Colloids and Surfaces B: Biointerfaces 146 (2016) 567–576

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Polyethyleneimine assisted-two-step polymerization to develop surface imprinted cryogels for lysozyme purification

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ARTICLE INFO

Article history:
Received 13 February 2016
Received in revised form 21 June 2016
Accepted 28 June 2016
Available online 29 June 2016

Keywords:
Two-step polymerization
Surface imprinting
Lysozyme
Cryogel
Polyethyleneimine

ABSTRACT

Surface imprinting strategy is one of the promising approaches to synthesize plastic antibodies while overcoming the problems in the protein imprinting research. In this study, we focused our attentions on developing two-step polymerization to imprint on the bare surface employing polyethyleneimine (PEI) assisted-coordination of template molecules, lysozyme. For this aim, we firstly synthesized poly(2-hydroxyethyl methacrylate-glycidyl methacrylate), poly(HEMA-GMA) cryogels as a bare structure. Then, we immobilized PEI onto the cryogels through the addition reaction between GMA and PEI molecules. After that, we determined the amount of free amine (NH_2) groups of PEI molecules, subsequently immobilized methacrylate functionalities onto the half of them and another half was used to chelate Cu(II) ions as a mediator between template, lysozyme and PEI groups. After the characterization of the materials developed by Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and the micro-computed tomography (μCT), we optimized the lysozyme adsorption conditions from aqueous solution. Before performing lysozyme purification from chicken egg white, we evaluated the effects of pH, interaction time, the initial lysozyme concentration, temperature and ionic strength on the lysozyme adsorption. Moreover, the selectivity of surface imprinted cryogels was examined against cytochrome c and bovine serum albumin (BSA) as the competitors. Finally, the mathematical modeling, which was applied to describe the adsorption process, showed that the experimental data is very well-fitted to the Langmuir adsorption isotherm.

1. Introduction

The lysozyme (EC3.2.1.17) is a glycoside hydrolase enzyme abundant in egg white [1]. As well-known, the lysozyme also exists in the spleen, tears, and milk and has a molecular weight around 14.3 kDa due to consisting of 129 amino acid residues, which are cross-linked by four disulfide bridges [2]. Moreover, the lysozyme breaks the cell wall of bacteria through hydrolyzing the β (1 → 4) glycoside bond between muramic acid and N-acetylglucosamine in mucopolysaccharides [3]. It has a quite a lot area of use as an agent due to its antibacterial feature [4]. The lysozyme, with potential for use in anticancer drug, can also be included in the treatment of diseases caused by HIV [5], ulcers and infections [6]. The lysozyme has been used mostly in the field the food industry as a protective chemical for the storage of meat, fish, milk, daily consumer goods, fruits, and vegetables [7]. Therefore, there is a high demand for this enzyme, in this respect, the efficient isolation and purification techniques for the lysozyme have attracted considerable attentions. The conventional lysozyme separation and purification methods are the ultrafiltration [8], precipitation [9], chromatography [10] and reverse micelles extraction methods [11]. Although all these methods are effective, they are relatively complicated, time-consuming and expensive methods [12].

The molecularly imprinted polymers (MIP) are considered as a good alternative against bio-receptors regarding cost and efficiency and are used today in a variety of diverse applications [13–15]. In contrast to biological molecules such as an enzyme, antibody and hormone receptors, the molecularly imprinted polymers have the significant advantages such as high mechanical and

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http://dx.doi.org/10.1016/j.colsurfb.2016.06.060
0927-7765/

chemical stability, ease of preparation, reusability and low cost of production [16,17]. The exciting features of MIPs have triggered the research activities and exponentially increased the applications for (bio)sensors [18], the solid phase extraction [16], affinity chromatography [13,14,19], enzymatic catalysis [15], enantioseparation [20], drug release [21] and so on. Although it is possible to imprint the molecules in any size [22], there are some drawbacks during protein imprinting by conventional methods. The first, the polymer layer is usually thicker that results in less recognition site per unit volume. The second is the limited mass transfer and the difficulty of the interaction of target molecules with the recognition site. Surface imprinting is one of the promising approaches to overcome these drawbacks [23]. In that method, a thin polymeric layer is formed on the surface of the bare polymer through a similar pathway to bulk imprinting. By this way, it is possible to imprint the template molecules to a very close location on the surface or near inside of the bare polymer [23,24].

The macroporous cryogels, a kind of hydrogels synthesized under the freezing point of the solvent are considerably preferable in the field of biotechnology and biochromatography [25] due to their features such as the short diffusion path, thin surface films, tolerability of transfer by conduction and low back pressure. Also, their interconnected porous structure allows to study at higher flow rate even for highly viscous mobile phase i.e. whole plasma, wastewater, etc. [25–28].

In this study, we focused on developing surface imprinted cryogels through two-step polymerization strategy. Herein, poly(2-hydroxyethyl methacrylate-glycidyl methacrylate) [poly(HEMA-GMA)] cryogel was synthesized as a bare polymeric layer at the first step. After immobilizing polyethyleneimine (PEI) onto the surface, the amount of free NH_2 groups of PEI was determined for inserting methacrylate group into the structure to further polymerization meanwhile Cu(II) ions chelation for template coordination. After binding the template molecules, lysozyme onto Cu(II) ions@PEI@Cryogel system, the second cryo-gelation was carried out through newly inserted methacrylate group in the presence of crosslinker to obtain surface imprinted cryogel network. Before optimizing the adsorption conditions from aqueous solution, the cryogels were characterized by using Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and the micro-computed tomography (μCT). Then, lysozyme purification from chicken egg white as a natural source was performed to demonstrate the applicability of the system. Finally, the selectivity of the cryogels for lysozyme molecules was evaluated in respect to potential competitors, such as cytochrome c (cyto c) and bovine serum albumin (BSA).

2. Materials and methods

2.1. Materials

2-Hydroxyethyl methacrylate (HEMA), ethylene glycol dimethacrylate (EGDMA), polyethyleneimine (PEI) (branched, a molecular weight of 25 kDa) and bovine serum albumin (BSA) were obtained from Aldrich (Munich, Germany). The acrylamide (AA), *N,N′*-methylene bisacrylamide (MBAA), sodium lauryl sulfate (SLS), copper(II) nitrate trihydrate and cytochrome c were obtained from Sigma (St. Louis, MO, USA). The glycidyl methacrylate (GMA) and lysozyme were obtained from Fluka (St. Gallen, Switzerland). The monomers and the lysozyme were kept refrigerated at 4 °C until use. *N,N,N′,N′*-tetramethylethylenediamine (TEMED) and ammonium persulfate (APS) were purchased from BioRad (Hercules, CA, USA). The APS was maintained within desiccator. The benzotriazole methacrylate was synthesized in the laboratory.

2.2. The preparation of the polymeric material

The functional monomers, GMA (250 μL) and HEMA (2.5 mL) were dissolved in 3.25 mL of deionized water (DIW). Separately, 1.2 mL of the crosslinker, EGDMA and 0.5 g of surfactant, SLS was dissolved in 12.8 mL of DIW. After mixing of both two phases, the mixture was cooled in an ice-bath for 15 min. The final monomer concentration was kept 19.75%, w/v for the cryogels. After addition of APS (10 mg) and TEMED (50 μL) as a redox initiator couple, the mixture was immediately poured between two glass plates and allowed to polymerize at −20 °C for 24 h. The cryogels obtained were cut into a small circular disc (o.d. 0.8 mm) and extensively washed with DIW to remove unreacted monomers and SLS molecules.

2.3. PEI immobilization onto the cryogels

The reactive glycidyl groups in the cryogels were utilized for direct PEI immobilization in single step treatment. For this aim, the cryogel samples were interacted with 25 mL of PEI solution (5%, w/v, pH: 10.6) at 100 rpm at 50 °C for 6 h. To quantify the amount of PEI immobilized onto the cryogels, potentiometric titration to the samples collected from the solution before/after immobilization process were performed by using 0.1 M HNO_3 solution as titrant. An extensive washing process was conducted to the PEI immobilized cryogels (PEI@Cryogel) to remove unbound, only physically adsorbed, PEI molecules. After that, the potentiometric titration for dried PEI@Cryogels was applied to quantify the available free NH_2 groups in the PEI molecules. For this aim, the dried PEI@Cryogels (approx. 0.5 g) were interacted with 25 mL of 0.1 M HNO_3 solution for 30 min and then filtered it out of the solution. After that, the amount of available free NH_2 groups was quantified through back-titration of the filtrate by using 0.1 M NaOH as a titrant. The primary amine groups were estimated using the appropriate mass balance.

2.4. The insertion of methacrylate groups onto PEI@Cryogel

In order to gain the polymerizable methacrylate functionalities to PEI groups, a benzotriazole mediated substitution reaction was performed between the methacrylic acid and PEI. First of all, the amount of the free NH_2 groups in the PEI structure was estimated through potentiometric titration as mentioned before. After that, the half of the amount of free NH_2 was used for the reaction between PEI and methacrylic acid. For this aim, we synthesized methacryloyl benzotriazole (MA-Bt) via the activation of methacrylic acid molecules by applying thionyl chloride ($SOCl_2$) assisted acylation reaction according to literature (with some modifications) [29]. Herein, 1.2 g of $SOCl_2$ was slowly dissolved in 12.5 mL of the benzotriazole solution in dichloromethane (0.1 g/mL) while stirring at 25 °C for 30 min. Then, the reaction was continued for 2 h after addition of 0.86 g of methacrylic acid. The white precipitate was then filtered off and washed with dichloromethane (4 × 12.5 mL). The organic solvents collected were washed with 2 M NaOH (4 × 12.5 mL) and dried with Na_2SO_4; then the solvent was removed under reduced pressure. The residue, MA-Bt was dissolved in absolute toluene with a final concentration 60.4 μmol/mL and kept at a dark environment in the fridge until use. According to potentiometric titration, the PEI@Cryogels have 2.32 mmol of free NH_2 groups/g cryogel. Based on this calculation, 19.18 mL of MA-Bt solutions was reacted with 1 g of PEI@Cryogels while stirring at 100 rpm at 25 °C for 6 h to introduce the methacrylate groups, which would be used for second-step polymerization. The cryogels were washed with ethyl acetate (4 × 20 mL) and then DIW (5 × 10 mL) for removing benzotriazole and unreacted MA-Bt molecules from the polymeric structure (MA-PEI@Cryogel).