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Preparation of poly(hydroxyethyl methacrylate) cryogels containing L-histidine for insulin recognition

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ABSTRACT

In the present study, affinity adsorption technique was studied for insulin adsorption. Firstly, insulin-imprinted supermacroporous cryogel was prepared for the insulin adsorption. *N*-methacryloyl-(L)-histidine methyl ester (MAH) was chosen as the monomer. Insulin was complexed with MAH, and insulin-imprinted p(HEMA–MAH) [insulin-(MIP)] cryogel was prepared by free radical polymerization with 2-hydroxyethyl methacrylate (HEMA), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) and ammonium persulfate (APS) in an ice bath. Then, insulin was removed from the cryogel by using 0.1 M glycine–HCl buffer (pH: 3.5). The characterization of the cryogel was carried out by using scanning electron microscopy (SEM) and swelling test. The equilibrium swelling ratios of the cryogels were found to be 8.56 ± 0.42 g H_2O/g polymer for p(HEMA) and 7.20 ± 0.36 g H_2O/g polymer for insulin-p(HEMA–MAH). Insulin adsorption experiments were performed under different conditions, such as flow rate, medium pH, initial insulin concentration and ionic strength. It was observed that insulin could be repeatedly adsorbed and desorbed with MIP cryogel without any significant decrease in the adsorption capacity.

1. Introduction

The continuous demand for increasingly pure biologically active preparations (low molecular weight compounds, biopolymers like proteins, DNA, viruses, cellular organelles and whole cells) requires rapid improvement of existing polymeric materials used in the bioseparation and development of new materials. These polymeric materials allowed rapid development in the isolation and purification of individual proteins and nucleic acids, as well as elucidation of their structure and function [1]. From the view point of modern polymer chemistry, gels have become new potential for these biological materials.

One of the new types of polymer gels with considerable potential in biotechnology is cryogels. Cryogels have recently been used as efficient adsorbents for separation and purification of biomolecules, such as proteins, plasmid, DNA and enzyme from unclarified feedstock with many advantages, including large pores, short diffusion paths, low pressure drops as well as very short times for both adsorption and elution [2–5]. Cryogels with metal affinity ligands, anion-exchange ligands, cation-exchange ligands and magnetic nanoparticles have also been prepared successfully [6–8].

The ability to selectively recognize a target molecule in a vast pool of similar molecules is essential to biological and chemical processes [9]. Recently, considerable attention has been drawn to the imprinting of biomolecules and proteins for their potential applications as biomaterials for separation, purification, biosensor, enzyme and antibodies [9–13]. Molecular imprinting is a method for creating recognition sites in synthetic polymer using a molecular template. Molecular imprinting polymers (MIP) are easy to be prepared and are stable and capable of molecular recognition [12,13]. These materials are prepared by the copolymerization of functional and crosslinking monomers in the presence of the target molecule which acts as a molecular template [14,15]. Initially, monomers form a complex with the imprint molecule, following polymerization. The functional groups are held in position by the highly crosslinked polymeric structure. The removal of the imprint molecule leaves behind the specific binding sites with the shape and the orientation of the functional groups complementary to those of the imprint molecule. Thus, a molecular memory is introduced into the polymer, which is capable of binding the analyte with high specificity [14]. The interactions between the template and recognition sites of the polymer can be achieved by non-covalent interactions, such as hydrogen bonds, hydrophobic, electrostatic interactions or reversible covalent interactions. Molecular recognition-based separation techniques have received much attention because of their high selectivity toward proteins [16]. However, polymer grinding into particles can cause the destruction of binding sites. Cryogels with their macroporous structure facilitating mass-transport of large molecules can be a good alternative to overcome these problem [17,18].

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Insulin is a small protein consisting of an A-chain and B-chain linked by two disulfide bonds, with an additional intra-chain disulfide bonds in the A-chain [19]. In the C-terminus of the B-chain, a high population of nonpolar amino acids is dominant. Insulin has a 6 kDa of molecular weight, an isoelectric point of 5.4, and the net charge on insulin monomers ranges from −2 to −6 in the pH range 7–11 [20]. By forming an antiparallel association with a neighboring insulin monomer, the insulin monomers constitute strongly bound dimers [21]. Insulin is the most important and indispensable regulatory hormone and has profound effects on metabolism by controlling glucose homeostasis [22]. When insulin is absent (or low), glucose is not taken up by body cells, and therefore, the body begins to use fat as an energy source [23]. Insulin-dependent diabetes mellitus is a syndrome of disordered metabolism with inappropriate hyperglycemia due either to an absolute deficiency of insulin secretion or a reduction in the biological effectiveness of insulin. Therefore, it is important to have methods of separation allowing an efficient and rapid purification of insulin. Protein separation techniques gain more importance at this point [24,25].

The main objective of this study is to prepare insulin imprinted cryogel for selective and efficient separation of insulin. The supermacroporous cryogel was chosen for this purpose. Poly(2-hydroxyethyl methacrylate-*N*-methacryloyl-(L)-histidine methyl ester) [p(HEMA–MAH), MIP] cryogel was prepared by free radical polymerization for the insulin imprinting, and the work was organized for investigation of insulin adsorption properties of this cryogel. This method makes use of a combinatorial selection strategy to enhance selectivity. Repeated use of the insulin-MIP cryogel was also discussed.

2. Materials and methods

2.1. Chemicals

L-Histidine methyl ester, methacryloyl chloride, 2-hydroxyethyl methacrylate, *N*,*N*′-methylenebisacrylamide (MBAAm), ammonium persulfate (APS) were supplied by Sigma Chemical Co. (St. Louis, MO). *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) was also obtained from Sigma. Insulin (Actrapid-HM, MW: 5734 Da) was purchased from Novo Nordisk. All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany).

2.2. Synthesis of MAH

Preparation and characterization of MAH were described previously [26]. The following experimental procedure was applied for the synthesis of MAH: 5.0 g of L-histidine methyl ester and 0.2 g of hydroquinone were dissolved in 100 mL of dichloromethane solution. This solution was cooled to 0 °C, and 12.7 g of triethylamine was added to the solution. 5.0 mL of methacryloyl chloride was poured slowly into this solution and solution was stirred magnetically at room temperature for 2 h. Then, hyroquinone and unreacted methacryloyl chloride was extracted with 10% NaOH. Aqueous phase was evaporated in a rotary evaporator at 30 °C. The residue (i.e., MAH) was crystallized from ether–cyclohexane mixture (1:1; v/v) and then dissolved in ethyl alcohol. ^{1}H NMR was used to confirm the synthesis of MAH structure. The ^{1}H NMR spectrum of MAH monomer was taken in $CDCl_3$ on a JEOL GX-400 300 MHz instrument. The residual non-deuterated solvent ($CHCl_3$) served as an internal reference. Chemical shifts are reported in ppm (δ) downfield relative to $CHCl_3$. ^{1}H NMR spectrum is shown to indicate the characteristic resonances from the groups in MAH monomer. These characteristic peaks are as follows: ^{1}H NMR ($CDCl_3$): δ = 1.99 (t; 3H, J = 7.1 Hz, CH_3), 1.42 (m; 2H, CH_2), 3.56 (t; 3H, O—CH_3) 4.82–4.87 (m; 1H, methin), 5.26 (s; 1H, vinyl H), 5.58 (s; 1H, vinyl), 6.86 (m; 1H, J = 7.4 Hz, NH), 7.82 (d; 1H, J = 8.4 Hz, NH), 6.86–7.52 (m; 2H, aromatic).

2.3. Preparation of insulin-imprinted cryogels

In the first part, insulin–MAH complex was prepared. Briefly, *N*-methacryloyl-(L)-histidine methyl ester (MAH, 3 μmol, 0.729 mg) was stirred in 1 mL of deionized water. Then, insulin (template molecule, 3 μmol, 17.5 mg/mL) was added into this solution. This mixture was incubated for 2 h for the formation of a complex between the insulin and the MAH monomer. Production of insulin imprinted cryogel was described as follows.

0.283 g/3.7 mL *N*,*N*′-methylenebisacrylamide (MBAAm) was dissolved in deionized water, and 1.8 mL HEMA was added to this solution, then the mixture was degassed to eliminate soluble oxygen under vacuum for about 5 min. The cryogel was produced by free radical polymerization initiated by *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED, 25 μL) and ammonium persulfate (APS, 20 mg). Insulin–MAH complex was added to this solution. The reaction mixture was then poured into a plastic syringe (5 mL, i.d. 0.8 cm) with closed outlet at the bottom. The polymerization solution in the syringe was frozen at −12 °C for 24 h and then thawed at room temperature. After washing it with 200 mL of water, the cryogel was stored in a buffer containing 0.02% sodium azide at +4 °C until use.

2.4. Removal of template molecule

In order to remove the unreacted monomers and other ingredients, insulin-MIP cryogel was washed with deionized water. Following the procedure, the template (i.e., insulin) was removed from the cryogel by using 0.1 M glycine–HCl buffer (pH 3.5). This solution was passed through the cryogel column by a peristaltic pump at room temperature for 2 h. This procedure was continued until no insulin was detected in the washing solution. The protein leak was followed by monitoring the absorbance in UV-spectrophotometer. The amount of insulin extracted from the cryogel was determined at 280 nm.

2.5. Characterization of the cryogel

The swelling degree of the cryogel (S) was determined as follows: cryogel sample was washed with water until washing was clear. It was sucked dry and then transferred to pre-weighed vial and weighed ($m_{\text{wet gel}}$). After drying it to constant mass in the oven at 60 °C, the mass of dried sample was determined ($m_{\text{dry gel}}$). The swelling degree was calculated as:

$$S = \frac{m_{\text{wet gel}} - m_{\text{dry gel}}}{m_{\text{dry gel}}} \quad (1)$$

The total volume of macropores in the swollen cryogel was roughly estimated by weighing the sample ($m_{\text{squeezed gel}}$) after squeezing the free water from the swollen gel matrix, then the porosity (P) was calculated as:

$$P = \frac{m_{\text{swolle gel}} - m_{\text{squeezed gel}}}{m_{\text{swollen gel}}} \times 100 \quad (2)$$

The surface morphology of the insulin-MIP cryogel was examined by using SEM. The sample was fixed in 2.5% glutaraldehyde for overnight. Then, the sample was dehydrated at −50 °C in lyophilization (Lyophilizer, Christ Alpha 1–2 LD plus, Germany). Finally, it was coated with gold–palladium (40:60) and examined through a scanning electron microscope (JEOL JSM 5600, Tokyo, Japan).