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Synthesis and characterization of a novel boronic acid-functionalized chitosan polymeric nanosphere for highly specific enrichment of glycopeptides

Xiajuan Zou[a,*], Dan Liu[a], Lijun Zhong[a], Bin Yang[a], Yaxin Lou[a], Yuxin Yin[a,b,**]

[a] Proteomics Laboratory, Medical and Healthy Analytical Center, Peking University, Beijing 100191, China
[b] Institute of Systems Biomedicine, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100191, China

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ABSTRACT

In this study we describe a method for highly specific enrichment of glycopeptides with boronic acid-functionalized chitosan polymeric nanospheres and matrix assisted laser desorption-ionization mass spectrometry (MALDI-MS). This is the first time chitosan has been used to create nanosphere support material for selective enrichment of glycopeptides by modification with glycidyl methacrylate (GMA) and derivatization with 3-aminophenylboronic acid (APB). Due to their multifunctional chemical moieties, these 20–100 nm chitosan–GMA–APB nanospheres have unique properties, such as good dispersibility, good biocompatibility and chemical stability, as well as augmented specificity with glycopeptides. Enrichment conditions were optimized by using trypsin digested glycoprotein horseradish peroxidase. The high specificity of chitosan–GMA–APB nanospheres was demonstrated by effectively enriching glycopeptides from a digest mixture of horseradish peroxidase and nonglycoproteins (bovine serum albumin (BSA)).

1. Introduction

Mass spectrometry (MS) is currently in widespread use for study of glycopeptides or glycoproteins (Pan, Chen, Aebersold, & Brentnall, 2011). Due to their low abundance (2–5%), the relatively low ionization efficiency of glycopeptides as compared with unmodified peptides, together with the signal dilution that results from the distribution of glycan species at each linkage site, glycopeptide ion signals are frequently "lost in the noise" in digests of purified glycoproteins. The challenge is even greater when analyzing glycopeptides from digests of complex protein mixtures. As a result, it is almost impossible to analyze substoichiometric quantities of glycopeptide without specific enrichment steps.

Several methods have been described for isolation of glycopeptides from digests of purified glycoproteins, and from more complex protein mixtures. The performance of glycopeptide enrichment depends strongly on the physico-chemical milieu used to support the affinity absorbents (Liu, Ma, & Li, 2008; Zhang, Lu, & Yang, 2010). Among current glycopeptide enrichment techniques, lectin affinity chromatography is the one which is most widely used (Hirabayashi, 2004). Concanavalin A (Con A) has frequently been used for enrichment of N-glycoproteins from diverse sources (Bunkenborg, Pilch, Podtelejnikov, & Wisniewski, 2004; Fan et al., 2004; Wang et al., 2006). Lectin has strong affinity for high mannose and hybrid N-glycans, has lower affinity for diantennary N-glycans, and no affinity for tri- and tetraantennary complex-type glycans (Cummings & Kornfeld, 1982). Another enrichment method is based on the hydrophilicity of the glycan moiety. Gel matrices such as cellulose or sepharose are used to extract the hydrophilic glycopeptides (Wada, Tajiri, & Yoshida, 2004). However, many non-glycosylated peptides, which contain several hydrophilic amino acids, demonstrate as hydrophilicity which is as strong as that of the glycopeptides. Moreover, glycogroups attached to some hydrophobic peptides may also render them unable to be retained with hydrophilic groups. Recently, hydrazide chemistry was used to selectively isolate, identify, and quantify N-linked glycopeptides in a very specific and efficient manner (Tian, Zhou, Elliott, Aebersold, & Zhang, 2007). This method uses oxidized cis–diol carbohydrate groups to bind glycopeptides covalently. However, this technique requires an additional oxidation step which clearly increases experimental time and sample complexity. Moreover this method does not provide structural information on the sugar due to the destruction and removal of the glycan moieties. Boronate affinity matrix has been introduced for the unbiased enrichment of both N- and O-glycopeptides (Sparbier, Koch, Kessler, Wenzel, & Kostrzewa, 2005; Xu et al., 2009), as diboronic acids are known to bind with high affinity with glycans and glycoconjugates containing cis–diol groups through reversible ester formation (Scheme S1).

Chitosan is a natural polymer obtained by alkaline deacetylation of chitin, which exhibits highly useful biological properties such as biodegradation in the human body, antibacterial activity,

* Corresponding author. Tel.: +86 10 82802676.
** Corresponding author. Tel.: +86 10 82805570.
E-mail addresses: zouxj@bjmu.edu.cn (X. Zou), yinyuxin@bjmu.edu.cn (Y. Yin).

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and wound-healing capacity (Muzzarelli, 2009, 2011). Chemical modification of chitosan is of particular interest as it does not change the fundamental skeleton of chitosan. It also maintains the original basic physicochemical and biochemical properties of chitosan while giving rise to new or improved properties (Jayakumar, Prabaharan, Reis, & Mano et al., 2005; Mourya & Inamdar, 2008; Prabaharan, 2008; Zou, Liu, et al. 2011; Zou, Zhong, et al. 2011). Cationic chitosan interacts electrostatically with the anionic glycosaminoglycans and proteoglycans (Muzzarelli, 2012). Chitosan shows strong hydrophilic behavior and can extract hydrophilic glycopeptides (Casal, Corzo, Moreno, & Olano, 2005).

Here we describe the highly specific capture and direct MALDI-MS analysis of glycopeptides employing boronic acid-functionized chitosan polymeric chitosan–GMA–APB nanospheres. This is the first time chitosan has been used to create nanosphere support material for selective enrichment of glycopeptides by modification with glycidyl methacrylate (GMA) and derivatization with 3-aminophenylboronic acid (APB). The properties and advantages of the chitosan–GMA–APB nanosphere are discussed in detail in light of their capacity for specific and sensitive enrichment of glycopeptides from tryptic protein digests.

2. Materials and methods

2.1. Materials and chemicals

Glycidyl methacrylate (GMA, for GC, ≥97.0%), and ammonium bicarbonate (99.5%) were purchased from Fluka (Buchs, Switzerland), 2,5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (CHCA), tris(2-carboxyethyl)phosphine (TCEP), trifluoroacetic acid (TFA), acetonitrile (ACN) (HPLC grade), horseradish peroxidase (HRP, 98%), and bovine serum albumin (BSA, >98%) were obtained from Sigma Aldrich (St. Louis, MO, USA). 3-aminophenylboronic acid (APB) was obtained from Beijing chemical factory (Beijing, China), Chitosan (Mw = 500,000 g/mol) was obtained from Qingdao Hecreat Bio-tech Company (Qingdao, China), Other chemical reagents were analytical grade.

2.2. Methods

2.2.1. Synthesis of boronic acid-functionalized chitosan polymer

Chitosan (0.5 g) was dissolved in diluted acetic acid with strong agitation. 0.5 mL GMA was added and the reaction mixture was heated for 2 h at 70 °C with 0.035 g ammonium persulfate and 0.035 g sodium thiosulfate as redox catalysts. The polymerization and grafting reaction was completed by raising the temperature to 80 °C for an additional 2 h. The suspension was then centrifuged and washed with a large volume of deionized water in order to extract the matrix product chitosan–GMA (2) (see Fig. 1).

The chitosan–GMA matrix (2) was dispersed in 0.5 g APB solution, prepared by dissolving APB in 20 mL of 1 M sodium carbonate solution following by the addition of 0.25 g sodium chloride for 6 h at 60 °C with stirring. The final boronic acid functionalized chitosan polymer (3) was centrifuged and washed with deionized water until pH neutral, dried at room temperature and ground into fine powder with a mortar.

2.2.2. Model proteins and peptide mixtures

Horseradish peroxidase (HRP), and bovine serum albumin (BSA) (Sigma) were dissolved in digestion buffer. Sequencing grade trypsin (V5111, Promega) was added to a concentration of 1 μg of trypsin/50 μg protein and digestion was carried out overnight at 37 °C. Digestion buffer 1, proteins were dissolved in a solution containing urea (8 M), EDTA (5 mM) and then incubated at 50 °C for 1 h, followed by dilution with 50 mM ammonium bicarbonate at pH 7.8 to give a final urea concentration of 2 M. Digestion buffer 2 proteins were dissolved and reduced in a solution containing urea (8 M), EDTA (5 mM), and TCEP (10 mM) and then incubated at 50 °C for 1 h. To avoid complications from over alkylation, cysteines were not "capped", and reformation of disulfides was prevented by the continuous presence of stable TCEP. The reduced proteins were then diluted with 50 mM ammonium bicarbonate at pH 7.8 to give a final urea concentration of 2 M.

Peptide mixture 1 contained peptides from tryptic digestion of 20 nM of the glycosylated protein HRP. Peptide mixture 2 contained peptides from tryptic digestion of 20 nM of the glycosylated protein HRP and nonglycosylated protein BSA in a molar ratio of 1:1, and 1:10, respectively.

2.2.3. Glycopeptide enrichment

Chitosan–GMA–APB nanosphere material (0.5 mg) was placed in a 0.5 mL Eppendorf tube, and then added to 100 μL of peptide mixture from tryptic digestion in binding buffer, and the entire suspension mixture was incubated on a platform shaker at 1200 rpm at 25 °C for 40 min. After centrifugation at 13,000 rpm for 1 min, the supernatant was discarded. Unbound peptides were eliminated by washing twice with 100 μL of binding buffer, followed by rapid washing with 100 μL of deionized water. 10 μL deionized water or more elution buffer (50% (v/v) ACN, 0.5% TFA) was then added to release the glycopeptides from the Chitosan–GMA–APB nanospheres over a period of 20 min. The glycopeptide enrichment on the chitosan–GMA–APB nanospheres was analyzed by MALDI-TOF-MS. Reproducibility of this technique was evaluated by repeating all enrichment experiments at least three times.

2.2.4. MALDI-TOF-MS process

Peptide-loaded nanosphere mixture or the elute (0.5 μL) was loaded onto a stainless steel target, and 0.5 μL of a mixture of 20 mg/mL DHB in 50% (v/v) ACN and 1% TFA was added as a matrix. The above mixture was aspirated and dispensed for at least 20 cycles. MALDI-TOF mass spectra were acquired on an AXIMA-CFP plus (KRATOS Analytical, Shimadzu Group Company, Japan) mass spectrometer equipped with a nitrogen laser (337.1 nm). Mass spectra were obtained in positive ion and linear mode with an acceleration voltage of 20 kV and an average of over 100 laser shots. Mass spectrometric data analysis was performed using Launchpad V 2. 4 Kompact MALDI software. Data analysis was carried out using Kompact MALDI software with default parameters. Each spectrum was externally calibrated with insulin (5734.62). Each spectrum was internally calibrated with HRP digestion fragment ions at m/z 3355.0, 4986.3.

2.2.5. Electron micrograph

SEM (scanning electron microscope) images were taken with a JSM-5600LV (JEOL Company, Japan) instrument.

3. Results and discussion

3.1. Product identification

We designed and synthesized the Chitosan–GMA–APB nanosphere for selective and efficient capture of glycopeptides, and the procedure for synthesis is presented in Fig. 1. Characterization of the derivative chitosan nanosphere is illustrated in Fig. 2 and Table S1. The properties of compounds 1–2 are identical to those described in our previous paper (Zou et al., 2011a,b).

The structure of these synthesized products was determined by FTIR with NEXUS-470 FTIR (Nicolet, USA), electron microscopy and elemental analysis. Chitosan provides functional groups such as primary amines and both primary and secondary hydroxyl groups in its monomers (Fig. 1). These two types of reactive groups that chitosan bears can be engrafted. The different functionalizations on

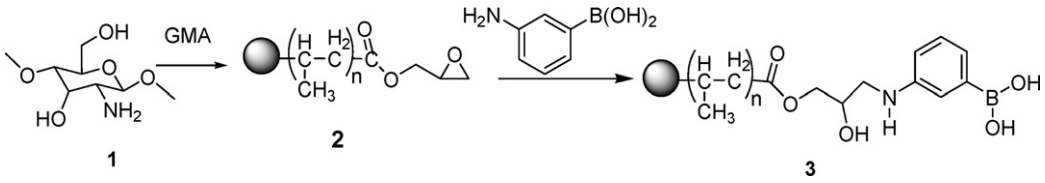

Fig. 1. Synthesis route.

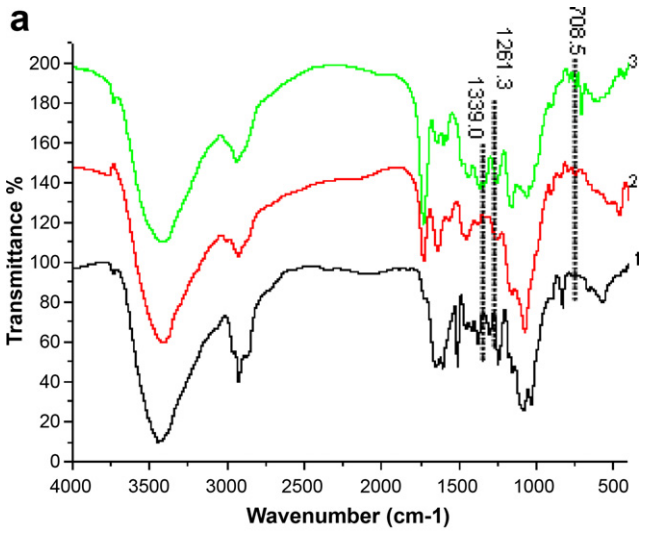

Fig. 2. Characterization of the title product. (a) FTIR spectra, (1) compound 1, (2) compound 2, (3) compound 3. (b) SEM pictures of compound 3.

the derivatized chitosan were analyzed by comparing FTIR spectra of the chitosan compounds 1, 2 and 3 with their characteristic absorptions (Fig. 2a and Table S1). The stretching peaks centered around 3420 cm^{-1} are due to H bonding of —OH and —NH_2 groups, and the peak at 1652 cm^{-1} is due to the amide group of the chitosan moiety. The strong sharp peak in the range of 1730.7 cm^{-1} together with the broad strong absorption centered at 1157 cm^{-1} may result from the ester in compounds 2 and 3. Moreover, as compared with compound 2, the presence of bands at 1339.0, 1261.3, and 708.5 are due to vibrations of B—O bonds in the nanosphere material itself is clearly consistent with boronic acid-functionalized chitosan material.

The morphology of the derivative chitosan was characterized optically by scanning electron microscopy (SEM) (Fig. 2b). The chitosan–GMA–APB consisted of regular microspheres of 20–100 nm.

The elemental analysis is listed in Table S2. ICP-MS (PE-Sciex DRC, PE, USA) was carried out to characterize the amount of boron loaded on the nanospheres, and this showed there was 10.5 mg/g of boron (average of three independent preparations). The method we describe here for the synthesis of chitosan–GMA–APB was highly reproducible and reliable.

3.2. Glycopeptides enrichment

The procedure for glycopeptide enrichment with chitosan–GMA–APB nanospheres consists of three steps which include loading of sample, washing for removal of nonspecifically adsorbed peptides, and direct evaluation by MALDI-TOF-MS. To investigate the specificity and efficiency of chitosan–GMA–APB nanospheres for enrichment of glycopeptides, HRP was chosen as a model glycoprotein as it has well-characterized glycosylation sites (Wuhrer, Hokke, & Deelder, 2004). A list of the theoretical tryptic glycosylated peptides derived from HRP and their molecular masses are shown in Table S3 (supplementary information). Evaluation of the binding selectivity of glycosylated peptides to the chitosan–GMA–APB nanospheres was performed by comparing the number of peaks of nonglycosylated tryptic peptides with those of the glycosylated peptides.

Fig. 3a shows the direct MALDI mass spectrum of the tryptic digestion of HRP (peptide mixture 1) without pretreatment. The presence of a number of nonglycopeptides in the spectrum significantly suppressed glycopeptides detection, and detected two weak of the theoretical glycopeptides (marked with asterisks). However after these digestions were enriched using chitosan–GMA–APB nanospheres as described, the MALDI-TOF mass spectrum showed detection of seven strong signals corresponding to glycopeptides (Fig. 3b, marked with asterisks).

3.3. Optimization of conditions for glycopeptide enrichment using chitosan–GMA–APB nanospheres

Various optimizations of the enrichment procedure were used for isolation of glycopeptides by chitosan–GMA–APB nanospheres, which included loading, washing, and elution. Previous research has shown that the formation of esters between boronate ion and glycopeptides requires basic pH conditions (Sparbier et al., 2005; Xu et al., 2009), and according to the work of Wang et al. (Springsteen & Wang, 2002; Yan, Springsteen, Deeter, & Wang, 2004), the formation of esters between boronate ions and diols is pH- and solvent dependent. In this work, we used a 50 mM phosphate buffer at pH 4.6, pH 6.0, pH 7.0, pH 7.8 and pH 8.5 as a loading buffer to investigate the effect of pH on the selective binding of glycopeptides to the nanospheres. Comparing Figs. 3b and 4a–d, the MALDI-TOF mass spectrum shows that only seven glycopeptides are detected at pH 4.6 (Fig. 3b). The intensity of the glycopeptides signals at m/z 3673 and 4224 are decreased under higher pH, and disappear at pH ≥ 7.0 (Fig. 4b). Moreover, some nonglycopeptides appear in the spectrum with the increase of pH. We investigated peptide mixture 2 containing peptides from tryptic digestion of 20 nM of the glycosylated protein HRP and nonglycosylated protein BSA in a molar ratio of 1:1 to examine whether highly abundant nonglycosylated peptides would affect the specificity or recovery of glycopeptides. In the original peptide mixture

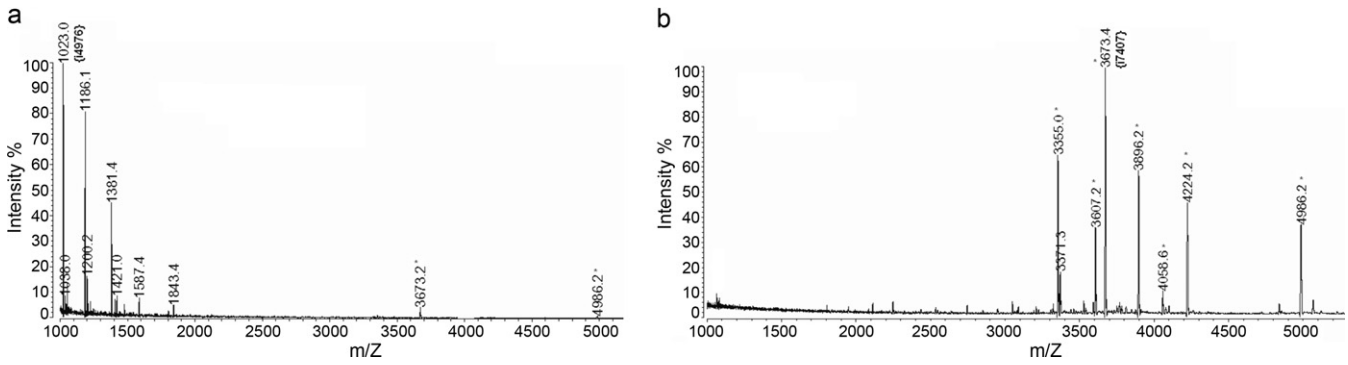

Fig. 3. MALDI-TOF mass spectra of (a) peptide mixture 1 without pretreatment (20 nM, 0.5 μL); (b) chitosan–GMA–APB nanospheres-enriched glycopeptides from peptide mixture 1 (20 nM, 100 μL) with a 50 mM phosphate buffer (pH = 4.6). *Glycopeptides. The data in parentheses represents intensity of the corresponding peak.

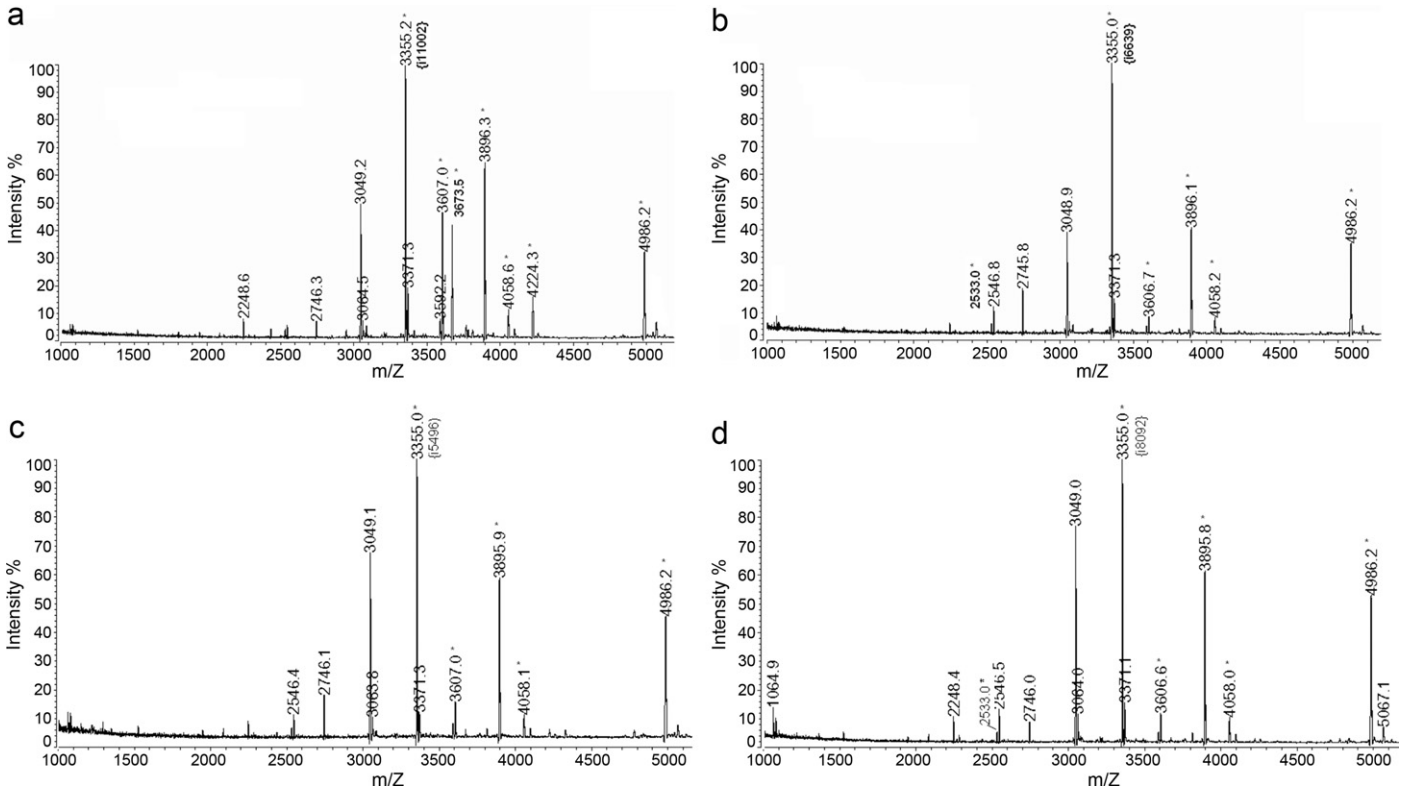

Fig. 4. Effect of sample pH on chitosan–GMA–APB nanosphere enrichment of glycopeptides. Shown are MALDI mass spectra obtained from 20 nM, 100 μL horseradish peroxidase (HRP) trypsin digest with 50 mM phosphate buffer at (a) pH = 6.0; (b) pH = 7.0; (c) pH = 7.8; (d) pH = 8.5. *Glycopeptides. The data in parentheses represent intensity of the corresponding peak.

(Fig. S1a), nonglycopeptides dominate the mass spectrum. Comparing Fig. S1b–g, there are some nonglycopeptides at pH 4.6 (Fig. S1b) but only glycopeptides are found at pH 6.0 (Fig. S1c). The intensity of the glycopeptide signal at *m*/*z* 3673 was decreased at higher pH, and this disappeared at pH > 7.8 (Fig. S2e). These results are consistent with the work by Wang et al. which showed the binding affinity between boronic acid and glycans and glycoconjugates which contain cis–diol groups is pH dependent. Therefore, when the boronic matrix is used for binding glycopeptides, the specific conditions must be taken into consideration, particularly the pH of the binding solution.

In an effort to determine the best conditions for use of this method, we have also examined the effect of buffer on the selective binding of glycopeptides to the nanospheres. Previous research used ammonium bicarbonate systems for buffering. We examined the effect of different concentrations of ammonium bicarbonate (pH 7.8) on the selective binding of glycopeptides to the nanospheres. A (0.50 M) concentration of ammonium bicarbonate buffer shows good selectivity in the enrichment of glycopeptides (Fig. S2). Holding the concentration of ammonium bicarbonate at 50 mM and adding concentrations of sodium chloride from 50 mM to 4 M into the solution, showed little effect on the enrichment efficiency (data not shown).

According to the HRP SwissProt entry P00433, for expected tryptic glycopeptides, there are three signals assigned as disulfide-linked glycopeptides (*m*/*z* 3606, 4223 and 4483). We tested two digestion buffers (see Section 2.2). Digestion buffer 2 was used to disrupt the disulfide bond and reduce the protein. Comparing Fig. S3a with Fig. 3b, it was found that two signals assigned as disulfide-linked glycopeptides (*m*/*z* 3606, 4223) disappeared in Fig. S3a. If the HRP was not digested completely (digestion time less than 4 h), these disulfide-linked glycopeptides (*m*/*z* 4483) can

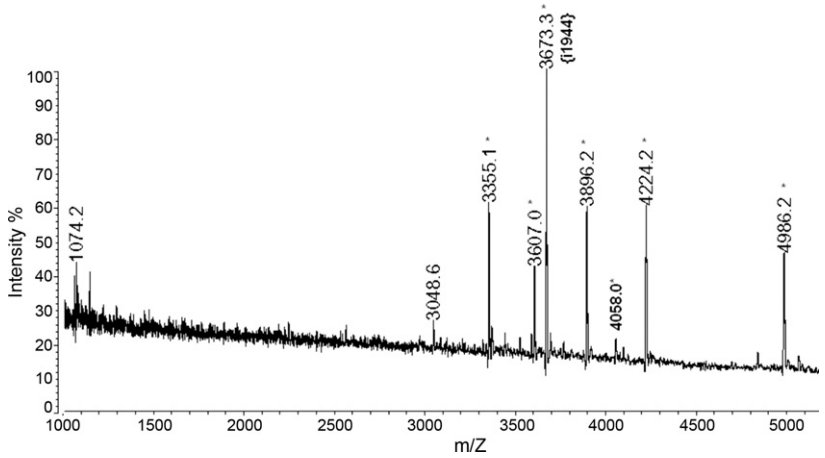

Fig. 5. Sensitivity assay. MALDI-TOF mass spectrum for glycopeptides enriched with horseradish peroxidase at a quantity of 1 fmol. *Glycopeptides. The data in parentheses represents intensity of the corresponding peak.

still be detected (Fig. S3b). This method thus can provide structural information which is consistent with the structure of the HRP SwissProt entry P00433.

Glycoproteins are often low-abundance components in real biological protein samples. We further reduced the ratio of tryptic digestion of HRP in peptide mixture 3–1:10 in order to test the performance of the nanosphere in enrichment of lower abundance glycopeptides. As shown in Fig. S4, a total of 5 glycopeptides were clearly present in the spectrum.

3.4. Sensitivity of glycopeptide enrichment

The sensitivity of glycopeptides enriched using the chitosan–GMA–APB nanosphere was investigated for tryptic digests of HRP. In the enrichment procedure, the volume of elution buffer was equal to that of the loading buffer, so that the molar concentrations of glycopeptides are nearly the same. As can be seen in Fig. 3, the originally undetectable glycopeptides ((20 nM, 0.5 μL; Fig. 3a) were easily detected after nanosphere enrichment (Fig. 3b). When the amount of HRP digest is as low as 1 fmol, the ion signals from the glycopeptides can still be detected with high S/N ratios (Fig. 5), which demonstrates the high detection sensitivity of this approach. This result may be explained by the fact that the presence of prominent nonglycopeptides severely suppressed the ionization of glycopeptides. Once the nonglycosylated peptides are eliminated by use of the chitosan–GMA–APB nanosphere, the sensitivity of detection of glycopeptides is significantly improved.

4. Conclusions

This report describes a highly specific method for capture and direct MALDI-MS analysis of glycopeptides which employs chitosan–GMA–APB nanospheres. For the first time, chitosan–GMA–APB nanospheres of diameter of 20–100 nm were synthesized for the selective enrichment of glycopeptides. This approach has several advantages.

First, the synthesis of these nanospheres is based on the fact that chitosan can be dissolved in aqueous acids. Grafting copolymerization of glycidyl methacrylate onto chitosan in liquid solution produces the nanosphere. These nanospheres have relatively great surface area and good dispersibility in buffer solution. The glycopeptides on the nanosphere may be directly detected by MALDI-TOF MS which avoids sample loss during elution.

Second, our study showed that MALDI-TOF-MS combined with use of chitosan–GMA–APB nanospheres is a simple and rapid approach for enrichment of glycopeptides. The loading time can be shortened to 10 min (or even to as little as 5 min, data not shown), and the whole procedure requires about 30 min. This method yields increased sensitivity and reproducibility without desalting steps that are mandatory for other MS-analysis techniques. With amounts of HRP digest even as low as 1 fmol, the ion signals from the glycopeptides can still be detected with high S/N ratios because the suppression effect from nonglycopeptides is eliminated by capturing and concentrating target glycopeptides. This simple and efficient method of glycopeptide isolation which we have described here will facilitate the study of large glycoproteins that generate numerous peptides upon enzymatic digestion.

A detailed examination of the factors which affect the binding between boronic acid and glycopeptides is important for the future design of boronic acid based sensors. The results we report indicate that binding between boronic acid and glycopeptides is pH- and solvent dependent.

The highly specific mechanisms of molecular separation using chitosan–GMA–APB nanospheres are based on electrostatic interaction between anionic glycosaminoglycan and proteoglycan, and chitosan which is cationic; hydrogen bonding between the hydroxyl group of chitosan and glycopeptides; strong hydrophilicities of chitosan with the hydrophilic glycopeptides; and the diboronic acid functional groups on the surface of the nanosphere which forms diesters with glycopeptides that contain cis–diol groups. Based upon these features which act in molecular separation, coupled with the unique properties of these nanomaterials which include generous surface area, good biocompatibility and chemical stability, we anticipate that these chitosan nanospheres will have broad applicability. In view of their ability to bind and concentrate many molecules, they will likely find use in pharmaceutical, biomedical, environment and biotechnological fields.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.05.090.

References

Bunkenborg, J., Pilch, B. J., Podtelejnikov, A. V., & Wisniewski, J. R. (2004). Screening for N-glycosylated proteins by liquid chromatography mass spectrometry. *Proteomics*, *4*, 454–465.

Casal, E., Corzo, N., Moreno, F. J., & Olano, A. (2005). Selective recovery of glycosylated caseinmacropeptide with chitosan? *Journal of Agricultural and Food Chemistry*, *23*, *53*(4), 1201–1204.

Cummings, R. D., & Kornfeld, S. (1982). Fractionation of asparagine-linked oligosaccharides by serial lectin–agarose affinity chromatography. A rapid, sensitive, and specific technique. *Journal of Biological Chemistry*, *257*, 11235–11240.

Fan, X., She, Y. M., Bagshaw, R. D., Callahan, J. W., Schachter, H., & Mahuran, D. J. (2004). Identification of the hydrophobic glycoproteins of Caenorhabditis elegans. *Anal. Biochem*, *332*, 178–186.

Hirabayashi, J. (2004). Lectin-based structural glycomics: Glycoproteomics and glycan profiling. *Glycoconjugate Journal*, *21*, 35–40.

Jayakumar, R., Prabaharan, M., Reis, R. L., & Mano, J. F. (2005). Graft copolymerized chitosan—Present status and applications. *Carbohydrate Polymers*, *62*, 142–158.

Mourya, V. K., & Inamdar, N. N. (2008). Chitosan-modifications and applications: Opportunities galore. *Reactive & Functional Polymers*, *68*, 1013–1051.

Muzzarelli, R. A. A. (2009). Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone. *Carbohydrate Polymers*, *76*, 167–182.

Muzzarelli, R. A. A. (2011). Chitosan composites with inorganics, morphogenetic proteins and stem cells, for bone regeneration. *Carbohydrate Polymers*, *83*, 1433–1445.

Muzzarelli, R. A. A. (2012). Chitosan, hyaluronan and chondroitin sulfate in tissue engineering for cartilage regeneration: A review. *Carbohydrate Polymers*, *89*, 723–739.

Pan, S., Chen, R., Aebersold, R., & Brentnall, T. A. (2011). Mass spectrometry based glycoproteomics – From a proteomics perspective. *Molecular & Cellular Proteomics*, *10*(1). R110.003251

Prabaharan, M. (2008). Chitosan derivatives as promising materials for controlled drug delivery. *Journal of Biomaterials Applications*, *23*, 5–36.

Sparbier, K., Koch, S., Kessler, I., Wenzel, T., & Kostrzewa, M. (2005). Selective isolation of glycoproteins and glycopeptides for MALDI-TOF MS detection supported by magnetic particles. *Journal of Biomolecular Techniques*, *16*, 407–413.

Springsteen, G., & Wang, B. (2002). A detailed examination of boronic acid–diol complexation. *Tetrahedron*, *58*, 5291–5300.

Tian, Y., Zhou, Y., Elliott, S., Aebersold, R., & Zhang, H. (2007). Solid-phase extraction of N-linked glycopeptides. *Nature Protocols*, *2*, 334–339.

Wang, L., Li, F., Sun, W., Wu, S., Wang, X., Zhang, L., et al. (2006). Concanavalin A-captured glycoproteins in healthy human urine. *Molecular & Cellular Proteomics*, *5*, 560–562.

Wada, Y., Tajiri, M., & Yoshida, S. (2004). Hydrophilic affinity isolation and MALDI multiple-stage tandem mass spectrometry of glycopeptides. *Analytical Chemistry*, *76*, 6560–6565.

Wuhrer, M., Hokke, C. H., & Deelder, A. M. (2004). Glycopeptide analysis by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry reveals novel features of horseradish peroxidase glycosylation. *Rapid Communications in Mass Spectrometry*, *18*, 1741–1748.

Liu, X., Ma, L., & Li, J. (2008). Recent developments in the enrichment of glycopeptides for glycoproteomics. *Analytical Letters*, *41*(2), 268–277.

Xu, Y., Wu, Z., Zhang, L., Lu, H., Yang, P., Webley, P. A., et al. (2009). Highly specific enrichment of glycopeptides using boronic cid-functionalized mesoporous silica. *Analytical Chemistry*, *81*(1), 503–508.

Yan, J., Springsteen, G., Deeter, S., & Wang, B. (2004). The relationship among pK_a, pH, and binding constants in the interactions between boronic acids and diols – It is not as simple as it appears. *Tetrahedron*, *60*, 11205–11209.

Zhang, L., Lu, H., & Yang, P. (2010). Specific enrichment methods for glycoproteome research. *Analytical and Bioanalytical Chemistry*, *396*, 199–203.

Zou, X., Zhong, L., Liu, D., Yang, B., Lou, Y., Peng, J., et al. (2011). Novel multifunctional chitosan–GMA–IDA–Cu(II) nanospheres for high dynamic range characterization of the human plasma proteome. *Analytical and Bioanalytical Chemistry*, *400*, 747L 756.

Zou, X., Liu, D., Zhong, L., Yang, B., Lou, Y., Hu, B., et al. (2011). Highly specific capture and direct MALDI-MS analysis of phosphorylated peptides using novel multifunctional chitosan–GMA–IDA–Fe (III) nanosphere. *Analytical and Bioanalytical Chemistry*, *401*, 1251–1261.