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Short communication

A Novel Surface Modification Using Tissue Factor Reconstituted in Phospholipid Vesicles for the Activation of Blood Coagulation

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Abstract

We describe a novel method to immobilize recombinant human tissue factor (rhTF) reconstituted in phospholipid vesicles. The rhTF vesicles were immobilized in a multilayer vesicle structure using cholesterol–DNA tethers spontaneously inserted into the lipid membrane. The properties of the rhTF vesicle surface modification were characterized by surface plasmon resonance biosensor technology. As an application of this surface modification, we investigated its use as a blood coagulation activating surface. The coagulation activating capacity of the surface modification was tested by exposure to human whole blood in a flow chamber. No increase in rhTF levels in the blood was found after passage through the flow chamber, indicating that the rhTF surface modification was stable. Thrombin–antithrombin (TAT) and prothrombin fragment (PF) 1 + 2 levels increased after exposure to the surface, and decreased in a concentration-dependent way upon addition of melagatran (a direct thrombin inhibitor), i.e., coagulation activity triggered by rhTF could be suppressed by anticoagulation. The results with this new thrombogenic surface are promising, and will be further developed into a useful tool for coagulation related investigations, e.g., characterization of anticoagulants and biomaterials.

Keywords

Tissue factor, tethered lipid vesicles, cholesterol–DNA, blood coagulation, flow chamber.

1. Introduction

Many common biomaterials trigger the intrinsic pathway of blood coagulation, e.g., by contact activation. However, the physiologically more relevant extrinsic pathway

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for blood coagulation is triggered by encryption of tissue factor (TF) on the membrane surface of endothelial and blood cells [1]. In the present study, we, therefore, aim at a surface modification where recombinant human TF (rhTF) is immobilised in a suitable phospholipid environment, mimicking cell membranes. Such a surface is relevant for many applications within the fields of haemostasis and biomaterials.

We describe a new lipid-based surface immobilization method of the transmembrane protein TF, together with its application as a pro-coagulant surface in a Badimon flow chamber [2] (Fig. 1a). This is to the best of our knowledge the first example of a lipid-based immobilization strategy for TF. In contrast, previous biosensor interaction studies have been performed using soluble TF (see, e.g., Refs [3–5]), which does not contain the transmembrane and the cytosolic parts of the protein, and the protein was covalently immobilised to the sensor surface. Recently, TF has also been adsorbed onto collagen in platelet adhesion studies [6]. The lipid-based immobilisation opens up for experiments, where, e.g., TF interactions with other proteins can be studied in the presence of lipid membranes of different composition. In the present application, however, the focus is not to characterise the interaction of TF with another specific protein, but instead to study its effect in a more complex whole blood environment. The immobilisation approach uses cholesterol–DNA tethers that insert spontaneously into the vesicle membrane when

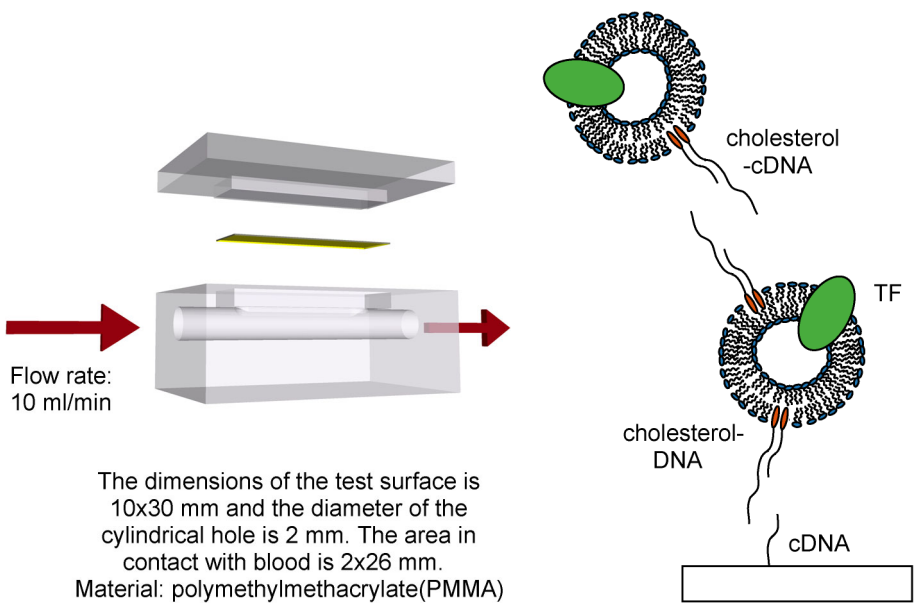

Figure 1. Schematic drawings showing (a) the blood flow chamber [2] (at a flow rate of 10 ml/min, the shear rate (200 s^{-1}) corresponds to a venous shear) and (b) the principle for the build-up of multilayer vesicle layers on the TF test surfaces with complementary DNA tags. This figure is published in colour at http://www.ingenta.com

added to the vesicle solution [7]. These vesicles are subsequently captured on complementary DNA strands immobilised on the chamber wall surface, or on an already immobilized layer of vesicles, and shows yet another application of this methodology (Fig. 1b) [7–9]. Multilayer vesicle structures, using this approach, have been described previously [10], and were recently applied to reconstituted membrane proteins [11]. The lipid-based surface preparation described here, i.e., the immobilisation of rhTF-containing vesicles, was developed using first the surface plasmon resonance (SPR)-based biosensor technique. Thereafter, a gold-coated silicon surface modified in a similar way as the sensor chips was placed in a Badimon flow chamber and the effect on recalcified whole blood passing through the chamber was studied. The pro-coagulant effect was determined by an ELISA technique from the level of the thrombin–antithrombin (TAT)-complex and prothrombin fragment (PF) $1 + 2$ in samples taken after passage over the rhTF-modified surface. In addition, the anticoagulant effect of a direct thrombin inhibitor was studied.

Promising results were obtained for this new type of thrombogenic surface containing rhTF, mimicking activation by the extrinsic pathway of the coagulation in whole blood. Furthermore, this surface could be a useful tool for investigations of the coagulation activity properties of fresh human blood and the further characterization of the effect of different anticoagulants.

2. Materials and Methods

For control experiments, vesicles were extruded using 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleyl-*sn*-glycero-3-[phospho-L-serine] (POPS) (Avanti Polar Lipids, Alabaster, AL, USA) (for details see Ref. [9]). Vesicles with rhTF (Innovin, DadeBehring, Eschborn, Germany) were reconstituted following the instructions by the supplier and frozen at −20°C. Aliquots were thawed at 37°C and vortexed. The vesicles were characterized by dynamic light scattering using a Brookhaven BI-90 particle sizer (Brookhaven Instrument, Holtsville, NY, USA); rhTF vesicles were not diluted, whereas extruded vesicles were diluted to 0.25 mg/ml in buffer. DNA tethers [7–9] (prototype kit from Layerlab, Gothenburg, Sweden) were added to the vesicles shortly before use; 10 µl of the DNA tether solution (10 µmol/l) to 200 µl of the rhTF vesicles or by mixing of the DNA tethers (0.4 µmol/l) with extruded vesicles (1:3).

The SPR experiments were performed using a Biacore 2000 instrument (Biacore, Uppsala, Sweden), Tris buffer (10 mmol/l Tris, 100 mmol/l NaCl, pH 7.4), and a flow rate of 5 µl/min. Neutravidin (Pierce, Rockford, IL, USA), diluted to 20 µg/ml in acetate buffer (10 mmol/l, pH 4.5) was immobilized covalently using an amine coupling kit (Biacore) onto a C1 Sensor chip (Biacore). Subsequently, biotin–DNA was injected over the neutravidin surface (2 µmol/l). DNA tags exposing the same sequence as the DNA strands on the surface, as well as DNA tags exposing the complementary sequence, were added to two aliquots of lipid vesicles (see above),

and the two solutions were injected one after the other over the surfaces such that a four-layer-structure of vesicles was assembled. Regeneration of the surface was straight-forward by applying a pulse of detergent (CHAPS), followed by an injection of water [7–9]. Factor VIIa (Novo Nordisk, Bagsværd, Denmark) was diluted to 24 μmol/l and stored frozen at −20°C.

Silicon wafers were precut and a gold film was deposited by thermal evaporation (adhesion layer of 1 nm of Ti (0.5 Å/s) followed by 40 nm of Au (0.8 Å/s); pressure 10^{-7} mbar). The gold-coated samples were cleaned in a mixture of ammonia (4%) and hydrogen peroxide (4%) at 80°C for 10–15 min and then immediately subjected to 30-min-exposures of biotin–BSA (Sigma-Aldrich, St. Louis, MO, USA) (0.1 mg/ml), neutravidin (0.1 mg/ml) and biotin–DNA (2 μmol/l) [7]. The immobilization of three layers of vesicles (30 min) was separated by addition (30 min) of more of the DNA tether (diluted to 0.5 μmol/l in buffer), to ensure that there would be enough anchoring points for the subsequent layer. Mixing was thorough, and once the lipids had been added, care was taken to avoid exposure of the sample surface to air. The running buffer was 10 mmol/l Hepes, 150 mmol/l NaCl, pH 7.4 (HBS-N, Biacore). Reference test surfaces for the flow chamber were prepared as described by Badimon *et al.* [2]. Frozen pig aortas were thawed, divided longitudinally, cleaned and cut into pieces of 3.5 × 1 cm. To obtain a collagen surface, the intima of the aorta pieces was first pulled off.

After informed consent blood samples were drawn from human healthy volunteers. No medication was allowed during seven days prior to blood collection. Directly after collection the tubes were turned gently to mix anticoagulant (10% of 0.109 mol/l (3.2%) sodium citrate solution) and blood.

The flow chambers (see Fig. 1a) were connected to a peristaltic pump and kept at 37°C during the experiment. After 30 min incubation at 37°C, directly before each experiment, 0.5 ml saline (when required containing the thrombin inhibitor (melagatran, AstraZeneca R&D, Mölndal, Sweden)) was added to each tube with 50 ml blood and gently mixed. Next 750 μl $CaCl_2$ (0.5 mol/l) was added to the tube, and after mixing, a sample was takwn for analysis of the free Ca^{2+} concentration in a blood as analyser. The recalcified blood was immediately pumped (10 ml/min) through the flow chamber to a waste container. Samples for analysis of TAT complex, PF 1 + 2 and rhTF were taken from the recalcified blood in the tube before and after the experiment, as well as after passage through the chamber (0.5–1 min runtime). All samples were anti-coagulated with 1 part stop solution (100 mmol/l EDTA, 30 μmol/l indomethacin, 0.129 mol/l sodium citrate, 1500 U/ml heparin and 1000 U/ml aprotinin (Trasylol)) to 9 parts of blood. Levels of TAT, PF 1 + 2 and rhTF in plasma samples were measured using sandwich enzyme-linked immunoassays (Enzygnost® TAT micro, Enzygnost® PF 1 + 2 (both from DadeBehring) and the Immubind Tissue Factor ELISA Kit, AmericanDiagnostica, Stamford, CT, USA, respectively). Platelets, white blood cells, red blood cells, hemoglobin and haematocrit were checked to be within normal ranges.

3. Results and Discussion

3.1. Assembly and Characterization of Multilayer Vesicle Layer by SPR

Vesicle multilayers were assembled onto DNA-modified SPR sensor chips. For rhTF vesicles, the response for four layers of immobilized vesicles was approx. 3000 response units (RU), whereas for plain, extruded POPC/POPS (4:1) vesicles, the response was approx. 6000 RU (Fig. 2). The result for the POPC/POPS vesicles is well in accordance with previous results [10], and the difference in the amount of material bound to the surface is likely mostly caused by differences in the size distribution of vesicles in the two types of lipid preparations. By dynamic light scattering, the extruded vesicles had a mean diameter of approx. 100 nm, whereas the rhTF vesicles were smaller, approx. 70 nm, and showed a much broader size distribution (the polydispersity was approx. 0.1 and approx. 0.5 for the extruded vesicles and the rhTF vesicles, respectively). Smaller vesicles diffuse more rapidly; therefore, the vesicle layers on the rhTF-modified surface are likely to be thinner, as reflected by the lower response in the SPR measurements. The incremental response for each added vesicle layer typically decreased as the number of layers increased, probably due to the decrease in SPR sensitivity at increasing distance from the surface [10]. The two types of vesicle multilayers were exposed to factor VIIa at concentrations ranging from 1 nmol/l up to 1 μmol/l. Significant binding of the protein was seen to the rhTF vesicle multilayer, but the amount bound was at all concentrations lower than the amount bound to the negatively charged control surface. It was concluded that factor VIIa was not a proper marker for the pres-

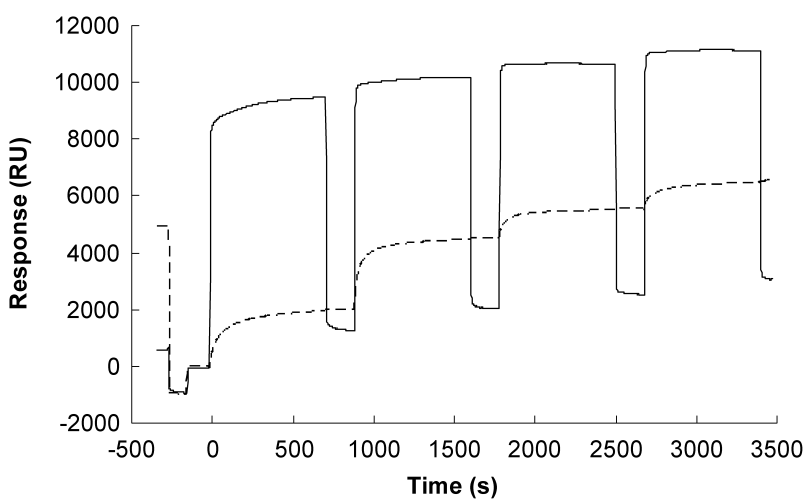

Figure 2. Sensorgrams obtained by SPR showing the assembly of 4-layer lipid vesicle structures. Extruded POPC/POPS (4:1) vesicles (dashed line) and rhTF vesicles (solid line) were used. Each experiment included a short injection of CHAPS, to regenerate the surface prior to the assembly of the multilayer lipid structure at $t = 0$ s. In the experiment with rhTF vesicles, there were large bulk shifts due to differences in bulk composition between the running buffer and the rhTF vesicle solution.

ence of exposed rhTF due to its inherent binding to negatively charged vesicles in general [7–9]. Note that the number of TF molecules required for the activation of coagulation (pmol/l in bulk) is much smaller than the number required to record a signal for the FVIIa binding (the detection limit for SPR assays is ng/cm^2).

3.2. Function of Multilayer Vesicle Layer in Blood Flow Experiments

Immobilization of rhTF vesicles to gold-coated test surfaces was achieved by a similar sequence of solutions as in the SPR experiments, prior to mounting of the surfaces in the blood flow chamber. Samples were taken from the blood before and after passage through the flow chamber. Plasma was analyzed with respect to levels of rhTF as a marker of the stability of the surface modification, and levels of TAT complex and PF 1 + 2, as markers of coagulation activation. The rhTF levels in the blood after passage over the TF-modified surfaces were slightly elevated for all types of surfaces. However, this was not due to release of rhTF from the surface, since control surfaces modified with neutral (POPC) or negatively charged (POPC/POPS 4:1) vesicles, also resulted in similar (apparent) increases in the measured rhTF levels without having been exposed, at any point, to the rhTF vesicles. TAT complex levels showed increases of 1.1 and 3.4 times in control experiments with POPC vesicles and POPC/POPS (4:1) vesicles, respectively. The increase for the negatively charged vesicles was comparable to the increase for the aorta piece (3.5 times). For rhTF vesicles the increase was 8.5 times the initial TAT levels. This clearly shows that the rhTF-loaded surfaces have a coagulation activating capacity better than the negatively charged phospholipids alone. The PF 1 + 2 levels increased 1.5 times for the aorta piece, 1.3 times for the negatively charged POPC/POPS (4:1), 1.8 times for the negatively charged POPC/POPS (4:1) with rhTF while the neutral POPC vesicles showed decreased PF 1 + 2 levels after the blood had passed over this surface. The higher levels of TAT and PF 1 + 2 downstream the rhTF-loaded surfaces compared to the aorta piece (Fig. 3a) were

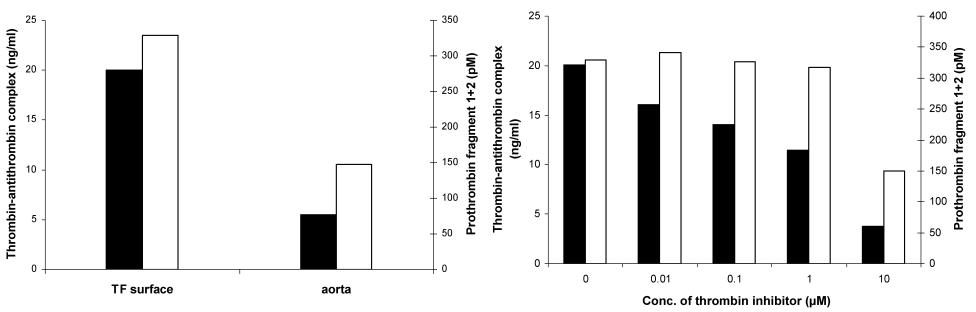

Figure 3. Levels of thrombin–antithrombin (TAT, black columns) complex and prothrombin fragment 1 + 2 (PT 1 + 2, white columns) detected after passage over the test surface (averages from duplicate samples). (a) Comparison between the TF test surface and the conventional aorta surface and (b) TAT complex and PT 1 + 2 levels obtained when adding different concentrations of a thrombin inhibitor (melagatran).

probably due to capture of the activation markers on the aorta piece. Both TAT and PF 1 + 2 levels showed a concentration-dependent inhibition with increasing concentrations of a thrombin inhibitor (Fig. 3b). The concentration required for 50% inhibition (IC_{50}) was estimated to be 1.4 μmol/l and 5.3 μmol/l for TAT and PF 1 + 2, respectively. These IC_{50} values are one order of magnitude higher than the IC_{50} obtained with the reference method based on aorta pieces (data not shown).

A disadvantage of using a commercial rhTF preparation as used in this study is that its exact phospholipid composition is not publicly known. The recommended reconstitution was, however, very simple; water is added to a lyophilized mixture of protein and lipids, and the suspension is thoroughly mixed. Nevertheless, this might not be optimal to obtain maximum performance of the immobilized layer, and we are presently reconstituting rhTF with defined vesicles to achieve control over the lipid membrane composition. We also note that for many applications of biomaterials in contact with blood, surface modifications which do not activate coagulation, or induce thrombus formation, are desired. This has been achieved, e.g., by the immobilization of poly(ethylene glycol) chains to surfaces [12, 13]. The present surface modification focuses on a controlled surface where rhTF is immobilized reconstituted in a lipid membrane that may or may not activate coagulation, depending on the composition. Such surfaces will find different applications, in particular for the detailed studies of TF interactions with other proteins in biosensor assays but also as controllable activating surfaces in haemostatic models. In particular, patterned substrates can be obtained. Here, a homogenous rhTF surface was applied to achieve the activation of pro-coagulant processes *in vitro*. Towards this end, further functionality will be added to the lipid-based surface modification described in the present communication, such that thrombi are formed on the actual test surface.

4. Conclusion

In conclusion, the present communication shows a proof-of-concept for a novel lipid-based surface modification using tethered lipid vesicles with reconstituted rhTF. We show that this surface modification is functional in whole blood experiments using a flow chamber, thus using conditions which are highly relevant for the development of thrombogenic surfaces activating the extrinsic pathway of the coagulation. The further development of this work is in progress and aims at surface modifications that do not only activate, but also support formation of thrombi on the test surface, thus providing new possibilities for investigating thrombotic processes *in vitro*.

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