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Immobilization of heparin on polysulfone surface for selective adsorption of low-density lipoprotein (LDL)

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ABSTRACT

A versatile method was developed to immobilize heparin covalently on polysulfone sheets (PSu) to achieve selective adsorption of low-density lipoprotein (LDL). This was achieved by activation of PSu with successive treatments of chlorodimethyl ether and ethylenediamine, and subsequent chemical binding of heparin with bifunctional linker molecules. A heparin density up to 0.86 μg cm^{-2} on a dense PSu film was achieved. The modified PSu films were characterized by attenuated total reflectance Fourier transform infrared spectroscopy and X-ray photoelectron spectroscopy. The hydrophilicity of the PSu film was improved greatly by covalent immobilization of heparin. The water contact angle of PSu film was decreased from 86.6 ± 3.7° to 50.5 ± 3.2° after binding of 0.36 μg cm^{-2} heparin. An enzyme-linked immunosorbent assay was used to measure the binding of LDL on plain and modified PSu films. It was found that the heparin-modified PSu film could selectively recognize LDL from binary protein solutions. Furthermore, it was possible to desorb LDL from heparinized PSu, but not from plain PSu, with heparin, sodium chloride or urea solution, which indicates a selective but reversible binding of LDL to heparin. The results suggest that heparin-modified PSu membranes are promising for application in simultaneous hemodialysis and LDL apheresis therapy.

1. Introduction

The abnormal elevated level of cholesterol in human blood has been confirmed to be a main risk factor for the process of coronary heart diseases, atherosclerosis and cerebral thrombosis. Cholesterol is carried in plasma by a series of lipoproteins, such as very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), intermediate density lipoproteins (IDL) and high-density lipoproteins (HDL). It is now believed that LDL is one of the major causative elements in the development of atherosclerosis [1,2]. High levels of LDL, especially coupled with low HDL concentrations, can promote cholesterol accumulation in the intra- and extracellular arterial wall that in turn leads to plaque formation causing various heart and vascular diseases [2–4]. Therefore, the reduction of LDL level in blood is used therapeutically to lower the risk of cardiovascular/cerebrovascular diseases [5–8].

In recent years, LDL has been efficiently eliminated by extracorporeal LDL apheresis systems [6–8]. Various general LDL apheresis procedures exist for routine clinical LDL elimination, such as unselective plasma exchange [9], semiselective double-filtration plasmapheresis (DFPP) [10], and there are other more selective techniques based on immunogenic or electrostatic interactions such as immunoadsorption using anti-LDL antibodies (LDL-Therasorb, Miltenyi Biotec, Bergisch Gladbach, Germany) [11], heparin-induced extracorporeal LDL precipitation (HELP, B Braun, Melsungen, Germany) [12,13], adsorption on dextran sulfate–cellulose (Liposorber, Kaneka Corporation, Osaka, Japan) [14], and direct adsorption of lipids from whole blood using polyacrylamide beads coated with polyacrylic acid (DALI-system, Fresenius, Bad Homburg, Germany) [15]. Normally, these apheresis systems, except for DALI, require the separation of blood cells and plasma by plasma filtration into a secondary circuit as a first step. For the HELP system, the process is even more complex and costly and a filtration is needed to remove the precipitated LDL complexes. Furthermore, a simultaneous LDL apheresis and hemodialysis procedure is required especially in treatment of patients with chronic renal failure and LDL-induced coronary heart disease [16]. In these cases, the membrane filtration must be applied during LDL apheresis procedures. Overall, procedures to carry out LDL apheresis are complicated by the additional burden for the patient. Simultaneous treatments of ESRD patients to continuously remove LDL during hemodialysis sessions without resorting to a combination of

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different methods would not only reduce costs, but probably also provide more safety and comfort for the patients.

For fabrication of LDL adsorbents, selection of an appropriate ligand and matrix is presently a major area of concern. Heparin, a highly sulfated polysaccharide, is known as one of the most efficient LDL ligands, which can interact with apolipoprotein B of LDL via electrostatic interactions [17,18]. For this reason different heparin-based adsorbents had been suggested, such as heparin-coupled Sepharose [8] and heparin-modified poly(vinyl alcohol) (PVA) granules [19]. Furthermore, heparin was used as ligand to prepare sensors for the measurement of LDL in blood [20,21].

Based on the complexity of the contemporary LDL apheresis procedures and the obvious advantage of a simultaneous LDL apheresis–hemodialysis treatment, we suppose that direct immobilization of a highly selective LDL ligand such as heparin on hemodialysis membranes may be useful for selectively adsorbing LDL in ESRD patients with hypercholesterolemia. As hydrophilized polysulfone (PSu) is currently the most widely used polymer for hemodialysis membranes, and enables efficient removal of small to medium-sized molecules [22,23], PSu was used here as matrix for heparin immobilization. The objective of this study was to explore different chemical routes to immobilize heparin on PSu films covalently with a high yield. Herein, a method to tether heparin onto a PSu surface is described, and the surface properties were analyzed by Fourier transform infrared spectroscopy (FTIR) and X-ray photoelectron spectroscopy (XPS) and water contact angle measurements. The ability of modified PSu to bind LDL selectively was studied by enzyme-linked immunosorbent assays (ELISAs). It was found that modification of PSu with heparin results in a material surface that can bind selectively and reversibly high quantities of LDL.

2. Materials and methods

2.1. Materials

Pure PSu granules were supplied by Fresenius Medical Care. Tetrahydrofuran (THF), tin(IV) chloride ($SnCl_4$), chlorodimethyl ether, ethylenediamine (EDA), 1-ethyl-3-(dimethyl-aminopropyl)carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), toluidine blue (TB), bovine serum albumin (BSA), human serum albumin (HSA, ~99%), primary antibody: anti-β-lipoprotein (LDL, antibody produced in goat whole antiserum), secondary antibody: anti-goat IgG (peroxidase antibody produced in rabbit), blocking buffer and Tween 20 were purchased from Sigma–Aldrich and used as received. LDL (5.8 mg ml^{-1}, Millipore), 3,3′,5,5′-tetramethylbenzidine (TMB, Care Roth GmbH), heparin (sodium salt, 166.0 U mg^{-1}, VWR International GmbH) and flat ELISA plates (Greiner Bio-One, Germany) were purchased and used as received. Water used in all experiments was deionized and ultrafiltered to 18 MΩ with a TKA MicroPure Water system.

2.2. Fabrication of PSu dense film

Pure PSu was dissolved in THF at about 25 °C for 24 h with vigorous stirring to form a 10 wt.% homogeneous solution. After air bubbles were removed completely, the solution was cast onto a clean glass plate using a casting knife with a 100 μm gate opening. The glass plate with the nascent film was directly dried for 24 h at 60 °C under vacuum. The dense film was peeled off and then dried for another 24 h at 80 °C under vacuum to ensure dryness before surface modification was conducted.

2.3. Immobilization of heparin on PSu surface

PSu was chemically activated according to the method of Higuchi et al. [24]. The PSu film was immersed briefly in a solution of chlorodimethyl ether, hexane and $SnCl_4$ at 25–30 °C for various reaction times (10 min–24 h) and then washed in methanol for 2 h. Thereafter, the chloromethylated PSu (PSu-CH_2Cl) was immersed into EDA at 25 °C to obtain amino groups for the subsequent chemical binding of heparin. Finally, EDA-modified PSu (PSu-NH_2) was submerged into heparin and EDC/NHS solution (5 mg ml^{-1} of heparin and EDC in citrate buffer solution: 0.2 M Na_2HPO_4 and 0.1 M citric acid, adjusted to pH 4.7 with 1 M NaOH, molar ratio of EDC to NHS = 1:1) for 24 h at 25–30 °C to bind heparin covalently. The modified PSu was taken out and washed three times with phosphate-buffered saline (PBS, 0.03 M Na_2HPO_4, 0.02 M KH_2PO_4, and 0.137 M NaCl, adjusted to pH 7.0 with 1 M NaOH).

2.4. Surface characterization

2.4.1. Heparin density on the modified PSu surface

The quantity of heparin bound to PSu surface was assayed by the toluidine blue (TB) colorimetric method according to the literature [25,26]. The assay is based on the fact that TB will irreversibly bind to a polyanion substrate (e.g. heparin-modified PSu). The amount of immobilized heparin can be calculated by comparison with a standard curve using soluble heparin of known concentration.

For a calibration curve, TB was dissolved in 0.01 mol l^{-1} hydrochloric acid containing 0.2 wt.% NaCl to prepare 0.005% TB solution. A series of heparin solutions with concentrations varying from 0 to 25 μg ml^{-1} were prepared by dissolving heparin in an aqueous 0.2 wt.% NaCl solution. Heparin standard solution (0.5 ml) was added to TB solution (0.5 ml) and then agitated for 30 s. Next, n-hexane (1 ml) was added, and the mixture was shaken well so that the heparin–TB complex was extracted into the organic layer. The non-extracted TB remaining in the aqueous phase was determined by measuring the absorption at 631 nm. A linear correlation between the concentration of heparin in the aqueous solution and the absorbance at 631 nm caused by the residual TB was obtained and used as a calibration curve to determine the quantity of immobilized heparin.

Accordingly, the heparin-modified PSu sheet film was cut into round size with a diameter of 1.35 cm and immersed in TB solution and incubated for 30 min. Subsequently, n-hexane was added and the mixture shaken well to ensure uniformity in treatment. After removing the PSu from the solution, the absorbance of aqueous layer at 631 nm was measured by UV–visible spectrophotometry. The quantity of immobilized heparin was calculated from the above constructed calibration curve. Each value was an average of five independent measurements.

2.4.2. FTIR and XPS

Attenuated total reflectance (ATR)-FTIR measurements were carried out on a Vector 22 FTIR (Brucker Optics, Switzerland) equipped with an ATR cell (KRS-5 crystal, 45°). Sixteen scans were taken for each spectrum at a normal resolution of 2 cm^{-1}. XPS spectra were recorded on a PHI-5000C ESCA system (Perkin-Elmer, USA) with Al K_α excitation radiation. The pressure in the analysis chamber was maintained at 10^{-6} Pa or lower during measurements. To compensate for the surface charging effect, all survey and core-level spectra were referenced to the C_{1s} hydrocarbon peak at 284.6 eV.

2.4.3. Water contact angle measurement

The hydrophilicity of the PSu film was characterized on the basis of water contact angle. Static contact angle was measured at room temperature on a contact angle goniometer (OCA20, Dataphysics, Germany) equipped with video capture. In a typical sessile drop method, a total of 2 μl of deionized water was dropped onto a