Journal of Chromatography A, 1216 (2009) 7558–7563

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Boronate functionalized magnetic nanoparticles and off-line hyphenation with capillary electrophoresis for specific extraction and analysis of biomolecules containing *cis*-diols

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ARTICLE INFO

Article history:
Available online 18 April 2009

Keywords:
Boronate affinity
Capillary electrophoresis
cis-Diol
Extraction
Magnetic nanoparticles

ABSTRACT

In recent years, functionalized magnetic nanoparticles (MNPs) have drawn continuously increasing attention due to their great potential for capturing biological molecules or species. However, functionalized MNPs as nanoextraction probes and the coupling with a separation platform for chemical analysis have not extensively investigated yet. In this study, boronate functionalized MNPs were synthesized and employed as extracting probes to capture and enrich *cis*-diol-containing biomolecules, and an off-line coupling method of the MNPs-based extraction with capillary electrophoresis (CE) was established by using pH junction, an on-line preconcentration technique in CE, as a bridge for the coupling. The prepared MNPs exhibited specific selectivity and sufficient capacity. The pH junction compressed a large injected sample volume into a much narrower sample zone and therefore significantly improved the detection sensitivity, solving the sensitivity mismatch between the MNPs-based extraction and CE. Experimental conditions for the pH junction and the desorption were optimized. Under the optimized conditions, the sensitivity was enhanced by 42-fold as compared with regular CE. N,N-dimethylformamide was found to be an effective desorption promoter, which reduced the desorption time to a few minutes. With the established method, riboflavin in a human urine sample was determined.

1. Introduction

Sampling/sample preparation is an important step in analytical approaches. Due to their peerless advantages especially the large surface-to-volume ratio, nanomaterials have been successfully applied as sample preparation support in many analysis tasks [1–5]. Among a variety of nanomaterials, magnetic nanoparticle (MNP) is a very useful one due to its easy manipulation in magnetic separation. Recently, functionalized MNPs as solid-phase extracting agents have increasingly obtained great attentions, particularly for the extraction of biological molecules or species [6–18]. Functionalized MNPs have exhibited significant strengths in three application areas: (1) integrated micro-total-analysis-system (μTAS) [7], (2) in vivo analysis especially single-cell analysis [8–10], and (3) combined with mass spectrometry (MS) for proteomic analysis [11–18]. However, few of these MNP-based extraction studies involve combination with a separation platform. Capillary electrophoresis (CE), as an important microscale separation tool for the analysis of biomolecules, features with high efficiency, high speed and low sample/reagent consumption. It is therefore of great interest to couple MNPs-based extraction with CE. However, such a coupling is challenging, since CE is generally associated with poor detection sensitivity due to the limited light-path while the amount of the analyte extracted by a small amount of MNPs is limited.

In this study, we present the use of boronate functionalized MNPs for specific extracting *cis*-diol-containing biomolecules and a strategy for its combination with CE. Poly-3-aminophenylboronic acid (poly-APBA) coated Fe_3O_4/SiO_2 core/shell magnetite nanoparticles were synthesized as the extraction probes. The extraction is based on covalent bond formation between the boronic acid and 1,2- and 1,3-*cis*-diols at a basic pH condition and reversible release of the *cis*-diol-containing compounds when changing the pH to an acidic condition. The prepared MNPs were characterized in terms of particles size, extraction specificity, extraction capacity and extraction equilibrium. To overcome the sensitivity mismatch issue, pH junction [19,20], an on-line preconcentration technique in CE, was proposed as a bridge to couple the MNPs-based extraction with CE. The pH junction step permits injection of a large sample volume into the capillary and focuses it into a much narrower zone, providing notably improved detection sensitivity. The focusing principle relies on differential electrophoretic velocity induced by significantly different pH values of the background electrolyte (BGE) and the sample zone, which matches well with the pH-controlled capture/release mechanism of the boronate

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doi:10.1016/j.chroma.2009.04.040

affinity extraction. Experimental conditions were optimized for the pH junction and the desorption procedure. Riboflavin (vitamin B_2), a 1,3-*cis*-diol-containing compound that plays significant roles in maintaining the health of humans and animals, was then used as a main analyte. The established method was finally applied to the extraction and determination of riboflavin in human urine sample.

2. Experimental

2.1. Reagents

Tetraethoxysilane (TEOS) was purchased from Sigma (St. Louis, MO, USA). 3-Aminophenylboronic acid (3-APBA) monohydrate and riboflavin were obtained from Alfa Aesar (Tianjin, China). Other chemicals were obtained from normal commercial sources. All chemicals were of analytical reagent grade or higher. Water was purified with a Milli-Q Advantage A10 System (Millipore, Milford, MA, USA), and was used to prepare all solutions.

2.2. Preparation of poly-APBA coated magnetic nanoparticles

The synthesis procedure is comprised of three steps: (1) synthesis of amino functionalized magnetic nanoparticles, (2) preparation of silica shell, and (3) encapsulation with poly-APBA polymer. The amino functionalized MNPs were synthesized according to a previously reported method [21]. Briefly, 1.0 g ferric trichloride hexahydrate, 6.5 g 1,6-hexanediamine and 2.0 g anhydrous sodium acetate were mixed with 30 mL glycol 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. To cover the amino functionalized MNPs with a silica shell, 0.9 mL TEOS and 5 mL ~8.5% ammonium hydroxide were added in 150 mL ethanol, and the mixture was left to react at room temperature for 20 min. Then 250 mg nanoparticles were added and the reaction was left for another 6 min. The resulting SiO_2/Fe_3O_4 nanoparticles were collected by a magnet at the wall and washed 3 times with ethanol and dried at 50 °C. To make a poly-APBA shell on the SiO_2/Fe_3O_4 nanoparticles, 80 mg SiO_2/Fe_3O_4 was mixed with 4 mL of 80 mM APBA and 4 mL of 100 mM ammonium persulfate and reacted at room temperature for 2 h. Finally, the poly-APBA/SiO_2/Fe_3O_4 nanoparticles were washed 3 times with ethanol, dried at 50° C, and then stored for further use.

2.3. Instruments

All CE separations were performed on a laboratory-rebuilt system with a high-voltage power supply and a laser-induced fluorescence (LIF) detector dissembled from a Unimicro TriSep-2100 pressurized capillary eletrochromatography (pCEC) system (Pleasanton, CA, USA). A bare fused-silica capillary of 70 cm length (50 cm effective length) × 75 μm I.D. × 375 μm O.D. from Yongnian Optical Fiber Factory (Hebei, China) was used as the separation column. Except the inlet and outlet ends, the main length of the capillary was placed in a cardboard box, the temperature within which was controlled around the ambient temperature by forced air delivered by an electric fan. The samples were injected by gravity through elevating the inlet end 30 cm or 10 cm higher relative to the outlet end for a certain time. Transmission electron microscopic (TEM) analyses were performed on a JEM-100 s system (JEOL, Tokyo, Japan) with an acceleration voltage of 80 kV. Samples for the TEM analyses were prepared by drop-wise applying water-dispersed NMPs onto copper grids and then drying naturally. IR spectra were taken on a Nicolet 6700 Fourier transform infrared (FTIR) spectrometer (Thermo Fisher, Franklin, MA, USA). A 1200 HPLC-diode array detection (DAD) system (Agilent Technologies, Waldbronn, Germany) was employed for the verification of the extraction specificity. The HPLC system was controlled by a LC 3D ChemStation (version B.02.01-SR1). A Zorbax Eclipse XDB-C8 analytical column (150 mm × 4.6 mm I.D., 5 μm particle size) from Agilent was used for the separation. The HPLC conditions were as follows: column temperature, 20 °C; mobile phase, methanol/50 mM acetic acid (60:40, v/v); flow rate, 0.5 mL/min; volume injected: 10 μL; and DAD wavelength, 275 nm. Fluorescence measurements were carried out on a RF-5301PC spectroflurophotometer (Shimazu, Kyoto, Japan). UV absorbance measurements were implemented on an UV-3600 spectrometer (Shimazu).

2.4. Measurement of extraction capacity

Direct UV absorbance and fluorescence measurements were carried out to determine the extraction capacity for catechol and riboflavin, respectively. The UV absorbance at 275 nm of a series of standard catechol solutions and the fluorescence intensity at 520 nm (excited at 450 nm) of a series of standard riboflavin solutions were measured to establish calibration curves. A certain amount of MNPs was added into 1 mL of 1 mg/mL catechol or 4 mL of 1 mg/mL riboflavin dissolved in 10 mM sodium carbonate buffer (pH 10.0). The mixture was dispersed by ultrasonication for 30 s and shaken on a rotator for 2 h. Then the MNPs were attracted to the tube wall using a magnet. The collected MNPs were washed for several times with totally 1 mL of 10 mM sodium carbonate buffer (pH 10.0). Then the extracted compounds were desorbed with 10 mM acetic acid of the same volume as the original samples and shaken on a rotator for 1 h. Finally, the MNPs were collected at the tube wall using a magnet and the eluent was transferred into a colorimetric cuvette for absorbance or fluorescence measurement. The extracted amounts were calculated according to the calibration curves, which were considered as the saturated capacity of the MNPs for test compounds under measurement.

2.5. Measurement of extraction equilibrium

The extraction equilibrium was investigated using indirect fluorescence measurement with riboflavin as the test compound. The fluorescence intensities at 520 nm (excited at 450 nm) of a series of standard solutions of riboflavin were measured to establish a calibration curve. 10 mg of MNPs was mixed with 4 ml of 0.57 μg/mL riboflavin solution. The mixture was dispersed by ultrasonication for 30 s and shaken on a rotator for a certain time. Then the MNPs were collected at the tube wall using a magnet and the supernatant was transferred into a colorimetric cuvette for fluorescence measurement under identical conditions. The riboflavin concentration in the supernatant was calculated by the calibration curve. The difference between the original concentration and the concentration found in the supernatant was used to calculate the amount of riboflavin extracted by the boronate functionalized MNPs at a certain extraction time.

2.6. CE conditions

At the beginning of each day, the capillary was rinsed with 1 M NaOH for 30 min followed with the BGE for 1 h. Between runs, the capillary was conditioned with 0.1 M NaOH for 3 min and the BGE for 6 min. A 20 mM sodium tetraborate buffer, pH 10.0, was chosen as the BGE. For normal CE, the riboflavin samples were prepared by diluting the stock riboflavin solution (0.1 mg/mL) with the BGE to get a certain concentration. While for pH junction, the stock solution was diluted with 20 mM phosphate buffer of certain pH to reach a certain concentration. The separation voltage was 15 kV. The excitation wavelength for the LIF detection was 473 nm while the detection wavelength was 520 nm.

2.7. Extraction

For each extraction, 2 mg poly-APBA/SiO_2/Fe_3O_4 nanoparticles were added to a 600-μL plastic microcentrifugal tube that contained 500 μL of 3 μg/mL riboflavin in 10 mM sodium carbonate buffer (pH 10.0). The tube was first immersed into ultrasonic bath for 30 s and then shaken on a rotator for a certain period. The MNPs were then collected at the tube wall by applying a magnet to the tube wall and rinsed with 500 μL 10 mM sodium carbonate buffer for 4 times. After washing, the MNPs were resuspended in 20 μL desorption buffer for certain period on a motor magnetic stirrer. The motor magnetic stirrer used in this work had a weak magnetic field and thus accumulation of the MNPs at the bottom of the vial due to the magnetic attraction was not observed. However, if a magnetic stirrer with a strong magnetic field is used, it is suggested to increase and carefully adjust the distance between the stirrer and the vial to avoid aggregation of the MNPs. Finally, the nanoparticles were trapped to the tube wall again and the desorption buffer was collected by pipetting carefully. The extraction and desorption experiments were carried out under ambient temperature.

For the extraction of urine sample, the pH of the urine sample was first adjusted to 10.0 with 2 M NaOH solution. For the extraction of riboflavin-spiked urine sample, 500 μL pH-adjusted urine sample was mixed with 3 μL of 0.1 mg/mL riboflavin. The extraction procedure was the same as above.

3. Results and discussion

3.1. Characterization of the magnetic nanoparticles

The size and morphology of the poly-APBA coated MNPs synthesized in this study was observed with TEM. A representative TEM image is shown in Fig. 1, which suggests that the MNPs were well-shaped nanoparticles. From the TEM images, the radium of the boronate functionalized MNPs was measured to be 60 ± 15 nm. Because TEM fails to recognize the Fe_3O_4 core, the SiO_2 shell, and the poly-APBA layer, the thickness of the SiO_2 shell and the poly-APBA coating is unknown at present.

FTIR spectroscopy was used to characterize the MNPs. For amino functionalized MNPs, a strong adsorption peak at 576 cm^{-1} was observed, which can be ascribed to Fe–O vibration, while two peaks around 1623 and 1048 cm^{-1} were observed, which can be ascribed to C–NH_2 vibrations. The presence of the later two peaks indicated the existence of –NH_2 group on the amino functionalized MNPs. For silica coated MNPs, a peak around 1066 cm^{-1} was observed, which is characteristic of the Si–O–Si bond. Since the absorption of B–C and B–O bonds is too weak as compared with the above-mentioned bonds, the presence of B on the surface of the final MNPs could not be confirmed by the FTIR spectroscopy.

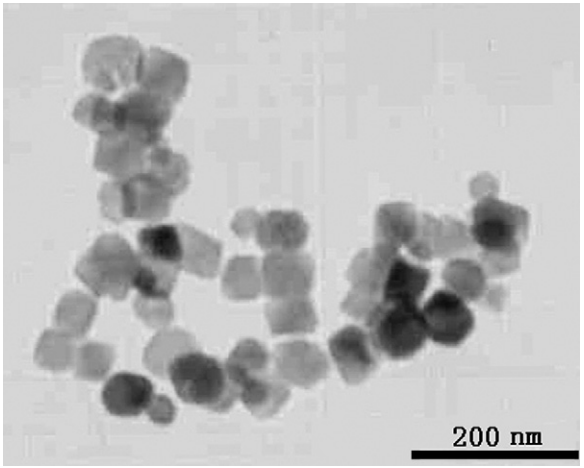

Fig. 1. TEM image of the boronate functionalized magnetic nanoparticles.

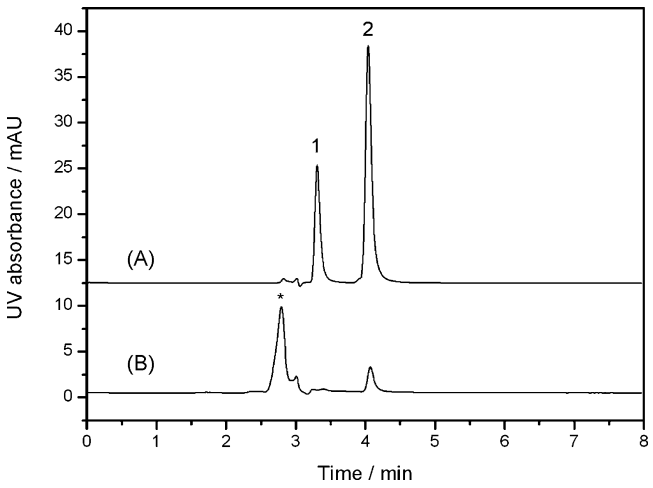

Fig. 2. HPLC chromatograms of (A) a mixture of catechol and quinol and (B) catechol extracted by the boronate functionalized MNPs from a mixture of catechol and quinol. Sample: (A) 24 μg/mL catechol plus 19 μg/mL quinol dissolved in 10 mM acetic acid; (B) 1 mg/mL catechol plus 1 mg/mL dissolved in 10 mM sodium carbonate solution, pH 10.0, extracted for 1 h, desorbed with 10 mM acetic acid for 1 h. Peak identity: *, baseline disturbance due to the presence sodium carbonate in the sample matrix; 1, quinol; 2, catechol.

3.2. Extraction selectivity

The prepared boronate functionalized MNPs exhibited specific selectivity to *cis*-diol-containing compounds over their non-*cis*-diol analogs. Representative evidence is shown in Fig. 2. HPLC analysis of the extracted analytes by the boronate functionalized MNPs from a mixture containing catechol (*cis*-diol-containing compound) and quinol (non-*cis*-diol analog) indicated that catechol was extracted by the boronate functionalized MNPs, whereas quinol was excluded. The specificity of the boronate affinity was also confirmed using nucleosides (*cis*-diol-containing compounds) and deoxynucleosides (non-*cis*-diol analogs) (data not shown).

3.3. Extraction capacity

The extraction capacity is an important value to evaluate the feasibility of the boronate functionalized MNPs for analytical purposes. As the extraction capacity depends on the nature of analytes, catechol (contains 1,2-*cis*-diol) and riboflavin (contains 1,3-*cis*-diol) were used as test compounds for the measurement. The capacity was determined to be 0.21 (±0.06) μg/mg MNP and 0.32 (±0.05) ng/mg MNP for catechol and riboflavin, respectively. The capacity for catechol was 650-fold higher than that for riboflavin. Generally 1,2-*cis*-diol-containing compounds have higher affinity to boronic acids as compared with 1,3-*cis*-diol-containing compounds. For example, the association constant of the complex of catechol and phenylboronic acid is 17,500; as a comparison, the association constant for 1,3-propanediol is 0.88 [22]. Therefore, the measured capacity values are reasonable.

3.4. Extraction equilibrium

The extraction time profile was investigated using indirect fluorescence measurement. As shown in Fig. 3, the extracted amount increased as increasing the extraction time within initial 45 min then kept constant after that. This means that the equilibrium was

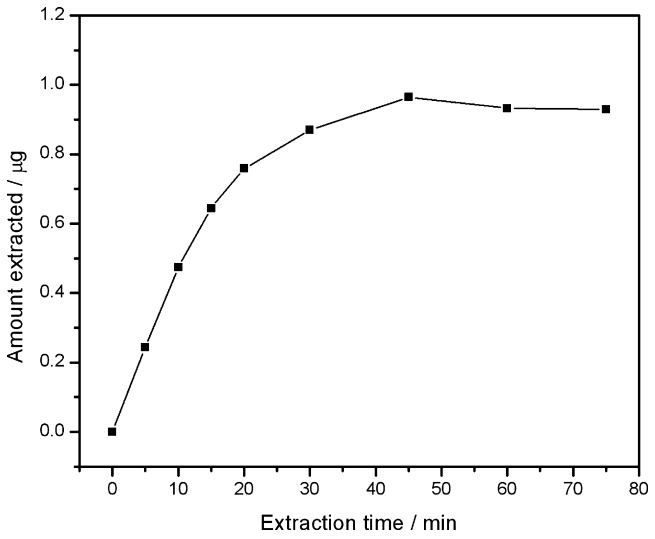

Fig. 3. Dependence of the extracted analyte amount on the extraction time. Sample: 0.57 μg/mL riboflavin in 10 mM sodium carbonate buffer, pH 10.0.

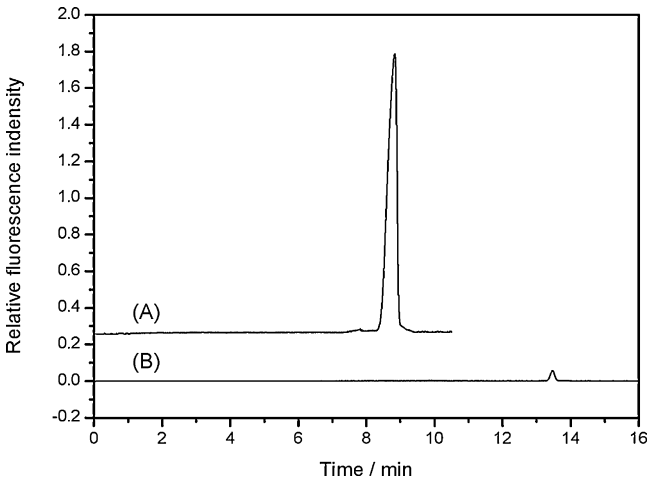

Fig. 4. Comparison of the peak of riboflavin in CE separation with (A) and without (B) pH junction. Sample: 5 μg/mL riboflavin in 20 mM phosphate buffer, pH 4.0; voltage, 15 kV; BGE, 20 mM sodium tetraborate, pH 10.0; gravity sample injection, (A) 30 cm for 120 s and (B) 10 cm for 10 s.

reached at 45 min and that the extraction time must be longer than 45 min for equilibrium extraction. Since extraction times of more than 45 min have been reported in the literature [11,13], such an extraction time is acceptable.

3.5. pH junction-CE method for off-line coupling with MNPs-based extraction

To facilitate the off-line coupling of CE with the MNPs-based extraction, we took full advantage of the pH-controlled capture/release mechanism of boronate affinity extraction and proposed using pH junction as a bridge for the coupling. For boronate affinity-based extraction, 1,2- or 1,3- *cis*-diol-containing compounds are captured at a high pH while they are released at a low pH. To separate hydroxyl compounds by CE, a high pH buffer is usually used to make the analytes charged. Therefore, it is clear that the low pH sample provided by the boronate affinity extraction matches well the pH junction technique in CE.

Experimental conditions for the pH junction-CE were optimized and the effectiveness of the pH junction was investigated. Sodium tetraborate buffer at pH 10.0 was used as the BGE, while phosphate buffers (20 mM) at pH 2.5, 4.0, 5.0 and 6.0, were used to prepare sample solutions to investigate the effect of sample pH on pH junction. The results indicated that pH 4.0 provided the best peak area and peak height for riboflavin (data not shown). Thus, pH 4.0 was chosen as the pH for desorption. With keeping the injection height at 30 cm, the injection time, namely 60, 90, 120, 150 and 180 s, was optimized among 60, 90, 120, 150, to 180 s. The peak width at half height obtained was plotted against the injection time. It was found that the peak width at half height increased as increasing the injection time. This means the sensitivity improvement is associated with loss in peak efficiency. However, increasing the injection time within the range of 90–120 s provided the lowest loss in peak efficiency. To compromise the sensitivity enhancement and the peak efficiency, 120 s was chosen as the best injection time for later experiments, which gave a sample zone of about 6.1 cm at a 30-cm height injection. Under the optimized conditions, the detection sensitivity was improved significantly by the pH junction. As illustrated in Fig. 4, the detection sensitivity was enhanced by 42-fold in term of corrected peak height (peak height/migration time), as compared with conventional injection.

3.6. Boronate functionalized MNPs vs bare MNPs

Experiments were carried out to verify the relationship between the extraction selectivity and the boronic acid ligand on the MNPs. Riboflavin in a solution was extracted by the boronate functionalized MNPs and bare SiO_2/Fe_3O_4 nanoparticles. The extracted analyte was analyzed by the established pH junction-CE method. As shown in Fig. 5, the boronate functionalized MNPs exhibited certain extraction capability to riboflavin, whereas bare SiO_2/Fe_3O_4 nanoparticles were not able to extract the analyte at all. This result suggests that the extraction capability was solely due to the boronate polymer coating on the MNPs.

3.7. Selection of desorption conditions

There are mainly four factors that dramatically influence the desorption, which include the pH, the composition of the desorbing

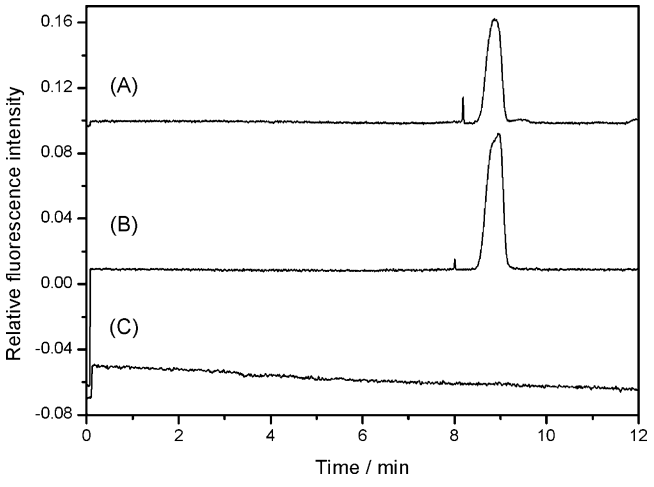

Fig. 5. Comparison of the electropherograms for (A) riboflavin standard, (B) riboflavin extracted with the poly-APBA/SiO_2/Fe_3O_4 nanoparticles, and (C) riboflavin extracted with the SiO_2/Fe_3O_4 nanoparticles. Sample: (A) 0.5 μg/mL riboflavin in 20 mM phosphate buffer, pH 4.0, (B) and (C) riboflavin extracted from 3 μg/mL riboflavin dissolved in 10 mM sodium carbonate solution (pH 10.0), extracted for 2 h and desorbed with 20 mM phosphate buffer (pH 4.0) for 1 h. Voltage, 15 kV; BGE, 20 mM sodium tetraborate, pH 10.0; sample injection, 30 cm for 120 s.

solution, the desorption time, and agitation. The desorption time depends on the desorption strength of the desorbing solution. The stronger is the desorbing solution, the shorter is the desorption time. Phosphate buffer of 20 mM was first used as the desorbing solution and three pH values, including 2.5, 4.0 and 6.0, were tested. The extraction time was set quite long (4 h) to ensure a nearly comparable amount was extracted each time. The desorption time was set 30 min. The corrected peak areas obtained at the three pH values were found nearly the same, indicating these pH values worked well comparably. Thus, pH 4.0 was chosen for later experiments due to its best performance in pH junction as mentioned above.

To find an appropriate desorbing solution and desorption time, a strategy of consecutive desorptions on a single extraction was employed. After the analyte was extracted from the sample by the boronate functionalized MNPs for one single time, a desorbing solution under investigation was applied to the MNPs for a desorption time predetermined. One or more consecutive desorption procedures were performed under the same conditions for the same MNPs. If the desorbing solution has a strong desorption strength, the carryover will be limited. Phosphate buffer (20 mM) at pH 4.0 was further examined and the desorption time was set at 6 min. An apparent peak for riboflavin was observed for a second desorption; the peak area was about two-third of that obtained by the first desorption. This means that the desorbing solution was weak. Therefore, a longer desorption time or a stronger desorbing solution is required for a better desorption efficiency.

To increase the desorption strength, we turned to add an organic solvent into desorbing solution to improve the solubility of the analyte. Isopropanol and N,N-dimethylformamide (DMF) were compared for this purpose. Isopropanol was found to be not compatible to the BGE (sodium tetraborate solution) and its presence in the desorbing solution gave rise to interruption of circuit. However, DMF turned out to be an effective desorption promoter. When 50% (v/v) DMF was added to the desorbing solution, a very small peak was observed for a second desorption; the peak area was only about 4% of that obtained by a first desorption. As compared with the desorbing solution in the absence of DMF, the addition of DMF dramatically improved the desorption strength; the corrected peak area provided by the first desorption increased by 8 times. An additional advantage of using DMF as desorption promoter is that the peak became sharper, which favors the concentration efficiency of pH junction. For extraction techniques, an ideal situation is that there is completely no analyte carryover while the desorption time is very short (for example, tens or even several seconds). In reality, however, there is usually a compromise. Clearly, the results we reported in this manuscript are far from the ideal situation. Since a recovery of 96% is acceptable, particularly for a preliminary study, however, we did not further increase the desorption time. Thus, 20 mM phosphate buffer containing 50% DMF (pH 4.0) was chosen as the desorbing solution and the desorption time was set at 6 min for later experiments in this study. While efforts to reduce both desorption time and carryover through optimizing structure of MNPs have been under way in the authors' laboratory.

3.8. Determination of riboflavin in human urine

Under the selected conditions for pH junction and sample desorption, the limit of detection (LOD) of the established method was measured to be 1 ng/mL or 2.7 nM ($S/N = 3$). This result was comparable to that obtained by CE-LIF (3 nM) [23] and better than that obtained by CE-light-emitting diode-induced fluorescence detection (20 ng/mL) [24]. Using the MNPs-based extraction-pH junction-CE method established in this study, riboflavin in urine from a healthy volunteer was extracted and determined. The standard addition method was employed to determine the riboflavin extracted from urine sample through spiking the sample with a certain riboflavin solution of known concentration and extracting and analyzing the spiked sample under the same conditions. The electropherograms for un-spiked and spiked urine samples are shown in Fig. 6. The concentration of riboflavin in the urine sample was determined to be 0.98 μg/mL, which is in the normal range (0.35–1.15 μg/mL) for healthy individuals [25].

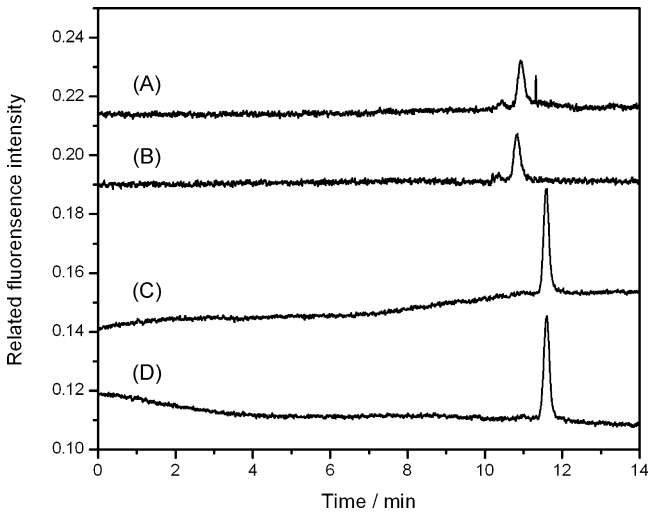

Fig. 6. pH junction-CE separation of the extracted riboflavin from (A and B) un-spiked urine; (C and D) urine spiked with 3 μL of 0.1 mg/mL riboflavin. The pH of the samples was adjusted to 10.0 and then extracted by the boronate functionalized MNPs for 2 h, desorbed with a 1:1 mixture of DMF and 40 mM phosphate buffer (pH 4.0) for 6 min. The CE conditions as in Fig. 4. The migration time shift between the results for the un-spiked and spiked samples was due to the drop of ambient temperature from the afternoon to the evening.

4. Conclusions

In this study, boronate functionalized MNPs have been synthesized and successfully employed as extracting probes to capture and enrich *cis*-diol-containing biomolecules, and an off-line combination of the MNPs-based extraction with CE has been established. The boronate functionalized MNPs exhibited specific selectivity and sufficient extraction capability to 1,2- and 1,3-*cis*-diol-containing compounds. As the sample size can be typically 500–1000 μL (though use of larger sample volumes is acceptable), while the extracted analyte can be rebuilt into a 20-μL volume, the enrichment factor can be at least 25–50. The pH-controlled capture/release mechanism of the boronate affinity allows for facile incorporation of a pH junction step in the CE separation, which effectively enhances the detection sensitivity. Since a lot of important biomolecules contain 1,2- or 1,3-*cis*-diols, such as carbohydrates, RNA, nucleosides, and glycoproteins, the boronate affinity MNP-based extraction should be a promising tool for bioanalysis.

Acknowledgments

We gratefully acknowledge the financial support of the General Grant (No. 20675038) from the National Natural Science Foundation of China and the Key Grant of 973 Program (No. 2007CB914102) and the International Science & Technology Cooperation Grant (2008DFA01910) from the Ministry of Science and Technology of China to Z.L. The work of P.D. is also supported by the Scientific Research Foundation of Graduate School of Nanjing University. The authors also gratefully thank Mr. Dan Ding's kind help for the TEM analysis.

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