ChemComm

COMMUNICATION

View Article Online
View Journal

Cite this: DOI: 10.1039/c4cc10285a

Received 24th December 2014,
Accepted 13th February 2015

DOI: 10.1039/c4cc10285a

www.rsc.org/chemcomm

Highly specific enrichment of *N*-glycoproteome through a nonreductive amination reaction using Fe_3O_4@SiO_2-aniline nanoparticles†

Ying Zhang,‡[ab] Meng Yu,‡[c] Cheng Zhang,[ab] Yali Wang,[a] Yi Di,[b] Changchun Wang*[c] and Haojie Lu*[ab]

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

N-Glycosylation is one of the most common protein post-translational modifications.[1] Changes in *N*-glycoprotein are associated with various disease progression, and many clinical biomarkers are *N*-glycoproteins.[2] Therefore, the highly sensitive identification of *N*-glycoproteome has the potential for use in disease diagnosis, prognosis, and the prediction of treatments, but there is still many challenges before it can be realized.[3] The human proteome database contains thousands of predicted *N*-glycosylation sites; however, only 5% of the *N*-glycosylation sites have been experimentally proven so far, mainly due to the inherently low availability of *N*-glycoproteome.[4] The selective enrichment of *N*-glycopeptides has become 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]

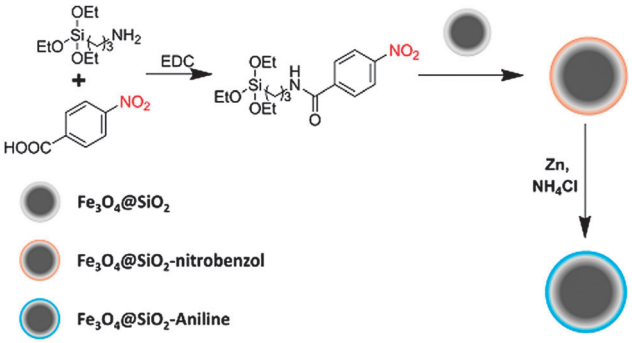
To meet the pressing need in MS-based *N*-glycoproteome research, numerous *N*-glycopeptides enrichment methods have been developed. Among these, hydrazide-chemistry-based solid phase extraction, which relies on the conjugation of the glycans to a hydrazide-functionalized solid support, is the most specific enrichment strategy.[6] However, this method requires a long conjugation time (12–16 h) to immobilize 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 required to remove nonspecific adsorptions inevitably gives rise to a loss of sample, 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 an amine group on the magnetic nanoparticles *via* a reductive amination reaction, which significantly shortens the conjugation time to less than 4 h.[8] However, the requirement for a reductive reagent to generate the stale bond between the *N*-glycopeptides and the amine-functionalized magnetic nanoparticles represents a major limitation of this method. To overcome this disadvantage, we have begun to investigate alternative methods.[9] Compared with the aliphatic Schiff base, the aromatic Schiff base offers better stability, because of its 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, this is the first time that the nonreductive amination reaction has been 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 was precisely designed and synthesized. The design requires that first, the aniline-functionalized nanoparticles should be easily prepared with high efficiency. Second, such composite particles should display a strong response to an 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 was then chromatographically purified, and

[a] Shanghai Cancer Center and Department of Chemistry, Shanghai 200032, P. R. China. E-mail: luhaojie@fudan.edu.cn
[b] Institutes of Biomedical Sciences and Key Laboratory of Glycoconjuates MOH, Fudan University, Fudan University, Shanghai, 200032, P. R. China
[c] State Key Laboratory of Molecular Engineering of Polymers and Department of Macromolecular Science, Laboratory of Advanced Materials, Fudan University, Shanghai 200433, P. R. China. E-mail: ccwang@fudan.edu.cn

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c4cc10285a

‡ Y. Z. and Y. M. contributed equally.



Scheme 1 Synthesis scheme for Fe_3O_4@SiO_2-aniline.

herein, 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 in 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.

The successful synthesis of these Fe_3O_4@SiO_2-aniline nanoparticles was proven by various means. The NMR spectra (Fig. S1, ESI†) clearly demonstrate the successful synthesis of the nitrobenzol silane coupling agents, and the detailed peaks attribution is shown in the ESI.† To prove the functionalization of the aniline group on the nanoparticles, Fourier transform infrared (FT-IR) spectroscopy was used to characterize the NBTES, Fe_3O_4@SiO_2 nanoparticles, and the Fe_3O_4@SiO_2-aniline nanoparticles, respectively, see Fig. 1(a). NBTES was characterized first; the peaks at 1640 cm^{-1} and 1520 cm^{-1} corresponding to the stretch vibration of C=O and the bending vibration of N–H proved the structure of NBTES.

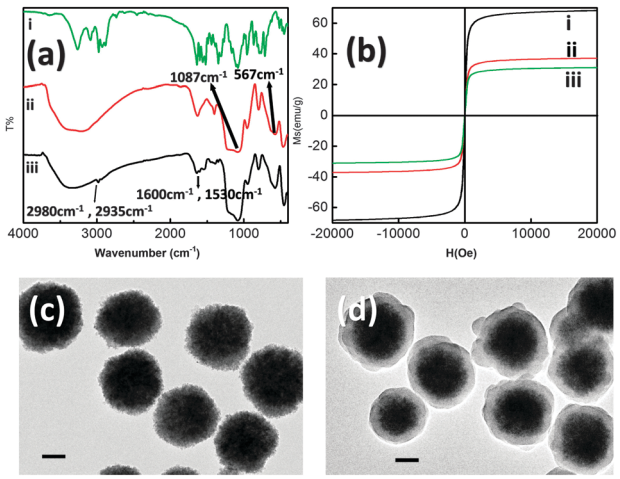
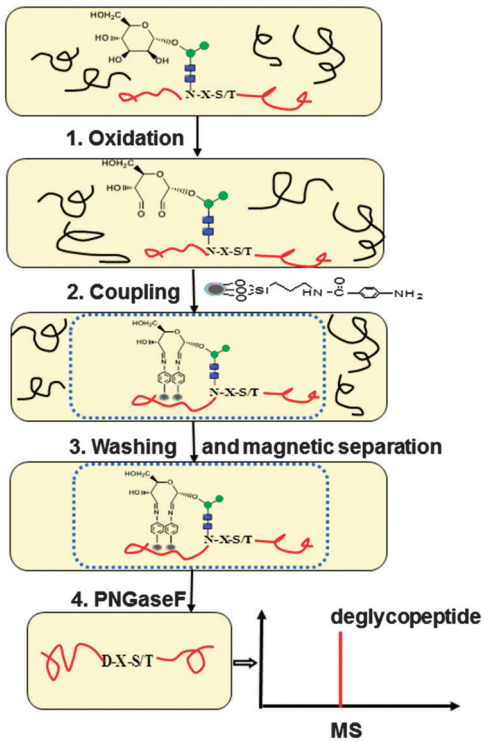


Fig. 1 (a) FTIR spectroscopy of (i) NBTES, (ii) Fe_3O_4@SiO_2 and (iii) Fe_3O_4@SiO_2-aniline; (b) magnetic hysteresis curves of (i) Fe_3O_4, (ii) Fe_3O_4@SiO_2 and (iii) Fe_3O_4@SiO_2-aniline; TEM images of (c) Fe_3O_4 and (d) Fe_3O_4@SiO_2 the scale bar is 100 nm.

For the 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 the Fe_3O_4@SiO_2 nanoparticles. Comparing the FT-IR spectrum of the Fe_3O_4@SiO_2-aniline with the Fe_3O_4@SiO_2, new absorption peaks at 1600 cm^{-1} and 1520 cm^{-1}, which are attributed to the vibrations of the aromatic ring and the 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, ESI†). 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 increased to +6.3 mV, indicating the successful reduction. The transmission electron microscopy (TEM) images (Fig. 1c and d) reveal the iron oxide microspheres are well encapsulated in a condensed, amorphous silica shell. Additionally, the superparamagnetic core (with a VSM of 35 emu g^{-1}, Fig. 1b) makes the Fe_3O_4@SiO_2-aniline material able to be readily separated from the solution phase within 30 s with the help of a magnet and then 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 an 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*-glycoproteins asialofetuin (ASF) with three known *N*-glycosylation sites. In a proof-of-concept experiment, the enrichment procedure was determined, and is illustrated in Scheme 2, and the proposed main reaction pathways of the nonreductive amination reaction are shown in Fig. S2 (ESI†). The comparison of this new method with the reductive amination reaction-based method and the traditional hydrazide chemistry-based enrichment methods is shown in Fig. S3, ESI.† In a typical enrichment procedure, the peptide digests of ASF were first oxidized by $NaIO_4$, then dissolved in a 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 a nonreductive amination reaction. Afterward, the Fe_3O_4@SiO_2-aniline with the captured *N*-glycopeptides was 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 non-specifically 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 70% methanol and 30% acetic acid (v/v) at 60 °C for 4 h, with all of the six theoretical deglycosylated glycopeptides peaks detected (Fig. S4–S6, ESI†). The optimized mass ratio of the solid phase to sample was determined as 1 mg of Fe_3O_4@SiO_2-aniline per 1 mg of protein digests (Fig. S7, ESI†). Then, we compared the effect of the reducing reagent on the enrichment performance,



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

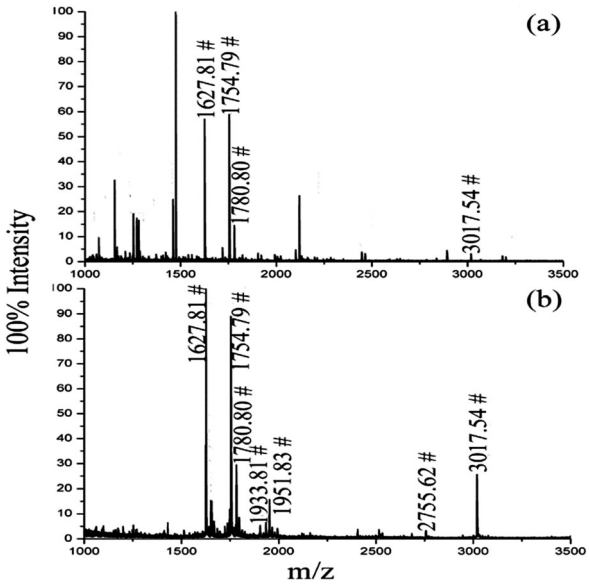


Fig. 2 MALDI-TOF mass spectra of a tryptic digest of ASF (a) before isolation but following deglycosylation by PNGase F and (b) after isolation by Fe_3O_4@SiO_2-aniline and then followed by deglycosylation by PNGase F. The symbols "#" represents the deglycosylated glycopeptides (including fragments from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

and found that the omission of the reducing reagent $NaBH_3CN$ did not hamper the enrichment performance (Fig. S8, ESI†). The reason for is that, different from the reaction between the aliphatic amine and aldehyde, the condensation product between the 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 the MS analysis.

The established new enrichment method shows remarkable selectivity toward *N*-glycoproteins, as detailed in the following. After optimization of the enrichment procedure, the ASF tryptic digests were subjected to the enrichment process, and ASF tryptic digests without enrichment were analyzed directly for 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 the deglycosylation by PNGase F, while peaks of the nonglycopeptides clearly dominated the spectrum (Fig. 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 (Fig. 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 by significant amounts of nonglycopeptides from MYO (Fig. S9a, ESI†). After enrichment, the signals of glycopeptides were easily detected with a clean background (Fig. S9b, ESI†). 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^{-1} (Fig. S10, ESI†). This sensitivity is 2–5 times better than our previously developed methods. The above results make us believe that this novel protocol can extract *N*-glycoproteome with remarkable specificity and high sensitivity.

The utility of this nonreductive amination reaction-based enrichment method is further demonstrated by profiling the *N*-glycoproteome of a normal human serum sample. The serum sample from a 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 then 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 the identified glycoproteins was as high as 71.8%. The detailed information is provided in Table S2 (ESI†).

It should be noted that only 1 μL of sample was used in our experiments, which is a very small sample volume. The results of a 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 method was established, with the following remarkable features: (1) the whole enrichment process is easy, and the enrichment time is greatly shortened compared with traditional hydrazide chemistry-based methods, and the addition of a reducing reagent and the desalting step after the coupling is omitted compared with the reductive amination reaction-based method; (2) the selectivity toward glycopeptides is impressive, 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 but effective separation of the glycopeptides after the coupling reaction is completed and in the washing steps. In view of these unique features in the separation of *N*-glycopeptides, we have reason to believe that this innovative method to be an efficient alternative *N*-glycoproteome extraction method.

This work was supported by the National Science and Technology Key Project of China (2012CB910602 and 2012AA020204), the National Science Foundation of China (Grant No. 21335002, 51073040, and 21375026), and Shanghai Projects (Grants Eastern Scholar, 11XD1400800, 13520720200, Shanghai Rising-Star and B109).

Notes and references

1 (*a*) D. Zielinska, F. Gnad, J. Wiśniewski and M. Mann, *Cell*, 2010, **141**, 897–907; (*b*) N. Tan, U. Bailey, M. Jamaluddin, S. Mahmud, S. Raman and B. Schulz, *Nat. Commun.*, 2014, **5**, 3099.
2 (*a*) J. Lowe, *Cell*, 2001, **104**, 809–812; (*b*) J. Marth and P. Grewal, *Nat. Rev. Immunol.*, 2008, **8**, 874–887.
3 (*a*) Y. Zhang, J. Jiao and H. Lu, *Clin. Proteomics*, 2014, **11**, 18; (*b*) B. Lepenies and P. Seeberger, *Nat. Biotechnol.*, 2014, **32**, 443–445.
4 G. Khoury, R. Baliban and C. Floudasa, *Sci. Rep.*, 2011, **1**, 90.
5 (*a*) Z. Xiong, H. Qin, H. Wan, G. Huang, Z. Zhang, J. Dong, L. Zhang, W. Zhang and H. Zou, *Chem. Commun.*, 2013, **49**, 9284–9286; (*b*) W. Chen, J. Smeekens and R. Wu, *Mol. Cell. Proteomics*, 2014, **13**, 1563–1572.
6 (*a*) H. Zhang, X. Li, D. Martin and R. Aebersold, *Nat. Biotechnol.*, 2003, **21**, 660–666; (*b*) Y. Tian, Y. Zhou, S. Elliott, R. Aebersold and H. Zhang, *Nat. Protoc.*, 2007, **2**, 334–339; (*c*) L. Zhang, H. Jiang, J. Yao, Y. Wang, C. Fang, P. Yang and H. Lu, *Chem. Commun.*, 2014, **50**, 1027–1029.
7 J. Chen, P. Shah and H. Zhang, *Anal. Chem.*, 2013, **85**, 10670–10674.
8 Y. Zhang, M. Kuang, L. Zhang, P. Yang and H. Lu, *Anal. Chem.*, 2013, **85**, 5535–5541.
9 Y. Zhang, M. Yu, C. Zhang, W. Ma, Y. Zhang, C. Wang and H. Lu, *Anal. Chem.*, 2014, **86**, 7920–7924.
10 (*a*) R. W. Layer, *Chem. Rev.*, 1968, **63**, 489–510; (*b*) S. I. Snovida, V. C. Chen and H. Perreault, *Anal. Chem.*, 2006, **78**, 8561–8568.
11 (*a*) M. Rohmer, B. Meyer, M. Mank, B. Stahl, U. Bahr and M. Karas, *Anal. Chem.*, 2010, **82**, 3719–3726; (*b*) K. Kaneshiro, Y. Fukuyama, S. Iwamoto, S. Sekiya and K. Tanaka, *Anal. Chem.*, 2011, **83**, 3663–3667; (*c*) Y. Cai, Y. Zhang, P. Yang and H. Lu, *Analyst*, 2013, **138**, 6270–6276.

