs (namely, NH_2-MNPs) to the pore surface monolith (see Fig 1, procedure B). Thus, the dispersion of NH_2-MNPs at 0.0025 wt.% was passed through the monolithic bed placed in a column oven at 60ºC, and at flow rate of 0.05 mL min^{-1}. Using this protocol, agglomeration phenomena of MNPs were not evidenced, which allowed to achieve a uniform covalent attachment amino-MNPs along the column length. Thus, the immobilization process was easily monitored visually by the colour change of the exiting dispersion. Thus, the surface saturation with MNPs was achieved when the dark brown color of this suspension leaving the ETFE tubing outlet was evidenced and the colour of the monolith turned deep brown. Fig. 2A shows the hybrid monolithic column with MNPs prepared in 100 mm of ETFE tubing. SEM images of the composite monolith were also obtained. As shown in Fig. 2B, clusters of MNPs covered the monolithic surface of globules were evidenced, which demonstrated the successful attachment of MNPs. Additionally, an EDAX analysis was used for characterization of MNP coating. Thus, the hybrid monoliths showed iron contents of *ca*. 7.5 wt%. The iron percentage found was higher than those obtained for other polymer monoliths modified with iron oxide NPs reported in literature (1% [18] and 3.7% [19]).

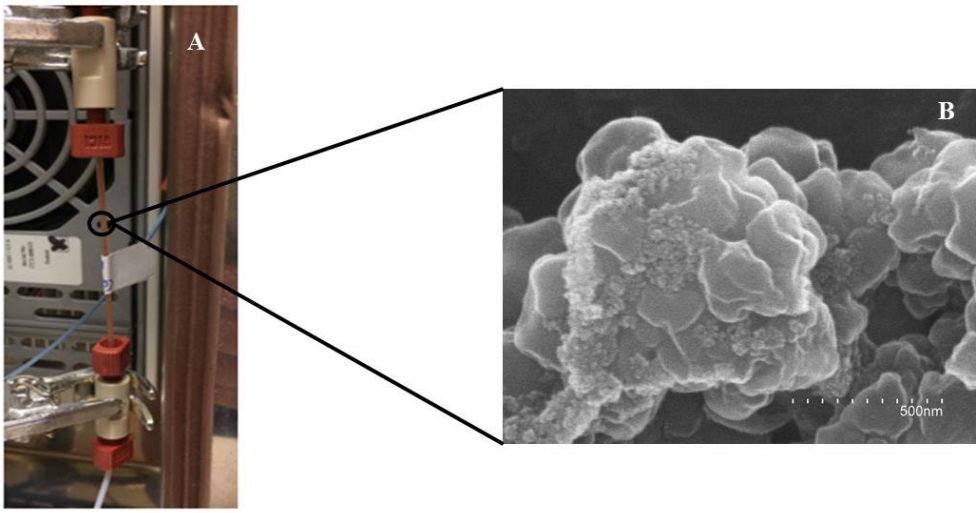

Figure 2. A) Photograph of hybrid monoliths prepared with amino-MNPs in an ETFE tubing (100 mm × 0.75 mm) with Luer-lock connectors. B) SEM micrograph corresponding to the hybrid monolith at 80 000 × magnification.

Measurements of backpressure were also done before and after the attachment of the amino-MNPs using either water or ACN. After immobilization of these NPs, there was a slight increase (*ca*. a 5%) in backpressure, which suggested that MNPs remain retained in the surface monolith. This fact remarks the satisfactory permeability of the prepared monolithic stationary phases in ETFE tubes.

3.2. Chromatographic evaluation of hybrid monolithic columns in ETFE tubing

In order to characterize the chromatographic features of hybrid monolithic columns and understand their retention mechanism, adenosine phosphates (AMP, ADP and ATP) were selected as probe compounds. Also, adenosine (AD), which does not contain phosphate functionalities, was used, and it was expected that would display little or no affinity for the iron oxide NP surface.

Isocratic conditions were initially tested by using mobile phases composed of phosphate buffer (pH 6.5) and ACN, which were adopted on the basis of previous works [31,32]. In particular, the phosphate content in the mobile phase was set at 5 mM, since higher concentrations were not tested due to the limited solubility of the salt at high ACN content. Fig. 3 shows the retention behaviour of model compounds on the hybrid monolithic column using mobile phases containing different ACN content. The composite column showed negligible retention or very weak retention of nucleotides using mobile phases containing ACN contents below 75%. However, further increase of ACN content resulted into a progressive increase in the retention of the compounds, and some such ATP were strongly retained. As it can be seen, the nucleotides eluted in order of the increasing number of phosphate groups and their interaction with the stationary phase. Besides, the behaviour shown in Fig. 3 was consistent with a general HILIC theory. As regards the mobile phase is hydrophilic, no or negligible retention is observed since polar analytes tend to remain in a mobile phase. However, when the mobile phase becomes enough hydrophobic, these analytes prefer the more polar stationary phase, which results in a retention enhancement.

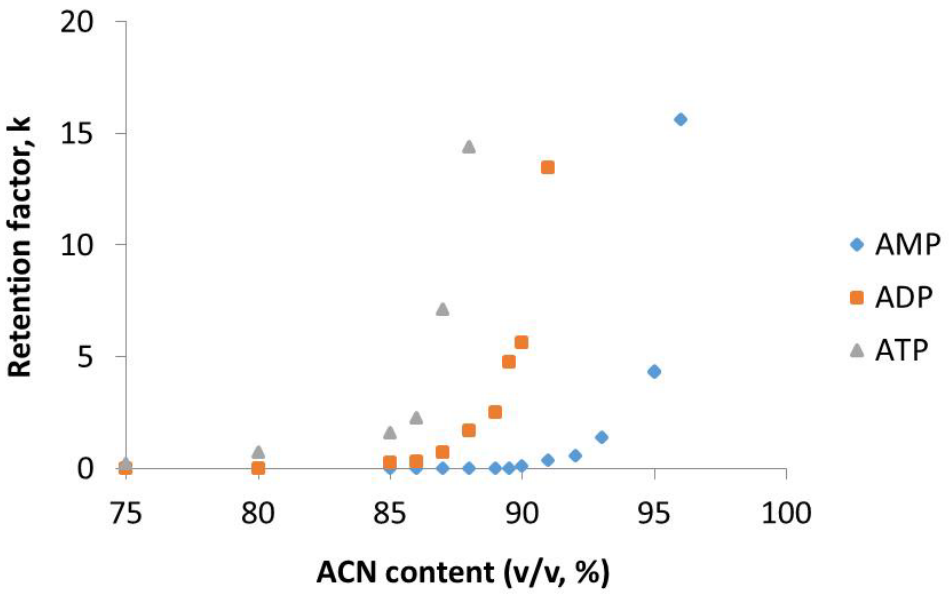

Figure 3. Influence of ACN content on the retention factors of model compounds. Conditions: hybrid monolith in ETFE tubing (100 mm × 0.75 mm); mobile phase, 5 mM phosphate buffer (pH 6.5) in different % (v/v) ACN; flow rate, 0.3 mL min^{-1}; UV detection at 230 nm; solutes (150 µg mL^{-1} of AMP, ADP and ATP).

The retention behavior depicted in Fig. 3 was also examined with respect to the mathematical models for partitioning (see Eq. (1)) and adsorption (see Eq. (2)). Therefore, the corresponding plots of the retention factors (*k*) and the content of the aqueous part of the mobile phase were compared in the HILIC region (75–95% ACN). As shown in Table 1, high correlations were obtained for both models, which is consistent with previous studies [33,34] that suggest that HILIC mode is a multimodal retention process. It is known that phosphorylated compounds interact strongly with iron oxide surfaces via Lewis acid-base interactions [35,36]. At sight of these results, we suppose that electron donor phosphate entities enter the water rich layer, and then these moieties exhibit a notable affinity to iron Lewis acid sites located on iron oxide NP surface.

Table 1. Comparison of partitioning and adsorption retention models (lin-log and log-log plots, respectively) of tested compounds in HILIC region.

Compound	Partitioning	Adsorption
	lin-log plot	log-log plot
AMP	0.978	0.979
ADP	0.983	0.982
ATP	0.982	0.982

Based on all these previous results, a complete separation of the three nucleotides under isocratic conditions was not possible due to the different strength of interaction of these compounds with the stationary phase. Thus, gradient elution conditions were applied to obtain a satisfactory resolution in a reasonable retention time. Baseline resolution between all the analyte pairs was achieved (Peak capacity (P_C) = 31 and global resolution (R_G) = 2.6) within 6 min (Fig. 4).

The performance of hybrid monolithic column was compared to that of previously reported for metal-oxide packed and other metal-oxide monolithic columns. The efficiencies, given in terms of peak widths (at half peak height), for the most retained compound (ATP) was about 15.8 s, which was better than that reported for columns packed with 3-μm titania particles (65.4 s of peak width for ATP for 30-min gradient) [37]. Also, the efficiencies found were higher than those obtained under isocratic elution conditions for titania-based capillary monolithic columns (23.3 [31] and 36.4 s [38] for ATP) or alumina-based monoliths in capillary format (19.5 s for ADP) [32].

Table 2. Reproducibility of composite monolithic columns in ETFE tubing for micro-bore LC separation of phosphorylated adenosines[a].

Parameter		Repeatability	Reproducibility	
		Run-to-run column (n = 3)	Column-to-column (n = 3)	Batch-to-batch (n = 3)
		RSD, %	RSD, %	RSD, %
AMP	t_R (min)	0.4	1.4	2.0
	Peak area	0.6	1.7	2.5
ADP	t_R (min)	1.4	1.7	2.6
	Peak area	1.6	2.8	3.8
ATP	t_R (min)	0.8	2.0	2.6
	Peak area	2.5	3.8	5.4

[a] The chromatographic data were obtained by injecting a test mixture nucleotides (150 μg ml^{-1}) in a hybrid monolith in ETFE tubing (100 mm × 0.75 mm i.d.) using the gradient elution conditions given in Fig. 4 (see Section 2.6 for details).

The reproducibility of preparation of hybrid monolithic columns was also investigated. The run-to-run repeatability was evaluated from series of three injections of a test mixture of APs, while the column-to-column reproducibility was evaluated with three columns prepared from the same polymerization mixture. The LC conditions used where those indicated in Fig. 4. As shown in Table 2, the relative standard deviation

(RSD, %) values of the retention times and peak areas were less than 2.5% in run-to-run repeatability, whereas the RSD values were below 3.8% in the column-to-column reproducibility. The batch-to-batch reproducibility was also estimated from three batches of three columns each. The same parameters investigated gave RSD values below 5.4%. These results indicated a satisfactory reproducibility in the fabrication process of the composites developed in this work.

To date, and despite the large significance for chromatographers, only a few studies related to the stability of monolithic columns are described in the literature [39,40]. The column stability of the hybrid monolith synthesized with amino-MNPs over time (two months of continuous use) was also evaluated. For this purpose, a total of 150 injections (each one of 12 min) and separations of the nucleotide test mixture were done by using the same column under the chromatographic conditions given in Fig. 4. The RSD values for retention times and peak areas were below 11%. Additionally, these stationary phases showed low backpressure (below 10 bar) and reasonable separation performance (P_C ~ 30) along this period of time, which showed the stability of the composite monoliths within the ETFE tubing.

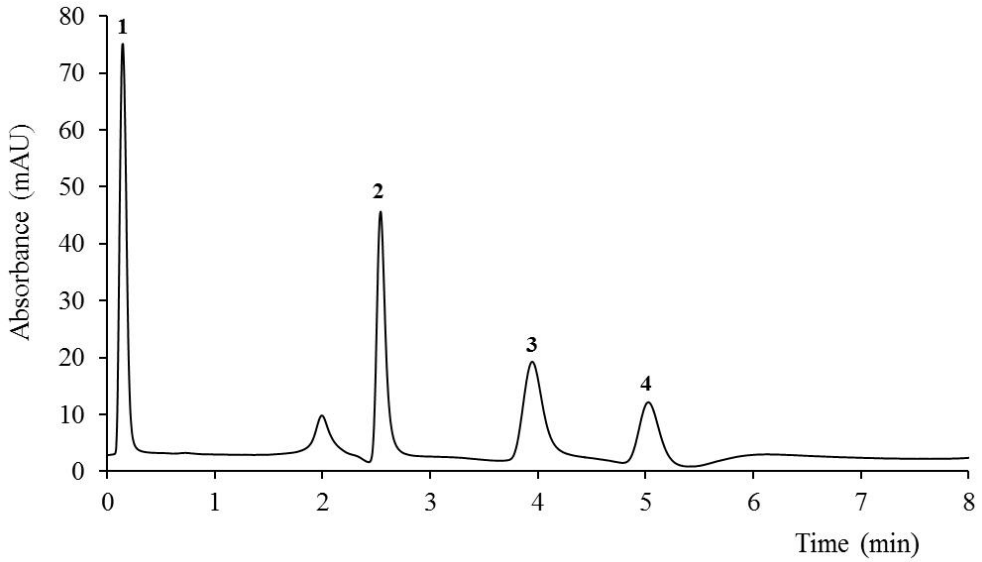

Figure 4. Separation of adenosine phosphates on hybrid monolith in ETFE tubing (100 mm ×0.75 mm) under optimized gradient elution conditions. Working LC conditions are given in section 2.6. Peak identification: 1, AD; 2, AMP; 3, ADP; 4, ATP.

The hybrid monolithic column was also tested for selective capture and extraction of phosphorylated compounds. For this purpose, a trypsin digest of β-casein, a protein that contains an anionic phosphoserine cluster in its structure [39] was used to evaluate its performance. Thus, the digested protein was loaded onto the hybrid column, treated following the procedure described in Section 2.7 and analyzed by MALDI-TOF MS. As shown in Fig. 5A, for the direct analysis of β-casein tryptic digest, only one phosphopeptide was detected since non-phosphorylated species dominate the spectrum. However, after enrichment, the signals of non-phosphorylated peptides significantly decreased and five phosphopeptides could be detected in the MS spectra (Fig. 5B). The detailed information of the identified phosphopeptides is displayed in Table S1. Additionally, the phosphopeptides from β-casein digest were analyzed with nano-LC-MS/MS (see Figs. S2 and S3), confirming the presence of these peptides identified in MALDI measurements.

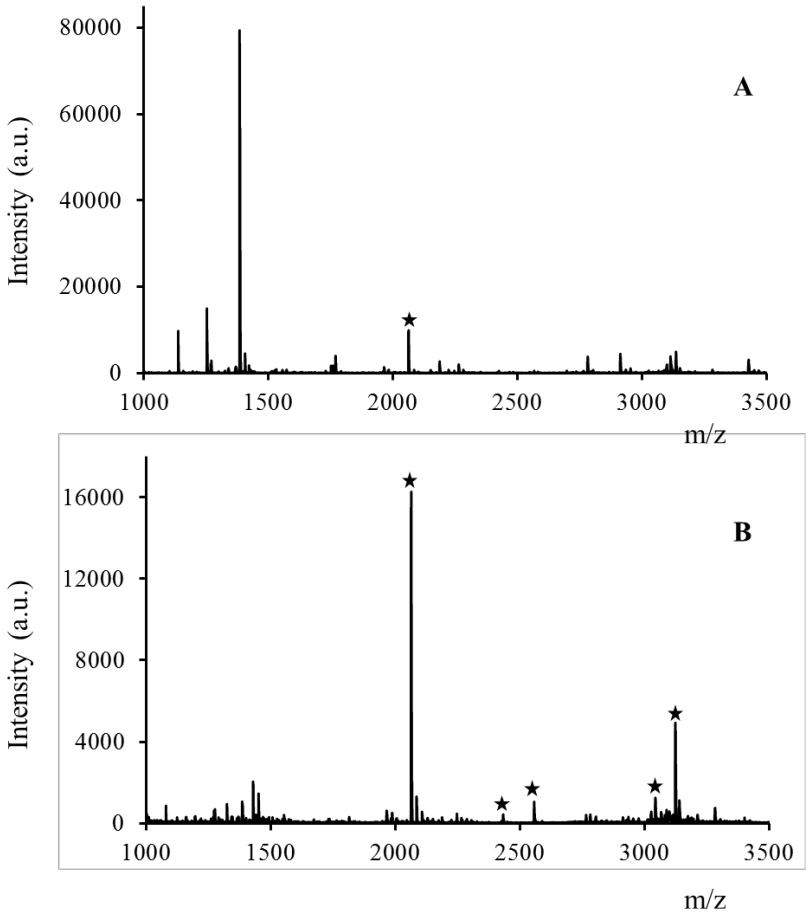

Figure 5. MALDI-TOF mass spectra of tryptic digest of β-casein without (A) and with phosphopeptide enrichment using the hybrid monolithic column (B).

4. Conclusions

In this study, MNPs have been incorporated into GMA-based monolithic columns prepared in ETFE tubing as housing support for microbore HPLC. From the two approaches adopted to introduce these NPs into the polymer monolith, the second one gave a proper and homogenous filling of the column with the amino-modified MNPs. The chromatographic performance and retention behavior of the resulting hybrid monoliths was evaluated using a set of phosphorylated adenosines as test analytes under HILIC conditions. It was found that the retention of these compounds was governed by surface adsorption and partition as retention mechanism. The resulting MNP-modified monolithic columns exhibited satisfactory column efficiency, low backpressures (< 10 bar), acceptable reproducibility and stability. In addition, the hybrid monoliths were suitably applied to the enrichment of phosphopeptides from a trypsin digest of β-casein. Taking into account the flexibility of synthesized hybrid monolithic columns, online coupling of these supports to conventional HPLC instruments could improve the purification and enrichment of phosphorylated compounds (*e.g.* phosphorylated peptides) prior to analysis, and thus extending its potential as tool in phosphoproteomic analysis.

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The authors declare no conflict of interest.

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