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Magnetic bead-based hydrophilic interaction liquid chromatography for glycopeptide enrichments

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ABSTRACT

Purification of glycopeptides prior to the analysis by mass spectrometry (MS) is demanded due to ion suppression effect during ionization caused by the co-presence of non-glycosylated peptides. Among various purification methods, hydrophilic interaction liquid chromatography (HILIC) has become a popular method in recent years. In this work, we reported a novel magnetic bead-based zwitterionic HILIC (ZIC-HILIC) material which was fabricated by coating a zwitterionic polymer synthesized by spontaneous acid-catalyzed polymerization of 4-vinyl-pyridinium ethanesulfonate monomer on iron oxide magnetic nanoparticles. The resulting magnetic ZIC-HILIC nanoparticles were shown to provide high specificity and high recovery yield (95–100%) for the enrichment of glycopeptides from a standard glycoprotein, fetuin, using a simple magnetic bar. In addition, we proposed a two-step HILIC enrichment strategy using magnetic ZIC-HILIC nanoparticles for a large scale analysis of glycoproteins in complex biological samples. Using this approach, we identified 85 N-glycosylation sites in 53 glycoproteins from urine samples. Two novel glycosylation sites on N^{513} of uromodulin and N^{470} of lysosomal alpha-glucosidase which have not yet been reported were identified by two-step HILIC approach. Furthermore, all these identified sites were confirmed by studies conducted using PNGase F deglycosylation and ^{18}O enzymatic labeling.

1. Introduction

Glycosylation is one of the most common protein post-translational modifications (PTMs) which is critical to cell growth control, cell migration, cell adhesiveness, tissue differentiation, and inflammatory reaction cascades [1,2]. It is estimated that more than 50% of eukaryotic proteins are glycosylated and the glycosylation sites are mainly on Asn residue with N-linked glycans or on Ser/Thr residue with O-linked glycans [3,4]. However, only low percentage of annotated glycoproteins is reported in database that may reflect the lack of robust analytical platforms for the study of protein glycosylation [5].

Mass spectrometry (MS) is one of the most powerful tools for protein identification including the identification of PTMs and protein expression profiling. Typically, protein mixtures or proteins purified by 2D gel electrophoresis were digested by trypsin and the digests were analyzed by MS or MS/MS to generate the information of peptide masses or peptide fragments through database matching. However, comprehensive analysis of glycoproteins in a complex sample by MS so far is still very challenging because it involves not only the determination of peptide sequence but also the determination of glycan branching points and linkages of the carbohydrate monomers [6]. Furthermore, direct identification of protein glycosylation in a complex protein digest by MS is still difficult due to the lower detection sensitivity of glycopeptides compared to non-glycosylated peptides. Thus, ion suppression effect could weaken the signal of glycopeptides when non-glycosylated peptides are co-present with glycopeptides, especially if the glycan is terminated with a negatively charged sialic acid moiety. Moreover, the high glycan heterogeneity leads to high complexities for structural identification and the spreading of the overall glycopeptide signal [6]. Thus, the common procedure for a comprehensive glycoprotein characterization requires purification of the glycoprotein, release of the glycans by either chemical or enzymatic cleavage, the analysis of glycosylation site on protein, and the analysis of glycan structure [7]. Removal of the glycan from glycopeptides prior to MS analysis can dramatically enhance the peptide ion signal and improve peptide backbone fragmentation under collision-induced dissociation (CID) since the positive charge borne by the deglycosylated peptide is more

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suitable for an MS detection than the native glycopeptides which bear negative charges [8]. Nevertheless, an efficient purification of glycopeptides from complex sample prior to MS analysis is the most critical step for pursuing distinct and enhanced MS signals.

Methods for enrichment of glycoproteins and/or glycopeptides from a complex mixture include lectin affinity chromatography, periodate oxidation followed by hydrazide coupling, boronate affinity chromatography, or glycosylation-specific antibodies [9–13]. Recently, hydrophilic interaction chromatography (HILIC) has become a popular method used for glycopeptide enrichment [14–17]. Using HILIC approach, glycopeptides can be enriched based on their relatively higher hydrophilicity compared to most tryptic peptides. The enriched glycopeptides can circumvent the suppression effect caused by abundant non-glycopeptides and provide enhanced MS signals [18,19].

Typically, HILIC is performed by the use of a hydrophilic stationary phase and a hydrophobic organic mobile phase, which was originally described by Linden et al. in 1975 [20]. In contrary to reversed phase liquid chromatography, hydrophilic compounds are retained longer than hydrophobic compounds. Therefore, HILIC is commonly regarded as normal-phase liquid chromatography (NP-LC). However, HILIC is compatible with water and water-miscible organic solvents, while NP-LC is only compatible with organic solvents, which is limited the use of NP-LC for biological samples [21,22]. The popular HILIC materials include siloxane [23], cation exchange [24,25], anion exchange [26], amide-based [27], zwitterionic (ZIC)-based [10], and saccharides [28] stationary phases available from commercial sources. Among these stationary phases, ZIC-HILIC has been shown better hydrophilic interaction toward glycopeptides than other stationary phases [29–32]. Currently, ZIC-HILIC has been widely used in glycopeptide enrichment as well as two-dimensional LC–MS/MS for comprehensive proteome studies [33–36]. Due to analytical benefits such as easy-to-operate, short operation time, and high loading capacity, some magnetic bead-based separation platforms which contained lectine and phenylboronic acid moieties have been developed for glycopeptides enrichment [37,38]. However, the development of HILIC-based magnetic beads has not been well documented. Currently, Resemann et al. developed magnetic ZIC-HILIC beads for enrichment of neutral and acidic glycopeptides in a poster abstract however the details about the beads fabrication and efficiency were not mentioned [39].

In this study, ZIC-HILIC magnetic beads were fabricated by a novel approach including the preparation of zwitterionic polymers *via* acid-catalyzed polymerization of 4-vinyl-pyridinium ethanesulfonate and the coating of these polymers on PAA-coated iron oxide nanoparticles. The efficiencies of ZIC-HILIC beads were demonstrated by the enrichment of glycopeptides from standard glycoprotein digests. The applicability of these beads toward real biological samples was further demonstrated by a large-scale analysis of N-glycosylation sites from urine samples.

2. Experimental

2.1. Materials

Acetonitrile (ACN), ammonium bicarbonate (ABC), 1,4-dithiothreitol (DTT), ethylene glycol, dimethylformamide (DMF) and formaldehyde (37% solution in H_2O) were purchased from J.T. Baker (NJ, USA). Iron (III) chloride hexahydrate, urea, and sodium cyanoborohydride were purchased from Riedel-de Haën (Seelze, Germany). Formic acid (FA), sulfuric acid, sodium hydroxide, 4-vinylpyridine, sodium 2-bromoethanesulfonate, trichloroacetic acid (TCA), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC hydrochloride), polyacrylic acid (average Mw ~100,000, 35 wt% in H_2O), fetuin (from fetal calf serum), formaldehyde-D_2 (20% solution in D_2O), iodoacetamide (IAA), and water-^{18}O (97 at.% ^{18}O) were purchased from Sigma–Aldrich (Steinheim, Germany). Ferrous chloride tetrahydrate was from Fluka (Buchs, Germany). Sequencing grade modified trypsin was obtained from Promeg (WI, USA). PNGase F was purchased from New England BioLabs (MA, USA). The water used in this study was obtained from Milli-Q® (Millipore) water purification system (MA, USA).

2.2. Synthesis of zwitterionic polymers

4-Vinyl-pyridinium ethanesulfonate was prepared by the reaction of 4-vinylpyridine (158 mg, 1.5 mmol) and sodium 2-bromoethanesulfonate (106 mg, 0.5 mmol) in ethylene glycol (3 mL) under refluxing for 5 h and the resulting 4-vinyl-pyridinium ethanesulfonate monomers were directly used for polymerization without further purification. The polymerization of the zwitterionic monomer was performed by the catalysis of concentrated sulfuric acid according to the procedures described previously but with some modifications [40]. Briefly, the synthesized 4-vinyl-pyridinium ethanesulfonate monomer solution was directly added to the concentrated sulfuric acid (100 μL) at ice-bath, the temperature was then raised to room temperature and continually stirred for 5 h until a reddish-brown sticky liquid was obtained. The sticky polymer was kept at 4 °C prior to the fabrication of ZIC-HILIC magnetic beads.

2.3. Fabrication of ZIC-HILIC magnetic beads

The preparation of iron oxide magnetic beads followed the procedure reported previously [41]. Briefly, $FeCl_3$ (1 M) and $FeCl_2$ (0.5 M) were dissolved in aqueous hydrochloric acid (0.4 M, 10 mL) at room temperature under sonication. When the solids were completely dissolved, aqueous sodium hydroxide (0.5 M, 100 mL) was slowly added with stirring at room temperature. The mixture was left to stir in an oil bath at 80 °C for 30 min. After cooling to room temperature, the magnetic nanoparticles were rinsed with aqueous hydrochloric acid (0.1 M) and ethanol to remove unreacted impurities and then resuspended in 100 mL of 80% ethanol. In order to efficiently immobilize the zwitterionic polymers on magnetic beads, the magnetic beads were pre-coated with polyacrylic acid (PAA) [42]. Briefly, 1 mL of the magnetic beads suspension was diluted with 9 mL of 30 mM phosphate buffer. The solution was then added with 10 mg of EDC hydrochloride and 50 μL of 1% PAA solution (prepared from PAA with average Mw ~100,000, 35 wt% in H_2O), and the mixture was vortexed for 30 min at room temperature. Then the magnetic beads were collected by magnetic force and the supernatant was removed. The beads were washed with 80% ACN in 0.1% formic acid (FA) (10 mL × 3) and re-suspended in 10 mL of 80% ACN solution containing 0.1% FA. Zwitterionic polymer solution (100 μL) was added to the bead suspension and the reaction mixture was vortexed at room temperature for 1 h. The polymer-coated beads were collected again by magnetic force and the supernatant was removed. The unbounded polymers were removed by washing with 80% ACN contained 0.1% FA (10 mL × 3), 45% ACN contained 0.1% FA (10 mL × 3) and 10% ACN contained 0.1% FA (10 mL × 3) consequently. The resulting modified beads were re-suspended in 5 mL of 80% ACN solution containing 0.1% FA and kept under 4 °C before use.

2.4. Trypsin digestion of fetuin and protein mixture from pregnancy urine

Fetuin (20 μg) was denatured in 8 M urea dissolved in 50 μL of 50 mM ammonium bicarbonate (ABC) at 37 °C for 30 min and then

reduced in 10 mM DTT under 37 °C for another 30 min. The reduced fetuin was alkylated by reacting with 20 mM IAA at room temperature in the dark for 1 h. The urea concentration was diluted to less than 2 M using 50 mM ABC. Sequencing grade modified trypsin was added to this protein solution at an enzyme-to-substrate ratio of 1/50 and incubated at 37 °C for 16 h. The resulting peptide mixtures were acidified by formic acid and kept at −20 °C prior to HILIC purification. In order to minimize interferences caused by impurities present in urine samples, urine sample was precipitated with TCA prior to trypsin digestion. Briefly, 250 μL of pregnancy urine was lyophilized and re-dissolved in 60 μL of deionized water containing 1% SDS and 10 mM DTT. The reaction mixture was incubated under 95 °C for 5 min. The denatured and reduced protein mixture was alkylated in 50 mM IAA under room temperature in the dark for 30 min. TCA was added to the solution till 20% (v/v) concentration and the solution was kept on an ice-bath. The white precipitate was spun down by centrifugation under 13k rpm for 10 min. The supernatant was then removed carefully and the precipitate was washed with de-ionized water (250 μL). The procedure was repeated twice and the resulting precipitate was re-suspended in 50 mM ABC (100 μL). Sequencing grade modified trypsin was added to the solution with a 1/30 enzyme/protein ratio and the mixture was incubated at 37 °C for 18 h. After the reaction, the product mixture was acidified by formic acid, lyophilized and kept under −20 °C prior to HILIC purification.

2.5. Enrichment and analysis of glycopeptides by ZIC-HILIC magnetic beads and LC–MS

The fetuin tryptic peptides (1 pmol) were dissolved in 100 μL of 80% ACN containing 0.2% FA. HILIC beads (0.5 mg) were suspended in 100 μL of 80% ACN solution containing 0.2% FA. The tryptic peptides were mixed with HILIC beads by pipetting to form a homogeneous suspension. The beads were centrifuged and placed on a magnetic separator. The supernatant was removed and the beads were resuspended in 200 μL of wash buffer containing 80% ACN containing 0.2% FA. After washing the beads several times, the bounded glycopeptides were eluted from HILIC magnetic beads using 20% ACN containing 0.2% FA for three times (50 μL × 3). The eluted fractions were combined and concentrated for LC–MS analysis. The enriched glycopeptides (2 μL) were subjected to the CapLC system (Waters, Milford, MA) utilizing a capillary column (75 μm i.d., 10 cm in length, MST, Taiwan) with a linear gradient from 0% to 60% acetonitrile containing 0.1% formic acid over 46 min at a flow rate of 300 nL/min. The separated glycopeptides were online analyzed under positive survey scan mode on a nano-ESI Q-TOF (Micromass, UK) instrument. The scan range was from *m*/*z* 400 to 2000 for LC–MS experiment. For LC–MS/MS experiment the survey scan range for precursor ion was from *m*/*z* 300 to 2000 and the product scan range was from *m*/*z* 50 to 2000.

2.6. N-glycosylation site identification by ZIC-HILIC magnetic beads, PNGase F deglycosylation and LC–MS/MS analysis

The lyophilized peptides obtained from ZIC-HILIC magnetic beads enrichment were re-dissolved in 50 mM ABC (50 μL). The solution was added with endoglycosidase PNGase F (0.1 μL) and incubated at 37 °C for overnight to remove the glycan moieties. For ^{18}O labeling experiment, the deglycosylation step was performed in 50 mM ammonium bicarbonate in water-^{18}O (97 at.% ^{18}O). ^{18}O atoms will be incorporated into the C-termini of the resulting Asp residues during the PNGase F deglycosylation. For urine sample, the glycopeptides were enriched through a two-step HILIC enrichment procedure. Briefly, the peptide mixtures purified from the first step HILIC enrichment according the above-mentioned procedure were deglycosylated with PNGase F deglycosylation reaction with or without ^{18}O atom incorporation. The deglycosylated peptide mixtures were subjected to the second step HILIC purification. Instead of the eluted fractions, the flow-through fractions were collected for MS analysis. The purified peptides were subjected to the same CapLC system as described above but with a linear gradient from 5% to 70% acetonitrile in 0.1% formic acid over 90 min at a flow rate of 300 nL/min. The separated peptides were online analyzed under positive survey scan mode on a nano-ESI Q-TOF (Micromass, UK), LTQ Orbitrap (Thermo Scientific, USA) instrument. The survey scan range for precursor ion was from *m*/*z* 300 to 1600 and the product scan range was from *m*/*z* 50 to 2000. The MS/MS raw data was processed into a MGF file format using Mascot Distiller v2.3.2.0 (Matrix Science, UK). The resulting MGF file was searched using the Mascot search engine v2.3 (Matrix Science, UK) with the following search parameters: (1) protein database was set to be Swiss-Prot; (2) taxonomy was set as *Homo sapiens* (human); (3) one trypsin missed cleavage was allowed; (4) the precursor and product ion mass tolerance was set at 0.4 Da/0.2 Da for Q-TOF instrument and 5 ppm/0.6 Da for Orbitrap instrument; (5) carbamidomethyl (C) was chosen for fixed

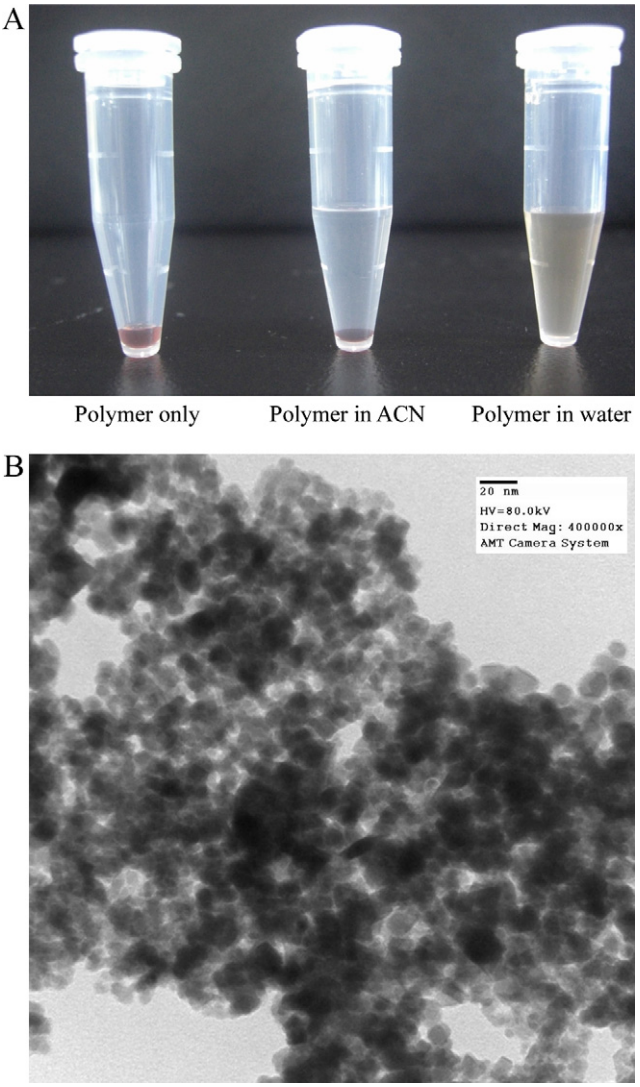

Fig. 1. (A) Solubility of zwitterionic polymer in organic solvent and aqueous solvent. Left: zwitterionic polymer was kept without solvent; middle: polymer aggregated in ACN; right: polymer was soluble in water. (B) TEM image of ZIC-HILIC magnetic beads. The dark particles, assigned as Fe_3O_4 nanoparticles, were surrounded with gray translucent polymers.

modification; (6) oxidation (M) and deamidated (NQ) were chosen for variable modifications (herein, the variable modification of deamidated (NQ) was used to determine the N-glycosylation sites); for ^{18}O introduction experiment, the deamidated (NQ) was replaced as deamidated $^{18}O(1)$(NQ); (7) proteins with scores above the significance threshold ($p < 0.05$) were shown as significant hits. All MS/MS spectra of identified peptides were further verified by manual interpretation. The deamidated residues at identified peptides with scores beyond the identity threshold were regarded as the identified N-glycosylation sites. These sites were further confirmed by comparison with the N-glycosylation sites of the corresponding proteins reported in UniProt database (http://www.uniprot.org/).

2.7. Recovery yield examination for HILIC magnetic beads by stable isotope dimethyl labeling and LC–MS analysis

Fetuin digest (2 pmol) was divided into two pools with equal amount (each contained 1 pmol), one pool was labeled with formaldehyde and sodium cyanoborohydride and the other was labeled with deuterium formaldehyde and sodium cyanoborohydride according to the procedure reported previously [43]. The hydrogen-labeled fetuin digest was purified with HILIC magnetic beads (dry weight 0.5 mg) according to above-mentioned procedure and the resulting eluted fraction was spiked into deuterium-labeled fetuin digest. The combined mixture was deglycosylated with PNGase F and the resulting peptides were analyzed by LC–MS/MS (ESI Q-TOF). The deglycosylated peptides were identified through MS/MS analysis and the relative abundance of H- and D-labeled deglycosylated peptide was determined from each corresponding precursor ions. The obtained ratios were regarded as the recovery yield of the enrichment method.

3. Results and discussion

3.1. Fabrication of zwitterionic HILIC magnetic beads

The preparation of zwitterionic monomers was achieved by the reaction of 4-vinylpyridine with sodium bromoethanesulfonate *via* S_N2 reaction. These 4-vinylpyridinium N-ethylsulfonates were polymerized *via* spontaneous polymerization catalyzed by concentrated sulfuric acid according to the protocol reported previously [40]. The resulting sticky polymers were soluble in aqueous buffer but aggregated in acetonitrile, which indicated that the polymer surface was extremely hydrophilic (Fig. 1A). The zwitterionic polymers were immobilized onto the surface of Fe_3O_4 magnetic beads which were prepared *via* co-precipitation method under alkaline condition [41]. In order to immobilize the zwitterionic polymers onto Fe_3O_4 magnetic beads effectively, the iron oxide beads were pre-coated with polyacrylic acid in the presence of carbodiimide to form a layer of hydrophilic surface [42]. After the removal of unbound PAA, the zwitterionic polymers coating on magnetic beads was carried out in 80% ACN contained 0.1% FA. Unbound zwitterionic polymers were removed by washing with 10% ACN solution containing 0.1%FA. According to the UV absorption measurement of unbound polymers ($\lambda_{max} \sim 285$ nm) about 0.35 mg of polymer was estimated to be coated on 11 mg of PAA-modified beads. The resulting magnetic beads bear many advantages including easy-to-operate, short operation time, and high capacity due to high surface-to-volume ratio and superparamagnetic properties of Fe_3O_4 particles. Immobilization of zwitterionic polymers on magnetic beads was further examined by transmission electron microscopy (TEM). As shown in Fig. 1B, the dark Fe_3O_4 nanoparticles were surrounded with gray translucent polymers. The TEM image also indicated that the zwitterionic polymers were heterogeneously coated on these Fe_3O_4 nanoparticles Furthermore, FT-IR measurements (Fig. 2) indicated the presence of the sulfonate stretching band at around 1040 cm^{-1} (Fig. 2) due to the formation of zwitterionic group through alkylsulfonic acid moiety [44,45]. These results confirmed the successful synthesis of ZIC-HILIC magnetic beads through the coating of zwitterionic polymer on Fe_3O_4 nanoparticles. Without PAA modification, the zwitterionic polymers can attach to the bare magnetic beads and show good specificity for glycopeptides enrichment. But polymers were sometimes released from beads and polymer signals were observed during LC–MS analysis. To achieve a robust immobilization, PAA was first immobilized onto magnetic bead surface and the positively charged zwitterionic polymer was attached to the PAA *via* electrostatic interaction. Since the zwitterionic moieties were regarded as "neutral", excess pyridinium moieties were required for providing positive charges. In this study, the 3:1 molar ratio of 4-vinylpyridine and 2-bromoethanesulfonate were used to prepare zwitterionic monomer. Without purification, the resulting zwitterionic monomers accompanied with excess vinylpyridine were polymerized to vinylpyridine–vinylpyridinium ethanesulfonate block copolymers. Therefore the zwitterionic copolymer can strongly attach to PAA layer through electrostatic interaction. After two month of storage at 4 °C, ZIC-HILIC magnetic beads still can enrich glycopeptides from fetuin digest without losing recovery yield and specificity.

The ionic strength effect caused by negatively charged PAA on the glycopeptides enrichment was also examined, as shown Fig. S11 in Supplemental information. Under 10 mM NaCl, ionic strength effect was neglectable, it may be due to the reason that PAA is not easy to be deprotonated under operation condition (pH 3–4) or the negative charges on PAA are mainly contributed to the attachment of co-polymer through electrostatic interaction of carboxylate–pyridinium. Therefore, the ionic strength effect on the glycopeptides enrichment could be minimized.

3.2. Glycopeptide enrichment using zwitterionic-HILIC magnetic beads

Application of ZIC-HILIC for glycopeptides purification was first examined using alpha-2-HS-glycoprotein (fetuin A) which contains three N-linked glycosylation sites, four O-linked glycosylation sites and a heterogeneous population of glycans [46] as the test standard. The glycopeptide enrichment can be easily operated using a bar magnet and the whole process can be completed within a minute. 20% ACN containing 0.2% FA is the optimized elution condition to achieve the best specificity and recovery yield without polymer detachment. The fetuin digest (without purification) and HILIC-purified glycopeptides were analyzed by LC–MS. As shown in Fig. 3A, the base peak ion chromatograms showed that without ZIC-HILIC purification, the ion signals of glycopeptides were hardly detected but the ion signals of glycopeptides (■) were dramatically enhanced after the samples were enriched by ZIC-HILIC magnetic beads. Furthermore, the combined mass spectra (Fig. 3B) indicated that almost all molecular ions larger than m/z 1100 were glycopeptides (■). In order to confirm these ions belonged to glycopeptides, MS/MS experiments were performed and the representative MS/MS spectra shown in Fig. 3C indicates the presence of typical glycan marker fragments (▲) (m/z 204 for HexNAc and m/z 366 for Hex-HexNAc)[38] in all glycopeptide ions. In addition, the glycopeptide signals were also validated by PNGase F deglycosylation. The deglycosylated peptides denoted by the round labels (●) were detected predominately in Supplemental Fig. S1 by LC–MS and their identities were further confirmed by LC–MS/MS. Four unique peptides which

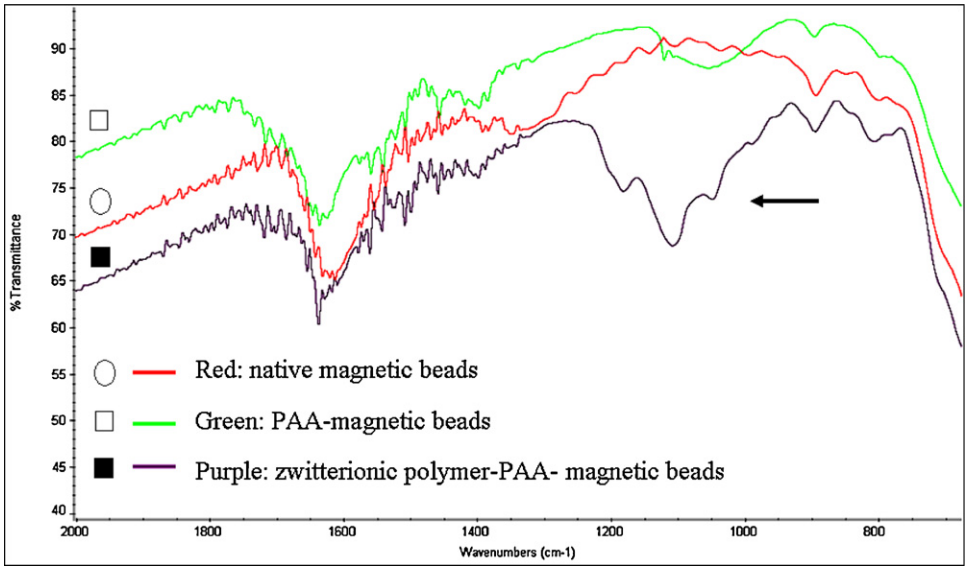

Fig. 2. Infrared spectra of polymer-coated beads. The red color is the IR spectrum of native Fe_3O_4 beads; the green color is the IR spectrum of PAA-coated Fe_3O_4 beads; the purple color is the zwitterionic polymer-PAA-coated Fe_3O_4 beads.

cover three fetuin N-glycosylation sites (N^{99}, N^{155} and N^{176}) were identified from these studies (Supplemental Table 1). When non-glycoprotein digest (bovine serum albumin, 6 pmol) was spiked into the fetuin digest (1 pmol), glycopeptides could still be specifically enriched by these ZIC-HILIC magnetic beads (data was shown in Supplemental Fig. S2) demonstrating superior specificity of ZIC-HILIC magnetic beads for glycopeptides enrichment. According to our data, the bare beads adsorbed most peptides nonspecifically and no any glycopeptides was obtained in the HILIC elute fraction. Without zwitterionic polymers, PAA modified beads can actually enrich glycopeptides possibly due to hydrophilic interaction between PAA and glycans. But the recovery yield and specificity of PAA beads toward glycopeptides enrichment were worse than those of zwitterionic polymer-PAA beads, data were shown in Fig. S10 in Supplemental information. In addition to glycopeptides enrichment, the ZIC-HILIC beads had been used to enrich phosphopeptides from milk digest, however, no significant phosphopeptide enrichment was observed. According to the previous report presented by Boersema et al. [29,47], the phosphopeptide enrichment was not observed with ZIC-HILIC may be due to the reason that outer negative charge in the zwitterionic moieties could repel the phosphate group, resulting in non-effective phosphopeptide enrichment.

3.3. Recovery yield test of ZIC-HILIC magnetic beads

The recovery yield examination of ZIC-HILIC magnetic beads for glycopeptides enrichment was carried out by stable-isotope dimethyl labeling [43]. Fetuin digest with and without HILIC purification were combined, subjected to PNGase F deglycosylation and then quantitatively analyzed by LC–MS. PNGase F deglycosylation was performed to minimize the errors arising from poor detection sensitivity and resolution. From the isotopic ratios of tryptic glycopeptides deduced from purified *versus* non-purified samples (Fig. 4), the recovery yields were estimated to reach 95–100% from 1 pmol of fetuin digest. Three replicated samples were examined and recovery yields of five individual deamidated peptides were determined. MS spectra for recovery yield measurement of other glycopeptides were shown in Supplemental Fig. S3 and their recovery yields were summarized in Supplemental Table S1. The strong hydrophilic interaction between zwitterionic polymers and high collection yield by magnetic bars has lead to high recovery yield for glycopeptides enrichment using ZIC-HILIC magnetic beads we reported in this study.

3.4. Two-step ZIC-HILIC strategy for N-glycosylation site identification of urine sample

The ZIC-HILIC magnetic beads were used to enrich glycoproteins in urine sample (250 μL). Without ZIC-HILIC purification, only one N-glycosylation site was obtained from 94 unique identified peptides when urine digest was deglycosylated by PNGase F and directly analyzed by LC–MS/MS, Mascot database searching and UniProt database matching. When ZIC-HILIC beads were applied for enrichment, some hydrophilic non-glycopeptides were detected after HILIC purification. To remove these hydrophilic non-glycopeptides which were co-eluted with glycopeptides, a two-step ZIC-HILIC strategy was proposed. The eluted fraction from the first step HILIC purification was subjected to PNGase F deglycosylation followed by the second step of HILIC purification. Since the glycosylated peptides were turned to hydrophobic after deglycosylation, instead of the elute fraction, the flow-through and wash fractions from the second step of HILIC were collected. By this manner, the glycosylated peptides could be separated from the native hydrophilic peptides which were co-eluted with the glycopeptides from the first step HILIC. Using this two-step HILIC strategy, more than 85 deamidated peptides can be specifically identified from the urine sample (250 μL) with few non-deamidated peptides. Proteins contained at least two deamidated peptides simultaneously identified by this method and confirmed by ^{18}O-labeling approach were summarized in Table 1 and the whole identified N-glycosylation sites were listed in Table S2 in Supplemental information. Almost all deamidated sites identified using two-step HILIC approach are consistent with the N-glycosylation sites well documented in UniProt database, except for two glycosylation sites, N^{513} of uromodulin and N^{470} of lysosomal alpha-glucosidase, which have not yet been reported. In order to confirm these novel N-glycosylation sites, $H_2{}^{18}O$ was applied for PNGase F deglycosylation reaction. The identified ^{18}O-labeling sites were found to be consistent with the deamidation sites identified from the enzymatic deglycosylation. The y_7 ion shown in the MS/MS spectrum of Fig. 5A of FALLMTNCYATPSSNATDPLK (M represented oxidated

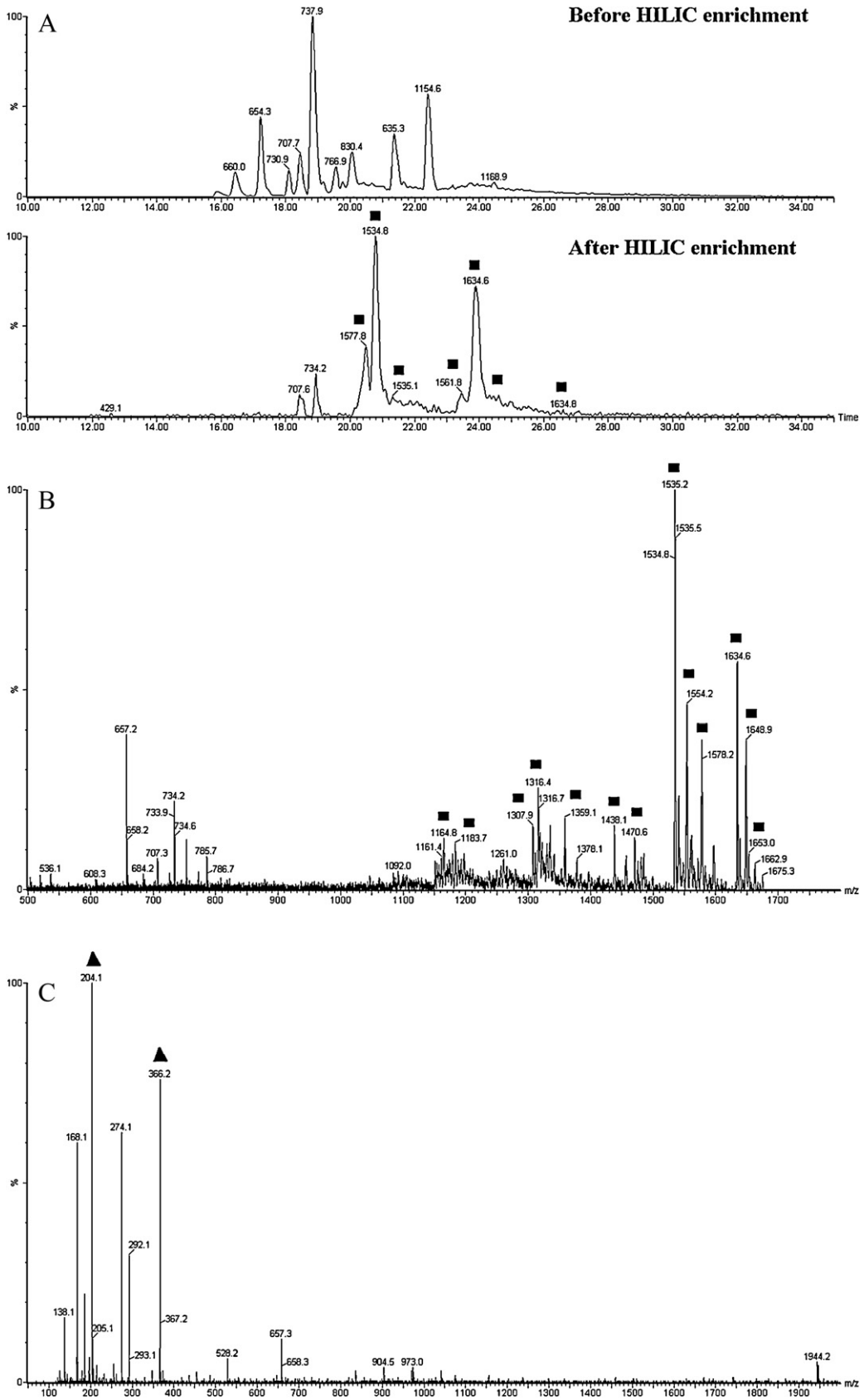

Fig. 3. (A) LC–MS chromatogram of fetuin digest with (up) and without (down) ZIC-HILIC beads treatment; (B) the combined MS spectra of the whole LC–MS chromatograms of ZIC-HILIC elution fraction; (C) selected MS/MS spectrum of ZIC-HILIC elution fraction. The glycopeptides were denoted by square labels (■) and the glycan marker fragments were labeled with ▲.

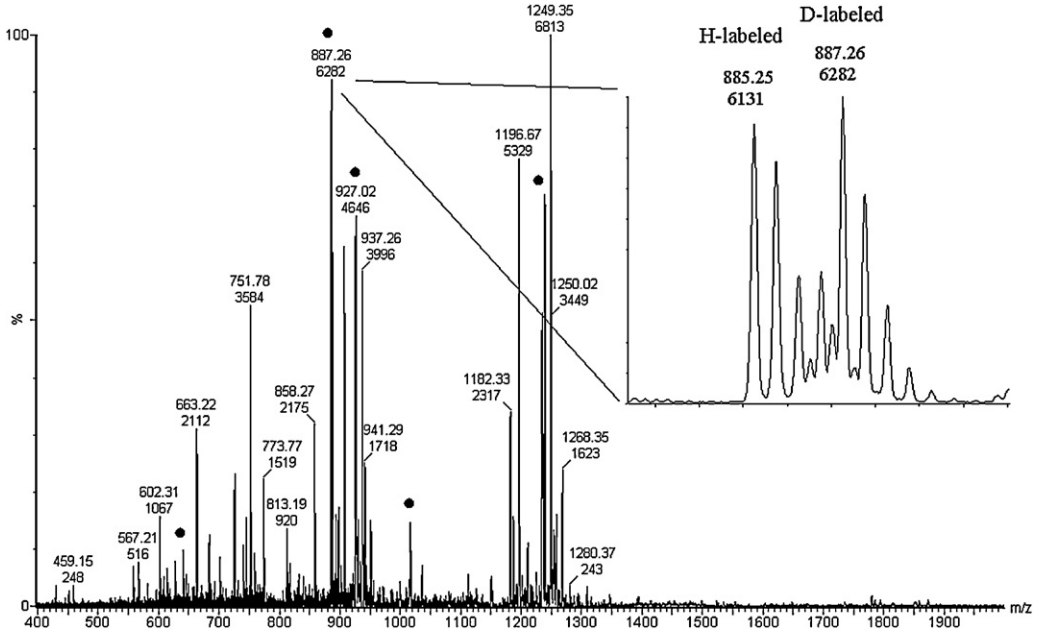

Fig. 4. LC–MS spectrum of enriched peptides (with H-labeling) spiked into unpurified peptide mixture (with D-labeling). The enlargement shows a pair of deamidated peptide (LCPDCPLLAPLNDSR) before (labeled with D) and after (labeled with H) ZIC-HILIC enrichment. The deglycosylated peptides denoted by the round labels (●).

Table 1
Summary of proteins contained at least two identified deamidated peptides. Deamidated peptides identified from urine samples by ZIC-HILIC enrichment/PNGase F deglycosylation and confirmed by ^{18}O enzymatic labeling.

Acc. no.	Protein name	glycopeptide sequence (Mascot score)	observed glycosylation site(s)	observed *m/z* (charge state)	observed ^{18}O labeled *m/z* (charge state)
P07911	Uromodulin	CNTAAPMWLNGTHPSSDEGIVSR (42)	N^{232}	834.38 (3+)	1251.75 (2+)
		QDFNITDISLLEHR (93)	N^{322}	851.42 (2+)	ND
		FALLMTNCYATPSSNATDPLK (113)	N^{513} [a]	1166.54 (2+)	1167.52 (2+)
P41222	Prostaglandin-H_2 D-isomerase	WFSAGLASNSSWLR (49)	N^{51}	791.88 (2+)	792.84 (2+)
		SVVAPATDGGLNLTSTFLR (110)	N^{78}	960.50 (2+)	961.51 (2+)
Q9BY67	Cell adhesion molecule 1	FQLLNFSSSELK (56)	N^{101}	707.36 (2+)	708.32 (2+)
		VSLTNVSISDEGR (86)	N^{113}	689.34 (2+)	690.42 (2+)
P01042	Kininogen-1	YNSQNQSNNQFVLYR (38)	N^{48}	938.44 (2+)	939.44 (2+)
		ITYSIVQTNCSK (89)	N^{205}	707.86 (2+)	708.85 (2+)
		LNAENNATFYFK (32)	N^{294}	716.85 (2+)	717.84 (2+)
P10253	Lysosomal alpha-glucosidase	LENLSSSEMGYTATLTR (72)	N^{140}	945.44 (2+)	946.67 (2+)
		GVFITNETGQPLIGK (74)	N^{470} [a]	787.92 (2+)	788.92 (2+)
		NNTIVNELVR (33)	N^{882}	586.81 (2+)	587.82 (2+)
Q08380	Galectin-3-binding protein	ALGFENATQALGR (53)	N^{69}	674.86 (2+)	675.85 (2+)
		EPGSNVTMSVDAECVPMVR (97)	N^{192}	1039.97 (2+)	ND
		GLNLTEDTYKPR (62)	N^{398}	704.36 (2+)	705.40 (2+)
		AAIPSALDTNSSK (37)	N^{551}	638.33 (2+)	639.32 (2+)
P01133	Pro-epidermal growth factor	VFLNGSR (28)	N^{104}	397.21 (2+)	398.21 (2+)
		GNNSHILLSALK (63)	N^{148}	423.23 (3+)	635.65 (2++)
		IITKENISQPR (35)	N^{596}	ND	651.36 (2+)
P02790	Hemopexin	SWPAVGNCSSALR (38)	N^{187}	703.33 (2+)	704.33 (2+)
		ALPQPQNVTSLLGCTH (56)	N^{453}	868.93 (2+)	869.93 (2+)

[a] Novel glycosylation sites.
ND represented not detected.

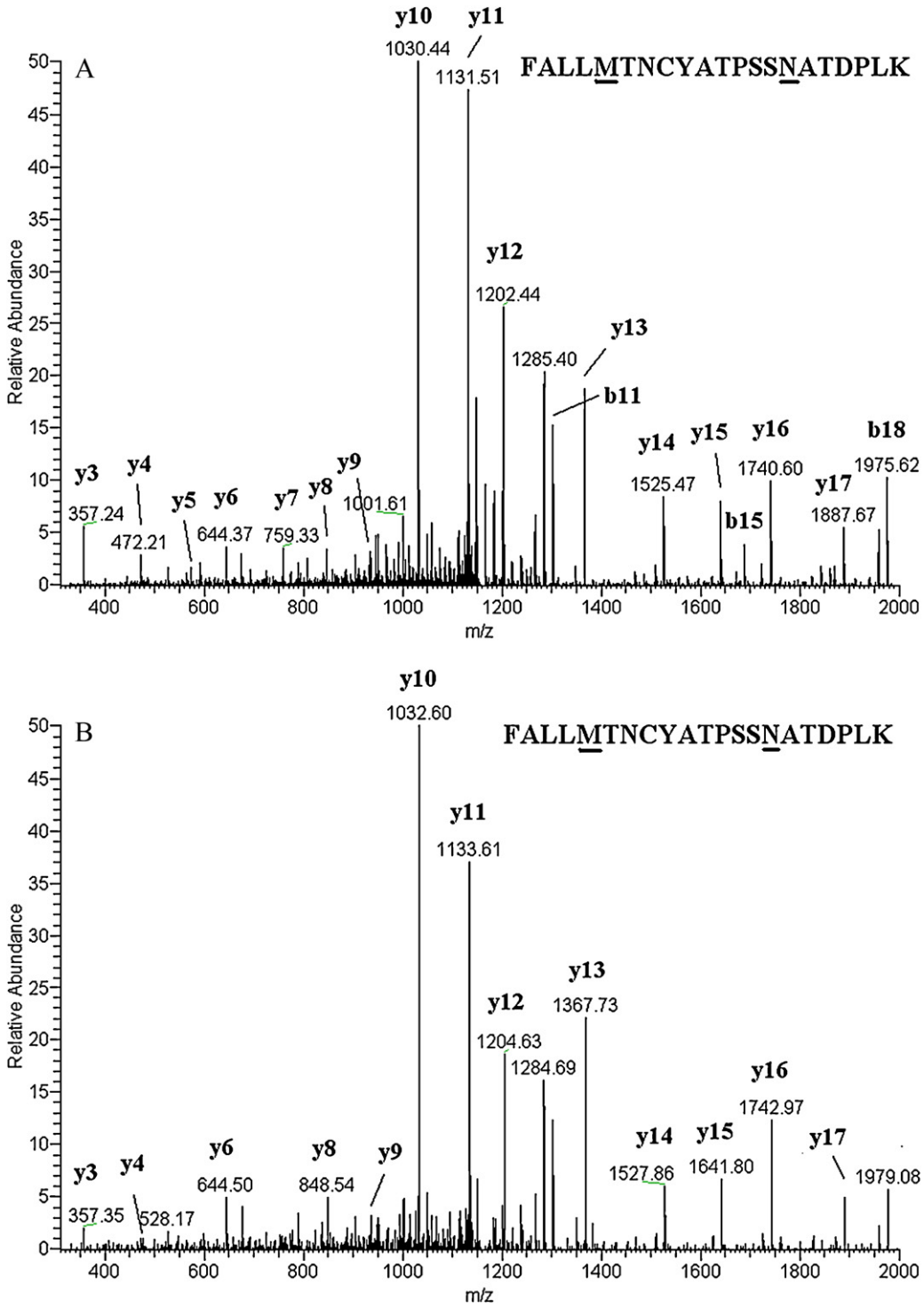

Fig. 5. The MS/MS spectrum of (A) FALLMTNCYATPSSNATDPLK and (B) ^{18}O-labeled FALLMTNCYATPSSNATDPLK. Here, C represented carbamidomethyl Cys, M represented oxidated Met and N represented deamidated Asn.

Met and N represented deamidated Asn) indicated the N^{513} site of uromodulin was deamidated (NQ) and each y_n ion ($n \geqq 7$) shown in the MSMS spectrum (Fig. 5B) of the ^{18}O labeled site were increased by 2 Da accordingly. The y_8–y_{17} ions of FALLMTNCYATPSSNATDPLK shown in Fig. 5B were 2 Da larger than the same y ion series shown in Fig. 5A but y_6 ion in Fig. 5B had the same m/z as y_6 ion shown in Fig. 5A. These data indicated that the N^{513} residue of uromodulin was deamidated and incorporated with an ^{18}O atom. This deamidated site was verified by three replicated urine samples. Therefore, N^{513} was assigned to be a novel N-glycosylation site of uromodulin. The glycosylation site on N^{470} of lysosomal alpha-glucosidase was also characterized by the same approach. The MS/MS spectrum of ^{18}O atom-labeled deamidated peptide, GVFITNETGQPLIGK, shown in Supplemental Fig. S4 confirmed the identified glycosylation site. As indicated in Table S2 in Supplemental information, most of the identified N-glycosylation sites

obtained using two-step HILIC approach could be validated by PNGase F/$H_2{}^{18}O$ approach. Totally, 85 N-glycosylation sites in 53 glycoproteins from 250 μL of urine sample were identified using these ZIC-HILIC magnetic beads.

4. Conclusion

The zwitterionic HILIC magnetic beads were fabricated by spontaneous polymerization and electrostatic adsorption of zwitterionic polymers onto Fe_3O_4 nanoparticles. The resulting ZIC-HILIC magnetic beads were shown to enrich glycopeptides through the hydrophilic interaction between glycans and the zwitterionic stationary phase. The simple operation procedure (less than 1 min), high specificity and recovery yield shown in this study indicated that these ZIC-HILIC beads are extremely useful for a high throughput and efficient isolation of low abundant glycopeptides from complex biological samples. Remarkably, the two-step ZIC-HILIC purification approach coupled with PNGase F deglycosylation and $H_2{}^{18}O$ enzymatic labeling facilitates the identification of N-glycosylation sites. We believe these beads hold great potential for glycoproteomics studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.12.057.

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