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Newly Developed Poly(Allyl Glycidyl Ether/Divinyl Benzene) Polymer for Phosphopeptides Enrichment and Desalting of Biofluids

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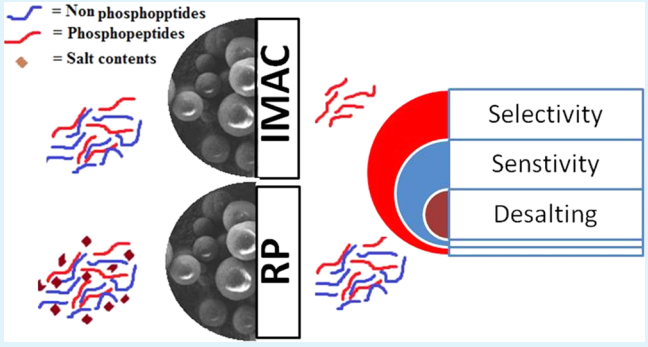
Supporting Information

ABSTRACT: The polymeric materials have contributed significantly in the area of bioanalytical science. The functionalization of polymeric backbone after its development brings unique selectivity towards the target biomolecules. In present work, the functionalities of choice have been introduced through the ring-opening of allyl glycidyl ether. The utility of polymer is widened through derivatizations to immobilized metal ion affinity chromatographic (IMAC) material for the phosphopeptides enrichment and Reversed Phase (C-18) for the desalting prior to MALDI-MS analysis. The polymer-IMAC in addition to Fe^{3+} is also immobilized with lanthanide ions like La^{3+}, Eu^{3+}, and Er^{3+}. The amount of Fe^{3+} immobilized is determined as 0.7928 mg/g. Spherical morphology with narrow particle size dispersion is revealed by scanning electron microscopy (SEM). The surface area, pore volume and size distribution is determined by nitrogen adsorption porosimetery. The elemental composition and purity level is confirmed by energy dispersive X-ray spectroscopy (EDX) data. The derivatization to IMAC and RP is evaluated by Fourier transform infrared (FT-IR) spectroscopy. The polymer enables the efficient phosphopeptide enrichment to equal degree from casein variants, non-fat milk, egg yolk, human serum, and HeLa cell extract. The identification of phosphorylation sites can lead to the phosphorylation pathways to understand the post-translational modifications. The identification with their sequence coverage is made using Mascot and Phosphosite Plus. It is sensitive to enrich the phosphopeptides down to 2 femtomoles with very high selectivity of 1:2000 with BSA background. These attributes are linked to the higher surface area (173.1554 m^2/g) of the designed polymer. The non-specific bindings, particularly the Fe^{3+} linked acidic residues are also avoided. Four characteristic phosphopeptides (fibrinopeptide A and their hydrolytic products) from fibrinogen α-chain are identified from the human serum after the enrichment, which have link to the hepatocellular carcinoma (HCC). The proportions of fibrinogen and their phosphorylation products enriched by poly(AGE/DVB)-IMAC open new horizons in the biomarker discovery.



KEYWORDS: *polymerization, IMAC (immobilized metal ion affinity chromatography), reversed phase, phosphopeptides, tryptic digests, desalting, MALDI-MS, selectivity*

INTRODUCTION

The extensive utility of polymeric beds in tips and columns has been a modern trend in chromatographic separations and subsequently the proteomics. The varying nature of functional groups on monomers leads to the diverse polymeric backbones. The polymers are often embedded with the range of functionalized materials for the targeted bindings, however, a significant percentage of functionalities get buried and become unavailable.[1] The selectivity and other desired properties are also introduced through new monomers; however, this approach is constrained because of the non-availability of relevant functional monomers. The problems are overcome by synthesizing a stable polymeric backbone and modify it through chemical derivatizations like on the epoxy endings.[2,3] The coupling of epoxy group to the amines, thiols and some other nucleophiles via epoxy ring-opening make it selective for capturing specific molecules. Additionally, the abundance of epoxy group on the surface causes the multipoint attachment of the bio-macromolecules.[4,5] Furthermore, the preparation of functional material by the copolymerization of different monomers with required functionalities also reduce the number of steps.

Thus functionalization of the polymer helps in selective binding of the molecules of interest such as phosphopeptides

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and phosphoproteins. The phosphorylated peptides and proteins have been of great significance as they are ready to lend a hand in understanding the disease status and act as biomarkers in biomedical research.[6,7] The mass spectrometric detection of the phosphorylated peptides has always been tricky because of the ionization suppression caused by its low abundance.[8,9] To overcome this problem, the enrichment of phosphopeptides prior to MS analysis has become a necessity to identify the peptides involved in different diagnostic works.[10−12] Among various techniques, immobilized metal ion affinity chromatography (IMAC) with different metal ions such as Fe^{3+}, Ga^{3+}, Zr^{4+}, and Ti^{4+} is predominant for the enrichment before analysis by MALDI-MS.[13−15] Immobilized metal ions capture the phosphopeptides through negatively charged phosphate groups of peptides.[16,17]

The digestion of protein to peptides is required for MS based peptidome methods.[18] During digestion addition of salts and presence of other contaminations makes its analysis tedious and results in the adduct formation. Because of this, it is cumbersome to assign the sequence and localize the phosphorylation sites when they get fragmented in mass spectrometer. To resolve the interferences, desalting of the sample is necessary to enhance the detection efficiency. Among different materials used for desalting are graphite,[19] polypropylene,[20] and more recently the diamond reversed phase.[21]

Different allyl monomers that have hydroxyl, epoxy, and ethylenic unsaturated groups as a comonomer are getting popular in the functionalized phases.[22−24] In present work, a new polymer of allyl glycidal ether is prepared by using the divinyl bezene as a cross-linker. The polymeric material is modified to IMAC and reversed phase by the epoxy ring-opening of allyl glycidal ether. Functionalized material has been used for the phosphopeptides enrichment from complex samples. With good selectivity and sensitivity because of the high surface area, poly(AGE/DVB)-IMAC is a useful addition to the polymeric IMAC materials. Knowing the importance of desalting, poly(AGE/DVB)-RP is synthesized and applied to the tryptic digest of standard protein, as well as the complex real samples.

EXPERIMENTAL SECTION

Chemicals and Instrumentation. The required chemicals and instruments are given in the Supporting information.

Synthesis of Polymer. Thermal polymerization of allyl glycidal ether (AGE) with divinyl benzene (DVB) was carried out by following the literature with minor modification.[25] A mixture containing 62.5 mg of initiator, 120 mL of ACN, and 2 mL of DVB was stirred at 60 °C for 4 h, with continuous nitrogen purging. After 4 h, 4.5 mL of AGE along with 100 mg of AIBN was added and temperature was raised to 70 °C for 16 h. The mixture was cooled down to room temperature and the polymeric product was collected through filtration after washing with 60 mL of each ACN and methanol.

Derivatization to IMAC. The iminodiacetic acid (IDA) solution was prepared by dissolving 10 g of IDA in Na_2CO_3 solution (pH 10). The prepared polymer was suspended in this solution with addition of 2−3% (w/v) of NaCl and 20 mL of ethanol. The suspension was stirred for 4 h at 75 °C. The product was finally collected by vacuum filtration and washed with deionized water. The Fe^{3+} ions were immobilized by incubating the polymeric beads with 0.5 M $FeCl_3$ solution and washed with deionized water to remove the excess metal ions.

Determination of Fe^{3+} Content of the IMAC Material. The metal uptake by poly(AGE/DVB)-IMAC was determined by shaking 0.25 g of the polymeric material with 20 mL of 25 ppm solution of $FeCl_3$ for 2 h. The supernatant was collected and the final concentration was determined using atomic absorption spectrometer. A calibration curve was plotted by using standard solutions of $FeCl_3$ of varying concentrations.

Derivatization to Reversed Phase. The synthesized polymer was treated with 5 g of octadecylamine in acetonitrile at 80 °C for 6 h. The final product was washed with acetonitrile, dried and stored in vacuum desiccator.

Protein Digestion. Phosphoproteins (α- and β-casein), lyophilized egg yolk, non-fat milk, and human serum were digested using trypsin. The detailed digestion protocol is given in Supporting Information.

Phosphopeptides Enrichment by Poly(AGE/DVB)-IMAC. The surface functionalities on poly(AGE/DVB)-IMAC material were activated by 200 μL of 80% ACN in 0.1% TFA for 10 min. Tryptic protein digest (50 μL, diluted up to 200 μL with 0.1% TFA) was incubated with the activated IMAC material for 30 min. The loaded polymer-IMAC was then washed with 80% acetonitrile in 0.1% TFA followed by deionized water, to wash out the non-phosphopeptides. The bound phosphopeptides were eluted by 20 μL NH_4OH (pH 11.5). Eluted fraction was added to 2 μL of the matrix solution and spotted onto the MALDI target plate. The spots were analyzed by Bruker Autoflex II MALDI-TOF/TOF-MS.

Desalting: Comparison to Commercial Aspire RP30 Tip. Poly(AGE/DVB)-RP was used as a desalting material for sample preparation in comparison to the Aspire RP30 desalting tip. The choice of Aspire RP30 for the comparison was made because of the packed polymeric bed. Initially poly(AGE/DVB)-RP was activated by 10 μL of ACN, followed by the equilibration with 10 μL of 5% ACN containing 0.1% TFA (a total of 2 mL solution was prepared by adding 100 μL of ACN in 1900 μL of 0.1% TFA). One hundred microliters of tryptic peptide digest was incubated with the polymeric RP. Unbound peptides were removed by washing with 10 μL of 5% ACN containing 0.1% TFA. The bound peptides were eluted with 10 μL of 50% ACN containing 0.1% TFA. The provided protocol was followed in case of commercial tips.

MALDI-MS Analysis. Peptides bound to the material, were analysed by MALDI-MS (Autoflex II MALDI-TOF-TOF, Bruker Daltonics, Bremen, Germany). All samples were measured in reflector mode. Data was collected by averaging 300 laser shots over the mass regions from 1 to 4 kDa. The peaks m/z were identified by using the biotool (Flex Analysis, version 3.0 (Bruker, Bremen, Germany), and sequence coverage of phosphopeptides was obtained from Microflex. MALDI-TOF MS and all further data processing were carried out by using Flex post analysis software.

RESULTS AND DISCUSSION

Characterization. Surface morphology of poly(AGE/DVB) phase is visualized by scanning electron microscopy (SEM) whereas elemental composition is found out by energy dispersive X-ray spectroscopy (EDX). The polymer beads are small spheres with narrow size distribution (Figure 1A). The elemental composition shows no impurities with carbon content 85.60% and oxygen 14.40% (Figure 1B). The surface area, surface volume, and pore size of poly(AGE/DVB) are found out by nitrogen adsorption porosimetry. The BET surface area comes out 173.1554 m^2/g, t-plot micropore volume 4.1×10^{-2} cm^3/g and adsorption average pore width (4 V/A by BET) as 21.1416 Å (Table S1). The nitrogen adsorption and desorption isotherms are given in Supporting Information Figure S1. The designing methodology and the selection of monomers have produced almost double surface area (173.1554 m^2/g) of poly(AGE/DVB) in comparison to the earlier reported polymeric material, that is, poly(GPE/DVB).

FT-IR analysis illustrates the polymerization by showing the functional groups and shifting patterns relevant to the derivatizations. The spectrum of the polymeric backbone, that

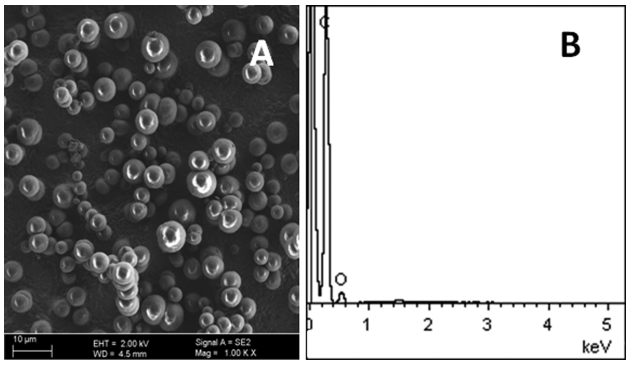

Figure 1. (A) SEM image of poly(AGE/DVB) and (B) EDX spectrum of poly(AGE/DVB).

is, poly(AGE/DVB) is compared with two derivatized forms, poly(AGE/DVB)-IMAC and poly(AGE/DVB)-RP (Figure S2). Attachment of iminodiacetic acid (IDA) to the polymer is confirmed by the band at 1120 cm^{-1} for C−OH group, which appears because of the opening of epoxy functionality of poly(AGE/DVB) on derivatization. The band at 1714 cm^{-1} indicates C═O stretch of carboxylic group of IDA. The bands at 3098 and 1433 cm^{-1} are for the O−H stretch and bend respectively. The attachment of octadecylamine (ODA) is evaluated by the band around 1465, 2850, and 2919 cm^{-1} for the long C-18 chain. C−H stretch at 1400 cm^{-1} and C−H bend at 2833 cm^{-1} is due to the C−H groups of octadecylamine. The rest of peak pattern is same as that for the precursor polymer.

Metal Content of Poly(AGE/DVB)-IMAC. The amount of the metal uptake, q_e (mg/g), is calculated by using the equation

$$q_e = \frac{(C_o - C_f)V}{W}$$

where C_o is the initial concentration of metal in solution, before the sorption (mg/L), C_f is the final concentration of metal in solution, after the sorption (mg/L), V is the volume of solution (L), and w is the amount of solid material (g). The final concentration of solution after metal loading is 15.09 ppm calculated from calibration curve. The amount of Fe^{3+} loaded on poly(AGE/DVB)-IMAC is calculated as 0.7928 mg/g.

Material Fabrication. The chemical derivatization of poly(AGE/DVB) is accomplished by the epoxy ring-opening. Iminodiacetic acid (IDA) is chosen as the chelating ligand because its carboxylate groups can coordinate to the loaded metal ions. Enrichment of phosphopeptides involves the interaction of phosphate groups to the immobilized metal ions. Different transition metals and lanthanides ions are acceptors of oxygen containing anions, such as phosphates. A well designed polymeric base material with immobilized metal ions can thus be a good enrichment solution to phosphopeptides from complex samples.

Phosphopeptides Enrichment from Tryptic Casein Digest. The feasibility of functionalized poly(AGE/DVB)-IMAC-Fe^{3+} to capture phosphopeptides is investigated using β-casein tryptic digest. Figure 2a represents the MALDI-MS spectrum of the raw digest without enrichment. Very few phosphopeptides are recorded and rests are suppressed because of the abundance of non-phosphopeptides. Figure 2b shows the enrichment of phosphopeptides by polymer-IMAC-Fe^{3+}, where different phosphopeptides along with their dephosphorylated segments are identified. The peaks are labeled with their protonated masses and the dephosphorylated segments. They

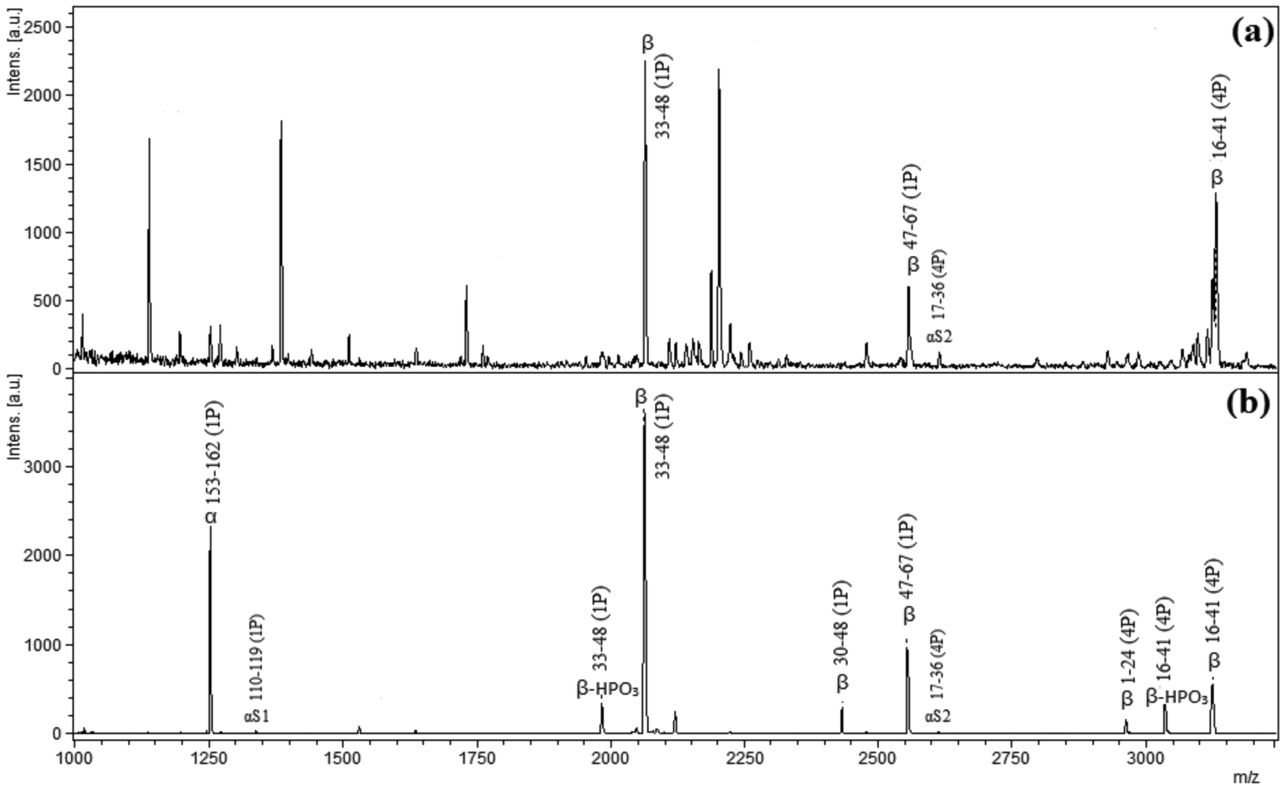

Figure 2. MALDI-MS spectra: (a) tryptic β-casein digest prior to enrichment and (b) eluted fraction after enrichment with poly(AGE-DVB)-IMAC-Fe^{3+}. Phosphopeptides are labeled with amino acid position and number of phosphate groups.

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Table 1. Phosphorylation Sites and the Sequence of Identified Peptides Enriched by Poly(AGE/DVB)-IMAC-Fe^{3+} and Poly(AGE/DVB)-RP

proteins	amino acid sequences	no. of phosphate groups	$[M + H]^+$
	α casein		
α1	KNMAINPS*KENL	1	1197.0
α2	LEIVPNS*AEERL	1	1224.4
α3	TVDMES*TEVFTK	1	1466.6
α4	VPQLEIVPNS*AEER	1	1660.5
α5	DIGSES*TEDQAMEDIK	1	1847.6
α6	DIGS*ES*TEDQAMEDIK	2	1927.6
α7	KYKVPQLEIVPNS*AEERL	1	1951.4
α8	KEKVNELS*KDIGS*ES*TEDQA	3	2247.5
α9	VNELSKDIGS*ESTEDQAMEDIK	1	2660.6
α10	KVNELSKDIGS*ES*TEDQAMEDIKQME	2	2867.4
α11	KNTMEHVS*S*S*EESIIS*QETYKQEKN	4	3132.3
α12	YLGYLEQLLR		1267.2
α13	LLYQEPVLGPVR		1383.8
α14	HQGLPQEVLNENLLR		1758.2
	β casein		
β1	KFQS*EEQQ	1	847.4
β2	KFQS*EEQQQ	1	975.6
β3	KFQS*EEQQQT	1	1104.5
β4	FQS*EEQQQTEDELQ	1	2061.6
β5	NVPGEIVESLS*S*S*EES*ITR	4	2352.6
β6	LVYPFPGPIHNS*LPQNIPPLTQTP	1	2431.5
β7	FQS*EEQQQTEDELQDKIHPF	1	2556.2
β8	KIEKFQS*EEQQQTEDELQDKIHPF	1	2779.4
β9	RELEELNVPGEIVES*LS*S*S*EESITRI	3	3122.3
β10	RELEELNVPGEIVES*LS*S*S*EESITR	4	2965.1
β12	LTDVENLHLPLPLLQSW		1688.0
β13	LYQEPVLGPVRGPFPIIV		1881.1
β14	LLYQEPVLGPVRGPFPIIV		1994.4
β15	FLLQEPVLGPVRGPFPIIV		2107.3
β16	DMPIQAFLLYQEPVLGPVR		2186.3
	ovalbumin		
Ov1	EVVGS*AEAGVDAASVSEEFR	1	2088.8
Ov2	FDKLPGFGDS*IEAQCGTS*VNVHS*S*LR	3	2901.4

are recorded at m/z 2061.9 (FQS*EEQQQTEDELQDK), 2431.5 (LVYPFPGPIHNS*LPQNIPPLTQTP), 2556.2 (FQS*EEQQQTEDELQDKIHPF), 2965.1 (RELEELNVPGEIVES*LS*S*S*EESITR), 3042.0, and 3122.3 (RELEELNVPGEIVES*LS*S*S*EESITRI).

Different IMAC materials have been used for the selective enrichment, but they face problem of non-specific bindings due to acidic peptides that are also co-purified. They are thus end-capped prior to the enrichment. However, in case of poly(AGE/DVB)-IMAC there is no such requirement of end-capping as acidic peptides are absent in the eluted fraction. Some of phosphopeptides from α-casein are also identified at m/z 1252.79, 1330.78, and 2616.56, because of the trace contamination. The information about the enriched peptides by poly(AGE/DVB) is included in Table 1. The appearance of abundant $[MH - H_3PO_4]^+$ or $[MH - 98]^+$ ions and a weaker $[MH - HPO_3]^+$ or $[MH - 80]^+$ ions in the spectrum indicate that peptide is phosphorylated on serine or threonine.

Furthermore, lanthanides have gained importance for their efficient phosphopeptides enrichment in different formats for example immobilized on IMAC, as metal oxides or composites.[26,27] Poly(AGE/DVB)-IMAC is thus immobilized with lanthanum, europium and erbium ions through their salt solutions. MS analysis of β-casein on these ions shows high efficiency with no loss of phosphopeptides in comparison to Fe^{3+} (Figure S3a–d). Therefore further studies are carried out on poly(AGE/DVB)-IMAC-Fe^{3+} facing no issue of non-specific bindings or acidic residues.

Selective Enrichment of Phosphopeptides from Protein Mixture. The study is expanded to complex protein mixtures with non-phosphoprotein background. Tryptic digests of α-, β-casein, and ovalbumin are added in different ratios to BSA protein as a background to investigate the selectivity of poly(AGE/DVB)-IMAC: mixture I, α-casein and β-casein (1:1); mixture II, α-casein, β-casein, and ovalbumin (1:1:1); mixture III, α-casein, β-casein, ovalbumin, and bovine serum albumin BSA (1:1:1:1); mixture IV, α-casein, β-casein, ovalbumin, and bovine serum albumin BSA (1:1:1:100). Figure 3 represents the enrichment of phosphopeptides from four mixtures having different ratios and thus the complexity levels. The quantity of BSA is increased hundred times to assess the selectivity of the material for phosphopeptides. At equimolar ratio of α-casein, β-casein, ovallbumin and BSA, phosphopeptides enriched from α-casein are 1197.009 (KNMAINPS*KENL) (αS2), 1466.61 (TVDMES*TEVFTK) (αS2), 1660.510 (VPQLEIVPNS*AEER (αS1), 1847.69 (DIGS*ESTEDQAMEDIK) (αS1), 1927.668 (DIGS*ES*TEDQAMEDIK) (αS1), and 1951.4 (KYKVPQLEIVPNS*AEERL) (αS1),

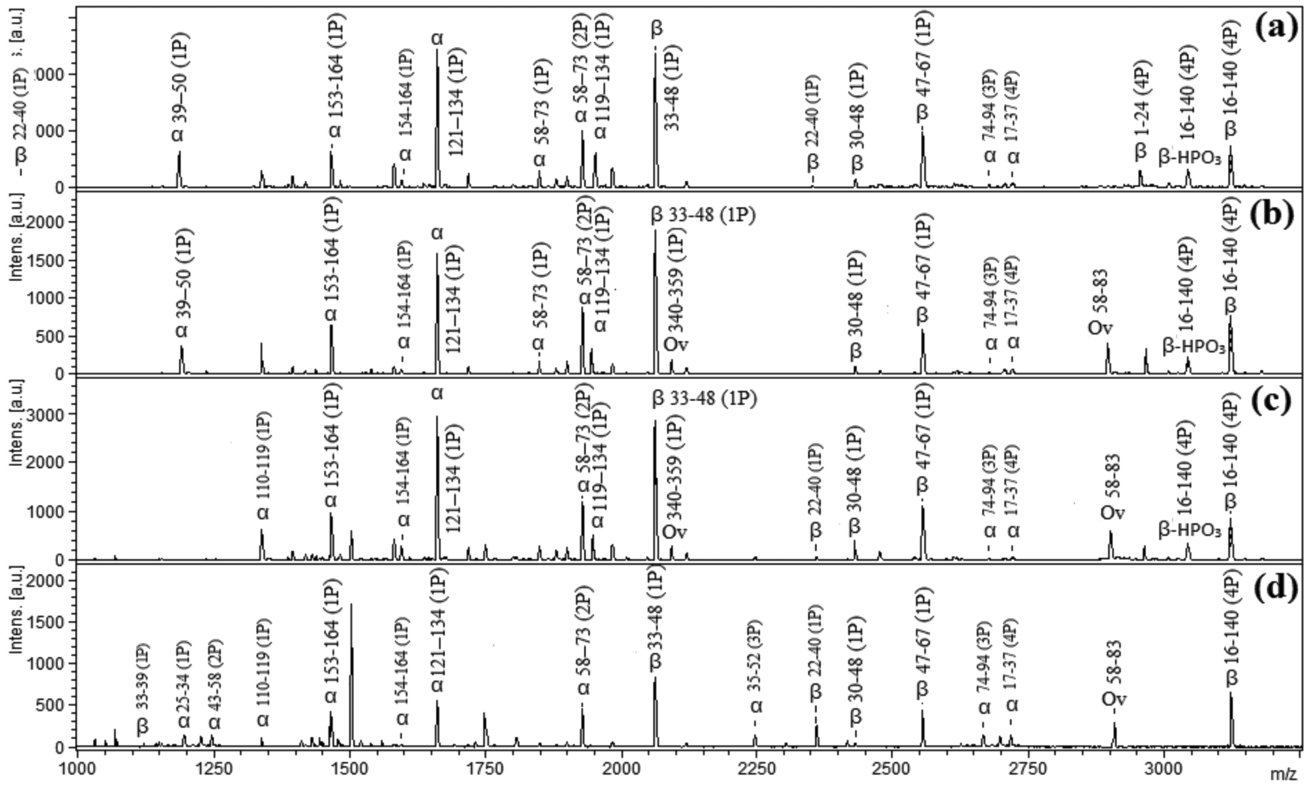

Figure 3. MALDI-MS spectra of selectivity study using poly(AGE-DVB)-IMAC-Fe^{3+}: (a) mixture I (α-/β-casein, 1:1), (b) mixture II (α-/β-casein/ovalbumin, 1:1:1), (c) mixture III (α-/β-casein/ovalbumin/BSA, 1:1:1:1), and (d) mixture IV (α/β-casein/ovalbumin/BSA, 1:1:1:100). *BSA is used without digestion. Identified phosphopeptides derived from tryptic digests of α-, β-casein, and ovalbumin are labeled with amino acid position and number of phosphate groups.

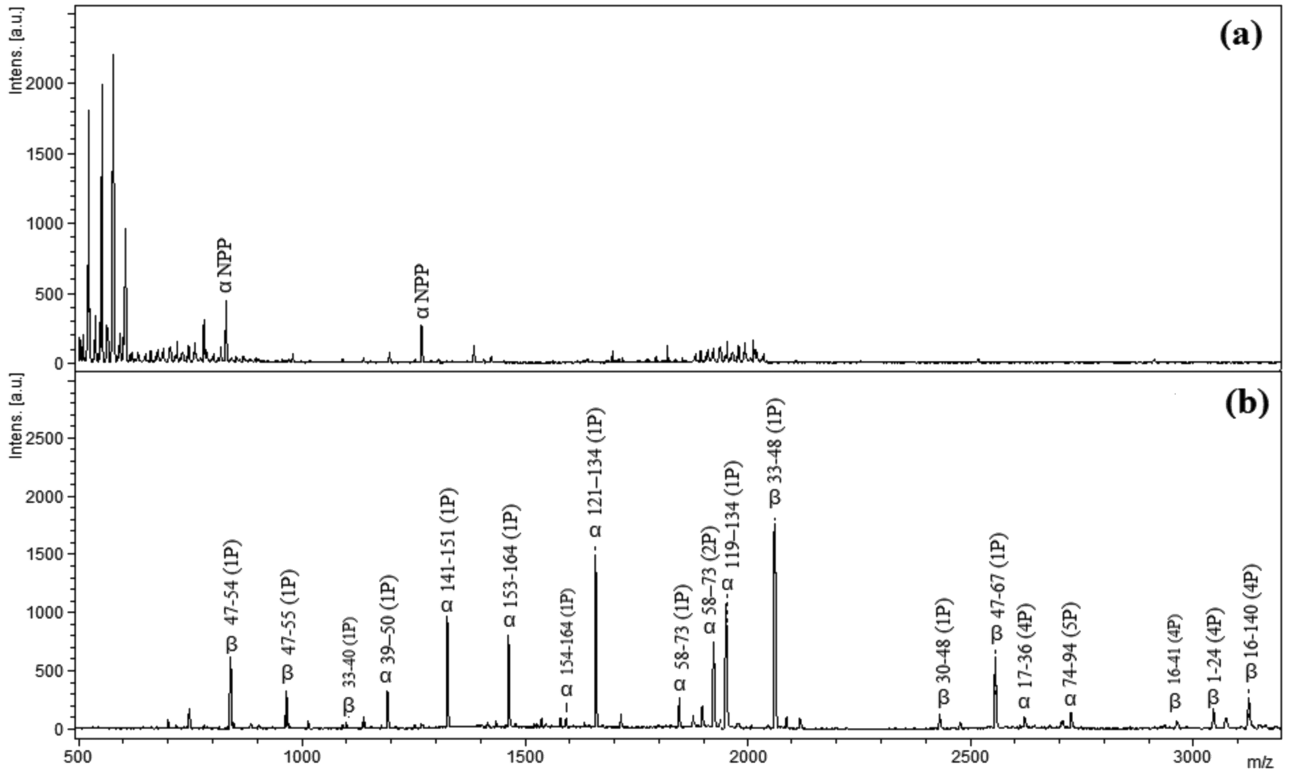

Figure 4. MALDI-MS spectra: (a) raw non-fat milk tryptic digest before enrichment and (b) eluted fraction after enrichment with poly(AGE/DVB)-IMAC-Fe^{3+}. Identified phosphopeptides are labeled with amino acid position and number of phosphate groups.

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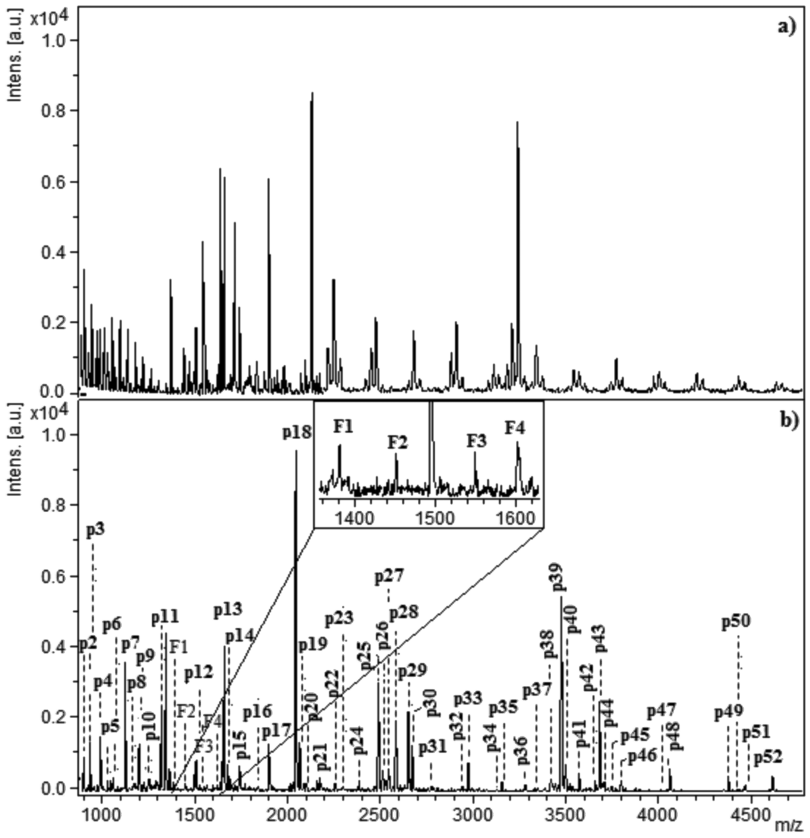

Figure 5. MALDI-MS spectra of tryptic human serum digest: (a) prior to enrichment and (b) eluted fraction employing poly(AGE-DVB)-IMAC. Identified four fibrinogen phosphorylated peptides are labeled as F1, F2, F3, and F4 and shown in inset.

whereas from β-casein are at m/z 2061.9 (FQS*EEQQQTEDELQDK), 2431.56 (LVYPFPGPIHNS*LPQNIPPLTQTP), 2556.2 (FQS*EEQQQTEDELQDKIHPF), and 3122.3 (RELEELNVPGEIVES*LS*S*S*EESITRI). The masses at m/z 2965.1, 3042.0 are with the dephosphorylated segments. The signals at m/z 2088.89 (EVVGS*AEAGVDAASVSEEFR) and 2901.0 (FDKLPGFGDSIEAQCGTSVNVHSSLR) are derived from ovalbumin digest. It is evident that the specificity of poly(AGE/DVB)-IMAC for phosphopeptides is still maintained without any decline even when the molar ratio of the phosphopeptides to the non-phosphopeptides/protein is very high (1:100).

To further investigate the selectivity of poly(AGE/DVB)-IMAC, the de-phosphorylated HeLa cell extract was spiked with β-casein digest (1 μL) maintaining ratio of 1:500, 1:1000, 1:1500, and 1:2000. Characteristic β-casein phosphopeptides are detected with complexity increased to 1500 folds (Supporting Information Figure S4a-c). Moving towards even higher complexity (1:2000), still one phosphopeptide at m/z 3124.98 can be detected (Supporting Information Figure S4d). Such extremely high selectivity is vital when working with complex biological fluids like serum.

Phosphopeptides Enrichment from Biofluids. The transformation from a semi complex mixture to the more complex real samples (non-fat milk and egg yolk) is the next task accomplished on poly(AGE/DVB)-IMAC material. The basic constituents of the commercially available non-fat milk are α- and β-caseins which have abundant phosphorylated sites. Normal bovine milk contains 30-35 grams of protein per liter, of which about 80% is arranged in casein micelles. There are number of salts and minerals that usually affect the enrichment efficiency. Therefore non-fat milk is considered a diverse complex sample and the protocol involves no depletion or prefractionation prior to the enrichment. Egg yolk is also a rich source of phosphoproteins. The lipovitellins comprise of lipid and metal storage and contain a heterogeneous mixture of about 16% (w/w) non-covalently bound lipid, most being the phospholipids. Phosvitin (phosphoprotein, 10% of the total protein content) contains high concentration of phosphate groups providing efficient metal-binding sites in clusters.

The direct analysis of these biofluids is difficult as there are varieties of salts in their digests. The direct MS spectrum of 2.5 $\times$ 10^{-6} M (1 μL) tryptic digest of non-fat milk is shown in Figure 4a. There is no prominent peak in the spectrum. After applying the digest to poly(AGE/DVB)-IMAC, the dominant peaks of mono- and multiphosphorylated peptides appear in the spectrum (Figure 4b).

Supporting Information Figure S5a shows the spectrum of raw tryptic digest of egg yolk, which is looking like a mess of different peptides. Because of the the ionization suppression, the masses cannot be identified. On applying the tryptic egg yolk digest to poly(AGE/DVB)-IMAC, different phosphorylated domains of both phosvitin and lipovitellin are enriched and can be identified (Supporting Information Figure S5b).

The serum level of fibrinogen and its hydrolytic products may reflect the expression and activation of enzymes including kinase, phosphatase, and protease.[28] Phosphorylated fibrinogen α-chain is linked to various diseases like ovarian carcinoma[29] and chronic kidney diseases.[30] Poly(AGE/DVB)-IMAC is applied to the digested serum prior to the enrichment, no phosphopeptide is detected (Figure 5a). Four characteristic phosphopeptides from fibrinogen α-chain are identified after

the enrichment (Figure 5b). Fibrinopeptide A (FPA) (F1-16)1P, (Ser 3), a 16-residue long peptide (1616 Da), is the segment that anchors on the thrombin surface during clotting. The other three phosphopeptides, (F1-15)1P, (F2-15)1P and (F2-16)1P (Table 2) are hydrolytic products of FPA. An

Table 2. Identified Phosphorylated Fibrinogen Fragments from Diluted Human Serum Employing Poly(AGE-DVB)-IMAC[a]

molecular mass (Da)			
theoretical	measured $[M + H]^+$	fibrinogen α-chain sequence	phosphorylation sites
1388.570	1389.902	D***S***GEGDFLAEGGGV	1
1459.509	1460.903	AD***S***GEGDFLAEGGGV	1
1544.680	1545.702	D***S***GEGDFLAEGGGVR	1
1615.600	1616.509	AD***S***GEGDFLAEGGGVR	1

[a]Phosphoserine residues are bold and italic.

altered ratio of FPA (F2-15) and FPA (F1-16) is detected in patients affected by hepatocellular carcinoma (HCC); the DS*GEGDFLAEGGGV peptide is up-regulated and ADS-EGDFLAEGGGVR peptide is down-regulated greatly.[31] The proportions of fibrinogen and their phosphorylation products offer new opportunities for basic research in exploring new frontiers in biomarker discovery. The current enrichment strategy with poly(AGE/DVB)-IMAC as an affinity sorbent can be a choice to explore the fibrinogen based biomarker identification strategies.

Using Mascot search engine (www.matrixscience.com) and Phosphosite Plus (www.phosphosite.org) online identification tools, 52 phosphopeptides from human serum are identified and listed in Supporting Information Table S2 with their protein identity, function that can be related to the disease.

Detection Limit and Reproducibility. Enrichment of target molecules present in lower quantity is often challenging. Therefore it is mandatory to investigate the sensitivity of synthesized polymeric IMAC material. Poly(AGE/DVB)-IMAC is incubated with different concentrations of β-casein digest (100, 50, and 2 fmol). The lowest detectable amount of phosphopeptides is 2 fmol in which three phosphopeptides can be identified (Supporting Information Figure S6c).

Reproducibility as an important requirement for the new material and protocol is determined using casein digest with three batches of poly(AGE/DVB)-IMAC-Fe^{3+} (Supporting Information Figure S7). The enriched phosphopeptides labelled as α and β are highly reproducible in three of the conducted experiments. Statistical evaluation of collected data for eight phosphopeptides provides the highest value of standard deviation 0.441 and the lowest 0.067 (Supporting Information Table S3).

Desalting by Poly(AGE/DVB)-RP and Comparison to Aspire RP 30 Tips. The hydrophilic phosphates are negatively charged at low pH and are attracted towards the reversed phase material. When dealing with the digestion of complex samples (along with the naturally occurring salts and minerals), various reagents are added which increase the salt concentration of the sample solution. These salts make the MALDI-MS analysis critical and peptide profile is less informative to deduce any conclusion about the sample composition. Moreover the co-crystallization of matrix with the analyte is also affected by the salts. Stressing on the need of desalting before MS analysis, in-house poly(AGE/DVB)-RP is synthesized. Poly(AGE/DVB)-RP works both in lower (tryptic casein digest) and higher salt concentrations (non-fat milk digest), without the loss of hydrophilic peptides. The desalting efficiency is compared with commercial desalting Aspire RP30 tips. The number of enriched and desalted content from tryptic casein digest is compared in the case of raw digest (Figure 6a), Aspire RP tips (Figure 6b) and poly(AGE/DVB)-RP (Figure 6c). The designed polymer has excellent retaining capability in comparison to the commercially available tips. The desalting of sample with high salt content, that is, tryptic digest of non-fat milk also shows the highest efficiency (Supporting Information Figure S8).

Standpoint of Poly(AGE/DVB) in Comparison to Literature and Poly(GPE/DVB). In literature polymeric phases are generally used in the form of monoliths or coated on metal oxide particles. Polymeric materials in combination with phosphoproteomics are quite limited. A comparison of poly(AGE/DVB)-IMAC with reported polymeric materials in phosphopeptides enrichment is given is Supporting Information Table S4. A polymer-based material, that is, poly(GPE/DVB) functionalized as IMAC/RP is recently published for the selective enrichment of phosphopeptides and desalting of samples.[32] Poly(GPE/DVB)-IMAC has shown high enrichment efficiency however it does not address the common issue of acidic peptides (non-specific bindings) enriched by Fe^{3+}. The limitation of IMAC-Fe^{3+} strategy is that acidic peptides (rich in carboxylic-acid of Glu and Asp) are enriched along with the phosphopeptides which affect the efficiency/binding sites. One of the solutions offered is to overload the column or material to allow competition among the peptides with successive enrichments using second column or fresh batch of material.[33] In poly(AGE/DVB)-IMAC-Fe^{3+} (173.1554 m^2/g), high surface area allows the overloading of material and also provide better selectivity than lanthanide immobilized on poly(GPE/DVB) (89.3824 m^2/g). Hence it tackles the issue of acidic peptide enrichment on Fe^{3+}. Moreover different lanthanide ions immobilized on poly(AGE/DVB)-IMAC material show no difference of performance. This demonstrates that change in material characterisitcs in combination with metal ions play role in the efficiency of enrichment. To prove the concept, no change is carried out in the enrichment protocol for poly(GPE/DVB)-IMAC and poly(AGE/DVB)-IMAC. Using same buffer conditions and samples, better enrichment is achieved by the later material (Supporting Information Table S5). Briefly, the selectivity achieved for poly(GPE/DVB)-IMAC is to ten folds complexity level whereas poly(AGE/DVB)-IAMC is selective up to the complexity level of 2000. In case of milk sample, 14 phosphopeptides are enriched by poly(GPE/DVB)-IMAC, whereas 20 phosphopeptides are enriched by poly(AGE/DVB)-IMAC. For the desalting prior to MS analysis, both polymeric materials are derivatized as RP and for casein mixture, poly(AGE/DVB)-RP has enriched much higher number of peptides (31 peptides) as compared to poly(GPE/DVB)-RP (26 peptides). The higher surface area does not let the abundant species occupy/saturate the binding sites of the polymer and thus space is still available to the low concentrated species. The increased number of species of interest are specifically bound and hence enriched.

CONCLUSION

The polymerization of AGE with vinyl monomer produces poly(AGE/DVB) with available epoxy group for subsequent functionalization. The polymeric IMAC material is selective to

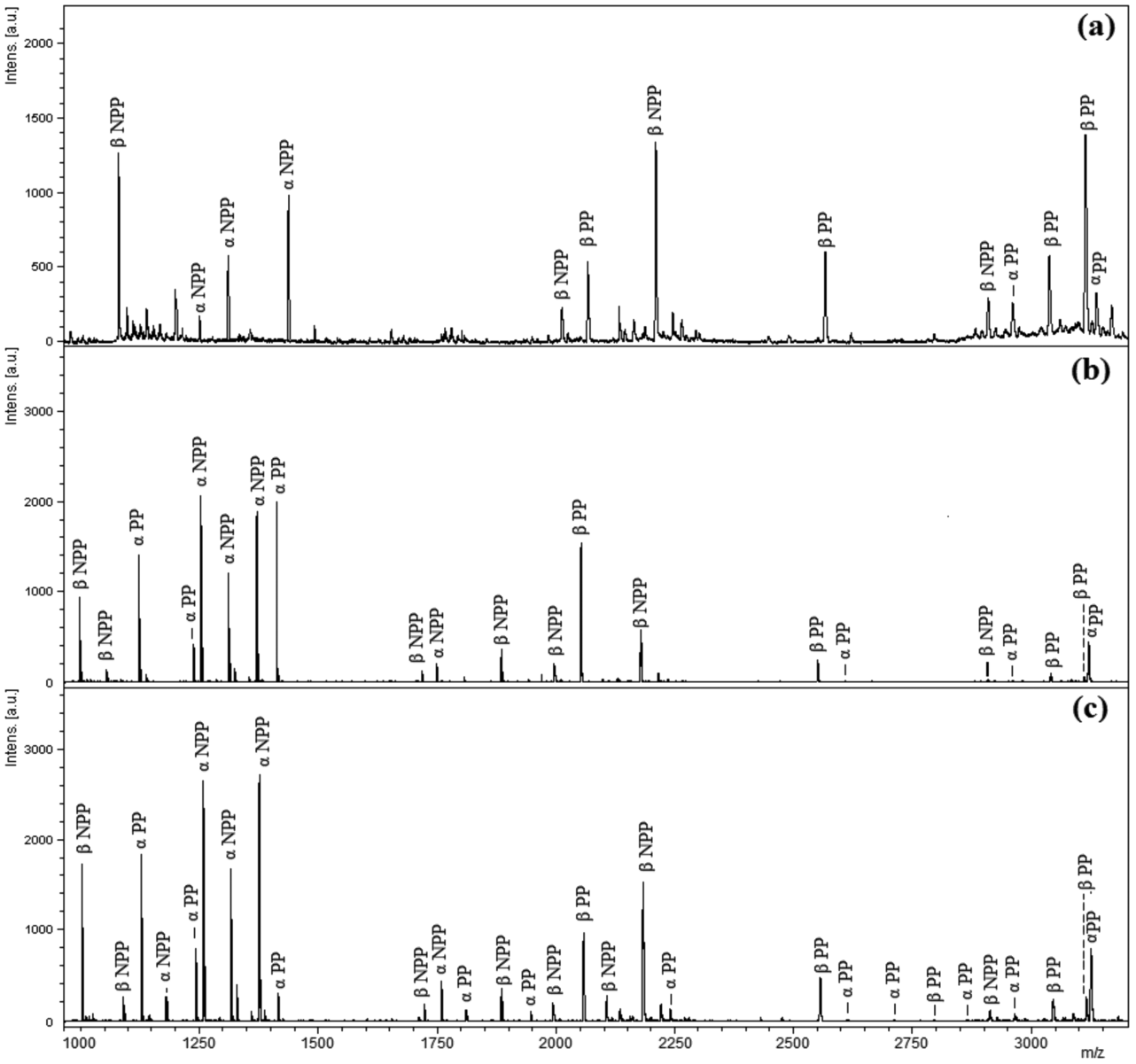

Figure 6. MALDI-MS spectra: (a) tryptic casein digest before enrichment, (b) eluted fraction after enrichment by Aspire RP 30 desalting tip, (c) eluted fraction after enrichment by poly(AGE/DVB)-RP. Both phosphopeptides and non-phosphopeptides derived from casein variants (α- and β-) are labeled with amino acid position.

phosphopeptides enrichment and selectivity is recorded to 1:2000 in complex background. The hydrophobic polymeric-RP shows excellent desalting ability in comparison to the commercially available polymer based Aspire RP 30 desalting tips. The functionalized poly(AGE/DVB) is not only able to enrich the mono phosphorylated and multiphosphorylated peptides but also their dephosphorylated fragments. The non-specific bindings especially of acidic residues are completely avoided. The desalting efficiency prior to the analysis eliminates the adduct formation. The phosphopeptides enrichment from standard sample (α-, β-casein) to complex real samples (non-fat milk, egg yolk, human serum) and the sensitivity down to 2 fmol, make this polymeric material a good addition to the modern day phosphoproteomic strategies. Four characteristic phosphopeptides (fibrinopeptide A and their hydrolytic products) from fibrinogen α-chain are identified from the human serum after the enrichment. The proportions of fibrinogen and their phosphorylation products open new horizons in the biomarker discovery.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information regarding the chemicals, instrumentation, protein digestion, IR spectra, reproducibility, selectivity, sensitivity, MALDI-MS spectra of egg yolk digest, desalting of non-fat milk, nitrogen adsorption porosimetry, identified phosphopeptides from tryptic serum digest and comparison to the literature studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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