

# Epitope imprinted polymer nanoparticles containing fluorescent quantum dots for specific recognition of human serum albumin

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Received: 20 November 2014 / Accepted: 18 February 2015 / Published online: 18 March 2015  
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**Abstract** Epitope imprinted polymer nanoparticles (EI-NPs) were prepared by one-pot polymerization of N-isopropylacrylamide in the presence of CdTe quantum dots and an epitope (consisting of amino acids 598 to 609) of human serum albumin (HSA). The resulting EI-NPs exhibit specific recognition ability and enable direct fluorescence quantification of HSA based on a fluorescence turn-on mode. The polymer was characterized by FT-IR, X-ray photoelectron spectroscopy, transmission electron microscopy and dynamic light scattering. The linear calibration graph was obtained in the range of 0.25–5  $\mu\text{mol}\cdot\text{mL}^{-1}$  with the detection limit of 44.3  $\text{nmol}\cdot\text{mL}^{-1}$ . The EI-NPs were successfully applied to the direct fluorometric quantification of HSA in samples of human serum. Overall, this approach provides a promising tool to design functional fluorescent materials with protein recognition capability and specific applications in proteomics.

**Keywords** Epitope imprinting · Human serum albumin · Quantum dots · Turn-on fluorescence · Fluorometry

**Electronic supplementary material** The online version of this article (doi:10.1007/s00604-015-1464-1) contains supplementary material, which is available to authorized users.

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## Introduction

Molecular imprinting technique has a history of more than 40 years and has an attractive method of artificial recognition [1, 2]. Molecularly imprinted polymers are capable of specific recognition based on the characteristics of the template molecule [3, 4]. A molecularly imprinted polymer mimics a biomolecular recognition mechanism and represent most promising materials for their high stability, ease of preparation and low cost [5]. The imprinting of small molecules [6, 7] has been successfully established, but the imprinting of proteins [8, 9] is still in development. The methods of protein imprinting can be classified into three main approaches: bulk imprinting, surface imprinting and epitope imprinting [10]. Among them, epitope imprinting becomes a new method for identification and separation of target molecule because of its simple preparation and low cost properties.

Epitope, at N or C-terminus of proteins, is one short part of the polypeptide chain. This short polypeptide rather than the entire protein was used as template to imprint and form imprinted polymer which can not only recognize the short polypeptide but also identify the corresponding protein. The advantages of epitope imprinting are as follows [10]: (1) Epitope as a template can avoid the difficulties from imprinting such as protein folding. Moreover, comparing to protein imprinting, the imprinted polymer increases the specific recognition ability to the template with the enhanced interaction force and reduces the adsorption of non-specific recognition. (2) Epitope imprinted polymer can be synthesized in organic phase because many peptides are stable in organic phase but proteins are not. (3) It is easy and cheap to achieve the template of short polypeptide compared with the corresponding proteins for some proteins.

Currently, there are reports on molecularly imprinted polymers coated fluorescent quantum dots [11, 12]. Quantum dots

have raised the attention as biosensor and identification element because of their properties such as high photoluminescence efficiency, strong resistance to photobleaching and emission wavelength tunable [13, 14]. Researchers have done some works about fluorescent molecularly imprinted polymer to detect the target proteins. For examples, Lin et al. [15] synthesized poly (ethylene-co-ethylene alcohol) creatinine-, albumin- and lysozyme-imprinted polymers nanoparticles via phase inversion when target molecules and hydrophobic quantum dots were mixed within the polymer solution. Tan et al. [16, 17] imprinted Mn-doped ZnS quantum dots for specific recognition of bovine hemoglobin. Our group developed CdTe [18] or modified CdTe [19] quantum dots coated molecularly imprinted polymer for recognition of the template proteins. Recently, our group prepared an epitope molecularly imprinted polymer where polymerization was performed on the surface of silica nanospheres embedded CdTe quantum dots for specific recognition and direct fluorescent quantification of the target protein bovine serum albumin [20].

In this study, epitope imprinted polymer nanoparticles (EI-NPs) were prepared by one-pot polymerization of N-isopropylacrylamide in the presence of CdTe quantum dots and an epitope of HSA. HSA of adults is created from liver and it contains 609 amino acids with a molecular weight of 66,000–68,000 Da. C-terminus dodecapeptides (consisting of amino acids 598 to 609) of HSA was selected as epitope. HSA has its prominent physiological functions. HSA extracted from human plasma can be used for blood volume reduction caused by a number of acute traumas, imbalance of water, electrolyte and colloid osmotic pressure [21, 22]. Many investigations about detection or separation of HSA have been reported [23–28], but a method including detection and separation of HSA is necessary. The objective of this work is to develop a new kind of fluorescent affinity material combining the merits of epitope molecular imprinting technique and fluorescence property of quantum dots to target protein HSA. With the high selectivity of epitope molecular imprinting technique and sensitivity of quantum dots, the EI-NPs can be applied in separation and direct fluorescence quantification of HSA from human serum sample.

## Experimental

### Materials

Chemical reagents and solvents used in these experiments were all analytical grade. Tellurium powder,  $\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$  and  $\text{NaBH}_4$  (purchased from Acros Organics, <http://www.inno-chem.com.cn/acros/>, New Jersey, USA) were used for the synthesis of CdTe quantum dots. 3-mercaptopropanoic acid (MPA, from Alfa Aesar, <http://www.alfachina.cn/>,

Tianjin, China) was used as stabilizer. Human serum albumin (HSA,  $\text{pI}=4.6$ ,  $\text{MW}=66.0$  kDa), lysozyme (Lys,  $\text{pI}=11.2$ ,  $\text{MW}=13.4$  kDa) and ovalbumin (OB,  $\text{MW}=45.0$  kDa,  $\text{pI}=4.7$ ) were purchased from Solarbio (<http://www.solarbio.cn/>, Beijing, China). Potassium persulfate (KPS), sodium dodecyl sulfate (SDS) were purchased from the Institute of Tianjin GuangFu Fine Chemicals (<http://guangfu.testmart.cn/>, Tianjin, China). N-isopropylacrylamide (NIPAM) was purchased from Acros Organics (<http://www.inno-chem.com.cn/acros/>, New Jersey, USA). N,N'-methylene bisacrylamide (MBA) was purchased from Sangon Biotech (<http://www.sangon.com/>, Shanghai, China). All peptides used in the experiments were synthesized by GL Biochem (<http://www.glschina.com/>, Shanghai, China).

### Preparation of CdTe quantum dots

According to the previous work [29], some modification had been made for the synthesis of water-soluble CdTe quantum dots. Manipulations in details are shown as follows: 0.363 g borohydride ( $\text{NaBH}_4$ ) was mixed with 0.0636 g tellurium powder. Tellurium powder was reduced by  $\text{NaBH}_4$  in water with magnetic stirring under the protection of nitrogen. Then, precursor NaHTe solution was achieved. Subsequently, a certain volume of fresh NaHTe solution was added to nitrogen-saturated mixture solution of  $\text{CdCl}_2$  and 3-mercaptopropanoic acid (MPA), which had been adjusted to  $\text{pH}=10$  with NaOH. The mixture was heated under a nitrogen environment at  $100^\circ\text{C}$  for 2.5 h. In the reaction system, the molar ratio of  $\text{CdCl}_2$  : MPA : NaHTe was 2 : 4.8 : 1.

### Preparation of the EI-NPs

30 ml of the mentioned CdTe quantum dots solution, 310 mg of N-isopropylacrylamide (NIPAM), 34 mg of N,N'-methylene bisacrylamide (MBA) and 10 mg of epitope template were added in a 100 ml three-neck flask. The template peptides were dissolved in 5 mL solution containing acetonitrile and deionized water with the volume ratio of 1:4. The flask was also installed with a reflux condenser, a magnetic stirrer and a nitrogen inlet tube. In order to deaerate oxygen, nitrogen was passed through for at least 30 min before the reaction began. When the temperature of water bath raised to  $70^\circ\text{C}$ , 35 mg potassium persulfate was added to initiate polymerization for 3 h. After completion of the reaction, the system was cooled down to room temperature. The product was centrifuged at high speed and washed with deionized water in order to remove excess CdTe quantum dots and other polymerized monomers. The achieved EI-NPs were washed by the eluant containing acetonitrile, methanol and deionized water with the volume ratio of 1:1:3 and deionized water, respectively, until no template was detected by HPLC. The preparation of non-

imprinted polymer nanoparticles (NI-NPs) was the same as above but without adding the template.

### Fluorescence analysis

A certain quantity of EI-NPs was mixed with a volume of protein solution. The mixture solution was placed on a rotary shaker for reaction in deionized water at 20 °C. Test the fluorescence intensity of the EI-NPs in the absence and presence of HSA, respectively. The imprinting factor was calculated by the correlation of the fluorescence changes and the concentration of HSA.

### Application to real samples analysis

In this experiment, human serum sample was used to demonstrate the applicability of the EI-NPs for recognition and quantitative fluorescence detection of HSA. The sample was provided by healthy volunteers.

The human serum sample was diluted 250-fold with deionized water and then the EI-NPs were added. After nonspecific binding proteins were removed, 10 % SDS was employed to elute the specifically adsorbed HSA. Finally, 10  $\mu\text{L}$  of each sample was used for SDS-PAGE analysis.

The human serum sample was diluted 1000-fold with deionized water and then was used to assess the quantitative fluorescence detection of HSA. The recovery was carried out by the standard addition method with  $5.0 \times 10^{-7} \text{ mol} \cdot \text{L}^{-1}$  to  $15.0 \times 10^{-7} \text{ mol} \cdot \text{L}^{-1}$  HSA.

## Results and discussion

### Selection of the epitope (template peptide)

Selection of the epitope is very important particularly when epitope plays as the template in the process of molecularly imprinted polymer preparation. Nishino et al. [30] chose exposed C-terminus nonapeptides of the proteins as the templates whose sequences and structures are known to prepare molecularly imprinted polymers. A certain length of peptides from exposed C-terminus of proteins were selected as the epitopes because they are less frequent target for post-translational modification, and they can represent a near-unique code for the identification of a specific protein [30]. The length of peptides is considered as an important parameter for specific identification: too short peptides are unable to determine a specific protein, too long peptides have complex structures that could impair the imprinting and the rebinding process [31].

According to the above criterion and considering the length and hydrophilicity of peptides, we chose three peptides from exposed C-terminus of HSA as the templates, whose

sequences [32–34] and 3D structures [35–37] (Fig. S1, Electronic Supplementary Material, ESM) are known: amino acids 601–609 AASQAALGL (nonapeptides), 598–609 KLVAASQAALGL (dodecapeptides), 596–609 EGKKLVAASQAALGL (pentadecapeptides). Each peptide was chemically synthesized. We prepared molecularly imprinted polymers respectively (ESM 2.1 & 2.2) and investigated the fluorescence intensity changes after they were interacted with target protein. Additionally, the imprinting factor was used to evaluate the selectivity of the EI-NPs. The imprinting factor was calculated from the following equation:

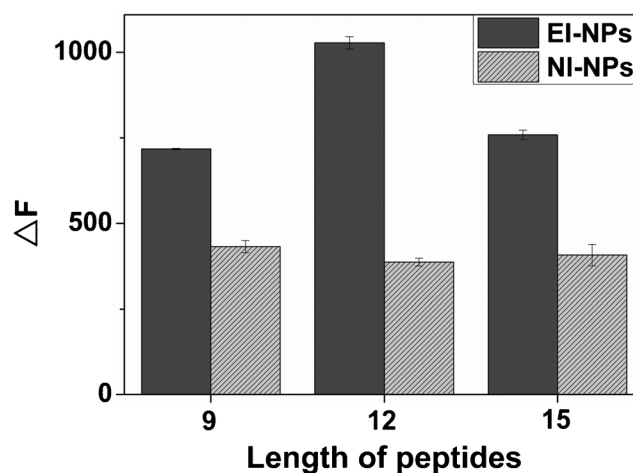
$$\text{Imprinting factor} = \Delta F_{(\text{EI-NPs}_s)} / \Delta F_{(\text{NI-NPs}_s)}$$

where  $\Delta F = F - F_0$ , and  $F$  is the fluorescence intensity in the presence of HSA,  $F_0$  is the initial fluorescence intensity in the absence of HSA. The experimental results were shown in Fig. 1, and the imprinting factor for nonapeptides, dodecapeptides and pentadecapeptides is 1.66, 2.65 and 1.86, respectively. These results indicated that the selectivity of dodecapeptides was higher than that of the others. So the dodecapeptides was selected as epitope of HSA.

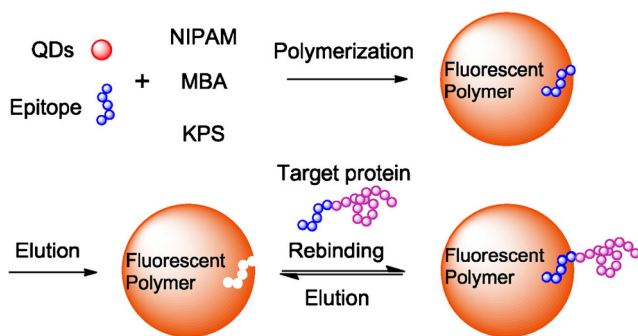
### Synthesis of the EI-NPs

The general method for the synthesis of the epitope molecularly imprinted material is illustrated in Fig. 2. In this protocol, polymerization was initiated with potassium persulfate and the molecularly imprinted nanospheres were prepared by one-pot method. It can be seen from Fig. 2 that the process of polymerization is simple. After removal of the template molecule, the material can be used for selective identification of the target protein.

The fluorescence stabilities of the EI-NPs in the absence and presence of HSA were studied (Fig. S2, ESM). The



**Fig. 1** Fluorescence enhancement and imprinting factor of EI-NPs with different length peptides as the template



**Fig. 2** Scheme for the preparation of the EI-NPs

reaction of EI-NPs with HSA reached equilibrium after 20 min, and we measured fluorescence at 30 min in experimental section of the fluorescence analysis. The results indicated that the imprinted material can remain stable for a long time in water, which guarantees the choices of the experimental conditions and stability of the experimental results.

### Characterization of the EI-NPs

FT-IR spectra of quantum dots, monomer NIPAM, EI-NPs and NI-NPs were shown in Fig. S3 (ESM). As shown in Fig. S3, the peak around  $1646\text{ cm}^{-1}$  was ascribed to the carbonyl of amide,  $1542\text{ cm}^{-1}$  was ascribed to the H from the amino of amide and the peak around  $2971\text{ cm}^{-1}$  was ascribed to the C-H stretching band. FT-IR spectra provided a direct proof for the experiment that the synthetic process of EI-NPs was successful and all raw materials were co-polymerized in the imprinted material.

X-ray photoelectron spectroscopy (XPS) was used to characterize the synthesized elements of the imprinted material. Before characterization, the product was purified three times to remove the unreacted monomers and excess quantum dots. As can be seen in Fig. S4 (ESM), the XPS survey showed the existence of CdTe quantum dots as element Cd appeared in the peak Cd  $3d_{5/2}$  at  $404.7\text{ eV}$  and Cd  $3d_{3/2}$  at  $411.4\text{ eV}$  and element Te appeared in the peak Te  $3d_{5/2}$  at  $571.8\text{ eV}$  and Te  $3d_{3/2}$  at  $582.2\text{ eV}$ . The peak of element N  $1s$  at  $399.4\text{ eV}$  proved the presence of product poly (N-isopropylacrylamide) (pNIPAM). Therefore, the analysis result of XPS demonstrates that

quantum dots were successfully coated in the pNIPAM microspheres in the polymerization process.

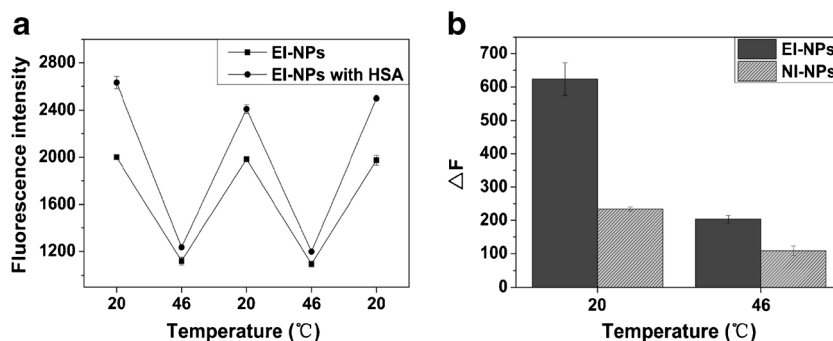
The shapes of EI-NPs and NI-NPs were examined by transmission electron microscopy (TEM). It can be seen from the Fig. S5 (ESM) that there is no significant difference in the particle diameter between EI-NPs and NI-NPs. The average particle size of the nanoparticles was about 300 nm. From this, it can be predicted for later experiment that the difference of recognition performance is from the effect of imprinting. It can be seen much clearly from the enlarged picture that CdTe quantum dots were distributed in the pNIPAM nanoparticles because the dark spots inside were the electron density contrast by the CdTe quantum dots.

Dynamic light scattering (DLS) was employed to determine the hydrodynamic radii ( $R_h$ ) of EI-NPs and NI-NPs at  $20\text{ }^\circ\text{C}$ . The DLS results (Fig. S6, ESM) showed that the mean hydrodynamic radii of EI-NPs and NI-NPs were nearly the same, about 240 nm. The sizes of the microspheres determined by DLS are somewhat bigger than those measured by TEM, which is because the nanoparticles on the TEM grid were induced by the dehydration of the pNIPAM during the evaporation of water.

### The thermo-sensitive properties of the EI-NPs

Poly (N-isopropylacrylamide) (pNIPAM) hydrogel is a well-known thermo-sensitive polymer, so thermo-sensitive property of the imprinted materials was also investigated. It shows in Fig. 3a that fluorescence intensity of the EI-NPs alone and EI-NPs with HSA at 20 and  $46\text{ }^\circ\text{C}$  changed regularly, but the fluorescence intensity of the raw quantum dots didn't change obviously (Fig. S7, ESM), which demonstrated the thermo-sensitive property of the imprinted material. Figure 3b shows the fluorescence response of the EI-NPs combined with HSA at different temperatures. As it is shown in the Fig. 3b, there are obviously different fluorescence changes of the EI-NPs with HSA under different temperatures. These results showed that the recognition ability of the EI-NPs could be adjusted by the temperature change.

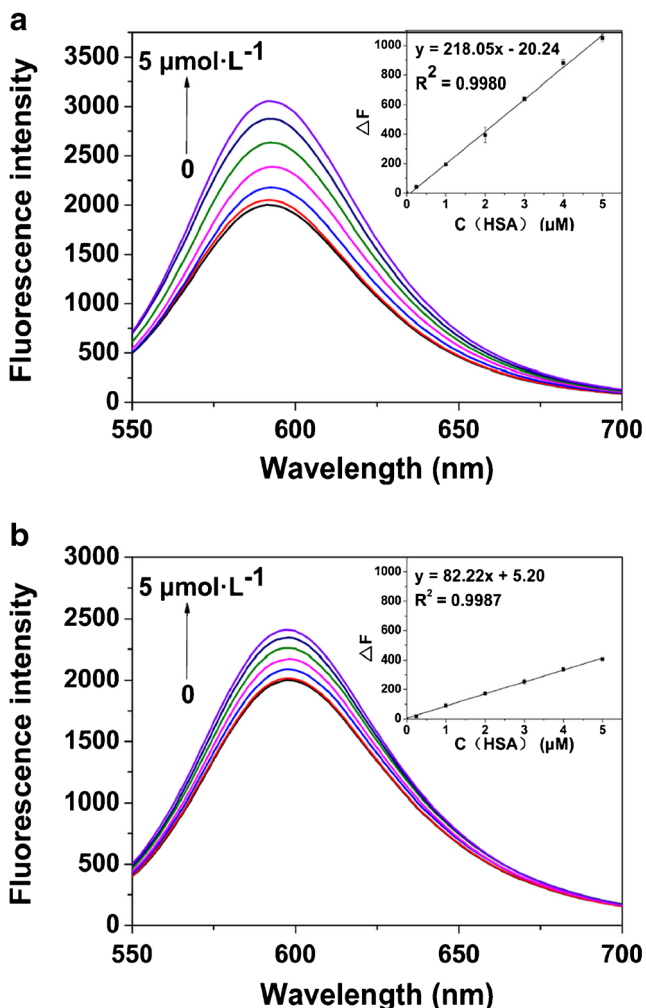
**Fig. 3** The fluorescence intensity of the EI-NPs without and with HSA (a) and fluorescence enhancements of EI-NPs and NI-NPs with HSA (b) at a temperature swing of 20 and  $46\text{ }^\circ\text{C}$



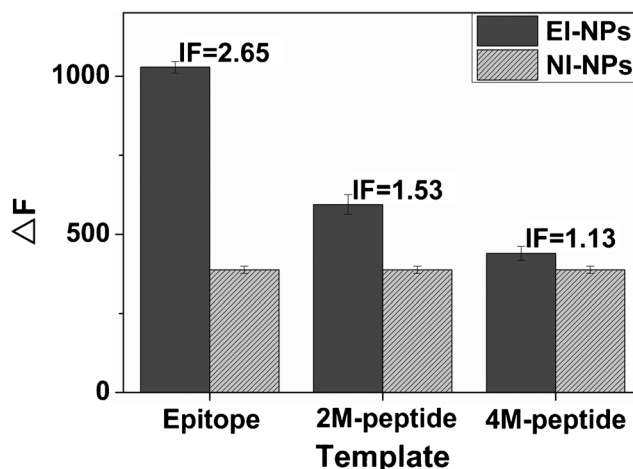
Fluorescence analysis of the target protein with the EI-NPs

The specific recognition ability of the imprinted materials was examined by comparing the fluorescence response between the EI-NPs and NI-NPs with the target protein. From Fig. 4, it can be seen that the fluorescence intensity of the imprinted materials gradually increased with the increasing concentration of HSA. For the EI-NPs, the imprinted site matches with the terminus of the target protein molecule, and consequently the fluorescence intensity increases more.

The fluorescence intensity of the EI-NPs increased probably because that HSA bonds directly to the surface of CdTe quantum dots by complexing between the hydrosulphonyl of cysteine residue and the surface of CdTe quantum dots, resulting in passivating the surface defects [38–40]. We studied the interaction of HSA and CdTe quantum dots (Fig. S8, ESM). It can be seen from the Fig. S8, within a certain range,



**Fig. 4** Fluorescence emission spectra of EI-NPs (a) and NI-NPs (b) with the addition of the indicated concentration of HSA. Insets were fluorescence enhancement of EI-NPs (a) and NI-NPs (b) with the addition of the indicated concentration of HSA

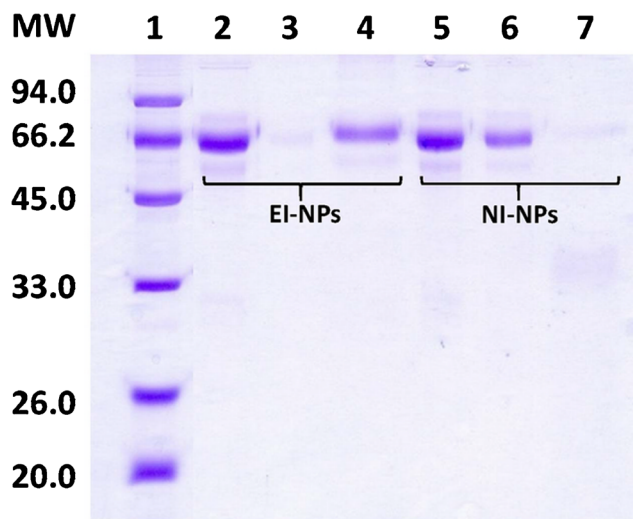


**Fig. 5** Effects of the epitope and the mutated sequence as templates on the imprinting factor of the imprinted polymers

the fluorescence intensity of the CdTe quantum dots is gradually increased as the protein concentration increases.

By comparing Fig. 4a and b, it can be seen that the impact of target protein on EI-NPs is significantly stronger than that of NI-NPs. The ratio of  $K_{EI-NPs}$  and  $K_{NI-NPs}$  is also able to calculate the imprinting factor.  $K_{EI-NPs}$  and  $K_{NI-NPs}$  are the slope of the line in insets of Fig. 4a and b, respectively.

Within the concentration range of  $2.5 \times 10^{-7} \sim 5 \times 10^{-6} \text{ mol} \cdot \text{L}^{-1}$ , the fluorescence increasing degree of the EI-NPs has a linear relationship with the concentration of target molecule HSA. The correlation coefficient was 0.998. The detection limit of the method was  $44.3 \text{ nmol} \cdot \text{L}^{-1}$ . The precision was 1.6 % (relative standard deviation), averaged over three measurements of  $3 \text{ } \mu\text{mol} \cdot \text{L}^{-1}$  HSA.



**Fig. 6** SDS-PAGE analysis of HSA by the EI-NPs from human serum. Lane 1, protein molecular weight marker; lane 2, 250-fold dilution of human serum; lane 3, remaining human serum after adsorption by the EI-NPs; lane 4, the eluent from the EI-NPs; lane 5, 250-fold dilution of human serum; lane 6, remaining human serum after adsorption by the NI-NPs; lane 7, the eluent from the NI-NPs. The injected amount of protein mixture:  $10 \text{ } \mu\text{L}$

**Table 1** Results for the determination of the HSA in human serum (1000-fold dilution)

Samples	HSA concentration ( $\times 10^{-7} \text{ mol} \cdot \text{L}^{-1}$ )		
	measured (mean $\pm$ SD, $n=3$ )		
	spiked	mean $\pm$ SD	recovery <sup>a</sup> (%)
human serum	0.0	7.31 $\pm$ 0.06	
	5.0	12.20 $\pm$ 0.34	97.88 $\pm$ 6.88
	10.0	17.30 $\pm$ 0.23	99.87 $\pm$ 2.26
	15.0	22.49 $\pm$ 0.11	101.19 $\pm$ 0.75

<sup>a</sup> Recovery (%) = [(total level of detected - level of endogenous)/level of spiked]  $\times$  100; The data were expressed as the mean  $\pm$  SD determined from triplicate independent experiments

### Specificity study

In order to investigate the specific identification ability of the imprinted materials, a series of protein mixed solution (lysozyme-HSA and ovalbumin-HSA) were prepared. Fig. S9a and Fig. S9b (ESM) show that, with the increasing ratio of  $C_{\text{lysozyme}} / C_{\text{HSA}}$  or  $C_{\text{ovalbumin}} / C_{\text{HSA}}$ , the fluorescence intensity of the EI-NPs was little affected. These phenomena confirmed that the imprinted sites of epitopes were formed in the process of imprinting and could specifically respond to the target protein.

### Effect of the mutated sequence of the template epitope

In order to study the effect of mutated sequence, two amino acid mutated (underlined) analogue (2 M-peptide, KG-VAASQAALLL, MW=1141.37 Da, PI=9.69) and four amino acid mutated (underlined) analogue (4 M-peptide, KLAF-ASQVFLGL, MW=1293.56 Da, PI=9.69) were synthesized and used as the control peptides [30]. We used epitope

peptide, 2 M-peptide and 4 M-peptide as the template to prepare the imprinted polymers, respectively, and tested the fluorescence response of the imprinted polymers to calculate the imprinting factor. It can be seen from Fig. 5 that the imprinting factors of the imprinted polymer nanoparticles with 2 M-peptide and 4 M-peptide were much lower than that of with the epitope peptide. The high imprinting factor of the EI-NPs with the epitope peptide confirmed the success of the epitope imprinting.

### Application to real sample analysis

The practical application of the EI-NPs was investigated by examining the separation and enrichment of HSA in 250-fold dilution human serum sample by SDS-PAGE gel electrophoresis. The experimental result was shown in Fig. 6. The content of HSA in the sample decreased dramatically after the actual sample was adsorbed by the EI-NPs. HSA strip appeared again in the eluent after washing the imprinted material with appropriate elution, which indicates the good separation capacity of the EI-NPs to the corresponding target protein molecule.

We applied this fluorescent material to determine the concentration of HSA in the actual samples and obtained a satisfied recovery by adding HSA to 1000-fold dilution human serum (Table 1). It can be seen that the recoveries were from 97.88 to 101.19 %, which indicate that this method was suitable for the determination of actual samples.

Many studies about the determination of HSA have been reported [23–26, 41], and different analytical methods for determination of HSA are summarized briefly in Table 2. Although some of the methods have high sensitivity, the method using epitope imprinting technique has high selectivity to interference proteins, and also can separate and enrich the target proteins.

**Table 2** Comparable methods for the determination of HSA

Methods/materials used	Analytical ranges	Samples	Advantages and disadvantages	References
Enzyme immunoassay	0.003–1 $\mu\text{g} \cdot \text{mL}^{-1}$	human urine	sensitive, but high cost	[23]
Quartz crystal microbalance	0.1–100 $\mu\text{g} \cdot \text{mL}^{-1}$	human urine	sensitive, but complex preparation	[24]
Eriochrome Cyanine R	0.5–12 $\mu\text{g} \cdot \text{mL}^{-1}$	human serum	Easy preparation, but can't separate different proteins	[25]
Constant-energy synchronous fluorescence technique	0.1–220 $\mu\text{g} \cdot \text{mL}^{-1}$	human serum and urine	no reagent was used, but can't separate different proteins	[26]
Fluorescent molecularly imprinted polymer thin films	0.5–2.5 $\mu\text{mol} \cdot \text{L}^{-1}$	not mentioned	high selectivity and separation ability, but complex preparation	[41]
EI-NPs containing quantum dots	0.25–5 $\mu\text{mol} \cdot \text{L}^{-1}$	human serum	high selectivity and separation ability, but washing template needs longer time	this study

\*1  $\mu\text{g} \cdot \text{mL}^{-1} \approx 0.015 \mu\text{mol} \cdot \text{L}^{-1}$ , 1  $\text{ng} \cdot \text{mL}^{-1} \approx 0.015 \text{nmol} \cdot \text{L}^{-1}$

## Conclusions

In this study, epitope imprinted polymer nanoparticles (EI-NPs) were prepared by one-pot polymerization of N-isopropylacrylamide in the presence of CdTe quantum dots. The synthesized EI-NPs using C-terminus dodecapeptides (consisting of amino acids 598 to 609) of HSA as the template could specifically recognize the target protein HSA. The fluorescence increasing degree of the EI-NPs had a linear relationship ( $R^2=0.998$ ) in a concentration range of HSA from  $2.5 \times 10^{-7} \text{ mol} \cdot \text{L}^{-1}$  to  $5 \times 10^{-6} \text{ mol} \cdot \text{L}^{-1}$ , and the detection limit was  $44.3 \text{ nmol} \cdot \text{L}^{-1}$ . The EI-NPs showed a good recovery of HSA from human serum (97 to 101 %). The EI-NPs were expected to be a good chemical probe in the selective recognition and fluorescence quantification of HSA in proteomics. Also this method is simple and low cost, which demonstrated its potential in practical applications.

**Acknowledgments** This work was supported by the National Basic Research Program of China (973 Program) (Nos. 2011CB707703 and 2012CB910601) and the National Natural Science Foundation of China (Nos. 21275078 and 21475069).

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