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Using thiophilic magnetic beads in purification of antibodies from human serum

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ABSTRACT

The rapid progress of biotechnology, immunology and molecular biology are requiring antibodies with higher purity and stronger activity. Therefore, the development of efficient technology, which is suitable for large-scale purification of antibodies at low cost, becomes much more urgent. In this study, we presented a novel method to isolate immunoglobulin G from human serum based on the utilization of thiophilic magnetic polymer beads. Micron-sized magnetic beads with paramagnetic properties were synthesized by microsuspension polymerization in the presence of modified magnetite. After the thiophilic ligand of 2-mercaptonicotinic acid was modified on the surface, these magnetic beads exhibited a strong specificity towards immunoglobulin G in a salt-independent manner. Then, antibodies could be directly isolated from human serum in batch-wise mode with the assistance of magnetic decantation. The purity of the isolated antibody exceeded 94%. Because isolation was performed in physiological conditions, bioactivity of the antibody was fully preserved (>99%). Prominent advantages of this method, such as strong specificity, rapid processing, mild conditions, conventional equipment and excellent reusability, make this non-chromatographic technology embody great potentialities to isolate the antibodies on a large scale.

1. Introduction

Widespread applications and increasing demands of antibody-based products put considerable pressure on purification methodologies [1–3]. A number of purification techniques such as centrifugal precipitation [4], protein A chromatography [5], ion exchange chromatography [6] and thiophilic chromatography [7] have been developed in recent years. However, disadvantages accompanied with the above conventional methods are not negligible, for example, tedious procedures and pretreatments, adsorbent contamination, ligand leakage and high cost. Thus new non-chromatographic techniques such as membrane filtration, preparative electrophoresis, aqueous two-phase systems, magnetic separation and affinity precipitation have been developed to overcome limitations of the affinity chromatographic method [8–11].

The advent of magnetic affinity separation techniques has opened up a new approach to antibody purification [12–15]. The rapid process of magnetic separation and specific fractionation of affinity chromatography are combined by this technology. This technique has attracted much attention and triggered immense research activities in various areas, including cell isolation, enzyme immobilization, immunoassay, drug targeting, etc. [16–19]. In order to conveniently tailor and design the surface structure of magnetic beads, many beads are prepared with organic polymers for their diversified functional groups. Suitable surface modification is the core technology for the functionalization of these magnetic carriers.

Selecting the appropriate ligand is very important for surface modification because it could endow magnetic beads with abilities to selectively recognize the target product. More than 70% of antibodies belong to immunoglobulin G (IgG). Consequently, conventional bioaffinity of ligands towards antibodies are mainly focused on protein A or protein G for their high specificity [20–22]. However, shortcomings of these bio-ligands, for example, harsh elution conditions, ligand leakage, high cost, difficult to sterilize and poor stability are becoming more and more prominent. Thus some pseudoaffinity ligands (metal chelates, immobilized histidine, thiophilic ligands, etc.) emerged for their high selectivity, reasonable cost, stable structure and convenient immobilization [23–25]. First proposed by Porath and Belew [26], thiophilic ligands have attracted great interest due to their excellent binding capacities and specificity towards IgG in thiophilic adsorption chromatography. More recently, a new type of thiophilic ligand, termed mercaptoheterocyclic ligand, exhibits a high adsorption capability to specifically capture IgG in a salt-independent manner [17]. Therefore, it is extremely promising for use in surface functionalization of magnetic beads.

In this study, we aim to develop a new non-chromatographic method to large-scale purification of antibodies using thiophilic magnetic beads. Micron-sized magnetic beads were prepared via microsuspension polymerization with vinyl acetate and divinylbenzene in the presence of modified magnetite nanoparticles.

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Subsequently, 2-mercaptonicotinic acid was immobilized on the surface of the divinylsulfone-activated magnetic beads. Finally, the specificity of these thiophilic magnetic beads towards IgG was carefully estimated. The recovery of IgG, the process of isolation and reusability of magnetic beads were also investigated.

2. Experimental

2.1. Materials

Divinylsulfone (DVS), 2-mercaptonicotinic acid and hexadecane (HD, 99%) were purchased from Alfa. Vinyl acetate (VAc) and divinylbenzene (DVB) were distilled under reduced pressure to remove the inhibitor and stored at 4 °C prior to use. 2,2-Azobis-(isobutyronitrile) (AIBN) was recrystallized and used as an initiator. Poly(vinylalcohol) (PVA124, 99% hydrolyzed, degree of polymerization: 2,400; Aldrich Chem. Co.), ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), ferrous chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$), ammonium hydroxide ($NH_3{\cdot}H_2O$, 25%, w/v) and oleic acid (OA) were all used as received.

2.2. Synthesis of magnetic beads

The OA-modified magnetic nanoparticles were prepared by the chemical coprecipitation method [27]. The magnetic beads were synthesized by microsuspension polymerization [28]. A typical recipe is described as follows. A mixture of VAc (8.0 g), DVB (2.0 g) and HD (0.6 g) was added to 2.0 g dried OA-modified Fe_3O_4 powder. After ultrasonic dispersion in an ice-cooled bath, AIBN (0.6 g) was added into the mixture and shook for 30 min to form the organic phase. The water phase was prepared with PVA124 solution (1.25%, w/v in relation to water). The above two phases were first mixed together with high-shear treatment at 3000 rpm to form polymerizable microsuspension. Then, the polymerization was initiated by increasing the temperature to 60 °C and proceeded for 24 h under constant stirring at 250 rpm. The resulted magnetic beads (PVAc-DVB) were separated by magnetic decantation and thoroughly washed with deionized water and ethanol to remove excess stabilizer and other impurities.

The prepared magnetic PVAc-DVB beads were converted into PVA-DVB beads by an alcoholysis reaction to obtain a hydroxyl-functionalized surface. A total of 10 g of PVAc-DVB particles were suspended in a 150 ml sodium hydroxide/ethanol (10%, w/w) solution and kept at 80 °C for 10 h. The resulted magnetic PVA-DVB beads were separated by magnetic decantation and repeatedly washed with water until neutral.

2.3. Preparation of thiophilic magnetic adsorbents

The heterocyclic ligand of 2-mercaptonicotinic acid (MNic) was selected as the pseudospecific affinity ligand. First, 1.6 ml divinylsulfone (DVS) and 0.64 g MNic were coupled together by Michael addition reaction in 30 ml NaOH solution (pH 13). Then, after 5 h at room temperature, 2 g of neutralized PVA-DVB beads were added into the above mixture and the pH value was adjusted to 13.1. Then, the reaction proceeded for another 19 h. The resulting thiophilic magnetic adsorbents were separated by magnetic decantation and thoroughly washed with deionized water to remove impurities. When not in use, the thiophilic magnetic adsorbents were kept at 4 °C in a 25 mM sodium phosphate buffer (pH 7.0) containing 0.1% NaN_3.

2.4. Isolation of IgG from human serum with magnetic decantation

The adsorption studies were carried out in batch-wise mode. The human serum samples were obtained from the local blood centre of Quanzhou city (Fujian, China). The serum was separated by centrifugation at 6000 × *g* for 10 min at room temperature and then frozen at −20 °C. The amounts of protein and IgG in the serum were determined to 91.4 mg/ml and 13.6 mg/ml, respectively. Before use, the serum was thawed for 24 h at 4 °C. In a typical adsorption experiment, the thiophilic magnetic adsorbents were suspended in a 20 ml, 25 mM PBS buffer (pH 6.0) at room temperature and incubated for 10 min. Then, the human serum sample was added and incubated for 30 min at 150 rpm. After completion of the adsorption, the supernatants were magnetically separated with the help of a handhold NdFeB magnet. In order to remove unbound proteins, an intensive washing procedure was performed with the adsorption buffer. Finally, the recovery of the adsorbed proteins was achieved by elution with a NaCl aqueous solution. The supernatants of each step were collected.

2.5. Characterization

The infrared spectrometry (FTIR) measurement was performed with a Nicolet Nexus^{TM} FTIR spectrometer at a resolution of 2 cm^{-1}. The spectra were recorded in the range from 4000 to 400 cm^{-1} in KBr pellets. The particle size and surface morphology of the magnetic microspheres were observed by scanning electron microscopy (SEM, JSM-6700F, Japan). Average particle size was determined with a particle size analyzer (MasterSizer 2000, Germany). Magnetization measurements were performed at room temperature in magnetic fields up to 100 kOe using a vibrating sample magnetometer (VSM, Mpms XL-7, Quantum Design). Ligand concentration of the thiophilic magnetic adsorbents using nitrogen stoichiometry was tested by a Vario ELIII Elementar. Magnetite content of the dried samples was measured by thermogravimetric analysis (TGA, Model TGA2050, TA Instruments). X-ray diffraction (XRD) patterns of magnetite particles and magnetic beads were obtained on a D8 Advance 2500 diffractometer made by Bruker.

All samples of corresponding supernatants were analyzed by a HPLC analyzer (Agilent 1100) with a gel column (protein KW-803) purchased from Shodex. An aqueous solution (pH 7.0) containing 50 mM sodium phosphate was selected as the mobile phase. Chromatographic separations were run at room temperature and the adsorbance was monitored at 280 nm. The flow rate was 0.7 ml/min. Components in human serum were assigned according to standard chromatograms of human serum analyzed in the column provided by Shodex (http://www.shodex.com/english/dc010206.html).

By use of the Bradford method [29], the amounts of adsorbed proteins were indirectly determined by mass difference, while that of desorbed proteins were directly tested. The purity of desorbed proteins was assayed by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) under reducing conditions using 12% separating gel and 5% stacking gel. The gel was stained for 30 min with Coomassie Brilliant Blue G-250 (0.25%, w/v) in acetic acid–methanol–water (10:5:85, v/v/v) and destained in ethanol–acetic acid–water (25:10:65, v/v/v). Electrophoresis was run for 4 h at 100 V. The biological activities of isolated IgG were assayed by nephelometry using a reagent kit for IgG (Elikan Biological Technology, Zhejiang, China).

3. Results

3.1. Preparation of magnetic beads with microsuspension method

The magnetic beads were prepared with VAc and DVB by microsuspension polymerization. The morphology of these magnetic beads is shown in Fig. 1. No evident morphology difference between responding particles was found in Fig. 1(A) or (B). All magnetic beads have a spherical structure and the average particle size of