Analytical and Bioanalytical Chemistry (2023) 415:1741–1749
https://doi.org/10.1007/s00216-023-04575-0

RESEARCH PAPER

Surface functionalization modification of ultra-hydrophilic magnetic spheres with mesoporous silica for specific identification of glycopeptides in serum exosomes

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Received: 30 September 2022 / Revised: 8 January 2023 / Accepted: 30 January 2023 / Published online: 15 February 2023

Abstract

Protein glycosylation of human serum exosomes can reveal significant physiological information, and the development of large-scale identification strategies is crucial for the in-depth investigation of the serum exosome glycoproteome. In this study, using surface functionalization techniques, an ultra-hydrophilic mesoporous silica magnetic nanosphere (denoted as Fe_3O_4-CG@mSiO_2) was synthesized for the quick and accurate detection of glycopeptides from HRP digests. The Fe_3O_4-CG@mSiO_2 nanospheres demonstrated outstanding enrichment capability, high sensitivity (5 amol/μL), good size exclusion effect (HRP digests/BSA proteins, 1:10,000), stable reusability (at least 10 times), and an excellent recovery rate (108.6 ± 5.5%). Additionally, after enrichment by Fe_3O_4-CG@mSiO_2, 156 glycopeptides assigned to 64 proteins derived from human serum exosomes were successfully identified, which demonstrates that the nanospheres have great potential for the research of the large-scale serum exosome glycoproteome.

Keywords Fe_3O_4-CG@mSiO_2 · Hydrophilic mesoporous nanospheres · Glycopeptide enrichment · Exosomes · Nano-LC–MS/MS

Introduction

Exosomes are a kind of extracellular vesicle including the cellular components (proteins, DNA, and RNA) that secrete them [1]. According to research, the post-translational modification of exosome proteins is closely linked to the incidence and progression of disease, and many exosome surface proteins and marker proteins are glycoproteins [2]. As a result, further research into the glycosylation modification of exosome proteins is critical. N-glycosylation plays important physiological and biological roles in the immune response, molecular recognition, cell adhesion, and signal transmission, among others [3–5]. Previous studies have found that aberrant glycosylation can influence the onset and progression of neurodegenerative diseases, diabetes, and various other conditions [6, 7]. Therefore, the investigation of protein glycosylation of exosomes is of great practical value and may have application in the clinical area.

In recent decades, mass spectrometry (MS) has become an efficient analysis tool for in-depth investigation of the glycoproteome [8, 9]. Nevertheless, MS-based methods still face some challenges, including low ionization efficiency, the occurrence of high-abundance non-glycopeptides, and the interference caused by salt, which severely suppresses the mass spectrometry signal of low-abundance glycopeptides [10]. Directly employing MS to characterize glycopeptides may produce poor results. Therefore, prior to MS analysis, it is important to design a method for specifically enriching glycopeptides from complex biological sample systems.

To date, substantial efforts have been devoted to developing strategies for glycopeptide enrichment before MS detection, including chemical hydrazide, boronate affinity

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chromatography, lectin affinity chromatography, and hydrophilic interaction liquid chromatography (HILIC) [11, 12]. Because of their simple operation, good reproducibility, superior compatibility with MS, and selective enrichment of multiple glycopeptides, the HILIC-based strategies have become the most commonly used methods [13–17]. Zwitterionic hydrophilic (ZIC-HILIC) materials stand out among a multitude of HILIC stationary phases because they include both positive and negative groups, which increases their hydrophilicity significantly [18–23]. Previously, our group employed a one-step hydrothermal method to prepare magnetic ZIC-HILIC Fe_3O_4-CG composites, which exhibited good glycopeptide enrichment capacity. However, the composites do not reveal effective size exclusion properties, so we have been eager to design a ZIC-HILIC material with good size exclusion capacity for the specific identification of glycopeptides [24].

Mesoporous materials have seen significant development in recent years due to their structural advantages, including huge specific surface area, simple synthesis methods, tunable pore size, and homogeneous pore channels [25, 26]. As a result, various functional mesoporous materials have been employed to adsorb low-abundance target peptides and exclude high-abundance proteins [27, 28]. For example, Zheng's group coated mesoporous polydopamine on a graphene oxide substrate, and then modified it with arginine. The composites produced have high glycopeptide enrichment capacity and can be efficiently used to enrich glycopeptides from biological samples [29]. Additionally, Wang's group developed a zwitterionic hydrophilic mesoporous silica material (denoted as Fe_3O_4@SiO_2@Au@mSiO_2@L-Cys) as a hydrophilic platform for glycopeptide enrichment [30]. However, the small specific surface area, cumbersome synthesis steps, large steric hindrance, and poor hydrophilicity of these HILIC mesoporous materials limit their further application. Thus, to boost glycopeptide enrichment efficiency, HILIC mesoporous materials with facile synthesis methods, large specific surface area, small steric hindrance, and strong hydrophilicity are urgently needed.

Herein, we designed an ultra-hydrophilic mesoporous silica magnetic nanosphere (denoted as Fe_3O_4-CG@mSiO_2, where C refers to cysteine and G refers to glutathione) for highly selective and sensitive glycopeptide enrichment. Firstly, ultra-hydrophilic bifunctional Fe_3O_4-CG nanospheres were synthesized through Fe–S interaction, which provides superparamagnetic properties for fast and efficient solid–liquid separation [31]. In addition, the nanospheres uniquely combine the properties of L-cysteine (Cys) and reduced glutathione (GSH), which overcomes the limitation of large steric hindrance and greatly enhances the hydrophilicity of the material. Afterwards, an ordered mesoporous silica layer was modified on the surface of the Fe_3O_4-CG to exclude large proteins and for specific capture of glycopeptides. The Fe_3O_4-CG@mSiO_2 nanospheres show great potential for use in large-scale glycoproteomics research and may contribute to the clinical diagnosis of illness in the future.

Materials and methods

Materials and chemicals

Ethylene glycol, iron chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), sodium acetate (CH_3COONa), acetonitrile (ACN), ammonium bicarbonate (NH_4HCO_3), phosphoric acid (H_3PO_4), and hydrochloric acid (HCl) were purchased from Aladdin. Bicarbonate (TEAB), CD_2O (20 wt%, 98% D), formaldehyde (CH_2O, 37%), cyanoborohydride ($NaBH_3CN$), horseradish peroxidase (HRP), bovine serum albumin (BSA), 2,5-dihydroxybenzoic acid (DHB), dithiothreitol (DTT), and iodoacetamide (IAA) were purchased from Sigma-Aldrich. Peptide-N-glycosidase F (PNGase F) was purchased from Genetimes Technology. Hexadecyl trimethyl ammonium bromide (CTAB), formic acid, sodium hydroxide (NaOH), Cys, GSH, and tetraethyl orthosilicate (TEOS) were purchased from Macklin Biochemical Co.. Human serum samples were acquired from the Affiliated Hospital of Medical School, Ningbo University. All deionized water was processed using a Milli-Q system.

Pretreatment of standard protein and serum

In 100 μL of deionized water, HRP (1 mg) and BSA (2 mg) were dissolved, respectively. The proteins were denatured in boiling water at 100 °C for 10 min after ultrasonication for 5 min. After cooling the denatured protein sample at room temperature, 100 μL of ammonium bicarbonate (50 mmol/L) was added along with the addition of trypsin at a protein-to-trypsin ratio of 40:1. The mixture was incubated for 16 h at 37 °C. The resultant solutions were stored at −20 °C.

To reduce viscosity, the serum sample was diluted with an equal volume of phosphate-buffered saline (PBS) solution. The diluted serum sample was centrifuged for 3 min at 4 °C at 3000×*g*. The supernatant was placed into 1 mL tubes and centrifuged for 45 min at 4 °C at 12,000×*g*. To obtain the serum exosomes, the supernatant was filtered through a 0.22 μm filter. The serum exosomes were suspended in an ice-cold buffer containing 50 mM Tris–HCl and 8 M urea and sonicated for 30 min for lysis. The exosome protein solution was mixed with 10 mM DTT and incubated at 37 °C for 4 h before alkylation in 20 mM IAA for 1 h in the dark. The proteins were then digested overnight at 37 °C with trypsin (enzyme/protein ratio 1:40). Finally, the tryptic digests were desalted, lyophilized, and stored for further use.

Preparation of Fe_3O_4-CG

Bifunctional hydrophilic Fe_3O_4-CG nanospheres were synthesized using a one-step solvothermal technique. The precise synthesis processes were consistent with those reported previously [32]. To begin, 1.35 g of iron chloride hexahydrate was dispersed in 75 mL of ethylene glycol. The blended solution was then mechanically agitated for 30 min, after which it was ultrasonically dispersed for a few minutes with 3.6 g anhydrous sodium acetate. Following that, 30.733 mg GSH and 6.058 mg Cys were simultaneously added to the combined solution and agitated for 2 h; the solution was then transferred to a reaction vessel. Finally, the solution was heated for 16 h at 200 °C. The product was cleaned three times with ethanol and deionized water followed by vacuum drying overnight.

Preparation of Fe_3O_4-CG@mSiO_2 nanospheres

The specific synthesis steps for Fe_3O_4-CG@mSiO_2 nanospheres were consistent with those reported previously, with minor revisions [33]. Briefly, 100 mg Fe_3O_4-CG nanospheres and 1 g CTAB were added to 100 mL deionized water and ultrasonically dispersed for 30 min until they were dispersed completely. Next, 100 mL of NaOH solution (10 mM) and 800 mL of deionized water were added to the solution, and the liquid obtained was stirred for 0.5 h at 60 °C. Afterwards, 2.5 mL of TEOS/ethanol (volume ratio of 1:4) was added to the solution and heated for 12 h at 60 °C. The finished product was washed three times each with deionized water and ethanol and then vacuum-dried for an entire night. To remove CTAB, the nanospheres were transferred to a muffle furnace and calcined for 4 h at 350 °C.

The procedure for glycopeptide enrichment from standard proteins and serum exosomes

The procedure for the enrichment of glycopeptide from standard proteins is exhibited in Fig. 1. Briefly, 0.25 mg of Fe_3O_4-CG@mSiO_2 was dissolved in 200 µL of loading buffer (95% ACN/3% TFA, V/V) containing 100 fmol/µL HRP digestion. Afterwards, the mixed solution was vibrated for 30 min at 37 °C. Then, the glycopeptide-loaded Fe_3O_4-CG@mSiO_2 was magnet-separated, and was washed three times in washing buffer (85% ACN/0.5% H_3PO_4, V/V). A centrifuge tube was then filled with 10 µL of eluent (30% ACN) and vibrated for 30 min at 37 °C. The eluent was then collected and analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS.

The process for enriching glycopeptides from serum exosomes was as follows. In brief, 0.5 mg of Fe_3O_4-CG@mSiO_2 was dissolved in 100 µL of loading buffer containing a predetermined quantity of serum exosomes. The mixed solution was then shaken for 30 min at 37 °C. After magnetic separation, the glycopeptide-loaded Fe_3O_4-CG@mSiO_2 was washed five times in washing buffer. Glycopeptides were collected after elution with 2 × 10 µL of the eluent. After lyophilizing the eluent and resolving it in NH_4HCO_3 solution (25 mM), PNGase F was added, and the mixture was vibrated for 16 h at 37 °C. The resulting liquid was lyophilized before detection using nanoscale liquid chromatography–tandem mass spectrometry (nano-LC–MS/MS).

Evaluation of the enrichment recovery

Two microliters of light-tagged HRP digestion was first enriched by Fe_3O_4-CG@mSiO_2 nanospheres, and the

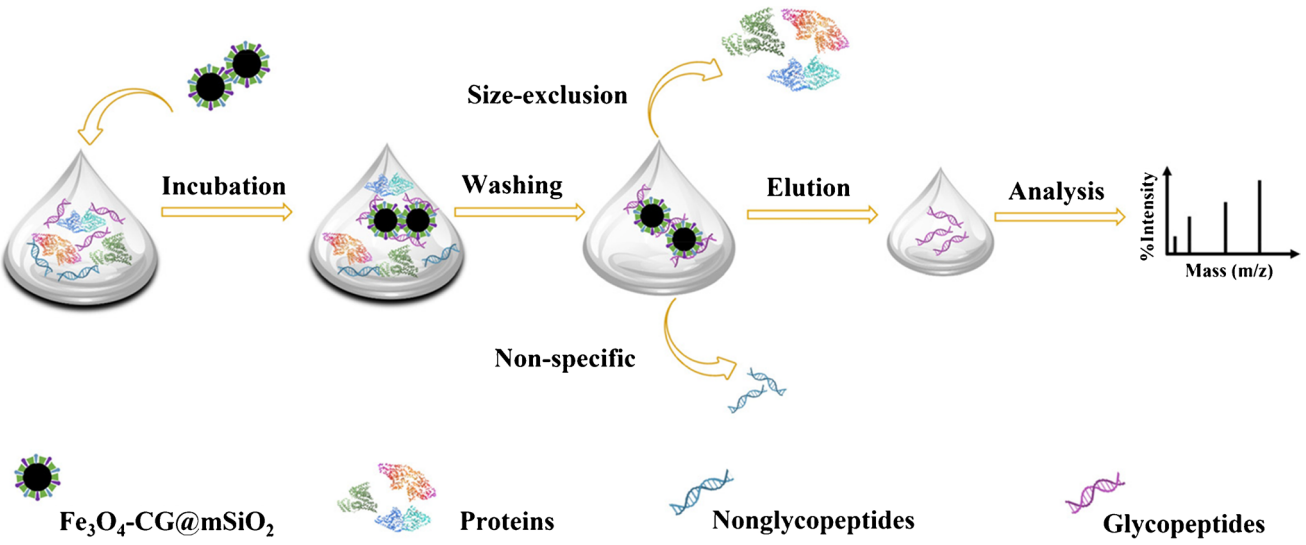

Fig. 1 The enrichment workflow of glycopeptides by Fe_3O_4-CG@mSiO_2

eluent was collected and combined with 2 μL of heavy-tagged HRP digestion. The resultant solution was then enriched with Fe_3O_4-CG@mSiO_2, and the eluent was used for MALDI-TOF MS analysis. The intensity ratio of light- and heavy-tagged glycopeptides was assessed in three parallel experiments to confirm the enrichment recovery.

Characterization of Fe_3O_4-CG@mSiO_2 nanospheres

The microscopic morphology of Fe_3O_4-CG@mSiO_2 was examined using transmission electron microscopy (TEM) images taken with a JEOL JEM-2100F microscope (Japan). The surface morphology and elemental composition of Fe_3O_4-CG@mSiO_2 were observed using scanning electron microscopy (SEM) images and energy-dispersive X-ray spectroscopy (EDX) obtained using a Hitachi S-4800 microscope (Japan). The crystal structure of Fe_3O_4-CG@mSiO_2 was studied using X-ray diffraction (XRD). GSH and Cys dual-functional Fe_3O_4-CG@mSiO_2 nanospheres were validated using Fourier transform infrared (FT-IR) spectroscopy.

Results and discussion

Preparation and characterization of Fe_3O_4-CG@mSiO_2

The procedure for the preparation of dual-functional hydrophilic mesoporous nanospheres (Fe_3O_4-CG@mSiO_2) is exhibited in Fig. 2. Two hydrophilic amino acids (Cys and GSH) were added during the preparation of Fe_3O_4, and the Fe_3O_4-CG nanospheres were synthesized by a one-step solvothermal method. Next, using a template technique and CTAB as a structure-directing agent, a layer of mesoporous silica was modified on the surface of the Fe_3O_4-CG. To verify whether the nanospheres were successfully prepared and assess their application potential in terms of enrichment of glycopeptides, we conducted the following characterizations and experiments.

SEM and TEM were used to determine the shape and size of the Fe_3O_4-CG@mSiO_2 nanospheres. The produced nanospheres, which are shown in Fig. 3a, have a nice spherical form and a diameter of between 200 and 300 nm. Additionally, as seen in the SEM image (Fig. 3b and c), the nanospheres are uniform in size and distribution. EDX

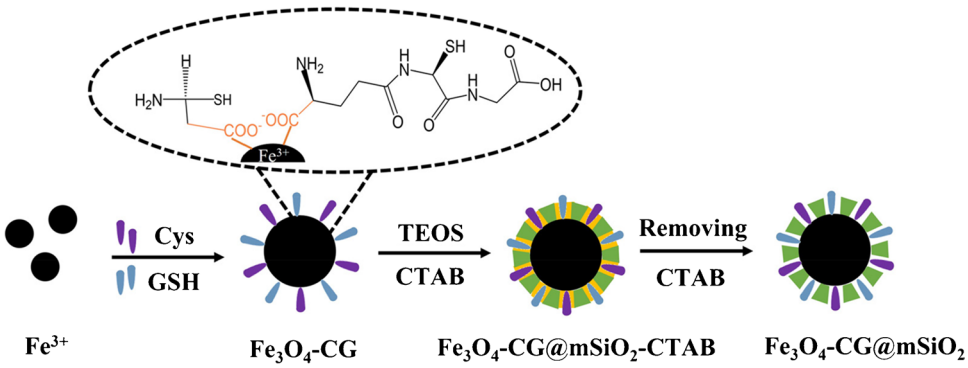

Fig. 2 Schematic representation of the preparation process for the Fe_3O_4-CG@mSiO_2

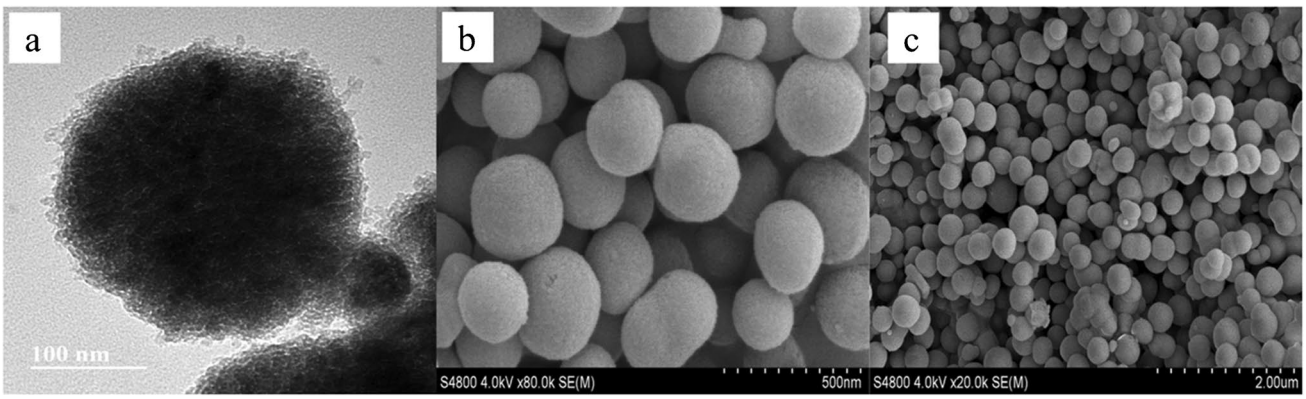

Fig. 3 TEM image (**a**) and SEM images (**b**, **c**) of the Fe_3O_4-CG@mSiO_2

was employed to observe the elemental composition of Fe_3O_4-CG@mSiO_2. As seen in Fig. S1, the existence of elements N, O, C, Fe, Si, and S demonstrated the successful synthesis of Fe_3O_4-CG@mSiO_2 nanospheres. The crystal structure of the nanospheres was characterized by XRD, and Fig. S2 shows that the locations of the six primary distinctive diffraction peaks are compatible with the earlier studies [34, 35]. FT-IR was used to verify that two hydrophilic amino acids and mesoporous silica were successfully coated on the surface of the Fe_3O_4 in turn. According to Fig. S3, the Fe–O stretching vibration was assigned to the peak at 575 cm^{-1}, and the Cys and GSH characteristic peaks were attributed to the peaks at 1260 cm^{-1}, 1534 cm^{-1}, and 1639 cm^{-1}. Si–O–Si symmetric and asymmetric stretching vibrations, respectively, were given the vibrational frequencies of 798 cm^{-1} and 1084 cm^{-1}. The outcomes demonstrated the function groups and layer were successfully modified.

Specific enrichment of glycopeptides from standard proteins with Fe_3O_4-CG@mSiO_2

HRP digestion was utilized as a standard sample to evaluate the enrichment capability of the Fe_3O_4-CG@mSiO_2nanospheres toward glycopeptides. For comparison, Fe_3O_4-CG was chosen to enrich glycopeptides under the same conditions. In Fig. 4a, before enrichment, only four characteristic peaks of glycopeptides appear in the spectrum. After enrichment by Fe_3O_4-CG, 16 glycopeptide peaks can be clearly observed, and the intensity and number of glycopeptide peaks are obviously increased. In addition, the spectrum background is clean, which confirmed the relatively lower interference. The effect is better, but still not ideal. After enrichment by Fe_3O_4-CG@mSiO_2, a total of 19 glycopeptide peaks can be observed, and the intensity of the glycopeptide peaks is further increased. This phenomenon confirmed the great enrichment performance of Fe_3O_4-CG@mSiO_2 nanospheres toward glycopeptides. Table S1 of the supplementary material contains the complete information for glycopeptides derived from HRP digestion after enrichment by Fe_3O_4-CG@mSiO_2.

We performed further tests to investigate the detection limit for assessment of the enrichment capability of Fe_3O_4-CG@mSiO_2 nanospheres. The majority of glycopeptides can be observed in Fig. 5a, when the concentration of the HRP digestion is 5 fmol/μL. The number of glycopeptide peaks decreased significantly when the HRP digestion concentration was further diluted to 0.5 fmol/μL; however, nine glycopeptides still remained on the spectrum. (Fig. 5b). After diluting the HRP digestion 10 times to 0.05 fmol/L, six glycopeptide peaks were seen (Fig. 5c). Four glycopeptides

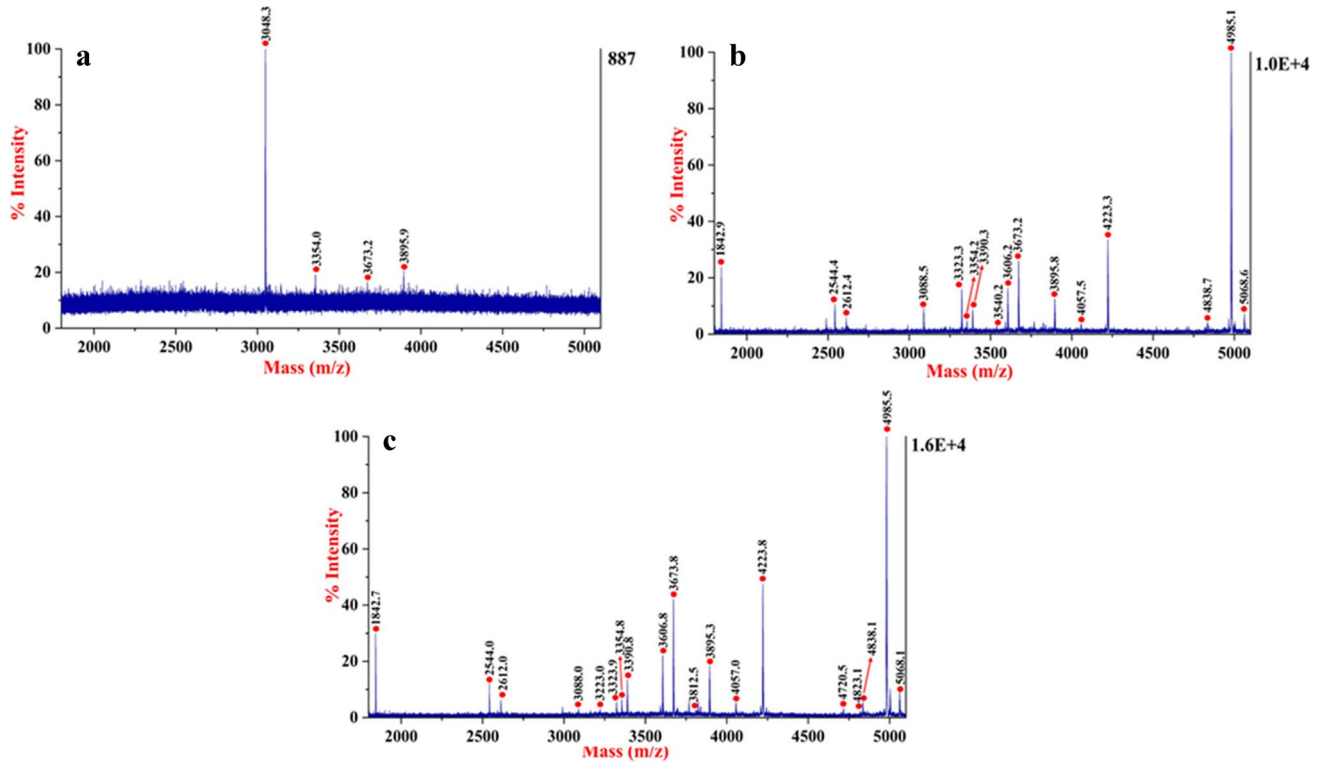

Fig. 4 MS spectra of 100 fmol/μL HRP digest (**a**) before enrichment and enriched by (**b**) Fe_3O_4-CG and (**c**) Fe_3O_4-CG@mSiO_2. Glycopeptide peaks are denoted as "●"

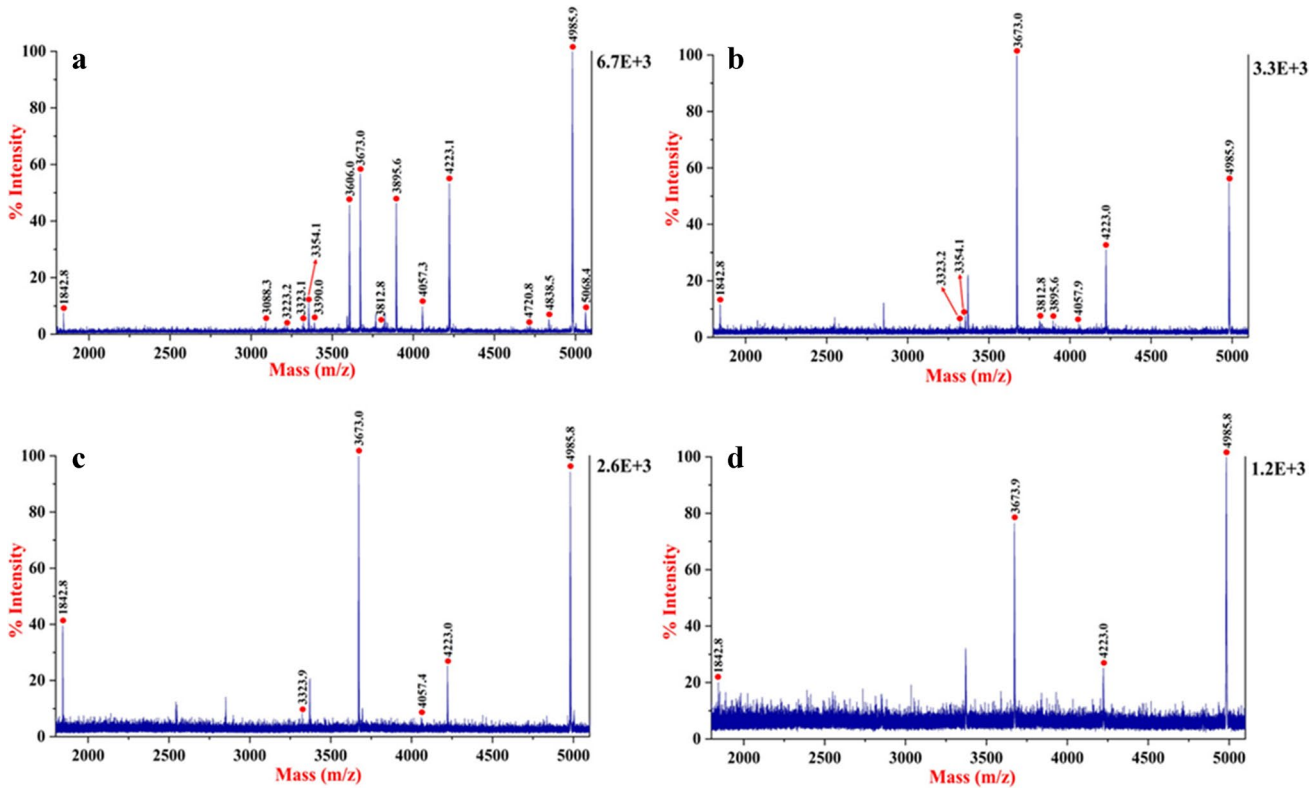

Fig. 5 MS spectra of glycopeptides enriched from different concentrations of HRP digests, (**a**) 5 fmol/μL, (**b**) 0.5 fmol/μL, (**c**) 0.05 fmol/μL, and (**d**) 0.005 fmol/μL, using Fe3O4-CG@mSiO$_2$. Glycopeptide peaks are denoted as "●"

were still detectable at concentrations as low as 0.005 fmol/μL, and just a few non-glycopeptides were visible on the spectrum (Fig. 5d).

Meanwhile, the size exclusion property of Fe_3O_4-CG@$mSiO_2$ nanospheres was demonstrated by adopting various proportions of BSA protein as interference mixed into β-casein digestion. As seen in Fig. 6a, when the ratio of BSA protein to HRP digestion was 1:500, most of the glycopeptide peaks could be observed, and the background of the spectrum remained clean and the peak intensity was still relatively high. When the ratio of BSA protein to HRP digestion increased to 1:1000, 12 glycopeptides could be identified. Further expanding the ratio of BSA protein (Fig. 6b), 11 glycopeptides could be captured with the Fe_3O_4-CG@$mSiO_2$ nanospheres when the ratio of BSA protein to HRP digestion was 1:5000 (Fig. 6c). Even when the ratio of BSA protein to HRP digestion reached 1:10,000, seven glycopeptides could still be enriched with the Fe_3O_4-CG@$mSiO_2$ nanospheres, and almost no non-glycopeptide peaks were present in the spectrum (Fig. 6d).

In addition, we designed experiments to investigate the reusability of Fe_3O_4-CG@$mSiO_2$ nanospheres through a cyclic experiment. As shown in Fig. S4a, after four cycles, 18 glycopeptide peaks could be captured. At the same time, after 7 and 10 cycles, we can find that there was no obvious difference in the peak number or peak intensity (Fig. S4b and Fig. S4c). The result demonstrated that the Fe_3O_4-CG@$mSiO_2$ nanospheres have superior reusability.

Finally, to assess the enrichment recovery of the Fe_3O_4-CG@$mSiO_2$ nanospheres toward HRP digestion, we used the stable isotope dimethyl labeling technique. The recovery rate was calculated as the ratio of the peak intensity of the light-labeled glycopeptide to the equivalent peak intensity of the heavy-labeled glycopeptide. The outcomes are displayed in Fig. S5. After three parallel experiments, we measured the average recovery of the Fe_3O_4-CG@$mSiO_2$ nanospheres as 108.6 ± 5.5%. The experimental results demonstrate that the material has good recovery capability.

Specific enrichment of glycopeptides from serum exosomes with Fe_3O_4-CG@$mSiO_2$

After the encouraging results of the preceding experiments, we assessed the use of the Fe_3O_4-CG@$mSiO_2$ nanospheres on actual biological samples. Exosomes have attracted significant research interest due to their widespread application in the clinical field. However, so far, the investigation of glycopeptides in serum exosomes is rare. Therefore, to verify the specific capture ability of the material for glycopeptides, we used the Fe_3O_4-CG@$mSiO_2$ nanospheres to

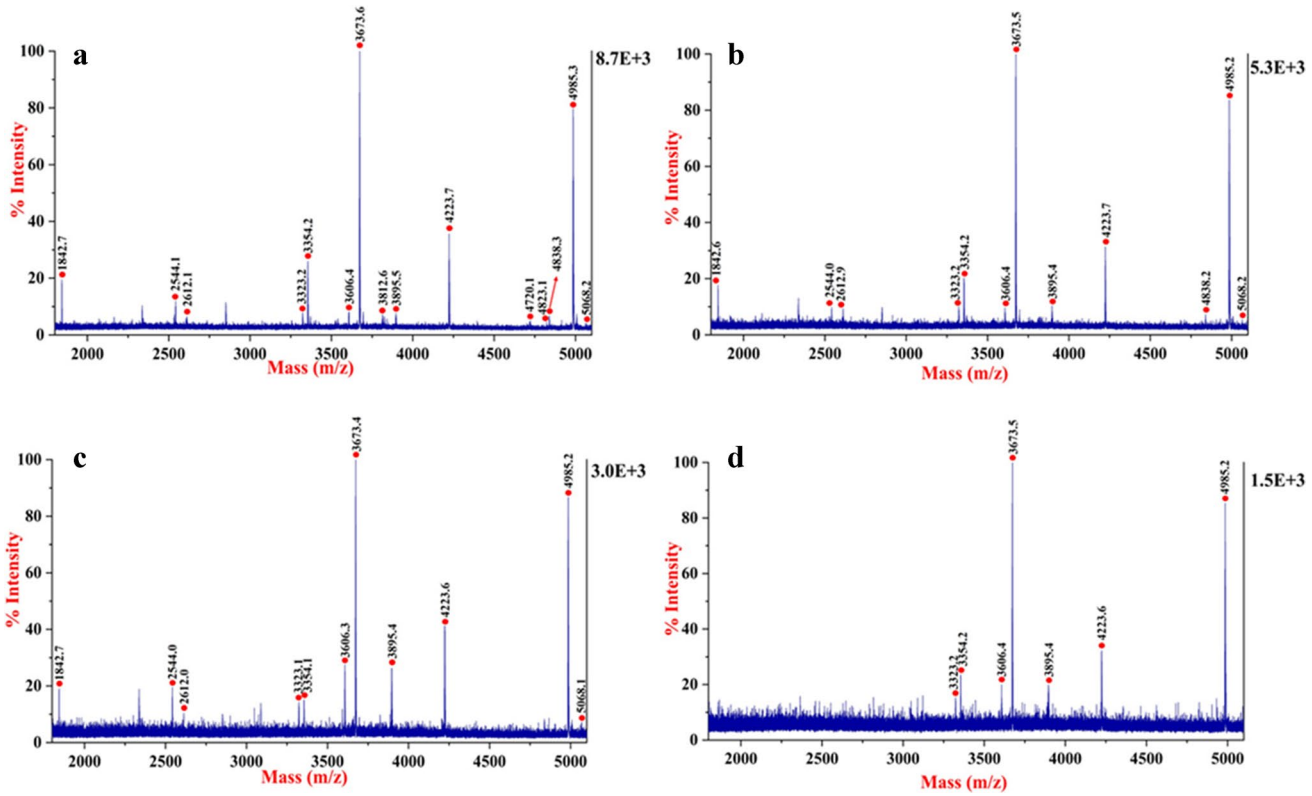

Fig. 6 MS spectra of enriched glycopeptides from a mixture of HRP digests and BSA proteins at molar ratios of (**a**) 1:500, (**b**) 1:1000, (**c**) 1:5000, (**d**) 1:10,000. Glycopeptide peaks are denoted as "●"

enrich glycopeptides in serum exosomes. After treatment by Fe_3O_4-CG@mSiO_2 nanospheres, we could detect 156 glycopeptides assigned to 64 proteins with nano-LC–MS/MS. Table S2 of the supplementary material contains the complete information for glycopeptides derived from the human serum exosomes after enrichment by Fe_3O_4-CG@mSiO_2. The superior specific capture capacity of Fe_3O_4-CG@mSiO_2 nanospheres makes it easy to efficiently capture glycopeptides from serum exosomes, which facilitates large-scale glycoproteomic research. Further, the nanospheres are likely to have great application value in clinical diagnosis in the future.

Conclusions

In summary, ultra-hydrophilic mesoporous silica magnetic nanospheres Fe_3O_4-CG@mSiO_2 were prepared via solvothermal and surface functionalization methods. The Fe_3O_4-CG@mSiO_2 nanospheres exhibited good glycopeptide enrichment effects due to the large specific surface area and small steric hindrance. Simultaneously, the Fe_3O_4-CG@mSiO_2 demonstrated superparamagnetic properties for fast and efficient solid–liquid separation. The Fe_3O_4-CG@mSiO_2 nanospheres showed remarkable performance of glycopeptides enrichment, including great sensitivity, the size exclusion effect, recovery rate, and sustained reusability. Moreover, the application of the Fe_3O_4-CG@mSiO_2 nanospheres for the enrichment of glycopeptides in serum exosomes achieved excellent results, with the successful identification of 156 glycopeptides assigned to 64 proteins derived from human serum exosomes. In short, the high capacity of Fe_3O_4-CG@mSiO_2 to specifically capture glycopeptides in standard digestion and serum exosomes will facilitate large-scale glycoproteome research and may have great potential in clinical diagnosis.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00216-023-04575-0.

Acknowledgements This research was funded by Ningbo major science and technology project (2022Z133), the medical health Project of Health Department of Zhejiang Province (2021KY1045), Ningbo public welfare science and technology project (2021S104).

Ethically approved human serum samples used in this research were collected with the consent of volunteers. The study was conducted with the approval of the experimental ethics committee of Ningbo University and its affiliated hospital.

Declarations

Consent for publication All of the authors have given approval for the final version of the manuscript.

Competing interests The authors declare no competing interests.

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