

# Covalently immobilized thrombomodulin inhibits coagulation and complement activation of artificial surfaces in vitro

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## Abstract

Thrombomodulin (TM) serves as the endothelial cell receptor for thrombin and alters its characteristics from pro- to anticoagulant. Additionally, it promotes the formation of activated protein C. We evaluated the conservation of the overall outcome of these functions in recombinant TM linked to artificial surfaces by incubation with human whole blood in vitro.

TM was covalently immobilized through poly(ethylene glycol) (PEG) spacers onto thin films of poly(octadecene *alt* maleic anhydride) covering planar glass substrates. TM binding to the polymer films was achieved after active ester formation at the carboxylic acid terminus of the PEG spacers and thoroughly characterized by HPLC-based amino acid analysis, immunofluorescence and ellipsometry. TM-coated samples were incubated for 3 h with freshly drawn whole human blood anticoagulated with heparin (5 IU/ml) using in-house developed incubation systems. The substantially reduced activation of blood coagulation (TAT) for TM-coated samples correlates well with the degree of contact activation (bradykinin and FXIIa formation) while no significant effects were observed for the platelet activation (PF4). Further, complement activation (C5a levels), was strongly diminished at the TM-containing surfaces. We conclude that the suggested method for preparation of TM immobilization may serve to prepare model substrates for studies on TM interactions but similarly provides a promising coating strategy for blood contacting medical devices.

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## 1. Introduction

The use of biomaterials contacting blood is challenged by blood coagulation and immune attacks. These blood reactions are initiated through characteristics of the surface of biomaterials which—despite of being non-toxic and inert—induce protein adsorption and blood cell adhesion patterns often provoking the activation of the body defence systems. Procoagulant processes still form the major barrier in a variety of demanding applications [1]. In that context, extensive investigations on the surface modification of biomaterials have been undertaken with the prospect of attaining hemocompatibility characteristics similar to that of the healthy endothelium. Intact endothelial cells constitute an

anticoagulant lining of the vessel wall with essential synthetic and metabolic functions concerning the hemostatic system [2,3]. After injury the damage of blood vessels exposes blood to tissue factor produced constitutively by cells beneath the endothelium [4]. Tissue factor can also be produced through vascular endothelial cells and monocytes, triggered through endotoxin [5,6]. It binds to factor VII or VIIa and promotes the formation of thrombin.

Numerous attempts to imitate the natural lining of the endothelium were undertaken by seeding endothelial cells on biomaterials but had limited success due to stability and vitality problems of the cells [7–9]. To avoid these problems molecular products or components of endothelial cells like heparin [10–17], plasminogen-activators (tissue- and urokinase-type [18–24]) and NO [25] were applied instead.

In that context, we recently suggested the use of thrombomodulin (TM) [26,27], one of the major

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anticoagulant components of the endothelial surface [28–30] with 30,000–100,000 molecules per cell [31]. TM is a transmembrane spanning protein but can also be cleaved from the membrane to circulate in a soluble form [32]. It binds to thrombin to form a 1:1 complex. Thrombin bound to TM consequently loses its procoagulant and proinflammatory functions: It cannot cleave fibrinogen or activate platelets and factor XIII [33,34]. Simultaneously, the TM–thrombin complex proteolytically activates protein C. The catalytic efficiency of thrombin to activate protein C is accelerated about 10,000-fold [35,36]. Activated protein C (APC) plays a pivotal role in controlling hemostasis [37–41] as well as inflammatory processes [42]. Activated protein C selectively inactivates blood coagulation factor Va and VIIIa. Another blood plasma protein—protein S—functions as a cofactor in this reaction. Protein C is inhibited through  $\alpha$ -1-antiprotease, the protein C inhibitor and  $\alpha$ -2-macroglobulin and has a half-life in plasma of about 20–30 min [43]. The TM–thrombin complex also inhibits fibrinolysis by activating the procarboxypeptidase TAFI (thrombin-activatable fibrinolysis inhibitor) [44] a property which formerly was connected to protein C [45]. TM containing a galactosaminoglycan moiety can additionally accelerate the inactivation of thrombin by antithrombin III and might also directly enhance plasminogen activation [46].

In view of the above-described functions of TM its application in the treatment of thromboembolic diseases is very attractive. Several studies provide evidence that the application of a recombinantly produced soluble variant of TM or the use of activated protein C can be advantageous for the patient in different ways [47].

Similarly, the localized use of TM as a modifying agent for biomaterials was expected to provide benefits for the hemocompatibility of medical devices. Experiments with this objective [48–50] have recently been applied to various biomaterials including poly(ethylene) (PE) [51], poly(tetrafluoroethylene) (PTFE) [26,27,52] and poly(etherurethane) (PEUU) [53]. These earlier studies utilized TM coatings on polymer bulk materials and verified the activity of the immobilized TM by biochemical binding assays. In extension of this work our study was focussed on the preparation of molecularly defined model substrates bearing covalently attached TM and on the evaluation of these samples by *in vitro* experiments using freshly drawn human whole blood to analyze the functional performance of chemically attached TM in a realistic set-up.

## 2. Materials and methods

The following samples were prepared and analyzed:

G: glass plate,

P: poly(tetrafluoroethylene) plate (PTFE),

M1: glass plate coated with poly(octadecene *alt* maleic anhydride) after tempering,

M2: M1 after autoclaving (hydrolysis of the anhydride moieties),

PEG: M1 after conversion with poly(ethylene glycol) spacer (MW 600),

TM: PEG after immobilization of TM.

Glass (G) and PTFE (P) were used as reference materials which had been thoroughly studied in blood incubation experiments before [54]. Further, in addition to the ultimate TM-immobilized polymer film, each step of the formation of the TM-bearing coating was analyzed.

### 2.1. Preparation of sample carriers and reference materials

The thin layered polymer substrates were prepared on standard glass discs ( $\text{Na}_2\text{O} \cdot \text{CaO} \cdot 6\text{SiO}_2$  [55]). The glass plates were cleaned in 50% ethanol and subsequently in Milli-Q water (Millipore Corporation, Molsheim, France) in an ultrasonic bath for at least 30 min and afterwards exposed to Caro's acid (an oxidizing solution consisting of sulfuric acid and potassium peroxodisulfate) for 1 h, thoroughly rinsed again with Milli-Q water and dried under a nitrogen stream. Subsequently, the glass carriers were treated overnight with a 10 mM solution of 3-aminopropyl-dimethylethoxysilane (ABCR, Karlsruhe, Germany).

Reference materials (glass plates: Floatglas, Berliner Glas KG, Berlin, Germany, and poly(tetrafluoroethylene) plates: PTFE Nüchritz, Nüchritz, Germany) were cleaned in ultrasonic bath, immersed into Caro's acid and rinsed with ethanol and Milli-Q water (glass) or extensively rinsed with detergent and Milli-Q water (PTFE), respectively. The reference materials were sterilized by autoclave before use.

### 2.2. Thin film formation

Glass plates prepared as described above (or similarly pretreated  $\text{SiO}_2$ -wafers) were spin-coated (RC5, Suess Microtec, Garching, Germany) with 0.08% poly(octadecene *alt* maleic anhydride) (PO-MA) (Polysciences Inc., Warrington, PA) solutions in tetrahydrofuran (Fluka, Deisenhofen, Germany) [56,57]. Stable covalent binding of the polymer films to the glass or  $\text{SiO}_2$  carriers and the reconstitution of the anhydride moiety were achieved by tempering at 120°C for 2 h. Afterwards, amino groups were generated by conversion of the polymer films with an aqueous 0.1 M solution of diamminobutane (Fluka, Deisenhofen, Germany) for 4 min followed by rinsing with Milli-Q water, 0.01 M HCl and Milli-Q for 1 min, respectively.

Subsequently, the carriers were kept at 120°C for 2 h and after cooling the substrates were immersed in 50 mM

polyethylene glycol (PEG) diacid  $M_r = 600$  (Fluka Chemie, Buchs, Switzerland), 50 mM *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 25 mM *N*-hydroxysulfosuccinimide (Fluka Chemie, Buchs, Switzerland) in PBS at pH 7.4 overnight. The samples were thoroughly rinsed with Milli-Q and dried under a nitrogen stream.

Prior to protein immobilization free carboxylic acid groups of the polyethylene glycol modified surfaces were again activated by 50 mM *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide and 25 mM *N*-hydroxysulfosuccinimide in PBS at pH 7.4 for 3 h. The activated surfaces were washed twice with Milli-Q water.

### 2.3. TM immobilization

TM (50  $\mu\text{g/ml}$ ) in PBS was exposed to the activated surfaces overnight at 4°C. The recombinant protein used in this study was prepared by Asahi Chemical Industry Co., Tokyo, Japan and obtained as a kind gift of Prof. Y. Ikada (Suzuka University of Medical Science, Japan). The TM-coated polymer films were washed five times with pure PBS and stored under PBS until incubation with blood for 16 h.

### 2.4. Characterization of the TM layer

#### 2.4.1. HPLC-based amino acid analysis

All glassware for amino acid analysis was cleaned with chromic acid before use. TM-coated samples were prepared as described above using glass coverslips fixed in home-built immobilization chambers to guarantee the contact of the protein solution with well-defined surface areas. The TM-coated carriers were subjected to vapor phase hydrolysis with 6 N HCl (Fluka, Deisenhofen, Germany) + 1% Phenol (Microselect >99.5%, Fluka, Deisenhofen, Germany), at 110°C for 24 h under reduced pressure [58–60]. Following hydrolysis the generated primary amines of the amino acids formed fluorescent isoindol derivatives with *ortho*-phthalaldehyde (OPA) (in 0.2 M  $\text{H}_3\text{BO}_3$  (99.5%, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) pH 10.2 with mercaptoethanol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany)) as described in [61]. Subsequent amino acid analysis of the hydrolyzates was performed with a HPLC system (Series 1100, Agilent Technologies, Böblingen, Germany) equipped with degasser, quaternary pump, auto-injector and fluorescence detector. Hydrolyzates and amino acids standards were separated on a reversed phase HPLC column (ZORBAX, SB-C18 3.5  $\mu\text{m}$ , 4.6  $\times$  150 mm<sup>2</sup>, Agilent Technologies, Böblingen, Germany) using a gradient. Quantification was accomplished using fluorescence detection at 455 nm with excitation at 335 nm comparing the results to a standard amino acid solution as reference. TM

amounts were determined numerically from amino acid quantities as described elsewhere [58].

#### 2.4.2. Ellipsometry

Immobilization of TM on PO-MA via PEG-spacers was also followed by ellipsometry using  $\text{SiO}_2$  wafers. Layer thickness values were determined after each step of the layer formation for carefully rinsed (Milli-Q water) and dried samples in at least four independent experiments. The thickness values were fitted using an optical four-layer model (Si/ $\text{SiO}_2$ /organic layer/ambient) based on data obtained from ellipsometric measurements with a single wavelength device (632.8 nm) ELX-02 (DRE Dr. Riss Ellipsometerbau GmbH, Ratzeburg, Germany) at a fixed angle of incidence (68°). The refractive index of PO-MA was determined independently to 1.5037 by spectroscopic multi-wavelength ellipsometry (M44 Woolam Inc., Lincoln, NE) and kept invariant for the evaluation of the layers compared in this study.

#### 2.4.3. Fluorescence microscopy

Surface-bound TM was further detected by immunostaining/fluorescence microscopy using plain PEG-modified surfaces as control. The sample surfaces were treated with PBS (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) containing 2 mM EDTA (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 0.03% Pluronic F58NF (BASF AG, Ludwigshafen, Germany) over night, then incubated for 30 min with an anti-human TM antibody (1:200, American Diagnostica, Greenwich, USA) followed by extensive rinsing and incubation with a TRITC labeled secondary antibody (1:50, donkey anti-sheep, Dianova, Hamburg, Germany). The surface was imaged using a confocal laser scanning microscope (TCS SP, Leica, Bensheim, Germany) with a 40  $\times$  oil immersion objective.

### 2.5. Hemocompatibility assessment of the TM-coated surfaces

#### 2.5.1. Experimental procedure of blood incubation

The experiments were done as described in [54]. Shortly, whole human blood (90 ml) was drawn by venopuncture from healthy donors (donor 1, 33 years, experiments 1–3 for all parameters except bradykinin, donor 2, 33 years, determination of bradykinin) who did not take any medication for more than 10 days with a 19-gauge needle into medical syringes (Braun, Melsungen, Germany). The study was approved by the Ethics Committee of the Dresden University Hospital, Dresden, Germany. Informed consent was obtained from each donor prior to blood donation. The syringe was pre-filled with a heparin portion (Heparin Sodium Salt Grade I-a: from porcine intestinal mucosa, Sigma-Aldrich, Steinheim, Germany) to yield a final

concentration of 5 IU/ml (blood/heparin solution vol/vol 19/1) and drained into the incubation chambers instantly after blood drawing. Heparin was used as an anticoagulant for these experiments even though a variety of heparin-induced reactions are well known [62,63]. Yet heparin does not block any activation cascade completely as EDTA or citrate do. Since blood out of one incubation chamber was used to determine the activation of different cascadic systems and cells this anticoagulant is the best alternative. Concerning the biochemical reactions it has to be underlined that heparin interacts not only with antithrombin to inhibit thrombin and FXa but other complex interactions occur with thrombocytes [64,65] and the complement system [66]. Heparin regulates C1 functional activity and supports the inhibitory effect of factor *H* just to name two of a number of inhibiting but also augmenting effects on the complement system.

To determine the amount of bradykinin formed upon HMWK cleavage an additional inhibitor had to be applied. The ACE inhibitor captopril ([2S]-1-[3-Mercapto-2-methylpropionyl]-L-proline, Sigma-Aldrich, Steinheim, Germany) was used (0.1 µg/ml) for that purpose.

The inhouse-built incubation chambers use two parallel test surfaces each, distanced by a cylindrical PTFE spacer [54]. The sample surface in this chamber type is 29 cm<sup>2</sup>, the filling volume app. 5 ml and the ratio of the blood-contacting sample surface to the PTFE spacer surface 6.1. To avoid sedimentation of blood cells during the incubation the chambers were turned slowly by a rotary construction with an epicyclic gear which was placed in an incubator (B6030, Heraeus, Hanau, Germany) at 37°C. The incubation time was 3 h or 20 min for the bradykinin determination, respectively.

#### 2.5.2. Blood analysis

Cell numbers (leukocytes, platelets, erythrocytes) were analyzed by means of a blood analyzer Coulter AcT<sub>diff</sub> (Beckmann-Coulter, Krefeld, Germany).

Commercial enzyme-linked immunosorbent assays (ELISA) were used to characterize coagulation activation (thrombin–antithrombin complex (TAT), Enzygnost TAT micro, Behring, Marburg, Germany), contact activation (Bradykinin, Peninsula Laboratories, San Carlos, USA; Factor XIIa, Shield Diagnostics, Dundee, UK), complement activation (C5a, Enzygnost C5a micro, DRG Diagnostica, Marburg, Germany) and platelet activation (platelet factor 4 (PF4), Diagnostica Stago, Asnières, France). Blood samples before or after incubation were mixed with specific inhibitors for each assay and centrifuged according to the respective instruction manuals. The gained plasma was frozen and stored at –70°C until analysis.

Hemolysis was tested according to the international standard ISO/TR 7405-1984 (F). Shortly, the blood was

centrifuged at 1500 × *g* at 4°C, 100 µl of the gained plasma was measured at 540 nm using the microtiter-plate photometer Anthos 2010 (Anthos Mikrosystems, Krefeld, Germany).

#### 2.5.3. Sample surface analysis after incubation

Adhesion and characteristic shapes of blood cells on the sample surfaces after blood incubation were investigated by scanning electron microscopy (SEM). The samples were washed with phosphate buffered saline (PBS, pH 7.4) to remove non-adherent cells. Subsequently, the adherent cells were fixed with glutaraldehyde (Serva Electrophoresis GmbH, Heidelberg, Germany) (2% in PBS) for 1 h, rinsed with PBS and dehydrated with a graded ethanol series [67]. Samples were critical point dried (CPD 030, BAL-TEC, Schalksmühle, Germany), gold coated with a sputter coater (SCD 050, BAL-TEC, Schalksmühle, Germany) and examined by means of a scanning electron microscope (XL 30 ESEM FEG, FEI-Philips, Eindhoven, Netherlands).

#### 2.5.4. Statistical analysis

Results are presented either as the mean with standard deviation or as individual data. The significance of differences was calculated based on the Student's *t*-test for paired samples. Differences were considered significant at the 95% confidence level ( $P < 0.05$ ).

### 3. Results and discussion

#### 3.1. Characterization of immobilized TM

The HPLC-based calculation of immobilized TM provided a mean surface concentration of  $159 \pm 20$  ng/cm<sup>2</sup>. The value was obtained from five independent experiments. This surface density is by far larger than on endothelial cells where the surface concentration was calculated to 2–8 ng/cm<sup>2</sup> (assuming 30,000–100,000 copies of TM per cell [31]).

The increase of the layer thickness with each modification step of the substrate was determined by ellipsometric measurements on four separate wafers and is shown in Fig. 1.

Starting from an initial value of  $1.7 \pm 0.4$  nm for the PO-MA layer the thickness increases to  $4.4 \pm 0.7$  nm with the formation of the PEG layer. The immobilization of TM adds  $1.8 \pm 0.8$  nm to a final height of  $6.2 \pm 1.3$  nm.

The surface density of reactive anhydride groups on the PO-MA film was determined to app.  $6 \times 10^{13}$  cm<sup>-2</sup> [57]. On the base of the TM amount determined by HPLC the amount of molecules can be determined to  $1.3 \times 10^{12}$  cm<sup>-2</sup>—that is one TM molecule on every 46th

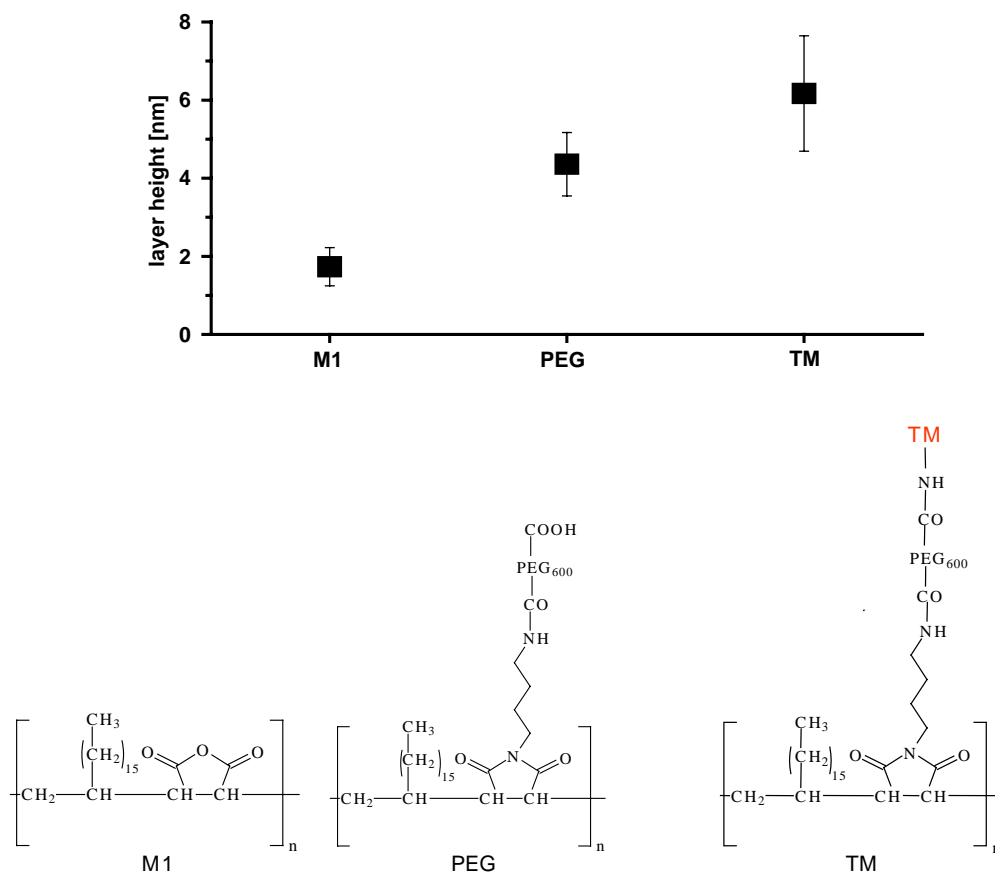


Fig. 1. Layer thickness measured by ellipsometry of surface M1 (poly(octadecene alt maleic anhydride) after tempering), PEG (M1 after conversion with poly(ethylene glycol) spacer (MW 600)), TM (PEG after immobilization of thrombomodulin). Mean with SD of measurements on four separate wafers. Below corresponding to the ellipsometric data a schematic presentation of the surfaces M1, PEG and TM.

anhydride group. As TM is not directly linked to the PO-MA film but through a three-step conversion ((1) diaminobutan, (2) PEG-diacid, (3) TM) the surface density of reactive sites available for TM binding can be expected to become substantially reduced due to incomplete conversion and/or bulkiness of the reactants. Also, multiple attachment of one TM through a number of surface moieties can be assumed.

Immobilized TM was also visualized after immunofluorescence staining. As can be seen in Fig. 2A the fluorescence is strongest on the surface with TM using the anti-TM antibodies. Unspecific adsorption of the anti-human TM antibody to the PEG surface is minimal as obvious from Fig. 2B. Unspecific adsorption of the secondary antibody to the TM as well as to the PEG surface also is negligible (Figs. 2C and D).

The immunofluorescence data indicate that the surface of the polymer film is evenly covered with the protein. There are no irregularities or imperfections of the layer on the sub-micron scale.

The composition of the TM molecule was confirmed by the amino acid analysis performed for protein

quantification and allows to predict a most probable orientation of the surface-bound protein. The TM structure can be divided into five distinct subunits: a NH<sub>2</sub>-terminal lectin-like domain (Ala<sup>1</sup>–Asp<sup>226</sup>) followed by six tandem epidermal growth factor (EGF) like domains (Cys<sup>227</sup>–Cys<sup>462</sup>), a serine/threonine-rich region (Asp<sup>463</sup>–Ser<sup>497</sup>), a transmembrane domain (Gly<sup>498</sup>–Leu<sup>521</sup>) and a carboxyterminal intracellular domain (Arg<sup>522</sup>–Leu<sup>557</sup>) [68,69]. The immobilization procedure stochastically links NH<sub>2</sub> groups of the protein (side chains of lysine) to the PEG spacer. TM contains eight lysine residues (confirmed by the relative amount of lysine obtained by the HPLC-based amino acid analysis described in 2.4). Five of these lysine units are located near the carboxyl terminus representing the cytoplasmic section of the protein [68,69]. Thus, with the applied immobilization technique there is a high probability to orient TM in a functionally active way. Based on that we may anticipate the ability of the immobilized TM to enhance the thrombin activity towards protein C which is related to a relatively small structural part of TM termed EGF 4–6 [34,70] (Fig. 3).

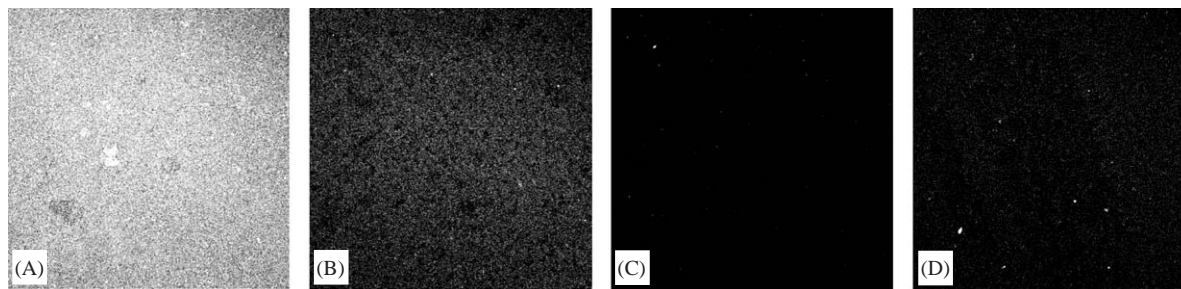


Fig. 2. Detection of immobilized thrombomodulin on surface using fluorescence labeled antibodies and laser scanning microscopy detection. First step: labeling with anti-human TM antibody second: incubation with TRITC labeled secondary antibody. Samples: (A) TM: Thrombomodulin immobilized onto PEG (MW 600) modified poly(octadecene alt maleic anhydride), labeled with first and second antibody. (B) PEG: PEG (MW 600) modified poly(octadecene alt maleic anhydride), labeled with first and second antibody. (C) TM labeled only with second antibody. (D) PEG labeled only with second antibody.

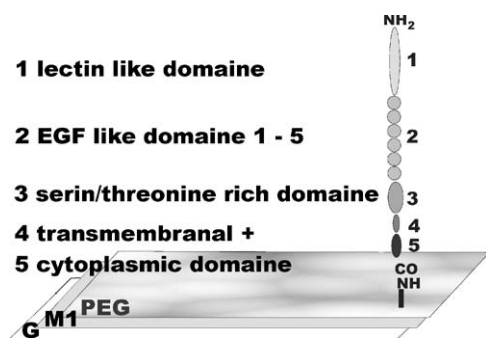


Fig. 3. Proposed scheme of thrombomodulin immobilization onto M1 (poly(octadecene alt maleic anhydride) after tempering) via PEG spacer using the lysin residues in the cytoplasmic domain of thrombomodulin.

### 3.2. Hemocompatibility data

The hemocompatibility assessment of the samples was performed in three independent experiments with blood repeatedly taken from one donor. The results are shown either as mean of 6 data points (2 of each experiment, 9 data points for the initial value, 3 for each experiment) with standard deviation or plotted as single points (■, ▼, ●: experiments 1–3) using a logarithmic scale which reflects the natural dynamics of biological processes best.

Fig. 4 shows the results of the TAT formation analysis. The initial values were below the detection limit for some of the samples. Activation of coagulation was highest with glass and M2 (poly(octadecene alt maleic acid)). The lowest results can be seen with the TM-coated sample. They were not significantly different from the initial values.

Glass activated the blood coagulation to a much higher extent than PTFE ( $P \leq 0.01$ ). The M2 surface exposing carboxylic acid functions also showed a high potential to activate the coagulation system. M2 was significantly more activating than M1 ( $P \leq 0.025$ ) which presents reactive anhydride surface groups to the blood but no free carboxylic acid functions. PEG displayed

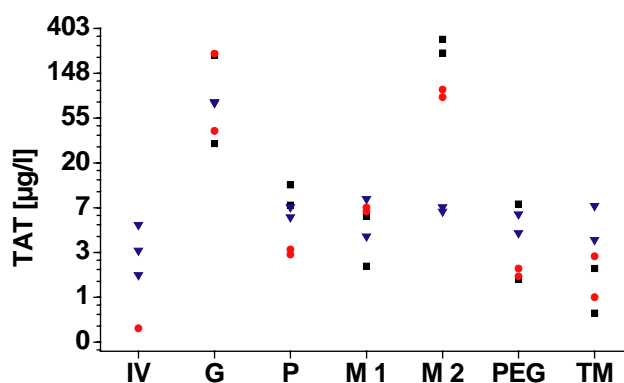


Fig. 4. Activation of coagulation after in vitro incubation (3 h) with heparinized (5 IU/ml) whole human blood measured as amount of TAT ( $\mu\text{g/l}$ ) (Thrombin-Antithrombin-complex, detected with ELISA assay). Samples: reference materials glass and PTFE, polymer substrates M1 (poly(octadecene alt maleic anhydride) after tempering) and M2 (M1 after autoclaving), modified materials PEG (M1 after conversion with poly(ethylene glycol) spacer (MW 600) and TM (PEG after immobilization of thrombomodulin) compared to the initial value (IV) in blood before incubation (results gained from 3 individual experiments; ■, ▼, ●: experiment 1–3,  $n = 2$  for each experiment).

less TAT formation (mean value) than M1, which was even more reduced on the TM-immobilized surface. Yet only the difference of M1–TM was significantly relevant ( $P \leq 0.05$ ). Surface-bound TM also reduced TAT formation compared to the reference material PTFE ( $P \leq 0.025$ ).

The lower amounts of thrombin formation using the TM-coated surface are in line with the well-known functional characteristics of TM. Supposedly the differences should be even more pronounced with the usage of blood containing less anticoagulant heparin which itself binds thrombin and acts as a competitive inhibitor for TM binding [71].

The bradykinin concentration in blood is considered to reflect the degree of contact activation in blood [72]. Bradykinin was determined in blood with addition of ACE inhibitor in two separate experiments in duplicate. The results are presented in Fig. 5. The initial value of bradykinin in blood was very low ( $0.04 \pm 0.04 \text{ ng/ml}$ ). It



significant reduction in C5a formation compared to M1 ( $P \leq 0.05$ ) and M2 ( $P \leq 0.025$ ) but only the TM immobilization reduced the C5a level considerably ( $P \leq 0.0005$  TM compared to PEG, M1 and M2;  $P \leq 0.001$  TM compared to glass).

Leukocytes can adhere to the biomaterials surface via ligand recognition of adsorbed plasma proteins like fibrinogen, von-Willebrand-factor and fibronectin. They may also be activated through soluble substances like complement fragment C5a. The adhesion to surfaces or the loss through destruction should be relevant in the leukocyte count. The percentage of leukocytes after blood incubation compared to the initial value is presented in Fig. 8. The decline was significant for the samples M1 and M2 with both only 64% of leukocytes of the initial value of  $5.7/8.5/5.9 \times 10^9/\text{ml}$ , respectively (experiments 1–3).

The activation of platelets on biomaterial surfaces can partly be realized through thrombin activation. Yet our in vitro set-up with a relatively high amount of heparin in blood blocked this reaction rather effectively. Direct activation paths of platelets on biomaterials through interaction of platelet glycoprotein with adhesion promoting proteins adsorbed on the biomaterials surface dominated the overall effect of platelet activation. The reduction of protein adsorption on the PEG surface led to a distinct reduction of formation of platelet factor 4 with the PEG surface.

A positive effect of the reduction of protein adsorption on the PEG surface was visible in the reduction of formation of platelet factor 4 with the PEG surface.

There was no significant positive effect of the TM immobilization on the activation of platelets as can be seen in Fig. 9.

The amount of platelets in blood was determined to 92–94% (mean value) of the initial value in blood for all

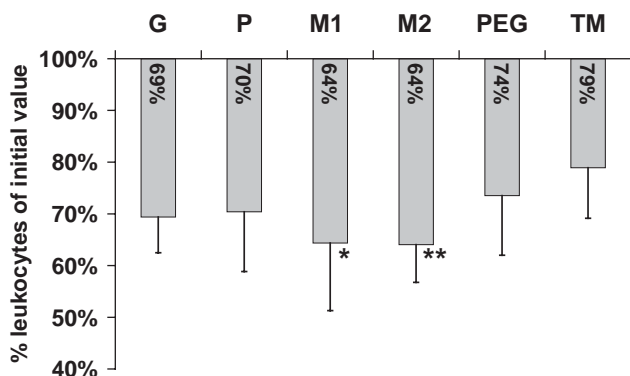


Fig. 8. Amount of leukocytes in blood after incubation with heparinized (5 IU/ml) whole human blood quantified with a Coulter AcT<sub>diff</sub> (see Materials and Methods), initial value before incubation is set as 100%, samples are displayed as percentage of IV,  $n=6$  mean with SD of 3 individual experiments with two repeats per sample) \* $P \leq 0.05$ , \*\* $P \leq 0.01$  compared to TM, other samples are not significantly different from the TM sample.

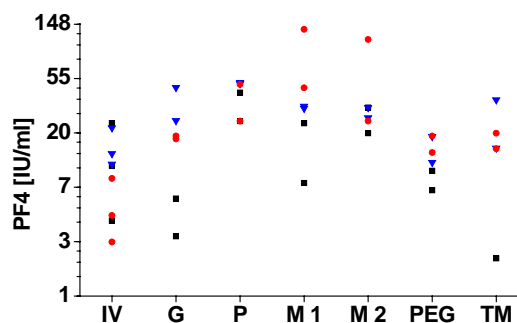


Fig. 9. Activation of platelets presented as PF4 (IU/ml) in blood (detected with ELISA assay) after in vitro incubation (3 h) of materials with heparinized (5 IU/ml) whole human blood. Materials: reference materials glass and PTFE, polymer substrates M1 (poly(octadecene alt maleic anhydride) after tempering) and M2 (M1 after autoclaving), modified materials PEG (M1 after conversion with poly(ethylene glycol) spacer (MW 600) and TM (PEG after immobilization of thrombomodulin) compared to the initial value (IV) in blood before incubation (results gained from 3 individual experiments; ■, ▼, ●: experiments 1–3,  $n=2$  for each experiment).

samples. No differences were detectable (data not shown).

The hemolysis of the samples after incubation was very low for all of the samples and showed no significant difference between the samples (data not shown).

The analysis of the surface after the blood contact by SEM is shown in Fig. 10. The surfaces were analyzed carefully on different spots. The reference material glass (A) showed more leukocyte adhesion than PTFE (B) which as a hydrophobic material displayed considerable more adhesion of platelets. The surface of M1 (C) was densely covered with leukocytes—correlating well with the drop of leukocytes in blood with an average of 64% of the initial value (Fig. 8). M2 (D) also displayed a similar drop in leukocyte numbers in blood after activation. Yet, the surface obviously induced an even higher activation of the cells indicated by stronger deformation which lead to a considerable loss of cell shape. The immobilization of PEG (E) can be expected to reduce the overall protein adsorption due to the hydrophilic, flexible structure of the PEG which, in turn, reduces cell adhesion. The samples with subsequent immobilization of TM (F) have a similar appearance. This observation was also reported for TM immobilized surfaces in [50].

#### 4. Discussion

TM, an integral part of the endothelial cells shows several characteristics that promise a significant improvement of the hemocompatibility of TM-coated biomaterials in blood contact. TM forms a complex with thrombin—the key-enzyme of blood coagulation cascade and by this inhibits its procoagulant properties.

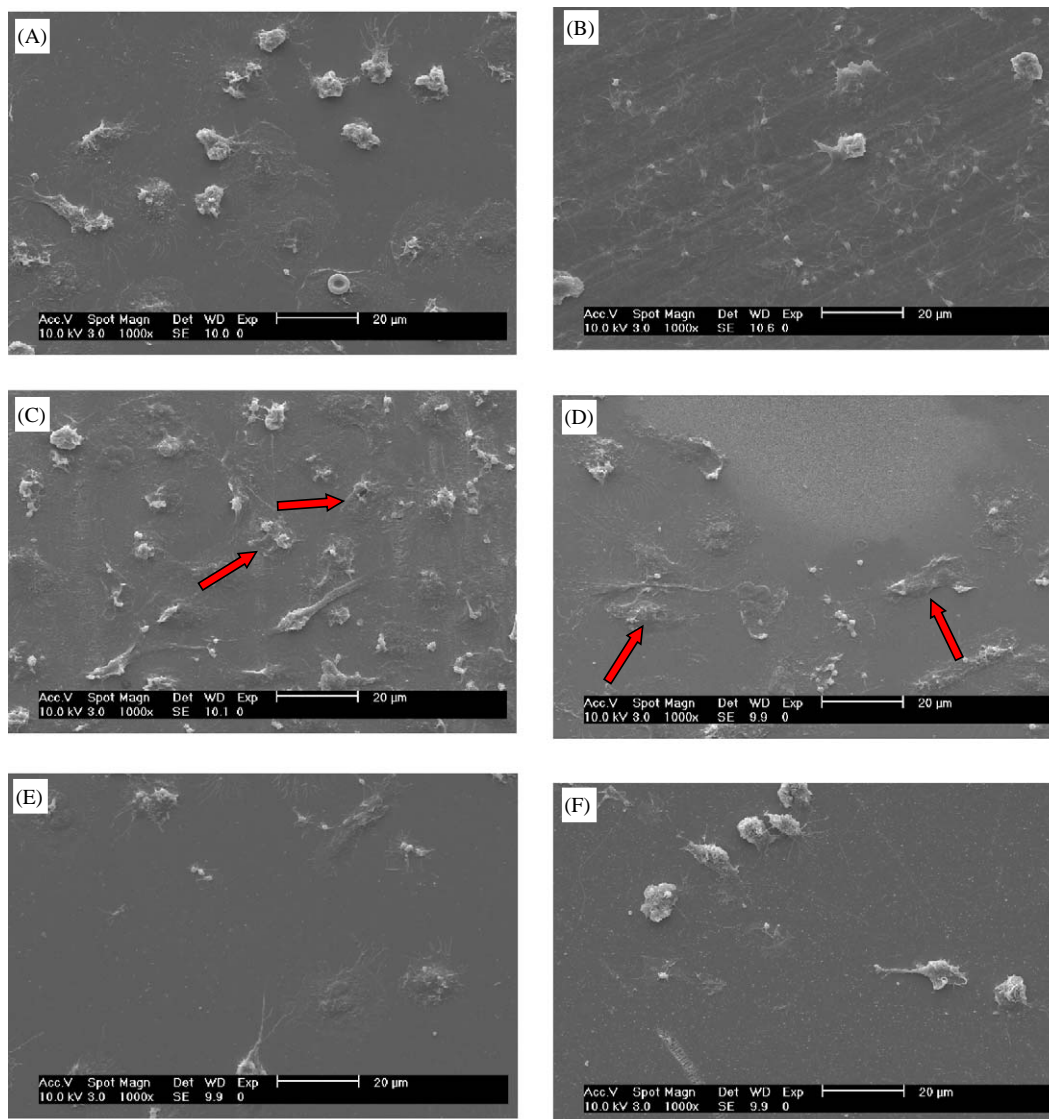


Fig. 10. REM analysis of surfaces after incubation with heparinized (5IU/ml) whole human blood for 3 h. (Processing of samples: fixation and drying are described in Materials and Methods.) These pictures show exemplarily the results of experiment 1 which corresponds to ■ in Figs. 4, 7 and 9—Material: (A) reference material glass G; (B) reference material PTFE P; (C) M1 (poly(octadecene alt maleic anhydride) after tempering); (D) M2 (M1 after autoclaving); (E) PEG (M1 after conversion with poly(ethylene glycol) spacer (MW 600)); (F) TM (PEG after immobilization of thrombomodulin).

Additionally, the amplified activation of protein C to APC adds to the positive effects of TM. APC inhibits the coagulation factors V and VIII and affects immune reactions [32–42].

Stable layers of covalently immobilized TM were prepared on top of solid supports coated with thin films of reactive maleic anhydride copolymers. TM binding to the polymer films was achieved after active ester formation at the carboxylic acid terminus of polymer-bound PEG spacers which was followed by ellipsometry (layer thickness), HPLC-based amino acid analysis and immunofluorescence. The amount of surface bound TM was determined to  $159 \pm 20 \text{ ng/cm}^2$  which is considerably more than reported to be found on the surface of

endothelial cells. Due to a favorable location of the lysine residues involved in this type of immobilization process the orientation of TM on the surface presumably resembles the natural one on endothelial cells facing blood and tissue, respectively.

This assumption is supported by the results of our study which indicate that the immobilized TM maintains its key characteristics and by a former study [26] and the results of Li et al. [52] who could demonstrate the activity of immobilized TM on PTFE using a chromogenic assay. The prepared substrates may on one hand help to unravel important physiological properties of TM without the interference of other components of endothelial cells. On the other hand, surfaces with

immobilized TM feature characteristics that can be most beneficial for different hemocompatibility aspects concerning biomaterials used in blood contact and may therefore be applied as bioactive coatings for medical devices.

Comparison of TM-coated samples with reference materials in hemocompatibility assays further enabled us to speculate on the impact of certain physicochemical surface characteristics on the initiation of coagulation and immunologic reactions *in vitro*.

The trigger of blood coagulation in *in vitro* set ups still remains speculative and is supposed to depend on a multitude of surface properties. Electrical charge, hydrophilicity/hydrophobicity and surface roughness are considered to be the most relevant. The so-called contact system of blood coagulation is thought to be initiated through an autoactivation process of FXII after binding to a negatively charged surface, where autoactivation of zymogen FXII occurs converting it to an active serine protease (FXIIa). FXIIa cleaves prekallikrein to kallikrein and FXI to FXIa [73,74]. Consequential bradykinin is cleaved from high molecular weight kininogen through FXIa, kallikrein or FXIIa (with different reaction velocities) [75]. FXIa activates FIX to FIXa which forms the intrinsic factor Xase complex with FVIIIa on a membrane surface. FXa forms the prothrombinase complex with its cofactor Va. The high procoagulant activity of glass could therefore be attributed to negative charges on the hydrophilic glass surface (water contact angle of app. 21° (proceeding) and 17° (receding), data not shown) [76,77]. Considering the high amount of bradykinin formed on the surface and also the elevated levels of FXIIa the activation of coagulation mainly in consequence of contact activation is presumable.

A strong formation of TAT is also shown by M2 which similarly exhibits a high density of negatively charged (acidic) surface groups (app.  $12 \times 10^{13} \text{ cm}^{-2}$  [57]) and displays elevated levels of bradykinin and to a lesser extent FXIIa. Although we cannot exclude definitively that other reactions mediated by the contact system lead to the elevated TAT level the direct activation of coagulation factors is most likely.

The other surfaces display little TAT formation or contact activation; only TM shows a noticeable decline in bradykinin content compared to the other surfaces. The decrease of TAT formation with the PEG surface compared to M1 and M2 can be attributed to a well-known prevention of protein adsorption on hydrophilic molecular cilia [78,79]. TM reduces the thrombin formation compared to PEG to a small extent. For the reported experiments we chose a standard screening approach for hemocompatibility parameters of materials which involves the anticoagulation of the applied blood with 5 IU/ml. Considering the low degrees of thrombin formation induced by the samples under

consideration the anticoagulant reactions of TM which inhibits further thrombin formation can be expected to be more distinct with higher thrombin formation e.g. derived from a release of TF in an *in vivo* situation. The formation of trace amounts of thrombin (either through the action of TF/VIIa or through the activation of the contact system as on some of our surfaces) could induce an amplification loop using thrombin's activating properties on FXI [80]. *In vitro* experiments using different or less anticoagulants can help to improve the prognostic value of the results [81].

The complete lack of bradykinin formation on the TM surface compared to an at least slight increase with the samples PEG, P and M1 implies a specific biochemical effect of TM. The activation of protein C and of thrombin-activatable-fibrinolysis-inhibitor (TAFI) might cause these effects through interactions with proteins of the contact system. Since bradykinin is a potent mediator of immune reactions a reduction of bradykinin formation is beneficial *in vivo*.

The relevance of the contact system has been investigated intensely *in vivo* indicating that it is also significant for the fibrinolytic and complement system [75]. Kallikrein was shown to cleave C5 to C5a and therefore activates the complement system [82]. It is probable that the high amount of C5a generated with glass and M1 can also be ascribed to the pronounced contact activation of these samples. The complement activation of PEG [83,84] can be attributed to the hydrophilic groups on the surface which are known to interact with complement proteins. These groups are also present on glass and further support C5a generation here.

Complement activation with the thrombomodulin samples is significantly suppressed compared to the PEG surface. We hypothesize that the thrombin/thrombomodulin complex itself can deactivate C5a through the activation of the thrombin-activatable-fibrinolysis-inhibitor (TAFI) [85]. We could show that this effect might be relevant in an *in vitro* setting for the hemocompatibility of biomaterials. Additionally, plasmin activates the complement system [86]. Since thrombomodulin helps in the activation of TAFI an inhibition of the complement system *in vivo* through TM might also be expected through an inhibitory effect on fibrinolysis.

Furthermore, the significance of APC for immunologic processes in blood was investigated and proved intensely during the last years [87–89]. APC inhibits under certain circumstances the activity of monocytes and through this can help to down-regulate the inflammatory potential at the site of the biomaterials-tissue contact.

The impact of immobilized TM on further relevant interactions of man-made materials with living matter can only be speculative since the parameters considered

in this study only cover a small sector of reactions that are influenced. For example, it was recently shown that the formation of intimal hyperplasia and restenosis is reduced with the application of recombinant TM [90]. TM may also inhibit fibrin-enhanced FXIII activation by competition for binding sites on thrombin as was shown by kinetic analysis using thrombin mutants [91].

We conclude that the combination of anti-coagulant and anti-inflammatory properties make TM a privileged candidate for the specific modification of biomaterials surfaces and anticipate that the multitude of the therewith induced blood reactions deserves further detailed investigation both *in vitro* and *in vivo*. The applied immobilization procedure established that TM stochastically attached to artificial surfaces through covalent binding of amine-terminated amino acid side chains can retain its functional characteristics, most probably due to the accumulation of lysine in the intracellular domain of the protein.

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