Electronic Supplementary Information

for

Magnetic nanoparticles with dendrimer-assisted boronate avidity for the selective enrichment of trace glycoproteins

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1. MATERIALS AND METHODS

1.1. Reagents and materials

PAMAM dendrimers (ethylenediamine core, generation 4.0 and 5.0), horseradish peroxidase (HRP), bovine serum albumin (BSA), transferrin (TRF), siapinic acid (SA) and protease cocktail inhibitor were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rat monoclonal IgG1 antibody against human α-fetoprotein (anti-AFP) was purchased from Shuangliu Zhenglong Chemical and Biological Research Laboratory (Sichuan, China). 4-Formylphenylboronic acid, sodium cyanoborohydride, anhydrous methanol, galactose, xylose and fucose were from J&K scientific (Shanghai, China). D-mannose was from Alfa Aesar China (Tianjin, China). N-acetylneuraminic acid (Neu5Ac) was purchased from Maya Reagent (Zhejiang, China). Glucose, phenylboronic acid (PBA), ferric trichloride hexahydrate, ethylene glycol, anhydrous sodium acetate, 1,6-hexanediamine, glutaraldehyde and 3,3,5′,5′-tetramethylbenzidine dihydrochloride (TMB) were from Sinopharm Chemical Reagent (Shanghai, China). D-fructose was obtained from Huixing Reagent (Shanghai, China). All reagents used were of analytical grade or higher. All commercially available reagents were used without further purification. Water used in all the experiments was purified by a Milli-Q Advantage A10 ultrapure water purification system (Millipore, Milford, MA, USA).

1.2. Instruments

Transmission electron microscopy (TEM) characterization was performed on a JEM-1010 system (JEOL, Tokyo, Japan). Fourier transform infrared (FT-IR) spectrometry was carried out on a Nicolet 6700 FT-IR spectrometer (Thermo Fisher, MA, USA). Thermogravimetric analysis (TGA) was performed using a Pyris 1 TGA instrument (PerkinElmer, USA) with a heating rate of 20 °C/min in the temperature range 25-700 °C under a nitrogen atmosphere. The adsorption isotherm measurements were performed with a NanoDrop 2000/2000C spectrophotometer (Thermo Fisher, MA, USA).

Capillary electrophoresis (CE) analysis was performed on a P/ACE MDQ system (Beckman Coulter, Fullerton, CA, USA) equipped with a diode array detector (DAD). A bare fused-silica capillary

(Yongnian Optical Fiber Factory, Hebei, China) of 50 μm i.d. × 60 cm (50 cm effective length) was used as separation column. The running buffer was 35 mM sodium tetraborate buffer (pH 10.0). At the beginning of each day, the capillary was sequentially rinsed at 20 psi with 1.0 M NaOH, water and running buffer for 20 min. Prior to each run, the capillary was sequentially rinsed at 20 psi with 0.1 M NaOH for 2 min and running buffer for 2 min. Samples were injected under pressure at 0.5 psi for 5 s. CE experiments were performed at 25 °C under a voltage of 20 kV. The UV absorbance was recorded at 260 nm.

MALDI-TOF MS analyses were carried out on a 4800 plus MALDI TOF/TOF Analyzer (AB Sciex, Darmstadt, Germany), a 200 Hz Nd:YAG laser, controlled by the 4000 Series Explorer Software V3.7.0. Spectra were acquired in the positive linear ion mode. A total of 500 laser shots per spot were accumulated for each spectrum. The accelerating voltage was 20 kV. 10 mg/mL SA in 0.1% trifluoroacetic acid: acetonitrile (50:50, v/v) was used as the matrix. Equivalent amounts (0.5 μL) of the sample and matrix were sequentially dropped onto the MALDI plate for mass spectrometric analysis. Data were processed using Data Explorer Software Version 3.7 (AB Sciex).

1.3. Preparation of dPBA-MNPs and PBA-MNPs

The procedure for the synthesis of dPBA-MNPs was comprised of three steps as shown in Fig.S1A: (1) Synthesis of amino-functionalized magnetic nanoparticles (AMNPs). The AMNPs were synthesized according to a previously reported method [1] with minor modification. Briefly, 2.0 g ferric trichloride hexahydrate was dissolved in ethylene glycol (60 mL) to form a clear orange yellow color solution and followed by the addition of anhydrous sodium acetate (4.0 g). The mixture was stirred vigorously (800 rpm) by a magnetic stirring for 30 min to form a yellow suspension. Then 13.0 g 1,6-hexanediamine was added, which turned the solution to clear red color. Finally, the solution was sealed in a PTFE-lined autoclave and reacted at 198 °C for 6 h. The resulting magnetic nanoparticles were rinsed with water and ethanol for 3 times each, and then dried at 50 °C in a vacuum overnight. (2) Preparation of PAMAM dendrimer modified MNPs (d-MNPs). 200 mg AMNPs were added to 40 mL anhydrous methanol containing 5% glutaraldehyde, the mixture was mechanically stirred (400 rpm) for 12 h at room temperature. The glutaraldehyde-activated MNPs

were washed three times with anhydrous methanol and then dispersed in 30 mL anhydrous methanol containing 0.5 g PAMAM dendrimer by ultrasound, the mixture was mechanically stirred (400 rpm) for 12 h at room temperature. 1% (w/w) sodium cyanoborohydride was added (100 mg every 4 hours) during the course of reaction. The d-MNPs were collected by a magnet, washed with water and ethanol for 3 times each, and then dried at 40 °C overnight. (3) Functionalization of the d-MNPs with 4-formylphenylboronic acid. 200 mg d-MNPs were dispersed in 40 mL anhydrous methanol containing 10 mg/mL 4-formylphenylboronic acid, the mixture was mechanically stirred (400 rpm) for 24 h at room temperature. Sodium cyanoborohydride was added (100 mg every 4 hours) during the reaction process. The resultant MNPs were magnetically collected, washed with water and ethanol, dried at 40 °C. The obtained dPBA-MNPs were stored for further use.

The procedure for the synthesis of PBA-MNPs was comprised of two steps as shown in Fig. S1B: (1) Synthesis of AMNPs, which is the same as in Fig. S1A. (2) Functionalization of the AMNPs with 4-formylphenylboronic acid. The procedure was the same as in Fig. S1A except that d-MNPs were replaced with AMNPs.

1.4. Characterization of MNPs

The size and morphology of the synthesized MNPs were characterized by TEM. As shown in Fig. S2, TEM images suggest that the MNPs were well shaped with a diameter of about 100 nm. As TEM failed to recognize the chemical substances attached on the Fe_3O_4 core, the three types of MNPs looked all the same under TEM.

FT-IR spectroscopy was also used to characterize the MNPs, and the spectra are also in shown Fig. S2. A strong adsorption peak at 580 cm^{-1} and two peaks at 1,642 and 1,048 cm^{-1} were observed for the three types of MNPs, which can be ascribed to Fe-O vibrations and the presence of the N-H and C-N, respectively. In addition, the peak at 1549 cm^{-1} for both d-MNPs and dPBA-MNPs is ascribed to N-H deformation vibration of -CONH-, which indicates the two types of MNPs were modified with PAMAM dendrimer. The peak at 1358 cm^{-1} for dPBA-MNPs alone was associated with the C-B vibrations, implying that 4-formylphenylboronic acid was present on the MNPs surface.

TGA was performed to estimate the organic content on the surface of MNPs. As shown in Fig. S2, the weight loss profile for AMNPs, d-MNPs and dPBA-MNPs exhibited two different stages within the range from 25 °C to 500 °C. All MNPs displayed a similar weight loss profile for the release of physically adsorbed solvent or water below 200 °C. There was a well-defined weight loss of 4.3%, 5.4% and 6.0% for AMNPs, d-MNPs and dPBA-MNPs from 200 °C to 500 °C, respectively. The increased weight loss suggests that the PAMAM dendrimer and 4-formylphenylboronic acid were successfully grafted on MNPs.

1.5. The selectivity of dPBA-MNPs and PBA-MNPs

The extraction and elution were carried out according to a previously reported method [2]. Briefly, for the extraction by MNPs, equivalent dPBA-MNPs or PBA-MNPs (2 mg) were added to mixtures (200 μL) containing different concentrations of adenosine and 1 mg/mL deoxyadenosine in 250-μL plastic microcentrifugal tubes. The tubes were shaken on a rotator for 1 h at room temperature. The MNPs were then collected at the tube wall by applying a magnet to the tube wall and rinsed with 200 μL 100 mM sodium phosphate buffer (pH 8.5) for 3 times each. After washing, the MNPs were resuspended and eluted in 15 μL 100 mM acetic acid solution for 1 h on a rotator. Finally, the dPBA-MNPs or PBA-MNPs were trapped to the tube wall again and the eluates were collected by pipetting carefully. The eluates were injected for CE analysis.

1.6. Measurement of adsorption isotherm

The measurement of adsorption isotherm was done according to a previously reported method [3] with minor modification. Briefly, equivalent dPBA-MNPs or PBA-MNPs (2 mg) were added to solutions (200 μL) of glycoprotein or adenosine at different concentrations in 250-μL plastic microcentrifugal tubes. The tubes were shaken on a rotator for 12 h at room temperature. The washing and elution procedures were the same as those in section 1.5. The amounts of glycoprotein or adenosine adsorbed by the MNPs were determined by measuring the amount of glycoprotein or adenosine in the eluates with the Nanodrop-2000C UV-vis spectrophotometer. UV absorbance was adopted at 260 nm for adenosine, 403 nm for HRP, 280 nm for TRF and anti-AFP.

1.7. Scatchard analysis

The Scatchard analysis was carried out according to a previously reported method [3]. The amount of glycoprotein or adenosine bound to the dPBA-MNPs or PBA-MNPs was plotted according to the Scatchard equation to estimate the binding properties of the MNPs. The Scatchard relationship can be established using the following equation:

$$Q_e / [S] = (Q_{max} - Q_e) / K_d$$

Where Q_e is the amount of glycoprotein or adenosine bound to the dPBA-MNPs at equilibrium; [S] is the free concentration at adsorption equilibrium; Q_{max} and K_d is the saturated adsorption capacity and the dissociation constant, respectively. By plotting Q_e/[S] versus Q_e, K_d and Q_{max} can be calculated from the slope and the intercept, respectively. The obtained Scatchard plot of the dPBA-MNPs binding exhibited one straight line, indicating that there was only a single type of binding site associated with the binding between glycoprotein or adenosine and the dPBA-MNPs or PBA-MNPs.

1.8. ACE analysis of the binding constants between phenylboronic acid and monosaccharides

As monosaccharides have no UV or visible absorbance, their binding constants with boronic acids cannot be measured by the above described adsorption isotherm-based method. The dissociation constants between phenyboronic acid (PBA) and monosaccharides were measured according the ACE method reported previously.[4] To ensure the binding constant data obtained by the two different methods are comparable, the dissociation constant between adenosine and PBA was also measured using the ACE method. As shown in Table 1, the K_d values for adenosine are in good agreement between the two methods.

1.9. Measurements of the lowest extractable concentration of HRP

The extraction, washing and elution procedures were identical to those in section 1.5 except for the amount of reactants used. Equivalent amounts of dPBA-MNPs (10 mg) were added to a series of 10-mL plastic microcentrifugal tubes which contained 9 mL HRP solutions at concentration ranging from 1 pg/mL to 10 ng/mL. To measure the concentrations of HRP in the eluates, 2 μL eluate was

mixed with 200 μL TMB staining solution and incubated for 10 min. After terminalizing the reaction by adding 20 μL of 1M H_2SO_4 solution, the obtained solutions were detected by UV absorbance at 450 nm within 20 min. A TMB stock solution was prepared by dissolving 3.0 mg TMB in 500 μL dimethyl sulphoxide. A staining solution was freshly prepared by adding 50 μL TMB stock solution and 2 μL H_2O_2 (30% v/v) to 4 mL of a 1:1 mixture of 0.1 M Na_2HPO_4 and 0.05 M citric acid.

1.10. Effect of competing monosaccharides on the extraction of glycoproteins by dPBA-MNPs

The extraction, washing and elution procedures were identical to those in section 1.5 except for adding monosaccharides to solution. Equivalent of dPBA-MNPs (2 mg) were added to a series of solutions (1 mL) of 100 mM sodium phosphate (pH 8.5) containing 1 ng/mL HRP without or with 1 mg/mL different monosaccharides including mannose, xylose, fucose, galactose, Neu5Ac, glucose and fructose. HRP amount in the eluates was determined with TMB colorimetric detection as stated in section 1.9. The same procedure was carried out for PBA-MNPs except that the sample contained 1 μg/mL HRP.

1.11. Selective extraction of glycoproteins by dPBA-MNPs

2 mg dPBA-MNPs were added to a 1.5-mL plastic centrifugal tube containing 1 μg HRP and 1 mg BSA dissolved in 1 mL 250 mM ammonium acetate buffer (NH_4OAc) containing 500 mM NaCl (pH 8.5). The tube was shaken on a rotator for 1 h. The dPBA-MNPs were then collected at the tube wall by applying a magnet to the tube wall and rinsed with 1 mL 250 mM NH_4OAc containing 500 mM NaCl (pH 8.5) and 1 mL 50 mM NH_4OAc (pH 8.5) for 3 times each. After washing, the dPBA-MNPs were transferred to a 200-μL plastic centrifugal tube and then resuspended in 15 μL 100 mM acetic acid solution for 1 h on a rotator. The obtained eluate was submitted to MALDI-TOF MS analysis.

1.12. Reusability of dPBA-MNPs

The reusability of dPBA-MNPs was tested in terms of the extracted HRP amount for five consecutive extractions. 5×2.0 mg dPBA-MNPs were used to extract the glycoprotein from 5×200 μL 1 mg/mL HRP solutions containing 100 mM sodium phosphate, pH 8.5. The extraction, cleanup and elution

procedures were identical to those in section 1.5. After elution for each extraction, the MNPs were further washed with 200 μL 100 mM acetic acid solution and 200 μL 100 mM sodium phosphate buffer (pH 8.5) for 3 times each. The eluates for five consecutive runs were detected at 403 nm and the relative standard deviation (RSD) for the UV absorbance was calculated.

1.13. Selective enrichment of glycoproteins in saliva samples

Whole saliva was collected from healthy adults in the morning, at least 2 h after the last intake of food. The mouth was rinsed with water immediately before the collection. The tube was placed on ice while collecting whole saliva. Protease cocktail inhibitor (1 μL per mL of saliva) was added to the collected saliva samples immediately to minimize protein degradation. The resultant samples were then centrifuged at 12 000 rpm at 4 °C for 10 min. The supernatant was collected and stored at -80 °C. Prior to analysis, the samples were thawed at 4 °C. The pH values were adjusted to 8.5 using 250 mM NH_4OAc containing 500 mM NaCl (pH 8.5) of equivalent volume. 2 mg of dPBA-MNPs were added to 200 μL saliva samples and the obtained mixtures were shaken on a rotator for 1 h at room temperature. After extraction and washing, the compounds extracted by the dPBA-MNPs were eluted with 15 μL 100 mM acetic acid solution for 1 h. The obtained eluates were submitted to MALDI-TOF MS analysis.

References

[1] L. Y. Wang, J. Bao, L.Wang, F. Zhang, Y. D. Li, *Chem. Eur. J.* 2006, **12**, 6341.

[2] L. Liang and Z. Liu, *Chem. Commun.* 2011, **47**, 2255.

[3] L. Li, Y. Lu, Z. J. Bie, H. Y. Chen and Z. Liu, *Angew. Chem. Int. Ed.* 2013, 52, 7451-7454.

[4] C. C. Lü, H. Y. Li, H. Y. Wang, Z. Liu, *Anal. Chem.* 2013, **85**, 2361.

2. Supporting Figures

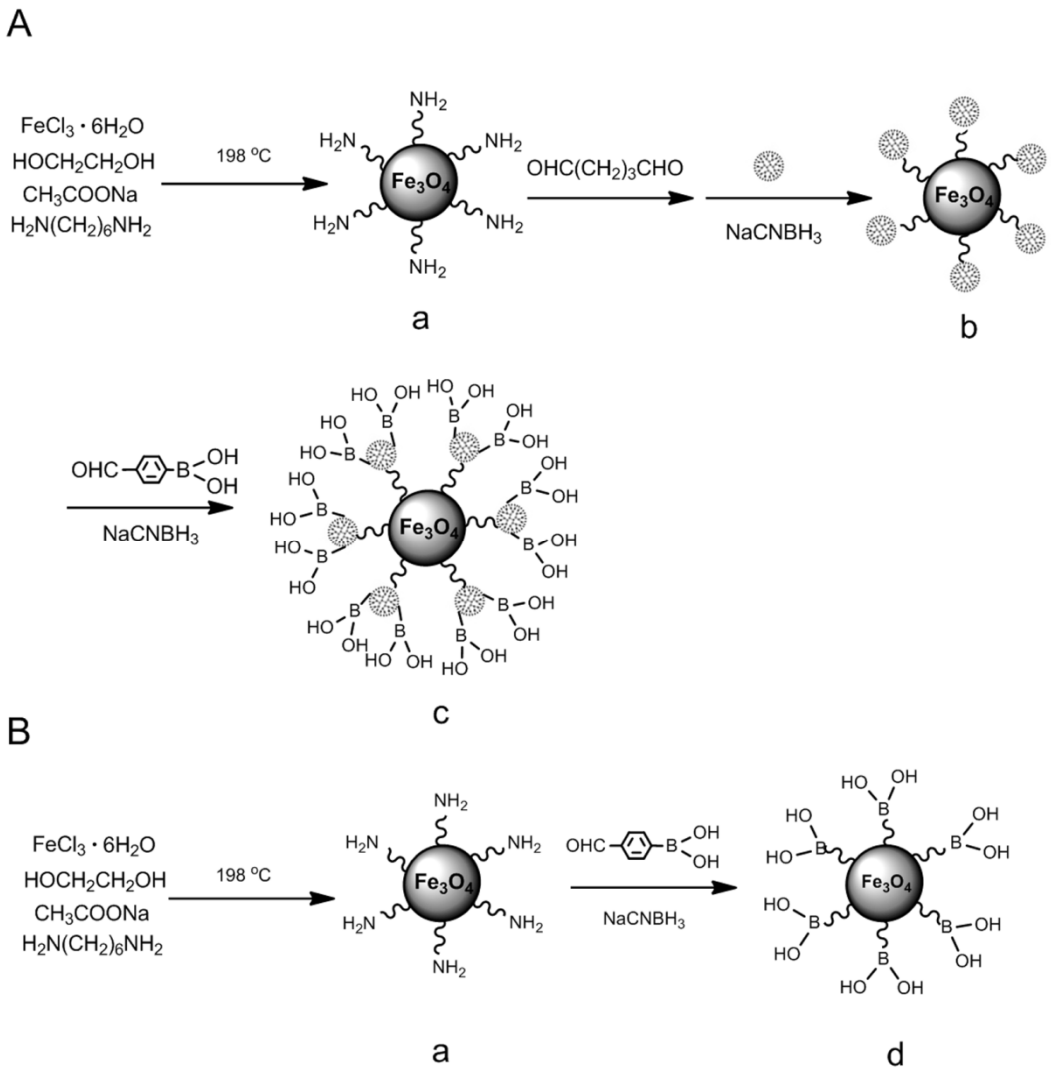

Fig. S1. Synthesis routes of dPBA-MNPs (A) and PBA-MNPs (B). a) AMNPs, b) d-MNPs, c) dPBA-MNPs, d) PBA-MNPs

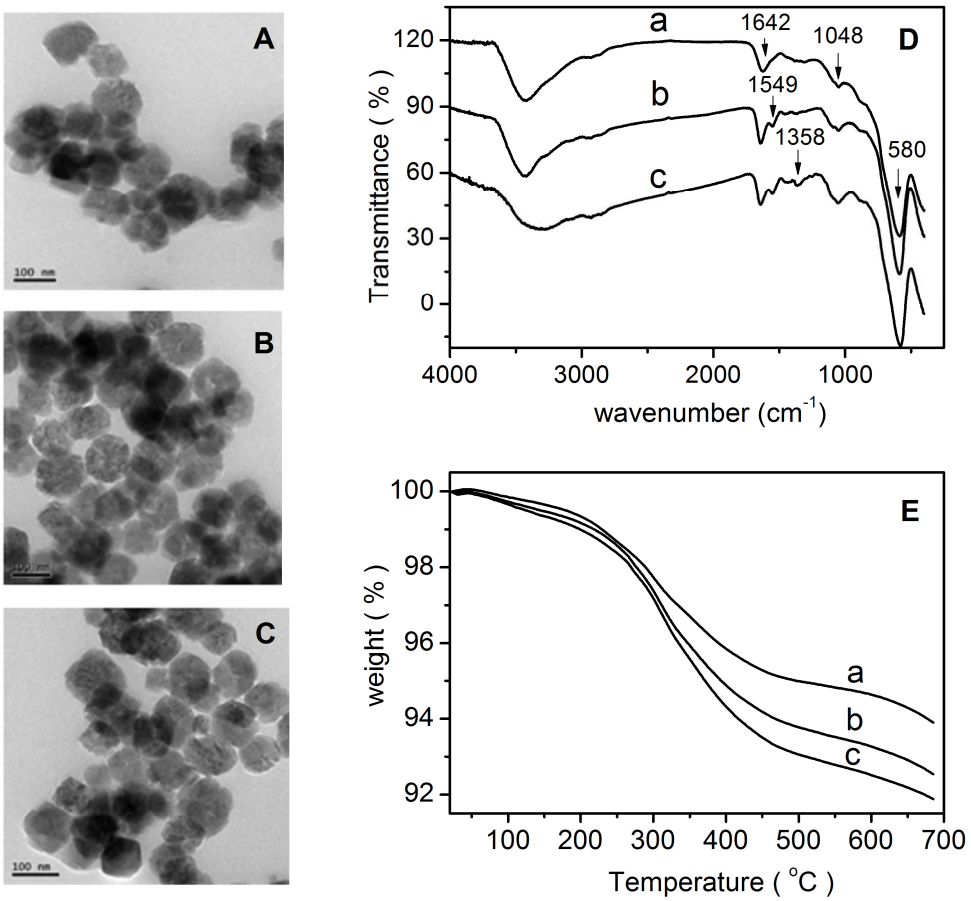

Fig. S2. TEM images for A) AMNPs, B) d-MNPs and C) dPBA -MNPs; D) FT-IR spectra and E) TGA curves of a) AMNPs, b) d-MNPs and c) dPBA-MNPs.

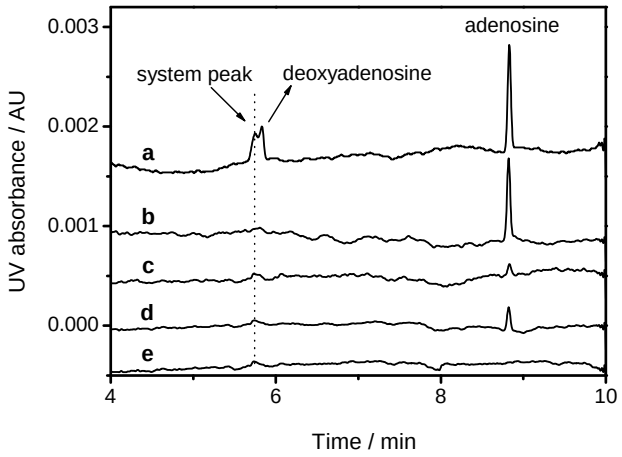

Fig. S3. CE analysis of a) a standard mixture of adenosine and deoxyadenosine (10 μg/mL each), b)-c) compounds extracted by dPBA-MNPs from 1:1 and 1:10 mixture of adenosine and deoxyadenosine, respectively; d)-e) compounds extracted by PBA-MNPs from 1:1 and 1:10 mixture of adenosine and deoxyadenosine, respectively. Deoxyadenosine concentration was kept constant at 1 mg/mL in b)-e).

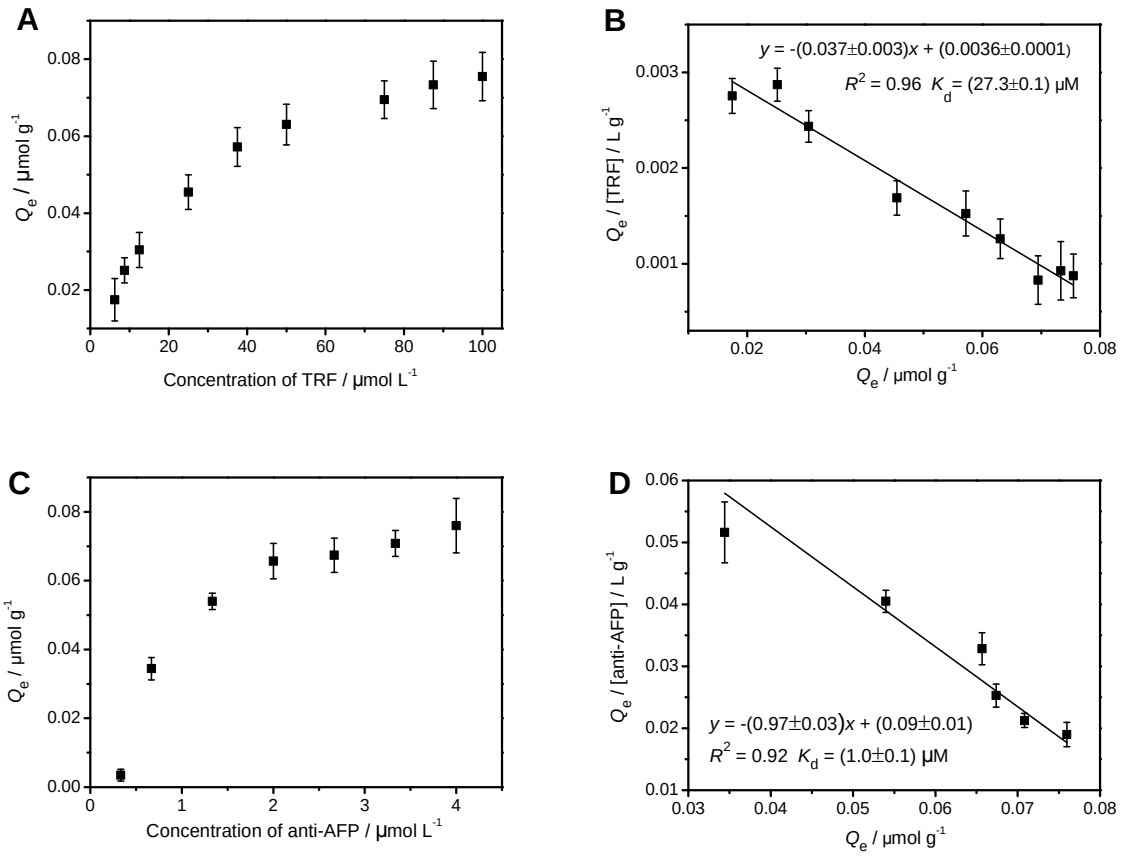

Fig. S4 Binding isotherms for the binding of dPBA-MNPs with TRF (A) and anti-AFP (C), and Scatchard plots for the binding of dPBA-MNPs with TRF (B) and anti-AFP (D).

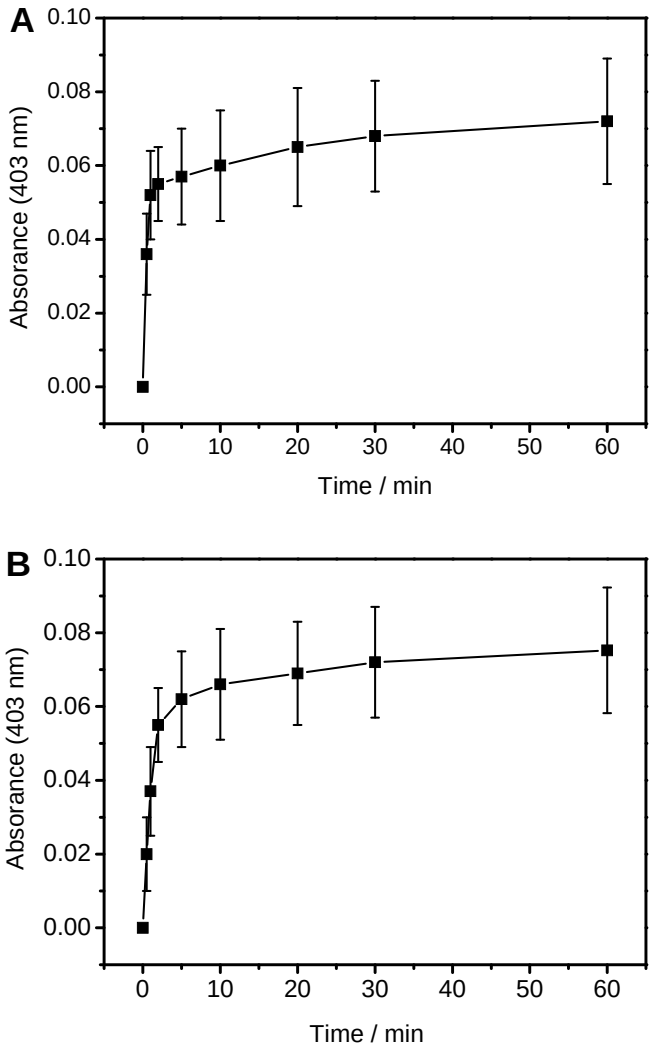

Fig. S5 Dependence of the signal intensity for HRP extracted on the extraction time (A) and desorption time (B). Sample: 1 mg/mL HRP in 100 mM sodium phosphate buffer, pH 8.5; desorption time in (A): 60 min; extraction time in (B): 60 min.

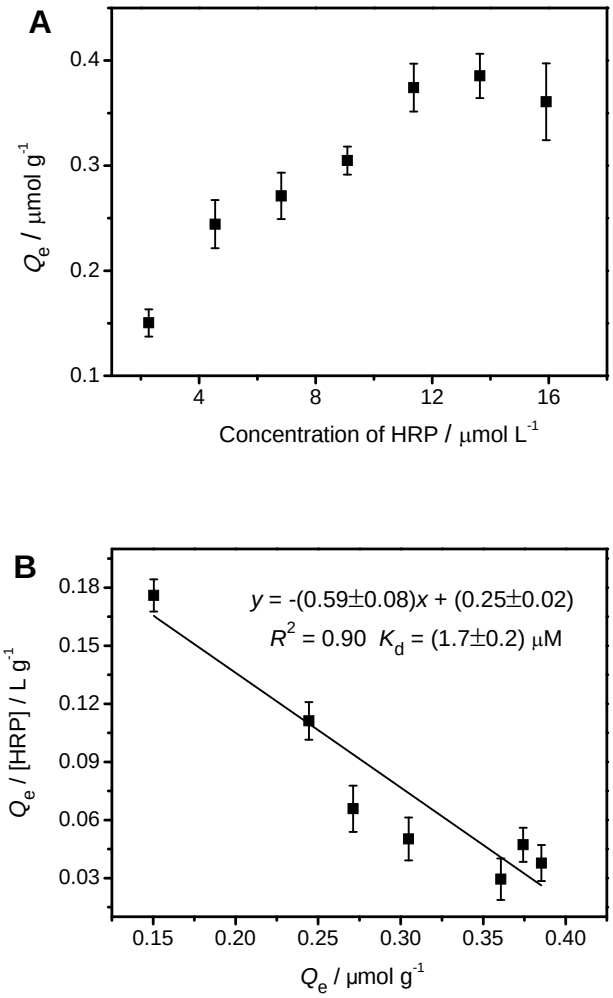

Fig. S6. Binding isotherms for the binding of dPBA-MNPs (G5) with HRP (A) and Scatchard plots for the binding of dPBA-MNPs with HRP (B).