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Magnetic molecularly imprinted nanoparticles for recognition of lysozyme

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ABSTRACT

Molecular imprinting is an attractive technique for preparing mimics of natural, biological receptors. Nevertheless, the imprinting of macromolecule remains a challenge due to their bulkiness and sensitivity to denaturation. In this work, we presented a method for preparing multifunctional lysozyme-imprinted nanoparticles (magnetic susceptibility, molecular recognition and environmental response). The magnetic susceptibility was imparted through the successful encapsulation of Fe_3O_4 nanoparticles. Selective lysozyme recognition depended on molecularly imprinted film. Moreover, it was also a hydrophilic stimuli-responsive polymer, which could undergo a reversible change of imprinted cavity in response to a small change in the environmental conditions. Thus, magnetic molecularly imprinted nanoparticles had high adsorption capacity (0.11 mg mg^{-1}), controlled selectivity and direct magnetic separation (22.1 emu g^{-1}) in crude samples. After preconcentration and purification with magnetic MIPs nanoparticles, a sensitive chemiluminescence method was developed for determination of lysozyme in human serum samples. The results indicated that the spiked recoveries were changed from 92.5 to 113.7%, and the RSD was lower than 11.8%.

1. Introduction

Molecular imprinting is a state-of-the-art technique for preparing mimics of natural, biological receptors. For the past few years, the interest and attention shown towards this field have been increased at an amazing pace, because of significant advantages of molecularly imprinted polymers (MIPs), such as predictable specific recognition, low cost, easy preparation, and high mechanical/chemical stability (Tan et al., 2008). Although MIPs have been successfully developed against a wide range of small molecules, the imprinting of macromolecules like protein has proven to be more difficult. The major problems are entrapment of macromolecular templates in polymers, unfavorable kinetics of the adsorption and desorption process, the production of heterogeneous sites and the sensitivity of biomacromolecule to denaturation (Bereli et al., 2008; Ouyang et al., 2008; Jin and Tang, 2009; Flavin and Resmini, 2009). To resolve these problems, surface polymerization has been proposed to prepare the MIPs film on a solid support substrate, which can improve mass transfer and reduce permanent entrapment of the template, but these methods also reduce the number of imprinting sites (Boninia et al., 2007; Wang et al., 2009a,b; Zhai et al., 2009).

Compared with conventional solid support substrate, magnetic nanoparticles (MNPs) have many superior characteristics such as small size, high surface-to-volume ratio, fast and effective binding to biomolecules and high magnetic susceptibility. It has attracted increasing attention in the field of biomedical and biotechnological applications, which include magnetic bioseparation and detection of biological entities, clinic diagnosis and therapy, targeted drug delivery and biological labels (Gupta and Gupta, 2005; Gu et al., 2006; Gao et al., 2009). When MNPs are coated with MIPs, these MIPs-coated MNPs can not only selectively recognize the analytes in the complex matrix, but also have more imprinting sites because of high surface-to-volume ratio of MNPs, which can improve the insufficiency of surface polymerization (Li et al., 2009a,b; Wang et al., 2009a,b). Meanwhile, the magnetic separation process can be performed directly in crude samples, which is especially useful for large-scale operation (Kan et al., 2009; Zhang et al., 2009; Lu et al., 2009). Therefore, combining magnetic separation and molecular imprinting would ideally provide a powerful analytical tool towards bioseparation application.

Nowadays, although some magnetic molecularly imprinted nanoparticles have been reported in the literatures (Tan et al., 2008; Zhai et al., 2009; Li et al., 2009a,b), the imprinted film is a rigid polymer with low flexibility. Smart hydrogels are stimuli-responsive materials, which can undergo a reversible volume change in response to a small change in the environmental conditions (Hayden et al., 2006; Hua et al., 2008, 2009). If this property can be introduced into molecularly imprinted film, not only the

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MIPs film can selectively recognize the template protein, but also this memory as well as the affinity to the target molecules can be controlled by the external stimulus, which plays an important role in the bioseparation.

Lysozyme (N-acetylmuramide glyconohydrolase) is considered as a self-defense enzyme, which is produced in serum, mucus, and many organs of vertebrates. Its common applications are as an important index in the diagnosis of various diseases including renal disease and leukemia. It also can be used as a cell disrupting agent in ophthalmologic preparations, as a food additive in milk products and as a drug for the treatment of ulcer and infections (Guo et al., 2007; Zheng et al., 2007). Furthermore, it has been demonstrated by animal experiment and in vitro cell chemotherapy that lysozyme can be used as a drug in the treatment of HIV infection (Siddhartha and Rein, 2009; Heriazon et al., 2009). Thus, lysozyme separation and purification from complex matrix is of great importance.

To the best of our knowledge, this article was the first attempt to synthesize the magnetic molecularly imprinted nanoparticles for recognition of lysozyme. The Fe_3O_4 nanoparticles were synthesized by the coprecipitation method and then coated with a thin MIPs film. This film was also the hydrophilic stimuli-responsive material, which was obtained using lysozyme as a template, methacrylic acid and acrylamide as functional co-monomers, and N,N′-methylenebisacrylamide as a cross-linker. The characterization, adsorption capacity and selectivity of these nanoparticles were investigated. And the response of the resultant nanoparticles to salt concentration was also studied. After preconcentration and purification with magnetic MIPs nanoparticles, a sensitive chemiluminescence (CL) method was developed for determination of lysozyme in human serum samples.

2. Materials and methods

2.1. Reagents and chemicals

Lysozyme (MW 14.4 kDa, pl 10.8) was used as the template and cytochrome *c* (MW 12.4 kDa, pl 10.2), ribonuclease A from bovine pancreas (MW 12.6 kDa, pl 7.8), and bovine serum albumin (MW 68 kDa, pl 4.9) were used as control proteins. The proteins, N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium persulfate (APS), N,N′-methylenebisacrylamide (MBAAm), tetraethyl orthosilicate (TEOS), methacrylic acid (MAA), γ-methacryloxypropyltrimethoxysilane (MPS) and acrylamide (AAm) were obtained from Sigma (St. Louis, MO, USA). Iron (II) chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$), iron (III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), cetyltrimethylammonium bromide (CTMAB), anhydrous ethanol, sodium citrate, luminol, and potassium ferricyanide were purchased from KeMiOu Chemical Reagent Company (Tianjin, China). Ultrapure water was obtained from a Milli-R04 purification system (Millipore, Germany). Serum samples were obtained from the authors and this study was approved by the Ethics Committee of Tongji Medical College.

2.2. Preparation of Fe_3O_4@SiO_2@MIPs(NIPs)

Fig. 1 illustrates the preparation method of Fe_3O_4@SiO_2@MIPs. The Fe_3O_4 nanoparticles were synthesized using the coprecipitation method. The resulting nanoparticles were coated with a thin SiO_2 film (Fe_3O_4@SiO_2). The experimental procedures were described in Supplementary data. Subsequently, modification of double bond can assure the tight growth of MIPs film on the surface of Fe_3O_4@SiO_2 nanoparticles. MPS (150 μL) were dispersed in 40 mL ultrapure water containing 10% acetic acid through mechanical stirring (700 rpm) for 5 h. Then, this solution and Fe_3O_4@SiO_2 nanoparticles (250 mg) were incubated at 60 °C for 5 h. The resulting product was obtained by magnetic separation and then washed with ultrapure water to remove unreacted chemicals.

For the coating of lysozyme-imprinted film, lysozyme (30 mg), MAA (36 mg), and AAm (564 mg) were dissolved in 10 mL of phosphate buffer (0.2 mol L^{-1}, pH 6.2) through mechanical stirring (100 rpm). Then, the modified nanoparticles (200 mg), MBAAm (100 mg), APS (10 mg), and TEMED (10 μL) were successively added to the mixture. Subsequently, the solution was deoxygenated by purging with nitrogen for 5 min and incubated at room temperature for 10 h. Finally, the Fe_3O_4@SiO_2@MIPs were washed with 100 mL NaCl solution (1 mol L^{-1}) and ultrapure water for five times to remove the entrapped template molecule, respectively. The complete removal of lysozyme from the MIPs was confirmed by a Shimadzu UV-2550 scan UV/vis spectrophotometer at 290 nm detection wavelength. The control nanoparticles (Fe_3O_4@SiO_2@NIPs) were prepared using the same procedure but without template lysozyme.

2.3. Characterization

The size and morphology of the Fe_3O_4@SiO_2@MIPs were measured using a FEI (United States) Tecnai G220 TEM instrument. The nanoparticles dispersed in anhydrous ethanol were cast onto a carbon-coated copper grid sample holder followed by evaporation at room temperature.

The particle size distributions of the Fe_3O_4@SiO_2 and Fe_3O_4@SiO_2@MIPs was performed on Delsa™ Nano particle analysis instrument (Beckman coulter, CA, USA).

Fourier transform infrared (FT-IR) spectra of the nanoparticles were recorded in the range 400–4000 cm^{-1} using a Bruker VERTEX 70 spectrometer (Germany) with KBr pellet technique.

The identification of crystalline phase of the synthesized nanoparticles was performed using an X-ray diffractometer (PANalytical BV Company x'Pert PRO) with monochromatized with Cu K_α radiation over the 2θ range of 20–80° at a rate of 4 °C min^{-1}.

Thermogravimetric analysis (TGA) was performed for powder samples (about 10 mg) using a Diamond TG/DTA instruments (PerkinElmer, USA) under a nitrogen atmosphere up to 1000 °C with a heating rate of 20 °C min^{-1}.

Magnetic properties were measured with a LDJ9600-1 (United States) vibrating sample magnetometry (VSM) at room temperature.

2.4. Protein adsorption experiments

Fe_3O_4@SiO_2@MIPs (NIPs) (100 mg) were firstly conditioned with phosphate buffer (0.2 mol L^{-1}, pH 6.2) containing 0.02 mol L^{-1} of NaCl at room temperature for 10 h, and then the equilibrium solution was replaced by 1 mL of the sample buffer solution with different initial concentrations of lysozyme in the range 0–2.0 mg mL^{-1}. The solution was shaken at room temperature for 2 h. The concentration of lysozyme in the supernatant solution was measured using the UV-spectrometer at 290 nm detection wavelength. The experimental data was presented as the adsorption capacity per unit mass (mg) of the nanoparticles, and the adsorption capacity (Q) was calculated from Eq. (1):

$$Q = \frac{(C_0 - C_s) \times V}{m} \quad (1)$$

In Eq. (1), C_0 (mg mL^{-1}) is the initial concentration of protein solution, C_s (mg mL^{-1}) is the protein concentration of the supernatant solution, V (mL) is the volume of the initial solution and m (mg) is the mass of Fe_3O_4@SiO_2@MIPs (NIPs).