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Heparin-modified polylactide as biodegradable hemocompatible biomaterial

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Several medical applications may benefit from biodegradable hemocompatible materials. Therefore a commercial biodegradable polylactide (RESOMER® R208; Boehringer-Ingelheim, Germany) was modified with heparin. The immobilization of heparin was performed by covalent binding with glutaraldehyde as spacer. Reaction conditions were varied to optimize the coupling of heparin. The efficiency of the immobilization and the biological activity of bonded heparin was estimated with a toluidine blue assay and factor Xa assay, respectively. It was shown that an immobilization at ambient temperature and a reaction time of 2 h yielded maximal heparin binding and biological activity. The hemocompatibility of the modified polylactide was tested after blood/material contact with an enzyme immuno assay (EIA) for GMP 140, a marker of platelet activation. The investigated materials were compared with polypropylene and Pellethane®. After modification it was found that high heparin binding and biological activity were correlated with very small activation of platelets.

1. Introduction

Polylactides are synthetic, aliphatic, biodegradable α-hydroxy acid polyesters. *In vivo* they are cleaved by hydrolytic deesterification into lactic acid. The lactic acid is incorporated in the tricarboxylic acid cycle and will subsequently be excreted as carbon dioxide [1]. These materials are frequently used for hard tissue replacement because of their biocompatibility and biodegradability [2, 3]. Several applications of biomaterials in contact with blood might benefit from the use of biodegradable, hemocompatible materials. For example, arterial prosthesis must have a certain porosity to allow the ingrowth of vascularized perigraft tissue and endothelial cells [4]. However, high porosity can result in bleeding through the graft interstices. Preclotting of vascular prosthesis with blood is one possibility to avoid hemorrhagic failure of the prosthesis [5]. The use of biodegradable biomaterials as one component of vascular grafts may produce an increasing mural porosity resulting in minimal blood loss and good healing properties [6]. Another undesirable property of biomaterials is their potential thrombogenicity. Initial adhesive interaction of platelets with foreign surfaces may trigger activation of the coagulation cascade [7–10]. Therefore, a further surface modification of biodegradable materials with anticoagulant compounds may produce an improved thromboresistance of the graft. Heparin is widely used to increase the hemocompatibility of biomaterials [11–13]. It was the aim of this study to develop a biodegradable material with excellent hemocompatibility on the base of polylactide and heparin.

2. Materials and methods

2.1. Materials

A commercial poly-(D,L-lactide) (RESOMER® R208; Boehringer-Ingelheim, Germany) was selected as material for the immobilization of heparin. The polymer was dissolved in chloroform. Cast sheets were prepared from a 5% polymer solution after evaporation of the solvent.

2.2. Methods

2.2.1. Immobilization of heparin

Porcine mucosa heparin (Serva Biochemistry, Germany, 174 IU/mg) was immobilized using glutaraldehyde (GA; Sigma, USA) as coupling agent by modification of the method of Peppas [14]. A 1:1 mixture of 12% aqueous solution of heparin and 6% aqueous solution of GA, adjusted with 0.1 M sulphuric acid to pH 5.2, were incubated with the polymer sheets.

2.2.2. Toluidine blue assay (TBA)

The amount of heparin immobilized on the surface was quantified by a modified toluidine blue colorimetric method [15]. Polymer sheets of 1 cm^2 were

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placed into 0.5 ml phosphate-buffered saline (pH 7.4, PBS) and 0.5 ml of 0.005% aqueous toluidine blue solution (Sigma, USA). After 5 min shaking, 1 ml of hexane was added. After mixing and phase separation the optical density (OD) of the aqueous phase was measured at 630 nm (SPEKOL 220, Carl Zeiss Jena, Germany). The amount of coupled heparin was estimated by comparison with a standard curve of known concentrations of heparin.

2.2.3. Factor Xa assay

The anticoagulant activity of immobilized heparin was analysed by a factor Xa chromogenic assay that measures the potentiating effect of heparin on antithrombin III (AT III) in the inhibition of factor Xa [16]. Therefore, prewetted polymer sheets (diameter 11 mm) were incubated in 0.075 ml AT III (0.04 IU/ml, Haemochrom Diagnostica, Germany). After 5 min 0.3 ml Factor Xa (0.32 IU/ml) and a further 5 min 0.3 ml 0.55 mM S-2222 (both Haemochrom Diagnostica, Germany) were added. The hydrolysis of the chromogenic substrate was stopped with 0.3 ml 20% acetic acid after 2 min. Aliquots of the solution were monitored spectrophotometrically at 405 nm (SPEKOL 220, Carl Zeiss Jena, Germany). The amount of active immobilized heparin was estimated by comparison with a standard curve of known activities of heparin.

2.2.4. GMP 140 assay

The hemocompatibility of the modifications of polylactide was studied by an EIA for GMP 140 displaying the activation of platelets on biomaterials [17]. The amount of GMP 140 was estimated after 30 min contact between platelets and materials using the monoclonal 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 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. The OD of aliquots of the substrate solution was measured at 492 nm (Anthos Plate Reader, Austria). The measured OD refers to the degree of platelet activation. Therefore, a low OD indicates a low platelet activation.

All experiments except immobilization and measurements were performed at 37 °C.

3. Results and discussion

Heterogeneous reactions such as solid/liquid systems need a sufficient contact between both phases. Fixed polymer sheets (diameter 9 cm) had to be covered with the reaction mixture during the whole immobilization process. To prevent a diffusion limited reaction of the reactants the reaction container was shaken. For geometric reasons, 20 ml of the reaction mixture were enough to ensure sufficient coverage of the polymer sheet. Increasing the volumes of the reaction mixture did not yield better results with respect to heparin binding and hemocompatibility (data not shown).

The immobilization of heparin was carried out for 1 h, 2 h and 3 h at ambient temperature (AT). Fig. 1a shows the total content of bonded heparin estimated by the TBA. It is clear from Fig. 1a that the binding of heparin was maximal after 2 h reaction. A shorter (1 h) or longer (3 h) reaction time caused decreased binding of heparin. Covalent binding of heparin may result in a loss of biological activity because of increased coupling of essential functional groups of heparin to the

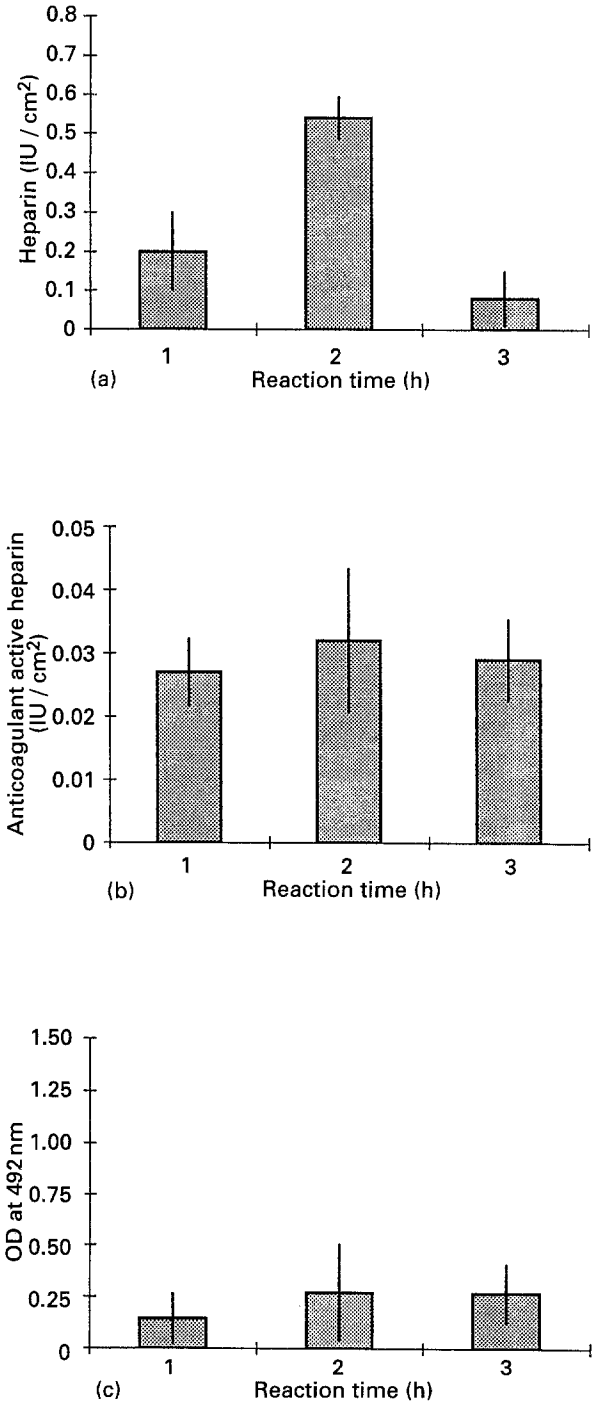

Figure 1 Total heparin content (a), anticoagulant activity of bonded heparin (b) and hemocompatibility (c) of heparin-modified RESOMER® R208 with respect to reaction time measured by the toluidine blue assay, factor Xa assay and GMP 140 assay, respectively (means ± SEM, $n = 5$).

surface. Therefore, the anticoagulant potential of bonded heparin was tested by the factor Xa assay (see Fig. 1b). It can be seen that the changes in the anticoagulant activity were less pronounced than the binding of heparin at different times. Nevertheless, the anticoagulant activity was maximal after 2 h reaction time. The hemocompatibility of heparin-immobilized RESOMER® R208, as indicated by the presence of GMP 140, is shown in Fig. 1c. In contrast to maximal heparin binding after 2 h the differences in the GMP 140 translocation with respect to reaction times were only small. It was observed that the OD was minimal for 1 h reaction time. Since both the anticoagulant activity and the prevention of platelet activation are desired effects of heparin immobilization, a 2 h reaction time was selected as optimal for further investigations.

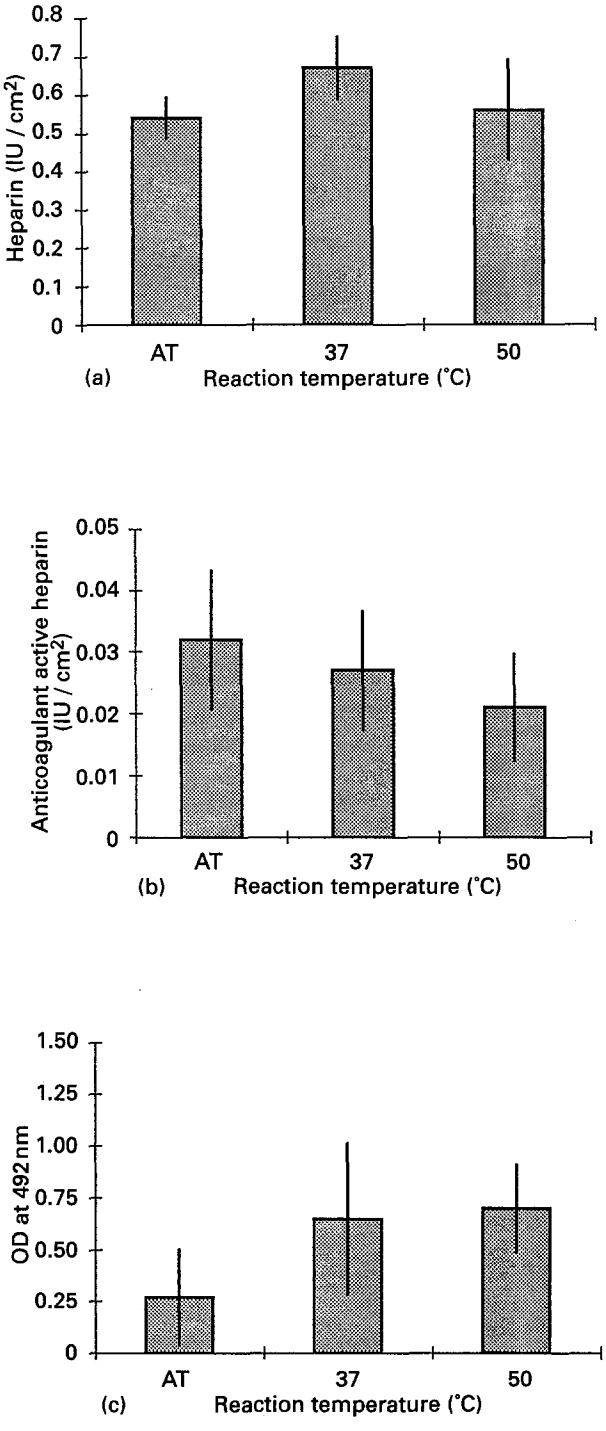

Figure 2 Total heparin content (a), anticoagulant activity of bonded heparin (b) and hemocompatibility (c) of heparin-modified RESOMER® R208 with respect to reaction temperature measured by the toluidine blue assay, factor Xa assay and GMP 140 assay, respectively (means ± SEM, $n = 5$).

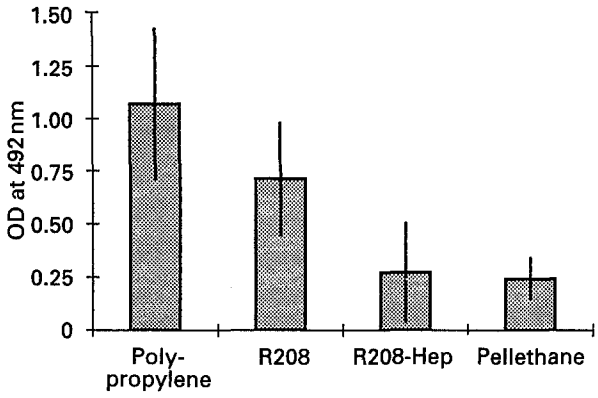

Figure 3 Comparison of the platelet activation on polypropylene, Pellethane®, non-modified and heparin-modified RESOMER® R208 (2 h, AT) measured by GMP 140 assay (means ± SEM, $n = 5$).

The effect of the reaction temperature was studied by investigations at ambient temperature, 37 °C and 50 °C at 2 h reaction time. Considering the low glass transition temperature $T_G \sim 50$ °C of RESOMER® R208, a higher reaction temperature was not applied because of possible changes to the mechanical and degradable properties of the material. In Fig. 2a it is demonstrated that an increase of the reaction temperature from ambient temperature to 37 °C caused a small rise in the heparin binding indicated by TBA results. However, further increase of the temperature close to the glass transition temperature yielded decreased binding of heparin. It is possible, for thermodynamic reasons that close to the glass transition temperature a rearrangement of macromolecular chains decreases the concentration of polarizable groups on the polymer surface [18]. Due to this process the binding of heparin might be hindered. Fig. 2b shows the results of the factor Xa assay. It is demonstrated that the rise in the reaction temperature diminished the anticoagulant activity of bonded heparin. Fig. 2c shows that the hemocompatibility of heparin-modified RESOMER® R208 also decreased with increasing reaction temperature with very small platelet activation at AT. Both results, however, show that the increase of reaction temperature deteriorates the biological properties of heparin. A possible explanation for this phenomenon is that an increasing binding of heparin (multipoint attachment) inhibits its anticoagulant properties by a loss of free essential functional groups [19]. This may also lead to decreased flexibility of the bonded heparin molecule that negatively influences the interaction with AT III and platelets [20].

In addition, the hemocompatibilities of non-modified and optimal heparin-modified RESOMER® R208 were compared with two reference materials polypropylene and Pellethane®. Fig. 3 shows the results

of these experiments. The pure RESOMER® R208 seemed to be more hemocompatible than polypropylene and less than Pellethane®. However, the hemocompatibility was substantially improved after binding of heparin, indicated by the low platelet activation on this surface.

4. Conclusions

In this study an improvement of the hemocompatibility of RESOMER® R208 was achieved by the covalent binding of heparin. An immobilization product with maximal binding of heparin and good hemocompatibility was obtained after 2 h heparinization at ambient temperature. In this case the RESOMER® R208 has a total heparin content of about 0.5 IU/cm^2, in which about 10% of the bonded heparin is biologically active.

Further investigations are planned to measure the permanence of anticoagulant properties as well as the degradation of heparin-modified polylactide.

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