Immobilization of the thrombin inhibitor r-hirudin conserving its biological activity

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Surface immobilization of the thrombin inhibitor r-hirudin was carried out on two different polymers. Linkage to poly(urethane-*graft*-acrylic acid) (PAC/PU) was done via carboxylic acid groups, using a water soluble carbodimide, while the immobilization on a modified poly[(ethene-*co*-vinyl acetate)-*graft*-vinyl chloride] (PVC/EVA) was achieved via the alcohol groups of the polymer using HDI as spacer. Direct immobilization of r-hirudin leaded to a remarkable loss of thrombin activity. As proved by means of protein chemical analysis, loss of activity was due to a selective coupling via the N-terminal amino group of r-hirudin, which is essential for its thrombin activity. Based on these results we developed an immobilization method via an ε-amino group of r-hirudin preserving full biological activity of the r-hirudin coated surface.

1. Introduction

When blood is exposed to a foreign body surface, a thrombotic clot is formed immediately at the surface which may cause vascular occlusion. Many of the reactions causing acute clotting and restenosis are thrombin mediated [1, 2]. In order to prevent these reactions, one approach consists of the immobilization of anticoagulants on the surface of the biomaterial. Heparin is by far the most frequently applied anticoagulant [3]. Another anticoagulant gaining clinical acceptance is r-hirudin, a recombinant protein consisting of 65 amino acid residues (6964 Da). r-Hirudin has several advantages over heparin, especially in the case of a combined application with stents, such as less allergenic activity, inhibition of neointimal hyperplasia and no deactivation by platelet factor 4 (PF 4) [4]. Since r-hirudin also blocks the active center of thrombin, it is additionally able to prevent thrombin mediated reactions considered to be responsible of restenosis [5]. r-Hirudin is the strongest known thrombin inhibitor forming a non-covalent complex with a 1 : 1 molar ratio [6]. The complex formation involves ionic interactions between the C-terminal region of r-hirudin and the anionic binding side as well as hydrophobic interactions between the N-terminal core fragment of r-hirudin and the active center of thrombin. In particular the N-terminal amino acid residues seem to be in an important position determinative for the thrombin activity [7].

2. Materials and methods

2.1. Polymer modification

Granules of Tecoflex® 60G were dissolved in tetrahydrofuran (THF, Riedel-de Haen) to a 10% (w/w) solution. This solution was casted in a melamine resin plate and the solvent was allowed to evaporate at room temperature for more than 3 days. After Soxhlet extraction with ethanol/hexane (21/79, w/w) overnight, followed by vacuum drying overnight at 60 °C, films with a thickness of about 0.4 mm were obtained. Tecoflex® films were treated for 30 s with an argon plasma using a microwave generator (a Hexagon Plasma Unit of Technics Plasma GmbH, Kirchheim, Germany) with a frequency of 2.45 GHz, a plasma power of 300 W and a gas flow rate of 16.8 ml/min at a pressure of 0.14 mbar. Photochemically induced graft-co-polymerization of acrylic acid on the modified poly(urethane) was executed in diethyl ether under nitrogen, which was initiated by an Excimer laser (308 nm, 1200 W, 10 min). Immobilization of r-hirudin was done with 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC, Aldrich) in a one-pot reaction [8]. PVC/EVA was functionalized by saponification of surface ester groups with aqueous potassium hydroxide solution. Free isocyanate groups were achieved by incubating the saponified PVC/EVA surfaces with hexamethylene diisocyanate (HDI, Aldrich) in absolute diethyl ether (1 : 10, w/w) for 5 days. After extraction in a Soxhlet under nitrogen in absolute diethyl ether for 8 h and drying in vacuum, free isocyanate groups were generated on the PVC/EVA surface (HDI-PVC/EVA).

2.2. r-Hirudin immobilization

For partial protection, r-hirudin was reacted with N-(methyl sulfonyl ethoxy carbonyloxy) succinimide (MSC-ONSu) (Aldrich) in 0.1 m sodium acetate buffer for 20 min [9]. Purification by gel chromatography and

subsequent ionic exchange chromatography delivered an exclusively N-terminal-protected r-hirudin ((MSC)-hirudin). All protein modification steps were monitored by high pressure liquid chromatography (HPLC) and capillary electrophoresis (CE). r-Hirudin and its derivatives were diluted to 1 mg/ml. Experiments were done with a BioFocus 3000 (Bio-Rad, Munich, Germany) in 0.1 M NaH_2PO_4/i-propanol (4/1; v/v) at pH 2.5. Protein immobilization was carried out in phosphate buffered saline (pH 7.4) contacting the HDI-PVC/EVA surface for 6 h with (MSC)-hirudin (reference: r-hirudin) solution of 2000 ATU/ml. After immobilization of the (MSC)-hirudin derivatives and intense rinsing with SDS-buffer (0.1%) the protecting group was cleaved by incubation with 10% aqueous piperidine for 2 h.

2.3. Analytical procedures

The amount of immobilized r-hirudin was determined by means of ^{125}I-radiolabeling experiments. ^{125}I-labeled r-hirudin was obtained using the Chloramin T method. Non-incorporated iodine was separated by means of a Sephadex column (Pharmacia, Sweden). After adsorption, the total radioactivity was measured by means of a gamma-counter.

The thrombin activity was determined by means of a commercially available thrombin time assay (Boehringer, Mannheim, Germany). Clotting time was determined by means of a coagulometer KC 10 (Amelung, Germany) equipped with an integrator CR-A (Amelung, Germany).

IR spectra were obtained with a Nicolet 710 spectrometer (Offenbach, Germany) using the attenuated total reflection (ATR) technique.

3. Results and discussion

3.1. Polymer modification

The poly(ether urethane) Tecoflex® (PU) and the poly[(ethene-*co*-vinyl acetate)-*graft*-vinyl chloride] (PVC/EVA) were functionalized. All modification steps were verified by means of infrared spectroscopy in attenuated total reflection mode (IR-ATR). In order to clarify modification procedures, IR-ATR spectra for PVC/EVA are shown in Fig. 1. After saponification, the hydroxyl band appeared at 3340 cm^{-1}, while the signals being characteristic of the ester carbonyl group at 1734 cm^{-1} and 1250 cm^{-1} were strongly reduced. Conversion of the saponified polymer with HDI delivered an isocyanate activated surface (HDI-PVC/EVA). Successful reaction was indicated by the appearance of the NH-bond at 3327 cm^{-1} and the characteristic signal of the isocyanate group at 2267 cm^{-1}.

3.2. Direct r-hirudin immobilization

As a first approach, we investigated the direct immobilization of unprotected r-hirudin via HDI to the modified PVC/EVA surface. r-Hirudin offers four primary amino groups for its linkage to a surface: ^{1}Val, ^{27}Lys, ^{36}Lys and ^{47}Lys. Since thrombin complex formation involves hydrophobic interactions between the N-terminal core

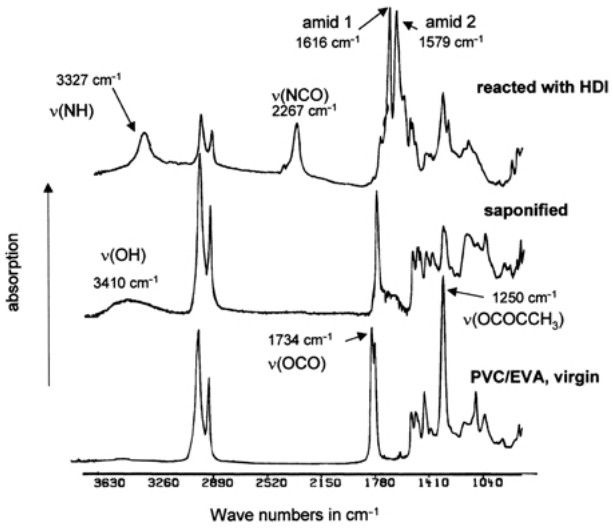

Figure 1 IR/ATR spectra of virgin (bottom), saponified (middle) and HDI activated (top) PVC/EVA monitoring the surface modification of the polymer.

fragment of r-hirudin and thrombin's active center, free amino groups may be critical for hirudin's thrombin activity. One main issue with hirudin immobilization is therefore to conserve physiological activity after protein linkage.

The results of this immobilization on PVC/EVA using a r-hirudin concentration of 2000 ATU/ml (anti-thrombin unit/ml) are shown in Fig. 2 in comparison to tissue culture styrene (TCPS) and Tecoflex®. The little prolongation of the thrombin time after immobilization strongly suggests that either immobilization did not occur or that r-hirudin lost its activity versus thrombin due to surface linkage. In order to confirm successful immobilization, the amount of surface bound r-hirudin was determined using ^{125}iodine labeled r-hirudin (^{125}I-hir). ^{125}I-Labeled r-hirudin was incubated with the HDI activated PVC/EVA surface; subsequently the radioactivity of the material was determined. The results show a significant increase in surface bound r-hirudin on HDI-activated surfaces compared to the non-activated ones (Fig. 3). The maximum surface concentration was determined to be 0.7 nmol/cm^2. The results as shown in Fig. 3 were compared with a blind sample (treatment of the polymer without HDI). This allows comparison of

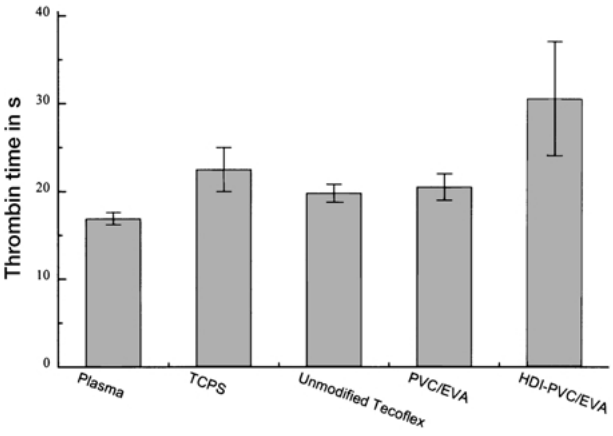

Figure 2 Blood clotting of r-hirudin coated PVC/EVA as determinated by means of thrombin time assay. As references are included TCPS PVC/EVA and the unmodified poly(urethane) Tecoflex ($n = 12$).

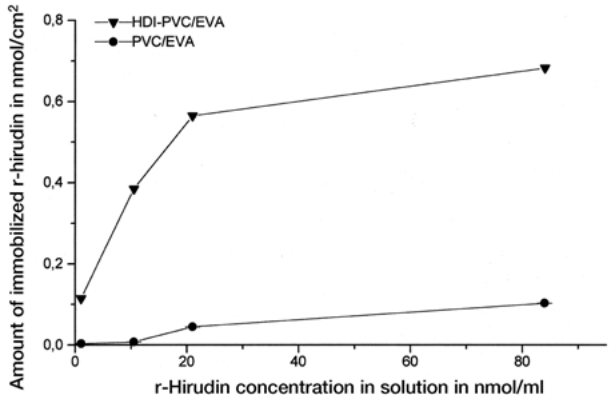

Figure 3 Amount of surface bound r-hirudin according to radio-assay using ^{125}I-hirudin as determinated for HDI-activated PVC/EVA surfaces vs. non-activated ones $(n = 12)$.

covalently versus adsorptively bound r-hirudin. While the amount of adsorptively bound r-hirudin (TCPS, non-activated PVC/EVA) was almost independent of the concentration of the incubation solution, the covalently bound r-hirudin increased proportionally to concentration. Due to the covalent linkage between the surface coating and r-hirudin, no leaching off of surface bound hirudin was determined. These experimental data prove successful linkage of r-hirudin to PVC/EVA and hint toward the fact that the loss of activity is due to deactivation during immobilization.

3.3. Bio-active immobilization of r-hirudin using (MSC)-hirudin derivatives

Therefore, amino protected derivatives of r-hirudin were synthesized by partial reaction of r-hirudin's amino groups with MSC-ONSu. Ion exchange chromatography yielded different fractions, which were determined to be (MSC)- [main fraction], $(MSC)_2$- and $(MSC)_3$-hirudin. All these derivatives showed a strong loss of thrombin activity (Fig. 4) similarly as the results after surface immobilization of r-hirudin. The fact that not even the (MSC)-hirudin showed significant thrombin activity confirms the conclusion that one amino group plays an essential role in the interactions with thrombin, and, at the same time, is preferentially reacted. The remaining free amino groups were subsequently blocked irreversibly by reaction with acetic acid anhydride. Cleavage of the MSC protection group delivered derivatives which were partially blocked. In contrary to (MSC)-hirudin derivatives however, the protection pattern was inverse: less reactive amino groups were blocked while the most reactive ones were free. Those derivatives showed thrombin activity being close to the activity of unmodified r-hirudin. The results, presented in Fig. 4, demonstrated that the N-terminal amino group had a superior chemical reactivity compared to the remaining three lysine ε-amino groups [10], but was critical for r-hirudin's thrombin inhibition capacity.

Therefore, an indirect immobilization strategy was chosen using an N-terminally protected r-hirudin derivative for immobilization. (MSC)-hirudin was subsequently immobilized on HDI-activated PVC/EVA (see Fig. 5) and on the modified poly(urethane) surfaces using a water soluble carbodiimide method [8] as shown in Fig. 6. On both polymer surfaces, thrombin activity was preserved after immobilization of (MSC)-hirudin and subsequent cleavage of the protecting group. Consequently, hirudin's activity is not depending on the immobilization method and tolerates a wide range of polymers, since the investigated PVC/EVA and the poly(urethane) Tecoflex® show distinguishable physical and chemical properties.

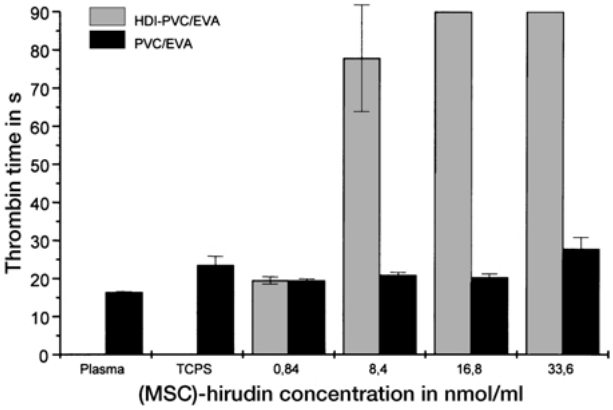

Figure 5 Blood clotting of r-hirudin coated PVC/EVA after cleavage of the protecting group as determinated by means of thrombin time assay. As reference is included TCPS $(n = 12)$.

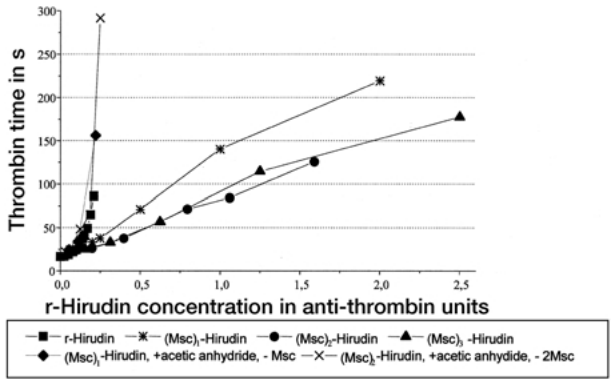

Figure 4 Blood clotting of r-hirudin derivates $(MSC)_x$-Hirudin derivates $(X = 0 - 3)$ in solution as compared to derivatives with acetic acid ester protection of the remaining amino groups and subsequent cleavage of MSC groups.

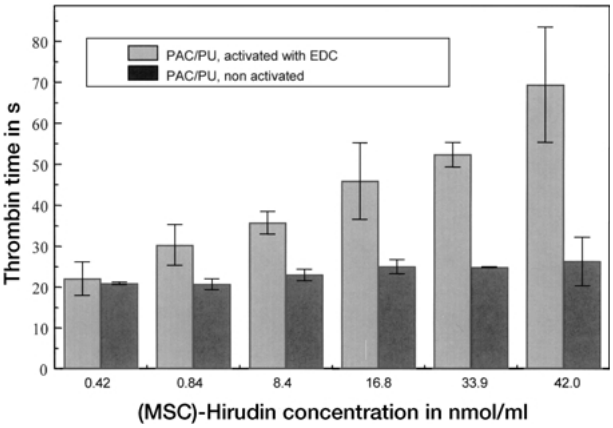

Figure 6 Blood clotting of r-hirudin coated PAC/PU after cleavage of the protecting group as determinated by means of thrombin time assay. As reference is included TCPS $(n = 12)$.

4. Conclusions

A generally applicable procedure for the bioactive coating of a poly(urethane) as well as of a modified poly(vinyl chloride) were described. Although direct immobilization of r-hirudin failed to be successful, immobilization of the (MSC)-hirudin derivative was carried out conserving r-hirudin's thrombin activity. Immobilization of r-hirudin via a temporarily protected r-hirudin derivative and subsequent deprotection on the surface allowed r-hirudin to maintain its thrombin activity. Surfaces prepared by covalent linkage of r-hirudin showed strongly enhanced prolongation of blood clotting due to inactivation of thrombin by surface bound r-hirudin. r-Hirudin coated surfaces may have a strong input in stent development [11] as well as for catheters with non-clotting properties.

Acknowledgments

This research project was supported by the ''Interdisciplinary Center for Clinical Research in Biomaterials and Tissue-Material-Interactions in Implants'' (BMBF project No. 01 KS 9503/9).

References

1. G. AGNELLI, *Cardiovascular Research* **31** (1996) 232.
2. S. M. SEILER, *Seminars in Thrombosis and Haemostasis* **22** (1996) 223.
3. S. W. KIM and H. JACOBS, *Blood Purification* **4** (1996) 357.
4. T. E. WARKENTIN, *Drug Safety* **17** (1997) 325.
5. J. DODT, *Angewandte Chemie* **107** (1995) 867.
6. T. J. RYDEL, K. G. RAVICHANDRAN, A. TULINSKY, W. BODE, R. HUBER, C. ROITSCH and J. W. FENTON II, *Science* **249** (1990) 277.
7. J.-Y. CHANG, *FEBS Letters* **164** (1983) 307.
8. J.-C. SHEEHAN, P. A. CUICKSHANK and G. I. BOSHART, *Journal of Organic Chemistry* **26** (1961) 2525.
9. G. J. TELLER and J. C. BALRETH-GEERS, *International Journal of Peptide and Protein Research* **7** (1975) 295.
10. A. TULINSKY, *Seminars in Thrombosis and Haemostasis* **22** (1996) 117.
11. J. LAHANN, D. KLEE, W. PLÜSTER and H. HÖCKER, *Biomaterials* **22** (2001) 817.

Received 21 September
and accepted 1 November 2000