Whole blood coagulation on protein adsorption-resistant PEG and peptide functionalised PEG-coated titanium surfaces

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Abstract

The aim of this study was to investigate whole blood coagulation on low blood plasma protein adsorbing surfaces. For this purpose, the polycationic graft copolymer poly(L-lysine)-*g*-poly(ethylene glycol) (PLL-*g*-PEG), PLL-*g*-PEG grafted with a cell adhesive peptide containing the amino acid sequence -Arg-Gly-Asp- (RGD), and PLL-*g*-PEG with a control peptide -Arg-Asp-Gly- (RDG) were adsorbed onto titanium (oxide), forming stable monomolecular adlayers through electrostatic attraction. Free oscillation rheometry and complementary techniques were used to measure the coagulation time and other interactions of the surfaces with native whole blood, recalcified platelet rich plasma (PRP), and recalcified citrated platelet free plasma (PFP). The results show that the uncoated titanium surfaces (reference) activated platelets and quickly triggered the coagulation cascade via the intrinsic pathway, whereas the PLL-*g*-PEG surfaces displayed a prolonged coagulation time, approximately 2-3 times longer compared to uncoated titanium. We hypothesize that blood coagulates outside the vascular system independent of low protein adsorption to or activation by surfaces, due to the absence of an active down-regulation of procoagulative processes by the vascular endothelium.

Introduction

Undesirable blood-material interactions are recognised problems in blood-contacting biomedical devices, such as catheters, vascular stents, artificial heart valves, and biosensors. It is well known that blood-contacting surfaces may activate the coagulation and complement systems, as well as trigger cellular responses [1, 2]. This results in serious complications. The surface mediated activation of the haemostatic system leads to platelet adhesion, activation, and thrombus formation that impairs the function of the device. Adsorbed proteins, amongst them FXII, fibrinogen, immunoglobulins, and complement factors are known to initiate cascade reactions, and hence artificial surface protein adsorption properties are believed to be crucial for blood compatibility.

One approach to avoid coagulation in e.g. cardiopulmonary bypass systems is to coat the blood-contacting surfaces with active molecules such as heparin that takes part in the elimination of thrombin, other coagulation factors, such as factor Xa, and suppresses the activation of complement [3-5]. However, long-term use of immobilised heparin may also introduce negative side effects such as surface clogging by plasma proteins, uptake of growth factors and fat, and finally calcification.

Another approach is to render surfaces protein adsorption and cell adhesion resistant (stealth surfaces). The hypothesis in this case is that the absence of adsorbed plasma proteins prevents active interactions between platelets and other blood cells with the surface, hence reducing the degree of blood activation. One common strategy to render interfaces protein adsorption resistant is to immobilise poly(ethylene glycol) in a dense brush form [6-13]. The resulting low protein adsorption and cell binding has been attributed to the high surface water retaining capacity,

charge neutrality, as well as steric repulsion and surface exclusion effects by the poly(ethylene glycol) brushes [14].

Titanium (Ti) is an often preferred biomaterial in bone and soft tissue applications, and occasionally also in blood contacting stents. The surface oxide (TiO_2) that is spontaneously formed in air and water has a point of zero charge (pzc) at pH 5-6. The net negative charge at a physiological pH is known to activate the intrinsic pathway of coagulation [15]. In recent publications, [9, 11, 12, 16] it was shown that polycationic poly(L-lysine)-*g*-poly(ethylene glycol) (PEG) attach strongly to negatively charged metal oxides by coulombic interactions via its positively charged poly-L-lysine backbone (PLL, $\text{pK}_a \sim 10.5$), thereby reducing the protein adsorption from blood serum to typically less than 5 ng/cm^2 . Recently, the same type of surface modification rendered titanium (oxide) surfaces resistant to human blood plasma at 37°C [17]. In the present work, whole blood coagulation was investigated by comparing titanium (TiO_2) without and with monomolecular coatings of PEG and PEG functionalised with either the common integrin receptor binding motif Arg-Gly-Asp (PEG-RGD); or a scrambled inactive (control) peptide Arg-Asp-Gly (PEG-RDG) [18, 19]. The coagulation times of native whole blood, recalcified citrated platelet rich plasma (PRP), and citrated platelet free plasma (PFP) were determined by free oscillation rheometry (FOR), and complementary techniques were used to study protein- and cell interactions with the surfaces. The results indicate that the often claimed protein- and cell binding resistance of PEG-coated surfaces is not a sufficient criterion to avoid a global blood coagulation *in vitro*, a hypothesis consistent with conclusions drawn by e.g. Sefton and Gemmell [20].

Materials and Methods

1. Poly(L-Lysine)-g-poly(ethylene glycol) graft co-polymers

Non-functionalised (PEG) and peptide-functionalised (PEG-peptide) poly(L-lysine)-g-poly(ethylene glycol) polymers were synthesised according to a previously published protocol [11, 17, 21]. The general architecture of the polymers (Fig. 1) used in this work was based on a PLL-backbone of approximately 120 L-lysine units (average value in view of the polydispersity of the polymer), a PEG side chain with approximately 47 ethylene glycol units (PEG MW ~ 2 kDa) and a grafting ratio g , expressed as the number of lysine monomers per PEG side chain, between 3.3 and 4.5. PEG polymers with optimised architectures have consistently shown the capacity to form dense PEG brush adlayers on negatively charged surfaces such as TiO₂, rendering them highly protein resistant in full serum [11] or heparinised plasma with typically 3 - 5 ng/cm² adsorbed mass [17].

For each polymer batch the grafting ratio, g , and the degree of peptide functionalisation were determined by NMR [17], protein adsorption in serum was monitored *in situ* by optical waveguide lightmode spectroscopy (OWLS) [11, 16] and in blood plasma *in situ* by null ellipsometry. Data related to molecular weight, grafting ratio g of the polymers, and the resulting grafted PEG-RGD and PEG-RDG peptide surface concentrations are summarised in Table 1.

2. Substrates

Preparation of titanium-coated silicon for XPS and in situ null ellipsometry measurements

Polished 3-inch silicon wafers (P-type, from Wacker-Chemie GmbH, München, Germany,) were coated with a 200 nm thick optically reflecting titanium metal by physical vapour deposition

(PVD) techniques at 6.7×10^{-7} mbar and with a deposition rate of 0.5 nm/s. The metal was of Specpure quality (Johnson Matthey Chemicals Ltd, UK). The titanium-coated silicon-wafers were cut with a diamond cutter to samples of 10 mm x 10 mm size. The samples were cleaned from macroscopic contamination or cutting dust by ultra-sonication in toluene and 2-propanol for 15 min each, dried in flowing nitrogen and stored in a clean, closed sample holder until needed.

CP titanium inserts for free oscillation rheometry (FOR)

The cup-shaped titanium inserts with outer diameter 11 mm, inner diameter 10 mm, and height 20 mm were manufactured from CP titanium rods (Ströms Mekaniska AB, Linköping, Sweden). The outer size and shape were adjusted to fit inside the reference Reorox 4 polyamide (PA) cups. In order to analyse the surface properties of the inner cup surface, the inserts were cut manually and cleaned using the same washing protocols as for titanium on silicon samples.

Surface characterisation of the substrate surface

AFM measurements on the titanium-coated silicon substrate were performed with a commercial scanning probe microscope (Nanoscope IIIA, Digital Instruments, Santa Barbara, USA). The measurements were made on radio frequency (RF)-plasma cleaned (Plasmaprep 100, Nanotech, Sweden) samples in ambient air. The surface topography and lateral force measurements were made simultaneously by operating the instrument in the contact mode. The arithmetic average R_a and the root mean square R_q were determined to be 8.3 ± 1.5 nm and 6.7 ± 1.2 nm, respectively. Scanning electron microscopy (SEM, LEO 1550 Gemini, Oberkochen, Germany) confirmed the appearance of the machined CP titanium inserts.

3. Buffers

The buffers used during the PEG/PEG-peptide synthesis work are described elsewhere [11, 17, 21]. During the present adsorption experiments, 10 mmol/L phosphate buffered saline (PBS) with a total ionic strength of 150 mol/L was prepared according to published protocols given by Tosatti *et al.* [17].

4. Blood and plasma

Heparinised (5 IU/mL) and citrated (0.011 mol/L) plasma were pooled from two apparently healthy donors at the Blood Transfusion Unit at Linköping University Hospital. The plasma was aliquoted in 2 mL vials and stored at -80°C until use.

Before use, plasma was thawed during three minutes at 37°C and temperature equilibrated for two minutes in 22°C.

To obtain citrated platelet free plasma (PFP), the plasma was filtered through a 0.22 µm sterile filter (MILLEX-GS, Millipore, Bedford, MA, USA).

Citrated whole blood was collected by drawing nine parts of venous blood from apparently healthy donors into Vacuette® blood collection tubes (Greiner Labortechnik GmbH, Kremsmünster, Austria) containing one part 0.129 mol/L sodium citrate. To obtain platelet rich plasma (PRP) the citrated blood was centrifuged for 20 min at 200 x g, followed by pipetting of the supernatant containing the platelets into new sample tubes. The platelet count in the PRP was adjusted to $200 \times 10^9/\text{L}$.

Native whole blood without anticoagulating substances was obtained by drawing venous blood from apparently healthy blood donors into S-Monovette® tubes (Sarstedt, Nümbrecht, Germany).

Hirudinised (Refludan[®], Aventis Pharma, Frankfurt, Germany) blood was obtained by drawing nine parts of blood into S-Monovette[®] tubes (Sarstedt, Nümbrecht, Germany) containing one part of hirudin (2000 antithrombin units/ml) with a final activity of 200 antithrombin units/ml.

Factor XII deficient native whole blood was generously donated by an individual with complete factor XII deficiency (factor XII levels <0.005 IE/mL in two samples collected with one year interval). The factor XII activity was determined by the laboratory of Clinical Chemistry at Karolinska Hospital in Stockholm, Sweden.

All blood donors gave their informed consent to the sampling, and the collection protocol was approved by the Ethical committee at Linköping University Hospital, Linköping, Sweden.

5. Surface preparations, analyses, and adsorption protocols

General

The lyophilised PEG and PEG-peptide polymers were dissolved to 1 mg/mL in PBS, pH 7.4, aliquoted and stored at -20°C until use. Before use, the polymer solutions were thawed during three minutes at 37°C followed by equilibration at experimental conditions (22°C) during two minutes.

The titanium-coated silicon samples or free oscillation rheometry titanium insert cups (see next section) were cleaned for 5 min in a radio-frequency oxygen plasma chamber (RF-plasma chamber) at an oxygen pressure of 0.1 mbar. The surfaces were then mounted inside an ellipsometer glass cuvette and PBS buffer was added. The system was allowed to equilibrate for 10 minutes at 22°C. The substrate ellipsometric angles (ψ) and (Δ) were determined (see below), and PBS replaced with a 1 mg/mL polymer solution. The polymer solution was removed after 15 min, and the cuvette rinsed 4 times in PBS. The ellipsometric angles were measured again after

10 minutes (n=6), and the adsorbed film thickness was calculated from the changes in the ellipsometric angles.

Subsequently, the PBS buffer was replaced for heparinised plasma during 30 min, and the thickness of the adsorbed protein layer determined after 4 rinsings in PBS buffer followed by 10 minutes of equilibration.

The inside of FOR titanium insert cups were polymer-coated following the applicable parts of the above described protocol.

XPS analysis of non-coated and polymer-coated titanium surfaces

The composition of the polymer layer on titanium was analysed by XPS according to a previously described protocol [17]. The analysed area was 6 mm² and the results represent a laterally averaged chemical composition, see Table 2, where the Scofield sensitivity factors were used [22]. The binding energies are relative to a main hydrocarbon peak (from residual contamination), set at 285.0 eV.

In situ null ellipsometry

In order to determine the PEG/PEG-peptide polymer deposition and protein adsorption upon whole plasma exposure 22°C onto titanium-coated silicon surfaces, *in situ* null ellipsometry was used (Rudolph Research AutoEL III, NJ, USA, with 70° angle of incidence). The samples were maintained at a fixed position in a glass cuvette and the measurements were made in the same spot for the duration of the experiment.

The fixed protein and buffer refractive indices at $\lambda=632.8$ nm were: $n_{\text{protein}} = 1.465$ and $n_{\text{buffer}} = 1.335$, respectively. The polymer refractive indices were assumed to be $n_{\text{PLL-g-PEG}}=1.62$ [9, 23].

The average thicknesses of the adsorbed layers were calculated according to the McCrackin algorithm [24]. The average adsorbed mass per unit area was calculated according to deFeijter [25] using $dn/dc = 0.169 \text{ cm}^3/\text{g}$, and a layer of 1 nm thickness then corresponded to approximately $70 \text{ ng}/\text{cm}^2$.

6. Biological assays

Free oscillation rheometry (FOR)

The coagulation time (CT) of native whole blood, citrated platelet free plasma (PFP), and citrated platelet rich plasma (PRP) was determined at 37°C , by free oscillation rheometry (FOR) with a four channel Reorox 4 instrument, softwares Reorox 4 bob v.011031 and Viewer v.2.13j from Global Hemostasis Institute MGR AB, Linköping, Sweden [26, 27].

CP titanium insert cups without and with PEG/PEG-peptide coatings, as described in section 5, were mounted inside the disposable polyamide (PA) sample cups (Global Hemostasis Institute MGR AB, Linköping, Sweden). The CT of the functionalised inserts were compared with those of uncoated inserts and reference PA cups.

Typical FOR tracings showing logarithmic damping (D) and frequency shift (Fq) as a function of time for native blood coagulation is shown in Figure 2.

The CT detection was based on a Pythagorean summation (Eq. 1) of changes in Fq and D upon coagulation and concomitant comparison of the result with a predefined weighted signal level C.

In the present study the “high sensitivity state detector” was used, corresponding to $C=0.01$ which in turn closely matches with the visual endpoint detection of blood coagulation [27].

$$C \leq \sqrt{(\Delta Fq^2 + \Delta D^2)}$$

Equation 1

During native whole blood coagulation studies, 500 μL native whole blood was added directly into the titanium cups and the CT was determined with the high sensitivity state detector. When PPP and PRP were used instead, 300 μL of the sample was mixed with 11 μL 0.5 mol/L CaCl_2 in each cup to obtain a free calcium concentration of approximately 1.2 mmol/L corresponding to the physiological concentration *in vivo*. The CT was subsequently measured as described above. In a separate series of experiment, inhibitors of the extrinsic pathway of coagulation; monoclonal anti-tissue factor antibodies (American Diagnostica Inc., Greenwich, CT, USA) and annexin V (Actiplate[®], Tau Technologies BV, Kattendijke, Netherlands), were used. Ten μL anti-TF (1 mg/mL) was then mixed into 490 μL native blood and incubated 5 minutes at 37 °C. The mixture was then put into the sample cups and placed in the instrument. The measurement was started and CT was measured. In experiments with annexin V, 12 μL annexin V (430 $\mu\text{g}/\text{ml}$) was mixed into 488 μL native blood and handled in the same way as the anti-TF treated blood.

Fluorescence Microscopy

Fluorescence microscopy and BODIPY[®] FL phalloidin staining of paraformaldehyde fixed cells were used to visualise the filamentous actin cytoskeleton and morphology of cells. The cells adhered from native whole blood to microscope cover slides coated with either PEG or PEG-peptide (Menzel-Gläser, Braunschweig, Germany), with the uncoated cover slides as reference. Blood was obtained from 2 blood donors and the analysis was performed in duplicate. The surfaces were modified similar to the silicon surfaces and stored in PBS until exposure to native blood during 10 min at 37 °C. The procedure is described elsewhere [28].

Flow cytometry

The platelet expression of P-selectin (CD62P), glycoprotein Ib (GPIb), and glycoprotein IIb/IIIa (GPIIb/IIIa) were studied by flow cytometry. Hirudin anticoagulated (200 AT units/mL) whole blood (500 μ L) was incubated at 37°C for 20 minutes in titanium cups that were coated with the different PEG polymers. Pure titanium inserts and PA cups were used as references. From each incubated sample, 10 μ L of whole blood was aspirated and diluted in 50 μ L HEPES buffer, pH 7.4 and 10 μ L anti-CD62P (mouse, monoclonal anti-CD62P-FITC, Immunotech, Marseille, France), anti-fibrinogen (chicken, polyclonal, Diapensia HB, Linköping, Sweden) or anti-vWF (chicken, polyclonal fab anti-vWF-FITC Diapensia HB, Linköping, Sweden) were added, as well as 10 μ L anti-GPIb-PE (mouse, monoclonal, Dako AS, Glostrup, Denmark) or anti-GPIIb-RPE (mouse, monoclonal, Dako AS, Glostrup, Denmark). Suitable negative antibody controls were used and anti-insulin-FITC (chicken, polyclonal, Diapensia HB, Linköping, Sweden) and anti-mouse-IgG (rabbit, polyclonal Dako AS, Glostrup, Denmark) were used to check the unspecific binding to platelets. The cell fraction expressing respective receptor protein was measured (Ortho Cytoron Absolute flow cytometer, Raritan, NY, USA.). The lysing solution contained anti-vWF-FITC, and anti-fibrinogen-FITC (chicken polyclonals, Diapensia HB, Linköping, Sweden).

Kallikrein measurements by S-2302 chromogenic substrate

PFP (40 μ L) and PBS (360 μ L) were added to titanium cups coated with either PEG- or PEG-peptide. After 10 min of incubation, 100 μ L of the solution from each cup was transferred to 1 mL plastic cuvettes. Then 200 μ L 4 mmol/L chromogenic substrate S-2302 (Chromogenix SpA, Milan, Italy) was added into the cuvette and incubated for 10 min. The reaction was stopped by the addition of 200 μ L citric acid (20% w/w). PBS was added to obtain a total volume of 1 mL.

The absorbance was measured at a wavelength of 405 nm with a spectrophotometer (UV-1601PC, Shimadzu Co, Japan). A mixture of 600 μ L PBS + 200 μ L S-2302 + 200 μ L citric acid was used as a blank reference.

7. Statistical methods

The ellipsometry results were assumed Gaussian distributed and presented as mean values $\pm$ SD. Comparisons between the measurement series were made by the two-tailed Student's t-test with Bonferroni correction.

The FOR results were also assumed normal distributed after test with normal probability plots and were evaluated using ANOVA based on unequal variances and the Bonferroni-Holm method for multiplicity correction.

Results

Surface characterisations

The SEM investigation of the CP titanium insert cups showed a surface structure typical for machined surfaces [29]. Titanium-coated silicon, and CP titanium insert cups were characterised by XPS in order to confirm the homogeneity of the monomolecular polymer coatings.

Table 2 summarises the chemical composition of the samples (RF-plasma cleaned) before and after PLL-g-PEG (PEG) adsorption on titanium-coated silicon and on machined titanium inserts. The results indicate that uncoated titanium inserts show in addition to titanium and oxygen, low amounts of nitrogen and sulphur. The signal at 396 eV indicate titanium present in the nitride form, as a result of the machining process.

After adsorption of PEG the adlayer composition showed strongly increased amounts of carbon and oxygen. The results are in agreement with previously published values [30]. On titanium insert surfaces, titanium nitride was observed at 396 eV, a second nitrogen at 400.1 eV, typical for the amine group of PLL. These nitrogen peaks were also observed on polymer-coated titanium on silicon surfaces.

In situ null ellipsometry was used to determine the thicknesses of the adsorbed polymer adlayers and to measure protein adsorption upon exposure to full heparinised plasma. Figure 3 shows that the thicknesses of the polymer layer was 1 nm on titanium. After incubation of the different surfaces in heparinised plasma, a 5-6 nm (350-420 ng/cm²) thick protein layer adsorbed onto uncoated titanium and 0.3-0.8 nm (21-56 ng/cm²) onto the PEG- and the PEG-peptide-coated surfaces. In the latter, the differences between the layer thickness before and after plasma exposure were not statistically significant, i.e. the adsorbed mass of proteins was below the detection limit of the ellipsometer.

Coagulation times

Free oscillation rheometry (FOR) was used to determine the coagulation time (CT) of native whole blood, FXII deficient native whole blood, recalcified citrated platelet rich plasma (PRP), and recalcified citrated platelet free plasma (PFP) upon exposure to the different surfaces (Figure 4). Normal native whole blood showed a significantly shorter CT in titanium than in PA cups, PEG-, PEG-RGD-, and PEG-RDG-coated titanium cups ($p < 0.05$). Factor XII deficient native whole blood showed a prolonged CT in all types of cups. Also the titanium CT became now comparable to the other surfaces. When recalcified PRP was used instead, the titanium CT was significantly shorter than that for cups coated with PEG-RGD or PEG-RDG ($p < 0.05$). PFP displayed a significant decrease in CT in titanium cups compared to PEG-RGD-coated cups ($p < 0.05$). Interestingly, the relatively protein resistant PEG surface did not significantly prolong the CT in any of the tested blood or plasma systems when compared to that of the reference PA surface.

Experiments were also made with the addition of coagulation inhibitors, annexin V that binds to negatively charged phospholipids on activated platelet membranes and suppress the coagulation cascade, and an anti-tissue factor antibody (anti-TF), which suppresses the extrinsic pathway of coagulation initiated via TF. The results are shown in Figure 5. Addition of annexin V ($n=4$) to native blood in PEG-coated sample cups increased CT (not significant), as compared to a control containing blood without additives ($n=4$). No significant differences in the CT for PEG-coated sample cups was observed upon the addition of anti-TF to native blood ($n=2$). No change in the CT was observed in titanium cups when annexin V treated native blood ($n=2$) and anti-TF treated native blood ($n=2$) were compared with blood without additives ($n=2$).

Fluorescence microscopy

In order to visualise platelet adhesion onto the different surfaces, fluorescence microscopy was utilised on uncoated or PEG-peptide-coated glass slides. The filamentous actin skeleton of the surface localised cells was stained and representative results are shown in Figure 6. Uncoated, negatively charged glass surfaces recruited and also activated many platelets which were extensively spread over the surface and displayed stress fibres. The RGD-functionalised titanium surfaces recruited large numbers of platelets which also displayed an extensive spreading. The PEG and the PEG-RDG surfaces, on the other hand, displayed only a few scattered surface-associated platelets with no apparent morphological signs of spreading and activation.

Flow cytometry

Flow cytometry was used to examine the expression of GPIIb/IIIa, GPIb, and CD62P integrin receptors on platelet membranes upon blood contact with the polymer-coated and non-coated surfaces. The results showed a relatively large donor variation (n=3). The expression of platelet CD62P and GPIb indicated surface related differences while the GPIIb/IIIa expression was low at all surfaces (Figure 7). The reference titanium insert cups induced a higher CD62P expression for all three donors while the other types of surfaces displayed low expression of CD62P. GPIb expression was elevated in titanium, PEG-RDG, and PA cups compared to PEG- and PEG-RGD-coated ones.

Kallikrein test (S-2302)

The kallikrein forming capacity in citrated PFP was studied using the chromogenic substrate, S-2302. The results (Figure 8) show that titanium surfaces induced formation of comparatively large amounts of kallikrein, which in turn accelerated the plasma and blood coagulation via the intrinsic pathway. The other surfaces in this study, i.e. titanium coated with PEG, PEG-RGD, or PEG-RDG, and the PA cups displayed comparably low kallikrein levels.

Discussion

Blood-material interactions that result in coagulation or complement activation continue to be a critical issue of blood-contacting devices. Despite large efforts in developing low activating interfaces the humoral responses often alters the subsequent blood cell adhesion in an unfavourable way thus limiting the usefulness of the device. One approach to avoid problems is to design functionally programmed surfaces that correspond in time and function with the changes in the host responses. Another extensively studied strategy is to prepare highly protein resistant surfaces and thereby minimise interactions with the cascade systems. In the present paper we investigated the coagulation behaviour of native whole blood, recalcified citrated platelet rich, and recalcified platelet free plasmas on low protein adsorbing PLL-*g*-PEG (PEG) coated titanium [17].

The results regarding surface composition and protein adsorption, are in agreement with a previously published work with the same type of surfaces [17]. Importantly, the blood plasma incubated surfaces did not bind antibodies against fibrinogen, high molecular weight kininogen (HMWK), and complement factor 3 (C3). In the present work, the bare (reference) titanium substrate adsorbed approximately 6 nm of plasma proteins and bound anti-fibrinogen and anti-HMWK into this layer, a result in agreement with the results by Kanagaraja *et al.* [31].

The PEG coating on titanium prolonged, in our study, the average coagulation time (CT) of recalcified citrated platelet free plasma (PFP) from approximately 12 minutes on reference titanium to 28 minutes. Thus, it can be concluded that in a coagulation system free from blood cells, the CT increased by more than a factor two in the presence of a monolayer of PEG. Similar coagulation times were observed for the PLL-*g*-PEG/PEG-RGD (PEG-RGD) and PLL-*g*-PEG/PEG-RDDG (PEG-RDDG) polymer adlayers, indicating none or low interaction between the

peptides and blood proteins and that the presence of the peptides did not change the resistance to protein adsorption of the PEG surfaces (Figure 4, left group of columns).

The above results agree well with the kallikrein forming capacity of the different surfaces (Figure 8), with a high kallikrein formation on titanium and lower levels at the other surfaces. This also supports the view that titanium is a contact activation surface, and hence displays a short CT in PFP.

When platelets were kept in the plasma, i.e. PRP, an overall reduction of the average CTs was observed. This was likely due to the presence of procoagulant phospholipids in activated platelet membranes. The phospholipids act as cofactors to coagulation factors and constitute a substrate on which activation complexes, convertases, are formed. This in turn increases the enzymatic turnover rate of the coagulation system. However, the relative differences in CT between the different surfaces remained similar to the PFP case (Figure 4) and strengthens the relevance of the results obtained with PFP.

When native whole blood was introduced a low activation of coagulation was demonstrated by the PEG-coated surfaces. The average CT for PEG-coated cups was prolonged about 18 minutes compared to titanium, i.e. more than 2.6 times. The PEG-RGD and PEG-RDG surfaces showed comparable coagulation times. The PEG-RDG surface was expected to possess a coagulation time similar to that of PEG, while the PEG-RGD surface was expected to display a shorter CT due to its demonstrated platelet binding capacity. In fact, a small decrease in the average CT was observed for this surface. Most surprisingly, the PA reference cup showed a longer CT than PEG-coated surfaces.

The reduced average CT for recalcified PRP and PFP systems compared to native whole blood is likely due to the addition of citrate, which results in a more procoagulant system after re-

calcification, as compared to a non-anticoagulated ditto. Therefore, the PRP and PFP results should only be compared with citrated plasma systems and definitely not with native whole blood. This demonstrates that studies involving blood-contacting artificial surfaces should be performed in non-anticoagulated whole blood since most anticoagulants affect the haemostatic systems, as shown by Schneider *et al.* [32].

To further evaluate the contribution from the contact activation process, native whole blood from a FXII deficient individual was used (Figure 4). The prolonged CT indicates that the contact coagulation process via FXII was, involved in the coagulation responses in the present study, especially for titanium where the maximum CT was prolonged about four times in FXII deficient blood. The average CT in the PEG system increased almost twice compared to native blood, strengthening the suspicion that the intrinsic pathway was involved in the *in vitro* coagulation process. Since the coagulation times of FXII deficient blood are in the same time range for all types of surfaces there seems to be either an activation process separated from the intrinsic pathway, or a very slow contact activation, that promotes coagulation in all blood samples that contact artificial surfaces.

To elucidate some possible mechanisms for the observed coagulation behaviour, annexin V and anti-tissue factor (anti-TF) were used to inhibit the coagulation process (Figure 5). Annexin V, blocks negatively charged phospholipids at platelet membranes or in microparticles. These phospholipids are necessary for an efficient coagulation cascade to proceed [33]. When annexin V was added to native blood in contact with PEG coatings, a prolonged CT from 27 minutes to 45 minutes was observed, indicating that the platelets were indeed activated during the normal whole blood coagulation process. However, this blocking of phospholipids seemed not effective in the case of pure titanium, probably due to a relatively massive contact activation with the

formation of kallikrein which in turn lead to thrombin formation that activated vast numbers of platelets. Therefore, annexin V was most likely depleted in this sample. The addition of anti-TF did not affect the coagulation times at all. The conclusion from the inhibition studies is that the extrinsic pathway via TF was not important for the initiation of the coagulation process and hence the activation of the intrinsic pathway and platelet activation determined the coagulation rate.

Flow cytometry highlighted the role of blood cells in the coagulation process (Figure 7). As expected, titanium activated platelets in the bulk solution, shown by the expression of the platelet activation marker CD62P. The PEG, PEG-RGD, PEG-RDG, and PA surfaces displayed low CD62P levels, indicating that the platelets were not activated in these systems. As mentioned, the results on titanium can be interpreted as an increased thrombin production via contact activation that in turn activated the platelets. Alternatively, the platelets became directly activated via interactions to titanium bound proteins, e.g. fibrinogen, C3, and HMWK. In fact, platelets have been observed to adhere and spread on titanium within 5 s after blood-material contact [34]. Regardless, the protein resistant PEG layers decreased the blood cell activation. We noticed that platelet binding and activation *per se* seemed not crucial for the coagulation kinetics, since the platelet binding PEG-RGD layer showed only a slightly shortened CT compared to the inactive reference PEG-RDG film.

The CD62P expression level in the present work may be an underestimation of the platelet activation according to Gemmell [35] since the activated platelets, were extensively bound onto the tested surfaces and may therefore be removed from the bulk solution during flow cytometry analysis. A possibly better estimation of the activation level would be to analyse soluble CD62P in the bulk phase .

Two other platelet activation markers, vWF and fibrinogen receptor expressions, were studied. Titanium, the PEG-RDG coating, and PA cups showed slightly increased vWF binding to activated GPIb, but no differences were observed between the different surfaces concerning fibrinogen binding to expressed GPIIb/IIIa. The increased vWF binding levels are a prerequisite for the later CD62P expression [36] and this seemed to be confirmed for titanium in the present study.

Previous observations during the use of GPIIb/IIIa blocking ligands indicate that platelet activation measured as the expression of CD62P depend on the expression of GPIIb/IIIa [37]. The present results did not show increased expression of activated GPIIb/IIIa on platelets in the whole blood that contacted the surfaces under study. However, Gemmell *et al.* faced the same phenomenon when they used anti-fibrinogen antibodies for blocking and suggested that this is due to a low number of activated platelets in the blood bulk phase while the activated platelets remain adhered to the activator surface [37].

In order to investigate effects like this, fluorescence microscopy was used to visualise stained filamentous actin cytoskeleton of adhered cells on hydrophilic glass, PEG, PEG-RGD, and PEG-RDG adlayers on glass, after incubations in native blood. The glass surface was used as a negatively charged surface that replaced the non-transparent titanium, thereby enabling light microscopy. The uncoated glass surfaces recruited a large number of activated cells, which were activated. Both the platelets and neutrophils showed prominent actin filaments and extensive spreading on glass. The PEG and PEG-RDG surfaces attracted low numbers of scattered and non-activated platelets and no neutrophils. The PEG-RGD adlayers showed spread platelets with stress fibres and spread neutrophils. Altogether, the fluorescence microscopy showed that cells did not attach to PEG and PEG-RDG surfaces while the PEG-RGD and glass surfaces showed an

extensive coverage with activated cells. The most immediate explanation for the observed low cell binding to PEG surfaces is its low protein adsorption capacity.

Why do then the coagulation process proceed also when both the contact- and platelet activations seem low? There are at least two possible explanations for this: It is possible but unlikely that the adhered PEG do not completely cover the underlying titanium surface. Since the coagulation process is an amplification cascade of events it may be enough if a few enzyme molecules become activated in order to initiate the coagulation. However, this is improbable since low amounts of kallikrein were formed on PEG surfaces. The other possible explanation is that the reduced haemostatic system cannot prevent the coagulation due to a lack of down-regulating processes governed by the endothelial cell lining, which is hard to mimic by artificial means. The luminal front of the endothelium faces the constantly circulating blood and maintains a non-thrombogenic system during normal conditions. The endothelial cells are the main regulators of the haemostasis and have a number of mechanisms to directly or indirectly affect the haemostatic balance [38]. This is illustrated by the increased risk of blood coagulation and thromboembolism during atrial fibrillation. The flow is disturbed, thereby attenuating blood contact with the endothelium. Successful electroconversion restores the flow and decrease the D-dimer level and fibrinopeptide A levels [39]. The lack of an *active* regulatory component in the present and other artificial systems may not be possible to replace with protein resistant surfaces. The combination of the two explanations above is of course also possible. In fact Basmadjian *et al.* [40] used mathematical modelling to investigate the possibility to completely block contact activation via FXII by the use of protein resistant surfaces. They concluded that it probably is impossible to find surfaces that lower the FXIIa levels to an extent that stops coagulation. They suggested that

the only way to achieve this is the use of active inhibition or down regulation of the procoagulative factors involved. The present data support such a hypothesis.

Finally, it is important that the present PEG coatings on titanium significantly reduced both the activation via the intrinsic pathway and the activation of platelets. Such a property in combination with the possibility to cover surfaces with specific functional groups, e.g. RGD-containing peptides, still make such surface modifications promising for a number of biotechnical and biomaterial applications.

Conclusions

This study shows that titanium accelerated coagulation via the intrinsic pathway and bound platelets that were activated. The PLL-*g*-PEG coating was resistant to protein adsorption and adhered few blood cells which displayed no morphological signs of activation. The non-adhesive PLL-*g*-PEG coating did not inhibit the coagulation process *in vitro* in native blood or citrated plasma, but reduced the activation of the systems. Activated platelets and membrane microparticles speed up the clot formation when present but no substantial differences in coagulation time were observed between a platelet binding PLL-*g*-PEG/PEG-RGD surface and the non-adhesive PLL-*g*-PEG/PEG-RDG. This suggests the presence of a procoagulant activity that proceeds in the blood bulk phase, which is independent of the surrounding artificial surface.

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Tables

Table 1. Molecular weight (MW) and grafting ratio g of the polymers and surface peptide concentration of the modified TiO₂ surfaces. The data was calculated from the polymer architecture (NMR) and the mass of adsorbed polymers (OWLS).

Polymer code	PLL- g -PEG	PLL- g -PEG/ 1.7% PEG-RDG	PLL- g -PEG/ 1.6% PEG-RGD
MW _{PLL-HBr} (g/mol) ^a	27.4K	25.7K	25.7K
MW _{PLL} (g/mol)	15.9K	15.7K	15.7K
Grafting ratio g	3.3	4.5	4.2
MW _{PEG side chain} (g/mol)	2.0K	2.0K	2.0K
MW _{PEG-VS} (g/mol)	-	3.4K	3.4K
MW _{Peptide} (g/mol)	-	1.4K	1.4K
Peptide-grafted side chains (in % of total PEG chains)	-	1.7	1.6
MW _{Polymer} (g/mol) ^b	96K	72K	76K
-RGD or -RDG peptide concentrations on TiO ₂ coated waveguides (pmol/cm ²)	0	0.7	0.7

a) PLL-HBr is the bromide salt of poly(L-lysine) used as educt in the synthesis

b) Calculated according to the formula published by Tosatti et al. [18]

Table 2. XPS binding energies ($E_B \pm 0.1\text{eV}$), components and normalised intensities¹ of cleaned and PLL-g-PEG coated titanium on silicon and CP titanium inserts.

Element	Assignment	Energy [eV]	Normalised intensities ^[1] titanium coated silicon		Normalised intensities ^[1] CP titanium inserts	
			RF-Plasma cleaned	PLL-g-PEG coated	RF-Plasma cleaned	PLL-g-PEG coated
C1s(1)	C-C, C-H contamination	285.0	8.1	25.8	5.5	26.4
C1s(2)	C-O	286.6				
C1s(3)	NHC(=O)	288.2				
O1s(1)	TiO ₂	529.9	63.5	52.2	58.9	50.5
O1s(2)	NHC(=O)	531.4				
O1s(3)	C-O	532.9				
Ti2p1/2	Ti(Metal)	454.1	28.4	19.5	28.4	18.8
Ti2p3/2	Ti(Metal)	459.7				
Ti2p1/2	TiO ₂	458.7				
Ti2p3/2	TiO ₂	464.4				
N1s(1)	NHC(=O) -C-NH ₃ ⁺	400.1	-	2.5	4.1	4.2
N1s(2)	TiN	396.1				
S2p(1)	Sulfate	169.4	-	-	3.0	-

¹ Experimental (overall) peak areas divided by the corresponding elemental sensitivity factors and expressed in % of the summed normalised peak areas.

Figure captions

Figure 1. Schematic diagram of the structure of a poly-L-lysine-*grafted*-poly(ethylene glycol) adsorbed on a titanium surface. The positively charged PLL-backbone interact electrostatically with the negatively charged oxide substrate at pH=7.4, while the more flexible PEG side chains form a brush and impart protein resistance; the PEG chains can also be end-functionalised with a peptide (Ψ).

Figure 2. A typical free oscillation rheometry (FOR) tracing, showing frequency and logarithmic damping recorded during coagulation of native blood in a polyamide (PA) sample cup. The coagulation time is denoted CT.

Figure 3. *In situ* ellipsometric layer thickness on titanium of adsorbed PEG and PEG-peptide (approx. 1 nm), and the concomitant low adsorption of blood plasma after 30 minutes of incubation at 22°C (0.3-0.7 nm). The results show net thickness of adsorbed layers and 1 nm corresponds to 70 ng/cm² (n=4, SD is shown as error bar). **** marks significant (p<0.0001) differences compared to the other indicated types of surfaces.

Figure 4. Summary of coagulation time (CT) measurements in normal native whole blood (n=10), FXII deficient native whole blood (n=1), recalcified citrated platelet rich plasma (n=3), and recalcified citrated platelet free plasma pooled from two donors (performed in duplicate). Normal native whole blood presented significantly shorter average CT in titanium cups compared to PA, PEG, PEG-RGD and PEG-RDG cups. Factor XII deficient native whole blood showed a clearly prolonged average CT compared to the normal blood. In titanium cups, the FXII deficient

blood CT was comparable to those of the other surfaces. Recalcified citrated PRP showed significantly shorter average CT in titanium cups compared to cups coated with PEG-RGD and PEG-RDG. Recalcified citrated PFP presented a significant decrease in CT for plasma that contacted titanium as compared to a PEG-RGD coating. * marks significant ($p < 0.05$) differences compared to the other surface types in the group (error bars show SD).

Figure 5. Coagulation time (CT), expressed as mean with SD as error bars, of native whole blood with added inhibitors, i.e. annexin V ($n=2$) and anti-TF ($n=2$), and native whole blood without additives ($n=2$) in reference titanium insert cups and corresponding experiments with PEG-coated titanium insert cups ($n=4$, $n=2$, and $n=4$, respectively). When annexin V was added to native whole blood in PEG-coated sample cups the average CT increased when compared to samples in contact with PEG without added annexin V. No significant difference in average CT was observed when anti-TF was added to native whole blood in cups coated with PEG. Titanium cups showed no difference in the average CT when annexin V or anti-TF was added.

Figure 6. Fluorescence microscopy images of platelets that adhered to PEG, PEG-RGD, PEG-RDG adlayers on glass and hydrophilic glass surface cleaned in a RF-chamber. The surfaces were incubated in native whole blood, adhered cells fixed in paraformaldehyde, and the filamentous actin cytoskeleton was stained with BODIPY[®] FL phalloidin. The magnification was 630x original magnification (bar in the lower right corner of the upper left picture represents 10 μm). The picture indicates that uncoated glass surfaces recruited large numbers of activated platelets showing marked stress fibres and neutrophils presenting prominent actin filaments and extensive spreading. The PEG and PEG-RDG polymeric surfaces attracted low numbers of apparently

scattered non-activated platelets and no neutrophils. PEG-RGD surfaces showed spread platelets with stress fibres and also spread neutrophils.

Figure 7. Percentage of platelets that expressed CD62P, glycoprotein Ib (GPIb) or glycoprotein IIb/IIIa (GPIIb/IIIa) after the incubation of native whole blood in titanium cups that were coated with either PEG, PEG-RGD, or PEG-RDG. References: titanium insert cups and polyamide cups (n=3). The reference titanium cups induced elevated CD62P expressions for all three donors, while the other surfaces showed comparably low expressions. The GPIb expression was elevated when hirudinised whole blood contacted uncoated titanium, PEG-RDG, and PA cups, as compared to cups coated with PEG or PEG-RGD. Fibrinogen binding to GPIIb/IIIa was not observed on any of the tested surfaces.

Figure 8. Kallikrein formation as analysed with a chromogenic substrate (S-2302) after incubation during 10 min of 10% citrated platelet free plasma in PBS in a FOR reference titanium insert cup, ditto with a coating of PEG, PEG-RGD or PEG-RDG, and a polyamide cup (n=4). Titanium induced the formation of comparatively large amounts of kallikrein, while the PEG, PEG-RGD, PEG-RDG, and PA cups displayed low kallikrein levels.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8