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Preparation of immuno-affinity membranes for cholesterol removal from human plasma

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Abstract

Anti-low density lipoprotein antibody (anti-LDL) immobilized polyhydroxyethylmethacrylate (pHEMA) based membrane was prepared for selective removal of cholesterol from hypercholesterolemic human plasma. In order to further increase blood-compatibility, a newly synthesized comonomer, methacryloylamidophenylalanine (MAPA) was included in the membrane formulation. p(HEMA–MAPA) membranes were produced by a photopolymerization and then characterized by swelling tests, SEM and contact angle studies. Blood-compatibility tests were also investigated. The water swelling ratio of the p(HEMA–MAPA) membrane increases significantly (133.2.9%) compared with pHEMA (58%). p(HEMA–MAPA) 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. The maximum anti-LDL antibody immobilization was achieved around pH 7.0. Immobilization of anti-LDL antibody was 12.6 mg/ml. There was a very low non-specific cholesterol adsorption onto the plain p(HEMA–MAPA) membranes, about 0.36 mg/ml. Anti-LDL antibody immobilized membranes adsorbed in the range of 4.5–7.2 mg cholesterol/ml from hypercholesterolemic human plasma. Up to 95% of the adsorbed LDL antibody was desorbed. The adsorption–desorption cycle was repeated 10 times using the same membrane. There was no significant loss in the adsorption capacity.

Keywords: Immunoaffinity membranes; Cholesterol

1. Introduction

Familial hypercholesterolemia is an autosomal dominant disorder, characterized by elevated levels of low density lipoprotein (LDL), tendon xanthomas and premature coronary artherosclerosis [1–3]. 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 low density lipoprotein 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 lipoproteins and cholesterol from plasma, continues to be used despite certain disadvantages. Total plasma 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

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plasma exchange. There are considerable losses of proteins with molecular masses 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 treatments for removal of LDL based on affinity adsorption have attracted considerable attention. Lupien et.al. have prepared heparin containing agarose beads as an affinity adsorbent and clinically applied it in selective cholesterol apheresis [10]. Stoffel and Demont have demonstrated an immuno-adsorption system containing anti-apoprotein B-antibodies immobilized on Sepharose® for the treatment of hypercholesterolemia [11]. This system was clinically applied by Borberg et al., who were able to perform more than 3000 successful treatment sessions of familial hypercholesteroleamic patients [12]. Behm et al. used dextran sulfate immobilized cellulose beads for selective cholesterol removal [13]. Promising results have been recently reported with a polyacrylate coated fractogel system [14]. Lopukhin et al. have used heparin and chytozane sulphate containing macroporous silica adsorbents for cholesterol removal from familial hypercholesterolaemic human plasma [15]. Pokrovsky et al. have removed LDL from human plasma by using anti-LDL immobilized Sepharose® beads [16]. Denizli and Piskin have used heparin immobilized polyhydroxyethylmethacrylate beads and they removed cholesterol effectively from hypercholesterolemic human plasma in an in-vitro system [17].

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 packaging materials (i.e., beads), the fouling, and particularly the slow flow-rate through the column [18]. One significant trend has been to replace the bead packing by porous membranes that allow operation at low pressure [19]. 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, large internal surface area, high chemical, biological and mechanical stabilities, hydrophilicity, low non-specific adsorption of blood proteins and the presence of functional groups for derivatization [20]. Due to these advantages, porous affinity membranes provide higher efficiency.

Of course, the most selective adsorption can be achieved with the adsorbents containing antibodies as the ligand. For these reasons, we have focused our attention on the development of anti-LDL antibody immobilized immunoaffinity membranes. In this study, we prepared an adsorbent containing anti-LDL antibody for selective removal of cholesterol from hypercholesterolemic human plasma. In order to further increase the blood-compatibility we included a newly synthesized comonomer i.e., methacryloyl-amidophenylalanine (MAPA) into the membrane formulation. 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 [21]. Due to this reason, MAPA comonomer was synthesized. p(HEMA–MAPA) membranes were produced by a photo-polymerization technique. Membranes were characterized by swelling studies, SEM and contact angle tests. Blood-compatibility tests of modified membrane were also investigated. Then these membranes were activated by CNBr, and anti-LDL antibody were covalently coupled to the membranes. Anti-LDL antibody immobilization onto the CNBr activated membranes from aqueous solutions containing different amounts of anti-LDL antibody and at different pHs are reported here. LDL coupling on the anti-LDL antibody immobilized membranes from human plasma and membrane regeneration are also studied.

2. Experimental

2.1. Materials

Phenylalanine was purchased from BDH (Poole, UK) and used as received. Cyanogen bromide (CNBr) was purchased from Sigma (Saint Louis, Missouri, USA). Methacryloylchloride was supplied from Aldrich (Milwakuee, USA). The monomer, 2-hydroxyethylmethacrylate (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 (AIBN) was provided from Fluka (Switzerland). Anti-human β-lipoprotein (LDL) (developed in goat, delipidized whole antiserum, Product No: L 8016) was obtained from Sigma. 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.

2.2. Synthesis of MAPA monomer

For the synthesis of MAPA monomer, the following experimental procedure was applied: 5.0 g of phenylalanine and 0.2 g of $NaNO_2$ were dissolved in 30 ml of K_2CO_3 solution (5%, v/v). This solution was cooled down to 0 °C. Methacryloylchloride (6 ml) 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. The organic phase was dried with $MgSO_4$ and evaporated in a rotary evaporator. The residue (MAPA) was crystallized in an ether–cyclohexane mixture.

2.3. Preparation of membranes

The p(HEMA–MAPA) membrane was prepared as described in the above section. HEMA (2 ml) containing 5 mg AIBN as a polymerization initiator and 400 mg of synthesized MAPA was mixed with 3 ml of 0.1 *M* $SnCl_4$. 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; 350 μm in thickness) with a perforator.

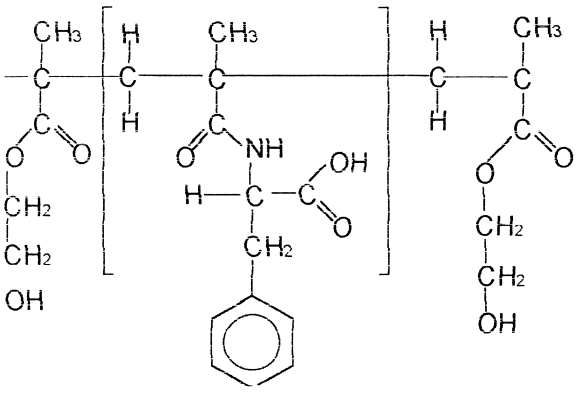

Fig. 1. Chemical structure of p(HEMA–MAPA) membrane.

The polymer chemical structure which is produced with HEMA and MAPA monomers is given in Fig. 1.

2.4. Characterization of membranes

2.4.1. Swelling test

Swelling ratios were determined in distilled water. The experiment was conducted as follows: initially dry membrane 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 membranes were calculated by using the following expression:

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

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

2.4.2. Contact angle measurements

The membranes were characterized by the air under water contact angle measuring technique. This device consists of a travelling goniometer with ×15 eyepieces a variable intensity light source and a micrometer-adjustable *X*–*Y* stage vertically mounted on an optical bench. The stage contains a plexiglass container in which a teflon plate is suspended. The

polymer sample is held on the underside of the teflon plate by means of small teflon clips. The container is then filled with triple distilled water and the plate with sample lowered into the container until the sample is completely immersed. A bubble of air with a volume of about 0.5 μl is then formed at the tip of the Hamilton microsyringe, below the surface, detached and allowed to rise to the polymer–water interface. The air bubbles were photographed at 25 °C within 5 min to reach equilibrium after contact with the membranes. The equilibrium contact angle (θ_{air}) was calculated from the height (h) and the width (b) of the air bubble at the membrane surface by using the following equation. The mean value of five contact angles measured on bubbles at different positions is considered. The reproducibility of contact angles is ±2%.

$$\theta_{air} = \cos^{-1}[(2h/b) - 1] \quad \text{for } \theta_{air} < 90° \qquad (2)$$

2.4.3. Microscopic observations

Microscopic observations and photographs of the the gold coated p(HEMA–MAPA) were performed by using a scanning electron microscope (SEM, Model: Raster Electronen Microscopy, Leitz-AMR-1000, Germany).

2.4.4. Blood compatibilty studies

2.4.4.1. Coagulation time (CT). Membranes were incubated in 0.1 *M* phosphate buffer solution (pH: 7.4) for 24 h at room temperature and washed on a glass filter with 0.5 *M* NaCl solution and distilled water. Fresh frozen human plasma (0.1 ml) was preheated to 37 °C for 2 min and then five pieces of membranes were added into this medium and mixed immediately. The clotting time was measured using the fibrometer method [22].

2.4.4.2. Activated partial thromboplastin time (APTT). Membranes were incubated in 0.1 *M* phosphate buffer solution (pH: 7.4) for 24 h at room temperature and washed on a glass filter with 0.5 *M* NaCl solution and distilled water. Fresh frozen human plasma (0.1 ml) was preheated to 37 °C for 2 min. Partial thromboplastin (0.3 ml, bioMerieux, Marcy-1'Etoile, France) was also preheated to 37 °C for 2 min and was added to preheated human plasma. Then, five pieces of membrane were transfered into this medium. Thirty seconds later, $CaCl_2$ (0.1 ml, 0.025 *M*) was added, then, the activated partial thromboplastin time (APTT) was determined using the fibrometer method [23].

2.4.4.3. Prothrombin time (PT). In order to determine prothrombin time (PT), a one-stage prothrombin method was used. Membranes were incubated in 0.1 phosphate buffer solution (pH: 7.4) for 24 h at room temperature. Fresh frozen human plasma (0.1 ml) was preheated to 37 °C for 2 min. The partial thromboplastin (0.3 ml, bioMerieux, Marcy-1'Etoile, France) was also preheated to 37 °C for 2 min and was added to preheated human plasma. Then, five pieces of membranes were added into this medium. Thirty seconds later, $CaCl_2$ (0.1 ml, 0.025 *M*) was transferred into the medium. After these operations, the prothrombin time was measured using the fibrometer method [22].

2.4.4.4. Cell adhesion studies. Heparinized human blood was incubated with five pieces of polymeric membranes for 1 h. It should be noted that prior to the blood contact, polymeric 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 count of samples were determined using a microscope.

2.4.5. CNBr activation

Prior to the 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 solutions (100 ml) with different initial concentrations (5–50 mg CNBr/ml distilled water) were prepared. The pH of this solution was quickly adjusted to 11.5 with 2 *M* NaOH. Membranes 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.

2.4.6. Anti-LDL antibody immobilization

The freshly CNBr activated 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 an anti-LDL antibody solution. In order to observe the effects of the CNBr initial concentration and pH on covalent coupling of anti-LDL antibody to the CNBr activated membranes, the CNBr initial concentration and the medium pH were varied between 10 and 100 mg/ml and 8.5–10.5, respectively. The initial concentration of anti-LDL antibody was 0.5 mg/ml. To obtain the effect of the anti-LDL antibody concentration on coupling, the initial concentration of anti-LDL antibody was varied between 0.05 and 0.50 mg/ml in which pH of the solution (containing 0.1 *M* $NaHCO_3$ + 0.5 *M* NaCl) was 9.5.

After coupling, the anti-LDL antibody immobilized 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 anti-LDL antibody immobilized on the CNBr activated membranes was determined by measuring the decrease of anti-LDL antibody concentration and also by considering the anti-LDL antibody adsorbed non-specifically (the amount of anti-LDL antibody adsorbed on the plain p(HEMA–MAPA) membranes), by the modified Lowry method. The amount of adsorbed anti-LDL antibody was calculated as:

$$q = [(C_i - C_t)V]/m \quad (3)$$

where q is the amount of anti-LDL antibody adsorbed onto unit volume of the membrane (mg/ml); C_i and C_t are the concentrations of the anti-LDL antibody 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 volume of the membrane (ml).

2.4.7. Cholesterol removal from human plasma

Cholesterol removal from human plasma on the plain and anti-LDL antibody immobilized membranes were studied in batch wise. The plasma with a total cholesterol initial concentration of 3.3 mg/ml was obtained from a patient with hypercholesterolaemia. 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. Plasma (10 ml), freshly separated from the patient, was incubated with the plain and anti-LDL antibody immobilized membranes at 20 °C for 4 h. Membranes containing two different amounts of anti-LDL antibody on their surfaces (i.e., 6.8 and 12.6 mg anti-LDL antibody/ml) were used. The amounts of cholesterol removed were determined colorimetrically by measuring the decrease in the cholesterol concentration in the plasma sample.

2.4.8. Desorption and repeated use

Desorption of cholesterol was studied in 0.1 *M* citric acid contained 0.02 *M* sodium phosphate (pH 3.0). Cholesterol adsorbed membranes were placed in this desorption medium and stirred (at a stirring rate of 600 rpm) for 30 min at room temperature. The total volume of desorption medium was 25 ml. The final cholesterol concentration in the aqueous phase was determined by using the same method which described in the previous section. The desorption ratio was calculated from the amount of cholesterol adsorbed on the membranes and the final concentration in the desorption medium.

In order to obtain the reusability of the anti-LDL antibody immobilized membranes, cholesterol adsorption–desorption procedure was repeated ten times by using the same adsorbent. It is worth to note that, after desorption of LDL, anti-LDL antibody release was also monitored.

3. Results and discussion

3.1. Membrane characteristics

p(HEMA–MAPA) membranes prepared in this study have a hydrophilic structure. They do swell in

aqueous solutions, but do not dissolve. The simple incorporation of water weaken the secondary bonds within the hydrogels. This enlarges the distance between the polymer chains and causes uptake of water. Compared with pHEMA (58%), the water swelling ratio of the poly(HEMA–MAPA) membrane increases significantly (133.2.9%). Several factors may contribute to this result. Reacting MAPA 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.

The scanning electron microscope (SEM) micrographs given in Fig. 2 show the surface and the cross-section of the p(HEMA–MAPA) membranes. As seen from the SEM photographs, the p(HEMA–MAPA) membranes have large pores (convective transport canals); the macropore dimensions are in the range of 5–10 μm. LDL is a large complex, of 100–300 Å in size [24]. Based on these data, it was concluded that the membranes had effective pore structures for efficient removal of LDL. The surface seems very rough and heterogeneous. In this case low diffusional resistance occurs due to the convective mass flow through the large pores. This facilitates mass transfer of LDL because of high inner surface area and also provides higher anti-LDL antibody immobilization.

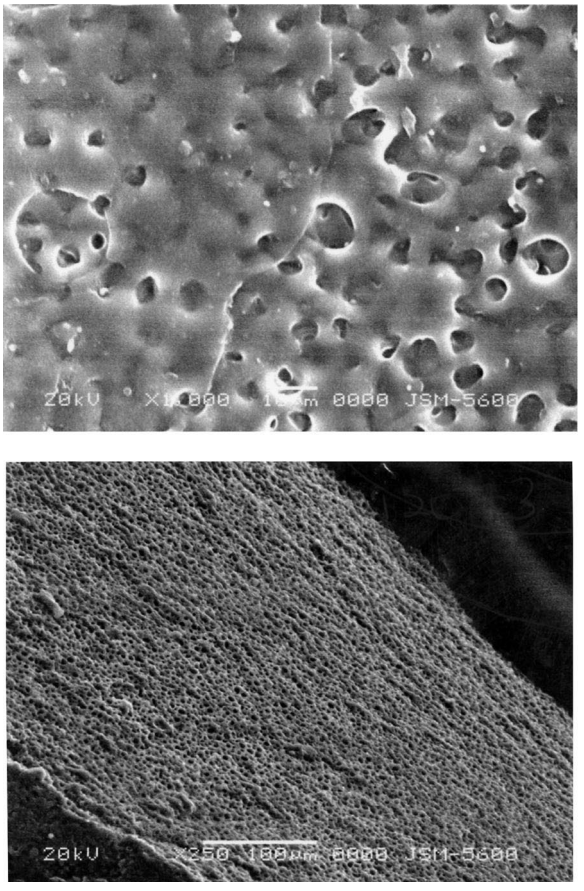

Fig. 2. The representative SEM micrographs of p(HEMA–MAPA): (a) surface; (b) cross-sectional area.

Characterization of the surface chemistry is a crucial issue in affinity technology [25]. Contact angle techniques are used routinely in the characterization of biomaterials to describe hydrophilicity or to estimate surface free energy. It is worth noting that the contact angles are measured through the water phase, so a relatively small contact angle values indicate a relatively more hydrophilic surface. There is a common opinion based on experimental evidence that blood-compatibility can be increased by rendering biomaterials in the sense of containing more hydrophilic groups in their active surface and/or within the material matrix. The equilibrium contact angle values were 45.3, 43.2 and 43.8° for the pHEMA, p(HEMA–MAPA) and anti-LDL antibody immobilized p(HEMA–MAPA) membranes, respectively. These values indicate that p(HEMA–MAPA) and anti-LDL antibody immobilized polymer surfaces are very wettable due to the orientation of hydrophobic phenyl groups through the bulk structure.

3.2. Blood compatibility studies

Table 1 summarizes the coagulation data. All the clotting times for p(HEMA–MAPA) membranes increased when compared with pHEMA membranes, although clotting times decreased when compared

Table 1
Coagulation times of human plasma (reported in seconds)

Experiments	APTT	PT	CT
Control Plasma	65.4	32.8	294
pHEMA	53.1	27.3	273
p(HEMA–MAPA)	62.3	30.8	285
p(HEMA–MAPA)/Anti-LDL	62.8	31.2	289

Each result is the average of six parallel studies.

with control plasma. However, these decreases are tolerable by the body [25]. Therefore, we concluded that the blood-compatibility of membranes was rather good, and the clotting times were quite reproducible comparing with the values reported in the related literature [25]. Consequently, in therapeutic affinity adsorption, the incorporation of MAPA 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 p(HEMA–MAPA) were 7.3 and 2.4%, respectively. Lost of leukocyte with pHEMA and p(HEMA–MAPA) were 5.8 and 1.9%, respectively. As seen here, there is no significant cell adhesion on the membranes. Surfaces modified with MAPA markedly reduced cell adhesion when incubated with human plasma. Therefore, incorporation of the MAPA 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 membranes are resistant to adhesion of blood cells (i.e., platelets and leukocytes). In conclusion, because of the good nonthrombogenic properties, microporous p(HEMA–MAPA) membranes seem to be very promising immuno-affinity adsorbent for biomedical applications such as extracorporeal immuno-adsorption therapy.

3.3. Anti-LDL antibody immobilization

Anti-LDL antibody was used as the affinity ligand for removal of LDL. Ligand leakage is a serious problem in biomedical applications due to undesired immunologic body reactions. Anti-LDL antibody leakage was investigated in phosphate-buffered saline solution consisted of 150 m*M* sodium chloride, 3 m*M* monobasic potassium phosphate, 7 m*M* dibasic potassium phosphate and 0.01% sodium azide, adjusted to pH 7.4 using sodium hydroxide pellets. Anti-LDL antibody leakage was not observed from anti-LDL antibody immobilized membranes. In the first part of this study, the effects of medium pH, initial concentrations of CNBr and anti-LDL antibody on the immobilization of anti-LDL antibody were investigated in batch adsorption-equilibrium studies.

The maximum anti-LDL-antibody immobilization was achieved around pH 7.0, which was assumed as optimal pH and used in the later part of this study. Note that both the three dimensional structure and the degree of ionization several groups on the anti-LDL antibody can change with pH. At pH 7.0, anti-LDL antibody probably has the most suitable structural properties for binding through CNBr activated sites on the surface of membranes. Significantly lower adsorption capacities were obtained below and above of pH 7.0.

High adsorption rates are observed at the beginning of adsorption process, and then saturation values (i.e., adsorption equilibrium) are gradually achieved within 60 min. Adsorption of anti-LDL-antibody molecules was rather fast, especially when the anti-LDL antibody concentration was high. This may be due to high driving force for mass transfer, which is the anti-LDL antibody concentration difference between adsorption medium (i.e, liquid phase) and the membrane (i.e, solid) phases, in the case of high initial anti-LDL antibody concentration.

Non-specific adsorption (adsorption on the p(HEMA–MAPA) membranes) was less than 0.74 mg anti-LDL antibody/ml, which may be considered as one of the advantages of using membranes as the

Table 2
Platelet and leukocyte adhesion with membranes

Substance	Platelet ($\times 10^{-3}$/mm^3)		Leukocyte ($\times 10^{-3}$/mm^3)	
	Initial/final	Loss (%)	Initial/final	Loss (%)
pHEMA	410/380	7.3	5.2/4.9	5.8
p(HEMA–MAPA)	410/400	2.4	5.2/5.1	1.9
p(HEMA–MAPA)/Anti-LDL	410/402	1.9	5.2/5.0	3.8

Each result is the average of three parallel studies.

carrier. The amount of anti-LDL antibody immobilized on the membranes increased by increasing the initial concentration of CNBr, up to 50 mg/ml, however, above this value, the effect was less pronounced. Note that a higher initial CNBr concentration corresponds to a higher number of activated sites on the carrier. Therefore, it was expected that the amount of anti-LDL antibody would increase with an increase in the initial CNBr concentration. Therefore, as expected, higher amounts of anti-LDL antibody are coupled on the CNBr activated p(HEMA–MAPA) membranes with higher number of activated sites. It should be noted also that, there is always a saturation capacity which depends on the number of functional groups on the matrix and the size of the ligand. However, the key factor in the performance of ligands immobilized on a solid surface is the ligand activity after coupling rather than the total number of ligands available for coupling. Binding anti-LDL antibody tightly to the surface on multiple interactions would certainly reduced effective utilization of active sites on the anti-LDL antibody.

Fig. 3 shows both non-specific adsorption and specific immobilization onto the p(HEMA–MAPA) membranes. The non-specific adsorption was very low, about 0.74 mg anti-LDL antibody/ml due to the hydrophilic nature of membranes. While specific immobilization of anti-LDL antibody first increased and then reached a saturation value (at 12.6 mg anti-LDL antibody/ml) at an initial anti-LDL antibody concentration of 0.4 mg/ml.

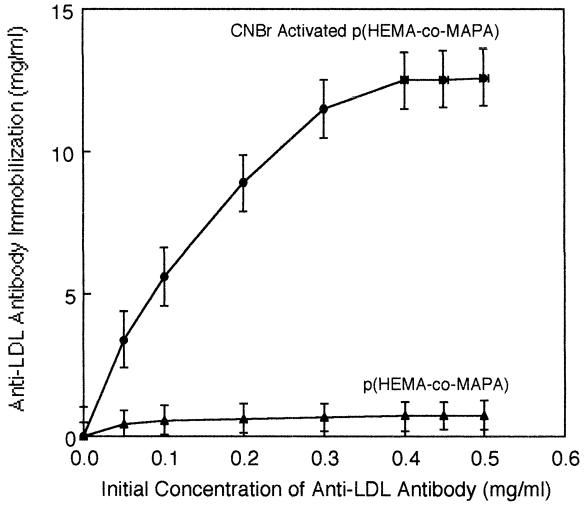

Fig. 3. Effect of anti-LDL antibody concentration on anti-LDL antibody immobilization onto p(HEMA–MAPA) membranes.

3.4. Cholesterol removal from human plasma

Fig. 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 plain p(HEMA–MAPA) which was about 0.36 mg cholesterol/ml for the 4 h incubation period (Curve A in Fig. 4). On the other hand, total cholesterol levels were significantly reduced when the anti-LDL antibody immobilized p(HEMA–MAPA) was used.

The anti-LDL antibody immobilized membranes containing 8.9 and 12.6 mg anti-LDL antibody/ml adsorbed 4.5 mg and 7.2 mg cholesterol/ml, respectively (Curves B and C in Fig. 4). As expected the cholesterol removal rate was much faster when the anti-LDL antibody content of the affinity adsorbent was higher. About 28 and 39% of the total cholesterol in the human plasma were removed when the anti-LDL antibody immobilized membranes contain-

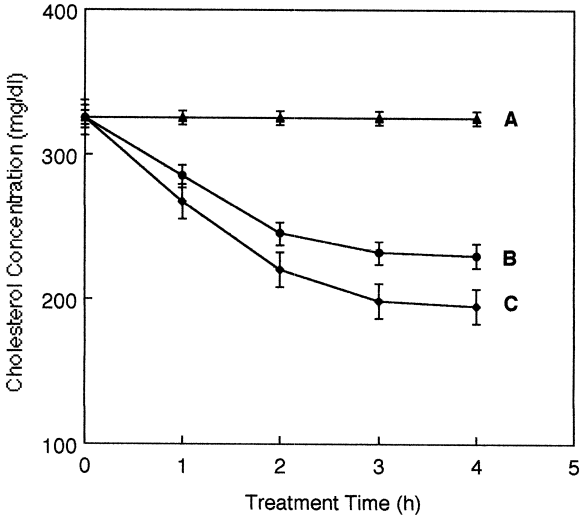

Fig. 4. Cholesterol removal from the hypercholesterolaemic human plasma (Total cholesterol initial concentration: 325.6 mg/dl). Curve A, adsorption onto plain membrane; curve B, adsorption onto membrane with 8.9 mg anti-LDL antibody/ml; curve C, adsorption onto membrane with 12.6 mg anti-LDL antibody/ml.

ing 8.9 and 12.6 mg anti-LDL antibody/ml, respectively, were used. The total cholesterol concentrations were reduced to about 2.3 and 1.95 mg/ml in 4 h, respectively, which represent a sufficiently low cholesterol level in hypercholesterolaemia. 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 Fig. 4, much higher adsorption values can be obtained for higher anti-LDL antibody loadings.

It is well known that the structure and composition of lipoproteins in normal and hypercholesterolemic patients may be different [24]. There are differences between their apolipoproteins as well as in the cholesterol content of the lipoprotein complexes. In this group of experiments, we have used fresh human plasma obtained from a healthy donor, in which the total cholesterol concentration was initially 2.1 mg/ml. The anti-LDL antibody immobilized membranes containing 8.9 and 12.6 mg anti-LDL antibody/ml adsorbed 2.4 mg and 3.2 mg cholesterol/ml, respectively. Figs. 4 and 5 show that, using anti-LDL antibody immobilized membranes, much more LDL can be removed from hypercholesterolemic human plasma than from normal human plasma.

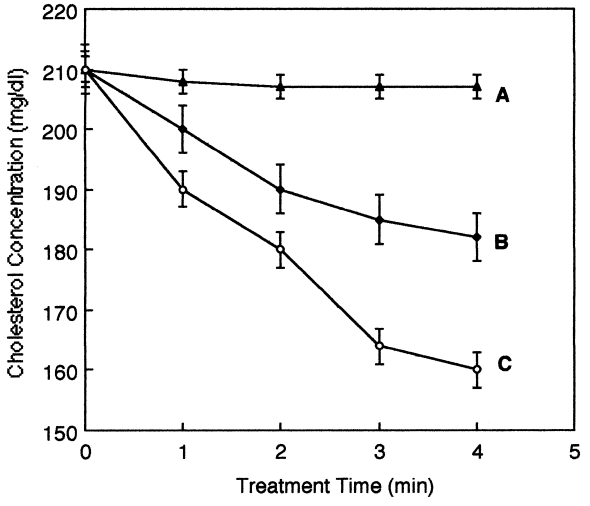

Fig. 5. Cholesterol removal from the normal human plasma (Total cholesterol initial concentration: 210 mg/dl). Curve A, adsorption onto plain membrane; curve B, adsorption onto membrane with 8.9 mg anti-LDL antibody/ml; curve C, adsorption onto membrane with 12.6 mg anti-LDL antibody/ml.

3.5. Comparison with related literature

Different affinity adsorbents have been reported in literature for cholesterol adsorption. Lopukhin et al. used macroporous silica beads as the carrier matrix, and immobilised heparin and chytozane sulphate as specific bioligand. They reported cholesterol adsorption capacities around 14.8–15.2 mg/g [15]. Pokrovsky et al. used commercial carrier made of Sepharose and monoclonal and polyclonal antibodies as the specific ligands [16]. Their maximum LDL binding capacities were in the range of 0.6–2.5 mg LDL per millilitre adsorbent. Denizli and Pişkin studied cholesterol removal from hypercholesterolemic human plasma and they reported maximum 4.7 mg cholesterol/g adsorption capacity with heparin immobilised PHEMA microspheres [17]. Tabak et al. used heparin immobilized agarose beads and they obtained in the range of 1.2–3.0 mg/g cholesterol adsorption capacity from healthy human plasma [24]. Ostlund reported 6–8 mg lipoprotein cholesterol per ml column volume with anti-LDL antibody attached commercially available Agarose beads from human plasma [26]. Smolik et al. were interested in determining the LDL adsorption capacity of dextransulfate immobilize cellulose affinity beads [27]. They achieved 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 [28]. Sinitsyn et al. used Sepharose beads carrying heparin and they reported adsorption values up to 12 mg cholesterol per gram polymer [29]. In this study, we demonstrated that anti-LDL antibody immobilized p(HEMA–MAPA) adsorbents exhibit comparable LDL binding capacities with affinity adsorbents including commercial media.

3.6. Desorption and repeated use

Desorption of LDL from the anti-LDL antibody immobilized membranes was also studied in a batch experimental set up. The anti-LDL antibody immobilized membranes loaded with LDL were placed within the desorption medium, and the amount of LDL desorbed in 1 h was determined. Elution buffer consisted of 0.04 *M* citric acid and 0.02 *M* sodium

Table 3
LDL Adsorption–desorption properties of anti-LDL antibody immobilized p(HEMA–MAPA) membranes

Anti-LDL antibody immobilized (mg/ml)	Adsorption (mg/ml)	Desorption (%)
Hypercholesterolemic human plasma		
8.9	4.5	97.2
12.6	7.2	96.8
Normal human plasma		
8.9	2.4	95.9
12.6	3.2	97.8

phosphate pH 3.0. Human plasma was used for repeated LDL adsorption–desorption cycles. Up to 95% of the adsorbed LDL was desorbed (Table 3). Note that there was no anti-LDL antibody release in this case which shows that anti-LDL antibody molecules are attached covalently to the p(HEMA–MAPA) membranes. This means that elution agent breaks down only the reversible bonds between anti-LDL antibody and LDL molecules. In addition, when citric acid was used as a desorption agent, anti-LDL antibody changes its three dimensional structure and release the bound LDL due to the decrease in interaction forces. With the desorption data given above we concluded that citric acid is a suitable desorption agent, and allows repeated use of these adsorbents used in this study.

For practical applications, the stability of the prepared adsorbent is very important. In order to verify the reusability of the anti-LDL antibody immobilized p(HEMA–MAPA) membranes, the adsorption–desorption cycle was repeated 10 times using the same membrane. There was no significant loss in the adsorption capacity of the membranes. The LDL adsorption capacity decreased only 2.8% after ten cycle. It was possible to reuse these adsorbents without significant losses in their LDL adsorption capacities. These results are encouraging and suggest that further studies should be done to develop p(HEMA–MAPA) immunoadsorbents.

4. Conclusions

The experimental results obtained proved the extistence of good adsorptive properties of anti-LDL antibody immobilized newly prepared p(HEMA–MAPA) membranes. Membrane based techniques has several potential advantages over conventional approaches. 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 ligand immobilized on the inner surface of the porous membrane, higher throughput and faster processing times onto the affinity membrane can be achieved. Microporous p(HEMA–MAPA) membranes, were produced by photo-polymerization of HEMA. A bioligand, anti-LDL antibody was then immobilized to these membranes to reach a loading up to 12.6 mg/ml, which resulted in a LDL adsorption of 3.2 mg/ml from hypercholesterolemic human plasma. It was possible to reuse these immuno-affinity membranes without remarkable reduce in the adsorption capacities.

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