Macromolecular Bioscience
www.mbs-journal.de

Tackling the Root Cause of Surface-Induced Coagulation: Inhibition of FXII Activation to Mitigate Coagulation Propagation and Prevent Clotting

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Factor XII (FXII) is a zymogen present in blood that tends to adsorb onto the surfaces of blood-contacting medical devices. Once adsorbed, it becomes activated, initiating a cascade of enzymatic reactions that lead to surface-induced coagulation. This process is characterized by multiple redundancies, making it extremely challenging to prevent clot formation and preserve the properties of the surface. In this study, a novel modulatory coating system based on C1-esterase inhibitor (C1INH) functionalized polymer brushes, which effectively regulates the activation of FXII is proposed. Using surface plasmon resonance it is demonstrated that this coating system effectively repels blood plasma proteins, including FXII, while exhibiting high activity against activated FXII and plasma kallikrein under physiological conditions. This unique property enables the modulation of FXII activation without interfering with the overall hemostasis process. Furthermore, through dynamic Chandler loop studies, it is shown that this coating significantly improves the hemocompatibility of polymeric surfaces commonly used in medical devices. By addressing the root cause of contact activation, the synergistic interplay between the antifouling polymer brushes and the modulatory C1INH is expected to lay the foundation to enhance the hemocompatibility of medical device surfaces.

1. Introduction

Blood becomes activated and begins to clot when being exposed to an artificial surface such as blood-contacting medical devices. Almost immediately after this, contact molecular components, i.e., proteins, adsorb to the surface. The first proteins to adsorb are those, which are present at higher concentrations, which favor the kinetics of the adsorption. These are later partially replaced with other proteins with higher binding affinity but at lower concentrations as dictated by the Vroman effect.[1] The strong binding of the latter drives conformational changes from native to denatured.[2–6] The adsorption of proteins and the following conformational changes are the true initiating event that translates the presence of the surface to a response from blood. First, albumin and fibrinogen expose sites for platelet adhesion and proteins of the complement system. But even if those are reduced, small amounts of factor XII (FXII) can adsorb and lead to coagulation and thrombus formation.[7–9] FXII is a zymogen that when activated into FXIIa, a protease, initiates coagulation via the contact system. When FXII adsorbs to the surface, it binds

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DOI: 10.1002/mabi.202300321

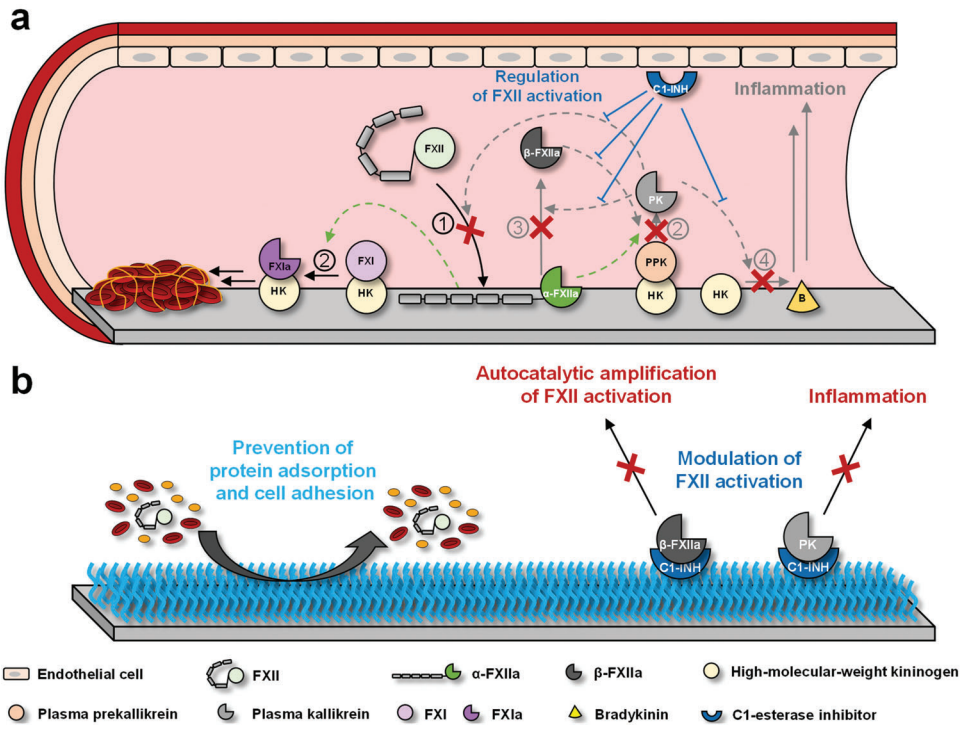

Figure 1. a) Contact activation on the surface of medical devices. The high interfacial energy of polymer surfaces in contact with blood results in the adsorption of FXII on the surface. Its adsorption occurs through the binding of its heavy chain leading to its unfolding and mechanical activation into α-FXIIa. Afterward, this enzyme cleaves surface-bound PPK into PK and surface-bound FXI into FXIa. The generation of FXIa is the beginning of the intrinsic coagulation pathway—a cascade of enzymatic reactions ultimately resulting in thrombus formation at the surface and dissemination of coagulation. PK cleaves FXII into more α-FXIIa and simultaneously it cleaves generated α-FXIIa into its low molecular weight fragment β-FXIIa. Since β-FXIIa does not exhibit the surface-affine heavy chain, it is released from the surface, where it can generate more PK disseminating coagulation. The interplay of PK and β-FXIIa is responsible for the autocatalytic activation of FXII. Moreover, these enzymes activate the complement system and the plasma bradykinin-forming pathway inducing a systemic inflammatory response. In nature, the most effective inhibitor that regulates these amplification reactions is C1INH. However, outside of the human body, the highly procoagulant surface might overwhelm the circulating inhibitor molecules leading to thrombus formation on the surface. b) Modulatory nanocoating system to regulate FXII activation developed in this work. Antifouling polymer brushes consisting of poly(HPMA-*co*-CBMAA) serve as a passive level to prevent protein adsorption and cell adhesion. In this way, no α-FXIIa will be generated by adsorption, unfolding, and mechanical activation of FXII. Additionally, the polymer brushes are decorated with C1INH, which modulates the autocatalytic amplification of FXII activation by inhibiting β-FXIIa and PK.

via its heavy chain resulting in unfolding and mechanical activation into α-FXIIa.[10] This enzyme is capable of cleaving surface-bound plasma prekallikrein (PPK) into plasma kallikrein (PK) and surface-bound FXI into FXIa, which starts the coagulation cascade leading to thrombus formation. PK generates more α-FXIIa by cleaving FXII and at the same time, PK cleaves the high molecular weight protease α-FXIIa into the low molecular weight fragment β-FXIIa. In contrast to the surface-bound α-FXIIa, β-FXIIa is missing the heavy chain, which results in its release from the surface.[10,11] The fact that FXIa remains on the surface, while PPK and PK can rapidly dissociate from it explains the high activity of β-FXIIa for PPK and the low one for FXI.[11] Thus, β-FXIIa is responsible for the autocatalytic activation of FXII through PK, activation of the complement system, and activation of the plasma bradykinin-forming pathway that induces a systemic inflammatory response,[12] while α-FXIIa is the main initiator of the intrinsic pathway of the coagulation cascade resulting in thrombus formation (**Figure 1**a).[9,10,13–15] By reducing the concentration of β-FXIIa molecules, not only can we prevent the activation of the complement system, but we can also inhibit the activation of the coagulation cascade.

In blood vessels, these amplification reactions are balanced by different inhibitors such as C1-esterase inhibitor (C1INH), antithrombin (ATIII), α_2-macroglobulin, and α_2-antiplasmin.[16–18] C1INH is the most active of these inhibitors with a 61-fold higher efficiency.[16,19] Its high efficiency to regulate the autocatalytic amplification of FXII activation can be attributed to the fact that it is a potent inhibitor for β-FXIIa, but a poor one for α-FXIIa.[16,17,19] However, the situation outside of the human body is different: contact with highly procoagulant surfaces inevitably overwhelms the circulating inhibitors resulting in thrombus formation at the surface,[20,21] which is the most undesirable event in blood-contacting medical devices and can lead to life-threatening complications. To date, anticoagulation is used in clinical practice to minimize these effects. However, the prevention of thromboembolic complications cannot be solely addressed by anticoagulation but requires addressing the root cause: the lack of hemocompatibility of the surface. Hence, the surface, which is the trigger of all upcoming events, has to be designed in a way that it does not cause severe activation of coagulation and platelet activation. At present, heparin coatings serve as the gold standard for enhancing the hemocompatibility of medical devices in contact with

blood. Their effectiveness is based on two key factors: the catalytic effect they have on the inhibition of thrombin by ATIII, similar to systemic heparinization,[12,22–24] and their ability to selectively adhere to plasma proteins, particularly C1INH.[12,23,25–28] However, their use could result in activation of coagulation through other routes, since they do not provide a sufficient barrier against protein adsorption.

In this work, we introduce a new type of coating to improve the hemocompatibility of blood-contacting medical devices. This coating tackles the two main challenges of surface-induced coagulation, protein adsorption, and contact activation, by combining a passive and a modulatory level (Figure 1b). The passive level consists of antifouling polymer brushes that provide a barrier against protein adsorption and activation of the contact system.[29–33] These brushes are highly dense hydrophilic polymers directly grafted from the surface. Their excellent antifouling properties originate from their stretched conformation (entropic barrier),[33–35] their high ability to catch water molecules (enthalpic barrier),[33,36] and their autophobic effect.[37,38] Moreover, polymer brushes made from, e.g., carboxybetaines exhibit kosmotropic groups, which prevent conformational changes of proteins,[39–45] allowing their functionalization with enzymes or their inhibitors.[46–48] The modulatory level is designed by functionalization of the passive level with C1INH, which was shown to reduce FXII activation in previous work.[49] C1INH on the surface leads to local capture and deactivation of β-FXIIa and PK, the promoters of surface-induced coagulation. Thus, it prevents the activation of the contact system by modulating the autocatalytic amplification mechanism of FXII. We selected poly(4-methyl-1-pentene) (PMP) membranes as the model surface for blood-contacting medical devices, specifically in extracorporeal membrane oxygenators. These membranes endure extended usage and often suffer from blood fouling, leading to reduced gas exchange capacity and triggering inflammation. Hence, there exists a significant unmet requirement for enhancing these membranes. The brushes were generated by surface-induced single electron transfer-living radical polymerization (SI SET-LRP) of *N*-(2-hydroxypropyl) methacrylamide (HPMA) and carboxybetaine methacryamide (CBMAA) directly from the surface of PMP membranes. As previously described, the antifouling poly(HPMA-*co*-CBMAA) brushes prohibit fouling from blood plasma (BP) and FXII.[46,47] The immobilization of C1INH will improve the hemocompatibility, while the capturing of β-FXIIa and PK do not interfere with the overall hemostasis. The translation of nature's blueprints to regulate the activation of FXII to a nonthrombogenic surface is a promising approach to enhancing the hemocompatibility of blood-contacting medical devices.

2. Results and Discussion

2.1. Antifouling Polymer Brushes as Passive Barrier to Protein Adsorption

In this work, we grafted poly(HPMA-*co*-CBMAA) brushes having 85% of the first comonomer and 15% of the second comonomer. These brushes have been previously shown to have excellent repellency to plasma protein,[46,47] a prerequisite to build hemocompatible surfaces that do not activate the contact system due to conformational changes of the adsorbed proteins signaling to coagulation factors or platelets. Additionally, the copolymer was selected to allow functionalization of the brushes through the carboxyl group of CBMAA, while not interfering with the overall antifouling behavior of the brushes stemming from both, HPMA and CBMAA. The brushes were grafted from PMP membranes modified with 11-(trichlorosilyl) undecyl-2-bromo-2-methylpropanoate initiator using photo-induced SET-LRP in dimethyl sulfoxide. The functionalization of the membranes was confirmed by X-ray photoelectron spectroscopy (XPS). The C 1s and N 1s spectra showed the characteristic bonds of [C—N], [C—O], and [O=C—N] related to HPMA and CBMAA, as well as [O=C—O] and [N^+—$(CH_3)_2$] related to CBMAA (Figure S1 in the Supporting Information). Additionally, the long-term stability of the polymer brushes was assessed by XPS after 2 years of storage in Milli-Q water (Figure S2 in the Supporting Information). The dry thickness of the brushes was selected to be 43.8 ± 0.87 nm. The brushes increased the wettability of the otherwise hydrophobic PMP membranes (Table S2 in the Supporting Information).[46,47] In line with previous work,[46] the brushes showed excellent antifouling properties, with Γ_{BP} = 17.7 ng cm^{-2}, which corresponds to over 89% decrease of protein adsorption compared to fouling surfaces (**Figure 2**b and Figure S3 in the Supporting Information).

2.2. Covalent Immobilization of C1-Esterase Inhibitor on Antifouling Polymer Brushes

C1INH was immobilized on the antifouling polymer brushes in order to build a means that is able to modulate coagulation at the onset of activation of the contact system. The immobilization was carried out using the switchable attraction protocol[46] to enable covalent bonding between the C1INH molecules and the protein-repellent polymer brushes. The carboxyl groups of CBMAA were activated using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/*N*-hydroxysuccinimide followed by contacting the activated brushes with a solution of C1INH (0.5 mg mL^{-1}) for 80 min at a flow rate of 10 µL min^{-1}. This allows the immobilization of C1INH without impairing the antifouling properties. Figure 2a shows representative immobilization kinetics monitored by surface plasmon resonance (SPR) in real-time. The contact of C1INH with the activated brushes resulted in fast adsorption and binding saturation was achieved after 80 min, resulting Γ_{C1INH} = 57.2 ng cm^{-2} (0.544 pmol cm^{-2}).

The C1INH-functionalized polymer brushes were challenged with undiluted BP to prove that their antifouling properties remain after functionalization. The C1INH-functionalized polymer brushes displayed Γ_{BP} = 31.5 ng cm^{-2} (Figure 2b and Figure S2 in the Supporting Information). Thus, the functionalized brushes decrease the adsorption of BP protein by 86% compared to bare gold indicating that the immobilization did not impair the antifouling behavior of the polymer brushes. Moreover, the kosmotropic properties of the antifouling polymer brushes may help to protect immobilized C1INH from conformational changes and deactivation. This is of great importance to create a coating with improved hemocompatibility.

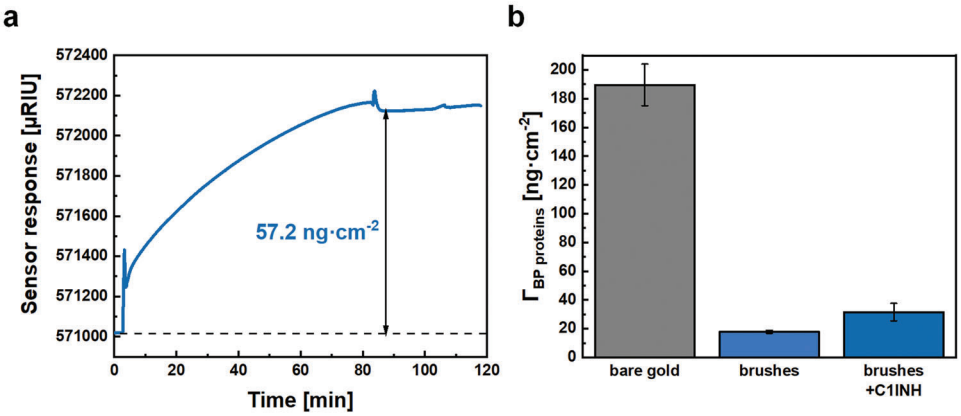

Figure 2. Immobilization of C1INH on antifouling polymer brushes. a) SPR sensogram of C1INH immobilization onto activated polymer brushes. b) Fouling from 100% BP on bare gold and on polymer brushes before and after the immobilization of C1INH.

2.3. Specific Capture of β-FXIIa and Plasma Kallikrein

The ability of the C1INH-coating system to modulate coagulation was evaluated by its specific capture of different coagulation factors, such as β-FXIIa, PK, α-FXIIa, FXIa, and FXII at physiologically relevant concentrations and monitored via SPR (**Figure 3**). A flow rate of 10 μL min^{-1} was selected as it provides an adequate timeframe for the molecules to reach the surface. In order to allow the comparison of the different captured coagulation factors, we calculated the ratio of the amount of captured factors to the amount of immobilized C1INH (Table S3 in the Supporting Information). The highest amounts were received for the capture of β-FXIIa and PK with a molar $\Gamma_{\text{coagulation factor}}/\Gamma_{\text{C1INH}}$ ratio of 26% and 15%, respectively. The capture of α-FXIIa was less with a molar $\Gamma_{\text{coagulation factor}}/\Gamma_{\text{C1INH}}$ ratio of 5%, while no capture of FXIa (0.7%) and FXII (0%) was observed. These results indicate that C1INH remained active after immobilization on the brushes and that it was selective to PK and activated FXII, while not interacting with FXII. This is crucial to not interfere with systemic hemostasis.

2.4. Interaction of C1INH Coating System with Human Blood in Dynamic Conditions

Chandler loop experiments were carried out to investigate the performance of the C1INH-coating system in contact with real human blood (**Figure 4**a and Figure S4 in the Supporting Information). To study the interaction with blood, it is essential to introduce a mild anticoagulation of the blood to slow down the initiation of coagulation. Without this precaution, the coagulation process would start within minutes. For this experimental setup, heparin emerged as the ideal anticoagulant choice due to its strong clinical significance. One noteworthy aspect is that at low doses, heparin permits the formation of fibrin, a sine qua non condition for the evaluation of anticoagulatory properties.[50,51] Uncoated PMP-membranes and PMP-membranes modified with poly(HPMA-*co*-CBMAA) brushes as well as with the C1INH-coating system (1 cm × 2 cm) were inserted in the Chandler loop and incubated with 15 mL heparinized blood (1 IU mL^{-1}) and rotated for 90 min at 37 °C with a rotation speed of 16 rpm. We chose particular parameters for

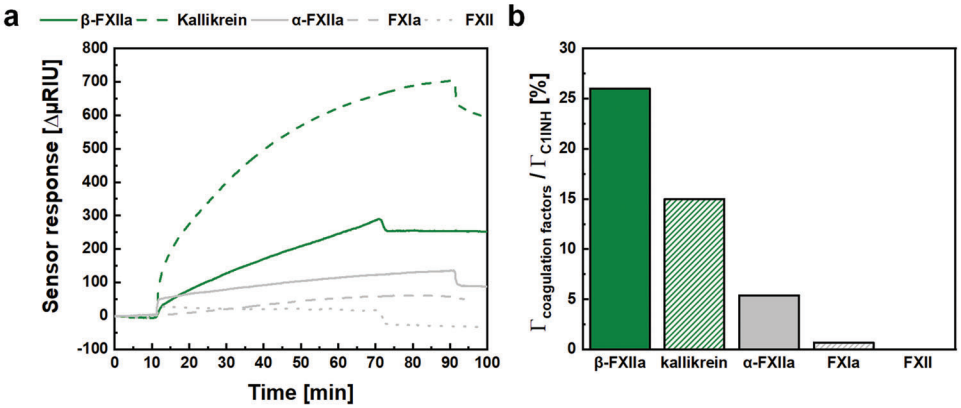

Figure 3. a) SPR sensograms demonstrating the capture of β-FXIIa (30 μg mL^{-2}), PK (50 μg mL^{-2}), α-FXIIa (30 μg mL^{-2}), FXIa (10 μg mL^{-2}), and FXII (30 μg mL^{-2}) by C1INH-functionalized polymer brushes. b) Comparison of the ratio of the amount of captured factors to the amount of immobilized C1INH.

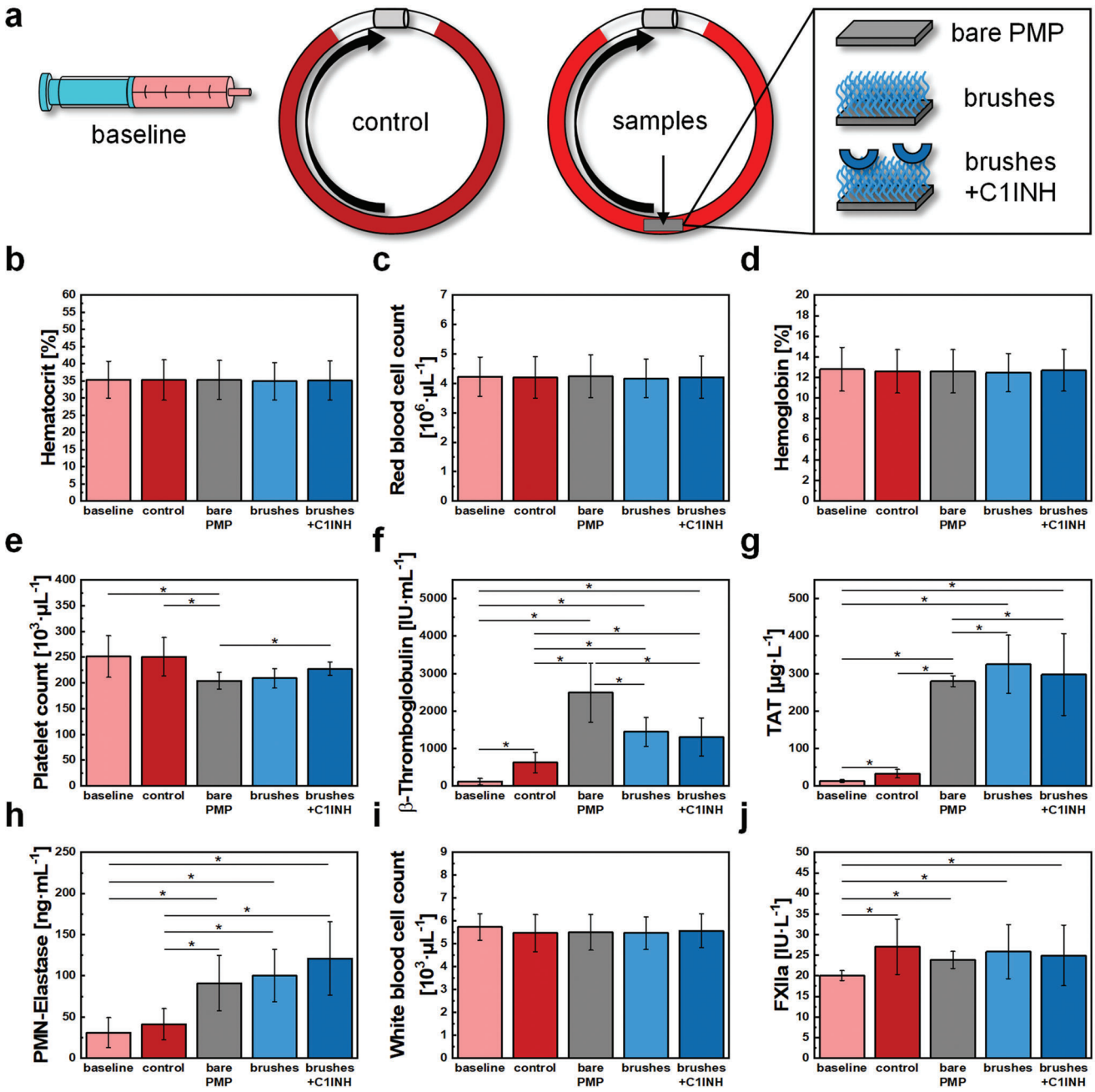

Figure 4. Dynamic blood experiments with heparinized human blood using a Chandler loop (37 °C, 90 min) and hemocompatibility analysis of uncoated and coated PMP membranes. a) Scheme of the Chandler loop experiments and visualization of the different samples. b) Hematocrit value. c) Number of red blood cells. d) Hemoglobin value. e) Number of platelets. f) Blood marker for platelet activation (β-thromboglobulin). g) Blood markers for coagulation (TAT). h) Blood markers for activation of neutrophils (PMN-Elastase). i) Number of white blood cells. j) Blood markers for the contact phase system (FXIIa). $n = 3$, statistical significance for $^*p < 0.05$.

the Chandler loop model, as outlined in Tables S1 and S4 in the Supporting Information. Our aim was to maintain low levels of wall shear stress and velocity. This deliberate choice allows us to isolate and study the impact of the coating on blood without the confounding influence of shear-induced blood activation.

The impact of the C1INH-coating system on activation of blood cells, complement system, and coagulation system was evaluated afterward by the determination of blood cell counts and hematological markers and comparison between the uncoated and coated samples.

The relative volume of red blood cells (Figure 4b) remains constant for all samples as well as the number of erythrocytes indicating that the coating did not produce any harm to red blood cells (Figure 4c). This is further supported by hemoglobin

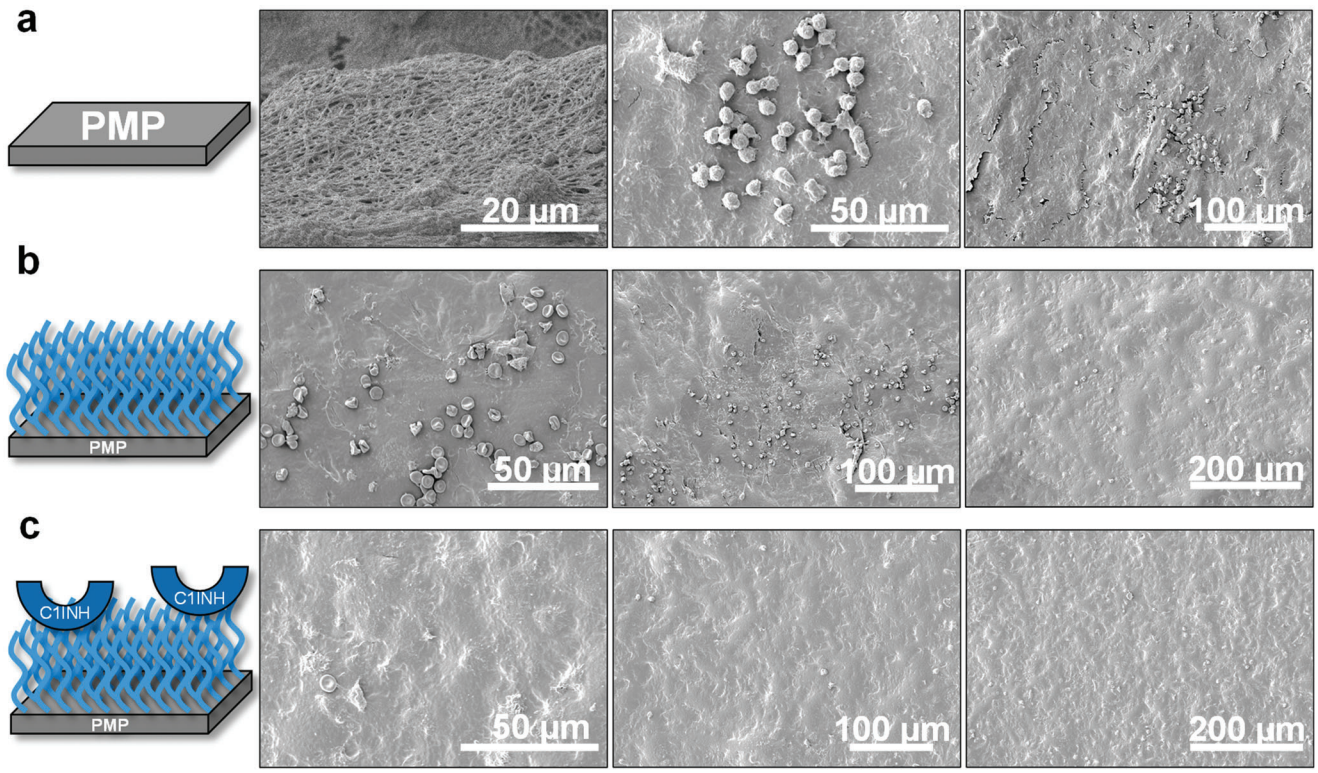

Figure 5. SEM images of the fibrin deposition and platelet adhesion on flat PMP substrates after Chandler loop experiments (donor 1). First row: uncoated PMP, second row: PMP coated with polymer brushes, third row: PMP coated with C1INH-functionalized polymer brushes. Additional SEM images for donor 2 and donor 3 are presented in Figure S5 in the Supporting Information.

levels (Figure 4d) not significantly higher than the one of blood immediately after blood withdrawal (baseline). Bare PMP membranes caused a 19% decrease in the platelet count (Figure 4e), which was accompanied by a 300% increase in the β-thromboglobulin level (Figure 4f) compared to the control samples. The β-thromboglobulin level indicates the activation of platelets, detrimental for hemocompatibility. The brush coatings (with and without C1INH) significantly reduced the activation as observed in the β-thromboglobulin levels (reduction by 42% for brushes and by 48% for C1INH-functionalized brushes). Thus, concealing the presence of PMP to platelets. The levels of thrombin-antithrombin complex (TAT) and polymorphonuclear (PMN) elastase (Figure 4g,h), markers of thrombin production and leukocyte activation, were increased compared to control. However, this was not accompanied by a reduction of the number of white blood cells (Figure 4i) nor an increase of FXIIa (Figure 4j). This suggests that even if PMP could cause some activation, the C1INH could quench FXIIa and prevent propagation, which is supported by scanning electron microscopy (SEM) images (**Figure 5** and Figure S5 in the Supporting Information) showing no thrombus adhered to C1INH-functionalized brushes, while strong fouling could be observed in bare PMP.

The blood collection procedures were approved by the research and ethics unit of the University of Tübingen (project approval number 287/2020BO2).

3. Conclusion

We have developed a coating system for blood-contacting medical devices that significantly improves their hemocompatibility. This system combines passive and regulatory properties to address the root cause of surface-induced coagulation, which is the activation of FXII. The passive level of the coating consists of antifouling poly(HPMA-*co*-CBMAA) brushes, while the modulatory level involves decorating these brushes with C1INH. The latter acts to quench β-FXIIa and PK, preventing further activation of FXII and the propagation of coagulation. Remarkably, the brush coatings (with and without C1INH) effectively prevent fouling by blood plasma proteins. Moreover, the C1INH coating system can capture β-FXIIa and PK at physiologically relevant concentrations, which is crucial for inhibiting the autocatalytic amplification of FXII activation. In vitro dynamic hemocompatibility studies using the Chandler loop demonstrate that the C1INH-coating system significantly enhances the hemocompatibility of PMP membranes. No clot formation was observed on the C1INH-functionalized PMP membranes after blood contact for 90 min (Figure 5). The synergistic interplay between the passive and modulatory levels of the coating prevents fouling by blood plasma proteins, inhibits FXII activation, and hinders the amplification and propagation of coagulation induced by the surface. We anticipate that the properties of the C1INH-coating system will have a profound impact on the hemocompatibility of

medical devices. However, translation of the antifouling polymer brushes to real devices might be challenging due to complex polymerization conditions. Therefore, future work could focus on the combination of the modulatory level with brush-like alternatives, e.g., ultrathin surface-attached hydrogels,[52] polymer-peptide-hybrids,[53–55] as well as other oligomeric,[47] polymeric,[56,57] or plasma-deposited[58] initiators for grafting from polymer substrates.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors acknowledge the financial support by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) via Schwerpunktprogramm "Towards an Implantable Lung" (Project numbers: 346972946 and AOBJ 640574), the German Federal Ministry of Education and Research (BMBF) with the projects AntiBacCat and Heart2.0 within the "Bio4MatPro-Competence Center for Biological Transformation of Materials Science and Production Engineering" program (grant nos. 031B1153A and 031B1154B), and IBEC which is a member of the CERCA Programme/Generalitat de Catalunya.

Open access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

β-FXIIa, C1-esterase-inhibitor, FXII activation, hemocompatibility, hemocompatible surface modification, polymer brushes

Received: July 10, 2023
Revised: September 1, 2023
Published online: September 29, 2023

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