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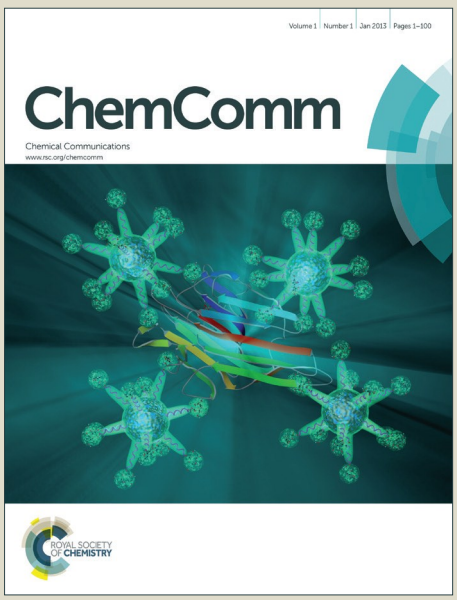

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Hydrazide functionalized monodispersed silica microsphere: a novel probe with tunable selectivity for versatile enrichment of phosphopeptides with different numbers of phosphorylation sites in MS analysis

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Hydrazide functionalized monodispersed silica microsphere (HFMSM) was developed for the enrichment of phosphopeptides for the first time. With the aid of the tunable selectivity of HFMSM, global enrichment or fractionation of phosphopeptides with different numbers of phosphorylation sites could be realized by simple modulation of the concentrations of formic acid in buffers.

As one of the most important post-translational modifications, protein phosphorylation regulates multiple biological processes, and increasing research interest on interpreting its role in biological processes and occurrence of various diseases has been attracted[1, 2]. Despite the extensive application of mass spectrometry (MS) in proteomic analysis, direct analysis of phosphorylated proteins/peptides by MS can hardly be realized because of their low stoichiometry, poor ionization efficiency and signal suppression by non-phosphorylated species with high abundance[3, 4]. Therefore, enrichment of phosphopeptides prior to MS analysis becomes necessary for their effective detection.

Comprehensive analysis of mono- and multi-phosphopeptides can provide integrated information of phosphoproteome for related researches, and global enrichment with subsequent simultaneous analysis covering both singly and multiply phosphorylated peptides offers a relatively fast and simple way for the comprehensive phosphopeptide analysis. However, co-existence of both mono- and multi-phosphopeptides may cause reciprocal interference during their ionization in MS[5], especially for multiphosphopeptides with lower ionization efficiency, as well as some monophosphopeptides in low abundance. As a comparison, such ionization interference can be significantly reduced in separate analysis of mono- and multi- phosphopeptides[6-8], and improved detection of species with low sensitivity may be realized. Therefore, incorporating separation of mono- and multi- phosphopeptides, or a finer fractionation of phosphopeptides with different numbers of phosphorylation sites in enrichment procedure should be of great help for obtaining more comprehensive information of phosphoproteome.

Multiple strategies have been adopted for enrichment of phosphopeptides. Among them, immobilized metal ion affinity chromatography (IMAC) and metal oxide affinity chromatography (MOAC) have been extensively investigated. Some of these metal-based affinity materials were used for global enrichment[9-12], while others showed preference for mono- or multi- phosphopeptides[6, 7, 13, 14]. Generally speaking, materials with stronger affinity towards phosphate groups could adsorb mono- and multi- phosphopeptides simultaneously, while those with weaker affinity showed obvious preference of multiphosphopeptides. However, few of these materials had adjustable affinity towards phosphopeptides, and their selectivity towards phosphopeptides with different numbers of phosphorylation sites was basically fixed. Recently, several amine-based (e.g. amino[15], quaternary ammonium[16], polyethyleneimine[17], polyarginine[18, 19], guanidyl[20, 21]) functional materials were developed for phosphopeptide enrichment[22]. Because the intensity of electrostatic attraction between amine-based functional groups and phosphate groups can be adjusted by modulating the degree of deprotonation of phosphate groups, amine-based functional materials are promising in developing enrichment materials with tunable selectivity, and their competence for multiple uses in phosphopeptide enrichment deserves in-depth exploration.

Hydrazide functionalized materials are well known for their capability of glycopeptide enrichment through the formation of hydrazone by hydrazide and oxidized saccharide groups[23], while their potential for phosphopeptide enrichment has not been exploited yet. Herein, the hydrazide functionalized mono-dispersed silica microsphere (HFMSM) with uniquely tunable selectivity was innovatively developed for phosphopeptide

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enrichment. Hydrazide, a kind of amine-based functional group, showed affinity towards phosphopeptides through electrostatic attraction and hydrogen bonding, facilitating the capture of phosphopeptides on HFMSM. The selectivity of HFMSM towards phosphopeptides could be simply adjusted by changing the concentration of formic acid (FA) in buffers. In detail, mono- and multi- phosphopeptides can be simultaneously enriched by HFMSM when using loading buffers with lower concentration of FA, while phosphopeptides with more phosphorylation sites were preferred when using loading buffer with higher concentration of FA. Through modulation of loading buffers and elution buffers, global enrichment of all phosphopeptides, separate enrichment of mono- and multi-phosphopeptides, and even finer fractionation of phosphopeptides with different numbers of phosphorylation sites can be realized by HFMSM, making it a versatile tool for phosphoproteome analysis.

The synthesis procedure of HFMSM is illustrated in Scheme 1. The monodispersed silica microsphere (MSM) was synthesized by controlled hydrolysis and condensation of tetraethyl orthosilicate (TEOS) for further functionalization, according to a modified Stöber method[24]. Amino functionalized monodispersed silica microsphere (AFMSM) was then prepared by grafting 3-aminopropyl triethoxysilane (APTES) with silanol groups on the surface of MSM. And finally HFMSM was obtained by treatment of AFMSM with methyl acrylate and hydrazine hydrate in order for the transformation of surface functional groups. The synthesized HFMSM was characterized by TEM, SEM, IR and elemental analysis. Uniformed sphere morphology of HFMSM with monodispersed diameter around 200 nm was observed in its TEM and SEM images (Fig. 1a-c). Appearance of characteristic absorbance peaks of amide bond in the range of 1640-1540 cm^{-1} in IR spectrum after hydrazinolysis reaction (Fig. 1d) and a nearly three-fold increase in the mass percentage of nitrogen element in HFMSM (2.16%) compared with AFMSM (0.78%) confirmed the successful functionalization with hydrazide groups.

The capability of HFMSM for tunable phosphopeptide enrichment was firstly tested using tryptic digest of a standard phosphoprotein β-casein (4×10^{-7} M). Before enrichment, the MALDI-Tof-MS spectrum were dominated by highly abundant non-phosphorylated peptides (Fig. 2a). For phosphopeptide enrichment by HFMSM, loading buffers containing 60% ACN and different concentration of FA were adopted for adsorption of phosphopeptides, and the MALDI-MS matrix solution (20 mg/mL DHB, 5% H_3PO_4, ACN/H_2O 1:1, v/v) was directly applied as elution buffer for the complete release of phosphopeptides adsorbed. When performing phosphopeptide enrichment in a loading buffer containing 0.02% FA, two monophosphopeptides and two multiphosphopeptides were enriched with almost no signal of non-phosphorylated peptides (Fig. 2b), while in loading buffer containing 1% FA, only two multiphosphopeptides could be identified (Fig. 2c). The varied selectivity of HFMSM towards mono- and multi-phosphopeptides could be ascribed to the different deprotonation degree of phosphate groups in loading buffers with different acidity. Phosphate groups were highly deprotonated in the loading buffer containing 0.02% FA (pH 3.2), and both mono- and multi- phosphopeptides showed electrostatic attraction towards hydrazide groups on HFMSM with sufficient intensity for their adsorption. However, in the loading buffer containing 1% FA (pH 2.4), phosphate groups were less deprotonated, causing the weakening of the electrostatic attraction and a more selective adsorption of multiphosphopeptides with more phosphate groups and higher affinity.

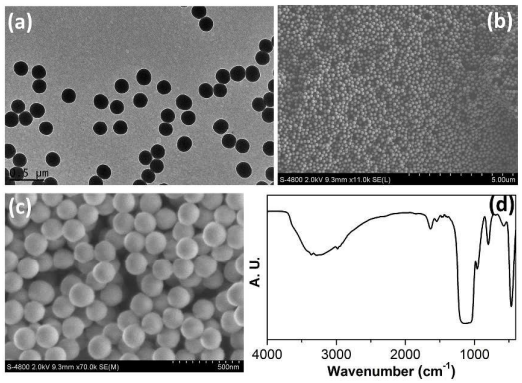
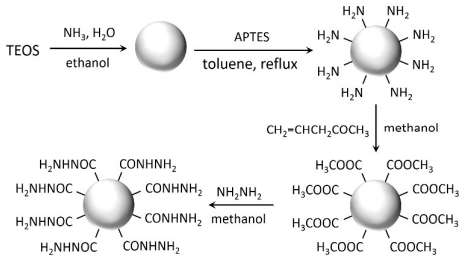


Fig. 1 (a) TEM image, (b and c) SEM images, and (d) FT-IR spectrum of HFMSM

Since both mono- and multi- phosphopeptides could be enriched in buffer containing 0.02% FA while only multiphosphopeptides could be concentrated in buffer containing 1% FA, a "subtraction" of buffers was applied for selective analysis of monophosphopeptides. The buffer containing 0.02% FA was adopted as loading buffer for adsorption of both mono- and multi- phosphopeptides. Then the monophosphopeptides were selectively eluted using the buffer containing 1% FA, while multiphosphopeptides still being adsorbed on HFMSM. It was confirmed that only monophosphopeptides were eluted and identified in the spectrum (Fig. 2d). Compared with the global analysis (Fig. 2b), one more monophosphopeptide (β2) with low sensitivity was detected, probably owing to the less ionization interference when performing selective monophosphopeptide analysis.

The enrichment efficiency of HFMSM for phosphopeptide enrichment was further evaluated. When the concentration of β-casein digest was as low as 1×10^{-10} M, one monophospho-peptide and one multiphosphopeptide were still detectable in



Scheme 1 Synthesis of HFMSM

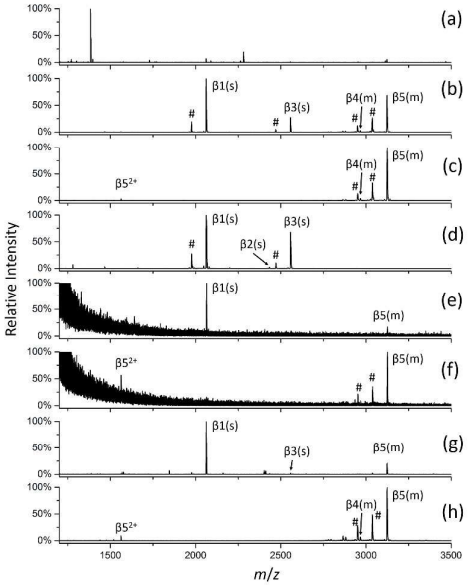


Fig. 2 MALDI-ToF-MS spectra of digest of β-casein (a) before enrichment and (b-h) after enrichment. Concentration of digest of β-casein was (a-d) 4×10^{-7} M, (e) 1×10^{-10} M, (f) 2×10^{-10} M, (g) 4×10^{-8} M, with 200-fold BSA digest in molar ratio, and (h) 4×10^{-8} M, with 1000-fold BSA digest in molar ratio, respectively. Loading buffer were (b, d, e, g) 60% ACN, 0.02% FA and (c, f, h) 60% ACN, 1% FA. Elution buffer were (b, c, e-h) matrix solution and (d) 60% ACN, 1% FA. Monophosphopeptides were marked with "s"; multiphosphopeptides were marked with "m"; "#" denoted dephosphorylated fragments.

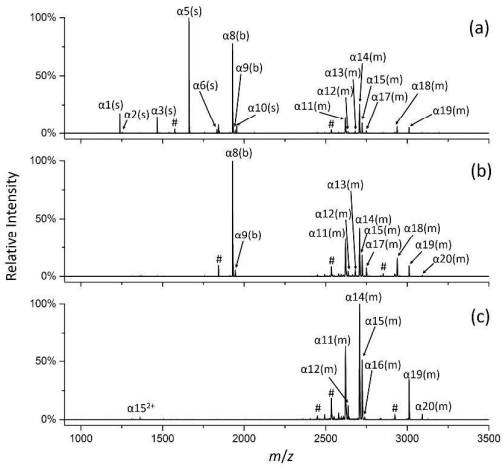


Fig. 3 MALDI-ToF-MS spectra of digest of α-casein after enrichment in loading buffers containing (a) 60% ACN, 0.02% FA, (b) 60% ACN, 0.5% FA, and (c) 60% ACN, 2% FA. All the phosphopeptides were eluted with matrix solution. Monophosphopeptides were marked with "s"; multiphosphopeptides with two and more phosphorylation sites were marked with "b" and "m", respectively.

global analysis (Fig. 2e), while the multiphosphopeptide could be identified at a concentration of 2×10^{-10} M with selective enrichment of multiphosphopeptide (Fig. 2f). Phosphopeptides could be extracted with no obvious non-specific adsorption from the mixture of tryptic digest of β-casein and BSA (molar ratio of 1:200 and 1:1000) in global enrichment and multi-phosphopeptide enrichment, respectively (Figure 2g and 2h), exhibiting the satisfactory specificity of HFMSM for phosphopeptide enrichment. Higher acidity in loading buffers not only weakened the affinity towards monophosphopeptides, but also diminished non-specific adsorption caused by acidic peptides with multiple carboxyl groups. As a result, increased tolerance of non-specific adsorption in selective multiphosphopeptide enrichment was observed.

The tryptic digest of α-casein containing more phospho peptides with varied numbers of phosphorylation sites were further applied for a more meticulous investigation of the tunable adsorption selectivity of HFMSM. When performing enrichment in the buffer with 0.02% FA, global enrichment of 6 monophosphopeptides and 10 multiphosphopeptides was observed (Fig. 3a), which was in conformity with its enrichment performance with β-casein digest. The selectivity of HFMSM towards multiphosphopeptides was also investigated in loading buffers with higher concentration of FA and acidity. As illustrated in Fig. 3b and 3c, in loading buffer containing 0.5% FA, multiphosphopeptides with two and more phosphorylation sites were enriched by HFMSM; while in loading buffer containing 2% FA, only multiphosphopeptides with more than three phosphorylation sites were extracted. As the FA concentration and buffer acidity increased, interaction between phosphate and hydrazide groups was weakened gradually, resulting in the gradual appearance of preference for multiphosphopeptides with more phosphorylation sites. In addition, detection of more multiphosphopeptides and enhanced MS responses of multiphosphopeptides with low sensitivity were also observed in Fig. 3b and 3c, indicating the decreased ionization competition and better detection efficiency in selective multiphosphopeptide analysis.

With the aid of gradually increasing selectivity towards phosphopeptides with more phosphorylation sites over buffer acidity, a finer fractionation of phosphopeptides with different numbers of phosphorylation sites could be realized. Phosphopeptides in digest of α-casein were firstly extracted in the loading buffer containing 0.02% FA, and another buffer containing 0.5% FA was then applied for selective elution of monophosphopeptides. As shown in Fig. 4a, a total of 8 monophosphopeptides, as well as only one biphosphorylated peptide with much lower sensitivity, were identified in the first fraction. Increases of both quantity and signal intensity of monophosphopeptides were also observed, compared with the result from global analysis (Fig. 3a). The HFMSM with multiphosphopeptides remaining adsorbed was then treated with buffer containing 2% FA. Biphosphorylated peptides were selectively eluted and identified in the second fraction without monophosphopeptides and multiphosphopeptides containing more phosphorylation sites (Fig. 4b). At last, other multiphosphopeptides with more phosphorylation sites could be eluted in the third fraction using matrix solution with the

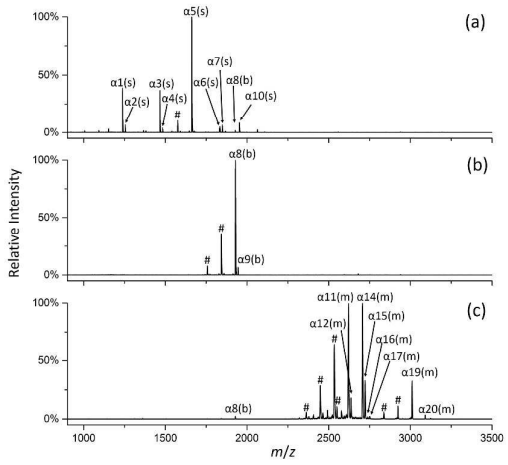


Fig. 4 MALDI-ToF-MS spectra of fractionation of phosphopeptides enriched from digest of α-casein. (a) The first fraction eluted by 60% ACN, 0.5% FA; (b) The second fraction eluted by 60% ACN, 2% FA; (c) The third fraction eluted by matrix solution. Monophosphopeptides were marked with "s"; multiphosphopeptides with two and more phosphorylation sites were marked with "b" and "m", respectively.

highest acidity (Fig. 4c). By the way of serial elution by elution buffers with increasing acidity, phosphopeptides containing different numbers of phosphorylation sites were separated successfully. The fractionation ability provides a possibility for the application of HFMSM as stationary phase for chromatographic separation of phosphopeptides with different numbers of phosphorylation sites using pH gradient.

To demonstrate its practicability for real sample analysis, HFMSM was further applied to trap phosphopeptides in the tryptic digest of nonfat milk with higher complexity. Both global and selective analysis were performed, according to the procedures mentioned before. A total of 6 monophospho-peptides and 8 multiphosphopeptides were detected in the global analysis (Fig. S1a, ESI), while 9 monophosphopeptides and 13 multiphosphopeptides were identified in the selective analysis of (Fig. S1b and S1c, ESI). The significant increase of quantity of phosphopeptides detected indicated the more sensitive detection of phosphopeptides in selective analysis, while global enrichment and analysis still remained an effective approach for rapid analysis.

In summary, HFMSM was developed and applied to versatile enrichment of phosphopeptides for the first time. HFMSM had tunable selectivity towards phosphopeptides with different numbers of phosphorylation sites. Both mono- and multi- phosphopeptides could be enriched by HFMSM in buffers with lower acidity, while only those with more phosphorylation sites could be extracted in buffers with higher acidity. Benefitting from its varied selectivity over buffer acidity, global analysis, selective analysis of mono- and multi-phosphopeptides, and finer fractionation of phosphopeptides with different numbers of phosphorylation sites could be realized by simple modulation of both loading and elution buffer. In addition, more efficient detection of phosphopeptides with low sensitivity was also observed in separate analysis, owing to the less interactive ionization interferences. We believed that HFMSM is promising in developing more efficient purification and chromatographic separation methods for phosphoproteome analysis, and it will open the door for versatile phosphopeptide enrichment with novel enrichment materials.

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