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Microporous Poly(hydroxyethyl methacrylate) based Affinity Membranes Carrying Heparin for Cholesterol Apheresis

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Abstract

In this study, the preparation of polymeric membranes using bioaffinity technology for application in blood filtration devices is described. Low molecular weight heparin (LMWH) attached poly(hydroxyethyl methacrylate) (PHEMA) based microporous affinity membranes were used for selective removal of cholesterol from hypercholesterolemic human plasma in-vitro. In order to further increase blood-compatibility of affinity membranes, aminoacid based comonomer N-methacryloyl-L-alanine (MAAL) was included in the polymerization recipe. PHEMAAL membranes were produced by a photopolymerization technique and then characterized by swelling test and scanning electron microscope (SEM) studies. In vitro blood-compatibility tests were assessed using modified activated partial thromboplastin time (APTT), prothrombin time (PT) and coagulation time (CT). The water swelling ratio of PHEMAAL increased significantly (133.2%) compared with PHEMA (58%). PHEMAAL membranes have large pores around in the range of 5-10 μm. All the clotting times increased when compared with PHEMA membranes. Loss of platelets and leukocytes was very low. LMWH loading amount was in the range of 87.5-147.2 mg/g. There was a very low non-specific cholesterol adsorption onto the PHEMA membranes, about 0.69 mg/g. LMWH attached PHEMAAL membranes adsorbed cholesterol in the range of 12.7-22.6 mg/g from hypercholesterolemic human plasma.

INTRODUCTION

Familial hypercholesterolemia (FH) is a genetic disorder that leads to severe cardiovascular complications in young adults [1]. FH is characterized by elevated levels of low density lipoproteins (LDL), tendon xanthomas and premature coronary atherosclerosis [2,3]. This abnormality is inherited as an incompletely dominant trait, which results in a deficiency of cell surface receptors to LDL and defective binding of LDL to these receptors [4]. High levels of LDL in plasma correlate directly with an increased risk for arteriosclerosis [5]. In addition to dietary and drug therapy, attempts have been made to remove cholesterol and LDL directly from plasma of patients, especially those resistant to drug therapy. Plasma exchange, the first approach to removing LDL and cholesterol from plasma, continues to be used despite certain disadvantages. Total plasma

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exchange is limited mainly by the effort required and by its high cost [6,7]. Cascade or double filtration plasmapheresis permit more selective cholesterol removal from plasma than does plasma exchange. There are considerable losses of proteins with molecular weights greater than 250 kD [8]. Also certain amounts of substitution solutions may be necessary. This treatment method therefore also has certain disadvantages in comparison to selective techniques [9].

Extracorporeal elimination is a method of LDL-lowering therapy effective in patients with homozygous or severe heterozygous FH after forms of therapy have failed [10]. Lupien et.al. prepared heparin containing agarose beads as an affinity adsorbent and clinically applied it in selective cholesterol apheresis [11]. Stoffel and Demont demonstrated an immunoadsorption system containing anti-apoprotein B-antibodies attached on Sepharose® for the treatment of hypercholesterolemia [12]. This system was clinically applied by Borberg et al., who were able to perform more than 3000 successful treatment sessions of familial hypercholesterolemic patients [13]. Behm et al. used dextran sulfate attached cellulose beads for selective cholesterol removal [14]. Promising results have been reported with a polyacrylate coated fractogel system [15]. Lopukhin et al. prepared heparin and chytozane sulphate containing macroporous silica adsorbents for cholesterol removal from familial hypercholesterolemic human plasma [16]. Pokrovsky et al. removed LDL from human plasma by using anti-LDL attached Sepharose® beads [17]. Denizli and Pişkin used heparin attached poly(hydroxyethyl methacrylate) beads and they removed cholesterol effectively from hypercholesterolemic human plasma in an in-vitro system [18].

There are advantages and disadvantages of the affinity adsorbents exemplified above. The hemoperfusion column is effective and widely used for diverse applications. However, it has several disadvantages, such as the compressibility of the column materials, the fouling, and particularly the slow flow rate through the packed bed column [19]. Another disadvantage is that it requires separation of plasma from blood to perfuse the plasma. Such a separation step is not only time consuming but also liable to contamination and increases therapeutic costs of patients. One significant trend has been to replace the bead packing by porous membranes that allow operation at low pressure [20]. Especially, when dealing with highly viscous mediums such as blood, contact with the membrane in a stacked-system is desirable because of high convective transport rates without cell damage. The desirable properties of affinity membranes are high porosity, high chemical, biological and mechanical stabilities, hydrophilicity, low non-specific adsorption of blood proteins and the presence of functional groups for derivatization [21]. Due to these advantages, porous affinity membranes provide higher efficiency.

Of course the selective adsorption can be achieved with the adsorbents containing biomolecules as the affinity ligand. For these reasons, we have focused our attention on the development of LMWH attached affinity membranes. In this study, we prepared an adsorbent containing LMWH for selective removal of cholesterol from hypercholesterolemic human plasma. To improve the blood-compatibility of affinity membranes, we included N-methacryloyl-L-alanine (MAAL) comonomer into the membrane formulation. A variety of approaches have been taken to improve blood compatibility of polymeric materials. One method of improving the biocompatibility of polymers for medical applications and restricting the toxicity of compounds released after degradation is to incorporate amino acid segments into their backbone [22]. Due to this reason, MAAL monomer was synthesized. PHEMAAL membranes were produced by a photo-polymerization technique.

Membranes were characterized by swelling studies and SEM. Blood-compatibility tests were also performed. Then, PHEMAAL membranes were activated by CNBr, and LMWH were covalently attached to the membranes. LMWH attachment onto the CNBr activated PHEMAAL membranes from aqueous solutions containing different amounts of LMWH are reported here.

EXPERIMENTAL

Materials

L-Alanine methyl ester hydrochloride (Cat. No: 330639) was supplied from Sigma (St Louis, MO, USA) and used as received. Cyanogen bromide (CNBr) was also purchased from Sigma. Methacryloyl chloride was supplied from Aldrich (Milwakuee, USA). The monomer, 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. Azobisisobutyronitrile was provided from Fluka (Switzerland). Low molecular weight heparin was supplied from Sigma Chemical Co. (Cat. No: H8537, St Louis, MO, USA) and used as received. All of the other chemicals used were reagent grade from Merck AG (Darmstadt, Germany). All 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. The resulting purified water (deionized water) has a specific conductivity of 18 MΩ. All glassware was soaked overnight in 4 M nitric acid.

Synthesis of MAAL Monomer

Preparation of N-methacryloyl-(L)-alanine (MAAL) had been reported in our previous article [23]. For the synthesis of MAAL monomer, the following experimental procedure was applied: 5.0 g of alanine and 0.2 g of $NaNO_2$ were dissolved in 30 mL of K_2CO_3 solution (5%, w/v). This solution was cooled down to 0°C. 6 mL of methacryloyl chloride was poured slowly into this solution under nitrogen atmosphere and then this solution was stirred magnetically at room temperature for 2 h. At the end of this chemical reaction period, the pH of this solution was adjusted to 3.0. Then the solution was extracted with chloroform ($CHCl_3$). The organic phase was dried with $MgSO_4$ and evaporated in rotary evaporator. The residue (MAAL) was crystallized in an ether-cyclohexane mixture.

Preparation of Membranes

Preparation of PHEMAAL membranes had been reported in our previous report [23]. Briefly, 2 mL of HEMA containing 5 mg AIBN as polymerization initiator and 100 mg of synthesized MAAL was mixed with 3 mL of 0.1 M $SnCl_4$ (pore former). The mixture was then poured into a round glass mould (9 cm in diameter) and exposed to ultraviolet radiation for 10 min under nitrogen atmosphere. The membrane obtained was washed several times with distilled water and ethyl alcohol, and cut into circular pieces (0.5 cm in diameter, thickness: 350 μm) with a perforator. Chemical structure of polymer which is produced with HEMA and MAAL is given in Figure 1.

Characterization of Membranes

Swelling ratio was determined in distilled water. The experiment was conducted as follows: initially dry membrane (diameter: 1 cm; thickness: 350 μm) were carefully weighed before being placed in a 50 mL vial containing distilled water. The vial was put into an isothermal water bath with a fixed temperature (25°C) for 2 h. The swollen membrane was taken out from the water periodically, wiped using a filter paper, and weighed. The water content of the membrane was calculated by using the following expression:

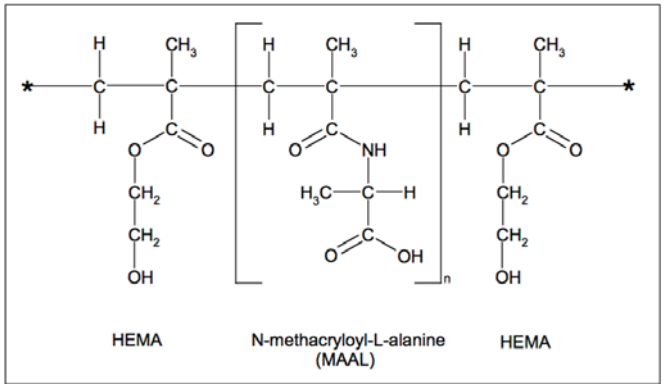


Figure 1. Molecular formula of PHEMAAL membrane.

$$\text{Swelling ratio \%} = [\,(W_s - W_o) / W_o\,] \times 100 \qquad (1)$$

where; W_o and W_s are the weights of membrane before and after swelling, respectively.

Microscopic observations and photographs of the gold coated PHEMAAL membranes were performed by using a scanning electron microscope (Model: Jeol JSM 5600).

In- Vitro Blood Compatibility Studies

In Vitro Coagulation Time Tests for Membranes

The nephelometry measurements including coagulation time (CT), prothrombin time (PT) and activated partial thromboplastin time (APTT), were performed with the coagulation instrument which measures the change of luminosity when light traverses the plasma sample. Membrane samples were rinsed until no detectable levels of impurities in the rinse solution (a period of two weeks was sufficient). Briefly the tested affinity membranes were incubated with healthy human plasma in a transparent plastic tube, and the reagents for each coagulation time test were added to the tube immediately.

Cell Adhesion Studies

Heparinized human blood was incubated with 5 pieces of membranes for 1 h. It should be noted that prior to the blood contact, membranes were washed with 0.1 M KCl solution in buffer until no further impurities (monitored by the absorbance at 280 nm) was detected in the washing solution. Blood samples were withdrawn from the beginning and the end of the operation, and the platelet and leukocyte counts of samples were determined using a microscope.

CNBr Activation

Prior to activation process, membrane pieces were kept in distilled water for about 24 h and washed on a glass filter with 0.5 M NaCl solution and water for the system to reach to equilibrium. CNBr aqueous solution (20 mg/mL) was prepared. The pH of this solution was quickly adjusted to 11.5 with 2 M NaOH. Membrane pieces were then added to this solution. The suspension was gently agitated at room temperature (25°C) and the activation procedure was continued for 60 min at a constant pH of 11.5. After the activation reaction, unreacted sites were quenched by washing with 0.1 M $NaHCO_3$ and any remaining active groups (e.g., isourea) on the surfaces were blocked by the treatment with ethanol amine (pH 9.1) and $FeCl_3$ solution for 1 h. Then, the activated membranes were washed four times with distilled water containing 0.5 M NaCl.

Low Molecular Weight Heparin Immobilization

The freshly CNBr activated PHEMAAL membranes were magnetically stirred (at 50 rpm) at a constant temperature of 25°C for about 4 h (i.e., equilibrium time) with 10 mL of a LMWH solution. pH of the solution (containing 0.1 M $NaHCO_3$ + 0.5 M NaCl) was 9.5. After coupling, the LMWH attached membranes were washed with 0.1 M borate buffer + 0.15 M NaCl (pH 8.8), with 2 M urea + 0.15 M NaCl, and finally with 0.1 M $NaHCO_3$ + 0.5 M NaCl (pH 9.5). The amount of LMWH attached on the CNBr activated membranes was determined by measuring the decrease of low molecular weight heparin concentration and also by considering the LMWH adsorbed non-specifically (the amount of LMWH adsorbed on the PHEMAAL membrane),

spectrophotometrically at 280 nm. The amount of adsorbed LMWH was calculated as:

$$q = [(C_i - C_t) . V] / m \quad (2)$$

Where, q is the amount of LMWH adsorbed onto unit mass of the membrane (mg/g); C_i and C_t are the concentrations of the LMWH in the initial solution and in the supernatant after adsorption, respectively (mg/mL); V is the volume of the aqueous phase (mL); and m is the mass of the membrane (mg).

Cholesterol Removal from Human Plasma

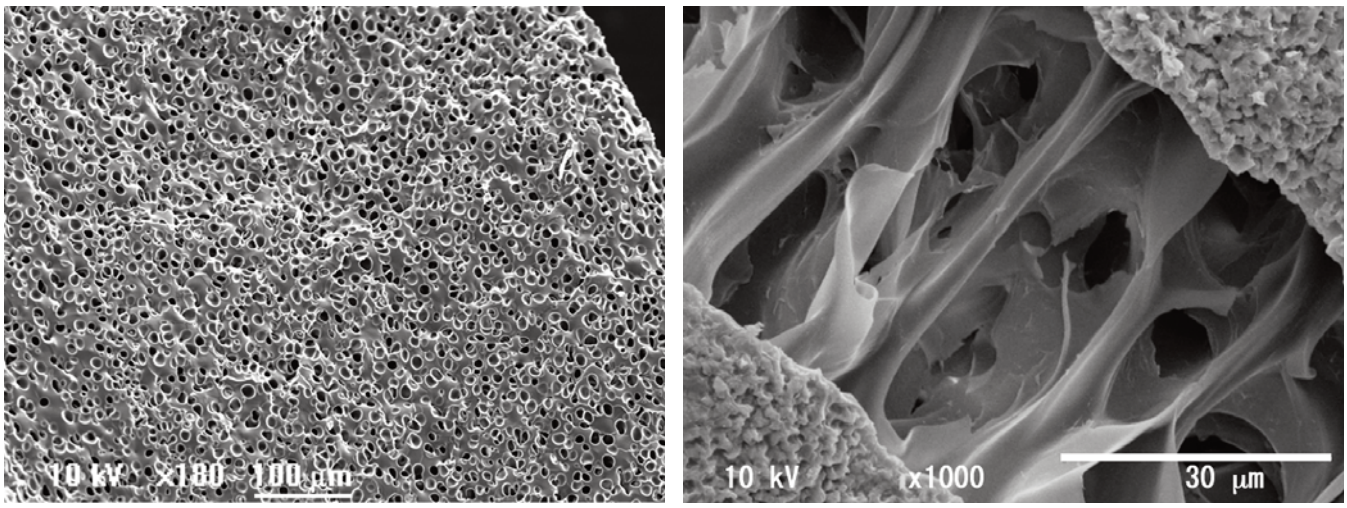
Cholesterol removal from human plasma on the PHEMA and the LMWH attached PHEMAAL membranes were studied in a batch wise. The plasma with a total cholesterol concentration of 2.98 mg/mL was obtained from a patient with hypercholesterolemia. Fresh frozen plasma was donated by the Blood Bank at the University Hospital (Hacettepe University, Ankara). Blood samples were centrifuged at 500 g for 30 min at room temperature to separate plasma. Plasma was filtered using 0.45 μm syringe filters (Model 245-0045 Nalge Co., Rochester, New York), and stored at 4°C. Sodium azide (0.1% w/v) was added to prevent bacterial growth. 10 mL of the plasma freshly separated from the patient was incubated with the PHEMA and the LMWH attached PHEMAAL membranes at 20°C for 4 h. The PHEMAAL membranes containing two different amounts of LMWH on their surfaces (i.e., 87.5 and 147.2 mg/g) were used. The amount of cholesterol removed was determined colorimetrically by measuring the decrease in the cholesterol concentration in the plasma sample.

RESULTS AND DISCUSSION

Membrane Characteristics

The scanning electron microscope (SEM) micrographs given in Figure 2 shows the surface structure and the cross-section of the PHEMAAL membranes. As seen from the surface and the cross-sectional surface photographs, the membranes have large interconnected pores (flow channels); the micropore dimensions are around in the range of 5-10 μm. The membrane surface seems very rough and heterogeneous. These large pores reduce diffusional resistance and facilitate mass transfer of cholesterol.

PHEMA belongs to a class of polymers known as hydrogels, which swell in contact with water. Hydrophilic PHEMAAL membranes prepared in this



(a) (b)

Figure 2. The SEM images of PHEMAAL membranes (a) Surface; (b) Cross-sectional area.

study swell in water, but do not dissolve. Compared with PHEMA (58%), the water swelling ratio of the PHEMAAL increased significantly (199.9%). Several possible factors may contribute to this result. First, incorporating MAAL actually introduced more hydrophilic functional groups into the polymer chain, which can attract more water molecules into polymer matrices. Second, reacting MAAL with HEMA could effectively decrease the molecular mass and reduce the crystallinity. Therefore, the water molecules penetrate into the polymer chains more easily, resulting in an improvement of polymer water swelling in aqueous solutions. Membrane thickness was measured as 350 μm.

Blood Compatibility Studies

A synthetic surface with improved biocompatibility would greatly improve the efficacy of blood-contacting devices. The first event after contact of polymeric surfaces with blood is the adsorption of proteins at the solid-liquid interface. Thereafter processes like the activation of the intrinsic coagulation, adhesion and aggregation of platelets and the activation of the complement system may take place, depending on the composition of the adsorbed protein layer and the conformation of the adsorbed proteins (Figure 3). The CT, APTT and PT tests were widely used for the clinical detection of the abnormalities of blood plasma. Table 1 shows the coagulation data. All the clotting times for PHEMAAL membranes increased when compared with PHEMA membranes, although all the clotting times decreased when compared with control plasma. However, these decreases are tolerable by the body [24]. Therefore, we concluded that the blood-compatibility of PHEMAAL membranes was

Table 1. Coagulation times of human plasma (reported in sec)*.

Experiments	APTT	PT	CT
Control Plasma	72.3	32.8	260
PHEMA	63.2	28.1	230
PHEMAAL	68.5	30.6	242

* Each result is the average of five parallel studies.

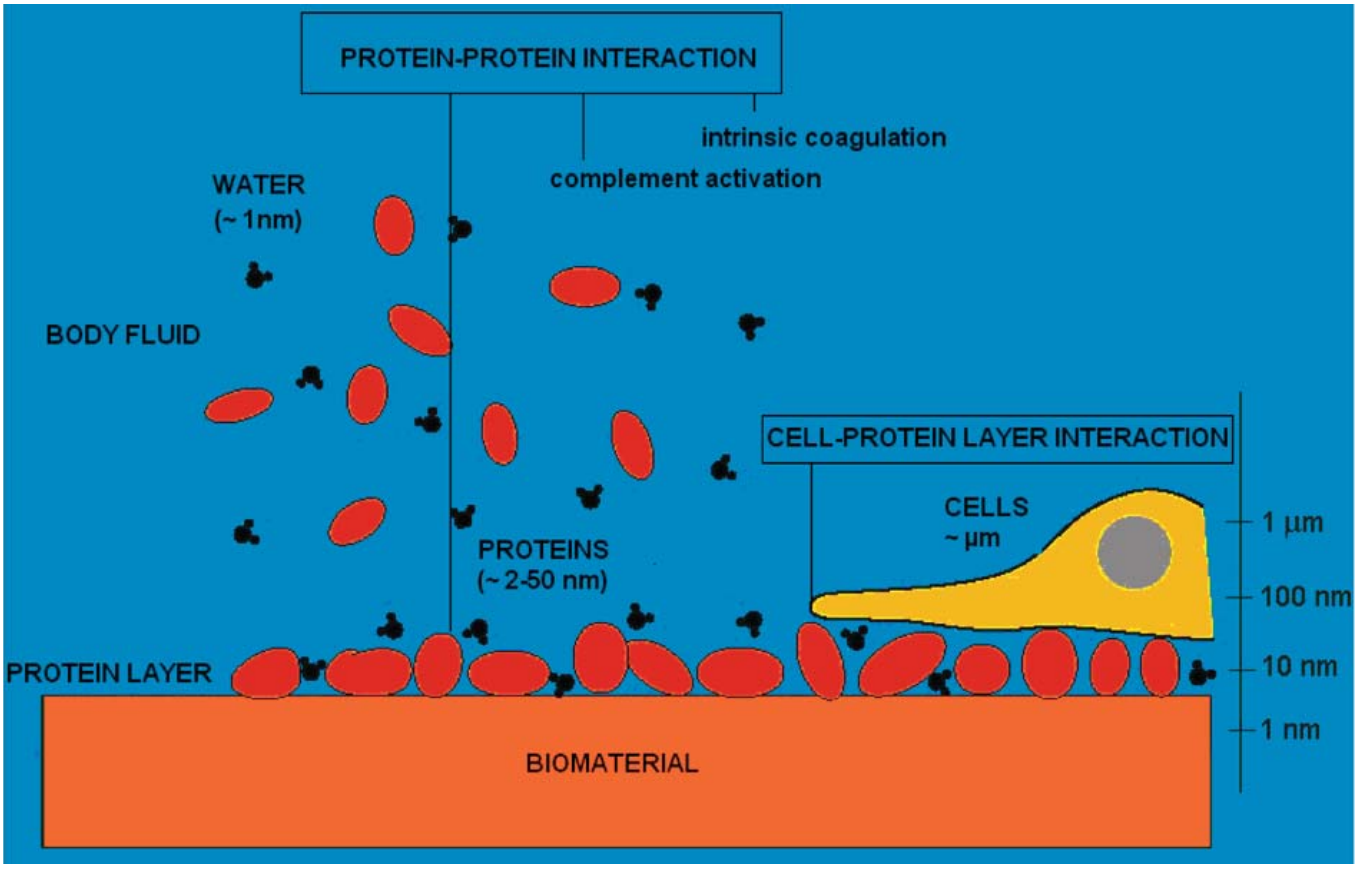


Figure 3. The role of adsorbed proteins in body fluid-biomaterial interaction [26].

rather good, and the clotting times were quite reproducible comparing with the values reported in the related literature [25]. Reference interval varies significantly among different reagent-instrument combinations. The approximate lower limit of normal is 10-12 seconds; the approximate upper limit of normal is 12-14 seconds. Critical values longer than 30 seconds is the most commonly used PT panic value in specialized coagulation laboratories according to the College of American Pathologists 1999 Survey CG2-C, but the value varies depending on the reagent-instrument combination and individual laboratory. With single factor deficiencies, the deficient factor has to be below 15% to 45% before the PT becomes prolonged, depending on the reagent. Consequently, in therapeutic affinity adsorption, the incorporation of MAAL as a comonomer may exert beneficial effects in two ways. First, improved blood-compatibility of the membrane support will reduce adverse reactions to treatment, and second, reductions in non-specific adsorption will reduce undesirable losses of beneficial proteins from treated blood.

Table 2, summarizes hematological data obtained from in-vitro blood assay. Loss of platelet with PHEMA and PHEMAAL membranes were 2.9% and 1.6%, respectively. Lost of leukocyte with PHEMA and PHEMAAL membranes were 8.6% and 6.9%, respectively. As seen here, there is no significant cell adhesion on the membranes. Surfaces modified with MAAL markedly reduced cell adhesion when incubated with human plasma. Therefore, incorporation of the MAAL groups in the structure of PHEMA has been found to improve the biocompatibility of membrane surfaces by limiting cell adhesion. These observations showed that surfaces of the affinity membranes are resistant to adhesion of blood cells. In conclusion, because of the good nonthrombogenic properties, PHEMAAL membranes seem to be very promising affinity adsorbents for biomedical applications such as extracorporeal affinity adsorption therapy for hypercholesterolemia.

Table 2. Platelet and leukocyte adhesion onto the PHEMAAL membranes*.

Substance	Platelet ($x10^{-3}/mm^3$)		Leukocyte ($x10^{-3}/mm^3$)	
	Initial/Final	Loss (%)	Initial/Final	Loss (%)
PHEMA	445/432	2.9	5.8/5.3	8.6
PHEMAAL	445/438	1.6	5.8/5.4	6.9

* Each result is the average of five parallel studies.

Cholesterol Removal from Human Plasma

Figure 4 shows the change of cholesterol concentration in the hypercholesterolemic plasma in the course of incubation. There was a very low non-specific cholesterol adsorption onto the PHEMA membranes which was about 0.69 mg/g for the 4 h incubation period (Curve A in Figure 4). On the other hand, total cholesterol level was significantly reduced when the LMWH attached PHEMAAL membranes was used.

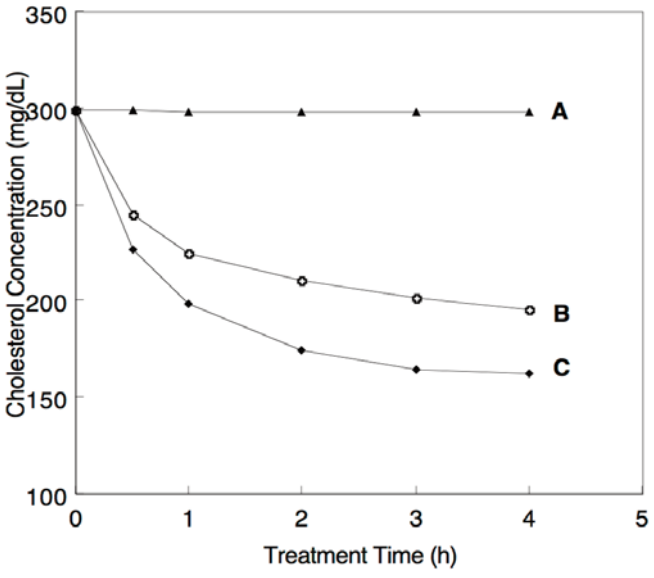


Figure 4. Cholesterol removal from the hypercholesterolemic human plasma (Cholesterol initial concentration: 298 mg/dL).

Curve A: adsorption onto PHEMA membrane; Curve B: adsorption onto PHEMAAL/LMWH membrane with 87.5 mg LMWH/g; Curve C: adsorption onto PHEMAAL/LMWH membrane with 147.2 mg LMWH/g.

The LMWH attached PHEMAAL membranes containing 87.5 and 147.2 mg LMWH/g adsorbed 12.7 mg and 22.6 mg cholesterol/g, respectively (Curve B and C in Figure 4). As expected the cholesterol removal rate was much faster when the LMWH content of the affinity adsorbent was higher. About 34.6% and 45.6% of the total cholesterol in the human plasma were removed when the LMWH attached PHEMAAL membranes containing 87.5 and 147.2 mg LMWH/g, respectively, were used. The total cholesterol concentration was reduced up to 162.4 mg/dL in 4 h, respectively, which represent a sufficiently low cholesterol level in hyper-cholesterolemia. Note that the adsorption values are not the equilibrium values (but the amounts of adsorption in 4 h), therefore they do not give the adsorption capacities of these affinity adsorbents. As easily projected from the curves in Figure 4, much higher adsorption values can be obtained for higher LMWH loadings.

Comparison with Related Literature

The specific ligands immobilized on the carrier matrix also play an important role in LDL-cholesterol adsorption. Many chemical and biological molecules have been extensively studied and applied as specific LDL-cholesterol ligands. Various supports have been also reported in literature. For example, Lopukhin et al used macroporous silica beads as the carrier matrix, and immobilized heparin and chytozane sulphate as specific bioligand. They reported cholesterol adsorption capacities around 14.8-15.2 mg/g [16]. Pokrovsky et al used commercial carrier made of Sepharose and monoclonal and polyclonal antibodies as the specific ligands [17]. Their maximum LDL binding capacities were in the range of 0.6-2.5 mg LDL per milliliter adsorbent. Denizli and Pişkin studied cholesterol removal from hypercholesterolemic human plasma and they reported maximum 4.7 mg cholesterol/g adsorption capacity with heparin immobilized PHEMA beads [18]. Tabak et al used heparin attached agarose beads and they obtained in the range of 1.2-3.0 mg/g cholesterol adsorption capacity from healthy human plasma [27]. Ostlund reported 6-8 mg LDL cholesterol per mL column volume with anti-LDL antibody attached commercially available Agarose beads from human plasma [28]. Smolik et al were interested in determining the LDL adsorption capacity of dextransulfate immobilized cellulose affinity beads [29]. They achieved a good selective LDL adsorption from whole blood and presented cholesterol adsorption capacities of 5-10 mg/mL gel. Schmidt used surface modified polyacrylate matrix and obtained 8-10 mg LDL-cholesterol per gram polymer [30]. Sinitsyn et al used Sepharose beads carrying heparin and they reported adsorption values up to 12 mg cholesterol per gram polymer [31]. Sellergren et al prepared a series of highly cross-linked molecular imprinted terpolymers in the presence of cholesterol acting as a template molecule [32]. Using a physiological relevant intestinal-mimicking solution of cholesterol, these polymers adsorbed ca. 17 mg cholesterol per gram dry adsorbent. Yu et al developed a new LDL-adsorbent by using graft polymerization technique [33]. Yavuz and Denizli reached 13.3-16.0 mg/g cholesterol adsorption capacity with anti-LDL antibody attached poly(HEMA-MAPA) beads [34]. They also used random and protein A oriented anti-LDL antibody bound PHEMA beads and reached 8.2 and 11.7 mg/g cholesterol adsorption amount, respectively [35]. Yavuz et al prepared cholesterol imprinted poly(HEMA-MAT) particles and obtained 16.23 mg/g cholesterol adsorption capacity [36]. In vitro study showed that this adsorbent could remove LDL-cholesterol at level of 4.5 mg/g from human plasma. In this study, we showed that PHEMAAL/LMWH adsorbents exhibit comparable LDL binding capacities with affinity adsorbents including commercial media.

CONCLUSIONS

The obtained results proved the existence of good adsorptive properties of LMWH attached PHEMAAL membranes. Membrane based affinity techniques have several potential advantages over conventional approaches [37]. The membrane cartridges require high flow-rates with a much lower operating pressure than a packed bed column. In this method, the molecule to be removed can be directly transported by convection to the LMWH attached on the inner surface of the porous membrane, higher throughput and faster processing times onto the affinity membrane can be achieved. PHEMAAL membranes were produced by photopolymerization of HEMA. A bioligand, LMWH was then attached to these membranes to reach a loading up to 147.2 mg/g, which resulted a cholesterol adsorption amount of 22.6 mg/g from hypercholesterolemic human plasma.

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