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A novel adsorbent for protein chromatography: Supermacroporous monolithic cryogel embedded with Cu^{2+}-attached sporopollenin particles

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ABSTRACT

The aim of this study is to prepare supermacroporous cryogels embedded with Cu^{2+}-attached sporopollenin particles (Cu^{2+}-ASP) having large surface area for high protein adsorption capacity. Supermacroporous poly(2-hydroxyethyl methacrylate) (PHEMA)-based monolithic cryogel column embedded with Cu^{2+}-ASP was prepared by radical cryo-copolymerization of 2-hydroxyethyl methacrylate (HEMA) with *N*,*N*′-methylene-bis-acrylamide (MBAAm) as cross-linker directly in a plastic syringe for affinity purification of human serum albumin (HSA). Firstly, Cu^{2+} ions were attached to sporopollenin particles (SP), then the supermacroporous PHEMA cryogel with embedded Cu^{2+}-ASP was produced by free radical polymerization using *N*,*N*,*N*′,*N*′-tetramethylene diamine (TEMED) and ammonium persulfate (APS) as initiator/activator pair in an ice bath. Embedded particles (10 mg) in PHEMA-based cryogel column were used in the adsorption/desorption of HSA from aqueous solutions. Optimum conditions of adsorption experiments were performed at pH 8.0 phosphate buffer, with flow rate of 0.5 mL/min, and at 5 °C. The maximum amount of HSA adsorption from aqueous solution was very high (677.4 mg/g SP) with initial concentration 6 mg/mL. It was observed that HSA could be repeatedly adsorbed and desorbed to the embedded Cu^{2+}-ASP in PHEMA cryogel without significant loss of adsorption capacity.

1. Introduction

Albumin has played an important role in the fluid management of acutely ill patients for more than 50 years. Some examples in the United States for licensed albumin therapy such as hypovolemia of shock, burns, hypoalbuminemia, cardiopulmonary bypass, hemodialysis, hyperbilirubinemia, sequestration of protein-rich fluids in acute peritonitis and extensive cellulitis have been summarized elsewhere [1]. Research on albumin separation has attracted considerable attention for its great potential in blood protein manufacture. Human serum albumin is isolated from human plasma by Cohn's method [2]. This method concerns the precipitation of proteins using ethanol with varying pH, ionic strength and temperature. However, this technique, which is the oldest method of industrial fractionation of proteins, is not highly specific and can give partially denaturated proteins [3]. Hence it is important to use cheap and efficient techniques for the removal of albumin. In respect of ease preparation, economy, adsorption capacity and stability, immobilized metal affinity chromatography (IMAC) offers several advantages when compared to other types of affinity chromatography, in particular immunoaffinity chromatography, e.g., ligand stability and protein loading [4–7]. IMAC is a sensitive technique for protein separation that enables distinguishing between proteins differing by only a single histidine residue on the surface [8–10]. Proteins interact mainly through the imidazole group of histidine and, to a lesser extent, the indoyl group of tryptophan and the thiol group of cysteine. Aromatic amino acids and the amino terminals of the peptides also contribute to retention of the protein in IMAC [11]. The low cost of metals and the ability to reuse adsorbents hundreds of times without any detectable loss of metal-chelating properties are the attractive features of metal affinity separation.

Sporopollenin obtained from *Lycopodium clavatum* is a natural biopolymer that has a constant chemical structure and contains only carbon, hydrogen and oxygen. It is highly resistant to both biological decay and chemical attack. It has a high capacity, and occurs naturally as a component of spore walls [12]. Architecturally, spores and pollen membranes have two layers, an inner one known as intine, made of cellulose and polysaccharides, and an outer one (exine) composed of a substance known as sporopollenin. Due to the constant mesh size, ready commercial availability, and constant molecular structure of sporopollenin, it has important

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advantages over synthetic resins [13]. Infra-red and ^{13}C NMR spectroscopic studies on sporopollenin derived from pteridophyta and spermatophyta have shown that sporopollenin has aliphatic, aromatic, hydroxyl, carbonyl/carboxyl and ether functions in various portions in its polymeric structure [14].

Cryogels are known as a novel generation of stationary phases in the separation science [15,16]. Cryogels which have several advantages such as large pores, short diffusion path, low pressure drop, and very short residence time for both adsorption and elution are a very good alternative to purification of biomolecules [17]. However, the adsorption capacity of the cryogels for proteins is low due to the interconnected supermacropores within the matrix (i.e., low surface area) [18,19]. Improving the binding capacity of supermacroporous cryogels has a great importance in a bioseparation process [20,21].

In this study, a novel continuous supermacroporous monolithic cryogel embedded with Cu^{2+}-ASP having large surface area for high protein adsorption capacity was prepared under the freezing-temperature. Pore structure and morphology of the embedded particles in the cryogel were studied by scanning electron microscopy (SEM).

2. Methods and materials

2.1. Materials

Hydroxyethyl methacrylate (HEMA) was obtained from Fluka A.G. (Buchs, Switzerland), distilled under reduced pressure in the presence of hydroquinone inhibitor, and stored at 4 °C until use. *N*,*N*′-methylene-bis-acrylamide (MBAAm) and ammonium persulfate (APS) were supplied by Sigma (St. Louis, MO, USA). All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany). *L. clavatum* spores (sporopollenin) with 20 μm particle size mesh and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were obtained from Fluka A.G. (Buchs, Switzerland). Human serum albumin (HSA, 98% pure by gel electrophoresis, fatty-acid free, 67 kDa) was purchased from Aldrich (Munich, Germany). Coomassie Blue, for the Bradford Protein Assay, was obtained from BioRad (Richmond, CA, USA). Water used in the experiments was purified using a Barnstead (Dubuque, IA, USA) ROpure LP® reverse osmosis unit with a high-flow cellulose acetate membrane (Barnstead D2731), followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion-exchange packed bed system.

2.2. Preparation of sporopollenin particles

10 g of *L. clavatum* spores was refluxed with 75 mL of acetone for 4 h to remove the hydrophobic layer, i.e., fat, to increase the hydrophilicity of the resin. The defatted spores were also treated with 2.0 M NaOH for 24 h to remove lignin like structure. After filtration, the sporopollenin residue was washed with hot water (150 mL) 5 times and then with hot ethanol 2 times, and dried at 60 °C under vacuum. The remaining membrane portion (sporopollenin) was used for further applications.

2.3. Preparation of Cu^{2+}-attached sporopollenin particles (Cu^{2+}-ASP)

The optimized adsorption conditions of Cu^{2+} onto sporopollenin particles (SP) were performed as described previously [22]. Briefly, 0.150 g of SP was treated with a Cu^{2+} solution [150 mg/L (pH 5.0), adjusted with HCl and NaOH] at room temperature for 2 h. A 1000-ppm atomic absorption standard solution was the source of the Cu^{2+} ions. The concentration of the Cu^{2+} ions in the resulting solution was determined with a graphite furnace atomic absorption spectrometer (GFAAS, Analyst 800/PerkinElmer, USA). The instrument response was periodically checked with known metal solution standards. The experiments were performed in triplicate. The Cu^{2+} concentrations in the initial and final solutions were used to calculate the amount of Cu^{2+} ions adsorbed.

Cu^{2+} leakage from the Cu^{2+} loaded SP was investigated in media whose pH varied between 4.0 and 7.0 and also in a medium-containing 1.0 M NaCl. The monolith was stirred for 24 h at room temperature. Then the concentration of Cu^{2+} ions in the supernatants was determined using an atomic absorption spectrophotometer.

2.4. Preparation of Cu^{2+}-attached sporopollenin particle embedded cryogel (Cu^{2+}-ASPEC) column

Preparation of supermacroporous monolithic cryogel embedded with Cu^{2+}-ASP is described as follows.

Monomers (50 mg MBAAm and 0.3 mL HEMA) were dissolved in deionized water (5 mL) and the mixture was degassed under vacuum for about 5 min to eliminate soluble oxygen. The cryogel was produced by free radical polymerization initiated by TEMED (20 μL) and APS (100 μL) (10%, w/v). After adding APS, the solution was cooled in an ice bath for 2–3 min. TEMED was added and the reaction mixture was stirred for 1 min. In this step, 10 mg of Cu^{2+}-ASP was added to the polymerization mixture. Then, the reaction mixture was poured into a plastic syringe (5 mL, i.d. 0.8 cm). The polymerization solution in the syringe was frozen at −12 °C for 24 h and then thawed at room temperature. For the removal of unconverted monomers and initiator, washing solutions (i.e., a dilute HCl solution and a water–ethanol mixture) were recirculated through the monolithic cryogel column, until cryogel column is clean. Purity of the monolithic cryogel was followed by observing the change of optical densities of the samples taken from the liquid phase in the recirculation system. After washing, the cryogel was stored in buffer containing 0.02% sodium azide at 4 °C until use. Illustration of cryogel embedded with Cu^{2+}-attached SP for HSA is represented in Fig. 1.

2.5. Characterization of cryogel

2.5.1. The porosity of Cu^{2+}-ASPEC column

For the measurement of volume and free water content of the cryogel sample, φ value was estimated. A piece of cryogel sample was saturated with deionized water, then it was immersed in water having volume V_1, after that the total volume of cylinder was measured as volume V_2. Water-saturated cryogel volume V_0 was calculated by the volume difference, i.e., $V_0 = V_2 - V_1$.

The mass of water-saturated cryogel, m_w, was weighted. After squeezing the cryogel sample to remove the free water within the large pores, the mass of the cryogel sample without free water, m_s, was weighted as described previously [20]. The porosity was calculated by the following formula:

$$\varphi = \frac{m_w - m_s}{\rho_w V_0} \quad (1)$$

where ρ_w is the density of deionized water. Then the cryogel sample was dried in the oven at 60 °C for 12–24 h to a constant and the dried cryogel mass m_d was determined, and the total water fraction (TWF) was calculated by the following formula:

$$\mathrm{TWF} = \frac{m_w - m_d}{\rho_w V_0} \quad (2)$$

2.5.2. Surface morphology

The morphology of a cross section of the dried cryogel was investigated by SEM. The sample was fixed in 2.5% glutaraldehyde