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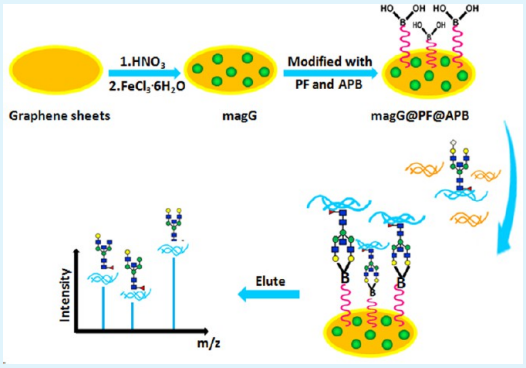
Multilayer Hydrophilic Poly(phenol-formaldehyde resin)-Coated Magnetic Graphene for Boronic Acid Immobilization as a Novel Matrix for Glycoproteome Analysis

Jiaxi Wang, Yanan Wang, Mingxia Gao,* Xiangmin Zhang,* and Pengyuan Yang

Department of Chemistry and Institutes of Biomedical Sciences, Fudan University, Shanghai 200433, China

Supporting Information

ABSTRACT: Capturing glycopeptides selectively and efficiently from mixed biological samples has always been critical for comprehensive and in-depth glycoproteomics analysis, but the lack of materials with superior capture capacity and high specificity still makes it a challenge. In this work, we introduce a way first to synthesize a novel boronic-acid-functionalized magnetic graphene@phenolic-formaldehyde resin multilayer composites via a facile process. The as-prepared composites gathered excellent characters of large specific surface area and strong magnetic responsiveness of magnetic graphene, biocompatibility of resin, and enhanced affinity properties of boronic acid. Furthermore, the functional graphene composites were shown to have low detection limit (1 fmol) and good selectivity, even when the background nonglycopeptides has a concentration 100 fold higher. Additionally, enrichment efficiency of the composites was still retained after being used repeatedly (at least three times). Better yet, the practical applicability of this approach was evaluated by the enrichment of human serum with a low sample volume of 1 μL. All the results have illustrated that the magG@PF@APB has a great potential in glycoproteome analysis of complex biological samples.



KEYWORDS: *boronic-acid-functionalized magnetic graphene, enrichment, glycoproteome, phenolic-formaldehyde resin, MALDI-TOF mass spectrometry*

INTRODUCTION

As one of the most significance and common post-translational modifications (PTMs), protein N-glycosylation plays a crucial role in various biological activities, for example, cell division, recognition, migration, and signal transduction in eukaryotes cells.[1−5] To further understand these bioprocesses, it is necessary to develop new techniques for precise and systematic identification of glycoproteins. Generally, mass spectrometry (MS) as a strategy has become a favored option in glycoproteome research due to its ultrahigh sensitivity, simplicity, and high-throughput in the analysis of glycosylation sites.[6−8] However, glycopeptides with low abundance and severe ion suppression effect often result in weak signals during the MS measurements. Thus, it is essential to enrich target glycans selectively from complex biological samples and determine their glycosylation sites.

In the past few years, the conventional approaches for glycopeptides enrichment are mainly based on lectin affinity chromatography,[9−11] hydrophilic interaction affinity chromatography,[12,13] size exclusion chromatography,[14] and hydrazine chemistry.[15−18] Among them, boronate-functionalized matrix has gained more potential in glycoproteome analysis since it is well-known that the reversible diester formation between boronic acid groups and cis-diol compounds. Until now, boronate-functionalized complex as the solid-phase extraction materials of glycopeptides have been widely reported, including mesoporous silica,[19] agarose resin,[20] monoliths,[21] polymer materials, and magnetic nanoparticles.[22,23] However, these methods often suffer from harsh conditions, cumbersome processes, and the interference of other nonglycopeptides. Consequently, it is preferable to develop a rapid, facile, and integrated method for the preparation of boronate-functionalized materials which can perform better in capturing the glycopeptides.

Since its discovery in 2004,[24] graphene has drawn widespread notices of scientists because of its excellent feasibility of associating with metals, organic compounds, and magnetic particles. Because graphene-based composites possess unique properties, including large specific surface area (2630 m^2/g)[25] and biocompatibility, they have been widely used as adsorbents in analytical chemistry.[26,27] For proteomics research, design of the graphene-based nanohybrids in a rapid and convenient way have become a hot topic in research. In recent years, as an effective functionalization method, grafting polymers on the

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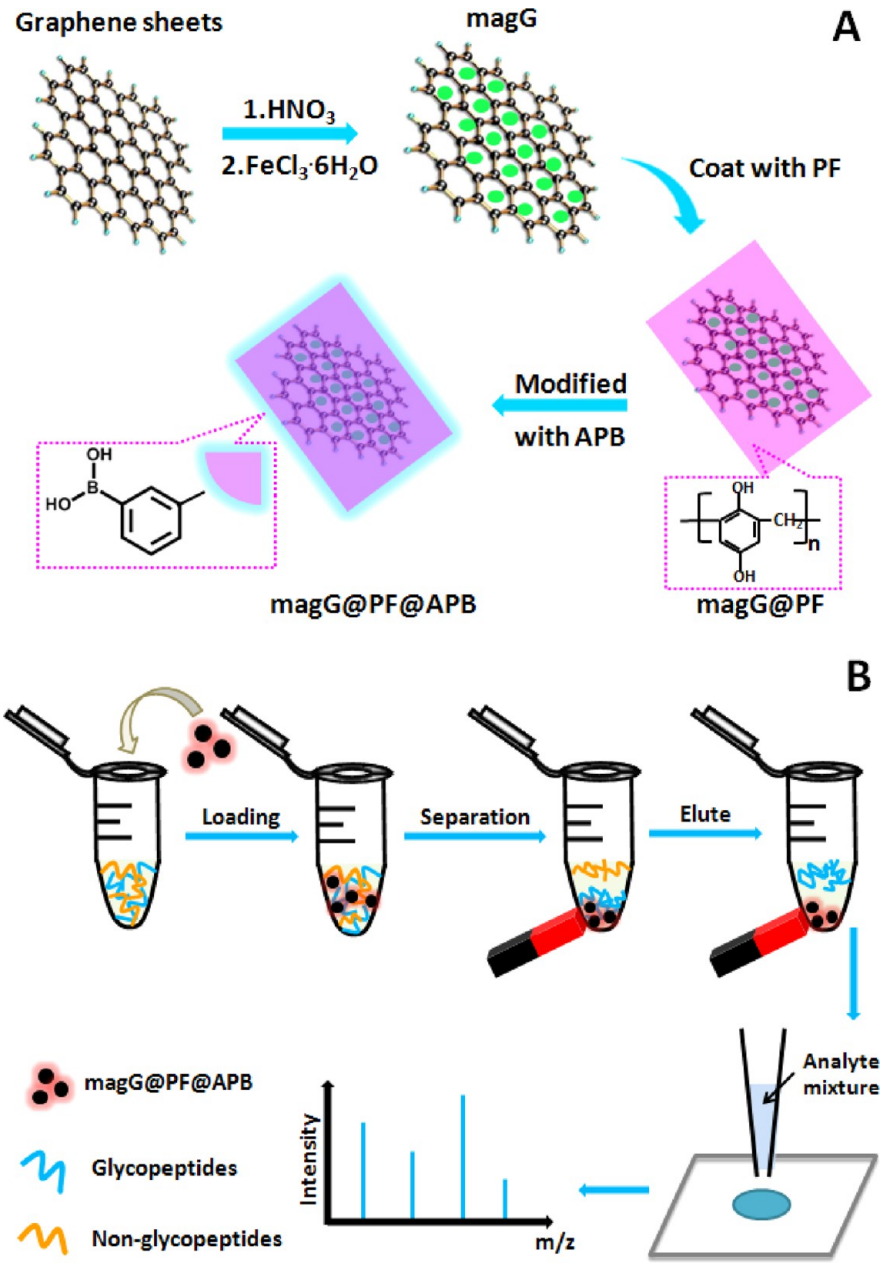
Scheme 1. (A) Schematic Illustration of the Synthetic Procedure for Preparation of magG@PF@APB and (B) Workflow of Glycopeptides Enrichment from the Biological Sample Using magG@PF@APB



magnetic graphene (magG) surface has been widely investigated by many groups.[28,29] To improve the hydrophilicity of the magnetic composites, one promising strategy is to adopt a phenolic-formaldehyde resin (PF) layer which can be easily coated on various kinds of substrates through the condensation polymerization of formaldehyde and hydroquinone.[30,31] Due to the splendid hydrophilicity and excellent environmental stability, it can be used as a promising linker in the glycoproteomics research.

Herein, for the first time, a facile strategy was established for putting boronic acid groups connection in graphene simply by employing phenolic-formaldehyde resin as the coupling connecter between the two parts (magG@PF@APB). The synthesis involves two steps, coating magnetic graphene with a PF layer via the condensation polymerization under acid solution and subsequent binding aminophenylboronic acid (APB) through $\pi-\pi$ stacking and hydrogen bonding interaction. The enhanced hydrophilic magG@PF@APB hold the features of well-defined 2D morphology and high loading amounts of boronic acid, and thus perform excellently in the selective enrichment and separation of glycopeptides. Besides, with the unique magnetic property, materials can be easily, rapidly, and completely recovered by employing an external magnet, which is beneficial for further MS analysis. Furthermore, the practical applicability of magG@PF@APB was evaluated by the enrichment of ultralow concentration glycopeptides in a tryptic digest of proteins from human serum. Due to the high selectivity, extreme sensitivity, and outstanding magnetic susceptibility, the prepared multilayer hydrophilic nanocomposites were anticipated to have excellent performance in the analysis of complex biological samples for glycoproteome research.

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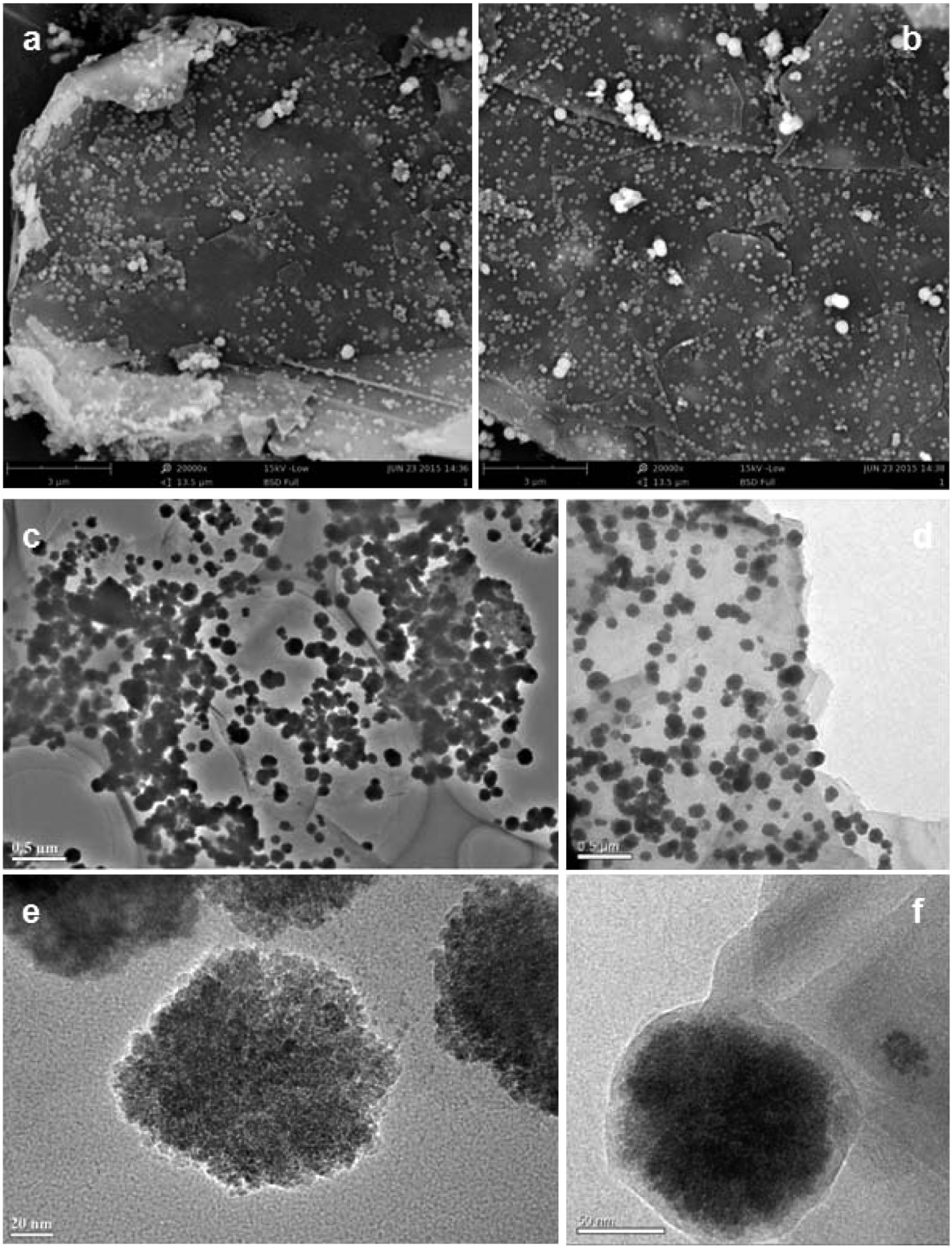

Figure 1. (a and b) SEM images of magG@PF@APB composites. TEM images of (c and e) magG and (d and f) magG@PF@APB composites.

EXPERIMENTAL SECTION

Materials and Chemicals. Horse-radish peroxidase (HRP), myoglobin (Myo), trifluoroacetic acid (TFA), TPCK-treated trypsin, 3-aminophenylboronic acid monohydrate (APB) and 2,5-dihydroxybenzoic acid (DHB) were from Sigma Chemical. Acetonitrile (ACN) and formaldehyde were ordered from Fisher Scientific. Peptide-*N*-glycosidase (PNGase F) was from New England Biolabs. Milli-Q water was used in all experiment process. Zhongshan hospital provided the human serum. All other reagents were obtained from commercial sources.

Synthesis of magG@PF@APB Nanoparticles. The synthesis approach for magG@PF@APB composites is depicted in Scheme 1A. First, the Fe_3O_4-modified graphene sheets (magG) were synthesized via a hydrothermal reaction.[32] The detailed experimental process was described in the Supporting Information. Next, phenolic-formaldehyde resin (PF) and magG@PF were prepared. Formaldehyde aqueous

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solution (30 mL, w/w = 37%) was added dropwise to a solution of hydroquinone (11 g) and HCl (4 mL) in deionized water (100 mL), and the mixture was stirred and maintained at 90 °C for 60 min. The desired products of PF were collected with a magnet, washed with anhydrous ethanol, and dried at 50 °C. PF (0.8 g) and magG (0.5 g) were dispersed in distilled water (60 mL), and then, 28% NH_4OH was added into the reaction solution until the final pH of the solution is greater than 10. The reaction under mechanical stirring at 60 °C for 1h. The magG@PF was gathered with an external magnet, washed using ethanol and water, and dried in vacuum. Finally, the magG@PF@APB nanoparticles were prepared. Fifty milligrams (50 mg) of APB was dissolved in 50 mM NH_4HCO_3. After ultrasonication for about 15 min, 50 mg of magG@PF were separated into 10 Eppendorf tubes. Each tube was mixed with 1 mL APB solution, which was stirred at 65 °C for about 3 h, and then, with the help of an external magnet, the supernatant was discarded. After removing the supernatant and adding the APB solution, we again stirred the mixture at 65 °C for about 3 h. As described above, the operation process was repeated three times to obtain the magG@PF@APB.

MagG@PF@APB Selective Enrichment of Glycopeptides from Standard Peptides and Human Serum. For glycopeptide enrichment, 100 μg magG@PF@APB was dissolved in 100 μL of 50 mM NH_4HCO_3 buffer (pH = 9.0); afterward, standard peptides or peptides mixture were added. After the addition, the reaction mixture was incubated within 45 min at 37 °C. We removed the supernatants with the aid of a magnet, and washed the composites with loading buffer three times. Subsequently, the enriched glycopeptides were eluted for 15 min using the buffer which was composed of 50% ACN and 1% TFA aqueous solution (v/v), analyzed by MALDI-TOF MS analysis with DHB matrix. The serum sample of healthy people was tested to confirm the enrichment effect of magG@PF@APB composites for glycopeptides. The detailed information was depicted in the Supporting Information.

Mass Spectrometric Analysis. In order, 1 μL of eluate and 0.5 μL of DHB aqueous solution were deposited on the plate and then dried in air. MALDI-TOF MS experiments were performed in positive ion mode on a 5800 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA) with the Nd:YAG laser at 355 nm, a repetition rate of 200 Hz and an acceleration voltage of 20 kV.

RESULTS AND DISCUSSION

Synthesis of magG@PF@APB. The synthesis approach for magG@PF@APB composites is depicted in Scheme 1A. In brief, the condensation polymerization of formaldehyde and hydroquinone was performed on magnetic graphene under acid condition; then, the immobilization of APB on magG@PF was performed through $\pi-\pi$ stacking and hydrogen bonding interaction.

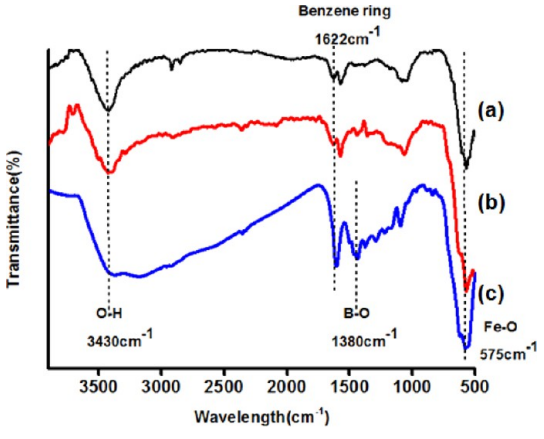

Figure 2. FT-IR spectra of (a) magG, (b) magG@PF and (c) magG@PF@APB composites.

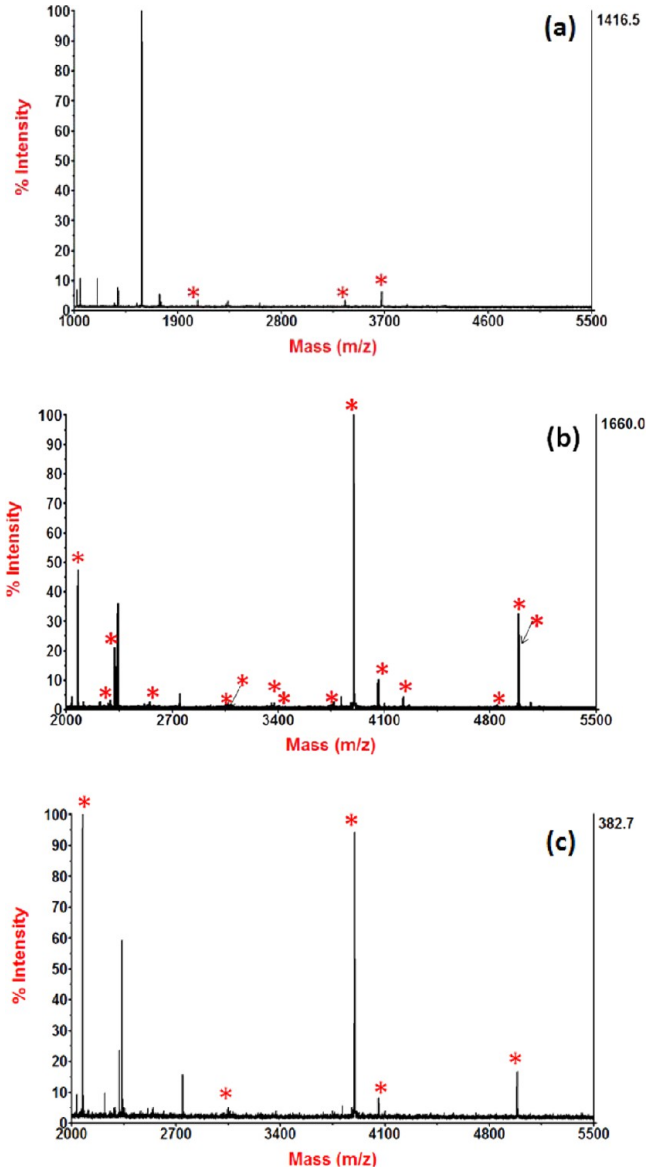

Figure 3. MALDI mass spectrum of peptides derived from HRP (a) before enrichment and (b) enriched by magG@PF@APB and (c) enriched by magG@PF; asterisks (*) indicate glycopeptides.

Characterization. Different techniques were employed to characterize the prepared magG@PF@APB. Figure 1a displayed the SEM image of the magG@PF@APB, showing the 2D structure of graphene with its characteristic crumple, which indicates that the graphene morphology was well preserved via modification of Fe_3O_4 and PF. Figure 1b suggested that after the hydrothermal reaction, plenty of magnetite nanobeads appeared on the graphene surface. Figure 1c,e displayed the TEM images of the magnetic graphene. After magnetic functionalization, the as-made Fe_3O_4 microspheres are uniform both in morphology and size and grafted onto the graphene sheets. The vertical boundary of graphene is observed, and meanwhile, the 2D structure is well preserved. In contrast, after modification with PF and APB (Figure 1d,f), the morphology of graphene was still unchanged because of its physicochemical characteristic. The layers of PF@APB with a thickness of around 10 nm is visible outside the magnetite

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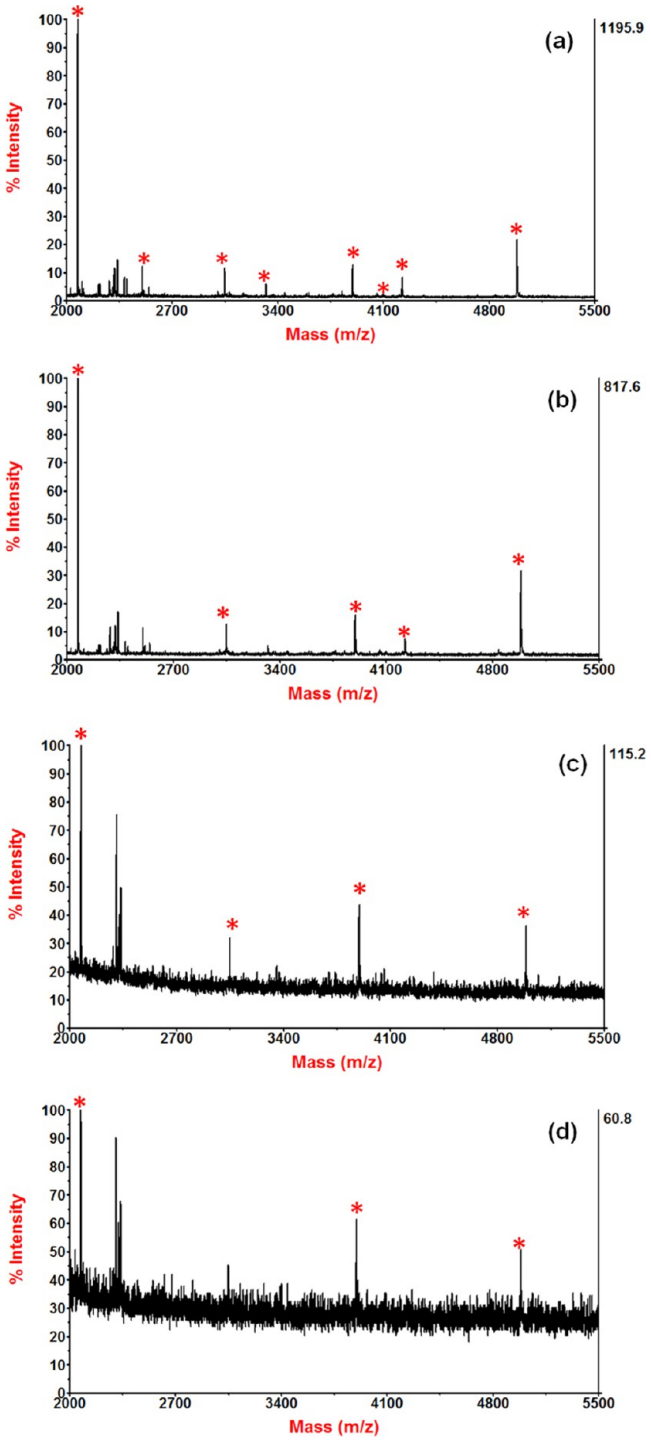

Figure 4. MALDI-TOF-MS spectra of different concentrations of the HRP tryptic digests after enrichment by magG@PF@APB: (a) 100 fmol/μL; (b) 50 fmol/μL; (c) 10 fmol/μL; (d) 1 fmol/μL.

microspheres. Figure S1 (Supporting Information) describes the EDX spectra of magG@PF@APB, showing the existence of Fe, B, O, and C element, also demonstrating that the boronic acid-functionalized composites were synthesized successfully. Besides, the magnetization of the material is strong enough for the effective magnetic separation under an external magnetic field within 5 s (Figure S2, Supporting Information).

FTIR was recorded for magnetic graphene before and after surface modification. In Figure 2, the peak at 575 cm^{-1}

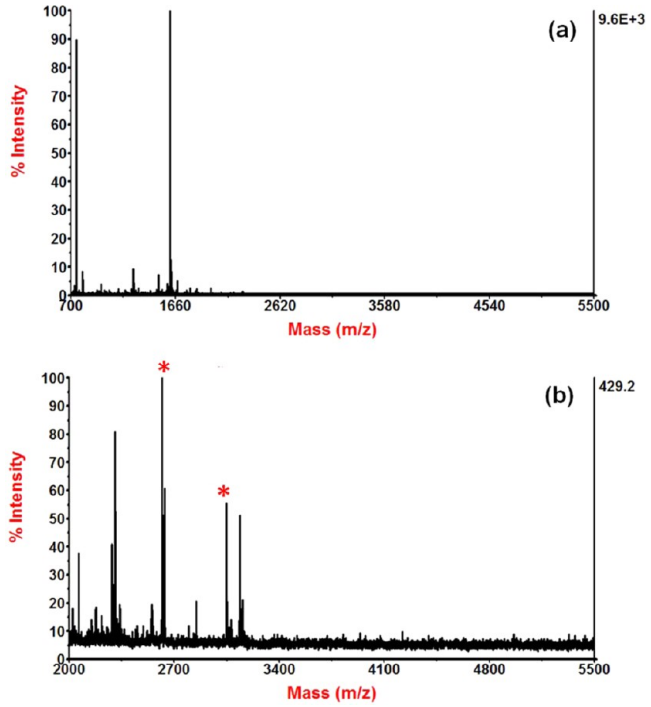

Figure 5. MALDI-TOF mass spectra of peptides derived from peptide mixture of HRP and Myo (a) before enrichment (in the molar ratio of 1:100) and (b) after enrichment by magG@PF@APB. Mass spectrometric peaks are marked as glycopeptides (*).

attributed to the Fe−O vibration of materials. The peak at 3430 cm^{-1} in Figure 2b, belonged to the stretching vibration of O−H, confirmed the existence of PF around graphene surface. Compared to the spectrum of magG and magG@PF, the FT-IR spectrum of magG@PF@APB exhibited the stronger IR band at 3430 cm^{-1} (Figure 2c) was attributed to O−H vibrations, while the peaks at 1650−1450 cm^{-1} could belong to the benzene ring vibration. Moreover, the materials modified with APB show a new peak at around 1380 cm^{-1}, which assigned to the deformation and stretching of B−O. Zeta potential measurements were performed for the as-prepared materials in deionized water. As we can judge from Figure S3 (Supporting Information), the zeta potential of graphene was −5.84 mV with the number of negatively charged groups. After magnetic functionalization and coating with PF, the potential increased to −2.8 mV because the large amount of positive charges from Fe_3O_4 and PF were attached to the surface of graphene. After modification with APB, the zeta potential decreased to −15.8 mV due to the negatively charged acidic hydroquinone hydroxyl groups and boronic acid groups. The XRD detection results (Figure S4, Supporting Information) show that the magG@PF@APB possess diffraction patterns similar to those of magnetite particles (JCPDS file no. 19-0629). And some representative peaks of magnetite at 2θ of 30.24, 35.54, 43.2, 53.5, 57.0, and 62.6° correspond to the specific (220), (311), (400), (422), (551), and (440), suggesting the crystal structure of the magnetite phase in the materials. The specific surface area of magG@PF@APB was also analyzed using the nitrogen adsorption−desorption isotherm. As is displayed in Figure S5 (Supporting Information), the Brunauer−Emmett−Teller (BET) surface area was estimated to be 76.288 m^2/g. In summary, the hydrophilic magnetic materials, combined with some merits

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(e.g., large specific surface area and abundant boronic acid groups), makes it an ideal candidate to separate glycopeptides from complex samples.

Investigation of the Performance of magG@PF@APB for Glycopeptides Enrichment in Peptidome Research. The procedure to capture glycopeptides from a tryptic digest of protein was displayed in Scheme 1B. For comparison, two kinds of magnetic composites (magG@PF and magG@PF@APB) were also used to fish glycopeptides from HRP enzymatic hydrolysate under the same enrichment condition. Namely, the standard peptides were incubated with materials for 45 min, and then, the materials were washed with NH_4HCO_3 buffer (pH = 9.0). The enriched glycopeptides were eluted and deposited on the MALDI target for MS analysis. HRP digest was directly measured without enrichment, the result show that only three peaks can match the glycosylated peptides, possibly due to the strong suppression by those nonglycosylated peptides (Figure 3a). Notably, after specific enrichment with magG@PF@APB (Figure 3b), the desired signals could be dramatically improved and dominated the spectrum, while five glycopeptides peaks can be observed after enrichment with magG@PF (Figure 3c), indicating that the APB shell plays the most important role in the highly selective enrichment of glycopeptides. Performance of the previously reported boronic acid-functionalized materials in the enrichment of glycopeptides are not comparable to those of our materials.[33,34] The results of the analysized glycopeptides by MALDI-TOF MS are listed in Table S1 (Supporting Information); the results proved that magG@PF@APB can be successfully employed for capturing glycopeptides.

The detection limit of magG@PF@APB composites was investigated by confecting a series of concentration gradients of a HRP digest solution. Figure 4 shows a low lever (100 fmol/μL) of HRP digests could be well spotted after enrichment by as-made composites. When HRP digest was diluted with an ultralow concentration of 1.0 fmol/μL, three MS signals of glycopeptides could still be observed. It may be that the lowest detection limit in the midst of the boronic acid-functionalized composite nanoparticles at present[19,35] can be credited to high surface area of graphene and the good water dispersibility.

To further test the selectivity of our as-made composites, large amounts of nonglycopeptides (Myo) blended with glycopeptides (HRP) at the molar ratio of 100:1. Glycopeptides could hardly be detected without enrichment (Figure 5a), while after treated with magG@PF@APB, the signals of glycopeptides are obviously enhanced and clearly observed (Figure 5b). Therefore, the new magG@PF@APB provided better specificity for glycopeptides while the background peptides are 100 folds than the target peptides. The results revealed that the composites had high selectivity, and excellent enriching specificity toward glycopeptides.

Whether magG@PF@APB can be recycled and reused for capturing glycopeptides was also investigated, the used materials treated by the NH_4HCO_3 buffer solution several times were recycled. The regenerated magG@PF@APB composites were reused for glycopeptide enrichment from HRP digest three consecutive times. After the composites were recycled three times, the MS spectra of glycopeptides after enrichment using as-made materials stayed almost identical to that of the first time (Figure S6, Supporting Information), confirming that the magG@PF@APB composites can be recycled and reused for capturing glycopeptides. With the purpose of examining the effectiveness and selectivity of the magG@PF@APB composites in practical samples, which were applied to capture the glycopeptides from human serum (1 μL). The peptides eluted were deglycosylated via PNGase F, then were measured by nano-LC-ESI-MS/MS. The detailed information on the glycopeptides enriched from human serum is displayed in Table S2 (Supporting Information). The results suggested that magG@PF@APB had an ability to selectively fish the glycopeptides from a natural sample.

CONCLUSIONS

In summary, boronic-acid-functionalized magnetic nanocomposites (magG@PF@APB) have been synthesized through a simple strategy, which involves the preparation of poly(phenol-formaldehyde resin)-coated magnetic graphene and subsequent post grafting method. Owing to the special interaction between abundant boronic acid groups and glycopeptides, the high surface area, and enhanced hydrophilicity, the materials were applied to capture N-linked glycopeptides with remarkable selectivity, low detection limit, biological compatibility, and good recyclability. On the basis of the above results, as a novel multilayer magnetic nanocomposite, the magG@PF@APB has proved to be very promising in the separation and identification of N-glycoproteome from extensive and complex biological samples.

ASSOCIATED CONTENT

Supporting Information

Additional experimental details; EDX spectrum of magG@PF@APB; zeta potential distributions and XRD patterns of magG, magG@PF, and magG@PF@APB materials; nitrogen adsorption−desorption isotherms of magG@PF@APB; MALDI mass spectrum of glycopeptides enriched from HRP using magG@PF@APB and detailed information on observed glycopeptides. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b04295.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: mxgao@fudan.edu.cn.

*E-mail: xmzhang@fudan.edu.cn.

Notes

The authors declare no competing financial interest.

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