Immobilization of human thrombomodulin onto PTFE

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Human thrombomodulin (hTM) is an endothelial cell-surface glycoprotein and has effective anticoagulant properties. This protein was immobilized onto polytetrafluorethylene (PTFE) surfaces to create biomaterials with enhanced haemocompatibility. The PTFE surface was functionalized by CO_2 plasma activation and subsequent vapour-phase graft polymerization of acrylic acid. Surface characterization after plasma treatment, grafting and hTM immobilization was achieved by attenuated total reflection-Fourier transform–infrared spectroscopy, X-ray photoelectron spectroscopy, zeta potential and wetting measurements. The activity of immobilized hTM was estimated using the protein C activation test.

1. Introduction

The need for haemocompatible materials for medical devices is constantly rising. There are numerous approaches to introduce antithrombogenic characteristics on a material used in medical devices with blood contact. One very promising approach is the idea to immobilize proteins with antithrombogenic or thrombolytic qualities. The immobilization of urokinase [1], hirudin [2] and thrombomodulin [3–5] on to polymers are examples of this approach.

Human thrombomodulin (hTM) is an endothelial cell-surface glycoprotein which has very effective anticoagulant properties [6, 7]. It binds to thrombin which then changes from a procoagulant protease into an anticoagulant [8]. The cleavage of fibrinogen, which leads to the formation of a fibrin clot, and the platelet-activating characteristics of thrombin, are inhibited. The thrombin inactivation of antithrombin III is activated. Most interestingly, the enhanced substrate specificity of thrombin for protein C with TM as a cofactor, is increased more than a thousand-fold. The activation of protein C causes the inactivation of blood coagulation factors Va and VIIIa and thus shuts down coagulation.

Polytetrafluoroethylene (PTFE) is a very inert biomaterial with unique chemical and physical–mechanical properties. Because of this it is widely used in cardiovascular surgery: 25% of the biomaterials contain PTFE [9] and 75% of vessel replacement with a diameter less than 10 mm are made from this material [10]. The covalent immobilization of hTM is intended to improve the blood compatibility and to permit the successful use as a small-diameter graft.

In this study, a method of surface functionalization for subsequent immobilization of hTM on to PTFE is suggested. The initiation of the graft polymerization with polyacrylic acid (PAAc) requires a special method, because the methods used for other materials are not effective with PTFE [11]. Microwave CO_2 plasma treatment in combination with vapour-phase grafting of PAAc, has been shown to be an effective preparation for the covalent coupling of human thrombomodulin.

2. Materials and methods

The PTFE films (25 mm × 20 mm × 0.5 mm; Nuenchritz GmbH, Germany) were treated with microwave CO_2 plasma [12] (Technics Plasma GmbH type 440G) at an operating frequency of 2.45 GHz, output power 250 W, gas flow rate 5–15 standard $cm^3\,min^{-1}$, pressure 0.05–0.15 mbar.

A vapour-phase acrylic acid (AAc) (Fluka, Neu-Ulm, Germany) graft polymerization followed. The probes were exposed to the vapour for 1–4 h. The amount of PAAc formed was constant after stirring in water at 75 °C for 24 h.

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The recombinant hTM (Asahi Chemical Industry Co., Tokyo, Japan, donated by Professor Ikada) is produced using Chinese hamster ovary (CHO) cells. It was immobilized using water-soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimid; WSC) (SIGMA GmbH, Deisenhofen, Germany). The PAAc-grafted-PTFE film was immersed in 10 ml WSC solution for 30 min at 4 °C, washed and then treated with hTM in phosphate buffer saline (PBS) solution (1 ml, pH 7.4). The reaction was performed for 24 h at 4 °C. Afterwards the samples were washed with PBS and freeze-dried.

After each treatment, chemical changes of the PTFE surfaces were characterized by attenuated total reflection-Fourier transform–infrared spectroscopy (ATR-FT–IR; IFS 66, Bruker) (KRS-5 crystal 60 mm × 20 mm × 4 mm; 45°; 15 reflections), X-ray photoelectron spectroscopy (XPS; Esca Lab I FG), zeta potential (EKA, A.Paar KG) and contact-angle measurements (sessile drop method) (G 40, Krüss GmbH).

The activity of immobilized hTM was estimated by the protein C activation test using Thrombin, Protein C and Boc-Leu-Ser-Thr-Arg MCA (all from Sigma) as fluorogenic peptide substrate [13–15]. The PTFE film with immobilized hTM was incubated with 1 ml 50 mM tris-HCl buffer (pH 8.0; 0.1 M NaCl; 5 mM $CaCl_2$; 0.1% bovine serum albumin) containing 2 NIH units of thrombin (Sigma) for 60 min at 37 °C. After removal it was washed with buffer and incubated with 0.5 ml 0.1 M protein C - tris-HCl buffer solution. After incubation at 37 °C for 60 min, the fluorogenic substrate *N*-t-Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin (MCA) was added. The amount of activated protein C can be measured by detecting the aminomethylcoumarin that is generated after the cleavage through the activated protein C. 200 µl 200 µM Boc-Leu-Ser-Thr-Arg-MCA tris-HCl buffer solution were added and the fluorescence was measured with a Perkin–Elmer LS-50 luminescence spectrometer after an incubation at 37 °C for 30 min. The excitation maximum was at 340 nm, the emission maximum was measured at 440 nm.

3. Results and discussion

3.1. ATR–FT–IR

The data are summarized in Fig. 1. New absorption peaks with maxima at 1881 and 1725 cm^{-1} can be attributed to COF and –CF = CF– groups which were generated through the plasma treatment.

The lowest process pressure led to the highest yield of COF groups after an optimum treatment time of 30 min. The characteristic bands for AAc occur at 3200 cm^{-1} (OH stretch), 1710 cm^{-1} (C=O stretch) and 800 cm^{-1} (COOH out-of-plane deformation). The decrease of the 1881 cm^{-1} absorbance band probably can be attributed to a reaction of AAc with COF groups.

The immobilization process only led to relatively insignificant changes in the spectrum, which is probably due to the small amount of immobilized protein and the information depth of the ATR–FT–IR method.

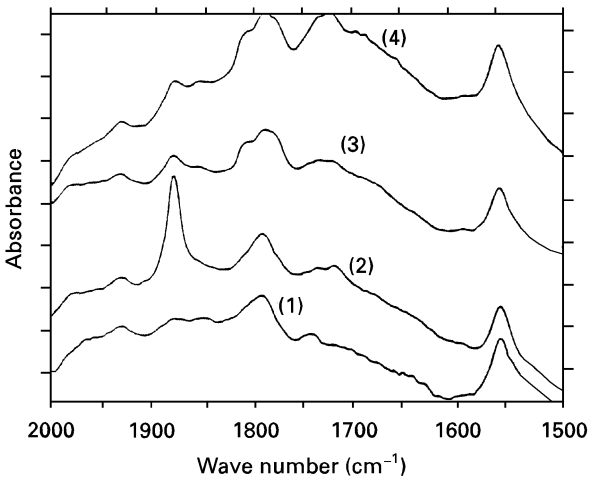

Figure 1 Absorption peaks after the treatments: (1) before treatment; (2) plasma treatment; (3) AAc graft polymerization; (4) hTM immobilization.

3.2. XPS spectroscopy

The XPS spectroscopy data showing the F/C, O/C and N/C atomic ratios are listed in Table I. The XPS survey spectra showed that treatment with CO_2 plasma not only leads to the oxidation of PTFE, but also to variations in the polymer chain. The F/C atomic ratio after each step of the modifications decreases from 1.69 to 0.83, while the O/C ratio increases from 0 to 0.14. The presence of amino acids on the modified PTFE surface after hTM immobilization is signified through the N-signal after this treatment. A strong increase of the peak intensity near 285 eV after the grafting process (attributed to CH_2), points to the fact that hydrocarbons (PAAc) are abundant on the surface of PTFE.

3.3. Zeta potential measurements

The plasma treatment and PAAc grafting lead to the formation of acidic surface sites. The relative positions of the isoelectric points (pH at which the zeta potential equals zero) indicate that the behaviour of the carboxylic groups generated directly on the PTFE after CO_2 plasma treatment is more strongly acidic than the carboxylic acid groups of PAAc.

3.4. Contact angle measurements

The water contact angles on the unmodified PTFE surface are very high, indicating the hydrophobic character of this material. After the plasma treatment only a minimal change in wettability resulted (Table II). A noticeable decrease of advancing and receding

TABLE I Surface composition of PTFE films: (1) untreated, (2) plasma treated, (3) PAAc grafted, (4) hTM immobilized

Atomic ratio	(1)	(2)	(3)	(4)
[F]/[C]	1.693	1.538	1.018	0.832
[O]/[C]	–	0.069	0.137	0.141
[N]/[C]	–	–	Traces	0.036

TABLE II Advancing, (Θ_a), and receding, (Θ_r), contact angles of water on untreated and modified PTFE surfaces (sessile drop method)

	Θ_a (deg)	Θ_r (deg)	$\Delta\Theta$ (deg)
Untreated	117 ± 2	91 ± 7	26
CO_2-plasma	114 ± 4	88 ± 1	26
PAAc-grafted	96 ± 5	58 ± 4	38
hTM-immobilized	77 ± 1	20 ± 3	57

contact angles after graft polymerization and hTM immobilization, due to an increase in hydrophilicity, can be measured. The contact angle hysteresis after PAAc grafting and hTM immobilization indicates the increase of chemical heterogeneity in these two modification steps.

3.5. Protein C activation assay

The hTM immobilized on the PAAc-grafted PTFE surface exhibits the expected activity in converting thrombin from a procoagulant protease to an anticoagulant form, when assessed by a protein C activation assay.

4. Conclusion

The described method for the immobilization of hTM on to PTFE provides a possibility for creating a potent antithrombotic biomaterial. The functionalization with CO_2 plasma and the subsequent grafting process are suited to permitting the covalent immobilization of human thrombomodulin. The success of each treatment was detected by the formation of COF and –C=C– groups after CO_2 plasma treatment, COOH groups after PAAc grafting, and amide, amine and N^+R_4 groups after hTM immobilization.

The continued research with respect to the covalent immobilization of hTM on to PTFE is dedicated to the following areas.

(a) Optimization of primary functionalization of PTFE by plasma treatment. The surface modification effect of plasma will be investigated by varying several process parameters. With that aim, a new research processor with unique features is being assembled.

(b) Characterization of the activity of the immobilized hTM. A quantitative analysis will answer the question: what percentage of immobilized hTM is still active and data on the speed of the ageing process are necessary before clinical experiments can be started.

(c) Investigation of the optimum combination of plasma treatment and vacuum-phase grafting parameters to obtain a surface that allows the successful and lasting covalent immobilization of a high amount of hTM.

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