Facile synthesis of aminophenylboronic acid-functionalized magnetic nanoparticles for selective separation of glycopeptides and glycoproteins†

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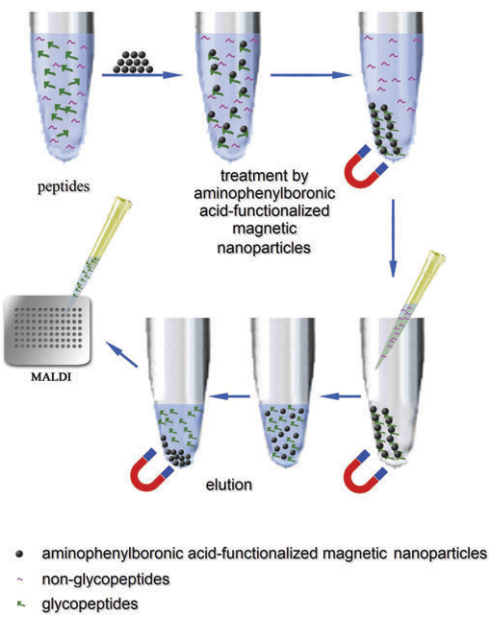
In this work, aminophenylboronic acid-functionalized magnetic nanoparticles were synthesized, and applied to selective separation of glycopeptides and glycoproteins.

Protein glycosylation is a broad and biologically significant post-translational modification, which is most commonly associated with secreted and membrane proteins.[1,2] The discovery and identification of glycosylated peptides and proteins, and the analysis of their glyco-structures are increasingly important in diagnosis and proteomics. A common approach to the assay of glycoproteins is to perform the proteolytic cleavage of glycoproteins, followed by enrichment treatment, or the release of glycans from glycopeptides using an exoglycosidase. Direct analysis by mass spectrometry (MS) and tandem MS (MS-MS) can identify peptides and proteins, and their corresponding modifications.[3] Several methods such as lectin-based affinity chromatography, hydrophilic interaction liquid chromatography and hydrazide chemistry have been widely developed for isolation and identification of N-linked glycopeptides in complex biological samples.[4–7] Besides, boronic acid affinity chromatography was also employed to isolate glycoproteins, and these methods have been applied in biological samples.[8] Recently, magnetic beads have been used for protein separation because they provide a simple and fast procedure for separating reacted protein from the rest of the reaction mixture by using an external magnet.[9] Commercial concanavalin A(Con A)-functionalized magnetic beads were developed to enrich glycosylated peptides and proteins.[10] Compared with Con A, boronic acid has more potential in the application for separation and enrichment of glycoproteins or glycopeptides, because of the covalent interaction with 1,2-*cis*-diol of glyco-structure. The interaction does not need a complex recognition motif consisting of several saccharides, which provides the possibility of capturing more kinds of glyco-structures (a more heterogeneous group of N-linked and O-linked oligosaccharides) (Scheme S1). More recently, phenylboronic modified electrodes were used for cell immobilization, in which poly- and oligosaccharides are present in outer cellular wall.[11]

Herein, a facile synthetic approach was developed for the preparation of aminophenylboronic acid-functionalized magnetic nanoparticles. As shown in Scheme 1, they were applied to selectively separate glycopeptides or glycoproteins with the help of an extra-applied magnetic field. The synthetic route to aminophenylboronic acid-coated magnetic nanoparticles is shown in Scheme S2. First, amine–magnetite nanoparticles were synthesized by solvothermal reaction. Second, they were further converted to aminophenylboronic acid magnetic nanoparticles through a two-step amidation reaction. Briefly, magnetite particles were first prepared by a one-pot method,[12] and then modified with hexanedioyl chloride and 3-aminophenylboronic acid.

Fig. S1 is a representative transmission electron microscopy (TEM) image of the synthesized amine-functionalized magnetic particles, and dark iron oxide particles of 50 nm mean diameter had a narrow size distribution. Fig. S2 shows a scanning electron microscopy (SEM) image of the synthesized amine-functionalized magnetic particles. As shown in Fig. S2, the particles are nearly spherical in shape and possessed



Scheme 1 The application of aminophenylboronic acid-coated magnetic nanoparticles for the removal of glycopeptides and glycoproteins with the help of an applied magnetic field.

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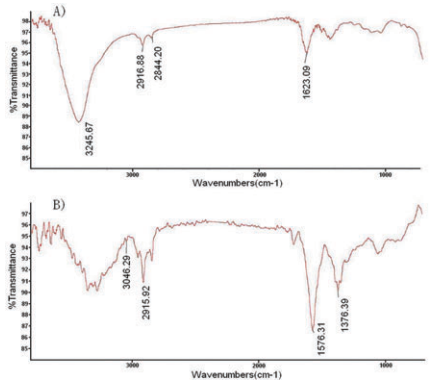

Fig. 1 FT-IR spectra of (A) the amine–magnetite nanoparticles and (B) the aminophenylboronic acid-functionalized magnetic nanoparticles obtained by reacting amine–magnetite nanoparticles with hexane diacyl chloride and further with aminophenylboronic acid.

smooth surfaces. Fourier-transform infrared (FT-IR) spectroscopy was employed to characterize the aminophenylboronic acid-functionalized magnetic nanoparticles before and after chemical modification (Fig. 1). Fig. 1B shows the FT-IR spectra of aminophenylboronic acid-functionalized magnetic nanoparticles. The peak at 1376 cm^{-1} is associated with the C–B vibrations, the peak at 1576 cm^{-1} shows the vibrations of phenyl, and peaks around 2850–3050 cm^{-1} were attributed to the C–H stretching model of alkyl chain and phenyl, implying aminophenylboronic acid was introduced onto the magnetite nanoparticles surface compared with the FT-IR spectra of amine-functionalized magnetic particles (Fig. 1A).

The quality and specificity of aminophenylboronic acid-functionalized magnetic nanoparticles were investigated by capturing the glycopeptides from the mixture of glycopeptides and non-glycopeptides, *i.e.*, the tryptic digests of asialofetuin (ASF), which is a standard glycoprotein. Fig. 2 shows MALDI-MS spectra of ASF digests (Fig. 2A), and the eluate of ASF digests after aminophenylboronic acid-functionalized magnetic nanoparticle treatment (Fig. 2B). Table S1 displays the molecular masses and proposed oligosaccharide composition of the glycopeptides from ASF digests after treatment by aminophenylboronic acid-functionalized magnetic nanoparticles. As shown in Fig. 2A and Table S1, many tryptic

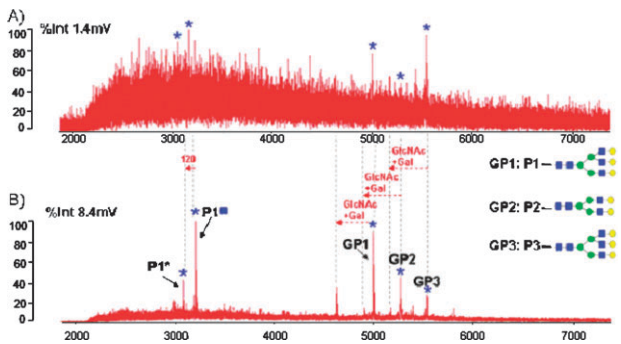

Fig. 2 Comparison of MALDI-TOF-MS spectra of ASF. (A) The spectrum of tryptic peptides from ASF. (B) The spectrum of eluate after aminophenylboronic acid-functionalized magnetic nanoparticle treatment of the tryptic digests of ASF. (P1* represents the $^{0,2}X_0$ cross-ring fragmentation ions).

peptides (also including incomplete digestion products) were observed in the MALDI-MS spectrum. The $[M + H]^+$ ions of glycopeptides were also observed in this spectrum but hardly detected (the peptide mixture was at a concentration of 5 ng μL^{-1}). Fig. S5A shows the spectrum of concentrated tryptic peptides from ASF (Fig. S5A) and the spectrum of the eluate after aminophenylboronic acid-functionalized magnetic nanoparticle treatment of the tryptic digests of ASF (Fig. S5B). Although the signal intensity and signal to noise ratio have been enhanced in both spectra, the interference from non-glycosylated peptides was reduced and glycopeptides were much easier to analyse in Fig. S5B. This might due to the fact that the existence of nonglycosylated peptides can suppress the mass spectrometric response to glycopeptides, making it hard to identify the low-abundance glycopeptides. Comparing the spectrum before and after treatment (Fig. 2A and B), the *m*/*z* 5004.2, 5544.8, 5278.0, 5179.8, 4913.2, 4638.9, 3220.5, 3100.5 are obviously separated from the ASF tryptic digest mixture. As depicted in the spectrum of eluates (Fig. 2B), GP1 (*m*/*z* 5004.2) was an observed glycopeptide indicating a composition of (GlcNAc)5(Man)3(Gal)3 for the attached N-glycan moiety on the V_{160}–R_{187} (glycosylation site Asn_{176}), which results in a mass increment of 1987 Da relative to the non-glycosylated theoretical peptide mass. This was in accordance with a previously published analysis of tryptic ASF digests,[13] which provided the proposed oligosaccharide triantennary composition of the carbohydrate side chain. The difference of 365 Da between MW of GP1 (*m*/*z* 5004.2) and $[M + H]^+$ ion at *m*/*z* 4638.9 exhibits the loss of galactose (Gal, mass of 162 Da) and the *N*-acetylglucosamine residue (GlcNAc, mass of 203 Da), and the two residues fall off the end of the glycan side chain. GP2 (*m*/*z* 5278.0, glycosylation site Asn_{99}) was also found in Fig. 2B which contains the biantennary carbohydrate side chain,[13,14] and the difference of 365 Da between GP2 and glycosylated $[M + H]^+$ ion at *m*/*z* 4913.2 revealed the mass loss of galactose (mass of 162 Da) and GlcNAc (mass of 203 Da) which fall off the end of the glycan side chain. GP3 (*m*/*z* 5544.8, glycosylation site Asn_{99}) also could be found in this spectrum. It is another glycopeptide of ASF which has the same glycosylation site as GP2,[13,14] yet it has oligosaccharide triantennary composition for the carbohydrate side chain and the peptide was modified with carboxyamidomethylation at cysteines, and the glycosylated fragment ion $[M + H]^+$ (*m*/*z* 5179.8) (the MW is 365 Da less than GP3) also could be found in this spectrum. Another two ions observed in Fig. 2B were the glycopeptide with GlcNAc residue attached to Asn_{176} ($[peptide + 204]^+$, *m*/*z* 3220.5) and the corresponding fragment ion $[peptide + CHCHNHAc]^+$ (or $[peptide + 84]^+$, *m*/*z* 3100.5), The latter ion corresponds to a $^{0,2}X_0$ cross-ring fragmentation of the connecting GlcNAc residue.[15] Comparing the spectrum before and after treatment, we could come to the conclusion that the glycopeptides were selectively isolated from the tryptic digests of ASF. To prove the effect of boronic acid structure, a control experiment was carried on, as shown in Fig. S6, the amine–magnetic nanoparticles had no isolating effect under our binding and eluting conditions.

The advantage of this enrichment process is that the aminophenylboronic acid-functionalized magnetic nanoparticles could not only remarkably separate glycopeptides

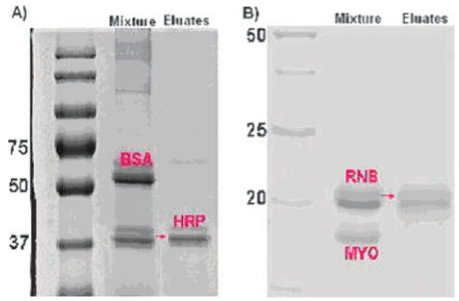

Fig. 3 Analysis of a mixture of model proteins without treatment and eluate after binding to boronic acid magnetic nanoparticles by SDA-PAGE. (A) mixture of BSA (2 μg) and HRP (2 μg). (B) mixture of MYO (2 μg) and RNB (2 μg).

from peptide mixture but also effectively enrich glycopeptides. As shown in Fig. 2B, the glycopeptide signals became stronger in the mass spectrum after the enrichment process. The intensities of the glycopeptides (with * marked) were increased from 176.5 (*m*/*z* 5004.2), 98.0 (*m*/*z* 5278.0), 152.9 (*m*/*z* 5179.8), 141.2 (*m*/*z* 4913.2), 196.1 (*m*/*z* 4638.9), 317.6 (*m*/*z* 3220.5), 207.8 (*m*/*z* 3100.5) to 3580.4, 1913.7, 603.9, 607.8, 1541.2, 4380.4, 1835.3, respectively. In this case, an intensity enhancement factor of 20, 20, 4, 4, 8, 14, 9 could be estimated for these glycopeptides, respectively. After the enrichment process, the signal-to-noise (S/N) ratios of the glycopeptides (with * marked) were increased from 5 (*m*/*z* 5004.2), 0 (*m*/*z* 5278.0), 0 (*m*/*z* 5179.8), 7 (*m*/*z* 4638.9), 8 (*m*/*z* 3220.5), 7 (*m*/*z* 3100.5) to 63, 27, 8, 18, 44 and 20, respectively. For MALDI-QIT-TOF MS, there was another item 100% Intensity (%Int.), which displays the value in millivolts of the largest peak in the spectrum. As shown in Fig. 2A and B, the %Int. were 1.4 mV and 8.4 mV, and after the enrichment process, the %Int. enhancement factor could be estimated as 6. Similar results were obtained by separation and analysis of another glycoprotein horseradish peroxidase (HRP) (Fig. S3 and S4).

The quality and the specificity of the aminophenylboronic acid-functionalized magnetic nanoparticles were demonstrated by the capturing of model glycoproteins as well, *i.e.*, horseradish peroxidase (HRP) and RNase B (RNB) which contain N-linked oligosaccharide structures of different N-glycan types. Non-glycoproteins, *i.e.*, bovine serum albumin (BSA) or myoglobin (MYO) were added to estimate the specificity of the nanoparticles in complex sample. As shown in Fig. 3A, there are two bands in the lane of the mixture, indicating HRP (2 μg) and BSA (2 μg), respectively. However, after treatment with boronic acid magnetic nanoparticles, only the band of HRP appeared in the lane of eluate without the band of BSA, which indicated a good specificity of the aminophenylboronic acid-functionalized magnetic nanoparticles. Moreover, as shown in Fig. 3B, the binding of the aminophenylboronic acid-functionalized magnetic nanoparticles with RNB (2 μg) is also efficient from the mixture of RNB (2 μg) and MYO (2 μg). HRP, a standard glycoprotein containing 9 glycosylation sites, was captured by magnetic beads, and the recovery is 77.78%. For RNB, the recovery is 58.39%, this probably has a relationship with the structure of the glycoprotein and the number of glycan side chains.

In summary, the synthesis of aminophenylboronic acid-functionalized magnetic nanoparticles was easy, low-cost, and timesaving. Moreover, the aminophenylboronic acid-functionalized magnetic nanoparticles were successfully applied to the enrichment of glycoproteins or glycopeptides. The specificity of these magnetic nanoparticles was also evaluated by capturing of different model glycopeptides or glycoproteins from mixtures containing non-glycomoleculars which were added as an interference. This method we have developed provides another efficient and convenient analysis approach to glycoproteomics.

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