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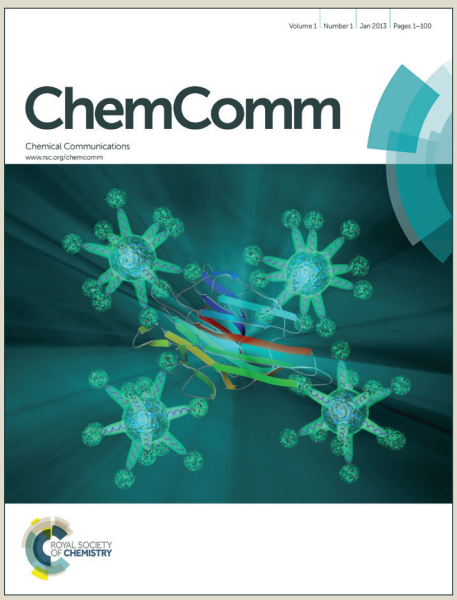

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Highly specific enrichment of N-glycoproteome through nonreductive amination reaction using Fe_3O_4@SiO_2-Aniline nanoparticles

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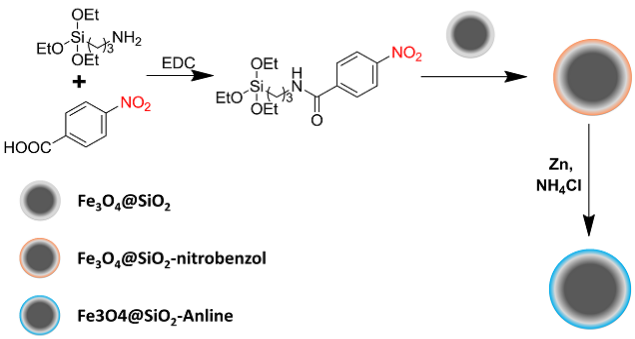
A novel method based on the conjunction of aldehydes from oxidized glycopeptides to aniline groups on the magnetic nanoparticles via nonreductive amination was reported for the highly selective enrichment of N-glycopeptides. For the first time, nonreductive amination reaction has been introduced into N-glycoproteome extraction; and correspondingly a new kind of aniline-functionalized nanoparticle was designed and synthesized.

N-glycosylation is one of the most common protein post-translational modification.[1] Changes in N-glycoprotein are associated with various of disease progression and many clinical biomarkers are N-glycoproteins.[2] Therefore, highly sensitive identification of N-glycoproteome has the potential for use in disease diagnosis, prognosis, and prediction of treatments, but there is still many challenges.[3] Human proteome database contains thousands of predicted N-glycosylation sites, however, only 5% of the N-glycosylation sites were experimentally proved so far mainly due to the inherently low abundance of N-glycoproteome.[4] The selective enrichment of N-glycopeptides became a prerequisite for mass spectrometry (MS)-based N-glycoproteome studies, because fishing out the N-glycoproteome before the MS analysis can make the characterization of N-glycoproteins more specific.[5]

To meet the pressing need in the MS-based N-glycoproteome research, numerous N-glycopeptides enrichment methods were developed. Among them, hydrazide chemistry based solid phase extraction, which relies on the conjugation of the glycans to a hydrazide-functionalized solid support, is the most specific enrichment strategy.[6] However, this method requires a long conjugation time (12-16 hrs) to immobilized the N-glycopeptides onto the solid phase, although a recent study showed the addition of aniline can accelerate the coupling time.[7] Meanwhile, the harsh washing process in order to remove nonspecific adsorptions inevitably gives rise to the loss of samples, limiting the sensitivity of this method. Recently, we have developed a new N-glycoproteome enrichment method based on the conjunction of aldehydes from oxidized N-glycopeptides to amine-group on the magnetic nanoparticles via a reductive amination reaction which significantly shortened the conjugation time to less than 4 hrs.[8] But the requirement of a reductive reagent to generate the stale bond between the N-glycopeptides and the amine-functionalized magnetic nanoparticles represents the major limitation of this method. Therefore, to overcome these advantages, we began to investigate alternative methods.[9] Compared with aliphatic Schiff base, aromatic Schiff base owns better stability because of the conjugation structure, and it can remain as a stable Schiff base in a wide pH range without reducing the C=N bond to C-N bond.[10] Also, it is documented that the aniline can react with the aldehyde group under nonreductive amination conditions without the addition of a reductive reagent.[11] The omission of the reducing reagent would significant simply the whole process and improve the enrichment sensitivity. To the best of our knowledge, it is for the first time that the nonreductive amination reaction is introduced into N-glycoproteome enrichment.

To develop such a new enrichment strategy, a novel aniline-functionalized nanomaterial that can capture N-glycoproteome through a nonreductive amination reaction were precisely designed and synthesized. First, the aniline-functionalized nanoparticle should be easily prepared with high efficiency. Second, such composite particles should display a strong response to the external magnetic field, because this feature would enable us to capture and release the particles easily. The synthesis of Fe_3O_4@SiO_2-Aniline is shown in scheme 1. A silane coupling reagent with a nitrobenzol group was synthesized through modifying the (3-aminopropyl)triethoxysilane with p-nitrobenzoic acid and chromatographically purified, denoted as NBTES. The Fe_3O_4@SiO_2 core-shell nanoparticle for modifying the NBTES silane coupling reagent was synthesized through a modified solvothermal reaction as previous work, and then the silica shell was coated on the surface of the Fe_3O_4 cores.[8] Subsequently, the NBTES was used to modify the as prepared Fe_3O_4@SiO_2 nanoparticles through a one-step silylation reaction to obtain Fe_3O_4@SiO_2-nitrobenzol. The final product of Fe_3O_4@SiO_2-Aniline was obtained by treating the Fe_3O_4@SiO_2-Nitrobenzol with zinc powder in ammonium chloride aqueous solution to reduce the nitrobenzol groups to aniline groups.



Scheme 1 Synthesis scheme for Fe_3O_4@SiO_2-Aniline.

The successful synthesis of this Fe_3O_4@SiO_2-Aniline nanoparticles was proved by various means. The NMR spectra (Figure S1) clearly demonstrate the successful synthesis of the nitrobenzol silane coupling agents, detailed peaks attribution was shown in Supplementary Information. To prove the functionalization of aniline group on the nanoparticles, Fourier transform infrared (FT-IR) spectroscopy was used to characterize the NBTES, Fe_3O_4@SiO_2 nanoparticles, and Fe_3O_4@SiO_2-Aniline nanoparticles, respectively, Figure 1 (a). NBTES was characterized first, the peaks 1640 cm^{-1} and 1520 cm^{-1} corresponding to the stretch vibration of C=O and bending vibration of N-H proved the structure of NBTES. For Fe_3O_4@SiO_2 nanoparticles, the strong IR band at 567 cm^{-1} is attributed to the vibration of the Fe-O and the adsorption peak at 1087 cm^{-1} is attributed to the Si−O−Si vibration, indicating the successful preparation of Fe_3O_4@SiO_2 nanoparticles. Compared the FT-IR spectrum of the Fe_3O_4@SiO_2-Aniline with the Fe_3O_4@SiO_2, new absorb peaks at 1600 cm^{-1} and 1520 cm^{-1}, which is attributed to the vibrations of aromatic ring and bending vibration of N-H, appeared in the FT-IR spectrum of Fe_3O_4@SiO_2-Aniline, indicating the successful modification of NBTES. The ZETA potential was measured to further verify the reduction of nitrobenzol group into aniline group (Table S1). The ZETA potential of Fe_3O_4@SiO_2-nitrobenzol was -16.3 mV. After the reduction by zinc powder, due to the positive electricity of aniline, the ZETA potential of Fe_3O_4@SiO_2-Aniline raised to +6.3 mV, indicating the successful reduction. The transmission electron microscopy (TEM) images (Figure 1c and d) reveal the iron oxide microspheres are well encapsulated in a condensed, amorphous silica shell. Additionally, the superparamagnetic core (with VSM as 35 emu/g, Figure 1b) make the Fe_3O_4@SiO_2-Aniline material can be readily separated from the solution phase within 30 s with the help of a magnet and quickly redispersed after removal of the magnetic field. This excellent magnetic response and redispersibility of Fe_3O_4@SiO_2-Aniline material would contribute to the efficient enrichment and separation of the N-glycoproteome.

The feasibility of introducing the nonreductive amination reaction into N-glycoprotein enrichment was demonstrated first by analyzing a model N-glycoprotins asialofetuin (ASF) with three known N-glycosylation sites. In a proof-of-concept experiment, the enrichment procedure is illustrated in Scheme 2; and proposed main reaction pathways of the nonreductive amination reaction is shown in Figure S2. The comparison of this new method between the reductive amination reaction-based method and the traditional hydrazide chemistry-based enrichment methods is shown in Figure S3. In a typical enrichment procedure, the peptide digests of ASF were first oxidized by $NaIO_4$, then dissolved in loading buffer and incubated with the Fe_3O_4@SiO_2-Aniline to couple the N-glycopeptides onto the Fe_3O_4@SiO_2-Aniline nanoparticles through nonreductive amination reaction. Afterward, the Fe_3O_4@SiO_2-Aniline with the captured N-glycopeptides were separated from the mixed solution using an external magnetic field, and then the nanoparticles were washed with different types of buffers several times to remove the nonspecifically adsorbed peptides and other impurities. Finally, the glycopeptides were released from the Fe_3O_4@SiO_2-Aniline with PNGase F for MS analysis. First, we focused on investigating the coupling condition including the reaction buffer, the reaction temperature and the reaction time, to efficiently conjugate the oxidized N-glycopeptides onto the Fe_3O_4@SiO_2-Aniline nanoparticles. We found that the conjugation reaction proceeded well in the 70% methanol and 30% acetic acid (v/v) at 60 °C for 4 hrs with all of the 6 theoretical deglycosylated glycopeptides peaks detected (Figure S4, S5 and S6). The optimized mass ratio of solid phase to sample is determined as 1 mg of Fe3O4@SiO2-Aniline per 1 mg of protein digests (Figure S7). Then, we compared the effect of the reducing reagent on the enrichment performance and found that the omission of the reducing reagent $NaBH_3CN$ did not hamper the enrichment performance (Figure S8). The reason is that, different from the reaction between aliphatic amine and aldehyde, the condensation product between aniline and aldehyde can form as a stable Schiff base without reducing the C=N bond to C-N bond. On the contrary, the addition of a reducing reagent requires an additional desalting process before MS analysis.

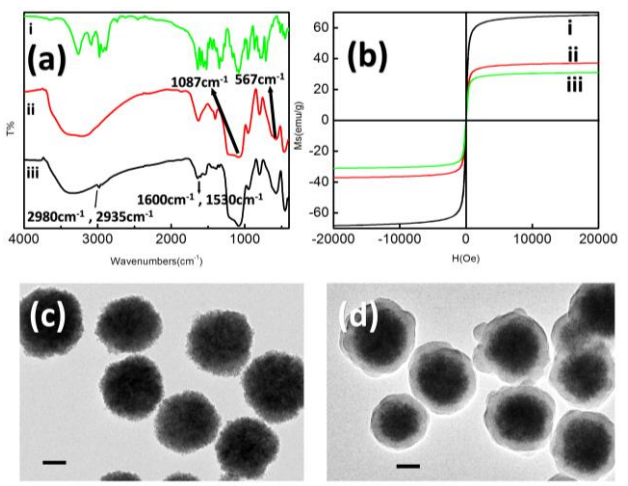
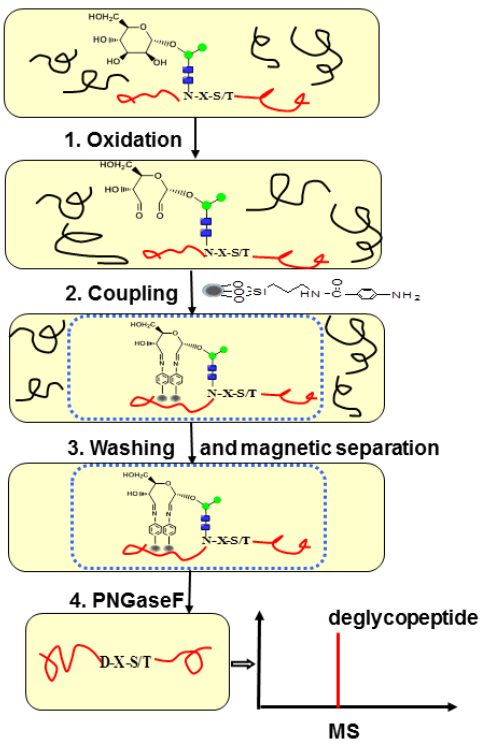


Figure 1 (a) FT-IR spectroscopy of (i) NBTES, (ii) Fe_3O_4@SiO_2 and (iii) Fe_3O_4@SiO_2-Aniline; (b) Magnetic hysteresis curves of (i) Fe_3O_4, (ii) Fe_3O_4@SiO_2 and (iii) Fe_3O_4@SiO_2-Aniline; TEM images of (c) Fe_3O_4 and (d) Fe_3O_4@SiO_2, The scale bar is 100nm.

The established new enrichment method shows remarkable selectivity toward N-glycoproteins as following. After optimization the enrichment procedure, the ASF try ptic digests were subjected to the enrichment process and ASF tryptic digests without enrichment were analyzed directly as a comparison. The standard N-glycoprotein ASF harbors three N-glycosylation sites at N_{99}CS, N_{176}GS, and N_{156}DS, , however, due to the presence of nonglycopeptides, only four deglycosylated N-glycopeptides at m/z 1627.81, 1754.79, 1780.80 and 3017.54 (including only two of the N-glycosylation sites) appeared in the spectrum after

deglycosylation by PNGase F, while peaks of the nonglycopeptides obviously dominated the spectrum (Figure 2a). After enrichment and deglycosylation by PNGase F, six predominant peaks of the deglycosylated N-glycopeptides from ASF were detected with a very clean background, covering all of the three known glycosylation sites (Figure 2b). The enrichment result clearly confirmed the selectivity of this method. To further investigate the selectivity of this method, a mixture of the tryptic digests of a standard nonglycoprotein myoglobin (MYO) and ASF was prepared at a molar ratio of MYO: ASF at 100:1 to mimic a real sample. Before enrichment, the signals of N-glycopeptides were hardly seen due to the interference of significant amounts of nonglycopeptides from MYO (Figure S9a). After enrichment, signals of glycopeptides were easily detected with a clean background (Figure S9b). As the mass spectrum revealed, the glycopeptides were selectively fished out from the mixture by the Fe_3O_4@SiO_2-Aniline. This result indicated the excellent enrichment selectivity of Fe_3O_4@SiO_2-Aniline toward N-glycopeptides. The selectivity is comparable to our previous developed method.[9] This new method also possesses high sensitivity at the nanogram per microliter level. To investigate the sensitivity of the established method, a series of diluted oxidized asialofetuin digest solutions were tested and the lowest detectable concentration was estimated to be at 5.0 ng/μL (Figure S10). The sensitivity is 2-5 times better than our previously developed methods. The above results make us believe that this novel protocol can extract the N-glycoproteome with remarkable specificity and high sensitivity.



Scheme 2 The enrichment and MS analysis scheme for N-glycopeptides using Fe_3O_4@SiO_2-Aniline **.**

The utility of this nonreductive amination reaction-based enrichment method is further demonstrated by profiling of the N-glycoproteome of a normal human serum sample. The serum sample from normal volunteer was provided by Fudan University Shanghai cancer center. The research followed the tenets of the Declaration of Helsinki and was approved by the Ethics Committee of Fudan University Shanghai cancer center. After reduction and alkylation, the serum sample was digested into peptides, followed by oxidation, enrichment by Fe_3O_4@SiO_2-Aniline, deglycosylation and 1D nano-LC-MS/MS analysis. In total, 156 unique N-glycosylation sites were found in 148 glycopeptides, which were assigned to 80 glycoproteins. The specificity of identified glycoproteins was as high as 71.8%. The detail information is demonstrated in Table S2. It should be noted that only 1 μL sample was used in our experiment, which is a very small sample volume. The results of real sample analysis clearly indicated the selectivity and sensitivity of this new method.

In summary, a new aniline functionalized nanoparticle Fe_3O_4@SiO_2-Aniline was designed and synthesized. Based on this nanoparticle, a novel N-glycopeptides enrichment methods was established with the following remarkable features: 1) The whole enrichment process is easy, the enrichment time is greatly shortened compared with the traditional hydrazide chemistry-based methods, and the addition of a reducing reagent and the desalting step after the coupling was omitted compared with the reductive amination reaction-based method; 2) The selectivity toward glycopeptides is distinguished, as demonstrated by extracting the N-glycopeptides from mixtures of nonglycopeptides at a 1:100 mole ratio and analyzing the N-glycoprotome from human serum with a very small volume; 3) The strong magnetic properties allow the simple while effective separation of the glycopeptides after the coupling reaction is completed and in washing steps. In view of these unique features in separation the N-glycopeptides, we have reason to believe that this innovative method will be an efficient alternative N-glycoproteome extraction method.

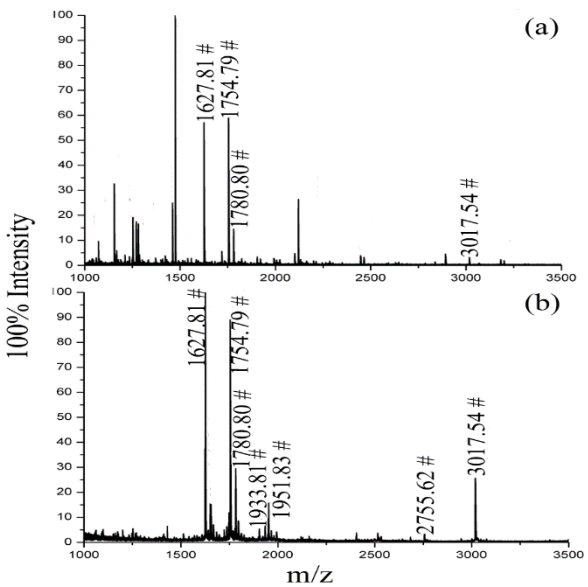


Figure 2 MALDI-TOF mass spectra of tryptic digest of ASF (a) before isolation but deglycosylation by PNGase F and (b) after isolation by Fe_3O_4@SiO_2-Aniline and then deglycosylation by PNGase F. The symbols "# represents the deglycosylated glycopeptides (including fragments from deglycosylated glycopeptides).

Notes and references

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