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Immobilized metal affinity chromatography using open tubular capillary for phosphoprotein analysis: Comparison between polymer brush coating and surface functionalization

Khadija El Idrissi[a,b], Saïd Eddarir[a,b], Caroline Tokarski[a], Christian Rolando[a,*]

[a] Université de Lille 1, Sciences et Technologies, USR CNRS 3290 Miniaturisation pour la Synthèse, l'Analyse & la Protéomique (MSAP), 59655 Villeneuve d'Ascq, France
[b] Université de Cadi Ayyad, Faculté des Sciences et Techniques Guéliz, BP 549 Marrakech, Morocco

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ABSTRACT

In the present article, open tubular-IMAC columns, functionalized by iminodiacetic acid (IDA) for the immobilization of Fe^{3+}, were prepared by in situ chemical modification of fused silica capillary using two chemistries, polymer brush coating and surface functionalization. One column was based on a poly-(glycidyl methacrylate) brush (GMA) and the other on 3-glycidoxypropyltrimethoxysilane (GLYMO). Phosphopeptide enrichment on the open tubular columns was evaluated on an α_{S1}, α_{S2} mixture and β casein peptides. The optimized enrichment protocol includes sample loading in a slightly acidic solution made with pure deionized water, a washing step with 10% acetonitrile, 0.1% formic acid, and an elution step with 50% acetonitrile, 0.1% phosphoric acid at pH 8.0. MALDI-TOF spectra generated from eluted fractions show several phosphorylated peptides. For example, 7 phosphorylated peptides of the α_{S1}, α_{S2} casein mixture were identified, including a pentaphosphorylated peptide. In terms of selectivity, the two proposed chemistries exhibit different behaviors: the GMA-IDA-Fe^{3+} IMAC polymer brush column elutes all phosphorylated peptides in one fraction independently of phosphorylation degree, whereas the GLYMO-IMAC polymer brush provides longer elution times for higher phosphorylation states. In particular, the pentaphosphorylated peptide was eluted after a 30 min elution versus 5 min for monophosphorylated species (isocratic gradient).

1. Introduction

Protein phosphorylation is a major post-translational modification [1,2] which is involved in all cellular processes, including signal transduction, gene expression and metabolism [3,4]. Approximately one-third of the thousands of proteins in a typical mammalian cell are believed to be phosphorylated and abnormal phosphorylation is recognized as a cause or consequence of many human diseases [5]. Three amino acids represent the main targets of phosphorylation: serine, threonine and tyrosine. Approximately 90% of all phosphorylated residues are serines, 10% are threonines, and 0.1% are tyrosines [6]. The detection of phosphoproteins requires specific analytical tools; for example, a phosphoproteome map can be obtained using gel electrophoresis combined with specific detection modes like dedicated fluorescent stains (e.g. Pro-Q Diamond) [7], ^{32}P radio labeling [8] or antibody-based western blotting detection [9]. However, phosphoprotein identification and phosphorylated site localization remain challenging tasks. To achieve these objectives, analytical strategies based on mass spectrometry analysis of enriched samples in phosphorylated compounds have been proposed [10–13]. In the literature, several methods relating to phosphopeptide or phosphoprotein enrichment have been described [14–16]; among them, Immobilized Metal Affinity Chromatography technique (IMAC), first introduced in 1975 by Porath et al. [17], is widely used either alone or combined with other separative steps [18]. The IMAC methodology is mainly based on interactions between phosphate group analytes and metal cations (e.g. Ga^{3+}, Cu^{2+}, Ni^{2+}, Fe^{3+}) [19,20] bound to a support material by chelating ligands. Among the support materials linked to solid matrices are agarose [21], cellulose [22] or silica [23]. Other approaches use synthetic polymer beads based on a GlycidylMethAcrylate/diVinylBenzene copolymer (poly GMA/DVB) for the selective isolation of phosphopeptides [24]. For example, Aprilita et al. [24] modified Poly(GMA/DVB) with IminoDiacetic Acid (IDA), and loaded Fe^{3+} ions to form an IMAC support material with high recovery rates and high phosphopeptide binding selectivity. Various methods for the immobilization of IDA onto a silica matrix have been reported [25]. Most of them start from 3-glycidoxypropyltrimethoxysilane

* Corresponding author. Tel.: +33 3 20 43 49 77; fax: +33 3 20 33 61 36.
E-mail addresses: Christian.Rolando@univ-lille1.fr, Christian.Rolando@cnrs-dir.fr (C. Rolando).

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(GLYMO)-activated silica by immobilizing GLYMO onto the silica and chemically linking IDA on a GLYMO molecule with an epoxy group. Liu and coworkers have used this technique on glass capillary support [26]; the functionalized open tubular column with Fe^{3+} ions showed promising results on *in vitro* phosphorylated proteins. Similar approaches based on open tubular IMAC-Fe^{3+} columns allowed the identification of one phosphorylated protein expressed in *E. coli* [27]. Another study employing Zr^{4+} or Ti^{4+} ions created by liquid phase deposition on phosphonate-grafted silica nanoparticle-deposited capillaries shown promising results regarding phosphopeptide enrichment [28]. A recent study proposed the same strategy for phosphopeptide enrichment starting by Surface Initiated Atom Transfer Radical Polymerization (SI-ATRP) of glycidyle methacrylate (GMA) followed by opening the epoxide with *N*,*N*,*N*′,*N*″,*N*″-pentamethyldiethylenetriamine, a reaction of the terminal amine with phosphorus oxychloride ($POCl_3$) and the immobilization of zirconium (IV) [29].

Open tubular columns offer a convective mass transfer process with lower back-pressure than bead-packed or monolithic columns and appear to be a good choice to handle complex biological mixtures without prior purification. Capillary columns used for separation are generally prepared using Porous Layer Open Tubular coating based on based on poly(styrene-divinylbenzene), also called the PLOT technique [30]. The poly(styrene-divinylbenzene) phase may be alkylated to introduce a different reactivity [31]. Chloromethylation or the incorporation of a reactive monomer like vinyl benzylchloride or glycidyl methacrylate allows the introduction of a metal chelator like iminodiacetic acid as described previously [24] (for a review see [32]). However, there are many reports of polymer brushes bearing a higher density of iminodiacetic acid groups as chelating agents grafted on surfaces or beads [16,33]. In order to combine both advantages, we developed an in-capillary polymer brush coating based on the approach described by Miller et al. [34] using glycidyl methacrylate and its functionalization using iminodiacetic acid as a chelating agent. In order to obtain high recovery rates and binding selectivity [24,26], we used Fe^{3+} cations to enrich and separate phosphopeptides. For comparison, we present the surface functionalization approach using 3-glycidoxypropyltrimethoxysilane and iminodiacetic acid in the same capillary. Both chemistries are much simpler than zirconium or titanium immobilization by phosphonate residues. The open tubular columns issued from these two functionalization procedures were evaluated using MALDI-TOF analysis on the peptides obtained from the well-described β-casein protein and a α_{S1}, α_{S2} casein mixture. Adapted from the literature [19,35–38], this work includes an optimized enrichment procedure and discussion on the composition of the different solvents used during each enrichment step.

2. Experimental

2.1. Materials

Fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA). Iminodiacetic acid (IDA), acetic acid, iron (III) chloride ($FeCl_3$), 2-bromo-2-methyl-*N*-3-[(trimethoxysilanyl)-propyl]-propionamide, formic acid (FA), acetonitrile (ACN), methanol, acetone, hydrochloric acid, sodium chloride, sodium hydroxide, ammonium hydroxide, phosphoric acid (PA), trifluroacetic acid (TFA), glycidyl methacrylate (GMA), 3-glycidoxypropyltrimethoxysilane (GLYMO), dihydroxybenzioc acid (DHB), α-casein, β-casein and bovine trypsin were purchased from Sigma-Aldrich (L'Isle d'Abeau, France). Deionized water was obtained from Milli-Q system (Millipore, Milford, MA, USA).

2.2. Preparation of an open tubular capillary column

To increase the density of silanol groups serving as anchors for the silanization, the siloxane groups on the inner surface of the raw fused-silica capillaries were hydrolyzed by a pretreatment process. Solutions were passed through a fused silica capillary column (20 μm i.d. × 360 μm o.d.), using a Model PHD 4400 syringe pump (Harvard Apparatus, Les Ulis, France) fitted with an 8 mL stainless steel syringe at the flow rate of 0.5 μL/min. For the pretreatment process, these solutions were injected into the column, one after the other: 0.2 M NaOH for 30 min, deionized water for 15 min, 0.2 M HCl for 30 min, deionized water for 15 min, acetone for 15 min, then the column was left at 80 °C overnight for drying (for a discussion of the etching conditions see [39]). A solution of 2-bromo-2-methyl-*N*-3-[(trimethoxysilanyl)-propyl]-propionamide prepared in acetone, the Atom Transfer Radical Polymerization (ATRP) initiator with a trimethoxysilane anchor, was synthesized using a procedure described in the literature [40]. To attach the initiator to the internal surface of the capillary, a solution containing 3 mg of the initiator in 100 μL of acetone (90 mM initiator) was passed through it for 15 min at 0.3 μL/min, then both of it ends were sealed and the column was kept at room temperature overnight. The column was finally washed extensively with methanol for 2 h and placed overnight in an oven at 80 °C. These two steps are very similar to those we developed previously to prepare monolithic columns in fused-silica capillary, except for the anchoring reagent, which was adapted for ATRP polymerization [41].

2.3. Preparation of open tubular Poly-(GMA) polymer brushes

A solution of glycidyl methacrylate (1.4 mL, 10 mmol) in methanol (1.1 mL), water (0.3 mL) and 2,2′-bipyridine (14.3 mg, 0.092 mmol) was degassed by bubbling dry Ar into it for 15 min. Copper (I) bromide (39 mg, 0.272 mmol) was added to this solution. The ratio of copper (I) bromide to 2,2′-bipyridine is nearly 3, which is ten times higher than is normally used (between 1/2 and 1/3). This inverted ratio combines several advantages: a low concentration of the active complex [Cu(I)Br, $(biPy)_{n=2\text{ or }3}$] as described recently by Tsarevsky et al. [42,43], a high concentration of bromide ions in the methanol/water solution which prevents the dissociation of the ATRP deactivator [Cu(II)Br, $(biPy)_{n=2\text{ or }3}$] as described by the same authors [44], and a reducing medium which favors a mechanism close to the activators regenerated by electron transfer atom transfer radical polymerization (ARGET ATRP) [45]. All three conditions preferentially lead to well-defined polymer brushes. To dissolve all the solids, the mixture was stirred for 5 min while continuing degassing, which led to a dark brown solution. This solution was sonicated for 2 min to ensure complete dissolution. The solution was then withdrawn with a syringe under argon and was passed through the 1 m-long column for 30 min with a 0.5 μL/min flow rate. This reaction is shown in Fig. 1. Then the two ends were sealed with classical 1/16″ (o.d.) UpChurch sleeves, fittings and connections (UpChurch Scientific, Oak Harbor, WA, U.S.A.), and the capillary was kept at room temperature for 24 h. Next, the capillary was rinsed with deionized water for 2 h to remove the unreacted chemicals and placed overnight at 80 °C to dry. Scanning electron microscope views of the uncoated 20 μm internal diameter capillary and the same capillary after surface-initiated living polymerization of glycidyl methacrylate polymer brush are presented in Fig. 2.

2.4. Preparation of the GMA-IDA-Fe^{3+} IMAC polymer brush column

The method used to attach iminodiacetic acid onto the surface of a fused silica capillary is a modified version of the one used by Bakry

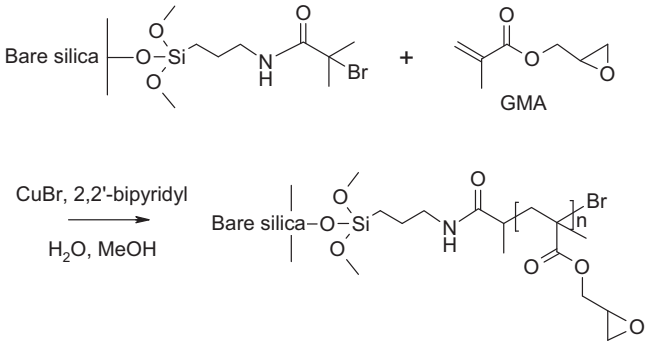

Fig. 1. General scheme for surface-initiated living polymerization of glycidyl methacrylate polymer brush.

et al. to prepare latex-coated capillary IMAC columns [46]. IDA (0.5 g) was dissolved in a 25 mL Na_2CO_3 solution (2 M) containing 250 mg NaCl. The pH was adjusted to 10.5 with NH_4OH. The capillary was loaded with this IDA solution at 40 °C using a 0.5 μL/min flow rate for 2 h and then left at 70 °C overnight. The capillary was then washed with deionized water (0.5 μL/min, 2 h). After the reaction, the IDA was neutralized with 0.1 M sodium hydrogen carbonate solution ($NaHCO_3$) to avoid hydrolysis of the methacrylate backbone (0.5 μL/mn, 2 h). Next a flow of N_2 was directed into the capillary. Then the capillary was loaded with an HCl solution (0.1 M) containing $FeCl_3$ (200 mM) with a 0.5 μL/mn flow rate overnight. This reaction is presented in Fig. 3. The excess of Fe^{3+} was removed by washing with deionized water (0.5 μL/mn, 2 h). The capillary was flushed and conditioned with an acetonitrile 10% v/v solution with 0.1% formic acid. Before use the column was cut in 15 cm pieces and the two ends were discarded.

2.5. Preparation of the GLYMO-IDA IMAC column

Several methods for the immobilization of IDA onto silica matrix have been reported in the literature [25]. Most of them start from GLYMO-activated silica by immobilizing the GLYMO onto the silica and then chemically linking the IDA on the GLYMO molecule with an epoxy group following a reaction similar to the

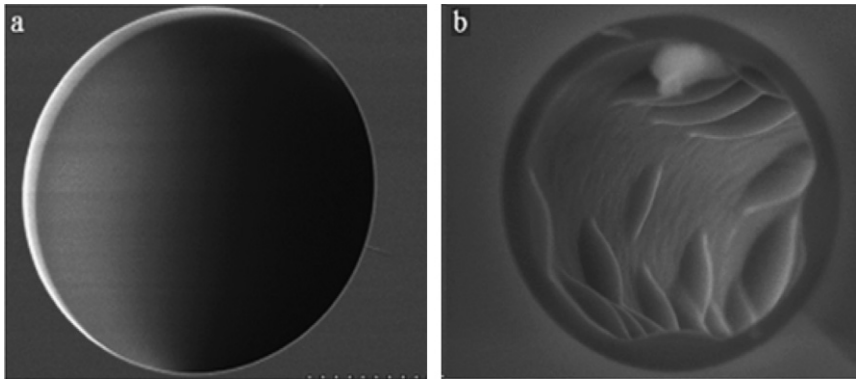

Fig. 2. Scanning electron microscope view (left panel) of uncoated 20 μm internal diameter capillary and (right panel) the same capillary after surface-initiated living polymerization of glycidyl methacrylate polymer brush.

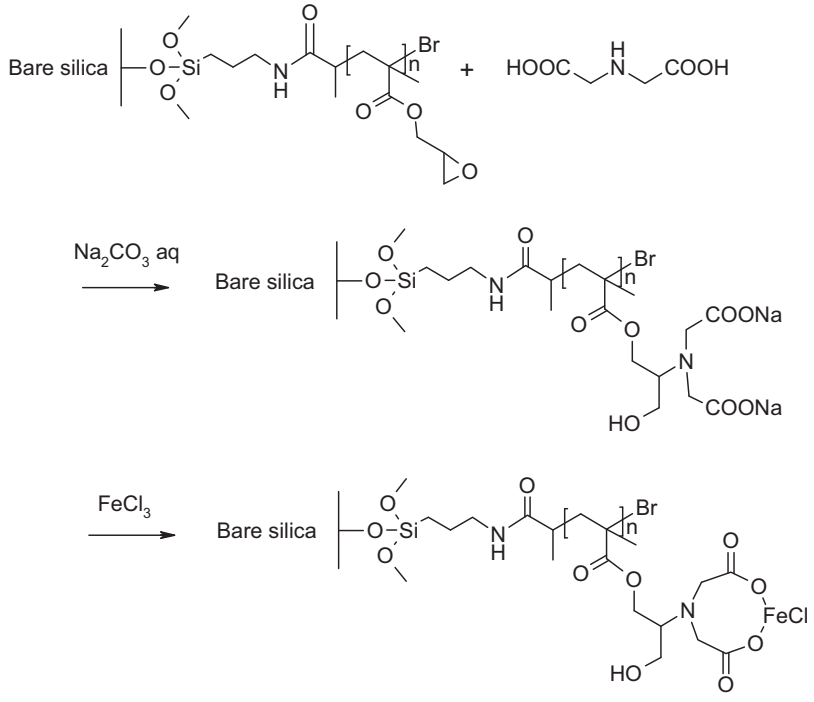

Fig. 3. Functionalization of the surface-initiated living polymerization of glycidyl methacrylate polymer brush with iminodiacetic acid and immobilization of Fe^{3+}.

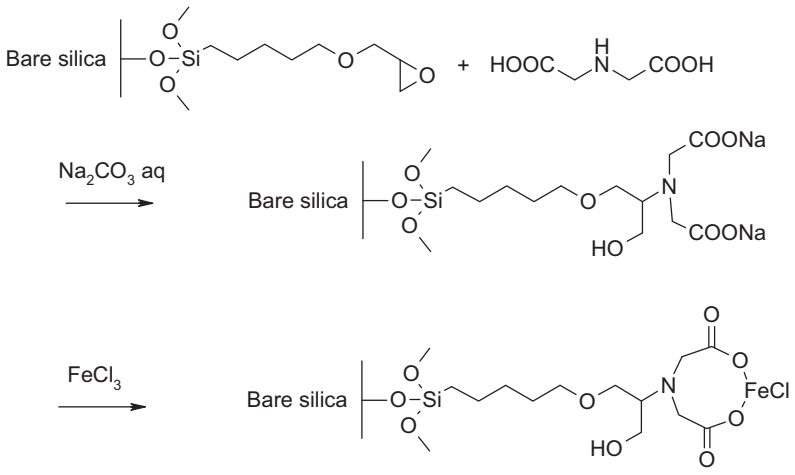

Fig. 4. Functionalization of the surface-initiated living polymerization of glycidoxypropyltrimethoxysilane polymer brush with iminodiacetic acid and immobilization of Fe^{3+}.

one described for GMA-IMAC. It is also possible to react GLYMO (3-glycidoxypropyltrimethoxysilane) with iminodiacetic acid in a basic medium to obtain the intermediate, which is not isolated but anchored directly to the activated silica surface under acidic conditions [26]. We chose to use the two steps approach which allowed us to use the best solvent and pH condition for each step. After pre-treatment of the capillary as described previously, a solution of GLYMO (3-glycidoxypropyltrimethoxysilane) in anhydrous THF (30%, v/v) was passed through a 1 m capillary for 5 h at room temperature with a 0.5 μL/mn flow rate. The reaction is presented in Fig. 4. Then the column was washed with THF overnight at the same flow rate (0.5 μL/mn) and dried with N_2. The other steps (functionalization with IDA, Fe^{3+} loading, washing) were similar to those for preparing an IMAC-based GMA column.

2.6. Protein digestion

One milligram of α- or β-casein was dissolved in 1 mL of a 100 mM ammonium hydrogen carbonate buffer (pH 8.0), then trypsin sequencing grade (Promega, Madison, Wisconsin, USA) was added at a protein/enzyme ratio of 50:1 (w/w), and the solution was incubated at 37 °C for 3 h. The digestion was quenched by freezing the solution to −18 °C.

2.7. Phosphopeptide enrichment

The open tubular IMAC was first washed with ACN with 0.1% FA (0.5 μL/min flow rate), and was equilibrated in the same manner with 50 μL H_2O. First, 15 μL of the protein digest solution in deionized water (pH 6.0) was loaded into the tip (flow rate, 0.5 μL/min). All non-phosphorylated peptides were washed with 15 μL of 10% ACN with 0.1% FA. One additional washing step was applied using deionized water. Phosphopeptides were eluted with the same flow rates in a NH_4OH solution (0.1 M, pH 10.5) and a 50% ACN solution acidified with 0.1% H_3PO_4 (adjusted to pH 8.0 with NH_4OH). Each experiment was repeated 3 times.

2.8. MALDI-TOF MS analysis and phosphopeptide identification

The eluted fractions were analyzed on a MALDI-TOF mass spectrometer (Voyager DE STR, ABSciex) equipped with a nitrogen laser (λ = 337 nm). One microliter of the eluted fraction was spotted on a stainless steel target. A volume of 1 μL of a saturated dihydroxybenzoic acid (DHB) matrix (50% ACN solution acidified with 0.1% TFA) was added on the eluted fraction spot on the MALDI target and was allowed to dry at room temperature. The analyses were carried out in positive reflectron mode with a *m*/*z* 700–4000 mass range of. Each mass spectrum was summed over 25 laser shots. The peptide monoisotopic masses were extracted from each MS and MS/MS spectrum using the Analyst software version 1.5 (AB Sciex). Calibrated peak list files (internal calibration on tryspin autolysis fragments or casein peptides ions) were submitted to local MASCOT software containing the SwissProt database (release 56.8 of 10 February 2009: 410,518 sequences) for protein identification. The peptide mass tolerance was set at 50 ppm. A tolerance of 2 missed cleavages was allowed. The interrogations were performed on the databases related to the other mammalia taxonomy. The parameters included ST-phosphorylation and Y-phosphorylation as variable modifications.

3. Results and discussion

3.1. MALDI-TOF MS mapping of β-casein phosphopeptides enriched with GMA-IDA-Fe^{3+} IMAC brush polymer column

Bovine α and β-caseins were chosen as model compounds because of their commercial availability and their well-known phosphorylation sites [47]. In particular, β-casein is commonly used to optimize phosphopeptide enrichment, as it contains two phosphopeptides (see P02666 Uniprot details, http://www.uniprot.org/uniprot/P02666): the monophosphorylated peptide FQSEEQQQTEDELQDK (position 48–63) at *m*/*z* 2061.82, which contains one phosphoserine in position 50, and the tetraphosphorylated peptide RELEELNVPGEIVESLSSSEESITR (position 16–40) at *m*/*z* 3122.26, which contains four phosphoserines in positions 30, 32, 33 and 34 (Table 1). Both sample preparation and sample analysis were optimized in terms of signal-to-noise ratio. In particular, a higher phosphopeptide production yield was observed by decreasing the digestion time from 12 h to 3 h. Linear MALDI-TOF analysis showed the complete disappearance of the intact protein and a maximum yield in peptides at this digestion duration (data not shown). It can be noted that the presence of phosphate groups hinders the MS analysis of phosphopeptides in positive mode because it reduces the ionization efficiency [48,49]. This reduced ionization can be solved by incorporating a small percentage of strong acid like phosphoric acid or TFA in the DHB matrix, which generally gives less suppression [35]. In the present study, we chose a DHB matrix with 0.1% trifluoro acetic acid (TFA) and not phosphoric acid as in the original paper in order to avoid any uncertainty on the source of the phosphate groups. Fig. 5

Table 1
Peptides and phosphopeptides identified in this study from β-casein using MALDI-TOF MS. The underlined amino acids are described as phosphorylated according to the SwissProt database annotations (Accession number P02666 for β-casein). AA means Amino Acids. Column A corresponds to β-casein peptides analysis without any enrichment (Fig. 5). Columns B to F corresponds to experiments on GMA-IDA-Fe^{3+} polymer brush column. In details, col. B corresponds to loading effluent fraction, β-casein was injected in 5% ACN (Fig. 6 A). Col. C corresponds to loading effluent fraction, β-casein was injected in deionized water (Fig. 6 B). Col. D corresponds to washing fraction with 10% ACN, 0.1% FA (Suppl. data Fig.1). Col. E corresponds to eluted fraction with NH_4OH (Fig. 6C). Column F corresponds to eluted fraction with 1% H_3PO_4 + 50% ACN (v/v) (Fig. 6 D). Columns G and H correspond to experiments on GLYMO-IDA-Fe^{3+} column. In details, col. G and H correspond to the eluted fraction using 1% H_3PO_4 + 50% ACN respectively during 5 min and 30 min elutions.

Mass MH+[a]	AA positions	Number phosphate groups	Sequences	GRAVY	A	B	C	D	E	F	G	H
3122.266	16–40	4	RELEELNVPGEIVESLSSSEESITR	−0.596[b]	×				×	×		×
2911.496	159–184	0	MHQPHQPLPPTVMFPPQSVLSLSQSK	−0.438	[c]	[c]	[c]	[c]				
2207.204	103–122	0	LQPEVMGVSKVKEAMAPKHK	−0.495	×	×	×	×				
2186.168	199–217	0	DMPIQAFLLYQEPVLGPVR	0.305	×	×	×	×				
2909.585	199–224	0	DMPIQAFLLYQEPVLGPVRGPFPIIV	0.269	[c]	×	×					
2061.828	48–63	1	FQSEEQQQTEDELQDK	−2.331[b]	×	×			×	×	×	×
1383.799	206–217	0	LLYQEPVLGPVR	0.283	×	×	×	×				
1138.640	113–122	0	VKEAMAPKHK	−1.030	×	×	×	×				
1013.524	121–128	0	HKEMPFPK	−1.625	×	×	×	×				
830.452	192–198	0	AVPYPQR	−0.929	×	×	×	×				
780.498	185–191	0	VLPVPQK	0.229	×	×	×	×				
748.370	123–128	0	EMPFPK	−0.983	×	×	×	×				
742.450	218–224	0	GPFPIIV	1.771	×	×	×	×				

[a] Calculated monoisotopic mass.
[b] GRAVY index of the non phosphorylated peptide.
[c] Peptides detected with Signal to Noise ratio (defined as monoisotopic peak intensity/noise intensity ratio) below 5.5.

shows the MALDI-TOF mass spectrum of 1 pmol of β-casein digest with these two improvements, and prior enrichment with an IMAC capillary column. Peptides with signal to noise ratio over 5.5 (Fig. 5, Table 1) represent approximately 60% of sequence coverage, including peptides and phosphopeptides. To improve phosphopeptide detection, their immobilization on the GMA-IDA-Fe^{3+} IMAC polymer brush coated column was optimized. During this enrichment step, non-phosphorylated peptides had to be eluted whereas phosphopeptides had to stay trapped on the column. For a given column, two factors are governing the hanging of the phosphopeptides: the pH of the eluting solution and the content of the organic solvent in the eluent [50]. In order to avoid non-specific binding of the hydrophobic peptides, 5% acetonitrile was included in the loading solution onto the IMAC microcolumn. But in this condition the monophosphorylated peptide 48–63 (m/z 2061.828) was not retained on the column as it is shown in Fig. 6A and Table 1 pointing its presence in the loading effluent fraction (see Table 1, column B). As predicted, this peptide was not detected in the elution fraction (data not shown); consequently, the addition of acetonitrile in the loading solution was abandoned in further experiments. When 1 pmol of β-casein digest in pure deionized water (pH 6.0) was loaded onto the IMAC microcolumn, no phosphorylated peptides were detected in the loading effluent fraction (Fig. 6B and Table 1, Column C). The absence of the phosphorylated peptides in this loading fraction implies that they were trapped on the column. The loading was followed by a first wash to remove the unbound material. Two conditions were tested; slightly acidic conditions (pure deionized water, pH 6.0) then acidic conditions (formic acid 0.1%, pH 3.0). With a 0.1% formic acid water solution, multiple peptides were not eluted. In order to completely wash the non-phosphorylated peptides, increasing amounts of acetonitrile were incorporated by 5% steps in the previous solution until the casein phosphopeptides were released from the IMAC capillary. The optimal washing condition consists in 10% acetonitrile/water (v/v), 0.1% formic acid solution (see Fig. S1 in supplementary data, Table 1). At this stage a third washing step with pure water was introduced to remove traces of acetonitrile. The phosphopeptides were eluted first using a strongly basic conditions (NH_4OH 0.1 M, pH 10.5) [37,38]. Other elution conditions were also evaluated like slightly basic conditions (0.1% H_3PO_4, 50% acetonitrile (v/v)

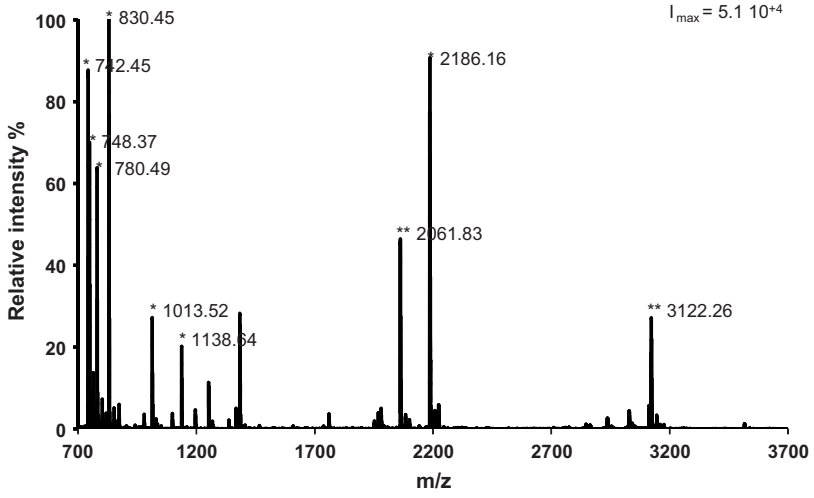

Fig. 5. MALDI-TOF mass spectrum of β-casein peptides (1 pmol). Peptides labeled with one asterisk are identified as β-casein peptides, peptides labeled with two asterisks are identified as β-casein phosphopeptides. The generated peaks are detailed in Table 1.

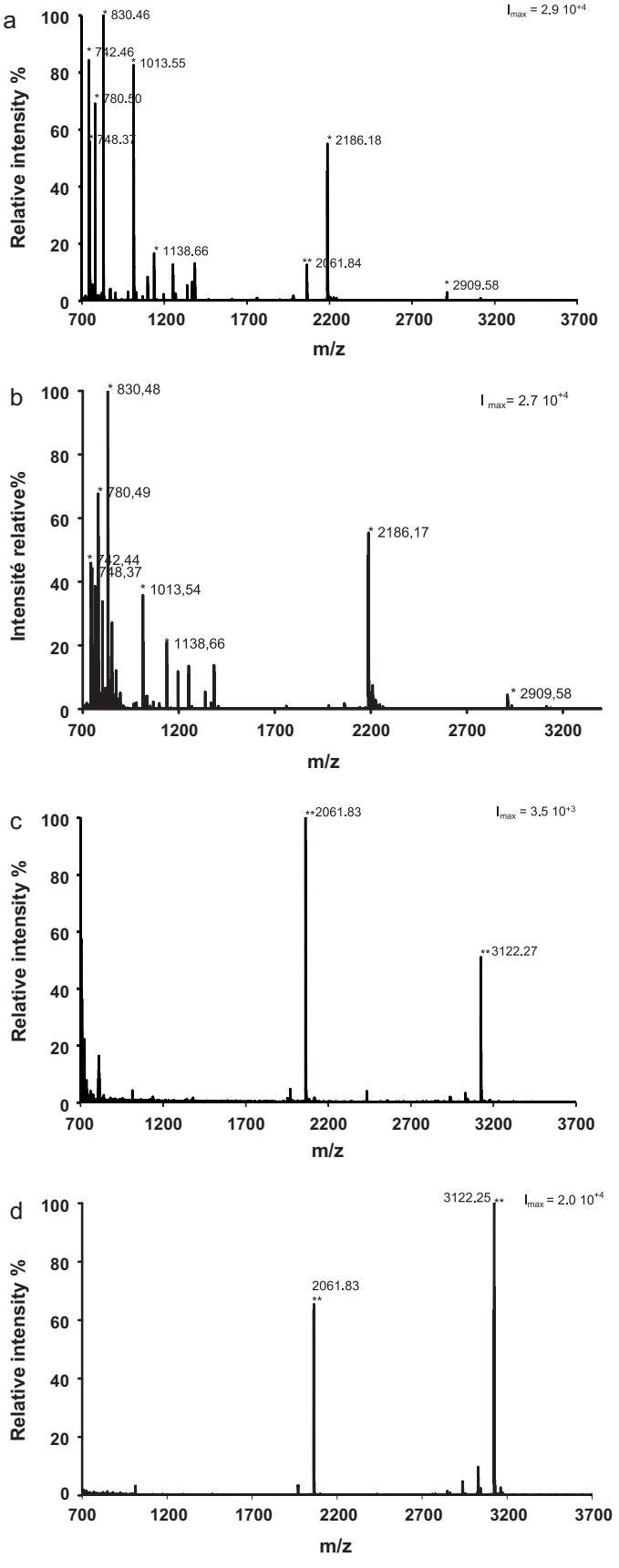

Fig. 6. MALDI-TOF mass spectra related to β-casein phosphopeptides enrichment on the 15 cm open-tubular capillary coated with a GMA-IDA-Fe^{3+} polymer brush (A) Loading effluent fraction resulting from injection of 1 pmol of β-casein peptides in 5% acetonitrile. (B) Loading effluent fraction resulting from injection of1 pmol of β-casein peptides in pure deionized water. (C) Eluted fraction using NH_4OH. (D) Eluted fraction using 1% H_3PO_4 + 50% ACN (v/v). Peptides labeled with one asterisk are identified as β-casein peptides, peptides labeled with two asterisks are identified as β-casein phosphopeptides. The generated peaks are detailed in Table 1.

adjusted to pH 8.0 with NH_4OH) [35,37,51,52] using excess phosphate and acetonitrile hydrophobicity to release the peptides. Both strongly and slightly basic conditions (Fig. 6C and D respectively) led to the elution of the mono and tetra phosphorylated peptides. In the following experiments we decided to use the slightly basic conditions (pH 8.0) which gave better intensities. The elution conditions proposed here are compatible with MALDI-TOF MS analysis without any desalting step before spotting. To complete this study, the phosphorylated peptides trapped using a 5% acetonitrile-based loading solution were eluted in basic conditions (NH_4OH 0.1 M, pH 10.5). Only the tetraphosphorylated peptide was detected in the MALDI-TOF mass spectrum (data not shown). These results show that multi-phosphorylated peptides bind more strongly to the IMAC than do monophosphorylated.

3.2. MALDI-TOF MS mapping of β-casein phosphopeptides enriched with a GLYMO-IMAC column

Using GLYMO is by far a simpler way to prepare an IMAC capillary column based on self-assembled monolayer chemistry. Unlike polymer brush chemistry, the functionalization of a capillary for GLYMO chemistry does not require excluding oxygen and it is straightforward starting from commercially available reagents. After activating the internal surface by acidic etching, the capillary is coated with GLYMO (3-glycidoxypropyltrimethoxysilane) and functionalized by IDA; then Fe^{3+} is loaded onto the capillary. One meter length capillaries are prepared in two working days and they are cut in ca 15 cm for analytical use. The spectra of the loading and washing fractions are very similar to those obtained for a polymer brush column and thus are not shown here. Using slightly acidic elution solution conditions (0.1% H_3PO_4, 50% acetonitrile (v/v) adjusted to pH 8.0 with NH_4OH), only monophosphorylated peptides were detected (Fig. 7A). However, after 30 min of elution, multi-phosphorylated peptides were observed (Fig. 7B). So unlike a GMA-IDA-Fe^{3+} IMAC polymer brush column which elutes mono and tetraphosphorylated peptides together, the GLYMO-IMAC polymer brush provides an opportunity to separate the peptides with different degrees of phosphorylation by applying different elution times.

3.3. MALDI-TOF MS mapping of α-casein phosphopeptides enriched with GMA-IDA-Fe^{3+} IMAC and GLYMO-IMAC columns

The optimized methodologies were applied to the study of another phosphoprotein, α-casein (a mixture of α_{S1}, α_{S2} caseins), for which trypsin digest contains a high number of phosphopeptides. The MALDI-TOF mass spectrum of 1 pmol of α_{S1}, α_{S2} casein digest prior any IMAC enrichment shows only three phosphorylated peptides, peptides 52–57, 119–134 and 121–134, which are monophosphorylated (see Supplementary data Fig. S2 and Table 2). Peptides 119–134 and 121–134 exhibit the same phosphorylated sites. The MALDI-TOF mass spectrum of the α-casein enriched in phosphopeptides with the GMA-IDA-Fe^{3+} IMAC polymer brush column using the slightly basic elution conditions shows seven phosphorylated peptides, whereas peaks related to non-phosphorylated peptides have a much lower intensity (see Supplementary data Fig. S3 and Table 2). Besides the monophosphophorylated peptides 119–134 (S1), 121–134 (S1) and 153–164 (S2), the diphosphorylated peptide 58–73, the tetraphosphorylated

Table 2
Peptides and phosphopeptides identified in this study from α_{S1}-casein and α_{S2}-casein using MALDI-TOF MS. The underlined amino acids are described as phosphorylated according to the SwissProt database annotations (Accession numbers P02662 for α_{S1}-casein and P02663 for α_{S2}-casein). AA means Amino Acids. Column A corresponds to α_{S1}-casein and α_{S2}-casein peptides analysis without any enrichment (Suppl. data Fig.2). Columns B and C correspond to the eluted fraction using 1% H_3PO_4 + 50% ACN respectively from GMA-IDA-Fe^{3+} polymer brush column (Suppl. data Fig.3) and GLYMO-IDA-Fe^{3+} column (Suppl. data Fig.4).

Mass MH+[a]	AA positions	Number phosphate groups	Sequences	GRAVY	A	B	C
α_{S1}-Casein peptides and phosphopeptides identified in this study							
1759.945	23–37	0	HQGLPQEVLNENLLR	−0.753	×		
1384.730	38–49	0	FFVAPFPEVFGK	0.867	×		
1927.691	58–73	2	DIGSESTEDQAMEDIK	−0.967[b]		×	×
2720.912	74–94	5	QMEAESISSSEEIVPNSVEQK	−0.781[b]		×	×
1337.681	95–105	0	HIQKEDVPSER	−1.755	×	×	×
1267.705	106–115	0	YLGYLEQLLR	0.070	×		
1951.952	119–134	1	YKVPQLEIVPNSAEER	−1.038[b]	×	×	×
1660.794	121–134	1	VPQLEIVPNSAEER	−0.536[b]	×	×	×
3207.593	140–166	0	EGIHAQQKEPMIGVNQELAYFYPELFR	−0.530	×		
2316.137	148–166	0	EPMIGVNQELAYFYPELFR	−0.137	×		
α_{S2}-Casein peptides and phosphopeptides identified in this study							
3132.195	16–39	4	KNTMEHVSSSEESIISQETYKQEK	−1.438[b]		×	×
769.349	52–57	1	VNELSK	−0.617[b]	×		
3008.029	61–85	4	NANEEEYSIGSSSEESAEVATEEVK	−1.080[b]		×	×
1367.695	96–106	0	ALNEINQFYQK	−0.891	×		
2709.407	107–128	0	FPQYLQYLYQGPIVLNPWDQVK	−0.355	×		
1466.612	153–164	1	TVDMESTEVFTK	−0.350[b]		[c]	×
1594.702	152–164	1	KTVDMESTEVFTK	−0.355[b]		[c]	
979.561	189–196	0	FALPQYLK	0.237	×		
1098.613	204–212	0	AMKPWIQPK	−0.800	×		
746.455	215–220	0	LNFLK	0.600	×		

[a] Calculated monoisotopic mass.
[b] GRAVY index of the non phosphorylated peptide.
[c] Peptides detected with Signal to Noise ratio (defined as monoisotopic peak intensity/noise intensity ratio) below 5.5.

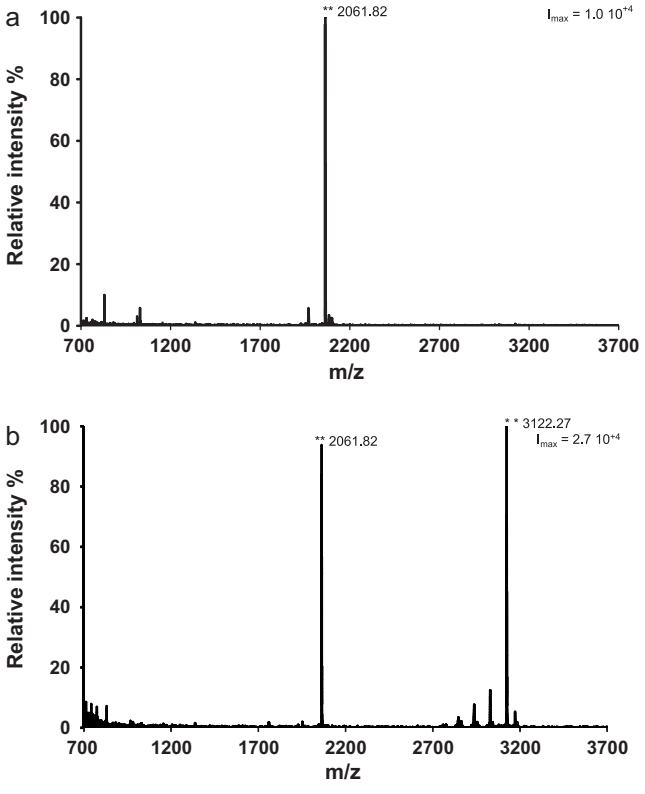

Fig. 7. MALDI-TOF mass spectra related to the enrichment on the 15 cm open-tubular capillary GLYMO-IDA-Fe^{3+}. (A) Eluted fraction using 1% H_3PO_4 + 50% ACN after 5 min of elution. (B) Eluted fraction using 1% H_3PO_4 + 50% ACN after 30 min of elution. Peptides labeled with two asterisks are identified as β-casein phosphopeptides. The generated peaks are detailed in Table 1.

peptides 16–39 and 61–85 and even, the pentaphosphorylated peptide 74–94 were observed as described in Table 2. Only one non-phosphorylated peptide was clearly detected on the MALDI-TOF mass spectrum. However, this peptide, whose sequence is HIQKEDVPSER, is very hydrophilic according to its GRAVY index (−1.755). HIQKEDVPSER also contains 2 glutamic acids and 1 aspartic acid, residues that are known to interact strongly with the IMAC phase [53]. For this highly phosphorylated sample, the GLYMO-IMAC column produced very close results (see Supplementary data Fig. S4 and Table 2). Due to its easier preparation, the GLYMO-IMAC column appears as the most adapted column for phosphopeptide analysis

4. Conclusion

In this work, relating to phosphopeptide enrichment using IMAC methodology, we compared two different column-coating methodologies, polymer brush coating and surface functionalization. One column was based on a poly-(glycidyl methacrylate) brush functionalized by iminodiacetic acid as a chelating agent. The second column used the functionalization approach using 3-glycidoxypropyltrimethoxysilane and iminodiacetic acid. Both columns used Fe^{3+} cations to enrich and separate phosphopetides. The open tubular columns issued from the two functionalization procedures were evaluated on an α_{S1}, α_{S2} mixture and β caseins phosphopeptide enrichments. The enrichment protocol was optimized using information gained from literature. The best results were obtained using the following protocol: (i) sample loading in slightly acidic solution made with pure deionized water (pH 6.0), (ii) washing step with 10% acetonitrile, 0.1% formic acid and (iii) elution step with 50% acetonitrile, 0.1% phosphoric acid pH 8.0. The results obtained with GMA-IDA-Fe^{3+} IMAC and GLYMO-IMAC open tubular columns show a high number of phosphorylated peptides, i.e. the 2 described phosphopeptides of the β-casein were detected, 7 phosphopeptides of α_{S1} and α_{S2} caseins were also

identified, including a pentaphosphorylated peptide. The GLYMO-IMAC polymer brush allows separating peptides with different phosphorylation states using different elution times.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.08.005.

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