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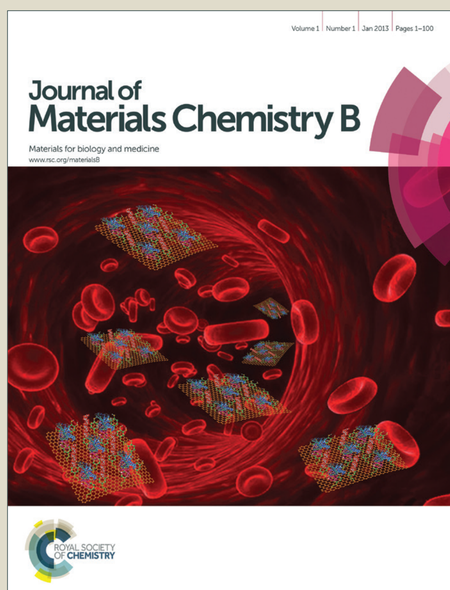

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ARTICLE TYPE

Efficient enrichment of glycopeptides using phenylboronic acid polymer brush modified silica microspheres

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Abstract

For the development of rapid glycopeptide enrichment materials, conventional monolayer phenylboronic acid (PBA) based materials inevitably encounter many puzzle problems, such as low loading efficiency, long incubation time, and unsatisfied selectivity. Expanding the materials from 1D monolayer to 3D polymeric matrix will be one of best candidates tackling these problems. In this work, a PBA-based polymer material (denoted as polyPBA@SiO$_2$) was developed, in which flexible PBA polymer brushes were immobilized on the surface of silica microspheres, constructing an ideal platform for the efficient enrichment of glycopeptides. This material exhibits strong interaction with glycopeptides in higher concentration of organic solvent than in aqueous solution, resulting in the maxium binding capacity of 60 mg/g. Moreover, higher selectivity for glycopeptides can be achieved with polyPBA@SiO$_2$ than with both monolayer PBA modified silica and commercial PBA-agarose. These unique features of PB-PBA@SiO$_2$ could be attributed to the synergistic effect of polyvalent interaction provided by polymer brush, specific interaction between PBA and glycopeptides and suppression of the non-specific binding of non-glycopeptides under high ACN concentration.

Introduction

Glycosylation is one of the most important post translational modifications of proteins[1, 2] and aberrant protein glycosylation has been associated with various diseases including cancer.[3-5] Enrichment of N-linked glycopeptides has been significantly important because they are crucial to further structural determination and functional elucidation of N-linked glycoproteins. Conventional approaches for glycopeptide enrichment are mainly based on lectin affinity chromatography,[6-9] hydrazide chemistry[10-13] or hydrophilic interaction affinity chromatography (HILIC).[14-17] These conventional methods, however, are limited by either low coverage of glycopeptides, loss of glycan information or medium specificity. Recently, phenylboronic acid (PBA) functionalized materials have attracted much attention in various biological applications due to reversible diester bonds between PBA and *cis*-diol-containing compounds.[18-23] Especially, PBA-containing materials have been utilized for glycopeptide enrichment in basic buffer solution.[24-27] However, these PBA-based methods suffer from non-specific binding resulting from hydrophobic interactions of non-glycopeptides in aqueous solution,[27, 28] leading to limited selectivity. Moreover, PBA-based method requires long incubation time because of slow formation of diesters between glycans and boronic acid. In order to improve the selectivity and fasten the binding of glycopeptides, it is of urgent need to develop novel method, which can strongly enhance the interaction between PBA glycopeptides and notably reduce the non-specific binding of non-glycopeptides.

Polymer brushes contain end-tethered polymer chains grafted on the support.[29] These flexible polymer chains stretch away from the substrate and facilitate multiple interactions between materials and targets.[21, 30] Moreover, some polymer brushes respond to various stimuli including solvent and pH.[31] N-linked glycopeptides contain branched, bulky, mobile oligosaccharide clusters,[32] and have higher hydrophilicity than their counterparts.[33] In HILIC mode, hydrophilic glycopeptides generally retain stronger on the polar matrix than the corresponding non-glycopeptides. Hydrophobic peptides tend to be eluted in the flowthrough and earlier fractions.[14, 34]

Herein, we designed and prepared PBA polymer brush modified silica microsphere materials (polyPBA@SiO$_2$). Furthermore, polyPBA@SiO$_2$ was used to separate glycopeptides from non-glycopetpides in HILIC mode. Improving retention of target glycopeptides and reduced binding of non-glycopeptides would be acchieved in HILIC mode. By combining the synergistic effect of polyvalent interaction between polymer brush and glycan, specific recognition of PBA toward saccharide residues and hydrophilic interaction of glycopeptides (Fig. 1A), highly selective and rapidly enrichment of glycopeptides would be obtained with polyPBA@SiO$_2$.

Experimental

Materials

All chemicals unless otherwise mentioned were purchased form Sigma-Aldrich (St. Louis, MO, USA). Sequencing grade modified trypsin was obtained from Promega (Madison, WI,

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USA). Acetonitrile (ACN) was purchased from Merck (Darmstadt, Germany). Formic acid (FA) was purchased from Acros Organics (Geel, Belgium). Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Commercial phenylboronate agarose (PBA-agarose) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of polyPBA@SiO_2

PolyPBA@SiO_2 was prepared via typical chain-transfer radical polymerization.[35] Porous silica microspheres with 30 nm pore size (1 g) were dispersed in anhydrous methanol (10 ml) containing 5 wt% 3-mercaptopropyltrimethoxysilane at room temperature for 24 h, generating chemically bonded -SH groups as the chain-transfer agent. After rinsing with methanol and 1,4-dioxane, polymerization of PBA was performed by dispersing the -SH bonded silica microspheres into a degassed solution of PBA (0.2 g) in 1,4-dioxane (10 mL) containingα, α'-azodiisobutyronitrile (10 mg) for 1 h at 80 ^oC. After washing with acetone, the polyPBA@SiO_2 were obtained through centrifugation (12000 r/min for 5 min), and then dried at 60 ^oC for 5 days.

Characterization of polyPBA@SiO_2

Characterization of prepared polyPBA@SiO_2 materials was performed for morphology and composition analysis. Fourier transform infrared (FTIR) spectrum was recorded with Vertex 80. The X-ray photoelectron spectroscopy (XPS) was performed with an ESCALab220i-XL electron spectrometer from VG Scientific using 300W AlKα radiation. The base pressure was about 3×10^{-9} mbar. The binding energies were referenced to the C1s line at 284.8 eV from adventitious carbon**.** High-resolution scanning electron microscopy (HRSEM) was taken on a Hitachi-S5500. N_2 adsorption–desorption experiments were measured on an automatic TriStar II 3020. Brunauer–Emmett–Teller (BET) surface areas were calculated from adsorption data in the relative pressure range from 0.01 to 0.2. Pore size distributions were analyzed from the adsorption branches of the isotherms. Thermo-gravimetric analysis (TGA) carried out on Perkin Elmer Pyris Diamond. 10 mg sample was analysed with a 10 ^oC/min heating rate from 50 to 750 ^oC under N_2 atmosphere.

Trypsin digestion of glycoproteins

Horseradish peroxidase (HRP) (1 mg) was dissolved with 100 μL 6 M urea in 50 mM ammonium bicarbonate (NH_4HCO_3). The protein solution was treated with 4 μL 50 mM dithiothreitol for 45 min at 56 ^oC. Then 5 μL 50 mM iodoacetamide was added and the mixture was incubated in the dark for 30 min at the room temperature. The mixture was diluted to 10 folds with 50 mM NH_4HCO_3 and digested with trypsin at an enzyme to protein ratio of 1:30.

Enrichment of glycopeptides

For glycopeptide enrichment from tryptic digests of standard glycoproteins, 1 mg of polyPBA@SiO_2 materials suspended in ACN was loaded into 20 μL GELoader tips (Eppendorf AG, Hambrug, Germany). After conditioning and equilibrating with 30 μL 50 mM NH_4HCO_3 and 85% ACN/50 mM NH_4HCO_3, 5 μL glycoprotein (12.5 pmol) tryptic digests were dried and redissolved with 30 μL 85% ACN/50 mM NH_4HCO_3. The redissolved peptides were loaded into the packed polyPBA@SiO_2 microcolumn. The microcolumn was washed twice with 30 μL 72% ACN/50 mM NH_4HCO_3. Glycopeptides were detached from column with 20 μL 50% ACN/0.1% FA. For comparison, commercial PBA-agarose and monolayer PBA@SiO_2 (See supporting information (SI)) were used to enrich glycopeptides under same condition applied to polyPBA@SiO_2 using HRP digests as samples. For C18 desalting, the performance was according to previous report. [36]

Nano-ESI-MS analysis

Peptide fractions eluted from polyPBA@SiO_2, PBA-agarose, C18 and monolayer PBA@SiO_2 were analyzed directly with a nano electrospray ionization-quadrupole time-of-flight mass spectrometer (ESI Q-TOF MS) (Waters, Milford, MA, USA) The MS analysis was under positive ion mode. Full scan MS data and MS/MS data were acquired at m/z 600-2000 and 100-2000, respectively. Collision energy was set in the range from 30-45 eV.

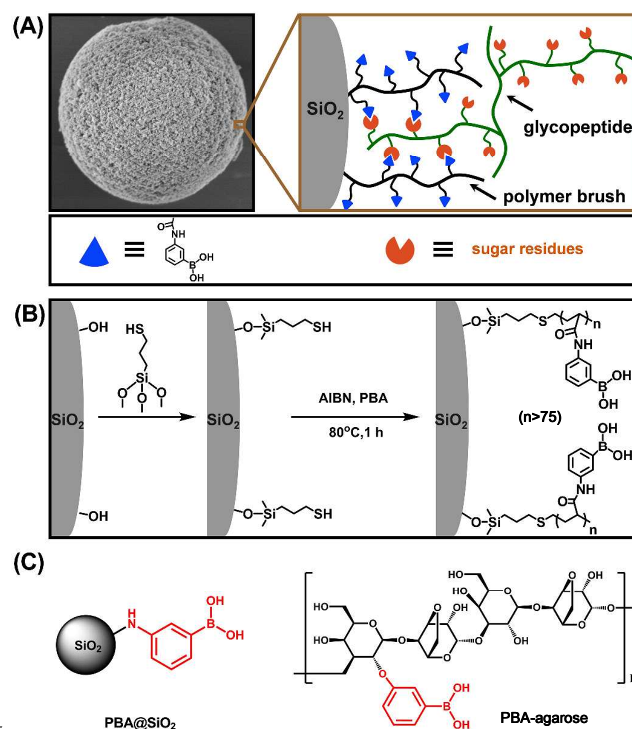

Fig. 1 Schematic illustration of multiple sites interaction between polyPBA@SiO_2 and glycopeptides (A), synthesis route of polyPBA@SiO_2 (B), chemical structures of two reference materials: PBA@SiO_2 and PBA-agarose (C).

Enrichment capacity

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To determine the enrichment capacity of polyPBA@SiO_2, various amounts of HRP digests in fixed volume (50 μL) were loaded into 1 mg materials packed GELoader tips. The flow-through was analyzed with Q-TOF MS until the glycopeptide signals were observed. The experiments were repeated triple times.

Results and discussion

Synthesis and characterization of polyPBA@SiO_2

The synthesis procedure of polyPBA@SiO_2 was illustrated in Fig. 1B. PolyPBA@SiO_2 was prepared via typical chain-transfer radical polymerization.[35] HRSEM images (Fig. 2) and N_2 adsorption (Fig. S1) results revealed that polyPBA@SiO_2 materials preserve similar morphology to that of silica microspheres and the pores in polyPBA@SiO_2 were not blocked. BET surface area of 70 m^2/g and pore volume of 0.55 cm^3/g were derived from N_2 adsorption experiment. These data proved that polyPBA@SiO_2 materials were not degraded after coating with polymers on the silica surface.

FTIR spectrum of polyPBA@SiO_2 exhibits obvious bands at about 1630 cm^{-1} and 1450 cm^{-1}, which corresponds to the characteristic groups in polyPBA. By comparison, no obvious bands can be found in the spectrum of SiO_2 (Fig. 3A). The XPS spectrum of polyPBA@SiO_2 shows four peaks with binding energies at about 173.3, 285.3, 400.3 and 533.8 eV, which can be ascribed to the B1s, C1s, N1s and O1s, respectively (Fig. 3B). Carbon content of 5.4% was measured from elemental analysis. TGA was used to further measure the relative composition of polyPBA@SiO_2 (Fig. S2). The sharp decline above 350 °C was resulted from the weight loss of polymer layer. The calculated surface coverage of polyPBA@SiO_2 was 0.12 μmol/m^2. These data suggested that PBA polymer brush was successfully grafted on the silica microspheres.

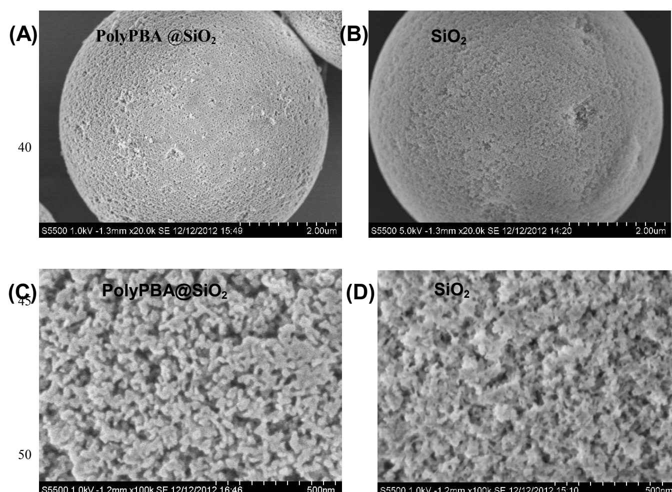

Fig. 2 High resolution scanning electron microscopy (HRSEM) images of polyPBA@SiO_2 (A) X 20 000, (C) X 100 000 and SiO_2 (B) X 20 000, (D) X 100 000.

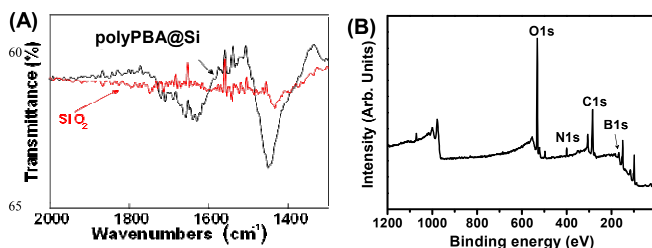

Fig. 3 Fourier transform infrared spectrum (A) and X-ray photoelectron spectroscopy of polyPBA@SiO_2 (B).

Retention of peptides on polyPBA@SiO_2

To test whether polyPBA@SiO_2 can retain glycopeptides, HRP digests in different concentration of ACN was loaded into the 1 mg polyPBA@SiO_2 packed solid phase extraction (SPE) micro-columns. The peptide sequence and glycan composition of HRP glycopeptides were listed in Table 1.[37] When 90% ACN/50 mM NH_4HCO_3 and 80% ACN/50 mM NH_4HCO_3 were used as loading buffer, only several non-glycopeptides were detected in their flowthrough. With the decrease of ACN concentration, 3 glycopeptides were found in 70% ACN flowthrough (Fig. S3C). When 50 mM NH_4HCO_3 solution was applied as loading buffer, 5 glycopeptides were detected in the flowthrough (Fig. S3D). These data suggested that strong interaction might be involved between glycopeptides and polyPBA@SiO_2. We speculated that the polyPBA@SiO_2 might be more lyophobic in 50 mM NH_4HCO_3 solution than in high concentration of ACN solution.

Table 1 Information of identified HRP glycopeptides

Amino acid sequence	Glycans	Observed m/z and charge	Position
NVGLNR	$Man_3GlcNAc_2Fuc_1Xyl_1$	921.85 (2+)	184-189
GLIQSDQELFSSPNATDTIP LVR	$Man_3GlcNAc_2Fuc_1$	1175.33(3+)	272-294
NQCR GLCPLNGNLSALVDFDLR	$Man_3GlcNAc_2Fuc_1Xyl_1$	1202.23(3+)	205-208, 237-254
GLIQSDQELFSSPNATDTIP LVR	$Man_3GlcNAc_2Fuc_1Xyl_1$	1224.55(3+), 1836.07(2+)	272-294
NQCR GLCPLNGNLSALVDFDLR	$Man_4GlcNAc_2Fuc_1Xyl_1$	1256.23(3+)	205-208, 237-254
LYNFSNTGLPDPTLNTTYLQ TLR	$Man_3GlcNAc_2Fuc_1Xyl_1$	1272.2(3+)	214-236
LHFHDCFVNGCDASILLDNTTSFR	$Man_3GlcNAc_2Fuc_1Xyl_1$	1299.36(3+)	69-92
MGNITPLTGTQGQIR	$Man_3GlcNAc_2Fuc_1Xyl_1$	1306.2(2+)	314-328
AAVESACPR QLTPTFYDNSCPNVSNIVR	$Man_3GlcNAc_2Fuc_1Xyl_1$	1407.59(3+)	115-123, 31-49
LYNFSNTGLPDPTLNTTYLQ TLR	$Man_6GlcNAc_4Fuc_2Xyl_2$	1662.06(3+)	214-236
SFANSTQTFFNAFVEAMDR	$Man_3GlcNAc_2Fuc_1Xyl_1$	1677.55(2+)	295-313

N*: Glycosylation site. C: disulfide bonds formed by the oxidation of two cysteine amino acids

To confirm our speculation, contact angles were measured on polyPBA grafted silicon wafer by using different liquid probes. Fig. 4 shows contact angles were about 67° and 65° when using 50 mM NH_4HCO_3 and 50 mM NH_4HCO_3/10 mM mannose as the probes, implying higher lyophobicity of polyPBA in aqueous solution. In contrast, the contact angle was about 33° when 85% ACN/50 mM NH_4HCO_3 was used, which indicates that the polymer surface became more lyophilic under the treatment of

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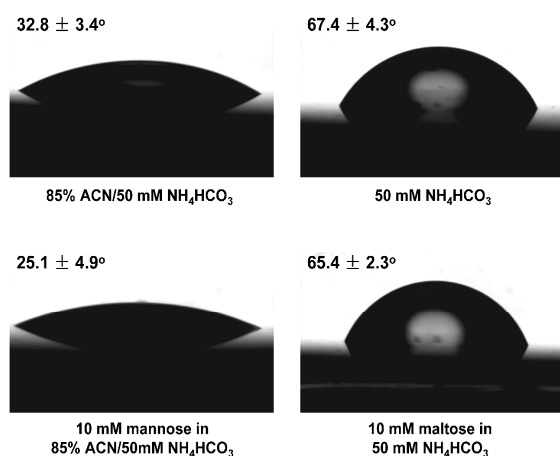

Fig. 4 Contact angle of polyPBA grafted silicon wafer under different conditions

high concentration of organic solvent. The presence of 10 mM mannose in the 85% ACN/50 mM NH_4HCO_3 buffer could induce more obvious change in the contact angle, which further decreased to 25°. This extra change can be attributed to the stronger interaction between monosaccharide (e.g. mannose) and PBA functional units, as well as the better compatibility of polyPBA@SiO_2 in high concentration of ACN. Considering N-linked glycopeptides comprise a large number of pentasaccharides in their glycans, which are supposed to exhibit much higher hydrophilicity than their counterparts, non-glycopeptides. The above results inspired us to deduce that polyPBA@SiO_2 had stronger binding to glycopeptides under higher concentration of organic solvent, while more lyophilic characteristic of materials can help to reduce the undesirable adsorption of non-glycopeptides.

The effect of acidic, neutral and basic solution to the retention of glycopeptides was also investigated. HRP digests dissolved with 75% ACN of pH 3, 7 and 9 were loaded into polyPBA @SiO_2 packed SPE microcolumns, respectively. It was found that glycopeptides were eluted with 75% ACN under both neutral and acidic solution (Fig. S4). On the contrary, the glycopeptides were well captured with 75% ACN in basic solution. This result demonstrated that polyPBA@SiO_2 bound strongly with glycopeptides in the higher pH solution even under high concentration of organic solvent, indicating affinity interaction existing between glycopeptides and boronate groups. Taken these results together, we concluded that both affinity interaction and hydrophilic interaction might be synergistically involved in the interaction between glycopeptides and the as-prepared polyPBA@SiO_2.

Enrichment of glycopeptides with polyPBA@SiO_2

The enrichment of glycopeptides was carried out under SPE mode because of microsphere morphology and flowthrough pore size of polyPBA@SiO_2. Tryptic HRP digests were used to qualitatively evaluate the enrichment selectivity. The captured peptides were characterized with ESI Q-TOF MS. Before enrichment, a large number of high-intensity non-glycopeptides dominated the mass spectrum and severely suppressed the signals of glycopeptides, leading to only 4 observed glycopeptide signals (Fig. 5A). After enrichment with polyPBA@SiO_2 materials, up to 27 HRP glycopeptide signals covering all known glycosylation sites were readily detected because the high-abundance non-glycopeptides and ion suppression effect were efficiently removed (Fig. 5D). HRP glycopeptides were characterized by comparing their molecular weights and fragment ion mass spectrum with those of literature. MS/MS mass spectra of the identified glycopeptides all had at least three indicator fragment ions of glycopeptides including oxonium ions m/z 163 (Hex+), m/z 204 (HexNAc+) and m/z 366 (Fig. S5 in SI). For comparison, commercial PBA-agarose and monolayer PBA modified SiO_2 (PBA@SiO_2, the synthesis and characterization of PBA@SiO_2 see SI) were used to bind HRP glycopeptides under the same condition as polyPBA@SiO_2. Enrichment selectivity of PBA@SiO_2 toward glycopeptides was impaired by several non-glycopeptides in the m/z range between 700-900 (Fig. 5B) even though incubation of PBA@SiO_2 and HRP digests for 30 min. PBA-agarose showed nonspecific adsorption toward non-glycopeptides, leading to co-elution of non-glycopeptides at m/z 959.5344, 1185.6184 and 1573.8035 with HRP glycopeptides (Fig. 5C). The above data demonstrated polyPBA@SiO_2 had higher enrichment efficiency for glycopeptides than commercial PBA-agarose and monolayer PBA@SiO_2.

When PBA@SiO_2, PBA-agarose and polyPBA@SiO_2 were used to enrich glycopeptides, they showed varied glycopeptides selectivity. We speculate that PBA@SiO_2 has low-density and close PBA groups modified on SiO_2, which to large extent leads to weak interaction with glycopeptides. For PBA-agarose, PBA on the rigid polysaccharide structures restricts the effective interaction between glycopeptides and materials, resulting in the limited selectivity. Compared with PBA-agarose and PBA@SiO_2, flexible PBA polymer chains on polyPBA@SiO_2 extend from the SiO_2 surface and provide multiple interactions between PBA and glycopeptides. Moreover, the end-grafted flexible chains might be more reactive than monolayer PBA close to SiO_2 surface.[30] These multiple interaction sites and more reactive end-grafted chains facilitate strong and selective interaction between glycopeptides and polyPBA@SiO_2. Meanwhile, the hydrophilic interactions of polyPBA@SiO_2 with non-glycopeptides were much weaker than that with glycopeptides containing multiple hydroxyl groups. Therefore, the enhanced affinity interactions and hydrophilic interaction provided by polyPBA@SiO_2 contributed to the isolation of glycopeptides and the improved selectivity of materials.

In order to test our speculation, flowthrough fractions from PBA@SiO_2 and polyPBA@SiO_2 were analyzed with MS. No HRP glycopeptide signals could be found in the mass spectra of flowthrough fractions from polyPBA@SiO_2 (Fig. S6 in SI). However, 3 HRP glycopeptides and a number of non-glycopeptides could be detected in the flowthrough fraction from PBA@SiO_2 (Fig. S6 in SI). This result further proved polyPBA@SiO_2 bound stronger and faster than momolayer PBA@SiO_2. [30]

Due to the multiple interactions between glycopeptides and polyPBA@SiO_2, we assumed that polyPBA@SiO_2 had high capacity toward glycopeptides. To determine the adsorption

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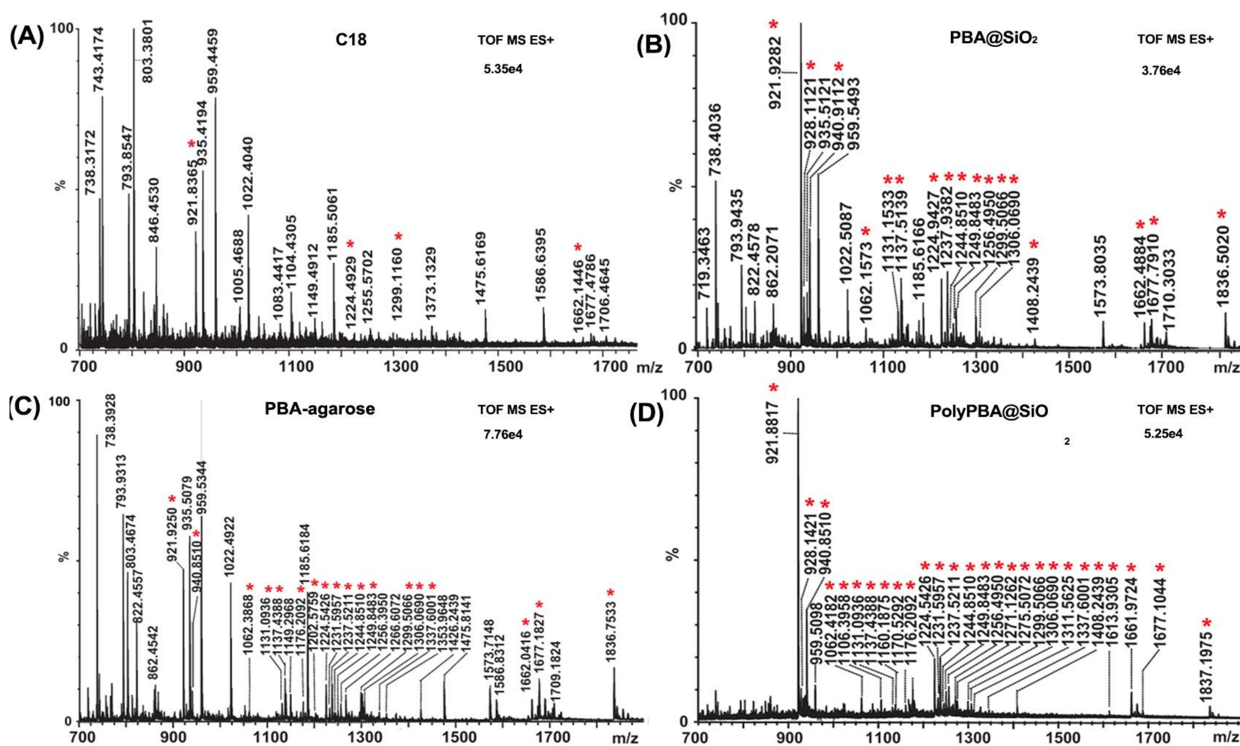

Fig. 5 Nano-ESI-Q-TOF mass spectra of tryptic HRP digests before enrichment (A) and after enrichment with monolayer PBA modified SiO_2 (B), commercial PBA-agarose(C) and (D) polyPBA@SiO_2 under the same conditions. Glycopeptides are labelled with *. The non-glycopeptides were labelled with their m/z values. polyPBA@SiO_2 showed much higher selectivity for glycopeptides than monolayer PBA modified SiO_2, commercial PBA-agarose.

capacity of PB-PBA@SiO_2, 1 mg materials were employed to bind various concentration of HRP digests in a fixed volume (50 μL). The flowthrough fraction was characterized with Q-TOF MS. Before reaching the adsorption capacity of PB-PBA@SiO_2, no glycopeptide signals were detected. When the loading mass of glycopeptides was overweighed the adsorption capacity of binding materials, the glycopeptide signals were observed. The binding capacity of PB-PBA@SiO_2 toward HRP glycopeptides were measured as was 60 mg/g, compared to 20 mg/g of monolayer PBA@SiO_2 (Fig. 6). The high binding capacity was attributed to the multiple sites interaction provided by polymer brush.

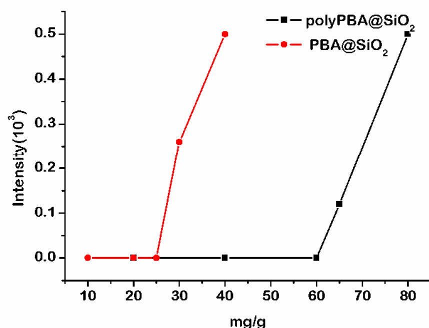

Fig. 6 Determination of adsorption capacity of polyPBA@SiO_2 and monolayer BA@SiO_2.

Conclusions

In summary, we developed a new strategy based on PBA polymer brush modified silica microspheres (i.e., polyPBA@SiO_2) to efficiently enrich glycopeptides. Our strategy demonstrated much higher enrichment selectivity and adsorption capacity toward glycopeptides than PBA@SiO_2 and PBA-agarose. We anticipate this new kind of glycopeptides capturing method may become a useful tool for glycoproteome. Further studies will introduce more functional groups including small peptides and oligosaccharides to polymer chains and enhance the interaction between glycopeptides and materials.

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Notes and references

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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