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Bioresorbable, heparinized polymers for stent coating: *in vitro* studies on heparinization efficiency, maintenance of anticoagulant properties and improvement of stent haemocompatibility

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Biodegradable poly-(D,L-lactide) RESOMER® R208 and poly(D,L-lactide-co-glycolide) RESOMER® RG756 were heparinized to improve the blood contacting properties of the materials. The immobilization of heparin was performed with glutaraldehyde as coupling agent. The efficacy of the surface modification was monitored with respect to the total amount of bound heparin measured by a toluidine blue assay, the anticoagulant potential estimated by a factor Xa assay, and the activation of platelets estimated by a GMP140 assay. It was found that a reaction at ambient temperature for 2 h resulted in optimal heparin binding, with high anticoagulant activity and low thrombogenicity. The storage of heparinized polylactide in saline solutions up to 8 days demonstrated the release of small quantities of heparin into the fluid. A further finding was that with prolonged storage the anticoagulant potential was improved, whereas the thrombogenicity decreased. Comparison of platelet activation on RESOMER® R208 as unmodified and heparinized material with polypropylene and Pellethane® revealed that heparinization of R208 substantially improved the haemocompatibility. Coating and subsequent heparinization of intravascular stents were carried out with RESOMER® RG756 because of its more appropriate mechanical properties. *In vitro* studies with blood under flow conditions demonstrated that platelet activation on Palmaz™ stents was considerably diminished after polymer coating and heparinization.

1. Introduction

Intravascular stenting is often used after angioplasty to prevent a reocclusion of the damaged vessel following dilatation. One problem inherent to stent implantation is a possible restenosis that is observed in a number of patients up to several months after treatment [1, 2]. The process of restenosis is attributed to myointimal hyperplasia as well as to thrombus formation on the metallic stent surface [2, 3]. The interaction of platelets with the stent surface may have considerable significance not only due to their involvement in thrombus formation, but also by the release of platelet derived growth factor that may be included in the stimulation of smooth muscle cell growth [2, 4]. Thus, the major disadvantage of metallic stents is their limited blood contacting properties [2, 3]. The use of polymeric stents with improved haemocompatibility may reduce thrombus formation and hence possible reocclusion of the vessel. However, those stents have to be more bulky than those made of metal to guarantee a comparable rigidity [2]. The construction of a composite device made of a metallic stent with a polymeric surface coating might overcome the problems involved in the application of both types of stents.

The application of biodegradable polymers such as polylactides and their co-polymers for stent coating may prevent the release of toxic substances, since the biocompatibility of polylactides (PLA), and polyglycolides (PGA) was reported to be good [5]. Choosing a bioresorbable polymer with a degradation time longer than three months an improved ingrowth of the

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stent is suggested because endothelization may be in good progress [2, 3]. Besides the good tissue compatibility of PLA/PGA little is known about its blood contacting properties. Indeed the haemocompatibility of polymers may be enhanced by covalent binding of heparin [6].

The aim of this study was twofold. First, it was tried to optimize the binding of heparin to PLA using a simple immobilization process with glutaraldehyde as coupling agent. The efficiency of the binding reaction was controlled by the measurement of the overall heparin immobilization, the anticoagulant activity of bound heparin and the activation of platelets on the heparinized surfaces. The maintenance of biological activity after heparinization was tested by a prolonged storage of heparinized PLA in physiological buffer. Second, Palmaz™ stents were coated with a polylactide–co-glycolide and the thrombogenicity of plain metallic, of polymer coated and heparinized stents was compared with respect to platelet activation under flow conditions in a closed loop system. Details are reported herein.

2. Materials and methods

2.1. Materials

2.1.1. Polymers and stents

Commercial biodegradable polymers (poly(D,L- lactide) RESOMER® R208 and poly(D,L-lactide–co-glycolide) RESOMER® RG756, Boehringer-Ingelheim, Germany) were selected as materials for the immobilization of heparin because degradation time of these materials was reported to be longer than three months [7]. Cast sheets of the polymers were prepared from a 5% solution in chloroform after evaporation of the solvent. For the polymer coating of stents (Palmaz™ stent P308M, Johnson & Johnson Interventional Systems Co., USA) the metallic bodies were immersed four times in a 5% polymer solution in chloroform for about 10 s with 24 h evaporation time between immersions.

The reference materials polypropylene and Pellethane® 2363-80 AE were kindly supplied by Dr W. Lemm (Free University Berlin, Klinikum Charlottenburg).

2.1.2. Blood

Blood was drawn from healthy volunteers who had had no medication for at least one week. The blood was collected in sodium citrate (3.13 g/100 ml) at a blood/citrate ratio of 9 : 1. Platelet-rich plasma (PRP) was prepared by centrifugation. Platelet count was adjusted to 200 000/µl.

2.2. Methods

2.2.1. Heparin immobilization

The immobilization of heparin was performed using porcine mucosa heparin (Serva Biochemistry, Germany, 174 I.U./mg) with glutaraldehyde (GA, Sigma, USA) as coupling agent [8]. 12% aqueous solution of heparin and 6% aqueous solution of GA (1 : 1) were mixed and adjusted with 0.1 M sulphuric acid to pH 5.2. Polymer sheets and the polymer coated stents, respectively, were incubated in this solution for 2 h and at the temperature indicated. The polymer samples were then washed thoroughly with distilled water.

2.2.2. Toluidine blue assay

The total amount of surface immobilized heparin was measured by a modified toluidine blue coloric method [9]. Sheets of the modified RESOMER® (1 cm^2) were incubated with 0.5 ml phosphate buffered saline (PBS) and 0.5 ml 0.005% aqueous toluidine blue solution (TB, Sigma, USA) for 5 min under shaking. After addition of 1 ml hexane the mixture was shaken for further 30 s, followed by a phase separation. The optical density of the aqueous phase was determined at 630 nm (Spectronic® Genesys™ 5 spectrophotometer, Milton Roy, USA). The amount of immobilized heparin was quantified by comparison with a standard curve of known concentrations of heparin.

2.2.3. Factor Xa assay

For controlling the anticoagulant activity of immobilized heparin a factor Xa chromogenic assay was developed. Prewetted RESOMER® sheets (d = 11 mm) were incubated in 0.075 ml antithrombin III (AT III, 0.04 I.U./ml) for 5 min. Factor Xa (FXa, 0.3 ml, 0.32 I.U./ml) was added and the mixture was incubated for 5 min. Thereafter the chromogenic substrate S-2222 (0.3 ml, 0.55 mM, all Haemochrome Diagnostica, Germany) was added. The hydrolysis of S-2222 was stopped with 0.3 ml 20% acetic acid after 2 min. The resulting chromophore was measured spectrophotometrically at 405 nm (Spectronic® Genesys™5 spectrophotometer, Milton Roy, USA). A comparison with a standard curve of known activities of heparin was used to estimate the amount of immobilized biologically active heparin per surface area.

2.2.4. GMP140 assay

The thrombogenicity of pure and heparin-modified RESOMER® was tested after blood–material contact with an enzyme immuno assay (EIA) for GMP140, a marker of platelet activation [10]. Briefly, after 30 min contact of PRP with the samples, platelet activation was assessed using the monoclonal mouse antibody CD 62 (Immunotech S.A., France) and the polyclonal sheep anti-mouse IgG peroxidase-conjugated antibody (Sigma Immuno Chemicals, St. Louis, USA). O-phenylene diamine (Sigma) and a 1 : 1 mixture of 2 M H_2SO_4 and 0.1 M Na_2SO_3 were used as chromogenic substrate and stopping reagent, respectively. Aliquots of the substrate solution were measured at 492 nm (Anthos Plate Reader, Austria).

2.2.5. Immunofluorescence microscopy

The degree of platelet adhesion and spreading on the different materials was further investigated with immunofluorescence microscopy. After 30 min contact of

PRP with the materials at 37 °C, samples were washed with PBS followed by a fixation with 3% paraformaldehyde. Labelling of platelets was performed with a monoclonal mouse antibody CD 41a (Immunotech., S.A., France), followed by polyclonal goat anti-mouse IgG antibody, conjugated with fluorescein (Jackson Immuno Research, USA). Immunofluorescence microscopy was carried out with a Jenamed Fluorescence Microscope (Carl Zeiss Jena, Germany).

2.2.6. Dynamic conditions

The haemocompatibility of stents was estimated *in vitro* with whole blood under flow conditions. For this purpose a closed-loop system was developed, consisting of a peristalic pump (Verder 505 RL, Watson-Marlow, USA) with three pump heads. Three silicone tubes of 400 mm length and 4.8 mm diameter were used, as well as a small blood reservoir for each tube. In order to prevent interaction of these materials with blood, these components of the flow system were immersed in serum albumin (1 mg/ml) for 24 h and washed afterwards with PBS. Experiments were carried out inserting either one plain, one polymer coated or one heparinized stent into the silicone tube. Whole blood was pumped through the tubes with a flow rate of 240 ml/min for 30 min. Thereafter the tubes were flushed with PBS and the stents were removed. Activation of platelets was quantified with the EIA for GMP140 as described above.

3. Results and discussion

The influence of the reaction temperature on heparin immobilization and its biological activity on RESOMER® R208 is shown in Fig. 1. It was found that a reaction temperature of 22 °C was optimal. Nevertheless lower heparin binding at 22 °C the anticoagulant activity of heparin was maximal, whereas the thrombogenicity indicated by the presence of GMP140 was minimal. The decreasing anticoagulant activity of heparin, as well as the increasing platelet activation with the rise in the reaction temperature, was most probably due to multipoint attachment of heparin to the substrate, since it is known that glutaraldehyde is a coupling agent [8]. Moreover, it is possible that crosslinking of heparin occurs to a certain degree. Both processes would reduce the molecular mobility of the heparin molecule inhibiting its biological function [11, 12].

Since there are almost no data in the literature on the haemocompatibility of PLA, experiments were performed to assess the relative thrombogenicity of PLA in comparison to polymers often applied in contact with blood. A comparison of RESOMER® R208 heparinized for 2 h at 22 °C with the non-modified R208, polypropylene and Pellethane® was carried out with the EIA for GMP140 (Fig. 2). It was found that the heparinized R208 possessed a similar low thrombogenicity like Pellethane®. This finding was confirmed qualitatively with immunofluorescence microscopy shown in Fig. 3. On the more thrombogenic materials polypropylene (Fig. 3a) and pure R208

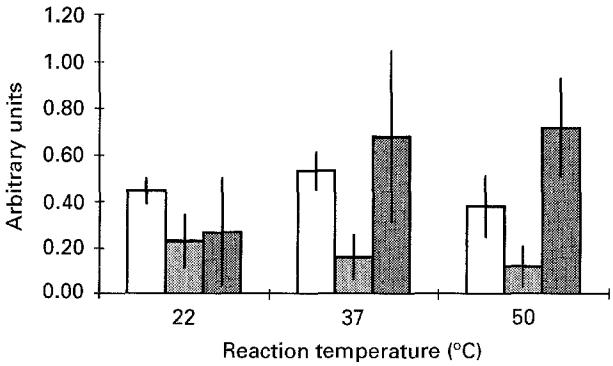

Figure 1 Total heparin content (TB) (□), anticoagulant activity of immobilized heparin (FXa) (▨) and platelet activation (GMP 140) (▩) of heparin-modified RESOMER® R208 with respect to reaction temperature (means ± S.E.M., $n = 5$; arbitrary units 1.0 = 1.0 I.U./cm² (TB), = 0.1 I.U./cm² (FXa), = 1.0 O.D./492 nm (GMP140))

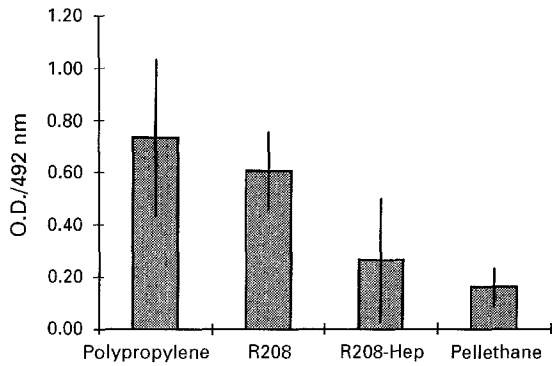

Figure 2 Comparison of platelet activation on polypropylene, non-modified and heparin-modified RESOMER® R208 (2 h, 22 °C) and Pellethane® measured by GMP140 assay (means ± S.E.M., $n = 5$)

(Fig. 3b) a high number of platelets was adherent. Most of these platelets were completely spread, although the extent of surface coverage was lower on R208 in comparison to polypropylene. In contrast to these materials the heparinized R208 (Fig. 3c) and Pellethane® (Fig. 3d) expressed excellent haemocompatibility, indicated by a very moderate adhesion of platelets with almost no formation of pseudopodia. Spreading of platelets was not observed. From these results obtained under static conditions, good haemocompatibility of heparinized R208 under flow conditions was concluded, as known from previous studies for Pellethane® [13, 14].

Because of the intended application of the heparinized RESOMER® as a surface coating for intravascular stents, a longer lasting stability and biological activity of the heparinization was needed. A first attempt was made to assess the stability of heparin immobilization *in vitro* by the incubation of treated foils in PBS at ambient temperature for up to 8 days. The results are shown in Table I. It was found that there was a continuous release of heparin (line 1). At the same time the heparin content of the surface estimated with the TB assay dropped to about 50% of the initial concentration (see line 2). More interesting, however, was the fact that the anticoagulant activity, measured with the factor Xa assay, increased from day

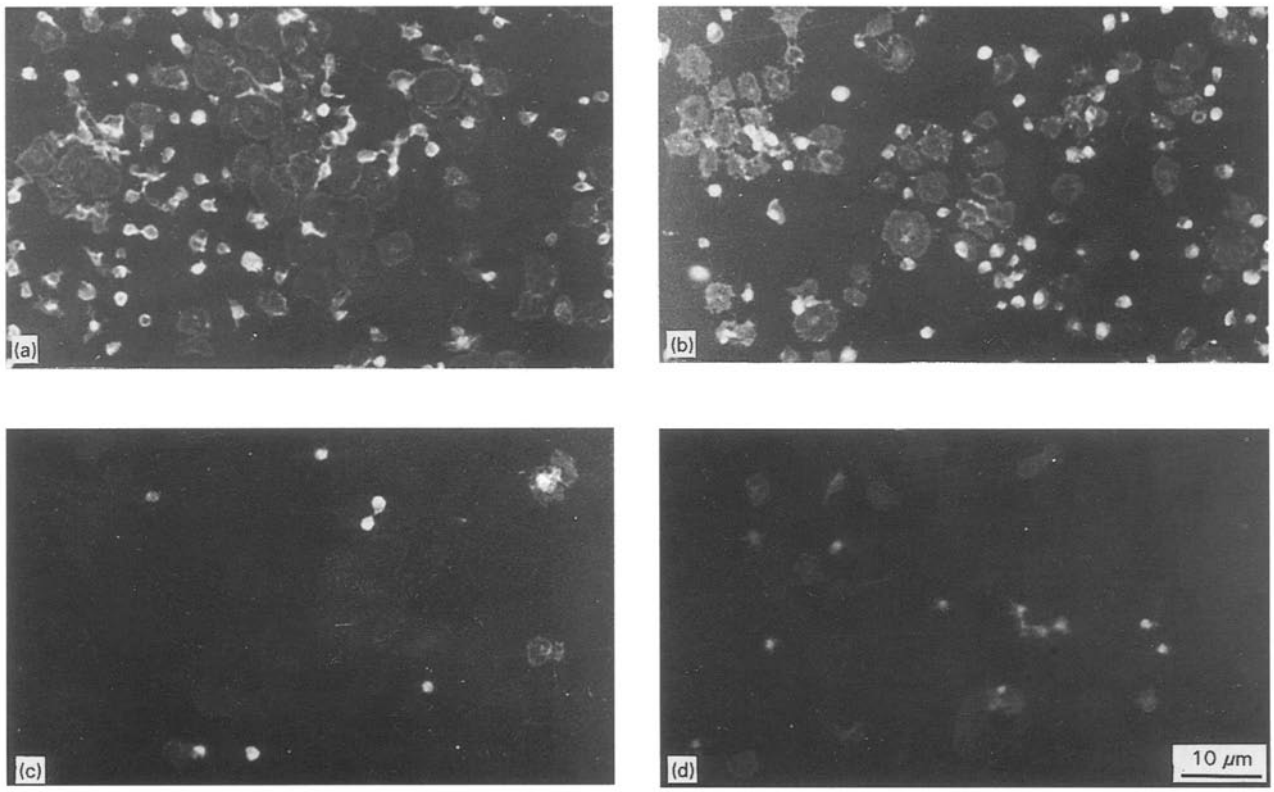

Figure 3 Immunofluorescence microscope images: (a) polypropylene; (b) non-modified R208; (c) heparin-modified R208 (2 h, 22 °C); (d) Pellethane® after contact with platelet rich plasma for 30 min.

TABLE I Total heparin content of supernatant and RESOMER® R208 surface (TB), anticoagulant activity of immobilized heparin (FXa) and platelet activation of heparin-modified RESOMER® R208 (GMP140) after incubation with phosphate buffered saline for up to 8 days (means ± S.E.M., $n = 5$)

Sample	Incubation time in PBS (days) 1	2	5	8
Heparin in supernatant (TB, I.U./ml)	0.41 ± 0.16	0.25 ± 0.09	0.28 ± 0.09	0.41 ± 0.14
Heparin on surface (TB, I.U./cm^2)	0.20 ± 0.12	0.24 ± 0.12	0.13 ± 0.05	0.14 ± 0.06
Anticoagulant heparin (FXa, I.U./cm^2)	0.010 ± 0.008	0.010 ± 0.008	0.009 ± 0.005	0.020 ± 0.004
Platelet activation (GMP140, O.D./492 nm)	0.45 ± 0.05	0.33 ± 0.10	0.20 ± 0.07	0.15 ± 0.05

5 to day 8 (line 3). In addition the thrombogenicity estimated by the GMP140 assay decreased (line 4). Therefore, it was assumed that even for longer contact with fluids, such as blood, the desired properties of the surface coating may be preserved. As a possible reason for the obvious improvement of the biological properties of the heparinized RESOMER® it is suggested that beginning degradation of PLA, and a breakdown of acetal bridges of the spacer (glutaraldehyde) leads to a better mobility of still attached heparin molecules. Better molecular mobility of heparin may improve its interaction with AT III and its antithrombogenic potential [12, 15], thus improving the haemocompatibility.

Dynamic studies measuring the amount of activated platelets with the GMP140 assay after contact with whole blood were carried out with RG756 as coating material for stents in a closed loop flow system. RG756 was used instead of R208 since the mechanical properties of R208 were not suitable for coating stents due to ruptures occurring upon expansion of stents. In comparative studies under static conditions it was proved that RG756 had similar heparin content and blood contacting properties to R208 (data not shown). In the dynamic experiments it was tested how coating of Palmaz™ stent with RG756 and subsequent heparinization changes the haemocompatibility of the stents. The results shown in Fig. 4 demonstrate that the plain Palmaz™ stent made of metal provokes considerable platelet activation on its surface. Even the pure polymer coating made of RG756 diminished the thrombogenicity of the stent, which was further decreased after heparinization.

In conclusion, the *in vitro* studies performed in this investigation suggest that it is reasonable to use

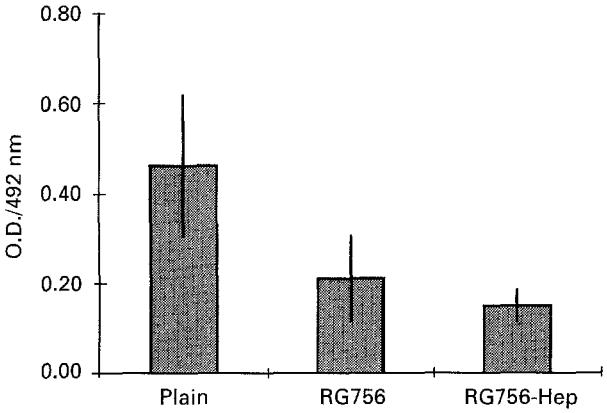

Figure 4 Comparison of platelet activation on plain metallic stent, RESOMER® RG756 coated and heparin-modified stent (2 h, 22 °C) measured by GMP140 assay (means ± S.E.M., $n = 5$)

degradable heparinized PLA/PGA as a surface coating for improving the biocompatibility of intravascular stents.

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