Facile synthesis of zwitterionic polymer-coated core–shell magnetic nanoparticles for highly specific capture of N-linked glycopeptides†

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Highly selective and efficient capture of glycosylated proteins and peptides from complex biological samples is of profound significance for the discovery of disease biomarkers in biological systems. Recently, hydrophilic interaction liquid chromatography (HILIC)-based functional materials have been extensively utilized for glycopeptide enrichment. However, the low amount of immobilized hydrophilic groups on the affinity material has limited its specificity, detection sensitivity and binding capacity in the capture of glycopeptides. Herein, a novel affinity material was synthesized to improve the binding capacity and detection sensitivity for glycopeptides by coating a poly(2-(methacryloyloxy)ethyl)-dimethyl-(3-sulfopropyl) ammonium hydroxide (PMSA) shell onto Fe$_3$O$_4$@SiO$_2$ nanoparticles, taking advantage of reflux-precipitation polymerization for the first time (denoted as Fe$_3$O$_4$@SiO$_2$@PMSA). The thick polymer shell endows the nanoparticles with excellent hydrophilic property and several functional groups on the polymer chains. The resulting Fe$_3$O$_4$@SiO$_2$@PMSA demonstrated an outstanding ability for glycopeptide enrichment with high selectivity, extremely high detection sensitivity (0.1 fmol), large binding capacity (100 mg g$^{-1}$), high enrichment recovery (above 73.6%) and rapid magnetic separation. Furthermore, in the analysis of real complicated biological samples, 905 unique N-glycosylation sites from 458 N-glycosylated proteins were reliably identified in three replicate analyses of a 65 μg protein sample extracted from mouse liver, showing the great potential of Fe$_3$O$_4$@SiO$_2$@PMSA in the detection and identification of low-abundance N-linked glycopeptides in biological samples.

Introduction

Protein glycosylation, one of the most significant post-translational modifications (PTMs) in the proteome, has great importance in multiple biological processes including signal transduction, intracellular transport, immune response and cell growth.¹–⁴ Abnormal glycosylation is associated with various diseases such as cancer and neurodegenerative diseases.⁵ To better understand these biological processes as well as to discover disease biomarkers, it is necessary to identify glycoproteins and determine their glycosylation sites. Currently, mass spectrometry (MS)-based techniques have become the most important and effective tools for analyzing and characterizing protein glycosylation. Unfortunately, the tremendous heterogeneity of each glycosylation site, inherent low abundance of glycopeptides and serious signal suppression caused by the co-existence of abundant non-glycosylated peptides still make the direct MS analysis of glycopeptides a challenge. Therefore, an efficient approach for glycopeptide enrichment from complex biological samples prior to MS analysis is imperative.

Hitherto, several materials and methods, including lectin affinity chromatography,⁶–⁸ boronic acid affinity chromatography,⁹–¹³ hydrazide beads,¹⁴,¹⁵ titanium dioxide,¹⁶ and hydrophilic interaction chromatography (HILIC) adsorbents,¹⁷,¹⁸ have been developed for glycopeptide enrichment. Among these methods, the enrichment strategy based on HILIC has been efficiently developed and has gained increasing popularity due to its simple operating process, high selectivity, excellent reproducibility, no bias and it allows reversible alterations in glycan composition. A variety of hydrophilic matrices, such as sepharose, cellulose, saccharides, ZIC-HILIC beads and metal–organic frameworks,¹⁹–²² have been used for the selective separation and extraction of glycopeptides. Nevertheless, the relatively low density of hydrophilic molecules on
the surface of traditional HILIC adsorbents has limited the specificity, detection sensitivity and binding capacity for glycopeptides. It was demonstrated that HILIC beads with a large amount of functional molecules bonded could further improve the enrichment performance.\(^{23}\) Thus, it is highly desirable for novel HILIC materials with abundant hydrophilic groups to improve glycopeptide enrichment efficiency.

In the last decade, magnetic nanoparticles have gained immense interest due to their unique biocompatibility, easy preparation, versatile modification and rapid magnetic response, and are widely used in magnetic resonance imaging, drug delivery, sensing, and proteomic research.\(^{24–28}\) A combination of magnetic nanomaterial and covalently bonded hydrophilic functional molecule could simultaneously achieve a simple and efficient capture of glycopeptides from a complex mixture by magnetic separation. Several types of functionalyzed magnetic nanoparticles (e.g., poly(4-vinyl-pyridinium ethanesulfonate)-coated Fe\(_3\)O\(_4\),\(^{29}\) Fe\(_3\)O\(_4@\)SiO\(_2@\)PEG-maltose\(^{23}\)) have been developed, which have shown great selectivity for glycopeptides. However, the synthesis procedures of these materials are tedious. Recently, Zou et al.\(^ {30}\) fabricated multilayer polysaccharide-shells-coated magnetic nanoparticles using a simple and convenient approach (namely, layer-by-layer), and the obtained composite material exhibited high selectivity, high detection sensitivity and large binding capacity for glycopeptide enrichment. Fang et al.\(^ {31}\) developed a one-pot method for the synthesis of chitosan-coated magnetic nanoparticles, and the thick chitosan shell was beneficial in improving the enrichment efficiency. Notwithstanding these successful examples, the design and synthesis of novel hydrophilic polymer-coated magnetic nanoparticles is attracting extensive attention for improvements in their detection sensitivity and binding capacity for glycopeptides.

Zwitterionic molecules-functionalized materials are well-known superhydrophilic and ultralow biofouling materials.\(^ {32}\) Zwitterionic groups on nanoparticles can bind water molecules more strongly via electrostatically induced hydration, in comparison with water retention materials based on hydrogen-bonding-induced hydration. A large number of zwitterionic groups enable the formation of a thick layer of water molecules, which greatly contributes to stronger hydrophilic interaction.\(^ {33–36}\) Among silica-based HILIC materials, ZIC-HILIC has shown the best enrichment performance in the analysis of protein glycosylation.\(^ {27,28}\) However, magnetic nanoparticles related to ZIC-HILIC materials have rarely been reported. Yeh et al.\(^ {29}\) prepared zwitterionic polymer-coated magnetic nanoparticles by employing spontaneous acid-catalyzed polymerization. When applied for glycopeptide enrichment, this material exhibited high selectivity and recovery. However, the synthesis process was tedious and the thickness of the polymer shell was difficult to control and it showed a lack of stability. Therefore, the synthesis of zwitterionic polymer-coated magnetic nanoparticles by a convenient, effective and robust method for glycopeptide enrichment would be very attractive.

Herein, we report a facile approach for the synthesis of zwitterionic polymer-coated magnetic nanoparticles (Fe\(_3\)O\(_4@\)SiO\(_2@\)PMSA) via reflux-precipitation polymerization for the first time. The thick zwitterionic polymer shell endows the composite not only with excellent hydrophilic surface properties, but also with abundant zwitterionic functional sites on the polymer chains for high detection sensitivity and large binding capacity for glycopeptides. Moreover, the unique magnetic property will facilitate the rapid and complete recovery of the composite. The specificity, detection sensitivity, enrichment capacity and enrichment recovery of Fe\(_3\)O\(_4@\)SiO\(_2@\)PMSA for glycopeptide enrichment have been evaluated using different biological samples. In addition, the practical applicability of Fe\(_3\)O\(_4@\)SiO\(_2@\)PMSA was demonstrated by the capture of low-abundance glycopeptides in a tryptic digest of proteins extracted from mouse liver. The outstanding selectivity, extreme sensitivity, excellent enrichment recovery of glycopeptides, and high magnetic susceptibility also demonstrated its great capability in glycoproteome analysis of real biological samples.

### Experimental section

#### Materials and chemicals

Iron(III) chloride hexahydrate (FeCl\(_3\)-6H\(_2\)O), ethanol, ethylene glycol (EG), ammonium hydroxide (NH\(_3\)-H\(_2\)O, 28 wt%), tetraethyl orthosilicate (TEOS) and sodium acetate (NaAc) were obtained from Tianjin Chemical Plant (Tianjin, China). γ-Methacyloxy-propyl-trimethoxysilane (MPS) (3-methacryloxy-propyl-trimethoxysilane), N,N\(^*\)-methylenebisacrylamide (MBA), methacrylic acid (MAA), trifluoroacetic acid (TFA), HPLC grade acetonitrile (ACN) and ammonium bicarbonate (NH\(_4\)-HCO\(_3\)) were purchased from Aladdin (Shanghai, China). 2,2-Dimethoxy-2-phenylacetophenone (DMPA) was obtained from Acros Organics (New Jersey, USA). PNGase F was obtained from New England Biolabs (Ipswich, MA). Human serum immunoglobulin G (human IgG), chicken avidin, horseradish peroxidase (HRP), trypsin (TPCK-treated), dithiothreitol (DTT), iodoacetamide (IAA) and 2,5-dihydroxy benzoic acid (DHB) were obtained from Sigma-Aldrich (St Louis, MO, USA). 2,2-azobisisobutyronitrile (AIBN) was supplied by Sinopharm Chemical Reagent Company (Shanghai, China). GELoader tips (20 μL) were purchased from Eppendorf (Hamburg, Germany). All the chemical agents were used without further purification. Purified water was obtained with a Milli-Q apparatus (Millipore, Bedford, MA, USA).

#### Synthesis of Fe\(_3\)O\(_4@\)SiO\(_2@\)PMSA nanoparticles

Fe\(_3\)O\(_4\) nanoparticles were first prepared by a solvothermal reaction, and then coated with a thin layer of SiO\(_2\) through a sol-gel process and subsequently modified with γ-MPS to obtain Fe\(_3\)O\(_4@\)SiO\(_2@\)MPS through the following steps. The procedure is described in detail in ESI.† A PMSA layer was coated onto Fe\(_3\)O\(_4@\)SiO\(_2@\)MPS nanoparticles by a one-step reflux-precipitation polymerization of MSA in a mixed solvent of acetonitrile and water, with MAA as the

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auxiliary monomer, MBA as the cross-linker and AIBN as the initiator. Typically, 50 mg of the as-prepared Fe₃O₄@SiO₂-MPS nanoparticles were ultrasonically dispersed in 80 mL of acetonitrile–water (3 : 1, v/v) in a dried 100 mL single-necked flask. Then, 200 mg MSA, 50 μL MAA, 200 mg MBA and 6 mg AIBN were added together into the flask. After ultrasonication for 20 min, the flask was submerged in a heating oil bath. The reaction mixture was then heated from ambient temperature to 95 °C within 30 min and reacted for 1.5 h in a micro-boiling state. The obtained Fe₃O₄@SiO₂-PMSA nanoparticles were collected by magnetic separation and washed with ethanol 3 times to eliminate excess reactants and self-polymerized PMSA. Finally, the product was dried under vacuum at 50 °C for 24 h.

**Synthesis of monolayer MSA molecules-functionalized nanoparticles (Fe₃O₄@SiO₂-MSA)**

Fe₃O₄@SiO₂-SH was first synthesized and this process is elaborated in ESI†. Then, Fe₃O₄@SiO₂-MSA was synthesized according to a previous study with a slight change. 42 Briefly, 50 mg Fe₃O₄@SiO₂-SH nanoparticles were evenly dispersed in 30 mL acetonitrile–water (3 : 1, v/v). Then, 5 mg MSA and 0.5 mg DMPA were added to the mixture, and then the mixture was irradiated using a Spectrolinker XL-1500 UV crosslinker (Spectronics Corporation, Westbury, New York) at room temperature for 15 min. The obtained Fe₃O₄@SiO₂-MSA was washed with ethanol 3 times and dried under vacuum at 50 °C for 5 h.

**Material characterization**

Field emission scanning electron microscopy (FE-SEM) images were collected on a JSM-7001F scanning electron microscope and transmission electron microscopy (TEM) images were obtained by a JEOL JEM-2000 EX transmission electron microscope (JEOL, Tokyo, Japan). Fourier-transform infrared (FT-IR) spectrum was obtained on a Thermo Nicolet 380 spectrometer using KBr pellets (Thermo Nicolet, Wisconsin, USA). Thermogravimetric (TG) analysis was performed under a nitrogen atmosphere with a heating rate of 10 °C min⁻¹ from room temperature to 700 °C (NETZSCH, Selb, Germany). The saturation magnetization curve was obtained on a Physical Property Measurement System 9 T (Quantum Design, San Diego, USA) at room temperature.

**Tryptic digestion of standard glycoproteins and proteins extracted from mouse liver**

1 mg human IgG and chicken avidin or HRP were respectively dissolved in 400 μL of buffer containing 100 mmol L⁻¹ NH₄HCO₃ and 8 mol L⁻¹ urea. After the addition of 10 μL DTT (1 mmol L⁻¹) and allowing to stand at 60 °C for 1 h, the protein was alkylated by 3.7 mg IAA at room temperature in the dark for 40 min. Then, the solution was diluted tenfold with NH₄HCO₃ (50 mmol L⁻¹) and digested with trypsin (protein : enzyme = 25 : 1, w/w) at 37 °C for 16 h. The tryptic digest was lyophilized and stored at -20 °C for further use.

Proteins from mouse liver were extracted following a literature procedure, 13 and the proteins (2 mg) were dissolved in 1 mL denaturing buffer containing 50 mmol L⁻¹ Tris-HCl and 8 mol L⁻¹ urea. After the addition of 20 μL DTT (1 mol L⁻¹) at 60 °C for 1 h, the proteins were alkylated by 7.4 mg IAA at room temperature in the dark for 40 min. Then, the solution was diluted tenfold with 50 mmol L⁻¹ Tris-HCl and digested with trypsin (protein : enzyme = 25 : 1, w/w) at 37 °C for 16 h. The peptide mixture was desalted by a C₁₈-SPE column, lyophilized and stored at -20 °C for further use.

**Selective enrichment of glycopeptides by Fe₃O₄@SiO₂@PMSA nanoparticles**

Fe₃O₄@SiO₂@PMSA nanoparticles were first washed and dispersed in 400 μL of loading buffer (ACN–H₂O–TFA, 86 : 13.9 : 0.1, v/v/v). Subsequently, 3 μg of tryptic digest of human IgG, chicken avidin, or HRP was added to it and moderately incubated at room temperature for 30 min. Then, the nanoparticles were separated from the mixed solution by applying a permanent magnet and washed with the loading buffer (400 μL) three times to remove non-specifically adsorbed peptides, and then the captured glycopeptides were eluted with the eluting buffer (ACN–H₂O–TFA, 30 : 69.9 : 0.1, v/v/v, 10 μL) twice and subjected to further MALDI-TOF MS analysis or deglycosylation for LC-MS/MS analysis.

For glycopeptide enrichment from complex samples, 3 mg Fe₃O₄@SiO₂@PMSA was incubated with tryptic digests of proteins extracted from mouse liver in 500 μL loading buffer (ACN–H₂O–TFA, 86 : 13 : 1, v/v/v). The mixture was incubated at room temperature for 40 min. After being washed with the loading buffer to remove non-specifically adsorbed peptides, the captured glycopeptides were eluted with the eluting buffer (ACN–H₂O–TFA, 30 : 69.9 : 0.1, v/v/v, 100 μL) three times. Finally, the eluate was lyophilized and deglycosylated for LC-MS/MS analysis.

**Selective enrichment of glycopeptides by HILIC-SPE tips**

The fabrication of HILIC-SPE tips and the enrichment procedure were carried out according to a previously described method with minor modification. 44 Detailed information is given in ESI†.

**Deglycosylation of N-linked glycopeptides by PNGase F**

The captured glycopeptides in the eluate were lyophilized and then redissolved in 40 μL NH₄HCO₃ (10 mmol L⁻¹). Then, 50 units of PNGase F were added to the solution and the mixture was incubated at 37 °C for 12 h.

**Mass spectrometry analysis**

All the MALDI-TOF MS experiments were carried out in a reflector positive mode with a pulsed Nd:YAG laser at 355 nm on an AB Sciex 5800 MALDI-TOF/TOF mass spectrometer (AB Sciex, CA). A 0.5 μL aliquot of the eluate and 0.5 μL of DBH matrix were successively dropped onto the MALDI plate for MS analysis. As a fine matrix, DHB was dissolved in ACN–H₂O–H₃PO₄ (70 : 29 : 1, v/v/v, 25 mg mL⁻¹). The mouse liver protein sample was analyzed by a Thermo Q Exactive mass spectrometer (Thermo, San Jose, CA) with a nanospray ion source and...
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lary analysis column (75 μm i.d.) packed with C18 AQ beads (3 μm, 120 Å, Daison, Osaka, Japan). The peptides were separated by a capillary analysis column (75 μm i.d.) packed with C18 AQ beads (3 μm, 120 Å, Daison, Osaka, Japan). The eluting buffers were 99.9% H2O with 0.1% FA (buffer A) and 99.9% ACN with 0.1% FA (buffer B). The gradient elution was performed as follows: from 0 to 4% buffer B (FA–ACN, 0.1 : 99.9) for 15 min, from 4% to 45% buffer B (FA–ACN, 0.1 : 99.9) for 100 min and from 45% to 80% buffer B (FA–ACN, 0.1 : 99.9) for 5 min. After eluting with 80% buffer B for 10 min, the separation system was equili-

bated by buffer A (FA–H2O, 0.1 : 99.9, v/v) for 15 min. The MS/MS spectra were obtained in a data-independent collision-induced dissociation (CID) mode, and the full mass scan was acquired from m/z 400 to 2000 with a resolution of 70,000. The 12 most intense ions with charge above 2 and intensity threshold higher than 104 were selected for MS/MS. The dynamic exclusion was set at 30 s.

Database searching

All the LC-MS/MS raw data were searched with MaxQuant (version 1.3.0.5) against a database (uniprot.Mouse, updated on July 9th, 2014). The mass tolerances were 20 ppm for initial precursor ions and 0.5 Da for fragment ions. Two missed cleavages were allowed for trypsin restriction. The cut-off discovery rate (FDR) for all peptide identifications including the glycopeptides was controlled below 1%. Only peptides with N! P-S/T were considered as N-linked glycopeptides.

Results and discussion

Fabrication and characterization of Fe3O4@SiO2@PMSA nanoparticles

The protocol for the synthesis of Fe3O4@SiO2@PMSA nanoparticles is illustrated in Scheme 1 (Route 1). First, an Fe3O4 nanoparticle was synthesized by a modified solvothermal reaction, and then modified with a thin layer of SiO2 shell via a sol–gel method. Subsequently, MPS was grafted on the Fe3O4@SiO2 nanoparticle to form abundant active double bonds for the subsequent polymerization. Finally, a robust layer of PMSA was coated on Fe3O4@SiO2-MPS core by one-step reflux-precipitation polymerization with MAA as the auxiliary monomer, MSA as the zwitterionic monomer and MBA as the crosslinker to form Fe3O4@SiO2@PMSA. For comparison, Fe3O4@SiO2 nanoparticles were also modified with a mono-

layer of MSA to obtain Fe3O4@SiO2-MSA.

As is widely known, it is difficult to synthesize hydrophilic polymer-coated nanoparticles by reflux-precipitation polymerization, to some extent due to some hydrophilic monomers (like MSA) being insoluble in commonly used solvents such as acetonitrile. Herein, an acetonitrile–water co-solvent was intro-

duced to dissolve the monomers, cross-linker and initiator. Because of their low solubility in the co-solvent, the formed oligomers precipitated from the homogeneous solution and were easily captured by Fe3O4@SiO2-MPS, thus an outer surface PMSA layer was obtained. To investigate the influence of the co-solvent on the subsequent polymerization, several mixed solvents with different ratios of ACN and water (9 : 1, 3 : 1, 1 : 1, v/v) were employed. As demonstrated in Fig. S1 (ESI†), no size increase was found when the ratios of ACN and water were 9 : 1 and 1 : 1. The main reason is that the mixed solvent with 90% ACN could not completely dissolve the monomer, which prevented the subsequent polymerization reaction. However, the co-solvent with a high ratio of water was unfavourable for the reaction due to the internal hydrogen bonding force. Moreover, the addition of MAA was also a sig-

ificant factor in the formation of core–shell structured nano-

particles because hydrogen bonding among MAA units and electrostatic bonding between MAA and MSA units make great contributions to the subsequent polymerization.

The representative TEM and FESEM images of Fe3O4, Fe3O4@SiO2, and Fe3O4@SiO2@PMSA nanoparticles are shown in Fig. 1. The Fe3O4 nanoparticles are uniform in both shape and size with an average diameter of ca. 280 nm. After coating with SiO2, a well-defined core–shell structure appeared and the surface was smoother than for Fe3O4 nanoparticles (Fig. 1b and d). The size slightly increased and the SiO2 layer was estimated to be 10 nm in thickness (Fig. 1c). The TEM image of Fe3O4@SiO2@PMSA nanoparticles (Fig. 1e) clearly indicates that a cross-linked PMSA layer has been successfully coated on the surface of Fe3O4@SiO2-MPS, and the thickness of the polymer shell is around 60 nm. Furthermore, the FESEM image of Fe3O4@SiO2@PMSA (Fig. 1f) with an obvious agglomeration compared to that of Fe3O4@SiO2 also reveals the successful coating of the PMSA.

The modification of Fe3O4 nanoparticles with a SiO2 shell is essential for the coating of the subsequent PMSA layer. We tried to directly coat a PMSA layer onto the Fe3O4-MPS surface without modification (Scheme 1, Route 2). Unfortunately, no final core–shell structure was obtained. We infer that the primary reason is that both the Fe3O4 and MSA become

Scheme 1 Schematic illustration of the synthetic procedure for the preparation of Fe3O4@SiO2@PMSA nanoparticles.
charged in the solvent and there is a strong mutual interaction between the core and the zwitterionic monomer, which subsequently seriously influences the dispersion stability of the Fe₃O₄ nanoparticles in the solvent. The Fe₃O₄ nanoparticles quickly subside without any further reaction. Therefore, a proper intermediate layer is required. The intermediate layer should not only ensure the good dispersibility of seed nanoparticles in the co-solvent but also give high accessibility to MSA for a further polymerization reaction. Taking all these inferences into consideration, due to its excellent hydrophilicity as well as its easily modified character, the silica layer was naturally introduced to obtain the coating layer of PMSA on the magnetic cores.

FT-IR spectroscopy was conducted to inspect the chemical structure of Fe₃O₄@SiO₂, Fe₃O₄@SiO₂-MPS and Fe₃O₄@SiO₂@PMSA. Compared to the FT-IR spectrum of Fe₃O₄@SiO₂ (581 cm⁻¹, νFe-O-Fe, 1091 cm⁻¹, νSi-O-Si), a new characteristic absorption peak (1730 cm⁻¹, νC=O) in the spectrum of Fe₃O₄@SiO₂-MPS (Fig. 2a) could demonstrate the successful grafting of MPS. The new characteristic peaks appearing at 1040 and 1190 cm⁻¹ are ascribed to the stretching vibration of O=S=O in the –SO₃⁻. The peak at 1550 cm⁻¹ corresponds to the bending vibration of N-H in the MBA. In addition, the absorption peak at 1730 cm⁻¹ attributed to the C=O stretching vibration is enhanced. All the evidences indicate the successful coating of PMSA on the surface of Fe₃O₄@SiO₂-MPS.

Energy dispersive X-ray (EDX) spectra and thermogravimetric analysis (TGA) were also recorded to confirm the introduction of PMSA. As shown in Fig. 2b, C, O, Si, and Fe are the main elements. The emergence of N and S elements indicates the successful modification with PMSA. The TGA curves show that the weight loss of 6.70% of Fe₃O₄@SiO₂ is attributed to the adsorbed water. It could be calculated that the weight loss of Fe₃O₄@SiO₂@PMSA nanoparticles is 35.5%, which demonstrates the presence of a high amount of PMSA on the composite nanoparticles.

The magnetic properties of three nanoparticles were studied using a vibrating sample magnetometer at room temperature (Fig. 2d). The magnetic hysteresis loop curves show that the three types of material have no obvious remanence or coercivity at room temperature, indicating that they all possess superparamagnetic character. The superparamagnetism derives from the small nanocrystals in the Fe₃O₄ cores, which behave as superparamagnets with a saturation magnetization (Mₛ) value of 64.3 emu g⁻¹. As a comparison, the Mₛ value of the Fe₃O₄@SiO₂ nanoparticles was 55.5 emu g⁻¹. After coating with the polymer layer, the Mₛ value strikingly decreased to about 33.6 emu g⁻¹. Fe₃O₄@SiO₂@PMSA nanoparticles were observed to have better dispersibility and stability in water compared to Fe₃O₄@SiO₂-MSA when no external magnetic field was applied. However, because of the high-magnetic response of Fe₃O₄ cores, the final product, Fe₃O₄@SiO₂@PMSA
microspheres, could be separated from the solution in only 30 s using a magnet.

Selective enrichment of glycopeptides from the tryptic digest of standard glycoproteins

On the basis of hydrophilic interaction chromatography, hydrophilic materials, i.e., immobilized polysaccharides and MOFs, have been reported to selectively enrich glycopeptides.46–48 Thus, to evaluate the practicability of Fe₃O₄@SiO₂@PMSA in the selective enrichment of glycopeptides, a tryptic digest of a standard glycoprotein (human IgG) was employed as the testing sample. The workflow of glycopeptide enrichment is presented in Scheme S1a.† As shown in Fig. 3a, only four glycopeptides with weak MS signal intensity and low signal-to-noise ratio (S/N) were detected in the direct analysis of IgG tryptic digest on account of the low abundance of glycopeptides and strong signal suppression by the abundant non-glycosylated peptides. However, after being enriched by Fe₃O₄@SiO₂@PMSA nanoparticles, the signals of non-glycosylated peptides were eliminated and twenty-six N-linked glycopeptides were detected and identified with high intensity and S/N ratio (Fig. 3b). For comparison, the tryptic digest of human IgG was also treated with Fe₃O₄@SiO₂-MSA; only eight glycopeptides could be detected with inferior signal intensity and S/N ratio (Fig. 3c). The enrichment efficiency of Fe₃O₄@SiO₂@PMSA was evidently superior to that of Fe₃O₄@SiO₂-MSA, which can be ascribed to the higher surface density of zwitterionic groups on the material and stronger multivalent hydrophilic interactions. The detailed structures and S/N ratios of the glycopeptides are listed in Tables S3 and S4 (ESI†), respectively. In addition, compared with the HILIC tips (Scheme S1b, S1c†), only seven glycopeptides could be noted and the intensity was relatively low (Fig. 3d) after enrichment. The enrichment performance of Fe₃O₄@SiO₂@PMSA is obviously better than that of the HILIC tips. Subsequently, on the one hand, all the enriched peptides were analyzed by MALDI-TOF MS/MS and the spectrum of a representative glycopeptide (m/z = 2763.8) with an explicit glycan structure is given in Fig. S2 (ESI†), indicating that the enriched peptides all appeared to be glycopeptides. On the other hand, the eluted glycopeptides were deglycosylated by PNGase F and only two deamidated peptides (m/z = 1158.54, 1190.52) were detected (Fig. 3e), which further confirmed that all the peaks appearing in Fig. 3b belonged to N-linked glycopeptides. This result indicates that Fe₃O₄@SiO₂@PMSA nanoparticles have high enrichment selectivity for human IgG enrichment. The surface functional group density of the magnetic composite nanoparticles is of great importance in biological enrichment. In our work, a high density of zwitterionic groups on the polymer chain is critical for achieving high performance in the specific enrichment of glycopeptides.

To further evaluate the universality of these nanoparticles for glycopeptide enrichment, tryptic digests of chicken avidin and HRP were also employed. A direct MALDI-TOF mass spectrum of chicken avidin is presented in Fig. 4c, and the MS signals of glycopeptides are severely suppressed by non-glycosylated peptides. Nevertheless, fifteen glycopeptides with enhanced intensity and S/N ratio dominate the MS spectrum of the treated sample (Fig. 4d, Table S2, ESI†). Moreover, compared to the mass spectrum of the direct analysis of the tryptic digest of HRP in Fig. 4a, fourteen glycopeptides (Fig. 4b, Table S1, ESI†) were identified after enrichment by Fe₃O₄@SiO₂@PMSA. These results indicate the high efficiency and selectivity of Fe₃O₄@SiO₂@PMSA for universal glycopeptide enrichment.

Fig. 3  MALDI-TOF MS spectra of (a) direct analysis of 0.5 pmol tryptic digest of human IgG; (b) after enrichment by Fe₃O₄@SiO₂@PMSA; (c) after enrichment by Fe₃O₄@SiO₂-MSA; (d) after enrichment by HILIC tips; and (e) deglycosylation by PNGase F.

Fig. 4  MALDI-TOF MS spectra of (a) direct analysis of 1 pmol tryptic digest of HRP, (b) after enrichment by Fe₃O₄@SiO₂@PMSA, (c) direct analysis of 1 pmol tryptic digest of chicken avidin and (d) after enrichment by Fe₃O₄@SiO₂@PMSA.
Evaluation of the detection sensitivity, binding capacity and enrichment recovery of Fe₃O₄@SiO₂@PMSA in glycopeptide enrichment

As the level of glycopeptides in a complex biological sample can be extremely low, the detection sensitivity of Fe₃O₄@SiO₂@PMSA towards glycopeptides was investigated. Tryptic IgG digests with low amounts of 50 fmol, 5 fmol and 0.1 fmol were treated by Fe₃O₄@SiO₂@PMSA as well as HILIC tips. As shown in Fig. 5a and d, several glycopeptides were clearly detected in 50 fmol of human IgG tryptic digest after enrichment by both Fe₃O₄@SiO₂@PMSA and HILIC tips. When the amount of human IgG was decreased to 5 fmol, fewer glycopeptides with lower S/N ratios could be detected when enriched by HILIC tips and Fe₃O₄@SiO₂@PMSA as well, as presented in Fig. 5b and e. When the total amount of human IgG was as low as 0.1 fmol, one peptide could still be detected after enrichment with Fe₃O₄@SiO₂@PMSA with a S/N ratio of 7.8 with an m/z of 2795.58. However, no glycopeptides could be identified after treatment with HILIC tips. Furthermore, the resulting detection sensitivity was also higher than for previously reported HILIC materials such as the branched PEG brushes hybrid hydrophilic magnetic nanoparticles (0.5 fmol), zwitterionic polymer brushes hybrid silica nanoparticles (10 fmol) and silica-based click maltose (30 fmol). 23, 44, 45

The lower detection limit may be attributed to the excellent hydrophilicity, absolute magnetic separation, and large amount of MSA groups as well as stronger multivalent hydrophilic interactions. This result shows that the prepared Fe₃O₄@SiO₂@PMSA nanoparticles significantly improve detection sensitivity for glycopeptides.

To inspect the binding capacity of Fe₃O₄@SiO₂@PMSA, different amounts of Fe₃O₄@SiO₂@PMSA (5–100 μg) were added to a fixed amount of human IgG digest (3 μg). After enrichment, the eluted fraction was analyzed by MALDI-TOF MS. The signal intensities of six selected glycopeptides reached a maximum when the added amount of the nanoparticles was 30 μg and the binding capacity was calculated to be 100 mg g⁻¹ (Fig. 6). The large binding capacity might be due to the high amount of zwitterionic groups and the strong multivalent hydrophilic interactions between the glycopeptides and the material.

A stable-isotope dimethyl labeling method 49 was utilized for evaluating the recovery yield of Fe₃O₄@SiO₂@PMSA for glycopeptide enrichment. Briefly, equal amounts of human IgG (3 μg) tryptic digest were labelled with light and heavy isotopes. The heavy-isotope-tagged digest was enriched with Fe₃O₄@SiO₂@PMSA according to the above-mentioned enrichment procedure. The resulting eluent was mixed and the mixture was re-enriched by Fe₃O₄@SiO₂@PMSA, and then the eluent was analyzed by MALDI-TOF MS. The recovery was calculated by the signal intensity ratio of heavy-isotope-labelled glycopeptides divided by light-isotope-labelled glycopeptides. The total recovery yield of six selected peptides was 73.6% (Table 1). This result indicates that the Fe₃O₄@SiO₂@PMSA nanoparticle is an ideal affinity material for the enrichment of glycopeptides.

Application in glycopeptide enrichment from a real complex biological sample

Inspired by the outstanding enrichment efficiency for standard glycoprotein tryptic digests, Fe₃O₄@SiO₂@PMSA nanoparticles were further applied to the analysis of the glycoproteome of mouse liver. 65 μg of proteins was extracted from a mouse liver
and incubated with Fe$_3$O$_4$@SiO$_2$@PMSA as well as with HILIC tips, and the eluent was lyophilized, deglycosylated and analyzed by nano LC-MS/MS, followed by database searching controlling the false discovery rate (FDR) for all peptide identifications including the glycopeptides at <1%. As a result, a total of 905 N-glycosylation sites from 458 N-glycoproteins were identified with a consensus sequence of N-\textbf{P-S/T} motif (Table S5, ESI†). The explicit data of three independent runs are shown in Fig. S3 (ESI†). In comparison, only 457 N-glycosylation sites from 269 N-glycoproteins were identified by HILIC tips. The differences in the structure of these materials contributed to their different performance in glycopeptide enrichment. This result clearly demonstrates that Fe$_3$O$_4$@SiO$_2$@PMSA nanoparticles have high selectivity and effectiveness in the enrichment of low-abundance N-linked glycopeptides from complex biological samples.

Conclusions

In summary, a facile, repeatable, and robust synthetic route for the preparation of a new HILIC material, Fe$_3$O$_4$@SiO$_2$@PMSA nanoparticles, with a well-defined core–shell structure was presented. Characterization results showed that the SiO$_2$ intermediate layer and the ratio of the co-solvent had strong effects on the successful preparation. The thick polymer layer improved the hydrophilicity and the amount of zwitterionic molecules on the polymer chains. Glycopeptide enrichment experiments confirmed that Fe$_3$O$_4$@SiO$_2$@PMSA nanoparticles have high specificity, extremely high detection sensitivity, large binding capacity and satisfactory recovery. In the selective enrichment of N-linked glycopeptides from tryptic digests of proteins extracted from a mouse liver, Fe$_3$O$_4$@SiO$_2$@PMSA shows great practicability in identifying low-abundance glycopeptides in minute amounts of complex biological samples. It can be expected that the as-synthesized Fe$_3$O$_4$@SiO$_2$@PMSA would hold great potential in glycoproteome research.

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Notes and references


