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Monosize microbeads for pseudo-affinity adsorption of human insulin

Müfrettin Murat Sarı*, Canan Armutcu, Nilay Bereli, Lokman Uzun*, Adil Denizli

Hacettepe University, Department of Chemistry, Biochemistry Division, 06532 Beytepe, Ankara, Turkey

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ABSTRACT

Affinity adsorption technique is increasingly used for protein purification, separation and other biochemical applications. Therapeutic molecules such as antibodies, cytokines, therapeutic DNA and plasma proteins must be purified before characterization and utilization. The aim of this study was to prepare micronsized spherical polymeric beads and to investigate the extent of their human insulin adsorption capability. Monosize poly(ethylene glycol dimethacrylate–N-methacryloyl-(L)-histidine) [poly(EDMA–MAH)] beads were prepared by modified suspension copolymerization. Functional monomer (MAH) was synthesized using methacryloyl chloride and L-histidine. The beads were characterized using scanning electron microscopy (SEM), Fourier transform infrared spectroscopy, swelling test and elemental analysis. MAH incorporation into monosize polymeric beads, having an average size around 2–3 μm, was estimated as 55.3 μmol MAH/g bead. Equilibrium swelling ratios of poly(EDMA–MAH) and poly(EDMA) beads were 65% and 55%, respectively. Adsorption experiments were performed under different conditions (i.e., pH, temperature, protein concentration and ionic strength). It was found that adsorption characteristics are strongly depend on these conditions. Maximum insulin adsorption capacity was achieved as 24.7 mg insulin/g poly(EDMA–MAH) beads. Results were well fitted to the Langmuir isotherm model. Compared with poly(EDMA–MAH), nonspecific insulin adsorption onto poly(EDMA) beads was very low (0.61 mg insulin/g bead) and can be negligible. It was observed that insulin could be repeatedly adsorbed and desorbed (at least 10 times) without significant loss in adsorption capacity.

1. Introduction

Insulin, a small protein consist of 51-amino acids distributed in two polypeptide chains and has 6 kDa of molecular weight, is the most important and indispensable regulatory hormone and it has profound effects on metabolism by controling of glucose homeostasis. It is secreted by groups of cells within the pancreas called islet cells. Insulin facilitates cell membrane for glucose absorption to convert into glycogen and causes cells in the liver, muscle, and fat tissue to take up glucose from the blood, storing it as glycogen in the liver and muscle, and stopping use of fat as an energy source. More glucose absorbed, then less amount locates in the blood. When insulin is absent (or low), glucose is not taken up by body cells, and the body begins to use fat as an energy source, for example, by transfer of lipids from adipose tissue to the liver for mobilization as an energy source [1]. As its level is a central metabolic control mechanism, its status is also used as a control signal to other body systems (such as amino acid uptake by body cells). In addition, it has several other anabolic effects throughout the body. Insulin functions in lowering the blood glucose level by increasing its rate of utilization by the cells in the way of glycolysis and subsequent metabolic pathways so as to burn them into energy for storage or immediate use [2]. Any disorder in insulin metabolism depending on glucose homeostasis directly causes serious diabetic illness. Unfortunately, there are more than 150 million people suffering from *Diabetes mellitus* worldwide, and this number will double by the year of 2025 [3]. Isolation, separation and purification of insulin to obtain as a purified raw material of diabetics drugs from various sources has great importance. Because, purified and treated insulin has been used therapeutically for the treatment of diabetes since 1922 [4]. Various studies aimed obtaining purified insulin have been performed recently [5–7] and it is now considered a well-described and thoroughly studied small protein [8].

Proteins should be purified before any treatment since the purity of a protein is a prerequisite for the studies related to its structure and function or its potential application. Purified proteins are extensively used as raw material in the drug industry, foaming, emulsifying and gelling agent in the food and cosmetic products such as hair-care solutions, photography supplies and in the medical diagnostic area because of their predisposition for surface activity [9]. Protein separation techniques and their biochemical and therapeutic application gain more importance at this point. Among the numerous methods, protein purification via adsorption

* Corresponding authors. Tel.: +90 312 2977963; fax: +90 312 2992163.
E-mail addresses: mufrettin@yahoo.com (M.M. Sarı), lokman@hacettepe.edu.tr (L. Uzun).

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is a widely investigated phenomenon having its origin in several different disciplines of science [10]. Protein adsorption and diffusion into solid support media is of significant importance in the fields related to separation science and biomedical research [11–13] and the separation and purification of proteins for medical, biotechnological and industrial purposes have been attempted by many methods including adsorption over many years, also some protocols have been proposed [14–19]. Although scientific and industrial applications with proteins have some difficulties and high complexity, a wide variety of protein separation and purification techniques are available today. Different types of chromatographic techniques have become dominant due to their high resolving power [20].

As well as proteins, the structure of the support used as adsorbent is the other important criteria for protein adsorption. Because, the specific applications of the supports with proteins are closely related to their physical and chemical characteristics such as particle diameter, porosity, toxicity, water uptake ability, biocompatibility, hydrophilicity and hydrophobicity [21]. Recently, adsorption techniques using natural or synthetic polymer based supports such as particles, membranes, hydrogels and monoliths varying in particle structure and size and the being of specific functional groups on their surface are considered to be an effective and economical method [22]. Also, adsorption of proteins onto a polymeric solid support material is the most general, easiest to perform and oldest protocol of physical immobilization methods and may have a higher commercial potential than other methods [23]. In the various techniques including adsorption such as gel filtration chromatography, dye-affinity chromatography, ion-exchange chromatography, immobilized metal-ion affinity chromatography, affinity chromatography, and hydrophobic interaction chromatography, the separation of a protein is dependent on its biological and physicochemical properties such as molecular size, net charge and other biospecific characteristics [24–28]. Especially, the preparation and applications of a wide variety of micronsized spherical beads have attracted considerable attention and have additional advantages required for protein adsorption because of their unusual physical and chemical properties owing to small size and large specific surface area, low-cost, high mechanical and thermal stability, effective protein adsorption, high uptake capacity and reusability due to easy mass transfer [29].

Pseudospecific ligands can be also used to purify a wide range of biomolecules [30]. Protein adsorption techniques using pseudospecific ligand can be favorable in much cases. Small aminoacid-molecules can be used as pseudo-specific ligands and may hold certain advantages as ligands for industrial bioaffinity separations since they are not likely to cause an immune response in case of leakage into the product. They are also much more stable than protein ligands because they do not require a specific tertiary structure for maintaining biological activity [31]. Especially, histidine has been used as a pseudo-specific ligand in affinity chromatography of proteins [32–34]. Pseudospecific ligand histidine interacts through its carboxyl, amino and imidazole groups with several proteins at around their isoelectric points and has shown particular efficiency in separation and purification of proteins [35,36]. For example, calf chymosine, myxaline and acid protease from *Aspergillus niger*, as well as catechol-2,3-dioxygenease have been purified on histidine-immobilized supports [37] and special interest has been the separation of IgG from different sources [38,39].

The main goal of the present work is the exploration of the possibility of specific insulin adsorption using a micronsized spherical polymeric solid support. Poly(ethylene glycol dimethacrylate-N-methacryloyl-(L)-histidine) [poly(EDMA–MAH)] beads are proposed as a new model polymeric support in order to perform this aim. In the first step of the study, N-methacryloyl-(L)-histidine (MAH), synthesized using methacryloyl chloride and L-histidine, was used as a co-monomer and/or pseudo-specific ligand for insulin molecules. Secondly, ethylene glycol dimethacrylate (EDMA) was directly copolymerized with MAH using suspension polymerization technique in order to produce poly(EDMA–MAH) beads. This approach, in which MAH acts as the ligand for the production and functionalization of the polymeric carriers, has several advantages over conventional methods. Coupling of a ligand to the adsorption matrix is an expensive, time consuming and critical step in the preparation of affinity adsorbent. Despite the most of the other well known bioaffinity applications based on the specificity of ligands mentioned above, comonomer MAH acted as pseudospecific ligand in this study and it is possible to incorporate it directly into polymeric backbone of the beads without further activation and ligand immobilization steps. In the third step of the study, prepared monosize poly(EDMA–MAH) beads were characterized, their insulin adsorption capacity and specificity were analyzed and compared with plain poly(EDMA) beads. Also, the effects of external stimuli such as pH, ionic strength and temperature on the insulin adsorption and reusability of the beads were investigated.

2. Experimental

2.1. Materials

Freeze-dried bulk preparations of recombinant human insulin containing Zn^{2+} were obtained from Novo Nordisk A/S (Bagsvaerd, Denmark). L-Histidine (98% pure thin layer chromatography) and methacryloyl chloride were supplied by Sigma (St Louis, MO, USA). Ethylene glycol dimethacrylate (EDMA) 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. Benzoyl peroxide was obtained from Fluka. Poly(vinyl pyrolidone) (PVP K-30) was supplied from Aldrich Chem. Co. (Milwaukee, WI, USA). Buffer and sample solutions were pre-filtered through a 0.2 μm membrane (Sartorius, Göttingen, Germany). All glassware was extensively washed with dilute nitric acid before use. All other chemicals were of reagent grade and purchased from Merck A.G. (Darmstadt, Germany). All water used in 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. Methods

2.2.1. Synthesis of MAH monomer

As mentioned above, the N-methacryloyl-(L)-histidine (MAH) monomer was used as both comonomer and pseudospecific ligand. In order to synthesize MAH, the following procedure was applied [40]: 5.0 g of L-histidine and 0.2 g of hydroquinone were dissolved in 100 ml of dichloromethane solution. This solution was cooled down to 0 °C and 12.7 g of triethylamine was added to the solution. Five milliliter of methacryloyl chloride was poured slowly into this solution, which was stirred magnetically at room temperature for 2 h. At the end of the chemical reaction period, hydroquinone and unreacted methacryloyl chloride were extracted with 10% NaOH solution. The aqueous phase was evaporated in a rotary evaporator. The residue (i.e. MAH) was crystallized in an ether-cyclohexane mixture and then dissolved in ethyl alcohol. The monomer was characterized by ^{1}H NMR. The characteristic peaks from the chemical groups in MAH monomer as follows: ^{1}H NMR ($CDCl_3$): δ 2.84 (*t*; 3H, J = 7.08 Hz, CH_3), 3.07–3.21 (*m*; 2H, CH_2), 4.82–4.87 (*m*; 1H, methyne), 5.26 (*s*; 1H, vinyl H), 5.58 (*s*; 1H, vinyl); 6.26 (*d*; 1H, J = 7.4 Hz, NH), 7.06–7.22 (*m*; 5H, aromatic), 10.09 (*s*; 1H, OH).