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Development of thrombus-resistant and cell compatible crimped polyethylene terephthalate cardiovascular grafts using surface co-immobilized heparin and collagen

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ABSTRACT

Short-term patency of polyethylene terephthalate (PET) cardiovascular grafts is determined mainly by the inherent thrombogenicity and improper endothelialization following grafts implantation. The aim of the present study was to immobilize heparin to develop thrombus resistant grafts. Additionally, collagen was co-immobilized to enhance the host cell compatibility. The synthetic woven and knitted forms of crimped PET grafts were surface modified by Denier reduction to produce functional carboxyl groups. The produced groups were used as anchor sites for covalent immobilization of heparin or co-immobilization of heparin/collagen by the end-point method. The modified surface was characterized using Fourier transform infrared spectroscopy and X-ray photoelectron spectroscopy. The biological activity of immobilized molecules was investigated *in vitro* using direct blood coagulation test, and "platelet deposition under flow condition. Furthermore, the biocompatibility of modified grafts with host cells was assessed using L929 cell as model. All modified grafts showed significant resistance against fibrin and clot formation. The number of deposited platelets on heparin-immobilized woven and knitted grafts obviously decreased by 3 fold and 2.8 fold per unit surface area respectively, while the heparin/collagen co-immobilized grafts showed only a decrease by 1.7 and 1.8 fold compared to unmodified PET. Heparin-immobilized grafts reported no significant effect on L929 cells adhesion and growth ($P > 0.05$), conversely, collagen co-immobilization considerably increased cell adhesion almost ~1.3 fold and 2 fold per unit surface area for woven and knitted grafts respectively. Our results emphasize that immobilization of heparin minimized the inherent thrombogenicity of the PET grafts. The simultaneous co-immobilization of collagen supported host cell adhesion and growth required for the grafts biocompatibility.

1. Introduction

Polyethylene terephthalate (PET) Dacron® grafts are promising alternatives for autologous grafts. The wide cardiovascular applications of PET grafts including; vascular prostheses [1,2], heart valve sewing cuffs [3,4], and surgical meshes [5] are associated with hemocompatibility problems [6,7]. One of the prominent shortcomings of PET is the substantial thrombogenic property that impaired ultimately the patency of the graft and hence, the patient's life quality. After implantation, the blood coagulation cascade is mostly triggered at the graft-blood interface by the adsorption of plasma proteins on the graft surface followed by activation of platelets and complements in blood serum, and finally leads to formation of non-soluble fibrin and thrombosis [6,7]. However, the previous studies suggest that the sequences of protein deposition, fibrin formation, and platelets aggregation on graft surface showed dependency mainly on the surface properties [7].

One potential strategy to reduce the thrombogenicity of prosthetic material is by immobilization of bioactive molecules to the graft surface. Recently, several studies have reported to the coating of heparin on surface as an effective method to create a thrombus-resistant graft [8–10]. Heparin, an anticoagulant non-branched mucopolysaccharide, prevents blood coagulation cascade by two ways; either directly by capture and inactivation of thrombin, or indirectly by stimulation of anti-thrombin enzyme (AT III). Thrombin is an enzyme that controls the conversion

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of soluble fibrinogen to precipitated fibrin fibers [11]. Additionally, a successful way to limit graft failure is to provide rapid, uniform, and complete coverage with an endothelial layer. Accordingly, cell growth on graft surface was enhanced using biomolecules such as insulin, transferrin, and peptides [12–14], whereas, tropoelastin, gelatin and collagen were used to stimulate cell adhesion and growth [15–17]. To the best of our knowledge, the covalent immobilization of heparin on woven and knitted forms of crimped PET cardiovascular grafts (Fig. 1) is rarely addressed. We aimed to improve the blood compatibility properties to reduce thrombosis and platelet deposition on two different forms of crimped PET grafts by surface modification.

Denier reduction was employed to selectively cleave the ester linkage within PET surface resulting in free carboxyl group as schematically shown in Fig. 2. Consequently, heparin was covalently immobilized to the resulting carboxyl group by the end-point attachment technique to maintain its chemical stability and bioactivity after immobilization process. To improve the endothelialization of cardiovascular PET grafts, the potential effect of collagen co-immobilization was evaluated. The surface modified PET was characterized by Fourier transform infrared spectroscopy (FTIR) and X-ray photoelectron spectroscopy (XPS). The biocompatibility of modified crimped PET grafts was confirmed *in-vitro* by direct blood coagulation test and platelet deposition under continuous flow condition. In addition, mouse L929 fibroblast cell line was used as model to assess the host cell adhesion and growth on the modified grafts.

2. Materials and methods

2.1. Materials

Woven and knitted forms of crimped Dacron®; polyethylene terephthalate PET grafts (14 mm internal diameter, 0,45 mm wall thickness, 30 cm length and 12 filaments per yarn bundle) were kindly provided by Vascutek GmbH, Germany. Heparin sodium salt; 196 USP (U/mg), 1-ethyl-3-(3-dimethylamidopropyl) carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (Sulfo-NHS), glutaraldehyde, toluidine blue, and ethylenediamine were purchased from Sigma Aldrich, Germany. Sodium hydroxide was supplied by Carl Roth, Germany. Paraformaldehyde, Tris buffer, and Giemsa stain were bought from Merck, Germany. Water soluble collagen MW 80.000 and 2-N-morpholinoethanesulfonic acid (MES) were acquired from Serva, Germany. Mouse L929 fibroblasts cells line were obtained from DSMZ, Braunschweig, Germany. Dulbecco's Modified Eagle's Medium (DMEM), Earle's Balanced Salt Solution (EBSS), gamma irradiated Fetal Bovine Serum (FBS), trypsin, streptomycin, penicillin, and amphotericin B were purchased from PAA Laboratories GmbH, Germany. L-glutamine was bought from VWR, Germany. All other used chemicals were of analytical reagent grade.

2.2. Methods

2.2.1. Surface modification of crimped PET grafts

In the present study, two different forms of crimped PET cardiovascular grafts, woven and knitted as shown in Fig. 1, were used for surface modification. All steps in the subsequent experiments were performed in triplicate for each form. Modified Denier reduction was used to introduce functional carboxyl groups on the surface of crimped PET grafts according to the method reported by Kissa [18] as schematically illustrated in Fig. 2. Briefly, slices (2×2 cm^2) from woven and knitted forms of crimped PET grafts were cleaned in 20 ml of 50% ethanol solution for 15 min in an ultrasonic water bath (Sonorex RK 100H, Bandelin, Germany). Subsequently, the grafts were rinsed several times with distilled water under stirring and then oven dried at 55 °C for 2 h. The cleaned grafts were dipped in 20 ml of 1% NaOH solution and incubated in a boiling water bath for 1 h. The modified grafts were rinsed with distilled water and oven dried at 55 °C for 2 h. The obtained grafts are abbreviated as PET-COOH.

2.2.2. Grafting of ethylenediamine onto PET-COOH

Ethylenediamine was grafted onto previously produced carboxyl group using the adapted zero-length cross linker reaction known as carbodiimide plus sulfo-NHS as previously reported [19]. Crimped PET-COOH were immersed in 15 ml of MES buffer (0.1 M, pH 5.5) and hydrated for about 2 h. Carboxyl group on the surface was activated by adding 0.1 M of EDC and 5 mM of sulfo-NHS and incubated under gentle stirring at 100 rpm/min (IKA C-MHGHS7, Germany) at room temperature for 3 h. A solution of 50 mM of ethylenediamine was slowly added to the activated PET-COOH. The conjugation between one terminal amino group of ethylenediamine and one activated carboxyl group on the surface of PET-COOH was completed overnight at room temperature while stirring. Afterwards, grafts were rinsed with distilled water under sonication for 5 min and dried under dust free-airflow. The obtained grafts are abbreviated as PET-NH_2.

2.2.3. Immobilization of heparin onto PET-NH_2

Heparin was covalently immobilized onto PET-NH_2 using homobifunctional cross-linker glutaraldehyde as described elsewhere [20]. Briefly, PET-NH_2 (2×2 cm^2) were immersed in 10 ml sodium phosphate buffer (0.1 M, pH 6.8) containing 150 μl of 50% glutaraldehyde and incubated overnight at room temperature under gentle stirring at 100 rpm/min. Afterwards, grafts were rinsed with distilled water under sonication for 5 min and dipped into 10 ml of heparin solution (1 mg/ml) in sodium carbonate buffer (0.5 M, pH 9.5) at 4 °C. The excess aldehydes and the formed Schiff bases were reduced by adding 500 μl Tris buffer (0.2 M, pH 8.5) and incubated at 4 °C for 1 h. Finally, the grafts were washed with sodium phosphate buffer and rinsed with distilled water under

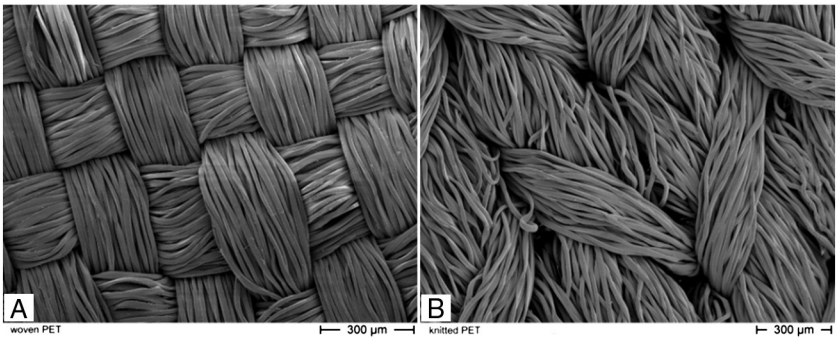

Fig. 1. SEM micrographs of unmodified woven (A) and knitted (B) forms of crimped PET grafts. The multifilament PET threads in woven grafts are fabricated in an over-and-under pattern, while PET threads in knitted grafts are looped.