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Oriented immobilized anti-LDL antibody carrying poly(hydroxyethyl methacrylate) cryogel for cholesterol removal from human plasma

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ABSTRACT

Low density lipoprotein (LDL) cholesterol is a major ingredient of the plaque that collects in the coronary arteries and causes coronary heart diseases. Among the methods used for the extracorporeal elimination of LDL from intravasal volume, immunoaffinity technique using anti-LDL antibody as a ligand offers superior selectivity and specificity. Proper orientation of the immobilized antibody is the main issue in immunoaffinity techniques. In this study, anti-human β-lipoprotein antibody (anti-LDL antibody) molecules were immobilized and oriented through protein A onto poly(2-hydroxyethyl methacrylate) (PHEMA) cryogel in order to remove LDL from hypercholesterolemic human plasma. PHEMA cryogel was prepared by free radical polymerization initiated with N,N,N′,N′-tetramethylene diamine (TEMED). PHEMA cryogel with a swelling degree of 8.89 g H_2O/g and 67% macro-porosity was characterized by swelling studies, scanning electron microscope (SEM) and blood compatibility tests. All the clotting times were increased when compared with control plasma. The maximum immobilized anti-LDL antibody amount was 63.2 mg/g in the case of random antibody immobilization and 19.6 mg/g in the case of oriented antibody immobilization (protein A loading was 57.0 mg/g). Random and oriented anti-LDL antibody immobilized PHEMA cryogels adsorbed 111 and 129 mg LDL/g cryogel from hypercholesterolemic human plasma, respectively. Up to 80% of the adsorbed LDL was desorbed. The adsorption–desorption cycle was repeated 6 times using the same cryogel. There was no significant loss of LDL adsorption capacity.

1. Introduction

LDL cholesterol is widely recognized as one of the major risk factors in the development of coronary heart diseases because of its ability to build up in the lining of arteries forming atheromas and fatty acid deposits [1–3]. The causative factor in high levels of LDL can be of genetic reasons, such as familial hypercholesterolemia [4,5].

A majority of the hypercholesterolemic patients can be effectively treated by reduced dietary intake and drug therapy to control plasma cholesterol levels, preventing severe heart diseases. However, a more aggressive approach is necessary in severe hypercholesterolemia [6]. Since the first plasmapheresis procedure implemented for homozygous familial hypercholesterolemia; a number of additional methods with different specificity and selectivity have been developed for the extracorporeal removal of LDL from the blood, including cascade filtration, heparin induced extracorporeal LDL precipitation (HELP), thermo-filtration, dextran induced LDL precipitation and direct adsorption of lipoproteins (DALI) [7]. Extracorporeal elimination is an effective and life saving procedure in hypercholesterolemia patients [8]. Of course the most selective binding affinity can be achieved by using immunoaffinity adsorbents containing antibodies as the ligand [9–12]. For instance; in case of high LDL levels in patients, antibodies against apoprotein B100, the main protein component of LDL, are used for the selective extracorporeal removal of harmful LDL without depriving the patients of the useful HDL and other blood components.

Antibody immobilization may be a critical step in the design of immunoaffinity adsorbents [13]. When antibodies are covalently immobilized onto adsorbents, their specific binding capacity usually decreases as compared to soluble antibodies [14]. This reduction is attributed to the random orientation of the antibodies on the surface of the solid support used, which disables the accessibility of the antigen binding fragments of the antibodies (Fab) towards their antigens. In normal coupling procedures, the target antibodies are immobilized at many different spots resulting random orientation of the antibodies on the matrix that might prevent the formation of the antibody–antigen complex [15].

More specific immobilization of antibodies through the Fc region of the heavy immunoglobulin chain would be very efficient in terms of providing orderly oriented immobilized antibody so that the antibody binding sites, located on the opposite part of the immobilization sites, become highly accessible to antigen binding. Protein A from *Staphylococcus aureus* has a unique capability of binding mammalian

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immunoglobulins through the Fc region [16]. Thus protein A can be employed for the orderly oriented immobilization of antibodies by formation of a protein A immobilized matrix followed by cross-linking of antibody with protein A in the matrix. The advantages of the orderly oriented immobilization process for biologically active proteins can be outlined as good binding accessibility and increased stability [16].

In biological applications, the conventional column chromatography techniques primarily use gel-beads with certain limitations such as high pressure drops and low flow-rates, lowering the efficiency in routine and scale-up applications [17]. In addition, the gel-bead column chromatography is not capable of applying highly viscous fluids such as human blood. Therefore, alternative chromatography techniques such as membranes, monoliths and cryogels [18–21] are applied in cases when gel-bead column chromatography techniques fail. Cryogels provide a potential solution in terms of their low pressure drop and lack of diffusion resistances utilizing macropores as compared to the traditional gel-bead columns [22–24]. Cryogel columns enable high flow-rates allowing voluminous elutions within shorter times. Whole blood can be applied on cryogel columns without any pre-treatment [25–29]. Cryogels are also cheap and thus they can be easily disposed of, eliminating cross-contamination between batches [30]. The PHEMA cryogels were selected for three good reasons: 1) They exhibit a low pressure drop, 2) they lack diffusion resistance and 3) viscous samples such as whole blood can be easily applied on them.

We have focused our attention on the development of anti-LDL antibody immobilized immuno-affinity PHEMA cryogels by combining the selectivity of immunoaffinity interaction with the biocompatibility and good flow properties of PHEMA cryogels. LDL removal performance of these immunoaffinity cryogels was reported here.

2. Experimental

2.1. Materials

Anti-LDL antibody (anti-human β-lipoprotein; Product No: L-8016) and protein A (from *S. aureus*, Cowan Strain I; Product No: P-6031) were obtained from Sigma (St. Louis, USA). 2-Hydroxyethyl methacrylate (HEMA) was obtained from Fluka A.G. (Buchs, Switzerland), which was later distilled under reduced pressure in the presence of hydroquinone inhibitor and stored at 4 °C until use. Cyanogen bromide (CNBr), N,N′-methylene-bis(acrylamide) (MBAAm), ammonium persulfate (APS) and N,N,N′,N′-tetramethylene diamine (TEMED) were also obtained from Sigma. All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany). Water used in adsorption experiments was purified by a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit having a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion exchange packed-bed systems.

2.2. Preparation of PHEMA cryogels

Preparation of cryogel is described elsewhere [20]. Briefly, monomers (1.3 mL of HEMA, and 10 mL of MBAAm) were dissolved in deionized water and the mixture was degassed under vacuum for about 5 min to remove soluble oxygen, yielding a total monomer concentration of 6% (w/v). The cryogel was prepared by free radical polymerization initiated by firstly addition of 20 mg of APS, 1 % (w/v) of the total monomers, into the solution of the monomers followed by cooling in an ice bath for 2–3 min, and then addition of 25 μL of TEMED, 1% (w/v) of the total monomers, which was stirred for 1 min. Then, the reaction mixture was poured into a plastic syringe (5 mL, internal diameter: 0.8 cm) with closed outlet at the bottom. The polymerization solution in the syringe was frozen at −12 °C for 24 h, which was then thawed at room temperature. After washing with 200 mL of water, the cryogel column was stored in a buffer solution containing 0.02% sodium azide at 4 °C until use.

2.3. CNBr activation

As adsorber materials anti-LDL antibody was used for random immobilization while protein A was used for orderly oriented immobilization. The PHEMA cryogel column was activated using cyanogen bromide in order to create reactive sites for protein immobilization, with either anti-LDL antibody or protein A. Prior to the activation process, the PHEMA cryogel column was kept in distilled water for about 24 h, which was then washed by passing a 0.5 M NaCl solution and 50 mL of 0.5 M sodium carbonate buffer solution (pH 10.5). An aqueous solution of CNBr (50 mg CNBr/mL) was carefully prepared in a fume hood and its pH was quickly adjusted to 11.5 using a 4 M NaOH solution and a pH meter (Mettler Toledo Gmbh, Switzerland). The pH of the CNBr solution was maintained between 10.5 and 11.5 during the activation reaction. The CNBr solution was passed from the cryogel column with a flow rate of 1.0 mL/min over 2 h at room temperature. After the activation reaction, the column was washed with cold sodium citrate buffer (0.1 M; pH 6.5) and the filtrate was discarded.

2.4. Random immobilization of anti-LDL antibodies

20 mL of an anti-LDL antibody solution (1.0 mg/mL) dissolved in 0.1 M sodium citrate buffer (pH 6.5) passed through a freshly activated PHEMA cryogel column at a 1.0 mL/min flow rate at 4 °C overnight. After immobilization, 2.0 M ethanol amine solution was recirculated for another 1 h in order to quench side reactions that may form between unreacted sites of the protein and any remaining active groups (e.g. isourea) on the cryogel surface. The amount of anti-LDL antibody immobilization was monitored by measuring the decrease in the protein concentration in the anti-LDL antibody solution by Bradford method. Non-specifically adsorbed anti-LDL antibody molecules on PHEMA were also considered. The amount of immobilized anti-LDL antibody was calculated as:

$$q = [(C_i - C_t).V] / m \quad (1)$$

where, q is the amount of anti-LDL antibody immobilized onto unit mass of the cryogel (mg/g); C_i and C_t are the concentrations of the anti-LDL antibody in the initial solution and in the supernatant after immobilization (mg/mL), respectively, V is the volume of the aqueous phase (mL), and m is the mass of the cryogel column (g).

2.5. Oriented immobilization of anti-LDL antibodies

Covalent immobilization of protein A onto freshly activated PHEMA cryogels was performed using the same procedure described in Section 2.4. The CNBr and protein A concentrations were 50 mg/mL and 1.0 mg/mL, respectively. The immobilization (0.1 M sodium citrate) buffer solution used was adjusted at pH 6.5. The amount of protein A immobilized onto the CNBr activated PHEMA cryogel was determined by measuring the decrease in the protein A concentration by the Bradford method, which was calculated with Eq. 1. The protein A-PHEMA cryogel was washed several times using 0.1 M glycine–HCl solution (pH 3.5) and the immobilization buffer in order to remove impurities. The anti-LDL antibody solution was prepared in a borate buffer solution (1.0 mg/mL, pH 7.5). The protein A immobilized PHEMA cryogel column was treated with the anti-LDL antibody solution for 2 h at room temperature to form an antibody protein A complex. Then, protein A was cross-linked to the adsorbed anti-LDL antibody by adding a solution of 0.1% (w/v) cyanamide (CH_2N_2), prepared in the immobilization buffer solution, into the protein A immobilized column and the reaction was carried out for about 30 min. After the reaction, the column was washed several times with a solution of 0.1 M NaCl and