Ligand-free strategy for ultrafast and highly selective enrichment of glycopeptides using Ag-coated magnetic nanoarchitectures†

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Selective enrichment of glycopeptides from complicated biological samples is essential for MS-based glycoproteomics, but still remains a great challenge. In this study, we report an unprecedented ligand-free strategy for the selective enrichment of glycopeptides by simply utilizing the multivalent interaction between glycopeptides and silver nanoparticles (Ag-NPs) coated magnetic nanoarchitectures. The composite microspheres were deliberately designed to be constructed with a high-magnetic-response magnetic colloid nanocrystal cluster (MCNC) core, a poly(methacrylic acid) (PMAA) interim layer and a Ag-NPs functional shell with high coverage. Taking advantage of the reversible interaction of glycans with Ag-NPs and the high magnetic susceptibility of the magnetite core, the MCNC@PMAA@Ag-NPs microspheres possess remarkable selectivity for glycopeptides even at a low molar ratio of glycopeptides/non-glycopeptides (1 : 100) with a very rapid enrichment speed (only 1 min needed) and a simple operation procedure using magnetic separation. Applying this approach, we identified 127 unique glycopeptides mapped to 51 different glycoproteins from only 1 μL rat serum samples. These results clearly demonstrated that the MCNC@PMAA@Ag-NPs have great potential for purifying and identifying the low-abundant glycopeptides in complex biological samples.

Introduction

Over the past decade, multifunctional magnetic nanoarchitectures have been demonstrated to hold great promise for their wide range of applications in the biomedical field.1–12 They allow mechanical sorting, trafficking, and other forms of intricate micromanipulation to be easily performed in complex biological systems, which is achieved simply via the application of an external magnetic field and is highly suitable for proteomics research.13–18 Glycosylation, one of the most common and important post-translational modifications of proteins, is involved in many physiological functions and biological pathways, including protein folding, intracellular sorting, secretion, uptake, and cell recognition.19 Currently, mass spectrometry (MS) has become a powerful tool for the analysis of glycopeptides or glycoproteins.20 However, the inherent low abundance of glycopeptides, the micro-heterogeneity of each glycosylation site and the ion suppression effect caused by the co-existence of nonglycopeptides make the detection of glycopeptides extremely difficult. Analyses of the SWISS-PROT database have revealed that at least 50% of all proteins are glycosylated. Nevertheless, the number of proteins annotated as glycoproteins is now only 10%. This clearly illustrates the great need for the comprehensive and in-depth glycosylation site mapping.21 As a result, different strategies have been developed for the selective enrichment of glycopeptides prior to MS analysis.

Among the enrichment techniques, lectin affinity enrichment based on the specific binding between a lectin and a distinct glycan is the most widely used.22–24 But the drawback is the weak affinity of lectins for the glycan segments and the biased collection of glycopeptides. Besides, hydrazide chemistry-based enrichment has also been frequently used recently.25,26 Typically, the cis-diol groups of carbohydrates on glycoproteins are oxidized to aldehyde groups, which are sequentially captured by hydrazide groups immobilized on a solid surface. Although this method shows good selectivity, the sample complexity is increased by the additional oxidation step and it is solely used for N-linked glycoprotein analysis so far. Boronic acid-based solid phase extraction is another useful method for the enrichment of both N-linked and O-linked glycosylation.27,28 It is based on the
formation of diester bonds between boronic acid and most glycans, but this process often requires a long incubation period. Similarly, these three methods all need a specific ligand, such as lectin, hydrazide or boronic acid to interact with the glycopeptides. Recently, hydrophilic interaction chromatography (HILIC)\textsuperscript{29–32} has become a popular approach for the separation and purification of glycopeptides utilizing the hydrogen bonding between the glycan moiety and the HILIC stationary phase. Compared with other methods, HILIC-based materials require a short fabrication process, have broad glycan specificity, good reproducibility as well as good compatibility with MS analysis.\textsuperscript{33} However, the present HILIC technique is limited by insufficient selectivity and analytical ability at trace level. Therefore, development of a new method for highly and rapidly selective enrichment of glycopeptides is of great importance.

In this study, we propose a novel ligand-free strategy for the selective enrichment of glycopeptides by using Ag nanoparticles (Ag-NPs) coated MCNC@PMAA magnetic nanoarchitectures. These composite microspheres were designed to be comprised of a high-magnetic-response MCNC core and a hydrophilic Ag-NPs modified poly(methacrylic acid) (PMAA) shell. To the best of our knowledge, this is the first report on the strong interaction between Ag-NPs and the glycan of glycopeptides. The as-prepared MCNC@PMAA@Ag-NPs composite microspheres possess both superparamagnetic properties and the ability to selectively fish the glycopeptides out of the pool of predominantly non-glycosylated peptides. The performance was evaluated of these composite microspheres for the selective enrichment of glycopeptides from both laboratory-prepared and real biological samples. The experimental results indicated that our approach possesses high specificity, ultrafast speed and great capability in the selective enrichment of low-abundant glycopeptides from complicated biological samples.

**Experimental methods**

**Materials**

Iron(III) chloride hexahydrate (FeCl\textsubscript{3} \textcdot 6H\textsubscript{2}O), ammonium acetate (NH\textsubscript{4}Ac), ethylene glycol (EG), anhydrous ethanol, trisodium citrate dihydroly, aqueous ammonia solution (25%), methacrylic acid (MAA) and silver nitrate (AgNO\textsubscript{3}, >99.8%) were purchased from Shanghai Chemical Reagents Company and used as received. N,N'-methylenebisacrylamide (MBA) was bought from Fluka and recrystallized from acetone. 2,2-Azobisisobutyronitrile (AIBN) was supplied by Sinopharm Chemical Reagents Company and recrystallized from methanol. γ-Methacryloyloxypropyltrimethoxy-silane (MPS) was obtained from Aladdin Reagent Company. Myoglobin (MYO, 95%), horseradish peroxidase (HRP, 98%), ammonium bicarbonate (ABC, 99.5%), dithiothreitol (DTT, 99%), iodoacetamide (IAA, 99%), α-cyano-4-hydroxycinnamic acid (CHCA, 99%), acetominic (ACN, 99.9%) and trifluoroacetic acid (TFA, 99.8%) were purchased from Sigma. Sequencing grade modified trypsin was from Promega. PNGase F was obtained from New England Biolabs. Bradford assay reagent was obtained from Bio-Rad. All these reagents were used as received without further purification. Deionized water (18.4 MW cm) used for all experiments was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

**Synthesis of MCNC@PMAA core–shell microspheres**

The magnetic MCNC@PMAA core–shell microspheres were prepared according to our prior work.\textsuperscript{34} Typically, 1.350 g of FeCl\textsubscript{3} \textcdot 6H\textsubscript{2}O, 3.854 g of NH\textsubscript{4}Ac and 0.4 g of sodium citrate were dissolved in 70 mL of ethylene glycol. The mixture was stirred vigorously for 1 h at 170 °C to form an homogeneous black solution, and then transferred into a Teflon-lined stainless-steel autoclave (100 mL capacity). The autoclave was heated at 200 °C and maintained for 16 h, and then it was cooled to room temperature. The black product was washed with ethanol and collected with the help of a magnet. The cycle of washing and magnetic separation was repeated several times. The final product was dispersed in ethanol for further use.

Modification of MCNCs with MPS was achieved by adding 40 mL of ethanol, 10 mL of deionized water, 1.5 mL of NH\textsubscript{3} \cdot H\textsubscript{2}O and 0.3 g of MPS into the MCNCs ethanol suspension and vigorously stirring the mixture for 24 h at 60 °C. The obtained product was separated by using a magnet and washed with ethanol to remove excess MPS. The resultant MCNC@MPS nanoparticles were dried in a vacuum oven at 40 °C till constant weight.

Coating a PMAA layer onto the MCNC@MPS nanoparticles was executed by distillation–precipitation polymerization of MAA, with MBA as the cross-linker and AIBN as the initiator, in acetonitrile. Typically, about 200 mg of MCNC@MPS seed nanoparticles were dispersed in 160 mL acetonitrile in a dried 250 mL single-necked flask with the aid of ultrasonication for 10 min. Then a mixture of 1.2 mL of MAA, 300 mg of MBA and 30 mg of AIBN were added to the flask to initiate the polymerization. The flask submerged in a heating oil bath was attached to a fractionating column, Liebig condenser, and a receiver. The reaction mixture was heated from ambient temperature to the boiling state within 30 min and the reaction was ended after about 80 mL of acetonitrile was distilled from the reaction mixture within 1 h. The obtained MCNC@PMAA microspheres were collected by magnetic separation and washed with ethanol in order to eliminate excess reactants and a few generated polymer microspheres.

**Synthesis of MCNC@PMAA@Ag-NPs core–shell–shell microspheres**

The method used to deposit Ag nanoparticles on MCNC@PMAA is similar to that of a previous report.\textsuperscript{35} Typically, 30 mg of AgNO\textsubscript{3} and 20 μL of butylamine were dissolved in 10 mL of ethanol, and then a 5 mL ethanol dispersion containing 5 mg of MCNC@PMAA was added to the above solution. The mixture was transferred into a polypropylene centrifuge tube and the tube was vigorously shaken for 1 h with the aid of a shaker at a speed of 150 rpm at 50 °C. The obtained MCNC@PMAA@Ag-NPs microspheres were collected by magnetic separation and washed with ethanol to remove excess AgNO\textsubscript{3}.
Trypsin digestion of standard proteins and protein mixture from rat serum

The standard protein (HRP or MYO) solution was denatured by incubating at 100 °C for 10 min. After cooling down to room temperature, the samples were treated with 10 mM DTT for 30 min at 57 °C and alkylated with 20 mM IAA at room temperature for 1 h in the dark. Prior to adding trypsin, the solution of the protein was diluted to 1 mg mL⁻¹ with 50 mM ABC buffer. Trypsin was added at an enzyme-to-substrate ratio of 1 : 40 (w/w) and the hydrolysis was allowed to proceed overnight at 37 °C. For the preparation of rat serum protein mixture digestion, 1 mL of rat serum was diluted with 40 μL deionized water and centrifuged at 12 000g for 10 min to remove the lipid. The supernatant was collected and the protein concentration was measured by the Bradford assay. The sample was then subjected to digestion as described above. And the obtained peptides were stored at −20 °C for further analysis. The experiment was performed in compliance with the relevant laws and institutional guidelines. The experiment has been approved by the local authorities of Shanghai, China.

Selective enrichment of glycopeptides with MCNC@PMAA@Ag-NPs

The obtained MCNC@PMAA@Ag-NPs was first washed with ethanol three times and then suspended in deionized water at 15 mg mL⁻¹. Tryptic digests of HRP and MYO or peptides mixture from rat serum were dissolved in 100 μL loading buffer (80% ACN containing 40 mM ABC), then 2 μL MCNC@PMAA@Ag-NPs was added and incubated for 1 min at room temperature. After that, the MCNC@PMAA@Ag-NPs microspheres with captured glycopeptides were separated from the mixed solutions by applying an external magnet. After washing with 200 μL loading buffer, the glycopeptides were released with the elution buffer (20% ACN containing 0.5% TFA) for the further MS analysis.

MALDI mass spectrometric analysis

For the analysis of glycopeptides enriched from the tryptic digests mixture of HRP and MYO, the eluate was deposited on the MALDI probe, and then 1 μL matrix solution (CHCA at 5 mg mL⁻¹ in 50% ACN containing 0.1% TFA) was deposited for MS analysis. MALDI-TOF mass spectrometry analysis was performed in positive reflection mode on a 5800 Proteomic Analyzer (Applied Biosystems, Framingham, MA, USA) with a Nd: YAG laser at 355 nm, a repetition rate of 400 Hz and an acceleration voltage of 20 kV. The range of laser energy was optimized to obtain good resolution and signal-to-noise ratio (S/N) and kept constant for further analysis. External mass calibration was performed by using standard peptides from MYO digests.

Nano-LC–MS/MS analysis of glycopeptides

For the analysis of glycopeptides enriched from rat serum protein mixture digestion, the eluate containing the enriched glycopeptides was lyophilized and redissolved in 50 mM ABC buffer, PNGase F was added to the peptides solution at a concentration of 1 μL PNGase F mg⁻¹ of crude proteins, and the deglycosylation was proceeded for 16 h at 37 °C. The deglycosylated peptides were then subjected to nano-LC–MS/MS analysis. Nano-LC–MS/MS was performed on an HPLC system composed of two LC-20AD nano-flow LC pumps, an SIL-20 AC auto-sampler and an LC-20AB micro-flow LC pump (Shimadzu, Tokyo, Japan) connected to an LTQ-Orbitrap mass spectrometer (ThermoFisher, San Jose, CA). Sample was loaded onto a CAPTRAP column (0.5 × 2 mm, MICHROM Bioresources, Auburn, CA) in 4 min at a flow rate of 25 μL min⁻¹. The sample was subsequently separated by a C₁₈ reverse-phase column (0.10 × 150 mm, packed with 3 μm Magic C₁₈-AQ particles, MICHROM Bioresources, Auburn CA) at a flow rate of 500 nL min⁻¹. The mobile phases were 2% acetonitrile with 0.1% formic acid (phase A) and 95% acetonitrile with 0.1% formic acid (phase B). To achieve proper separation, a 90 min linear gradient from 0 to 45% phase B was employed. The separated sample was introduced into the mass spectrometer via an ADVANCE 30 μm silica tip (MICHROM Bioresources, Auburn CA). The spray voltage was set at 1.8 kV and the heated capillary at 180 °C. The mass spectrometer was operated in data-dependent mode and each cycle of duty consisted of one full-MS survey scan at a mass range of 385–2000 Da with a resolution power of 100 000 using the Orbitrap section, followed by MS/MS experiments for the 10 strongest peaks using the LTQ section. The AGC expectation during full-MS and MS/MS was 1 000 000 and 10 000, respectively. Peptides were fragmented in the LTQ section using collision-induced dissociation with helium and the normalized collision energy value set at 35%. Previously fragmented peptides were excluded for 60 s.

Database search

Tandem mass spectra were extracted by BioWorks version 3.3.1 sp1 (ThermoFisher, San Jose, CA). All MS/MS samples were analyzed using Sequest (version 28; ThermoFisher, San Jose, CA). Sequest was set up to search the rat proteome database from UniProt release 2010_08 assuming the digestion enzyme to be trypsin. The sequence was searched with the following criteria: carboxymethylated cysteines (fixed), oxidized methionines (variable), and an enzyme-catalyzed conversion of asparagine to aspartic acid at the site of carbohydrate attachment (variable), at most 2 missed tryptic cleavage sites, 20 ppm error tolerance in MS and 1.0 Da error tolerance in MS/MS. Trans-Proteomic Pipeline® (version 4.3.1) was used to validate MS/MS based peptide and protein identifications. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Characterization

Field-emission transmission electron microscopy (FE-TEM) images were taken on a JEM-2100F transmission electron microscope at an accelerating voltage of 200 kV. Field-emission scanning electron microscopy (FE-SEM) was performed with a Hitachi S-4800 scanning electron microscope at an accelerating voltage of 20 kV. Samples dispersed at an appropriate concentration were cast onto a glass sheet at room temperature and
sputter-coated with gold. XRD patterns were collected on an X0Pert Pro (Panalytical, The Netherlands) diffraction meter with Cu KR radiation at $\lambda = 0.154$ nm operating at 40 kV and 40 mA. Thermogravimetric analysis (TGA) measurements were performed on a Pyris I TGA instrument. All measurements were taken under a constant flow of nitrogen of 40 mL min$^{-1}$. The temperature was first increased from room temperature to 100 °C and held until constant weight, and then increased from 100 °C to 800 °C at a rate of 20 °C min$^{-1}$. Magnetic characterization was carried out with a vibrating sample magnetometer (VSM) on a Model 6000 physical property measurement system (Quantum, USA) at 300 K. The zeta potentials were measured by a Malvern Nano-HT Zetasizer at 25 °C.

Results and discussion

The detailed fabrication procedures of MCNC@PMAA@Ag-NPs core–shell–shell microspheres with MCNC as core, cross-linked PMAA shell as interim layer and Ag nanoparticles as functional shell are schematically illustrated in Scheme 1a. Briefly, citrate-stabilized MCNC cores with an average diameter of about 300 nm were first synthesized using a modified solvothermal reaction. Then the as-prepared MCNCs were modified by MPS to form the available double bonds on the surface of MCNCs in order to facilitate the encapsulation of the robust PMAA layer by distillation–precipitation polymerization.$^{34}$ The PMAA interim layer is critical for the ultimate application of MCNC@PMAA@Ag-NPs in the selective enrichment of glycopeptides. It not only provides abundant carboxyl groups on the surface of the MCNC@PMAA microspheres, but also shields the contact of the MCNC core with the peptide mixture and provides a highly hydrophilic surface for the sake of decreasing non-specific binding. The deposition of Ag nanoparticles onto the surface of MCNC@PMAA was achieved by a simple in situ reduction of Ag$^+$ in the presence of butylamine.$^{35}$

Representative TEM images of MCNCs and MCNC@PMAA core–shell microspheres are shown in Fig. 1a and b. The MCNCs had an average diameter of ca. 308 nm, and were uniform both in shape and size. After encapsulating with PMAA, the size of the composite microspheres increased to around 380 nm, indicating that the shell thickness was about 36 nm. The obtained MCNC@PMAA microspheres possessed a well-defined core–shell structure and superior dispersibility in many polar solvents. The hydrodynamic diameters ($D_h$) and their distribution of MCNCs and MCNC@PMAA microspheres in water provided additional evidence for the successful coating of the PMAA layer (Fig. S1†). The average $D_h$ and PDI (polydispersity index) for MCNCs and MCNC@PMAA were 332 nm and 0.091, and 586 nm and 0.072, respectively, which meant that the size distributions were very narrow. The existence of a large amount of carboxyl groups on the surface endows the composite microspheres with a highly negative surface charge (the zeta potential of MCNC@PMAA was $-45.2$ mV, lower than that of MCNCs ($-30.8$ mV)), enabling them to strongly interact with positively charged silver ions through robust electrostatic attraction and serve as the nucleation sites. To deposit silver nanoparticles onto the surface of MCNC@PMAA only requires the incubation of the MCNC@PMAA seed microspheres in the ethanol solution of AgNO$_3$ with butylamine as the reducing agent. During the reaction process, a mass of Ag$^+$ was first adsorbed onto the surface of MCNC@PMAA via electrostatic interaction, and then the anchored Ag$^+$ was reduced by butylamine to act as the nucleation centers for the growth of the ultimate Ag nanoparticles shell. This facile method only needs a moderate reaction temperature (50 °C) and a very short reaction time (less than 1 h). Moreover, the feeding amount of the Ag resource (AgNO$_3$) and the reducing agent (butylamine) can be reduced to as low as 10 and 18 mM respectively. Besides, individual pure Ag nanoparticles were not found in the solution, which exhibits rather high reaction efficiency.

The typical TEM image of MCNC@PMAA@Ag-NPs (Fig. 1c) proved that the Ag NPs were successfully coated on the surface of the MCNC@PMAA microspheres. The average $D_h$ (698 nm) and PDI (0.126) give powerful evidence for coverage of the Ag-NPs shell and the great dispersibility of the composite microspheres in water (Fig. S1†). Because of the far fewer carboxyl groups exposed on the outer surface, the zeta potential of MCNC@PMAA@Ag-NPs ($-21.5$ mV) is much higher than that of MCNC@PMAA ($-45.2$ mV). The SEM image shown in

![Scheme 1](image-url)

**Scheme 1** Schematic illustration of (a) the synthetic procedure for the preparation of MCNC@PMAA@Ag-NPs and (b) the selective enrichment process for the glycopeptides using MCNC@PMAA@Ag-NPs microspheres.

Fig. 1 Representative TEM images of (a) MCNCs, (b) MCNC@PMAA (c) MCNC@PMAA@Ag-NPs and (d) SEM image of MCNC@PMAA@Ag-NPs.
Fig. 1d further confirms the existence of Ag NPs shell with high coverage and also indicates that the size of Ag NPs is about 10–30 nm. Energy dispersive X-ray (EDX) spectra and powder X-ray diffraction (PXRD) were recorded to identify the composition of the resulting microspheres. Ag, Fe, and O were the three main elements found in the EDX spectrum (Fig. 2a), indicating that the obtained microspheres were composed of the target composition and have no other impurities. It is necessary to point out that the Cu peaks are derived from the carbon coated copper TEM grid. As shown in Fig. 2b, prior to the deposition of Ag nanoparticles, the MCNCs and MCNC@PMAA microspheres showed a simple PXRD pattern (the peaks are marked with “M”), which could be well ascribed to the typical cubic structure of Fe3O4 (JCPDS card no. 19-629). The average crystallite size in these microspheres can be further calculated by the strongest peak at 35.4° using Scherrer’s formula, the data was around 10.8 nm. After the deposition of Ag NPs, the PXRD pattern for the synthesized MCNC@PMAA@Ag-NPs microspheres was noticeably different from the former pattern, five distinct XRD peaks were clearly observed at 2θ values of 38.1°, 44.3°, 64.4°, 77.4° and 81.5° (marked with “Ag” in the spectrum), which is well indexed to the typical crystallographic planes of the cubic phase of Ag (JCPDS card no. 04-0783). Owing to the fact that the signal coming from Ag is much stronger than that coming from the Fe3O4, the diffraction peaks ascribed to Fe3O4 could hardly be seen in the spectrum of MCNC@PMAA@Ag-NPs except for the strongest peak at a 2θ value of 35.4°. According to the Scherrer’s formula, the average size of the Ag NPs was estimated to be about 22.4 nm by using the strongest peak at 38.1°, which agrees well with the data obtained from the TEM and SEM results.

To quantitatively determine the composition of the composite microspheres, thermogravimetric analysis (TGA) was executed. The TGA curves of MCNC@MPS and MCNC@PMAA (Fig. 3a) show the Fe3O4 weight percentage of these microspheres (the organic components decomposed in high temperature while the inorganic components remained). The 17.8 wt% weight loss of MCNC@MPS is attributed to that of the citrate stabilizer and a small amount of MPS. After the encapsulation of the PMAA layer, the Fe3O4 content declined to 36 wt%. When Ag NPs were introduced to the system, the Fe3O4 and Ag components of MCNC@PMAA@Ag-NPs will all remain their weight during the calcination process. Through calculation, the Fe3O4 content is about 12.4% in weight while the content of Ag can reach up to 65.5 wt%. The magnetic properties of the three kinds of microspheres were studied using a vibrating sample magnetometer (VSM) (Fig. 3b). The magnetic hysteresis curves showed that all the three microspheres have no obvious remanence or coercivity at 300 K, indicating that they all possess a superparamagnetic feature. The superparamagnetism is coming from the small nanocrystals in the MCNC cores, which behave as superparamagnets. As a control, the saturation magnetization (Ms) value of the MCNCs was measured; it reached 70.6 emu g⁻¹. Upon the coating of the PMAA and Ag-NPs layer, the Ms values for the MCNC@PMAA and MCNC@PMAA@Ag-NPs were reduced to 32.3 and

![Fig. 2](https://example.com/f2.png) (a) EDX spectrum of MCNC@PMAA@Ag-NPs and (b) X-Ray diffraction patterns of (i) MCNCs, (ii) MCNC@PMAA, (iii) MCNC@PMAA@Ag-NPs.

![Fig. 3](https://example.com/f3.png) (a) TGA curves and (b) magnetic hysteresis curves of (i) MCNC@MPS, (ii) MCNC@PMAA and (iii) MCNC@PMAA@Ag-NPs.
Accordingly, the Fe$_3$O$_4$ content of these two composite microspheres was estimated to be 37.6 wt% and 14.1 wt% respectively, which agrees well with the TGA results. In addition, the final product of MCNC@PMAA@Ag-NPs microspheres could be separated from the solution in only 20 s when the magnetic field was applied.

The Ag–O interaction has been investigated in detail in coordination chemistry$^{37,38}$ and catalysis.$^{39}$ Glycan, which contains a large amount of hydroxyl groups, ought to have relatively stronger interaction with Ag via the multivalent interaction (Scheme 2). Actually, the multivalent interaction is a synergetic binding between the functional ligand with multiple paws and the specified surface.$^{40}$ In biological systems, most multivalent interactions occur between protein receptors and carbohydrate ligands through hydrogen-bonding and hydrophobic interactions.$^{41–43}$ Compared with weak affinity binding between one ligand and one binding site, i.e. monovalent interaction, multivalent interactions provide greater avidity and specificity, and therefore play unique roles in a broad range of biological activities. Nonetheless, the interaction of glycan on Ag nanoparticles is still comparable with that of water molecules on Ag nanoparticles due to the high molecular weight of glycopeptides. Thus, it provides a possibility to realize the enrichment and eluting process for glycopeptides simply through altering the water content of the solvent.

This thought was then testified using the standard glycoprotein HRP as the model. During the loading and washing process, solvent with low water content (80% ACN aqueous solution containing 40 mM ABC) was used to maximally remove non-glycopeptides from the surface of Ag NPs while glycopeptides could be retained via the Ag-glycan multivalent interaction. After the separation of the microspheres using an external magnetic field and washing with 200 μL loading buffer, the trapped glycopeptides were released by using a small volume of elution buffer with high water content (20% ACN containing 0.5% TFA, 5 μL), and this solution was used for MALDI-TOF MS analysis (Scheme 1b). For comparison, direct analysis of the digest of standard glycoprotein HRP was also performed, as the result presented in Fig. 4a. Without the enrichment procedure, the signals of the non-glycopeptides (mainly at the $m/z$ range of 1000–2000) were much stronger, and their presence greatly suppressed the signals intensity of the glycopeptides (mainly at the $m/z$ range of 2000–5500). However, after selective enrichment with MCNC@PMAA@Ag-NPs (Fig. 4b), the signals of the glycopeptides could be dramatically enhanced and clearly observed. The detailed information of the detected glycopeptides from tryptic digests of HRP by MALDI-TOF mass analysis are listed in Table S1.† What’s more, the incubation time only needs to be 1 min and the whole enrichment process can be completed within 10 min. Additionally, MCNC@PMAA without anchored Ag nanoparticles was used as a control, the enrichment result (Fig. S2†) indicated that the MCNC@PMAA had no binding of the glycopeptides. That is to say that the Ag nanoparticles are exclusively responsible for the observed enrichment.

To further evaluate their ability to selectively capture glycopeptides in complex samples, large amounts of MYO-derived tryptic peptides were added to the tryptic digests of HRP (the molar ratio of MYO to HRP is 50 : 1). As shown in Fig. 5a, due to the presence of high-abundance non-glycopeptides (from the...
MYO), no glycopeptides were detected before enrichment. However, after incubation with MCNC@PMAA@Ag-NPs (Fig. 5b), the glycopeptides from HRP could be easily detected, with a very clean background in the mass spectrum. Even when the amount of non-glycopeptides was 100 times as that of glycopeptides, the glycopeptides could be sensitively detected (data shown in Fig. S3†).

With its great selectivity in mind, we used rat serum to further examine the performance of MCNC@PMAA@Ag-NPs for identifying the glycoproteins in real complex samples. The proteins extracted from rat serum were digested with trypsin and incubated with MCNC@PMAA@Ag-NPs for enriching glycopeptides. The eluted glycopeptides were then deglycosylated by PNGase F and the deglycosylated peptides were subjected to nano-LC–MS/MS analysis. The identity of the N-glycosylated peptide was confirmed by the presence of a consensus Asn-Xaa-(Ser/Thr) sequence, in which Xaa can be any amino acid except proline, and by deamidation on the asparagine residues using PNGase F and the deglycosylated peptides were subjected to nano-LC–MS/MS run, 463 nonredundant peptides were identified, within the 245 nonredundant glycopeptides, 127 unique glycopeptides mapped to 51 different glycoproteins have been identified (Table S2†). Almost all deamidated sites identified using this approach are consistent with the N-glycosylation sites well documented in the UniProt database. Besides, for this total analysis process, only 1 μL rat serum was used. All these results demonstrated that the MCNC@PMAA@Ag-NPs based enrichment strategy is highly selective and sensitive for the identification of glycoproteins in real biological samples.

Conclusion
In summary, well-defined core–shell–shell magnetic composite microspheres comprising a 300 nm sized MCNC core, a PMAA interim layer and an Ag-NPs outer shell were successfully synthesized. The resulting MCNC@PMAA@Ag-NPs microspheres possess high coverage of Ag nanoparticles and high magnetic susceptibility. We found that, with the help of the multivalent interaction between Ag nanoparticles and the glycan of glycopeptides, an ultrafast enrichment speed (within only 1 min) and high specificity (a molar ratio as low as 1 : 100 of glycopeptides/non-glycopeptides could be achieved) could be simultaneously realized. The real sample test further proved that the MCNC@PMAA@Ag-NPs as a novel enrichment nanomaterial is extremely useful for the efficient enrichment of low-abundant glycopeptides from complex biological samples. We also expect that this study can inspire the investigation of the interaction between glycan and other nanomaterials.

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Notes and references