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Short communication

Heparin-immobilized polyhydroxyethylmethacrylate microbeads for cholesterol removal: a preliminary report

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Abstract

Heparin-attached polyhydroxyethylmethacrylate (PHEMA) microbeads were investigated for specific removal of cholesterol from human and rabbit plasma. PHEMA microbeads were prepared by a suspension polymerization technique and activated by cyanogen bromide (CNBr) in an alkaline medium (pH 11.5). Heparin was then immobilized by covalent binding onto these microbeads. Cholesterol adsorption onto PHEMA microbeads containing two different amounts of immobilized heparin, i.e., 57.3 and 122.7 mg/g, from both hypercholesterolaemic human and rabbit plasma was investigated. The non-specific cholesterol adsorptions on the plain PHEMA microbeads were 0.47 mg/g and 0.30 mg/g from human and rabbit plasmas, respectively. About 35% and 32% of the cholesterol was removed from human and rabbit plasmas, respectively, when the heparin-immobilized PHEMA microbeads were used.

1. Introduction

Familial hypercholesterolaemia (FH) is an autosomal disorder characterized by high levels of low-density lipoprotein (LDL)-cholesterol in plasma which correlate directly with an increased risk for arteriosclerosis [1]. Arteriosclerosis in such forms as coronary heart disease and cerebrovascular disease are among the major causes of morbidity and mortality in all industrial countries. In addition to dietary and drug therapy, attempts have been made to remove cholesterol and LDL directly from plasma of patients suffering from FH. Plasma exchange, the first approach to removing lipoproteins-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 [2]. Cascade or double filtration plasmapheresis permit more selective cholesterol removal from plasma than does plasma exchange. This method also has certain disadvantages [3].

Extracorporeal treatment for removal of LDL based on affinity sorption has attracted considerable attention. Lupien et al. [4,5] have prepared heparin-containing agarose beads as an affinity sorbent system and clinically applied this in a selective cholesterol apheresis. In 1981, Stoffel and Demont [6] have demonstrated an immunoadsorption system containing anti-apoprotein B-antibodies immobilized on Sepharose for the treatment of hypercholesterolaemia. This system was clinically applied by Borberg et al. [7], who were able to perform more than 3000 successful

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treatment sessions of familial hypercholesterolaemic patients. Odaka et al. [8] and Behm [9] have used dextran sulphate-immobilized cellulose beads for selective cholesterol removal. Promising results have been recently reported with a polyacrylate-coated fractogel system [10]. Lopukhin et al. [11] have used heparin- and chytozane sulphate-containing macroporous silica sorbents from rabbit and familial hypercholesterolaemic human plasma. Pokrovsky et al. [12] have removed LDL from human plasma by using polyclonal and monoclonal anti-LDL antibody-immobilized Sepharose beads.

There are advantages and also disadvantages of the affinity sorbent systems exemplified above. Of course the most selective adsorption can be achieved with the sorbents containing monoclonal antibodies as the ligand. However, they are highly expensive and immunogenic, which means that they can not be used in direct hemoperfusion systems; rather they need a plasma separation system (e.g., plasmapheresis, apheresis) or other means of immunoisolation. Dextran sulphate is a much cheaper ligand, but with relatively low specificity to the LDL-cholesterol system.

Heparin is a class of mucopolysaccharides, termed glycosaminoglycans, and its structural subunit is a disaccharide composed of hexuronic acid, either D-glucuronic or L-iduronic, linked by a 1,4-glucosidic bond to glucosamine [13]. It has been very widely used as anticoagulant in many biomedical applications both in free and immobilized forms [14–16]. It is also well known that heparin rather specifically interacts with the LDL-cholesterol system [17]. Its blood-compatibility may be considered as an important advantage in direct-blood-contacting hemoperfusion columns. Therefore, heparin has been an attractive ligand in the related applications. Note that heparin-immobilized affinity sorbents have been applied in combination with a plasma separation system, mainly because of the limited blood-compatibility of the carrier system (e.g., agarose).

This short report describes our recent effort to prepare a bioaffinity sorbent containing heparin for selective removal of cholesterol. We have selected polyhydroxyethylmethacrylate (PHEMA)-based microbeads as the basic carrier matrix, by considering possible applications of these sorbents in direct hemoperfusion extracorporeal therapy [18,19]. It should be noted that PHEMA is one of the most widely used hydrophilic polymers in medicine, and has found a wide variety of biomedical applications due to its high biocompatibility [20–22]. We have produced PHEMA microbeads by a suspension polymerization technique and activated the hydroxyl groups on these microbeads by CNBr, and then heparin molecules were covalently coupled to the microbeads through the active sites. Cholesterol removal both from human and rabbit plasma was investigated.

2. Experimental

2.1. Preparation of PHEMA microbeads

The basic monomer, 2-hydroxyethylmethacrylate (HEMA), was purchased from Sigma (St. Louis, MO, USA) and was purified by vacuum distillation under a nitrogen atmosphere. The comonomer ethyleneglycoldimethacrylate (EGDMA, Merck, Darmstadt, Germany) was used as the crosslinking agent. The polymerization initiator was 2,2′-azobisisobutyronitrile (AIBN) (BDH, Poole, UK). The dispersion medium was a saturated aqueous solution of magnesium oxide (MgO) (Sigma).

PHEMA microbeads were prepared by a suspension polymerization technique [23]. Polymerization was carried out in an aqueous dispersion medium containing magnesium oxide which was used to decrease the solubility of the monomer HEMA in the medium. The monomer phase containing HEMA, EGDMA and AIBN was added to the dispersion medium within a laboratory type reactor (i.e., a two-neck flask with a volume of 500 ml) provided with a blade type stirrer. In order to produce polymeric microbeads of about 200 μm in diameter and with a narrow size distribution, the HEMA/EGDMA ratio, the monomer phase/dispersion phase

ratio, the amounts of EGDMA and AIBN, and the agitation speed were 1:3 (v/v), 1:10 (v/v), 0.33 (mol EGDMA/mol HEMA), 0.0015 (mol AIBN/mol HEMA), 600 rpm, respectively. Polymerization was carried out at 70°C for 3 h and then at 90°C for 1 h. After cooling, the polymeric microbeads were separated from the polymerization medium and residual MgO was removed by washing with a dilute HCl solution. The microbeads were also washed with water and ethanol, and then dried in a vacuum desiccator at room temperature.

2.2. CNBr activation

Prior to the activation process, the PHEMA microbeads were kept in distilled water for about 24 h and washed on a glass filter with 0.5 *M* NaCl solution and water in order to remove impurities. A cyanogen bromide (CNBr, Sigma) aqueous solution (100 ml, 20 mg CNBr/ml distilled water) was prepared. The pH of this solution was quickly adjusted to 11.5 with 2 *M* NaOH while it was magnetically stirred. One gram of PHEMA microbeads was then added to this solution and the activation procedure was continued for 60 min at a constant pH of 11.5. After the activation reaction, in order to remove the excess activation agent, the PHEMA microbeads were washed with 0.1 *M* $NaHCO_3$ and any remaining active groups (e.g., isourea) on the surfaces were blocked by treatment with ethanol amine (pH 9.1) and $FeCl_3$ solution for 1 h. Then, the activated PHEMA microbeads were washed four times with distilled water containing 0.5 *M* NaCl.

2.3. Heparin immobilization

Heparin (low molecular mass, 6000) was purchased from Sigma. One gram of the freshly CNBr-activated PHEMA microbeads was magnetically stirred at 50 rpm at a constant temperature of 20°C for about 2 h (i.e., equilibrium time) with 50 ml of a heparin solution. In order to observe the effect of the initial heparin concentration on covalent coupling of heparin to the activated PHEMA microbeads, it was varied between 2 and 4 mg/ml. The medium pH was 7.4 (phosphate buffer).

After coupling, the heparin-immobilized PHEMA microbeads were washed with 0.15 *M* NaCl and with 0.1 *M* $NaHCO_3$. The amount of heparin immobilized on the CNBr-activated microbeads was determined by measuring the decrease of heparin concentration and also by considering the heparin molecules adsorbed non-specifically (the amount of heparin adsorbed onto the plain PHEMA microbeads) and spectrophotometrically at 280 nm [23,24].

2.4. Cholesterol removal from human and rabbit plasma

Cholesterol removal from human and rabbit plasma on the plain and heparin-immobilized PHEMA microbeads was studied batch-wise. The plasma with an initial total cholesterol concentration of 269.48 mg/100 ml was obtained from a patient with hypercholesterolaemia. A male white rabbit of four months age was used as a test animal. Hypercholesterolaemia was achieved by feeding him with a cholesterol-containing synthetic diet [25], consisting of casein (25%), coconut oil (10%), crude fibre (10%), minerals (8.2%), vitamin mix (0.5%), cholesterol (0.25%, Merck) and corn starch to 100%. The initial cholesterol concentration in the plasma obtained from the rabbit was 225 mg/100 ml. Blood samples were centrifuged at 500 *g* for 30 min at room temperature to separate plasma. Volumes of 10 ml of the plasma freshly separated from the patient's blood and from the rabbit's blood were incubated with a 100 mg of the plain and heparin-immobilized PHEMA microbeads at 20°C for 4 h. PHEMA microbeads containing two different amounts of heparin on their surfaces (i.e., 57.3 and 122.7 mg heparin/g PHEMA) were used. The amounts of cholesterol removed were determined colourimetrically by measuring the decrease in the cholesterol concentrations in the respective plasma samples [26].

3. Results and discussion

We have used human plasma samples obtained from a hypercholesterolaemic patient, in which the total cholesterol concentration was initially 269.48 mg/100 ml. The plain and the heparin-immobilized PHEMA microbeads containing two different amounts of heparin (57.3 mg/g and 122.7 mg/g PHEMA) were incubated with the samples of this plasma for 4 h at room temperature. Fig. 1 shows the change of cholesterol concentration in the plasma during the incubation. There was a very low non-specific cholesterol adsorption onto the plain PHEMA microbeads which was about 0.47 mg cholesterol/g PHEMA for the 4 h incubation period (curve A in Fig. 1). On the other hand, total cholesterol levels were significantly reduced when the heparin-immobilized PHEMA microbeads were used. The PHEMA–heparin microbeads containing 57.3 and 122.7 mg heparin/g PHEMA adsorbed 3.09 mg and 4.71 mg cholesterol/g PHEMA, respectively (curves B and C in Fig. 1). As expected the cholesterol removal rate was much faster when the heparin content of the affinity sorbent was higher. About 23% and 35% of the total cholesterol in the human plasma was removed, respectively, with heparin-immobilized PHEMA microbeads containing 57.3 and 122.7 mg heparin/g PHEMA. The total cholesterol concentrations were reduced to about 210 and 175 mg/100 ml in 4 h, respectively, which are sufficiently low cholesterol levels in hypercholesterolaemia [27]. 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 sorbents. As can be easily concluded from the curves in Fig. 1, much higher adsorption values can be obtained with longer incubation periods. However, even the values presented above are values comparable with those reported in the related literature [8,11,12,28].

We have also studied cholesterol removal from plasma samples obtained from a hypercholesterolaemic rabbit (Fig. 2). Note that the total cholesterol concentration was initially 225 mg/100 ml. The PHEMA–heparin microbeads containing 57.3 and 122.7 mg heparin/g PHEMA were incubated with this plasma. Very low non-specific cholesterol adsorption onto the plain PHEMA microbeads was observed (0.30 mg cholesterol/g PHEMA, curve A in Fig. 2). Cholesterol levels in the rabbit's plasma were

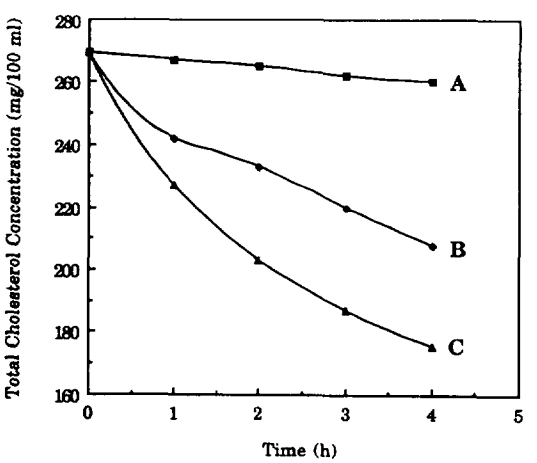

Fig. 1. Cholesterol removal from hypercholesterolaemic human plasma (initial cholesterol concentration: 269.48 mg/100 ml): curve A, adsorption onto plain PHEMA; curve B, adsorption onto PHEMA with 57.3 mg heparin/g PHEMA; curve C, adsorption onto PHEMA with 122.7 mg heparin/g PHEMA.

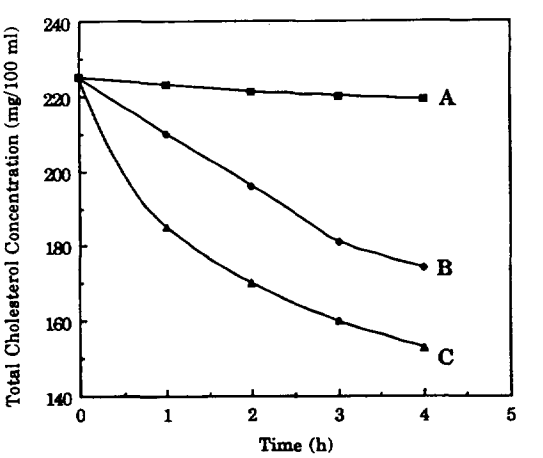

Fig. 2. Cholesterol removal from hypercholesterolaemic rabbit plasma (initial cholesterol concentration: 225 mg/100 ml): curve A, adsorption onto plain PHEMA; curve B, adsorption onto PHEMA with 57.3 mg heparin/g PHEMA; curve C, adsorption onto PHEMA with 122.7 mg heparin/g PHEMA.

significantly decreased, very similar to the data obtained with the human plasma. About 23% and 32% of the cholesterol in the rabbit's plasma was removed, respectively, by using heparin-immobilized PHEMA microbeads containing 57.3 and 122.7 mg heparin/g PHEMA. The total cholesterol concentrations were reduced to about 174 and 153 mg/100 ml in 4 h, respectively. The cholesterol removal increased with increasing amount of immobilized heparin. The microbeads containing 57.3 and 122.7 mg heparin/g PHEMA adsorbed 2.6 mg and 3.6 mg cholesterol/g PHEMA (curves B and C in Fig. 1), respectively.

As conclusion it can be said that cholesterol levels in blood plasma can be reduced within safe limits with relatively fast adsorption rates and rather selectively by using heparin-immobilized PHEMA microbeads developed in this study. Further ex vivo studies with animal models are under investigation.

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