Interplay of platelets and contact activation boosts blood coagulation on biomaterials

Der Einsatz von Biomaterialien in Anwendungen mit Blutkontakt führt häufig zur Aktivierung von Blutgerinnungs- sowie Immunreaktionen, was oft mit unerwünschten gesundheitlichen Konsequenzen für die betroffenen Patienten verbunden ist. Obwohl einige Zusammenhänge zwischen Oberflächeneigenschaften von Materialien und Blutaktivierungsprozessen aufgeklärt werden konnten, entziehen sich wichtige Phänomene bisher noch einer präzisen mechanistischen Beschreibung. Dies betrifft auch die Aktivierung des in vielen Anwendungen dominierenden Prozesses der Blutgerinnung. Molekular definierte Materialoberflächen und genau kontrollierte Inkubationsexperimente mit humanem Blut erlauben es, den Einfluss bestimmter Oberflächeneigenschaften auf Reaktionen des Blutes wie die Aktivierung von Enzymen der Blutgerinnungskaskade, sowie die Adhäsion und Aktivierung von Zellen (Thrombozyten, Leukozyten) zu klären.
Unser Projekt dient in diesem Zusammenhang der Untersuchung von verschiedenen Aktivierungsmechanismen der Blutgerinnung an Materialoberflächen. Unter Nutzung von Zweikomponenten-Alkylthiolmonoschichten (SAM) mit verschiedenen funktionellen Endgruppen (–CH_3 und –COOH) wurde eine Variation der Oberflächenhydrophilie bzw. -ladung erreicht. Die hydrophoben Oberflächen, die aus –CH_3 terminierten Alkylthiolen gebildet wurden, zeigten eine starke Thrombozytenadhäsion, während die negativ geladenen –COOH-terminierten Oberflächen eine ausgeprägte Aktivierung des sogenannten „Kontaktsystems“ (einer Protein-Kaskadenreaktion) aufwiesen. Überraschenderweise wurden an Oberflächen, die ausschließlich –CH_3- bzw. –COOH-Gruppen aufwiesen, eine vergleichbare Bildung von Thrombin, dem Endprodukt der Blutgerinnungskaskade, nachgewiesen, während auf binären Monoschichten mit Anteilen beider funktioneller Gruppen (50 % bzw. 83 % –COOH) eine signifikant erhöhte Thrombinbildung beobachtet wurde. Diese Ergebnisse lassen auf einen ausgeprägten synergistischen Effekt der Kontaktaktivierung und der Thrombozytenadhäsion auf die Thrombusbildung an Biomaterialoberflächen schließen. Ein Zusammenspiel von plasmatischen und zellulären Mechanismen scheint die notwendige Voraussetzung für eine starke Blutgerinnung an Materialoberflächen zu sein. Diese neue Erkenntnis sollte bei der Gestaltung von Biomaterialien zur Anwendung im Blutkontakt bzw. bei der Auswahl von adäquaten Testsystemen künftig besondere Beachtung finden.

Keywords
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Introduction

Blood without the contact to healthy endothelium has a tendency to coagulate. It does so at wound sites as well as when contacting non-physiological surfaces like biomaterials used in medical devices. In recent years the development of medical products with enhanced hemocompatibility proceeded [1] yet nevertheless in most cases systemic anticoagulation is needed to avoid thrombus formation. This process is a highly regulated sequence of events that encompasses plasmatic coagulation enzymes (activators and inhibitors) as well as blood platelets [2, 3]. *In vivo* coagulation initiation is mainly connected to subendothelial layers.

Concerning the initiation of this process induced by biomaterials there are many theories – and as many questions still remain [4]. Material surface characteristics are the main clue to get more answers: roughness, hydrophilicity and charge being responsible for protein adsorption processes, enzyme activation and cell adhesion. It is known that negative surface charges promote the activation of the coagulation factor FXII in the coagulation cascade [5]. It is also known that certain proteins present their cell recognition sequence upon adsorption and through this are activators of platelets [6]. A mechanistic understanding of activation processes induced at the blood-material interface is necessary for specific steps ahead in the rational design and testing procedure of hemocompatible materials.

Within this project binary self assembled monolayers of alkylthiols (SAMs) with various ratios of $–CH_3$ and –COOH terminations were used for studying the relevance of hydrophobic and negatively charged surfaces for the initiation of blood coagulation. These uniform and well defined surfaces show a strong similarity concerning roughness and therefore enable a direct correlation of blood activation processes with parameters related to charge and hydrophilicity [7].

Methods

For the preparation of SAM surfaces glass slides or silicon wafers were cleaned extensively and coated with an 80 nm gold layer. We prepared SAM surfaces from single solutions ($–CH_3$ and –COOH terminated alkylthiols C_{10}–COOH, C_{10}–CH_3) by chemisorption from solutions in absolute ethanol while the mixtures (17 %, 34 %, 50 %, 83 % –COOH, balance $–CH_3$) were prepared using dry tetrahydrofuran as a solvent. A surface characterization using physico-chemical methods included IRRAS-FTIR, AFM, XPS and water contact angle measurements [8].

The interaction of blood with the model surfaces was tested using whole blood as well as blood plasma (platelet rich plasma and platelet poor plasma). The activation of the coagulation enzyme FXII to FXIIa or prothrombin to thrombin on the surfaces was tested using citrated plasma and a chromogenic or a fluorogenic substrate respectively. The adhesion of platelets was determined using platelet rich plasma as well as after *in vitro* incubation with whole blood. This type of incubation was done using custom made screening chambers that enable the incubation of the surfaces with fresh whole human blood (heparinized for anticoagulation) at 37 °C for 2 hours without air contact and without cell sedimentation. After the incubation the blood and the incubated surfaces are tested for parameters concerning the activation of coagulation, platelet and leukocytes.

Results

The characterization of the model surfaces showed that the surface hydrophilicity was finely tuned and related to the content of methylene groups (Table 1). The XPS analysis of oxygene with the rising content of –COOH terminated alkylthiols also showed that the surfaces well related to the theoretical expectations (data not shown).

$X_{COOH,\ solution}$	θ_{adv} [°] ± SD
0	111 ± 3.5
17	102 ± 2.4
34	92 ± 7.7
50	87 ± 6.4
66	76 ± 11
83	51 ± 9.3
100	21 ± 7.1

Table 1:
Water advancing contact angles (θ_{adv}) of C_{10}–COOH and C_{10}–CH_3-SAMs in degree; measured values with standard deviation (SD)

The adherence of platelets from platelet rich plasma correlated well with the hydrophilicity of the surface. The most hydrophobic surface (0 % COOH = $–CH_3$) showed the strongest adhesion of platelets as can be seen in Fig 1.

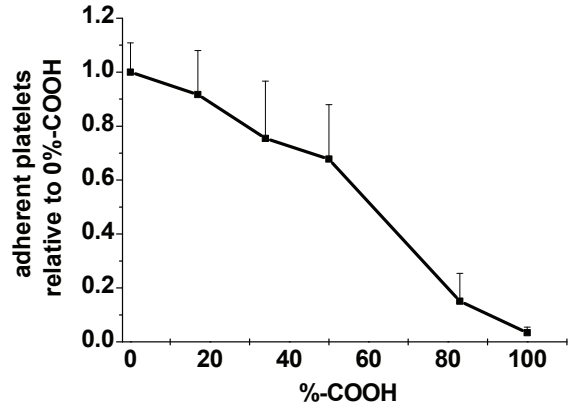


Fig. 1:
Adherent platelets on surfaces after incubation with PRP for 45 min determined by photometrical measurement of platelet derived lactate dehydrogenase (LDH)

The cell surface density dropped gradually and on the surface presenting only –COOH surface groups no platelets adhered. Especially the adsorption of the plasma protein fibrinogen but also of other proteins that interact with specific cell surface receptors like glycoprotein (GP) Ib/IX/V (binds to von-Willebrand-factor) and GPIIb/IIIa (αIIbβ3) is supposed to be relevant for cell adhesion. Using radiolabelled fibrinogen in blood plasma we were able to show that the adsorption of fibrinogen actually was prominent on $–CH_3$ terminated surfaces compared to –COOH (data not shown) being possibly an explanation for the high adhesion rates on the $–CH_3$ surface.

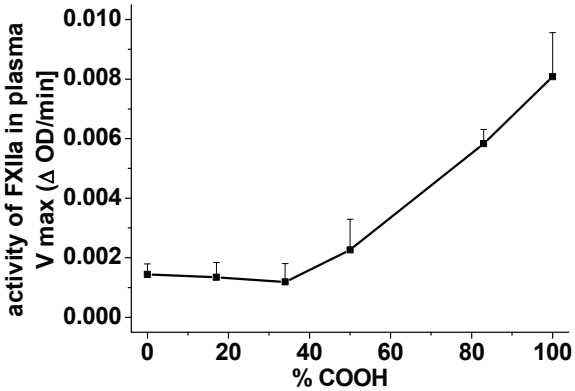


Fig. 2:
Activity of FXIIa in liquid phase (blood plasma) after incubation with heparinized plasma for 1 min using the chromogenic substrate S2302 and kinetic determination. Results are displayed as V_{max}. Statistics: liquid phase 83 %, 100 % C_{10}–COOH: $P < 0.05$ different versa all other surfaces

The activation of the plasmatic coagulation cascade *in vivo* can be initiated via FXIIa or FVIIa/TF. On biomaterials' surfaces the activation of FXIIa is regarded to be relevant [5]. This activation probably is attributed to a conformational change of FXII on negatively charged surfaces leading to an auto-activation of this enzyme. On the SAM surfaces tested a relevant activation was seen on

surfaces with a high amount (100 %, 83 %) of negatively charged surface –COOH groups while no relevant differences were to be seen for the surfaces with 50 % –COOH terminated thiols and for lower –COOH ratios.

Considering these results a high activation of coagulation in whole blood can be expected for the surface with either prominent platelet adhesion ($–CH_3$) or with a strong activation of the plasmatic coagulation (–COOH).
But unexpectedly the activation of coagulation in blood did not scale with either one of these parameters but peaked at the surfaces with a combination of carboxylic and methylene surface groups. We found significantly elevated levels of coagulation activation on the surfaces with 50 % and 83 % –COOH as can be seen in Fig. 3.

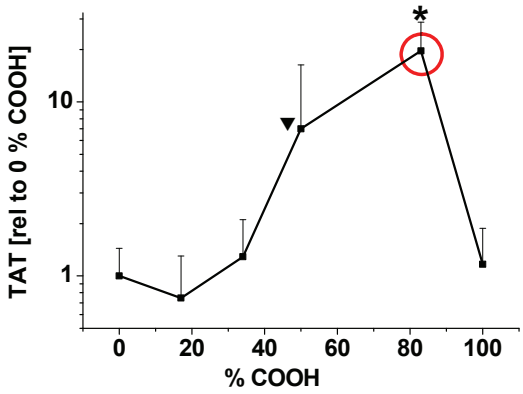


Fig. 3:
*Coagulation activation determined as TAT (thrombin-antithrombin-complex) formation in plasma after whole blood incubation with SAM surfaces. TAT detected by ELISA assay, values are related to the 0 % C_{10}–COOH surface *)P < 0.001 different to all other surfaces*
▼ P < 0.05 different to all other surfaces

Elevated thrombin levels would forcedly lead to blood clotting and indeed clot formation was macroscopically visible on the surface with 83 % –COOH. This can also be seen on surfaces after blood contact: Fig. 4 shows that the surfaces with only $–CH_3$ and with growing content of COOH (until 50 %) have a dense layer of adherent platelets. For the surface with 83 % –COOH clot formation is visible (white patches). The surface without $–CH_3$ groups does not show any adherent platelets at all (100 % –COOH).

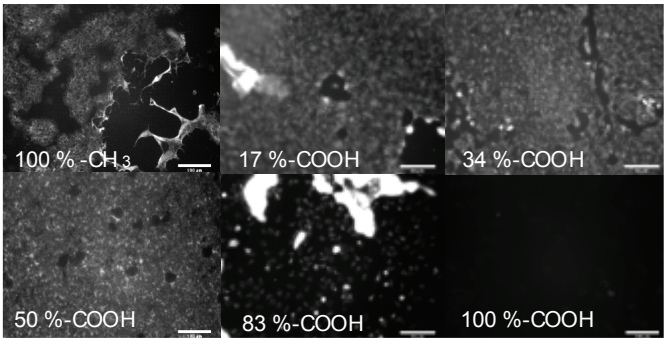


Fig. 4:
Fluorescence microscopic analysis of SAM surfaces (FITC-labelled anti-CD 41a on platelets) with rising content of C_{10}–COOH terminated thiols (diminishing content of C_{10}–CH_3 terminated thiols) after incubation with whole blood

Obviously neither the adhesion (and activation) of platelets nor solely the activation of the coagulation cascade via FXII activation is sufficient to considerably boost thrombus formation.
For further discrimination of the crucial reactions on the material surface we tested the activation of the enzyme thrombin in plasma and in platelet rich plasma. While in plasma all of the essential enzymes of the coagulation cascade and a necessary amount of phospholipids (part of the platelet cell membrane required for the

assembly of the coagulation cascade enzyme complexes) are included, platelet rich plasma additionally contains functional platelets with the ability to significantly enhance coagulation through the allocation of procoagulant phosphatidylserine. This procoagulant lipid can be found on activated platelets after special enzymes modified the composition of the non activated platelet membrane.

The time needed for formation of a predefined level of thrombin (the so called *onset time*) was measured using a fluorogenic substrate and is shown in Fig. 5. Supporting our previous results in whole blood the shortest onset time – meaning the fasted formation of thrombin – was determined for platelet rich plasma at 83 % –COOH, followed by $–CH_3$ and –COOH. The onset time for 100 % –COOH was higher than for 83 % –COOH, verifying the supporting effect of adherent platelets which are not present on the 100 % –COOH surface. Incubation with platelet poor plasma resulted in considerably higher onset times. This almost equals the onset time for not activated plasma.

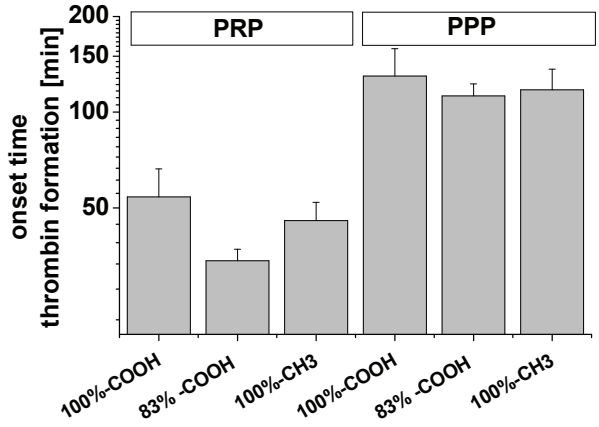


Fig.5:
Formation of thrombin in plasma with platelets (platelet rich plasma PRP) and without platelets (platelet poor plasma PPP) measured using a fluorogenic substrate. Determination of the time (onset time) until thrombin formation exceeds certain level for SAM surfaces with 100 %, 83 % and 0 % –COOH terminated alkyl thiols.

According to the above described findings we propose a scheme of the main processes occurring on our different monolayers. The interplay of ongoing mechanisms is summarized in Fig. 6. The first picture shows a monolayer with carboxylic terminated thiols (100 % –COOH). This surface is a strong activator of the contact system. With a decreasing percentage of negatively charged alkanethiols reduced levels of FXII activation were found – the intensity of FXII activation scaled well with the amount of negative charges on the surface (Fig. 2). This observation of a direct dependence of FXII activation on the amount of negative charged surface groups is supported by others [9, 10].
The second scheme shows reactions on the surface with only methylene terminated thiols. Here on the other hand a strong platelet adhesion was seen when incubated with PRP, correlating well with an enhanced adsorption of fibrinogen to this surface compared to the charged hydrophilic surface. Especially fibrinogen adsorption to biomaterials is considered to be a significant factor for hemocompatibility. The observed enhanced adsorption of fibrinogen on our hydrophobic $–CH_3$ and lower amounts on –COOH-modified surfaces, is in accordance with literature [6].

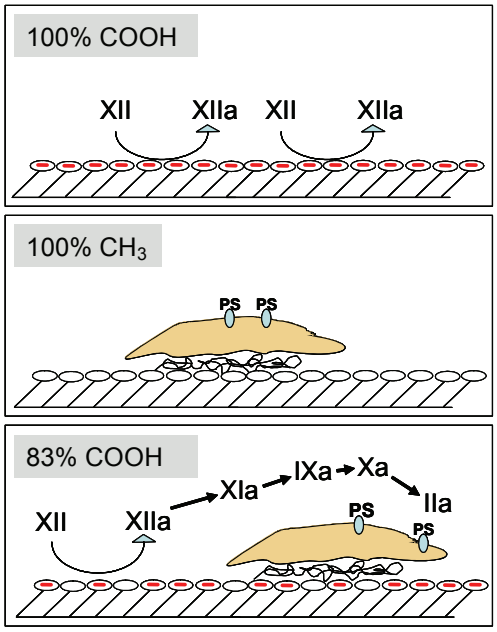
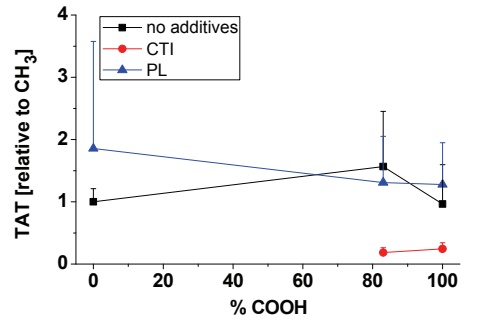


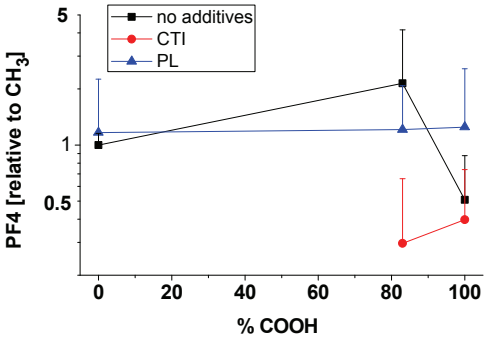
Fig. 6:
Scheme of reactions on biomaterials surfaces with charged groups (100 % –COOH), hydrophobic groups (100 % –CH_3) and intermediate with a predomination of charged groups (83 % –COOH) Description see text.

The third surface pictured in the scheme represents a monolayer with 83 % –COOH. Trace amounts of thrombin probably are generated early on through the activation of the contact system. The reaction velocity of thrombin formation strongly depends on cofactors. It was found that phospholipids potentiate the catalytic efficiency 1000-fold. The allocation of phosphatidylserine on the platelet membrane establishes a membrane with a procoagulant surface. This is necessary to support the formation of enzyme complexes turning prothrombin into an active enzyme. A high efficiency is necessary for substantial thrombin formation since thrombin half life in plasma is only about 10-15 s. We thus state that an intriguing interaction of trace activation of thrombin through contact activation and the following propagation on activated platelets can be observed on this surface.

An additional assay enabled us to confirm the above postulated contact activation on –COOH surfaces by inhibiting FXII activation through the addition of the specific inhibitor corn trypsin inhibitor (CTI) to whole blood just before blood incubation. Additionally we tested the influence of phospholipids (PL) with the capability to mimic activated platelets. The results of blood incubation with the addition of either an inhibitor to FXIIa (CTI) or an equivalent of activated platelets (PL) are shown in Fig. 7 (activation of coagulation: formation of TAT (a); activation of platelets: release of PF4 (b)).
The formation of thrombin (TAT), being the final product of the coagulation cascade initiated by contact activation, was lowered on 83 % –COOH after CTI addition to levels minor to –CH_3 terminated surfaces. Even the already reduced thrombin formation on –COOH is lowered to an additional extent by CTI addition.
FXIIa inhibition also lowered platelet factor 4 (PF4) release on 83 % –COOH to levels equal to 100 % –COOH, where no platelet adherence is observed. The addition of CTI did not change the PF4 levels on the pure –COOH surface signifying that no activation of platelets on this surface depending on contact activation is observable. This surely can be attributed to the total absence of platelets there as the effect is very strong on the 83 % –COOH surface.



a)



b)

Fig. 7:
Coagulation activation determined as TAT (thrombin-antithrombin-complex) formation (a) and platelet activation determined as PF4 release (platelet factor 4) (b) in plasma after whole blood incubation (2 h, 37 °C) with SAM surfaces using either fresh whole human blood or blood with the addition of a specific inhibitor to FXIIa (CTI) or phospholipids (PL) being an equivalent to an activated platelet surface. TAT + PF4 detected by ELISA assay.

The addition of phospholipids to whole blood before the incubation on the other hand induced clot formation even on the COOH-terminated surface, as can be seen in Fig. 8, substantiating the assumption that the procoagulant potency of platelets can be explained already by their phospholipid surface. The TAT formation was elevated only slightly on this surface. Interestingly the addition of PL to the surface with 100 % $–CH_3$ lead to an additional increase of TAT formation.

PF4 release was increased on –COOH with the addition of PL. This can be attributed to the elevated activation of coagulation leading to an additional enhancing effect of thrombin on platelets.

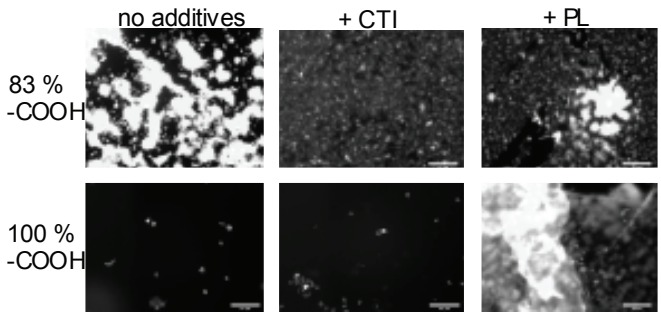


Fig. 8:
Fluorescence microscopic analysis (FITC-labelled anti-CD 41a on platelets) of SAM surfaces with rising content of 83 % or 100 % –COOH terminated thiols after incubation with fresh whole human blood or of blood with the addition of a specific inhibitor to FXIIa (CTI) or phospholipids (PL) being an equivalent to an activated platelet surface.
The length of bars is 65 µm.

Conclusions

Neither the adhesion of platelets at biomaterial surfaces without a concurrent activation of coagulation nor the activation of coagulation without activated platelets is sufficient for a potential coagulation initiation on biomaterials' surfaces. Yet the presence of few platelets was sufficient to propagate coagulation substantially if simultaneous initiation was achieved by contact activation enzymes. As our results show, this synergistic effect can be attributed to the procoagulant lipid surface of adherent platelets on one side and traces of activated enzymes like FXa and thrombin formed through the activation of the contact system on the other side. Generated thrombin consecutively boosts platelet activation considerably. This activation possibly leads to a stronger support of coagulation therefore pushing thrombin formation above the necessary threshold.
These results provide evidence that surface design as well as reliable testing of hemocompatible materials need to consider several aspects as the cascadic reactions in blood do not react linearily. The coexistence of surface characteristics leading to contact activation and platelet adhesion has to be minimized for biomaterials with a good hemocompatibility.

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