



A molecularly imprinted polymer-coated nanocomposite of magnetic nanoparticles for estrone recognition

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ABSTRACT

In this study, we synthesized Fe₃O₄ magnetic nanoparticles coated estrone-imprinted polymer with controlled size using a semi-covalent imprinting strategy. In this protocol, the estrone–silica monomer complex (EstSi) was synthesized by the reaction 3-(triethoxysilyl)propyl isocyanate with estrone, where the template was linked to the silica coating on the iron oxide core *via* a thermally reversible bond. The removal of the template by a simple thermal reaction produced specific estrone recognition sites on the surface of silica shell. The resulting estrone-imprinted polymer coating Fe₃O₄ magnetic hybrid nanoparticles exhibit a much higher specific recognition and saturation magnetization. The hybrid nanoparticles have been used for biochemical separation of estrone.

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1. Introduction

In more recent years, magnetic nanoparticles (MNPs) have been studied for biomedical and biotechnological applications, including targeted drug delivery, MRI contrast enhancement, biosensor, rapid environmental and biological separation, and concentration of trace amounts of specific targets, such as bacteria or leukocytes and proteins [1–6]. For many of these applications, surface modification of MNPs is a key challenge. In general, surface modification can be accomplished by physical/chemical adsorption or surface coating of specific ligands, depending on the specific applications. When modified with a specific functional polymer, for example, the molecularly imprinted polymer (MIP), these magnetic nanoparticles coated MIP could be used to separate and concentrate chemicals more conveniently with the help of an external magnetic field. In this study, we focused on the development of a new methodology for preparing MNPs attached functional moieties of specific recognition with tailor-made properties through molecular imprinting technique.

The molecular imprinting technique is an attractive method for the generation of polymer-based molecular recognition elements tailor-made for a given target or group of target molecules [7,8]. The technique involves polymerization of functional monomers and a cross-linker around a template. Extraction of the template

leaves behind recognition sites of functional and shape complementarity to the template. MIP has been used in a variety of applications, such as separation media [9], mimicking antibody [10], chemical and biochemical sensing [11]. Therefore, by combining MNPs coated artificial receptor tailor-made with specific recognition and magnetic property can be an ideal candidate for the multifunctional nanomaterial toward bioseparation. The mechanical/chemical stability, low cost, ease of preparation of molecularly imprinted materials have attracted extensive research interest. However, they suffer from some drawbacks in certain applications, such as the heterogeneous distribution of the binding sites, low binding capacity and selectivity, poor site accessibility and slow binding kinetics. The development of molecular imprinting nanotechniques will provide a potential solution to overcome these problems [12–21]. Nanosized molecular imprinted materials (MIP nanoparticles [12–15], MIP nanocapsule [16], MIP nanowire [17–19], MIP nanotube [20,21]) having a small dimension with high surface-to-volume ratio are expected to improve the removal of template molecules, the binding capacity and fast binding kinetics over normal imprinting materials. By incorporating magnetic iron oxide, the superparamagnetic composite MIP beads with an average diameter of 13 μm were prepared by Ansell and Mosbach using suspension polymerization in perfluorocarbon liquid in the first time [22]. The magnetic MIP nanowires for theophylline using a nanopores alumina template was reported by Li et al. [23]. Tan recently reported bovine serum albumin surface-imprinted sub-micrometer particles (500–600 nm) with magnetic susceptibility through miniemulsion polymerization [24].

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In this work, we report the synthesis of the MIP-coated hybrid nanoparticles by “semi-covalent” imprinting technique [25] through a thermally reversible covalent bond and application for biochemical separation of estrone. Estrone is one of naturally estrogenic hormones, can be toxic and carcinogenic even at low levels. It has been reported that estrone influenced the normal development and maturation of female, and was suspected to induce cancer and has often been identified as the major contributor to the endocrine disrupting activity observed in environmental water samples [26,27]. In order to prevent these uncontrolled effects on human health and deleterious effects on the aquatic environment, it is of great significance to develop new adsorbents for the separation of estrone. Therefore estrone was selected as template molecule in this study. Chang et al. [28] reported the use of a thermally reversible covalent bond for imprinting estrone on silica spheres with diameter of 1.5–3 μm . In this study, based on an approach modified from Chang, we bring together the Fe_3O_4 magnetic core coated with molecularly imprinted silica films of nanosize. Compared to traditional MIPs nanoparticles, the molecularly imprinted magnetic hybrid display several advantages: (i) the superparamagnetic iron oxide core enables the particles replace the centrifugation step with a magnetic separation, and facilitates the application of magnetic MIP in immunoassay and magnetically stabilized-fluidised-bed separation. (ii) The semi-covalent imprinting can be looked upon as a hybrid approach in which the imprinting is covalent, but the rebinding is noncovalent in nature. (iii) There are no randomly distributed functional groups and the binding sites are more uniform in nature [25]. (iv) The template removal by hydrolysis leaves the binding sites in the silica shell during the imprinting step, and the template molecules can reach the imprinting sites easily and quickly during the rebinding step.

2. Experimental

2.1. Materials

3-(Triethoxysilyl)propyl isocyanate, 3-aminopropyl triethoxysilane, tetraethoxysilane (TEOS), dibutyltin dilaurate (DBDU) were purchased from Alfa Aesar Chemical Company. Estrone and testosterone propionate were obtained from Sigma. $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, ammonium hydroxide (25%) were purchased from Tianjin Chemicals Ltd. THF and DMSO were used after

purification by standard methods. Other chemicals were used as received without further purification.

2.2. Preparation of Fe_3O_4 MNPs

$\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (1.72 g) and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (4.72 g) was dissolved in 80 mL of deaerated highly purified water contained in a three neck flask with vigorous stirring (800 rpm) under nitrogen. As the temperature was elevated to 80 °C, 10 mL of ammonium hydroxide was added drop by drop, and the reaction was maintained for 30 min. The black product was separated by putting the vessel on a Nd–Fe–B permanent magnet and the supernatant was decanted. The black precipitate was washed for six times with highly purified water to remove the unreacted chemicals, then the black product Fe_3O_4 was dried in the vacuum.

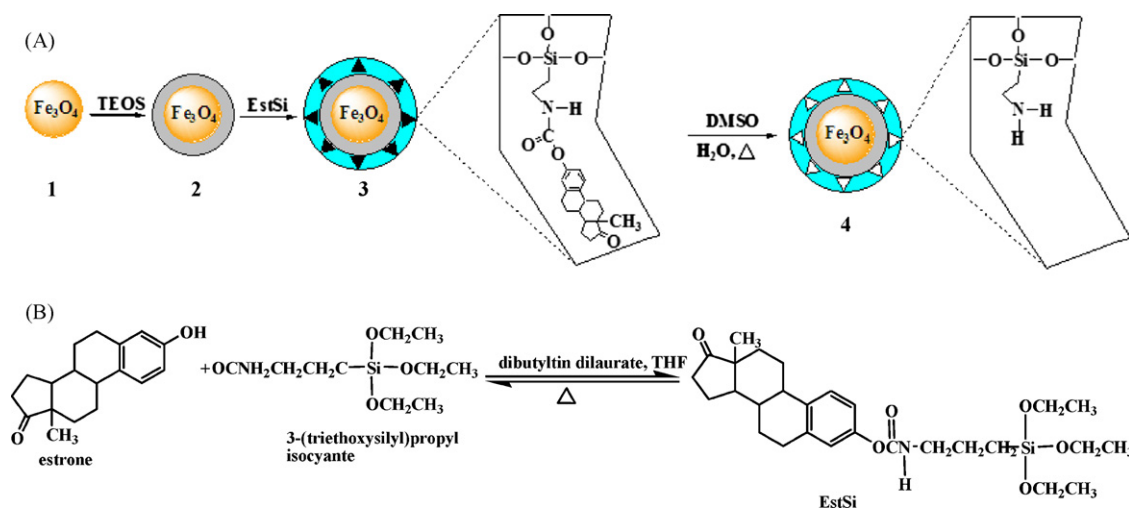
2.3. Synthesis of the $\text{Fe}_3\text{O}_4@/\text{SiO}_2$ MNPs

300 mg superparamagnetic magnetite nanoparticles were dissolved in 50 mL 2-propyl alcohol and 4 mL of highly purified water by sonication for 15 min, followed by the addition of 5 mL ammonium hydroxide and 2 mL TEOS sequentially. The mixture was reacted for 12 h at the room temperature under a continuous stirring. The resultant product was collected by an external magnetic field, and rinsed with highly purified water for six times thoroughly, and dry to powder in the vacuum.

2.4. Preparation of estrone-imprinted MNPs and a control silica-coated MNPs

EstSi (Scheme 1B) was synthesized according to the Chang et al.'s method [28]. The $\text{Fe}_3\text{O}_4@/\text{SiO}_2$ MNPs (1 g) was dissolved into acetate buffer (0.1 mol L⁻¹, pH 5.2), then EstSi (0.12 g) was added. The mixture was reacted for 12 h at room temperature with a mechanic stirring. The product was separated by the magnetic field and washed with acetone, then dried to powder. This imprinting MNPs (0.3 g) were added to a solution of DMSO (10 mL) and water (2 mL). The mixture was stirred for 3 h at 180 °C. Finally, estrone-imprinted MNPs with specific sites was separated by magnetic field and dried in vacuum oven at 25 °C for a week.

Control silica-coated MNPs were synthesized in the almost same manner for the preparation of estrone-imprinted MNPs except for 3-aminopropyl triethoxysilane was used in place of EstSi.



Scheme 1. (A) Multistep synthesis of estrone-imprinted MNPs. Fe_3O_4 MNPs (1) were prepared by coprecipitation method and the MNPs surface was then transformed to silica shell by a sol-gel process using TEOS to give $\text{Fe}_3\text{O}_4@/\text{SiO}_2$ MNPs (2). The $\text{Fe}_3\text{O}_4@/\text{SiO}_2$ MNPs reacted with a template-silica monomer complex (EstSi) to produce silica surface functionalized with estrone-imprinted polymer (3). After remove the template estrone by simple thermal reaction, estrone-imprinted polymer coated MNPs (4) were obtained. (B) Synthesis of EstSi.

2.5. Binding experiment

Estrone-imprinted MNPs (20 mg) and control silica-coated MNPs (20 mg) were added to the solutions of estrone in chloroform (10 mL) at various concentrations respectively. After incubating for 24 h, the estrone-imprinted MNPs and control silica-coated MNPs were isolated by an external magnetic field, and rinsed with THF and chloroform. The filtrate was concentrated to dryness by evaporation of the solvent before HPLC analysis. The amount of estrone bound to the MNPs was determined by HPLC.

Reverse phase HPLC analysis was performed on a Shimadzu LC-20A system (Shimadzu, Kyoto, Japan) equipped with a ODS column and a UV–vis detector (set at 254 nm for all the compounds). The data were collected and analyzed using LCsolution software. The eluent is methanol at the flow rate of 1.0 mL min^{-1} . For each analysis $20 \mu\text{L}$ of sample was injected.

2.6. Characterization

A Tecnai G² 20 S-TWIN microscope was used to obtain transmission electron microscope (TEM) images of Fe_3O_4 MNP and estrone-imprinted MNPs. For TEM analyses, samples were prepared by placing one or two drops of nanoparticle solution onto the carbon-coated copper grid and drying it in air at room temperature.

Fourier transform infrared (FT-IR) spectra were recorded on a AVATAR 360 (Nicolet Corp., USA) and samples were dried at 80°C in vacuo oven for at least 12 h prior to fabrication of the KBr pellet.

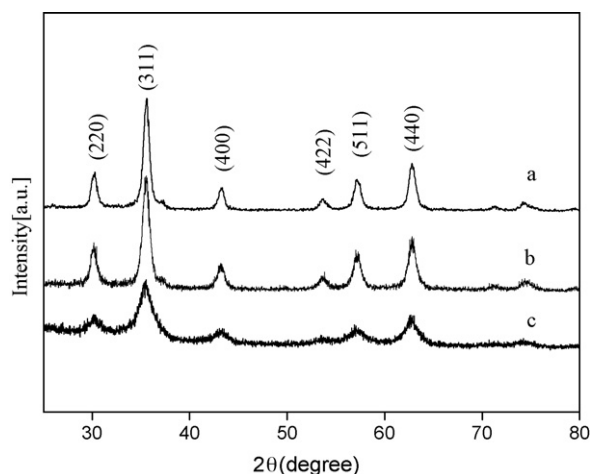


Fig. 1. XRD patterns of Fe_3O_4 (a), $\text{Fe}_3\text{O}_4@SiO_2$ (b) and estrone-imprinted polymer coated MNPs (c).

In this context, 2 mg of each sample was thoroughly mixed and crushed with 100 mg of KBr, and the mixture was used for pellet fabrication. Fifty scans of the region between 400 and 4000 cm^{-1} were collected for each FT-IR spectrum recorded.

^1H NMR spectra of EstSi was investigated by Varian Mercury Vx-300.

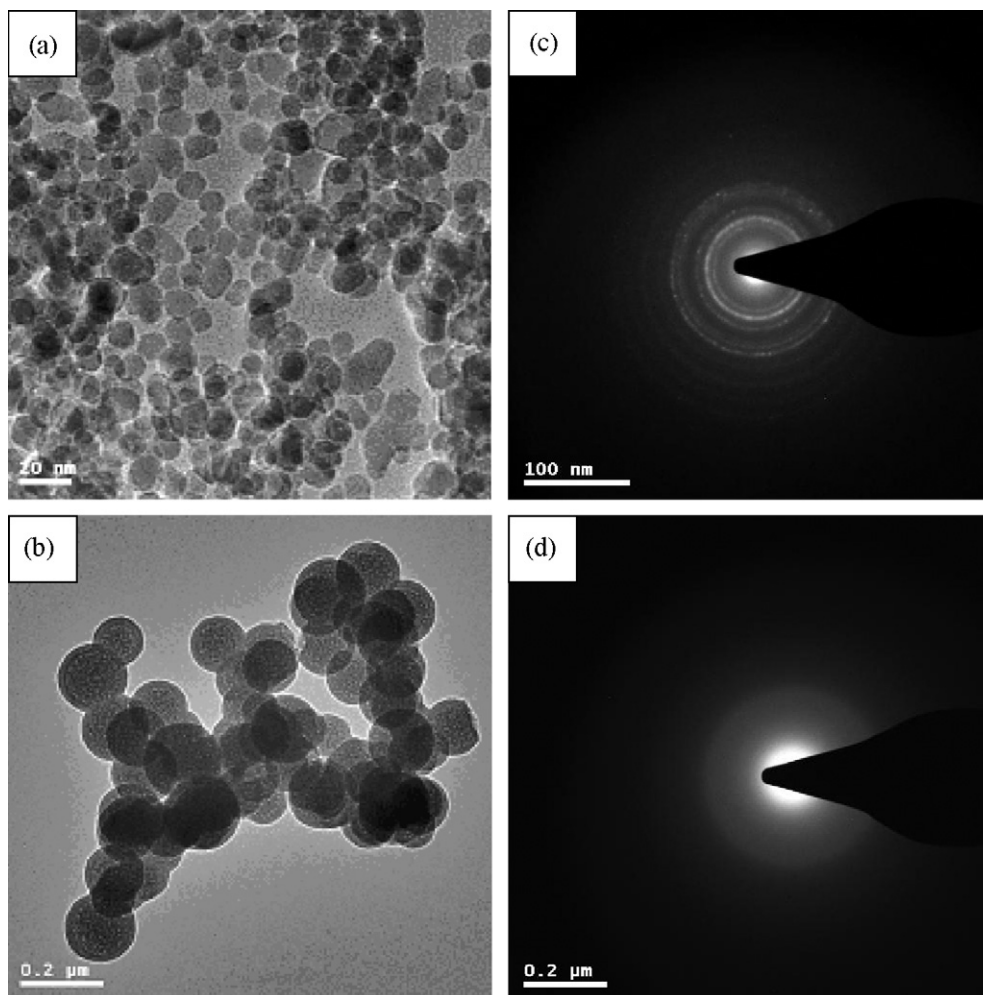


Fig. 2. TEM images of Fe_3O_4 MNPs (a) and estrone-imprinted MNPs (b), and SAED of Fe_3O_4 MNPs (c) and estrone-imprinted MNPs (d).

The identification of crystalline phase of the synthesized nanoparticles was performed by a Rigaku D/max/2500v/pc (Japan) X-ray diffractometer with Cu K α source. The 2θ angles probed were from 10° to 80° at a rate $4^\circ/\text{min}$.

The magnetic properties were analyzed with a vibrating sample magnetometer (VSM) (LDJ 9600-1, USA).

3. Results and discussion

3.1. Preparation of imprinted magnetic nanoparticles

The synthesis of the MIP-coated MNPs via a multistep procedure is illustrated in Scheme 1A, which involves synthesis of Fe_3O_4 MNPs, silica-shell deposition, MIP-functionalized onto the silica surface, and final extraction of estrone and generation of the recognition site. Fe_3O_4 MNPs were synthesized by modifying the procedure as reported by Kang et al. [29]. The MNPs surface was then transformed to silica shell by a sol-gel process [30,31] using tetraethoxysilane (TEOS) to give $\text{Fe}_3\text{O}_4@\text{SiO}_2$. Superparamagnetic nanoparticles having a silica shell provides good biocompatible, non-toxic coating as well as a hydrophilic surface. Furthermore, the silanol group on the silica coated can be easily modified to link bioconjugators by the sol-gel method with interesting biofunctionalities [32–34]. In this study, we used a thermally reversible bond for the preparation of template-silica monomer complex, which allowed us to remove the template by simple thermal reaction and simultaneously introduce functional groups into the cavity on the surface of MNPs. The silica coating MNPs $\text{Fe}_3\text{O}_4@\text{SiO}_2$ reacted with EstSi to produce silica surface functionalized with MIPs.

EstSi (Scheme 1B) was synthesized by the 3-(triethoxysilyl)propyl isocyanate with estrone in the presence of dibutyltin dilaurate according to the Chang et al.'s method [28]. The reaction

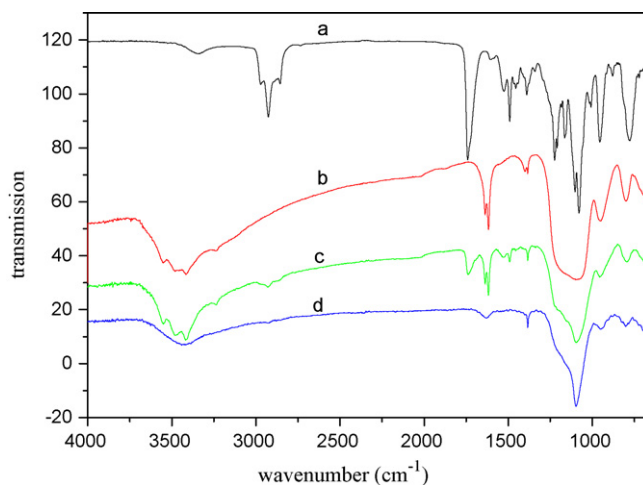


Fig. 3. FT-IR spectra of EstSi (a), $\text{Fe}_3\text{O}_4@\text{SiO}_2$ (b), molecularly imprinted MNPs (c) before and (d) after extraction of estrone.

occurred between the isocyanate group of 3-(triethoxysilyl)propyl isocyanate and a phenol moiety of estrone, forming a thermally cleavable urethane bond.

The thermal cleavage of the urethane bond was investigated by ^1H NMR. EstSi was dissolved in $\text{DMSO}-d_6$ and its ^1H NMR spectra at room temperature was investigated by Varian Mercury Vx-300. The aromatic ring proton peak appeared at 7.270, 6.835, 6.777 ppm, and the NH peak appeared at 7.675 showed the urethane is formed. The thermally cleavable urethane bond is stable at room temperature and undergoes reversible cleavage at elevated temperature and then the MIP coating was prepared by a sol-gel reaction of the EstSi.

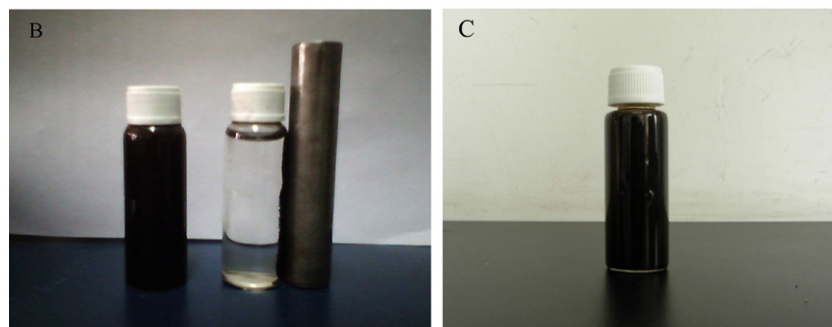
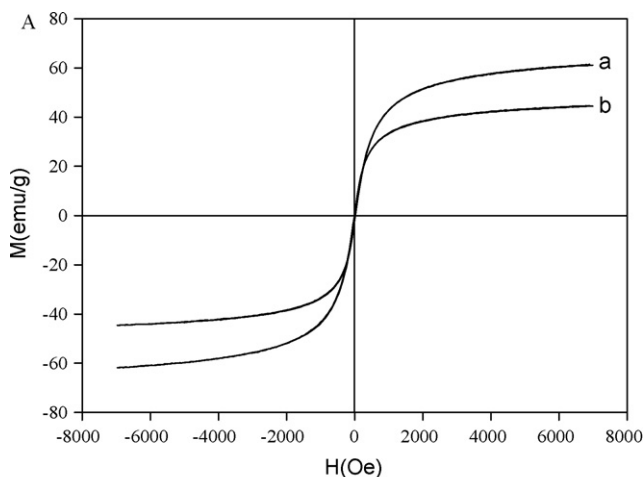


Fig. 4. (A) Magnetization curve at 298 K with (a) $\text{Fe}_3\text{O}_4@\text{SiO}_2$ and (b) estrone-imprinted MNPs. (B) Separation of estrone-imprinted MNPs by a magnet. (C) Redispersion of estrone-imprinted MNPs after removing the magnet.

To extract the imprinted estrone molecules from the core-shell MNPs, the estrone-imprinted nanoparticles were heated at 180 °C in a mixture of DMSO and water, and the dissociated isocyanato group in silica shell was converted to an amino group by its reaction with H₂O. The control MNPs were prepared with 3-aminopropyl triethoxysilane and TEOS in the absence of a template molecule.

3.2. Characterization of imprinted magnetic nanoparticles

The structural properties of synthesized MNPs were analyzed by X-ray power diffraction (XRD). As shown in Fig. 1, XRD patterns of the synthesized Fe₃O₄, Fe₃O₄@SiO₂ and estrone-imprinted polymer coated MNPs display several relatively strong reflection peaks in the 2θ region of 20–70°, which is quite similar to those of Fe₃O₄ nanoparticles reported by other group. The discernible six diffraction peaks in Fig. 1 can be indexed to (220), (311), (400), (422), (511) and (440), which match well with the database of magnetite in JCPDS (JCPDS Card: 19-629) file. However, it is insufficient to exclude the possibility of γ-Fe₂O₃, there are probably two types of iron oxide particles in this dispersion: maghemite and magnetite [35]. The trace amounts of maghemite could be attributed to the oxidation of Fe₃O₄ to γ-Fe₂O₃ during the coprecipitation and silanization process [36–38]. Because they have similar magnetic properties, the identification is not important in the present study.

Transmission electron microscopy (TEM) revealed that the diameter of Fe₃O₄ MNPs was in the range of 6–16 nm (Fig. 2a), while the average diameter of estrone-imprinted MNPs increased to about 150 nm (Fig. 2b) with a relatively narrow size distribution. The selected area electron diffraction (SAED) patterns are taken from Fe₃O₄ MNPs and estrone-imprinted MNPs. It can be seen from SAED, the presence of the ring pattern indicated that Fe₃O₄ MNPs are polycrystalline (Fig. 2c), but estrone-imprinted polymer coated MNPs are non-crystalline (Fig. 2d).

The extraction of the estrone and the presence of the molecule imprinted sites were confirmed by FT-IR spectroscopy measurements taken before and after removal of imprinted estrone (Fig. 3). The strong peaks at 1078 cm⁻¹ (Fig. 3a), 1099 cm⁻¹ (Fig. 3b), 1095 cm⁻¹ (Fig. 3c) and 1097 cm⁻¹ (Fig. 3d) are attributed to the stretch of Si–O–Si, indicating the formation of silica film. The peak was shifted from 1078 cm⁻¹ to 1097 cm⁻¹. The typical peak of 1741 and 1737 cm⁻¹ represent the stretching vibration of carbonyl groups of the urethane bond and estrone respectively. These peaks disappeared after the extraction of the template molecules. The characteristic peak of –NH₂ at 3428 cm⁻¹ verified the successful introduction of the functional groups in the imprinted cavities.

Fig. 4A shows the plots of magnetization versus magnetic field (M–H loop) at 25 °C for the Fe₃O₄@SiO₂ and estrone-imprinted MNPs respectively. It is apparent that there is no hysteresis, both remanence and coercivity are zero, suggesting that the samples are superparamagnetic. The saturation magnetization (*M_s*) values obtained at room temperature were 61.68 emu g⁻¹ and 44.63 emu g⁻¹ for Fe₃O₄@SiO₂ and estrone-imprinted MNPs respectively. The theoretical specific saturation magnetization of bulk magnetite is reported to be 92 emu g⁻¹ [39,40]. The decrease in magnetization value can be attributed to the small particle surface effect such as magnetically inactive layer containing spins that are not collinear with the magnetic field [41]. The saturation magnetization of estrone-imprinted MNPs was reduced to 44.63 emu g⁻¹ in comparison with the bulk Fe₃O₄, but remained strongly magnetic at room temperature and allowed for as effective magnetic separation carrier. Fig. 4B and C shows the separation and redispersion process of estrone-imprinted MNPs. In the absence of an external magnetic field, a dark homogeneous dispersion exists. When an external magnetic field was applied, the black particles were attracted to the wall of vial and the dispersion became clear and transparent. The superparamagnetism of estrone-imprinted MNPs

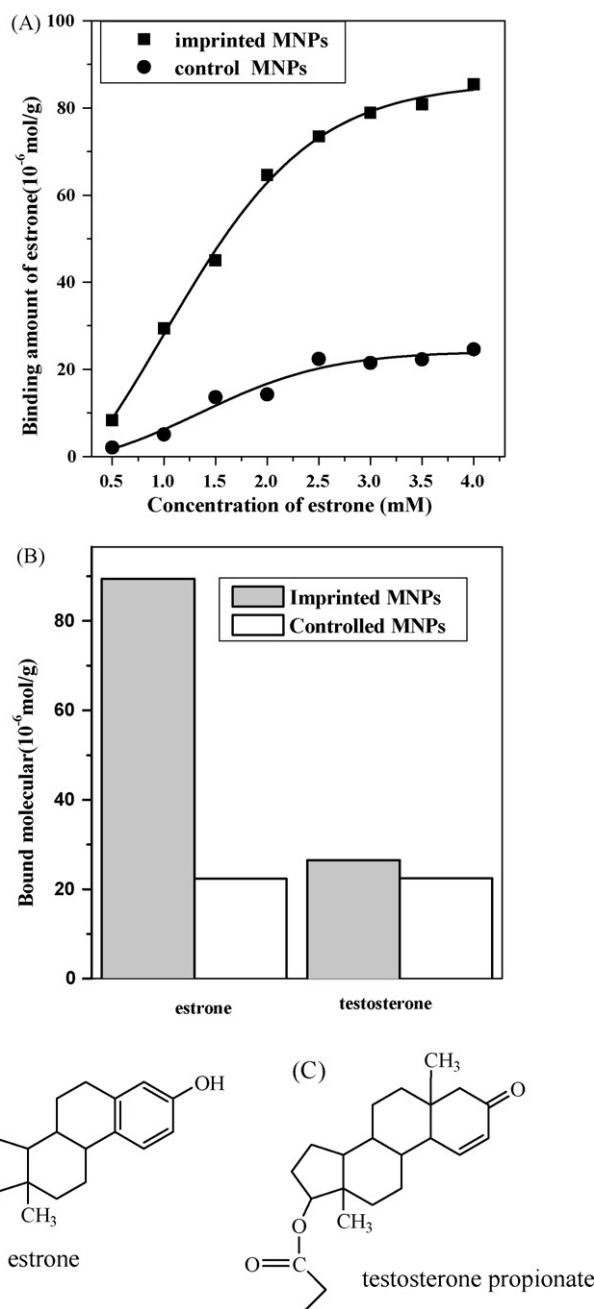


Fig. 5. (A) Amount of estrone bound by the estrone-imprinted MNPs (■) and control MNPs (●). (B) Amount of rebinding estrone and testosterone, filled bars represent estrone-imprinted MNPs, while empty bars correspond to control silica-coated MNPs. (C) The structures of estrone and testosterone propionate.

prevents MIP from aggregating and enables them to redispersed rapidly after the magnetic field is removed (Fig. 4C).

3.3. Binding properties of imprinted magnetic nanoparticles

The recognition ability of the imprinted magnetic nanoparticles toward the template estrone was investigated. The estrone-imprinted MNPs (20 mg) was added into the solutions of estrone in chloroform (10 mL) at various concentrations. After incubating for 24 h, then the MNPs was collected by an external magnetic field instead of a complex centrifugal separation, the supernatant was concentrated to dryness by evaporation of the solvent. The amount of the estrone adsorbed by estrone-imprinted MNPs was measured the residual estrone in the filtrate by HPLC, while the amount of

bound molecule of the control silica-coated MNPs was determined by the same manner. As shown in Fig. 5A, the estrone-imprinted MNPs had much higher recognition ability than the control MNPs at all concentration ranges.

The saturation binding data were further processed with Scatchard equation to estimate the binding properties of imprinted magnetic nanoparticles. The Scatchard equation was as follows:

$$\frac{Q}{C_e} = \frac{Q_{\max}}{K_d} - \frac{Q}{K_d}$$

where Q was the amount of estrone bound to estrone-imprinted MNPs at equilibrium, Q_{\max} was the apparent maximum number of binding sites, C_e was the free analytical concentration at equilibrium and K_d was the dissociation constant. The values of K_d and the Q_{\max} can be calculated from the slope and intercept of the linear line plotted in Q/C_e versus Q .

The Scatchard analysis for MIPs was performed. It was observed that the Scatchard plot was a single straight line, which indicated the binding sites of imprinted magnetic nanoparticles were identical. The “semi-covalent” imprinting technique [25] combines the advantages of both covalent and non-covalent imprinting techniques, which takes covalent imprinting technique to assemble sites that bind the target molecules in a non-covalent fashion.

The linear regression equation for the linear region is $Q/C_e = 40.54 - 0.2210Q$ ($R^2 = 0.9984$). From the slope and the intercept of the straight line obtained, the values of K_d and Q_{\max} were 4.525 mmol/L and 183.4 μ mol/g respectively.

We also investigated the specific recognition ability of estrone-imprinted MNPs for testosterone propionate which is structural analogue of estrone, under the same conditions. From the amounts of rebinding estrone and testosterone, estrone-imprinted polymer coated MNPs exhibited high selectivity for imprinting molecule estrone compared to the structural analogue testosterone propionate. The controlled MNPs showed low binding values for both estrone and testosterone (Fig. 5B). The adsorption of testosterone is due to unspecific adsorption. The results confirmed clearly the effectiveness of the molecular imprinting because the estrone-imprinted MNPs showed efficiently specific recognition ability to the template estrone in comparison with structural analogue testosterone propionate.

4. Conclusions

In conclusion, we explored synthesis of estrone-imprinted polymer coated Fe_3O_4 magnetic nanoparticles that exhibit a much higher specific recognition and saturation magnetization. This work provides a platform to prepare molecularly imprinted polymer modified magnetic nanoparticles with high affinity, selectivity and capacity to nearly any target molecules. We believe that the imprinted polymer coating magnetic nanoparticles can be one of the most promising candidates for various applications, including chemical and biochemical separation, cell sorting, recognition elements in biosensors and drug delivery.

Acknowledgements

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