Linköping University Medical Dissertations
No. 1131

Imaging methods for haemostasis research

Lars Faxälv

Linköping University
FACULTY OF HEALTH SCIENCES

Division of Clinical Chemistry

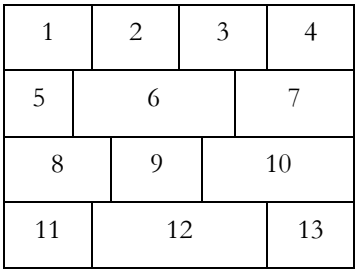
Department of Clinical and Experimental Medicine

Linköping University, Sweden

Linköping 2009

During the course of the research underlying this thesis, Lars Faxälv was enrolled in Forum Scientium, a multidisciplinary doctoral programme at Linköping University, Sweden.

Cover images:



1: *In vitro* thrombus formation on surface covered with fibroblasts (fluorescence microscopy)
2: *In vitro* thrombus formation at low shear, large thrombus (fluorescence microscopy)
3: Platelet adhesion and fibrin formation on hydrophilic glass (DIC microscopy)
4: *In vitro* thrombus formation at high shear (confocal microscopy)
5: Platelet adhesion to polystyrene (fluorescence microscopy)
6: Time-lapse images from Imaging of coagulation method
7: Platelet adhesion to patterned hydrogel with immobilized fibrinogen (fluorescence microscopy)
8: Platelet adhesion in cone-and-plate setup (fluorescence microscopy)
9: *In vitro* thrombus formation at low shear (fluorescence microscopy)
10: 3D visualization of adhered platelets
11: *In vitro* thrombus formation at low shear, large thrombus (fluorescence microscopy)
12: Imaging of coagulation setup
13: Platelets loaded with Ca^{2+} probe Fluo-4 (fluorescence microscopy)

Imaging Methods for Haemostasis Research

ISBN: 978-91-7393-621-7

ISSN: 0345-0082

Printed in Sweden by LiU-Tryck, Linköping 2009

To Sara and Anja

Abstract

Blood is a vital part of the human physiology; a transport system that brings nutrients and oxygen to sustain living cells and simultaneously facilitates the removal of carbon dioxide and other waste products from the body. To assure the continuity of these functions, it is of uttermost importance to keep the flowing blood inside the vascular system at any cost. The principal components of the haemostatic system are the blood platelets and the plasma coagulation system, both working in concert to create a blood stopping haemostatic plug when a vessel is ruptured. In modern health care, methods for treatment and diagnostics often implicate the contact between blood and artificial materials (biomaterials). Biomaterial surfaces may activate platelets and the coagulation cascade by exposing a surface that during blood contact shares certain characteristics with surfaces found at the site of vascular injury. Therefore it is of great importance that the mechanisms behind the interactions between foreign surfaces and blood are studied in order to minimize, and if possible, prevent unnecessary reactions that may lead to thrombosis.

This thesis describes two important methods to study blood – surface interactions in terms of surface induced plasma coagulation and platelet adhesion/aggregation. The method 'Imaging of coagulation', a coagulation assay based on time-lapse image capture of the coagulation process was developed during the course of this work. The use of images enables the method to answer questions regarding where coagulation was initiated and how fast coagulation propagates. Such questions are highly relevant in the study of blood-biomaterial interactions but also in general haemostasis research. *In vivo,* platelet adhesion and aggregation are events that always proceed under flow conditions. Therefore we also developed a cone-and-plate flow model to study these mechanisms under similar conditions *in vitro*. The cone-and-plate setup was found to be a flexible platform and was used for both blood compatibility testing of potential biomaterials as well as for general haemostasis research.

With the above mentioned methods we tested the haemocompatibility of glycerol monooleate (GMO), a proposed substance for use in biomaterial applications. It was found that GMO did not activate coagulation to any great extent either in plasma or in whole blood.

Surface induced coagulation and platelet adhesion was also studied on PEG-containing hydrogels and compared with hydrogels constructed from three different non-PEG-containing monomers. It was concluded that all the grafted hydrogels, in particular those produced from the monomers 2-hydroxyethyl methacrylate (HEMA) and/or PEG- methacrylate (PEGMA), demonstrated good haemocompatibility.

Supported phospholipid bilayers were used to investigate the relationship between surface charge and procoagulant activity. The coagulation process was studied in a straightforward manner using the imaging of coagulation setup. We concluded that the content of negatively charged 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS) in the bilayer must exceed ~ 6% for the bilayer to exert procoagulant activity.

The physiological role of factor XII in human haemostasis and thrombosis was investigated in the imaging of coagulation setup and the cone and plate setup by the use of surfaces with thrombogenic coatings. We found that tissue factor initiated coagulation could be greatly accelerated by the presence of contact activating agents in a platelet dependent manner.

In conclusion, the method 'Imaging of coagulation' and platelet adhesion/aggregation in the cone-and-plate flow model are both versatile methods with many possible applications. The combination of the two methods provides a solid foundation for biomaterial and haemostasis research.

List of papers

Paper I

Imaging of blood plasma coagulation and its propagation at surfaces
Lars Faxälv, Pentti Tengvall, Tomas L. Lindahl
Journal of Biomedical Materials Research Part A, 2008, 85A, (4), 1129-1134

Paper II

Glycerol Monooleate - Blood Interactions
Emma M. Ericsson, Lars Faxälv, Anna Weissenrieder, Agneta Askendal, Tomas L. Lindahl and Pentti Tengvall
Colloids and Surfaces B: Biointerfaces, 2009, 68(1), 20-26

Paper III

Blood compatibility of photografted hydrogel coatings
Lars Faxälv, Tobias Ekblad, Bo Liedberg, Tomas L. Lindahl
Submitted to Biomaterials

Paper IV

Activation of blood coagulation at charged supported lipid membranes
Lars Faxälv, Jasmin Hume, Tomas L. Lindahl, Bengt Kasemo, Sofia Svedhem
In manuscript

Paper V

The role of coagulation factor XII in propagation of coagulation
Lars Faxälv, Sofia Ramström, Kristina Soutukorva, Pentti Tengvall, Tomas L. Lindahl
In manuscript

Papers not included in this thesis

Paper VI

Photografted hydrogel microarrays for controlled platelet adhesion

Tobias Ekblad, Lars Faxälv, Tomas L. Lindahl, Bo Liedberg

In manuscript

Paper VII

Thrombin binding to GPIbα facilitates thrombin receptor PAR1 cleavage and platelet activation

Lars Faxälv, S. Nylander, R. Chaireti, F. Pettersson, Tomas L. Lindahl

In manuscript

Paper VIII

CD44-Deficiency Reduces Antithrombin, Factor VIII and Factor X and Promotes Blood Clots with Compact Structure

Lars Faxälv, Sara Sjöberg, Marcus Andersson, Anders Sellborn, Tomas L. Lindahl, Alexandra Krettek

In manuscript

Table of contents

1. Introduction 2
2. Haemostasis 4
2.1 Platelets and platelet plug formation - Primary haemostasis 6
2.2 Coagulation - Secondary haemostasis 10
2.3 Biomaterial – Blood interactions 18
3. Aim 24
4. Materials and Methods 26
4.1 Blood sampling and plasma preparation 26
4.2 Imaging of coagulation 26
4.3 Cone-and-plate flow model 32
4.4 Fluorometric methods 37
4.5 Additional methods 38
5. Summary of Papers 40
5.1 Paper I 42
5.2 Paper II 43
5.3 Paper III 44
5.4 Paper IV 46
5.5 Paper V 48
6. Concluding remarks 50
Tack 52
7. References 54

Abbreviations

ADP	Adenosine 5'-diphosphate
AMC	7-Amido-4-methylcoumarins
ATP	Adenosine 5'-triphosphate
CEA	2-carboxyethyl acrylate
CTI	Corn trypsin inhibitor
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
EGMEMA	Ethylene glycol methyl ether methacrylate
FITC	Fluorescein isothiocyanate
FOR	Free oscillation rheometry
FT-IR	Fourier transform infrared spectroscopy
GMO	Glycerol monooleate
GP	Glycoprotein
HEMA	2-hydroxyethyl methacrylate
HMWK	High molecular weight kininogen
HSA	Human serum albumin
LED	Light emitting diode
PAR	Protease activated receptor
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEGMA	Poly(ethylene glycol) methacrylate
PFP	Platelet-free plasma
PMMA	Poly(methyl methachrylate)
POEPC	Palmitoyl-oleoyl-glycero-3-ethyl-phosphocholine
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
POPS	1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine]
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PS	Phosphatidylserine
PS	Polystyrene
QCM-D	Quartz crystal microbalance with dissipation
RF	Radio frequency
SIPGP	Self-initiated photografting and photopolymerization
SPR	Surface plasmon resonance
TF	Tissue factor
VWF	von Willbrand factor

Chapter 1

1. Introduction

Blood is a vital part of the human physiology; a transport system that brings nutrients and oxygen to sustain living cells and simultaneously facilitates the removal of carbon dioxide and other waste products from the body. Blood borne cells and enzymatic systems are also involved in the body's defense to keep foreign substances and organisms from causing harm. To assure the continuity of these functions, it is of uttermost importance to keep the flowing blood inside the vascular system at any cost. The acute response mechanisms that are initiated to stop bleeding when blood vessels are injured are all comprised in the term haemostasis, from Greek meaning blood-stop. Since the blood is contained in a pressurized system, the temporary vessel repair mechanisms of haemostasis have to act tremendously fast and efficient in order to prevent excessive bleeding. However, such a potent system also requires the addition of strong regulatory mechanisms to keep it in a perfect balance so that the haemostatic response is not activated at the wrong time or in the wrong place. If the haemostatic system is too effective, if the control mechanisms fail or if the haemostatic mechanisms respond to triggers that are not specific to a vessel injury, the potent haemostatic system can also lead to unwanted thrombosis with subsequent vessel occlusion or embolism, ultimately causing substantial physiological damage. This is also the case when artificial materials come into contact with blood and the haemostatic mechanisms mistakenly identify the material surface as a vessel injury. This is an important issue today since biomaterials are commonly used in the clinic for both diagnostic and treatment purposes.

The principal components of the haemostatic system are the blood platelets and the coagulation system, working in concert to create a blood stopping haemostatic plug. Both platelets and the coagulation system are well known to rapidly respond to artificial surfaces, and therefore it is of great importance that the mechanisms behind the interactions between foreign surfaces and blood are studied in order to minimize, and if possible, prevent unnecessary reactions in terms of thrombosis when blood and biomaterials meet.

In this thesis I will present the development of methods for blood – material interaction studies as well as the main results that can be attributed to these methods. The method for imaging of coagulation (described in Paper I) was found to be a very versatile platform for coagulation studies. The method is compatible with almost any kind of surface and has been extensively used throughout my research (Paper I–V). The development of a flow model to study platelet adhesion and function during flow conditions did also make a great contribution to my work (Paper II, III and V).

Chapter 2

2. Haemostasis

The haemostasis is a complex and efficient system that keeps the blood in balance between bleeding and thrombosis. In a healthy individual the blood is maintained in a flowing state during its passage through the vascular system, but always ready to rapidly form a haemostatic plug that will prevent unrestricted bleeding when a vessel is ruptured (Figure 1a). The normal haemostatic response occurs after a vessel has been damaged and the response can be divided into a primary and secondary stage. The primary stage includes the adhesion of platelets to the damaged vessel wall (Figure 1b), and platelet activation facilitating the recruitment of more platelets to the damaged area. These platelets form a platelet plug that initially stops the bleeding (Figure 1c) (reviewed in (1)). The second stage in the haemostatic plug formation is the coagulation process, initiated by exposed tissue factor on the damaged vessel wall. Coagulation is driven through a complex enzymatic pathway that involves several zymogen-to-enzyme conversion steps and allows the reaction to undergo great intrinsic amplification. The final step in this cascade is the conversion of soluble fibrinogen into an insoluble fibrin network. The coagulation process is accelerated by activated platelets and will ultimately form a stabilizing fibrin network that will secure the platelet plug until the vessel wall is restored (Figure 1d) (reviewed in (2)).

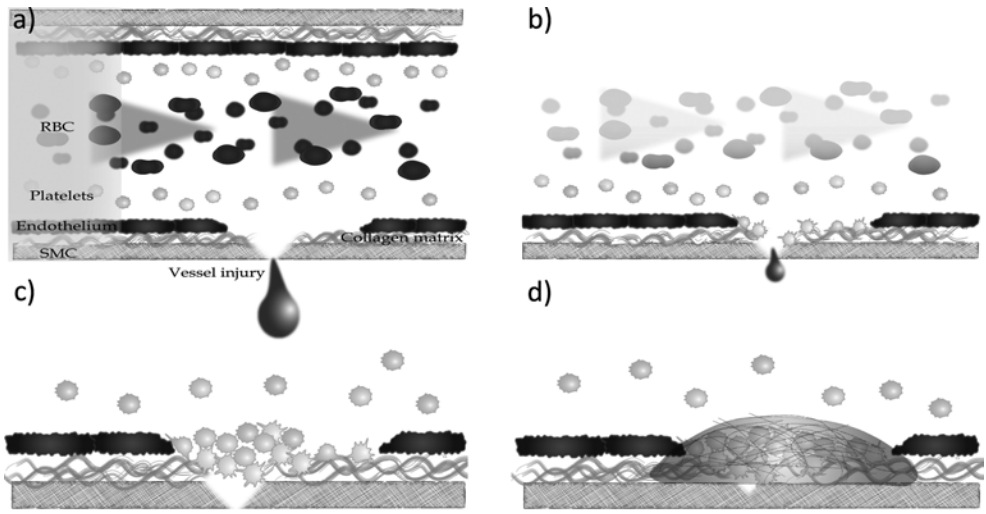


Figure 1. The sequential events following vessel injury: a) Schematic illustration of the vessel, b) platelet adhesion to collagen in the subendothelial matrix, c) platelet aggregation and activation; forming a platelet plug that initially stops bleeding, d) fibrin network formation (coagulation) stabilizes the platelet plug.

After the haemostatic system has played its part and the vessel integrity is restored by vessel repair mechanisms, the clot needs to be cleared in order to regain unobstructed blood flow. This is taken care of by the fibrinolytic system that digests the fibrin network into small soluble fragments that can be transported away by the blood flow. The pathway of fibrinolysis share similarities with the coagulation cascades and the key protein plasminogen is activated by zymogen-to-enzyme conversion (reviewed in (3, 4)).

2.1 Platelets and platelet plug formation - Primary haemostasis

The platelet

The platelets are small non-nucleated cells circulating in our vascular system with a primary function to initially arrest bleeding from a damaged vessel by rapidly creating a platelet plug. Platelets and not replicate by cell division but originate from the bone marrow where they are shed off into the blood stream from the large megakaryocytes (5). Platelets are the second most abundant cellular component in healthy blood, and the platelet concentration is in the range of 150 – 350 $\times 10^9$/L (6). The average lifespan of circulating platelets is estimated to between 7 and 10 days (7). The platelet surface is packed with functional receptors to facilitate platelet activation and interactions with subendothelial matrix, other blood cells and other platelets. The platelet also contains several granules that upon activation can fuse with the platelet membrane and thereby release their content into the surrounding media. The granules are categorized into the larger α-granules, the smaller dense granules and lysosomes. α-granules contain a large variety of biological active substances involved in haemostasis, e.g. platelet activation and adhesion molecules, plasma coagulation factors and fibrinolysis proteins. The dense granules mainly contain ions and smaller signal molecules such as adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP) and serotonine. Lysosomes contain acid hydrolase, cathepsin and lysosomal membrane proteins (8). Recent findings furthermore suggest the existence of two distinctly different types of α-granules that are selectively released depending on activation pathway. Most interestingly, these subpopulations contain either pro-angiogenic or anti-angiogenic mediators such as vascular endothelial growth factor (VEGF) or endostatin, respectively (9). A rapid and effective response from the platelets is vital. To ensure this function the platelet are very sensitivity towards external stimuli and can undergo a fast activation process that is enhanced by intrinsic activation feedback loops in the form of autocrine and paracrine signaling (reviewed in (10)). A schematic illustration of the platelet, including principal adhesion, activation and aggregation receptors is presented in Figure 2.

Platelet adhesion

Following vessel wall injury, platelets rapidly adhere to exposed elements in the subendothelial matrix; initiating the first step in the haemostatic response leading to the formation of a platelet plug. This primary adhesion is mediated by the synergistic function of several receptors on the platelet surface. Collagen is the main component of the subendothelium matrix and is also considered to play the principal role in the adhesion process. Nine types of collagen have been identified in the vessel wall but the main constituents have been determined as collagen type I and type III (11).

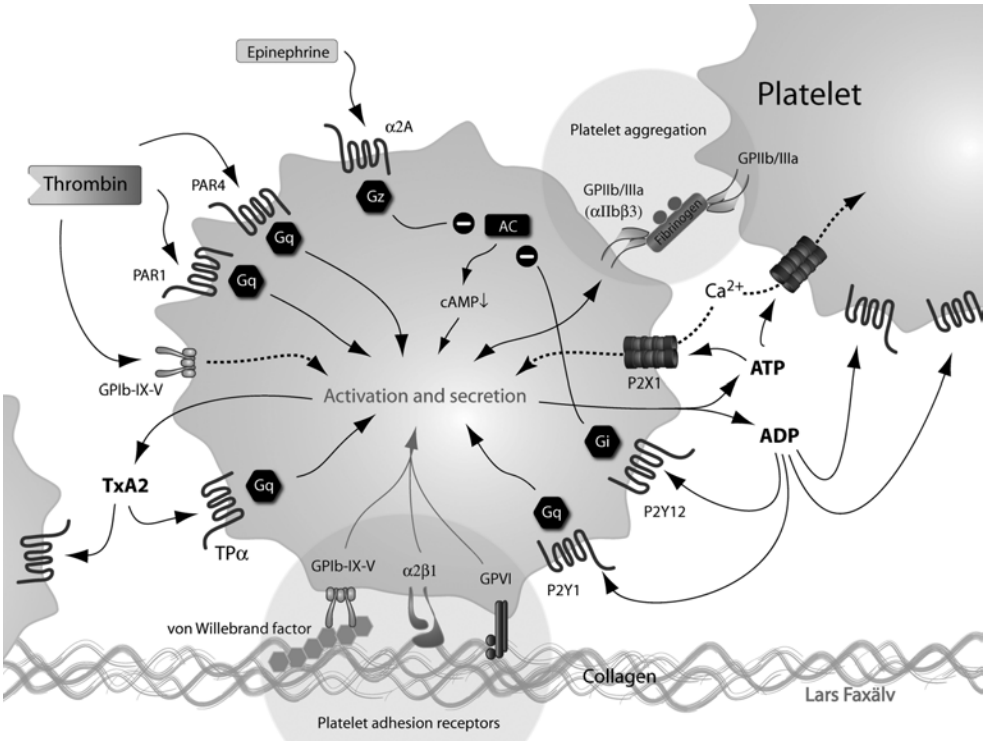


Figure 2. A schematic illustration of the platelet and its main functional receptors. Platelets adhere to collagen in the damaged vessel wall through adhesion receptors. The adhesion receptors can contribute to platelet activation and thereby stimulate the release of autocrine activators such as ADP and TxA2. Upon activation the platelets are capable of forming platelet aggregates by linking to fibrinogen molecules with the GPIIb/IIIa receptor. Thrombin, produced by activation of the coagulation cascades can also activate PAR1 and PAR4 by proteolysis of the receptor.

The local flow conditions are also important in determining the platelet behaviour. Blood is always moving inside the vessels, but the conditions can vary from almost stagnant to extremely high flow rates. The velocity of blood, in relation to the stationary vessel wall, gives rise to shear forces in the moving blood that can be described by a velocity gradient. Shear forces will lead to a higher concentration of erythrocytes and other large blood cells in the middle of the high-velocity flow, and the platelet concentration will be highest closer to the vessel wall where the fluid velocity is lower (12, 13). The highest shear rate, or velocity gradient, is always found closest to the vessel wall (12). Under high shear conditions, the initial tethering and arrest of platelets from the flowing blood to collagen is facilitated by the glycoprotein (GP) Ib-IX-V receptor, a heterotrimer composed of two disulfide linked GPIbα-GPIbβ in complex with two GPIX and one GPV (14, 15). The binding is mediated by von Willebrand factor (VWF), a multimeric plasma protein that rapidly binds to exposed collagen. Long multimers of VWF can contain several binding sites for the GPIb-IX-V adhesion receptors and upon binding GPIb-IX-V is capable of

outside-in signaling leading to platelet activation (16, 17). The GPVI receptor binds directly to collagen via the specific Gly-Pro-Hyp peptide repeat sequence and has an important role in platelet activation by outside-in signaling (18, 19). The GPVI activation has been found to be mediated by the Fc receptor γ-chain, also involved in platelet interaction with immunoglobulins (20, 21). The integrin $\alpha_2\beta_1$ adhesion receptor (also known as GPIa/IIa) can bind to collagen directly. Although it has been indicated that the $\alpha_2\beta_1$ integrin is not involved in the initial tethering of platelets to collagen under high shear, it is still considered important for firm adhesion and securing the platelets (22). $\alpha_2\beta_1$ has also been proposed to be involved in platelet spreading on the collagen surface (23). The integrin $\alpha_2\beta_1$ receptor must be activated by inside-out signaling to attain a state of high affinity for its ligand (24). The two collagen receptors GPVI and $\alpha_2\beta_1$ are both essential for platelet adhesion and aggregation on collagen in flow conditions, blockade of either receptor will abolish the platelet plug forming capabilities (25).

Platelet aggregation

Once a primary layer of adhesive platelets have covered the exposed subendothelial matrix, subsequent adhesion will continue but in the form of aggregation, where platelets from flowing blood bind to adhered platelets at the wound site. The mechanism of platelet aggregation is mainly attributed to the integrin $\alpha_{IIb}\beta_3$ (also known as GPIIb/IIIa) receptor. The main ligand of integrin $\alpha_{IIb}\beta_3$ is fibrinogen but the receptor also have affinity for multimeric vWF, vitronectin, fibronectin and thrombospondin (26). The mechanism of aggregation involves two integrin $\alpha_{IIb}\beta_3$ receptors on different platelets that bind to the same fibrinogen molecule. The $\alpha_{IIb}\beta_3$ receptor has a low and a high affinity state for binding fibrinogen. The high affinity state is achieved by inside-out signaling when platelets are activated (27). The $\alpha_{IIb}\beta_3$ receptor can also mediate outside-in signaling by binding fibrinogen (28).

Platelet activation

In order for the recruitment of platelets to continue during the aggregation phase it is necessary that the activated state of the adhered and aggregated platelets can be propagated to newly arriving platelets. This propagation of activation is executed via potent autocrine and paracrine signaling pathways. Activated platelets will secrete abundantly of ADP and ATP from dense granule that in turn may activate neighboring platelets via ADP and ATP sensitive receptors. ADP is a ligand to the receptors $P2Y_1$ and $P2Y_{12}$, located on the platelet surface (29, 30). The P2Y receptors are seven transmembrane spanning proteins that transmit outside-in signaling by coupling to G-proteins (31). The P2X1 receptor is a ligand gated cation channel and was not until the year 2000 disclosed as a specific ATP receptor. Its main function has been suggested as potentiating mechanism for other activation pathways (32).

Following initial platelet activation, the signal molecule thromboxane A_2 (TxA_2) is rapidly synthesized and released by platelets. The target for TxA_2 is the TPα receptor, a seven transmembrane spanning receptor that is coupled to G-proteins on the cytosolic side. TxA_2 is a lipid signal molecule synthesized by arachidonic acid metabolism, and the synthesis can be effectively inhibited by aspirin treatment, resulting in the attenuation of platelet activation (33, 34).

During thrombus formation, the simultaneous activation of the coagulation cascade results in generation of the key coagulation enzyme thrombin. Thrombin has a dual role in haemostasis, as it in addition to facilitating the fibrin network formation also has a function as an activator of two distinct platelet receptors, namely the protease activated receptors (PARs) 1 and 4. Thrombin acts on the PARs by cleaving off a small peptide from its exposed extracellular part of the N-terminus. The cleavage results in a new tethered N-terminus that functions as a ligand and thereby mediates the activation of the receptor. The outside-in signaling event is further propagated on the intracellular side by coupled G-proteins (35, 36). Although PAR1 and PAR4 couple to the same G-protein subtypes, PAR1 and PAR4 apparently differ in terms of affinity for thrombin and the duration of intracellular signalling. It has been proposed that PAR1 has a higher affinity for thrombin than PAR4 (37-39); however, there is evidence that PAR4 is also activated by thrombin at low concentrations (39). Furthermore, GPIbα binds thrombin with high affinity and has been proposed as a cofactor for the PAR activation mechanism by co-localization of thrombin with the PAR receptors, thus facilitating their cleavage (40).

A large number of the platelet signaling receptors are coupled with G-proteins on the cytosolic side and such receptors are therefore termed G-protein coupled receptors (GPCRs). There are several types (and subtypes) of G-proteins in platelets and the most prominent are Gq, Gi, Gz and G_{13}. Their respective activation will propagate through different intracellular signaling pathways and lead to differential activation responses (reviewed in (41)). The platelet receptors are an exceptionally important part of the functionality of the haemostatic system and deficiency of functional platelet receptors often result in bleeding disorders (reviewed in (42)).

2.2 Coagulation - Secondary haemostasis

Coagulation is the transformation of flowing blood into a stable gel, and the transformation is the result of complex enzymatic mechanisms that initiate when blood comes in contact with extravascular surfaces. The gelling of blood is accomplished when soluble fibrinogen molecules undergo enzymatic cleavage and assemble into insoluble fibrin fibers. The growing fibrin fibers will create a fine interconnected network and trap blood cells, forming a gel of blood termed coagulum or clot. The intricate enzymatic process of blood coagulation was first described in fullness during the year 1964. Davie and Ratnoff presented their theory of blood coagulation as a "waterfall sequence" of coagulation factor activation (33). Independently, and almost at the same time, very similar conclusions were drawn by Macfarlane who published his idea of the coagulation mechanisms as an "enzyme cascade"(43). Both publications describe how the activation of factor XII leads to a sequential activation of five coagulation factors in stepwise order, ending with the enzymatic cleavage of fibrinogen by the enzyme thrombin. This cascade theory laid the foundation for modern coagulation research. The functional enzymes in the coagulation cascades are termed coagulation factors and are assigned a specific roman number. To distinguish between an activated coagulation factor from the zymogen, the activated factor number is suffixed with an "a". Five of the zymogens involved in the coagulation cascades are vitamin K-dependent serine proteases, factor VII, factor IX, factor X, prothrombin and protein C, all containing a characteristic domain with γ-carboxyglutamic acid residues (Gla domain) (44). The Gla domain have also been recognized to mediate the binding of the enzyme to phospholipid bilayers in a Ca^{2+} dependent manner (45). Inhibition of the γ-carboxylation of coagulation factors results in drastic attenuation of coagulation, and has for a long time been used in anticoagulation therapy by the administration of warfarin [reviewed in (46)].

The two pathways of the coagulation cascade

There are two separate initiation mechanisms for the enzymatic coagulation cascade, both leading to the same endpoint; fibrin network formation. The two pathways following these initiation mechanisms are termed the intrinsic and extrinsic pathway. The names are derived from the fact that all functional components of the intrinsic pathway are blood borne, whereas the triggering component of the extrinsic pathway, tissue factor, is not found in blood, but in extravascular tissue. Although there are major differences in the activation mechanism and the subsequent enzymatic steps, both the intrinsic and extrinsic pathways converge into the common pathway, ultimately leading to fibrin formation. Extensive research in the area has with time revealed that these pathways are highly interconnected and also dependent on platelet surface association for proper function (for review see (47)).

Tissue factor activation

The extrinsic pathway is the main contributor to physiological activation of coagulation *in vivo*. The pathway is initiated by tissue factor (TF), an integral membrane glycoprotein found in extravascular tissue (48, 49). TF can bind the blood born factor VII (FVII) as well as its active form, factor VIIa (FVIIa). FVII is mainly found in circulation as the inactive zymogen, but reports state that 1-2 % of the total amount is in the activated form, FVIIa (50). TF itself has no enzymatic function, but serves as a cofactor and facilitates rapid auto-activation of FVII and equally important, dramatically enhances the enzymatic activity of FVIIa (51). The TF:FVIIa (extrinsic Xase) complex can activate both factor X (FX) and factor IX (FIX) into their active forms (50), and as part of an initial amplification loop, both FX and FXI contribute to further initiation of coagulation by feedback activation of more FVII to FVIIa (52, 53). Also, an inhibiting mechanism of FXa has recently been proposed, due to the fact that FXa remains reversibly bound to the TF:FVIIa complex after activation, hence blocking further activation of remaining FX (54). The small amounts of FXa that are initially formed lead to a limited conversion of the zymogen prothrombin to the enzymatically active thrombin. The minor amount of thrombin produced through the extrinsic Xase pathway is however insufficient for complete fibrin formation but is enough to activate platelets through PAR receptors as well as converting the cofactors factor V (FV) and factor VIII (FVIII) in the intrinsic pathway to their active forms. The activated FVIIIa can in turn form a complex with FIXa, previously activated by the extrinsic pathway. The FIXa:VIIIa complex (intrinsic Xase), preferentially assembles on the phospholipid membrane of activated platelets (55, 56), where it effectively activates FX at a far greater conversion rate than the extrinsic Xase complex (57). The rapidly forming FXa will thereafter form the prothrombinase complex together with FVa, also located on the phospholipid membrane (58). The FIXa:VIIIa and the FXa:FVa complexes are both highly efficient and will in short time raise thrombin concentration levels sufficiently to facilitate fibrin network formation. An additional amplification step constitutes the feedback activation of FXI by thrombin, subsequently generating activated FIX, and thereby boosting the ongoing activation of the intrinsic pathway (59). This amplification mechanism is however only considered relevant in a setting with a low level TF exposure (60), e.g. after tissue factor pathway inhibitor (TFPI) shuts down further TF-dependent activation (61). The activation of factor XI by thrombin has been proposed to occur on the platelet surface since both factor XI and thrombin can bind to the platelet receptor GPIbα (62).

The entire coagulation pathway, comprised of multiple steps of zymogen to enzyme conversions are indeed very nested and complex. However, FX activation, the point of convergence for the extrinsic and intrinsic pathways, has been deemed as the rate limiting step for thrombin generation (63). Once the massive thrombin generation has started, soluble fibrinogen is rapidly converted into soluble fibrin molecules that immediately start

to assemble into fibrin fibers in a favorable reaction. Activation of the extrinsic pathway by tissue factor and the follow enzymatic coagulation cascade are visualized in Figure 3.

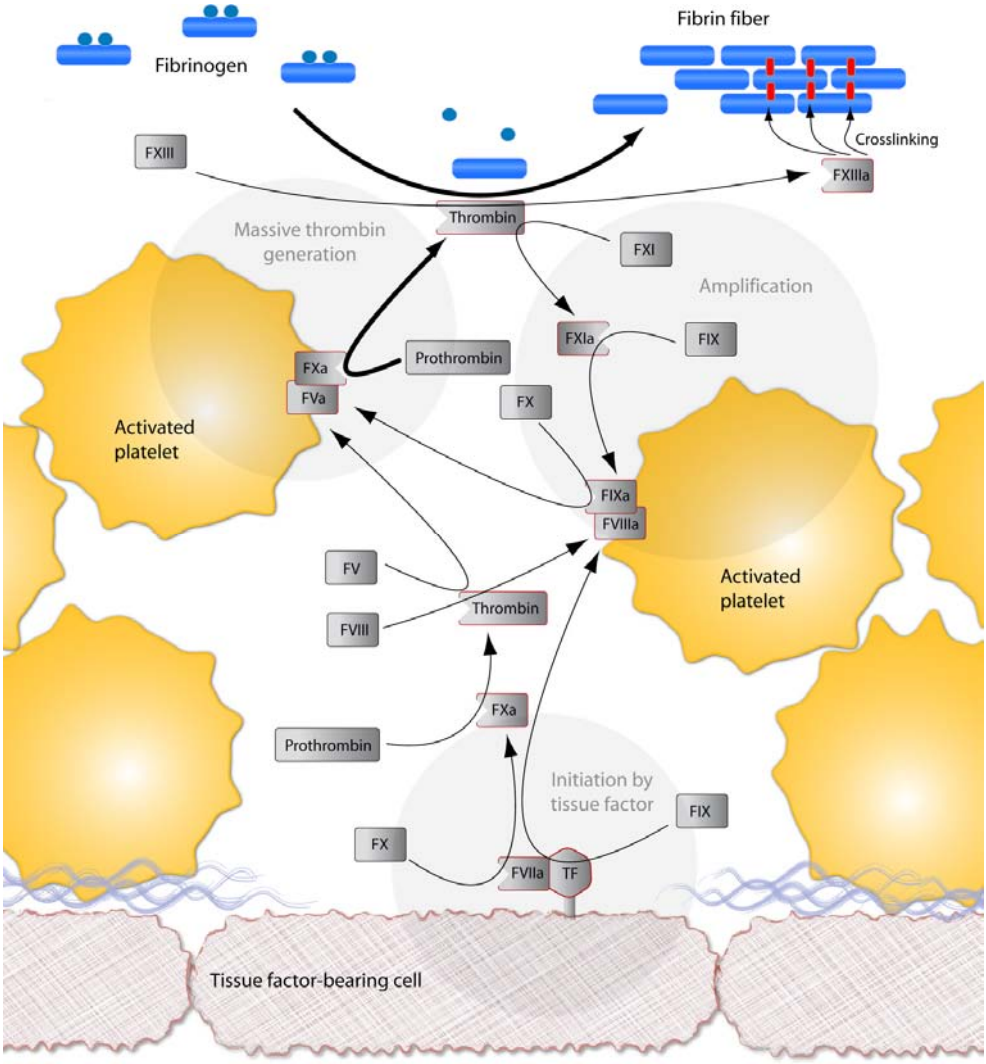


Figure 3. Tissue factor bearing cells in the damaged vessel wall can initiate the extrinsic coagulation cascade. Small amounts of thrombin are produced by factor Xa which in turn will active part of the intrinsic pathway (FXIa and FXIa/FVIIIa complex) leading to a large scale production of the very effective prothrombinase (FXa/FVa) complex on the surface of activated platelets. The prothrombinase complex will generate massive amounts of thrombin from prothombin. Thrombin cleaves off the fibrinopeptides from the fibrinogen molecule, making the molecule able to polymerize into a fibrin fibre. Finally the fibrin fibres are stabilized by the cross-linking activity of factor XIIIa.

Factor XII – contact activation pathway

The other pathway that leads to thrombin formation is by the intrinsic pathway, also termed the contact activation pathway; the name derived from its intriguing initiation mechanism where factor XII undergoes auto-activation triggered by the contact with a surface interface. Ratnoff et al was the first to discover factor XII in 1955 and named it Hagman factor, from the name of the first studied patient with factor XII deficiency. The deficiency was brought to attention when neither blood nor plasma from Mr. Hagman coagulated properly during incubation in test tubes (64). These primary findings have lead to the unveiling of the contact activation system, regulated by three plasma proteins; factor XII, prekallikrein (PK) and high molecular weight kininogen (HMWK). The pathway is initiated as factor XII binds to a surface and thereby undergoes spontaneous cleavage of its single peptide chain, rendering the zymogen into a fully active serine protease comprised of two polypeptide chains held together by a disulfide bond (65). Three separate sites on the factor XII molecule have been characterized and proposed to facilitate its important interactions with surfaces (66-68). The activated factor XII on the surface converts both factor XI and prekallikrein to their activated forms, factor XIa and kallikrein respectively. Factor XII is also a substrate for kallikrein and the pair thereby forms a short reciprocal activation loop, enabling rapid activation of the intrinsic pathway (69). Factor XI is not the exclusive substrate for factor XIIa but can also be activated by thrombin and factor XIa (70), and it is the activation by thrombin that is considered to be the physiological relevant pathway since deficiency of factor XI but not that of factor XII results in bleeding tendencies (71, 72). Factor XI activity are enhanced by association with the platelet membrane (73), and it has been demonstrated that the GPIb-IX-V receptor facilitate binding of factor XI to the platelet surface (74). Also, the interaction of factor XI and prekallikrein with surfaces are to a large extent mediated by HMWK. The dual function of HMWK; forming complex with factor XI and prekallikrein and binding to surfaces are both crucial for its procoagulant role in coagulation. It has been shown that inhibition of one or the other function by directed monoclonal antibody blocking results in a complete loss of the procoagulant property of HMWK (75). HMWK is also cleaved during the contact activation process by kallikrein, liberating bradykinin (76), a peptide mediator that induces anti-thrombotic responses in the vascular tissue (77). The intrinsic pathway activation is propagated downstreams by factor XIa, subsequently activating the vitamin K-dependent zymogen factor IX (78). Facor IX then activates factor X, the factor that links the intrinsic and extrinsic pathways. Prothrombin can then be converted into active thrombin by the activated factor Xa. However, this initial thrombin formation is limited to proceed at a slow rate since both factor IXa and factor Xa lack their respective cofactor, factor VIIIa and factor Va. Thrombin, but also factor X can convert the pro-cofactors into active cofactors, facilitating the formation of the FIXa:FVIIIa (intrinsic Xase) and the FXa:FVa (prothrombinase) complex on a phospholipid surface (79, 80). This complex formation is essential for rapid formation of thrombin; its necessity reflected by a ~50,000 fold increase in enzymatic activity for factor XIa (79), and a

~1,000 fold increase for factor Xa (58). As both complexes become functional, large amounts of thrombin will be generated, converting fibrinogen into fibrin, ultimately resulting in fibrin network formation.

The physiological role of factor XII in haemostasis and thrombosis have for a long time been a subject for investigation, especially since deficiencies in factors VIII, IX and XI from the intrinsic pathway cause bleeding disorders (81), while a deficiency in the first factor in this pathway, factor XII, does not cause any bleeding (64, 72). Recently a patophysiological role for FXII in thrombosis was proposed as it was showed that FXII-deficient mice do have defective thrombus formation in arterial beds and are protected against thromboembolism induced by collagen and epinephrine (82). The authors furthermore speculated that FXII-driven thrombin generation might proceed on the platelet surface.

Naturally occurring surfaces that can facilitate the autoactivation of factor XII have also been thoroughly investigated. A recent report presented results that indicate that FXII might be activated by polyphosphate anions released by activated platelets (83). Another exogenous source of contact activation may be the lipopolysaccharides found on the surface of bacteria during sepsis (84, 85). Exposed collagen is also a proposed endogenous activator of FXII, and although reported results have pointed both in favor and against this concept, the consensus is that collagen type I, that is found in the subendothelial matrix can facilitate autoactivation of FXII (82, 86, 87).

Fibrin network formation

The formation of an insoluble network of fibrin is the final endpoint of both coagulation pathways. Fibrin is converted from fibrinogen by the proteolytic activity of thrombin, cleaving off two different peptides from the fibrinogen backbone. The peptides are named fibrinopeptide A and B (FPA and FPB) and are present in two copies on each fibrinogen molecule. FPA is initially cleaved off at a considerably faster rate than FPB, and it has been determined that the polymerization reaction is only dependent on the FPA cleavage. The fibrin molecules assemble to form protofibrils, which are then stabilized by the secondary cleavage of FPB. The protofibrils will join in parallel to form fibrin fibers that are slightly twisted. The lateral growth is limited to a width of about 100 nm, due to stretching of the fibrin molecule along the outer surface of the fiber (88). The fibrin formation process is sensitive to environmental factors and changes in chemical or physiological conditions can affect the final network structure. There are a several known factors that influence the fibrin network structure. The fibrin polymer fibers can be gathered in thicker or thinner bundles during formation, thus affecting the porosity of the final network (89). Thrombin, fibrinogen and ion concentrations are some factors that have previously been reported to influence fibrin network structure. Another factor that plays a part in determining the network structure is the concentration of plasma proteins. It is believed that a high concentrations of plasma proteins will spatially restrict the fibrin

fiber extension (88). Thinner fibrin fibers do not interact with visible light to the same extent as thicker fibers and therefore fibrin networks comprised of thin fibers generate less light scattering. This makes the fine network seem almost transparent whereas the coarse network, built up by thicker fibrin bundles, is opaque (90). In the final stage of the coagulation process the fibrin network is stabilized by the crosslinking action of the thrombin-activated factor XIII (reviewed in (91)). Factor XIII is a transglutaminase that introduce ε-(γ-glutamyl)lysin crosslinks between fibrin molecules (92). The physiological function of factor XIII is important, and patients that are deficient in factor XIII suffer from bleeding tendencies (93). Factor XIII also mediates the incorporation of fibronectin into the fibrin network, a process that will increase both size and density of the fibrin fibers (94).

Inhibitors of the coagulation system

The thrombus formation process is a composite mechanism with great capacity for intrinsic amplification. The amplification will ensure a fast enzymatic route that will prevent excessive blood loss in the event of vessel injury. However, for such a powerful pro-thrombotic system there is also a need for regulatory functions, to stop thrombus growth from spreading too far away from the site of injury. Protein C is activated by thrombin to activated protein C (APC), the mechanism dependent on the association of thrombin and protein C with the endothelial cell membrane protein, thrombomodulin. APC, together with its cofactor protein S, functions as an inhibitor of coagulation by inhibiting the cofactors factor Va and factor VIIIa through enzymatic cleavage (95). It has been found that thrombin is an allosteric enzyme that shows two different conformations depending on Na^+ binding. The thrombin conformation where Na^+ is bound yields a higher efficiency towards activation of fibrinogen and the PAR receptor while the conformation without Na^+ is slower. The thrombin activation rate of the coagulation inhibitor protein C is however not affected and is the same for both conformations (96). Another inhibitor of the coagulation system is the tissue factor pathway inhibitor (TFPI), which is able to inhibit both factor Xa and the TF-factor VIIa complex (97). Antithrombin (AT) is a protein that can trap thrombin and thus inhibit thrombin's enzymatic capabilities. Heparin binding enhances the activity of AT mediated inactivation of thrombin profoundly by colocalization of the two proteins (98). Several of the inhibitors that are involved in the coagulation process belong to the serpin protein superfamily (reviewed in (99)), that inhibit their target protease by forming a covalent bond to its catalytic site (100).

Procoagulant properties of platelets

Coagulation and platelet activation have turned out to be tightly integrated processes. Thrombin, the key enzyme in the coagulation cascade, is a potent platelet agonist, acting on the platelet PAR receptors 1 and 4, and conversely, activated platelets are known to enhance and contribute to the overall coagulation process via several independent mechanisms (reviewed in (2, 36)). In this aspect, it has been found that the platelet

phospholipid membrane acts as one of the main contributors to coagulation by catalyzing several enzymatic steps in the coagulation cascade. The major constituents of the platelet membrane are phosphatidycholine (PC), 38%; phosphatidylethanolamine (PE), 27%; sphingomyelin (SM), 17% and phosphatidylserine (PS), 10%. However, in the resting platelet membrane a considerable asymmetry is evident and almost no detectable PS and only 20% of the total PE is presented on the outer leaflet of the platelet surface (101). This asymmetry is maintained by the continuous action of aminophospholipid translocase, actively transporting PS and PE to the intracellular membrane leaflet by a flip-flop mechanism (102). The activation of the platelet will however lead to a rapid loss of asymmetry, exposing PS and more PE on the outer surface of the platelet. The mechanism is attributed to scramblase, a protein facilitating bidirectional movement of phospholipids between the two leaflets (103). The scrambled membrane will expose a slightly anionic surface due to negative net charge of PS. Together with Ca^{2+} the surface efficiently binds the coagulation factor complexes and thereby increase their activity significantly (reviewed in (104)). However, the relationship between PS exposure and procoagulant activity is far from clear (105), and more research on the subject is necessary. Another procoagulant mechanism is the concomitant release of α-granule content during platelet activation, the release resulting in a drastic increase in the local concentration of coagulation factors as well as fibrinolysis inhibitors at the site of the growing thrombus (106).

Clot retraction

After the clot has been formed, the activated platelets incorporated in the clot rearrange and contract their intracellular actin/myosin cytoskeleton. The intracellular actin network is connected to the internal part of the integrin $\alpha_{IIb}\beta_3$ fibrinogen receptor. Following coagulation, the external part of $\alpha_{IIb}\beta_3$ will have bound to the fibrin network of the clot, and therefore, as a result of platelet contractile force on the fibrin network, the formed clot will compact on itself and hence reduce its total volume. The mechanism is termed clot retraction, and it is considered that its main physiological function is to clear the obstructed vessel for renewed blood flow (107). Disrupting the fibrin binding capability of platelets with the use of integrin $\alpha_{IIb}\beta_3$ inhibitors (see Figure 4) results in a complete loss of clot retraction (108, 109). Commonly used integrin $\alpha_{IIb}\beta_3$ inhibitors are abciximab, eptifibatide and tirofiban. Clot retraction can also be inhibited by the use of cytochalsin E, a cell permeable metabolite of fungal origin that prevents intracellular actin rearrangement (110-112).

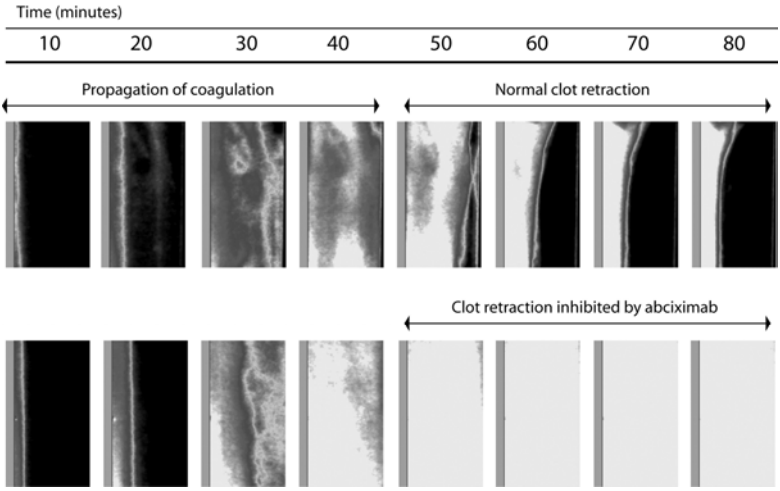


Figure 4. Normal clot rertaction (above) following coagulation in PRP. The addition of abciximab (below), an integrin $\alpha_{IIb}\beta_3$ blocking monoclonal antibody fragment, will lead to complete inhibition of clot retraction.

Fibrinolysis

After the haemostatic plug has served its purpose by preventing blood loss during the vessel repair process, it is cleared from the site by an enzymatic process termed fibrinolysis. The fibrinolytic system is a short enzyme cascade system, which ends in the degradation of the fibrin network into soluble products by the function of the key enzyme plasmin. Initiation of the fibrinolytic system can be accomplished by either tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). tPA is secreted by endothelial cells and is in the presence of fibrin a strong activator that converts the zymogen plasminogen to its active form, plasmin. uPA is mainly found in urine, but has been shown to be secreted by several different cell types. uPA, in contrast to tPA, is not dependent on the presence of fibrin to convert plasminogen into plasmin (3, 4). Both tPA and uPA exist in single chain precursor molecules (sctPA and scuPA) that can undergo enzymatic cleavage by plasmin. scuPA behaves as a zymogen and its enzymatic activity is increased by ~200% by the cleavage, whereas both sctPA and tPA functions with approximately the same efficiency. Interestingly, scuPA can also be activated by the contact activation enzymes factor XIIa and kallikrein in the intrinsic pathway of coagulation (113). The architecture of the fibrin network has been shown to affect the fibrinolytic process, and the degradation process proceeds drastically slower for thinner fibrin structures than for thicker fibrin structures (114). The fibrinolytic system has to be constrained by regulatory mechanisms to prevent it from interfering with the vital process of haemostatic plug formation. This is accomplished in numerous ways but the principal inhibitors are plasminogen activator inhibitor (PAI-1) (115), α2-macroglobulin (116), C1-inhibitor (117) and thrombin activated fibrinolysis inhibitor (TAFI) (118).

2.3 Biomaterial – Blood interactions

The haemostatic system (described in previous sections) is always in a delicate balance between unstopped bleeding and thrombosis. The balance can however be disrupted by, for example, a state of disease or when blood comes into contact with the surface of a foreign material. In modern health care, treatment- and diagnostic methods often implicate the contact between blood and artificial materials (biomaterial). Treatments including venous catheters, stents, vena cava filters, vascular grafts and prosthetic heart valves are a few examples of biomaterials that are introduced into the vascular system and brought into contact with blood. This contact between blood and biomaterial is also constantly present during procedures when blood is handled outside the body, e.g. during cardiopulmonary bypass, in dialysis machines, during blood transfusions or simply, when blood is drawn by venipuncture and used for *in vitro* experiments. The artificial material surface may activate both blood cells (platelets and white blood cells) and the enzyme mediated coagulation cascade by exposing a surface that during blood contact shares certain characteristics with surfaces found at the site of vascular injury. This may ultimately lead to unwanted thrombus formation at the artificial surface (for example see Figure 5), a serious adverse effect that is usually prevented by aggressive antiplatelet and/or anticoagulation therapy. Three examples of such therapies are: high doses intravenous heparin during cardiac surgery with cardiopulmonary bypass (119), dual antiplatelet therapy after implantation of metal stents in the coronary vessels (120), and continous warfarin treatment of patients with prosthetic heart valves(121). The extent to which artificial materials affect and provoke the haemostatic mechanisms is heavily dependent on the design and characteristics of the biomaterial, but also where in the vascular system it is implanted (for review see (122, 123)). Surfaces inert to platelet adhesion and activation of coagulation can be achieved through surface modifications. Immobilized heparin or poly(ethylene glycol) (PEG), are the most widely used surface modifications in this context (124, 125).

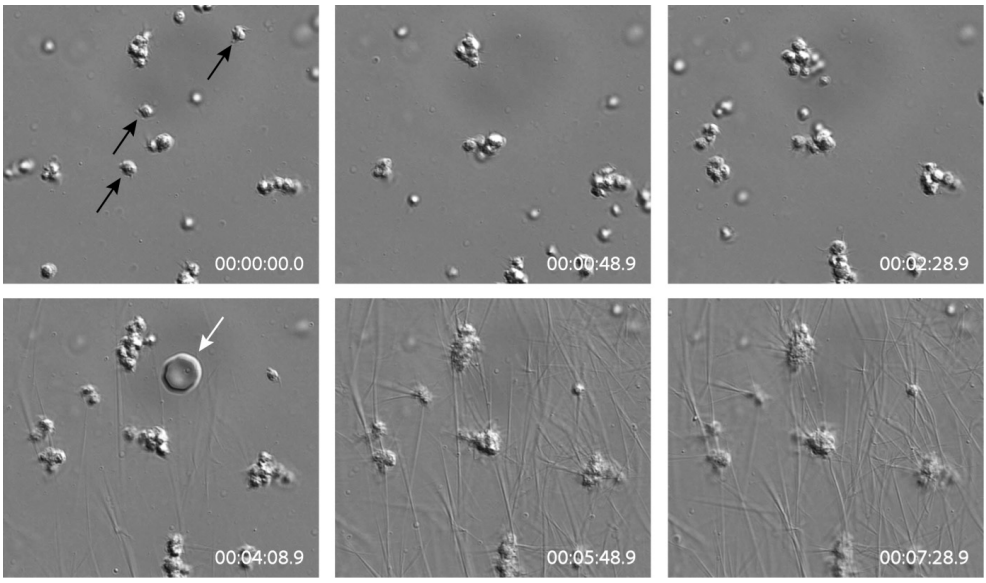


Figure 5. Time-lapse images visualizing the adhesion of platelets, platelet aggregation and ultimately fibrin network formation on a hydrophilic glass surface. Black arrows indicate single platelets and the white arrow indicate a red blood cell. The duration of the experiment was 8 minutes. Images were acquired by differential interference contrast (DIC) microscopy.

Plasma protein adsorption

The adsorption of blood plasma proteins to biomaterial surfaces is usually a rapid process that within seconds generates a biologically active surface that may interact with other blood borne mechanisms (126, 127). Both platelet adhesion and activation of the coagulation cascade are mediated by plasma proteins. Blood is a very complex fluid containing a large variety of molecules with different characteristics and functionalities. Proteins are one of the main constituents of blood plasma and approximately three hundred distinct proteins have so far been documented, with plasma concentrations ranging from 35-50 mg/mL for serum albumin to only 0-5 pg/mL for interleukin 6 (128). The dynamics of protein adsorption are strictly related to the chemical and physical properties of the surface, protein and solvent, and the exothermic process is observed as a decrease in Gibbs free energy (G), i.e. $\Delta_{ads}G=\Delta_{ads}H-T\Delta_{ads}S$ <0, where H=enthalpy, S=entropy and T=temperature. This decrease generally arises from increased system entropy explained by dehydration of parts of protein and/or adsorbent surface, interactions between charged groups at the interface, and/or conformational changes in protein structure. The different modes of interaction result in selectivity between surface and proteins, i.e. proteins preferentially adsorb on surfaces exposing certain properties (129). Proteins can adsorbed to form a covering monolayer on the surface, resulting in a protein layer with a thickness of 2-10 nm (130). Accumulation of proteins at the surface may yield a concentration of surface-associated proteins that is up to 1000- fold higher

than the concentration of the protein in solution (131). Adsorbed proteins are not always bound indefinitely to the surface, and the composition of proteins may be subject to change over time. This phenomenon is termed the Vroman effect and has been observed to occur preferentially on negatively charged hydrophilic surfaces (132). Wettability (or hydrophilicity) has been characterized as a key determinant for the protein adsorption process, and it is generally accepted that hydrophobic surfaces adsorb more proteins than hydrophilic surfaces (131). The hydrophobic surface allows interaction with hydrophobic domains and residues in the protein, the process assisted by an entropy gain during the subsequent release of unfavorably organized water at the surface (129). Surface hydrophilicity can be determined by static and dynamic water contact angle measurements and a surface is deemed hydrophobic when generating a contact angle with water of more than 65° (133). Protein and surface charge are also important factors in the adsorption process, both dependent on the pH of the solute. Maximal adsorption occurs when surface and proteins possess opposite net charges, i.e. at a pH between the isoelectric points (pI) of the surface and the protein (129). Conformational changes in protein structure have also been suggested as a potential driving mechanism for protein absorption, especially under circumstances when hydrophobic interaction and electrostatic attraction is not present. However, adsorption induced conformational changes have been reported in a variety of proteins and may also affect the biological activity of the protein (131). The effect of flow conditions on protein adsorption has been studied; however, increasing shear does not seem to affect the adsorption process, not even high shear rates of 2700 s^{-1} (134).

Platelet adhesion and activation

Adsorbed proteins on artificial surfaces can be recognized by platelet adhesion receptors, potentially leading to the subsequent adhesion, spreading and activation of the platelet. The natural plasma soluble platelet adhesion and aggregation molecules fibrinogen, fibronectin, vitronectin, immunoglobulins and von Willebrand factor (VWF) are all potential mediators of platelet adhesion and have therefore been thoroughly studied in this aspect (Figure 6 presents a schematic drawing of biomaterial induce platelet adhesion and aggregation). Among these candidates, fibrinogen has been shown to be the key protein in the adhesion process (135, 136), whereas fibronectin, VWF and immunoglobulins only show supporting effects that may be related to platelet activation (137, 138). Furthermore, the amount of adsorbed fibrinogen required to facilitate platelet adhesion is extremely small, ~ 7 ng/cm^2 (136). Platelet adhesion to surface-bound fibrinogen is however intriguing, since the fibrinogen receptor integrin αIIbβ3 is required to be activated into a high affinity state by inside-out signaling to effectively bind fibrinogen in solution (139). This mechanism is however overridden as integrin αIIbβ3 in its "low affinity" state evidently is capable of binding surface adsorbed fibrinogen (140). It is speculated that this is facilitated by a change in the conformation of fibrinogen when adsorbed to surfaces. The kinetics of platelet adhesion to artificial surfaces have been

revealed to be very rapid and initiation may take place in < 5 s for hydrophobic surfaces and <30 s for hydrophilic surfaces (141). However, this is not very surprising with the physiological function of platelets in mind: to rapidly adhere and aggregate at the site of vessel injury. The direct effect of wettability on platelet adhesion and activation has been studied with the use of hydrophilic-to-hydrophobic gradient surfaces, with water contact angles ranging from 40-90°. From this study it was evident that platelets in plasma adhered in increasing numbers with decreasing wettability (142). This is no surprise since fibrinogen is retained to a greater extent by hydrophobic surfaces than by hydrophilic surfaces (126). Prolonged exposure of thrombogenic materials to blood *in vivo* has showed that platelet numbers in blood decrease with surface exposure time. This discovery has been used to measure the actual thrombogenic effect of different biomaterials in animal models (123).

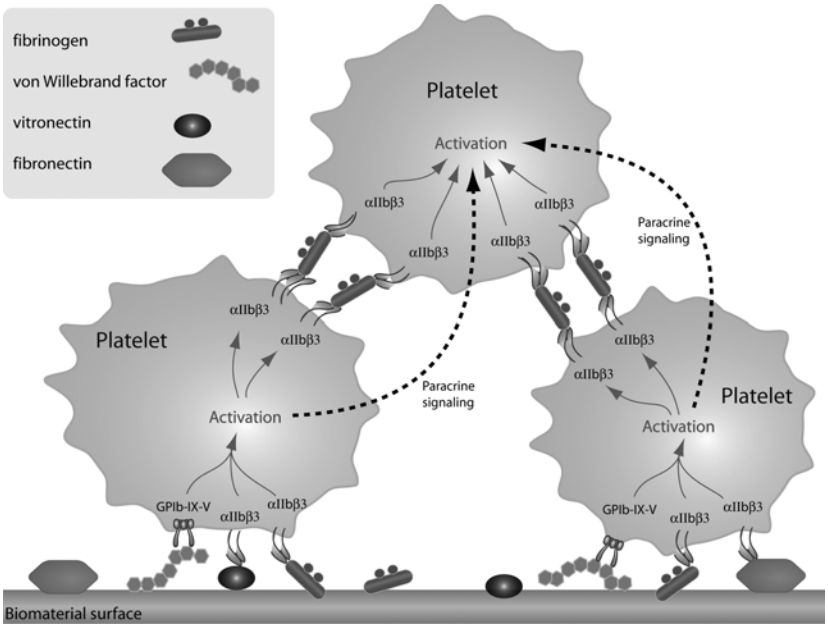


Figure 6. Platelets adhere to artificial surface through interaction with surface adsorbed adhesion molecules like fibrinogen, von Willebrand factor, vitronectin and fibronectin.

Contact activation of plasma coagulation

Although the physiological role of factor XII in haemostasis is debated, as congenital factor XII deficiency does not result in any type of bleeding diathesis (64, 72), it is however clear that factor XII has a great impact on coagulation during the contact between blood and artificial materials. Factor XII activation has for a long time been thought to exclusively undergo autoactivation on surfaces exposing a negative net charge

(anionic surfaces). However, after the recent work of Zhuo et al. (143, 144) it is evident that autoactivation also can occur on hydrophobic surfaces, but only in neat factor XII buffer solutions. It has been hypothesized that factor XII in plasma is competitively excluded from hydrophobic surfaces by other proteins with higher affinity to the surface, a process that has been named "adsorption-dilution effect" (69). Although this was an interesting discovery, it has no real impact on blood compatibility testing of biomaterials under physiological conditions. Blood contact will always entail competitive adsorption between the many plasma proteins, and contact activation of factor XII in plasma will therefore only occur at negatively charged hydrophilic surfaces. The underlying mechanism facilitating the conversion of the inactive zymogen factor XII to the active enzyme factor XIIa on anionic surfaces, independent on other enzymatic activity, is still not very well understood, but it is suggested to be facilitated by conformational changes in the protein structure upon surface adsorption (145, 146). After initiation by factor XII the contact activation is effectively amplified by the presence of prekallikrein and HMWK. Prekallikrein is located at the anionic surface when bound to its surface binding cofactor HMWK. The co-localization of prekallikrein with factor XIIa on the surface facilitates the cleavage of prekallikrein to kallikrein, which then can participate in reciprocal activation of factor XII. The intrinsic pathway is from factor XII propagated downstream by activation of factor XI, which also is colocalized to the surface by HMWK (reviewed in (147)). The mechanisms of contact activation are presented in Figure 7. Contact activation in plasma by a hydrophilic glass surface will lead to fibrin formation in a matter of minutes (see Paper I).

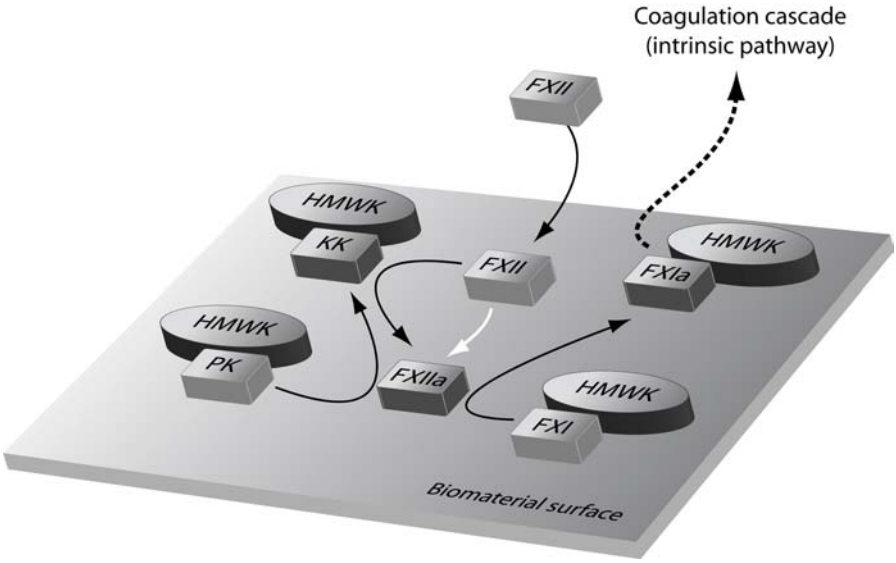


Figure 7. The initiation of the intrinsic pathway through autoactivation of factor XII (contact activation) at a biomaterial surface. Factor XII activation is accelerated by feedback activation of kallikrein. High molecular weight kininogen functions as a cofactor for kallikrein and factor XI by facilitating the binding to surfaces. White arrow indicates autoactivation of factor XII (FXII).

Complement deposition on biomaterial surfaces

Although not the core of this work, it is important to mention biomaterial surface susceptibility to humoral immune complement proteins. According to theory, nucleophilic surfaces, exposing $–NH_2$ (amino groups) or –OH (hydroxyl groups), but not negatively charged surfaces bind complement factor 3b (C3b). C3b in turn binds factors Bb and P forming an active alternative convertase C3bBbP, that subsequently cleaves C3 in solution to C3a and C3b (148). An amplification mechanism is thereby formed. The surface binding of C3b was recently shown to also be mediated by pre-adsorbed proteins (149). In yet another study, it was shown that negatively charged surfaces, such as silica and spontaneously oxidized titanium facilitate C3b deposition (150), leading to the hypothesis that complement binds to denatured proteins on surfaces regardless of surface charge. The alternative complement activation pathway is considered to be of greatest importance, and especially in blood contacting applications where a large area of the biomaterial is in contact with blood, such as cardiopulmonary bypass and dialysis apparatus (148). There are several known connections between the coagulation-, complement- and fibrinolytic systems, although this is outside the scope of this thesis.

In vitro experiments- the absence of vascular endothelium

The onset of platelet activation and plasma coagulation is not only dependent on some external activator. When blood flows in the vascular system it is in constant contact with the endothelium that covers the lumen of the blood vessels. The endothelium is far from a passive component in the haemostatic system and there are several independent mechanisms that attenuate both procoagulant and platelet activities. The endothelium expresses heparan sulfate that facilitate the inhibition of thrombin by antithrombin. Thrombomodulin, a protein involved in the inactivation of factor Va and VIIIa through activation of protein C, is also expressed on the endothelial cell surface. Endothelial cells also synthesize and release tissue factor pathway inhibitor (TFPI), protein S and annexin A5, all participating in anticoagulant mechanisms. In addition, prostacyclin, prostaglandin E_2 and NO are secreted and suppress platelet activation (reviewed in (151, 152)).

Therefore, when blood is collected and intended for use in *in vitro* experiments, it is important to bear in mind that there are many potent inhibitory mechanisms of the haemostatic system that are excluded, and this imbalance will ultimately lead to activation of both platelets and coagulation. This is also an important consideration for the application of prosthetic vascular grafts, where in some cases considerable lengths of the the inside vessel wall, covered with endothelial cells, are replaced with artificial materials.

Chapter 3

3. Aim

The primary aim for this work was to develop relevant methods to study blood – biomaterial interactions in terms of platelet adhesion/aggregation and plasma coagulation.

The secondary aim was to use the developed methods to evaluate different materials that could potentially be used in blood contacting applications.

The newly developed methods also offered an opportunity to study aspects of the haemostatic mechanisms that had not been accessible for in vitro study before. To this end we also developed surfaces that mimicked the surface of the damaged vessel wall.

Chapter 4

4. Materials and Methods

4.1 Blood sampling and plasma preparation

Blood collection and preparation was for the great majority of experiments performed as described below. Blood was drawn from healthy volunteers using a 0.8mm Venoject® needle from Terumo (Leuven, Belgium) and collected in 4.5 mL or 7.5 mL S-Monovette® tubes from Sarstedt (Nümbrecht, Germany), filled with 1/9 of 130 mM sodium citrate. The S-Monovette® sampling tubes have in a previous study proved to generate minimal activation of blood in terms of plasma and whole blood coagulation (153). To acquire platelet-rich plasma (PRP), whole blood was first cooled for 15 minutes at room temperature and then centrifuged at 140×g or 180×g for 20 minutes for 4.5 mL and 7.5 mL tubes, respectively. The platelet-rich supernatant was transferred into another container, where it was stored in room temperature until used in the experiment. Platelet-poor plasma (PPP) was acquired in the same way as PRP but with centrifugation at 2500×g for 20 minutes regardless of tube size. Platelet-free plasma (PFP) was obtained by filtering PPP through a 0.20 μm pore size disposable Minisart® filter from Sartorius (Göttingen, Germany). In experiments where coagulation was required, citrated blood and plasma was recalcified by adding 17.4 mM $CaCl_2$ (final conc.) immediately before the experiment started.

4.2 Imaging of coagulation

A variety of different methods are today available for the study the coagulation process, and although they utilize different principles of detection, most methods measure coagulation as a bulk event without regards to where coagulation was initiated or the kinetics describing the propagation of coagulation from the initiation point. The method 'Imaging of coagulation' that was developed in this project is a coagulation assay that includes a two dimensional spatial parameter in the analysis of the coagulation process and hence, can answer questions regarding where coagulation was initiated and how coagulation propagates. Such questions are highly relevant in both haemostasis research and in the study of blood-biomaterial interactions.

Setup and image acquisition for coagulation studies

The principle of detection in this method is based on the quantitative difference in scattering of incident light off the soluble fibrinogen molecules and the insoluble fibrin network. The insoluble fibrin network scatters incident light extremely well compared to soluble fibrinogen and hence, as the fibrin network starts to form it will become detectable as an increased intensity in scattered light. As artificial surfaces can activate coagulation through contact activation (described in the previous chapter), it was of great necessity to find a suitable transparent vehicle in which the coagulation process could be studied without risking interference from surface-induced contact activation at the inner walls of the vehicle. Disposable PMMA spectrophotometry cuvettes from Kartell (Noviglio, Italy) were found to meet these criteria and could therefore successfully be used in the method. PMMA had in a previous study been found to be a rather inter material in terms of contact activation (124). Depending on the study, different surfaces were fitted vertically along one side of the PMMA cuvette (see Figure 8, left), making the surface induced coagulation observable as a process starting from the surface followed by a horizontal propagation phase across the cuvette. To facilitate studies of multiple cuvettes simultaneously, a cuvette holder which carried four cuvettes was developed. This was of great importance for the efficiency of the method as it allowed a higher throughput of experiments and made comparative measurements more accurate. The cuvette holder was fitted with a white light LED illumination array, providing stable illumination of the cuvttes from below (see Figure 8, right).

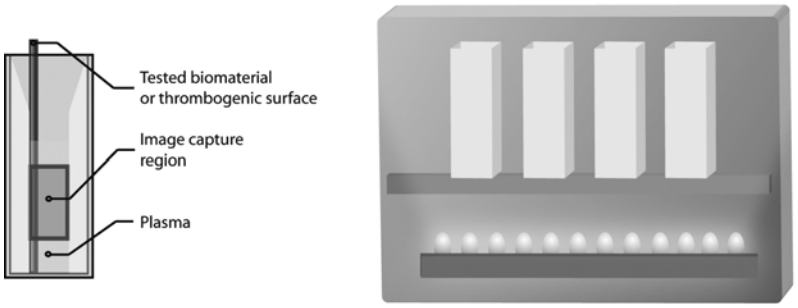


Figure 8. To the left: Experimental surface in PMMA cuvette. To the right: cuvette holder for four cuvettes fitted with a white light LED illumination array, providing illumination of the cuvttes from below.

The image capture system consisted of a high resolution EOS 400 digital SLR camera from Canon (Tokyo, Japan), delivering images with a resolution of up to 3888 x 2592 pixels.* The camera was controlled by EOS Utility (Canon), a camera bundled time lapse software. The spatial resolution in the experiments with four simultaneously captured cuvettes was for this camera ~ 50 μm. The experiment was usually started by the addition of recalcified PRP or PFP to the cuvette, immediately followed by the start of time lapse image acquisition. For most experiments images were captured with an interval of 15 s. Depending on coagulation rate such experiment usually generated between 200 and 800 captured images, i.e. 50 to 200 minutes. Captured images were directly transferred to a PC for further analysis.

Setup for AMC enzyme substrate measurements

Minor modification of the imaging setup made it possible to measure surface-induced enzyme activity by the use of 7-Amido-4-methylcoumarin (AMC) fluorogenic substrates. The slight modification entailed an addition of a UV-LED (370 nm) cartridge that was inserted into one of the four cuvette fittings. The UV-LED illuminated the sample cuvette from the side, but due to the limited intensity of the UV-LED it was only possible to run one sample at the time. The digital camera was fitted with a 400 nm long pass filter. Both factor XIIa sensitive substrate Boc-Gln-Gly-Arg-AMC (in Paper IV) and thrombin sensitive substrate Z-Gly-Gly-Arg-AMC was used successfully in this modified setup.

Image data processing

In order to extract useful information from the captured image data, several software functions were written in Matlab® (v. 7.2) from The Mathworks Inc. (Natick, USA) that in sequential steps processed and analyzed the image data. A flowchart of the data processing functions are presented in Figure 9. The initial step in the analytical process was to use a function that merged the images into continuous video sequences. Individual video sequences were rendered for each cuvette, only containing the region of interest for further analysis. This process made the subsequent handling and analysis of image data considerable faster and allowed data storage to be more efficient. The video rendering function also created a separate file containing information about the time points for each image capture. The image capture time points were required for subsequent analysis.

* image capture was performed with a Powershot S3 IS (Canon, Japan) in a previous version of the setup (used in Paper I and Paper III).

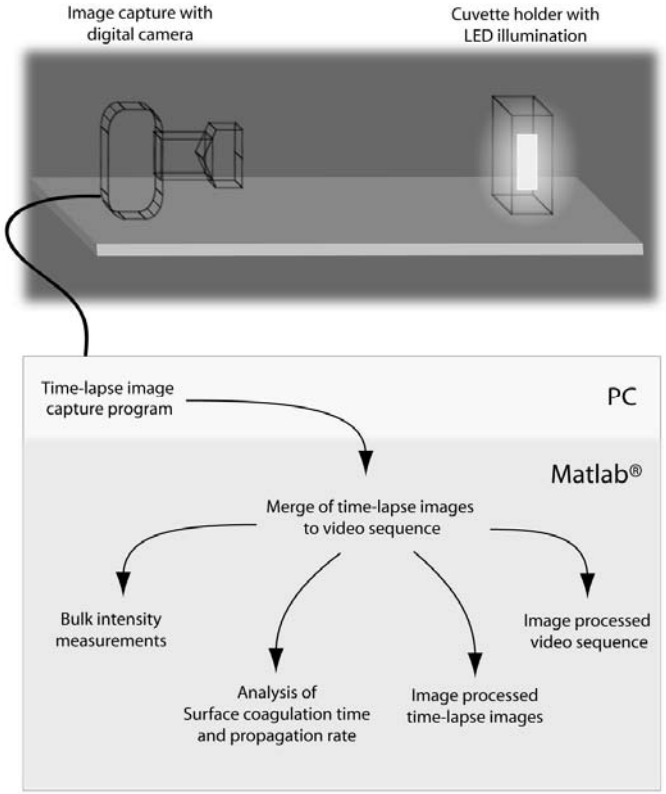


Figure 9. Flowchart of the image data processing steps.

Bulk coagulation measurements

This function was designed to measure the coagulation process in a user specified region of the video image. The average intensity in the specified region was calculated for each video frame in the sequence, which was presented in the function as a graph of intensity versus time. The function could be executed in three different modes, depending on the circumstantial requirements. These modes included absolute measurement, absolute measurement with baseline subtraction, and relative measurement. The absolute measurements made use of the direct measured intensities while the relative measurement were standardized to always range between 0 and 100%. This function was used in Paper I to determine the detection level for coagulation in the imaging setup. This was accomplished by comparing coagulation times detected by a ReoRox4 rheometer from Medirox (Nyköping, Sweden) with the corresponding intensity in the imaging setup. The ReoRox4 measures coagulation by detection of viscoelastic changes in plasma during coagulation. The ReoRox4 has in turn been calibrated against the reference method visual inspection of tilting tube in water-bath technique (154). From the results of these

comparative measurements it was concluded that an intensity increase of 40% correlated well with the coagulation times determined with the ReoRox4. Detection of coagulation times in the imaging of coagulation setup are illustrated in Figure 10. The bulk intensity function was also used in Paper IV to measure AMC fluorescence from the cleavage of the factor XIIa sensitive substrate.

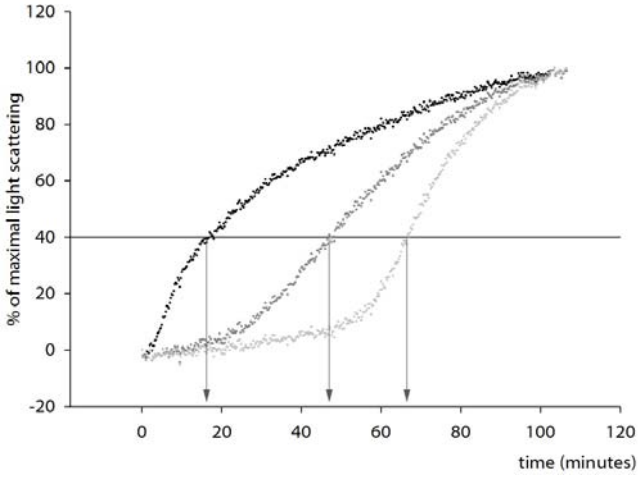


Figure 10. Relative light scattering intensity at different locations in the cuvette during a coagulation experiment in PFP. Black dots: close to the initiating tissue factor coated surface, gray dots: middle of cuvette, light gray dots: at the opposite side of the cuvette from the initiating surface. Vertical arrows indicate the corresponding coagulation times (at 40 % of maximal intensity) for the three measured regions.

Surface coagulation time and propagation of coagulation

The most frequently used function for analysis of the video data was a function to determine the initial coagulation at the surface and the rate with which coagulation propagated out from the surface. The main principle of the function was to calculate individual coagulation times for each pixel in the video image. The coagulation time closest to the surface, termed 'surface coagulation time', was calculated as the mean coagulation time in the column of pixels adjacent to the surface. Propagation rates were calculated in two steps. The first step calculates the mean coagulation time in consecutive pixel columns stepwise progressing out from the surface. The result from this calculation is a propagation profile, i.e. a graph with 'coagulation time' as a function of 'distance from surface' (for example see Figure 11). Such graphs offer good insight in the propagation phase of coagulation but are difficult to compare quantitatively, therefore a second calculation step was introduced, in which linear regression was applied to short intervals (usually 0.25 mm) of the propagation profile. The propagation rate (mm/min) was then calculated as the reciprocal slope from the linear regression. It should be noted that the

calculations of propagation rates were dependent on a user defined length scale in the image. This was accomplished by setting a reference distance in the image equal to the defined width of the the cuvette. This function has been used to analyze image data in all enclosed papers (Paper I-V). In order to obtain correct measures of the propagation rate in PRP it was necessary to inhibit clot retraction during the experiment. This was accomplished by inhibiting the contractile function of the actin cytoskeleton by the addition of cytochalasin E (Paper I) or by inhibiting fibrinogen binding to integrin αIIbβ3 by abciximab (Paper V).

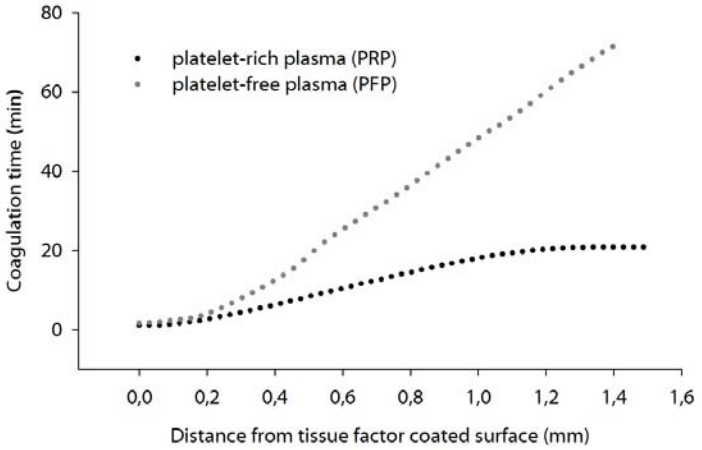


Figure 11. Propagation profiles of the coagulation process in PRP and PFP, initiated by tissue factor coated surfaces.

Visualization of coagulation by image processing

For presentation and publication purposes, two functions were written to facilitate the visual enhancement of the coagulation process. One function generated image processed time lapse images and the other function rendered image processed video sequences of either single or dual cuvettes. Both functions utilized the same image processing steps (visualized in Figure 12 and 13). The basic principle for the image enhancement was to subtract the background (first image frame) from all video frames in the sequence, and as a second step, to convert the images into grayscale and apply a user defined color map. The color map was used to visually enhance the range of different intensities presented in the image, except for those individuals suffering from color blindness. The image processing step was not strictly necessary for all experiments. In PFP, the initial light scattering is very low and thus coagulation in PFP yields intensity signals of considerable magnitude. In PRP however, the platelet will contribute with a great amount of initial

scattering, which will lead to lower contrast in the images. Image enhancement was therefore required and also used in PFP experiments to maintain consistency. To visualize standard coagulation experiments it was desirable to generate 4 to 10 time lapse images or a video sequence of approximately 30 s.

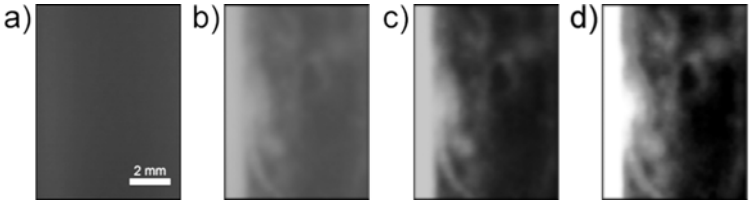


Figure 12. Image processing steps for enhanced visualization of the coagulation process. The coagulation experiment in PRP was initiated by a hydrophilic glass surface. Raw captured image frames at a) 0 min and b) 40 min. c) Image after background subtraction and, d) applied color map to image c).

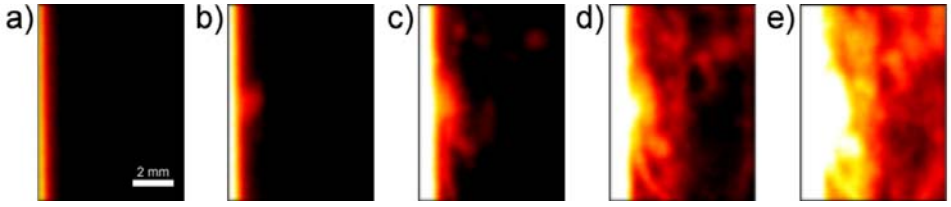


Figure 13. Time-lapse images after image enhancement; visualizing the coagulation process in PRP. Coagulation was initiated by a hydrophilic glass surface. Images after a) 10, b) 20, c) 30, d) 40 and e) 50 minutes.

4.3 Cone-and-plate flow model

Platelet adhesion and aggregation are events that *in vivo* always proceed under flow conditions. Blood is always in a flowing state in the body and this will generate a high degree of contact between circulating platelets and vessel walls, or in the case of biomaterial implantation, the biomaterial surface. Therefore, when studying platelet adhesion and aggregation *in vitro*, it is important to simulate these conditions as accurately as possible.

During the course of this work we developed a cone-and-plate flow model in which blood flow is generated by a rotating cone in very close proximity to an experimental surface. One of the main advantages with this type of flow model is that it requires very small blood volumes (80-200 μL) for standard experiments. Considerably larger blood volumes are required for experiments performed in the commonly used parallel plate flow chamber (155). Hence, more experiments can be performed in the cone-and-plate flow

model with blood from a single donation, which is of importance for comparative measurements. Also, there a no need for cleaning or exchanging tubes in between experiments, which allows for a high throughput. The main disadvantage with the cone-and-plate flow model is that the same blood is circulating over the surface throughout the duration of the experiment, and hence no dilution of activated cells or enzymes is present as it would be in *in vivo* conditions or in a parallel plate flow model.

Cone-and-plate setup

The setup consisted of a fixed RW 20 digital controlled motor from IKA (Staufen, Germany) with an attached aluminum cone (constructed in-house). The cone has a horizontal contact angle of 5° and is during experiments coated with Parafilm® M to avoid cleaning of the cone between experiments. Rotational speed of the cone could be adjustable in the range 60-2000 rpm. The support for the experimental surface is comprised of a z-axis adjustable stage equipped with an outlet connected to a vacuum pump. The outlet is located directly beneath the cone and the experimental surface is placed on an O-ring on top of the vacuum outlet to secure the surface to the stage. A drop of blood (80-200 μL) is placed on the experimental surface and the stage is raised so that the surface is within close proximity of the tip of the cone (Figure 14). Thereafter the experiment was immediately started by initiating cone rotation.

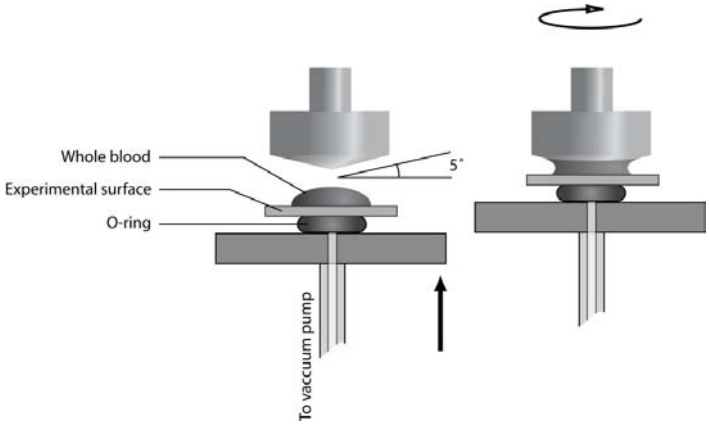


Figure 14. Schematic drawing of the cone-and-plate flow model setup. The experimental surface is fixed on an O-ring that interfaces with an outlet connected to a vacuum pump. The stage with the experimental surface is elevated within close proximity of the cone. Rotation of the cone will generate whole blood flow on the surface.

Shear rate

The shear rate in a liquid can be described by a linear velocity gradient between a stationary surface and parallel surface at specified distance *(d)* moving at a constant velocity *(v)*. The design of the cone-and-plate entails that under ideal conditions the shear rate is equal over the entire experimental surface (156) at a specific angular velocity *(ω)*, given by:

$$\dot{\gamma} = \frac{v}{d} = \frac{r\,\omega}{r\,\tan\alpha} = \frac{\omega}{\tan\alpha} = \frac{2\pi f}{\tan\alpha}$$

f is the frequency (revolutions per second).

The ideal conditions require that there is no gap between cone and surface. However, this is not feasible in reality since the experimental surface would be damaged by the cone and the cellular components of blood would be lysed due to mechanical stress in the contact zone. Therefore, the additional distance of the gap *(h)* should also be included in the equation, and the new relationship is given by:

$$\dot{\gamma} = \frac{v}{d} = \frac{r\,\omega}{h + r\,\tan\alpha} = \frac{\omega}{\frac{h}{r} + \tan\alpha} = \frac{2\pi f}{\frac{h}{r} + \tan\alpha}$$

The resulting equation is however not independent of the radial distance from the cone center, and shear rates will therefore decrease towards zero at the absolute center. Also, shear rates will be lower over the entire surface and dependent on both the gap between cone and surface, and the radial distance (see Figure 15). In the described setup the gap has been measured and was found not to exceed 0.1 mm. With regard to this, it should be noted that the shear rate generated by this setup is not exact, and reproducible results are best acquired by limiting measurements to the area that is further out than 3 mm from the cone center.

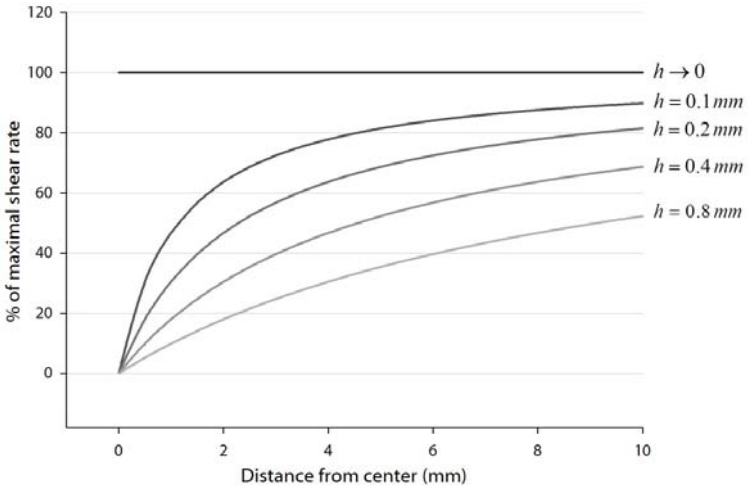


Figure 15. The influence of the gap *(h)* on shear rate, presented as percent of shear rate produced by the ideal cone-and-plate setup where *h*=0. In reality *h* is in the range 0.05-0.10 mm.

Applications of the cone-and-plate flow model

The cone-and-plate setup has mainly been used to study platelet adhesion from citrated whole blood. Usually 100 µL blood was applied to the surface and the cone was rotated to generate shear rates of 100s^{-1} or 1000-1500 s^{-1}, simulating low and high shear conditions respectively. Figure 16 shows a representative experiment performed on hydrophobic and hydrophilic polystyrene at a shear rate of 1000 s^{-1}. Platelet adhesion experiments were performed on hydrogel coatings proposed for biomaterial applications in Paper III. The cone-and-plate setup was also used to test surface integrity; in Paper II where a glycerol monooleate coating was tested at various shear rates to estimate the reduction in coating thickness shearing induced.

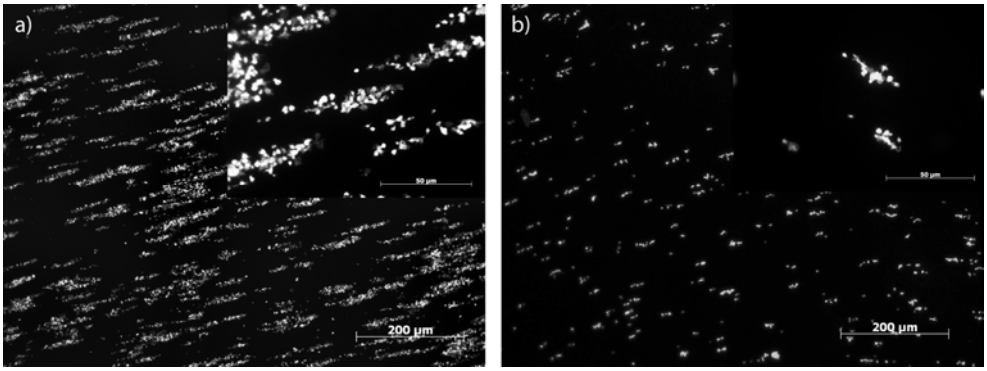


Figure 16. Platelet adhesion on a) hydrophobic polystyrene and b) hydrophilic polystyrene after exposure to flowing whole blood for two minutes at a shear rate of 1000 s^{-1}. Platelets were stained with Alexa Fluor® 546 phalloidin.

We also utilized the cone-and-plate in the development of an *in vitro* thrombus formation assay. As the cone-and-plate setup is an 'open' flow model, we could allow coagulation to proceed without risking that any pump, tubing or flow chamber would be blocked by coagulated blood. By using reacalcified whole blood and surfaces with thrombogenic coating we successfully induced thrombus formation on the coated surfaces. This method was used in Paper V.

The standard procedure for evaluating platelet adhesion/aggregation or thrombus formation in the cone-and-plate setup was by the use of a fluorescence staining and image capture in a fluorescence microscope. Following exposure to blood flow in the cone and plate setup platelets were stained either with the cytosolic stain Celltracker® Green (5-chloromethylfluorescein diacetate) or the F-actin stain Alexa Fluor® 546 phalloidin. After thrombus formation experiments, additional staining was performed to visualize fibrin network (anti-fibrinogen [FITC]), white blood cell nucleus [DAPI] and red blood cells (anti-glycophorin C [Alexa Fluor® 647]).

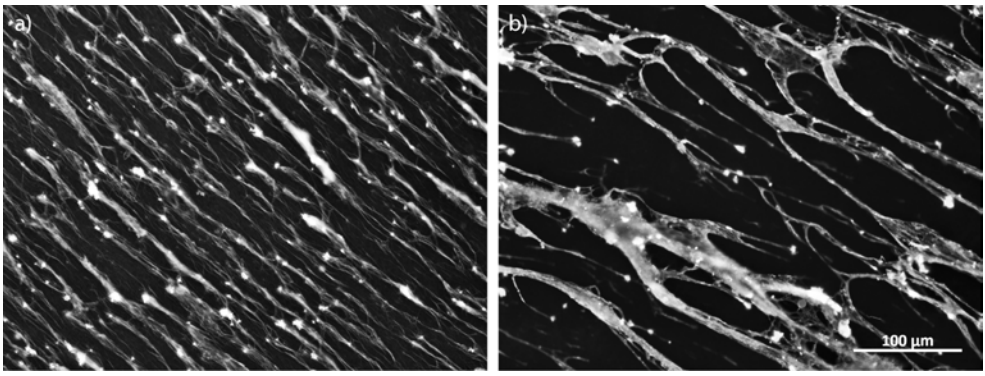


Figure 17. *In vitro* thrombus formation experiments performed on a surface with thrombogenic coating consisting of tissue factor, fibrinogen and von Willebrand factor. The surfaces were exposed to recalcified whole blood at a shear rate of a) 100 s^{-1} and b) 1500 s^{-1} respectively. Fibrin network structure differs greatly between the two shear rates. Platelets are visible as brighter aggregates. Platelets were stained with Alexa Fluor® 546 phalloidin, fibrin network with anti-fibrinogen [FITC], white blood cell nuclei with DAPI and red blood cells with anti-glycophorin C [Alexa Fluor® 647].

To obtain quantitative data from the captured microscopy images, a function in Matlab® was written that performed analysis of platelet surface coverage as well as size distribution and quantity of objects in the images.

4.4 Fluorometric methods

Fluorogenic measurements of factor XIIa and thrombin activity were useful tools to quantitatively analyze the dynamics of contact activation and thrombin generation. The experiments were carried out in 96-well plates which allowed multiple samples to be analyzed simultaneously and in addition only required small sample volumes (~ 100 µL). The enzymatic activity was measured from the cleavage of peptide conjugated 7-Amido-4-methylcoumarin (AMC). The AMC molecule will exhibit fluorescent properties when the amide bond to the peptide is cleaved (excitation: 380 nm, emission: 460 nm) (157, 158). Boc-Gln-Gly-Arg-AMC was used in Paper V for measuring factor XIIa activity (159) and the substrate Z-Gly-Gly-Arg-AMC was used in Paper V for measuring thrombin activity (160). Measurements were performed in a Fluoroskan Ascent plate reader (Thermo Electron Corporation, Waltham, MA). The fluorogenic substrate measurements are compatible with both PFP and PRP which makes it a valid method for investigating platelet dependent contribution to contact activation and thrombin generation. Calibrated thrombin generation was performed and analyzed with Thrombinoscope® reagents and analysis program from Thrombinoscope BV (Maastricht, The Netherlands). The Thrombinoscope® software performs advanced analysis of the thrombin generation kinetics and calculates several useful parameters (160). However, the analysis program was only compatible with experiments where thrombin generation was initiated by strong activators, rapidly generating massive amounts of thrombin. In experiments where strong activators were absent, the magnitude of thrombin generation

was estimated in the sample by calculating the mean fluorescence intensity increase rate over a specific time period, usually 30 or 60 minutes. Factor XIIa generation was always evaluated by measuring the mean fluorescence intensity increase rate. It should be noted that we found the Boc-Gln-Gly-Arg-AMC fluorogenic substrate not to be entirely specific to factor XII and the substrate could also be cleaved by thrombin. This could however be circumvented by restricting the use of the Boc-Gln-Gly-Arg-AMC substrate to experiments with citrated plasma.

4.5 Additional methods

Free-oscillation rheometry (FOR) was performed with the ReoRox4 from Medirox (Nyköping, Sweden). The ReoRox4 is an instrument that can detect coagulation in whole blood and plasma samples by measuring changes in viscoelasticity. This method was used in Paper I to correlate coagulation times in plasma detected by the ReoRox4 to recorded intensity changes in the method for imaging of coagulation. The method was also used in Paper II and Paper V to measure whole blood coagulation.

Flow cytometry is a method to quantitatively measure cell surface antigens by the use of fluorescently labeled antibodies. The cells are suspended in liquid and pushed through a micro capillary where bound fluorophores are excited by laser radiation and the emission measured by photomultiplier tubes. The narrow capillary forces the cells to pass through the exciting laser one by one, and a fluorescence signal from each single cell can be recorded. Flow cytometry was used in Paper V to measure platelet bound factor XI and XII.

Several methods for surface characterization were also used; contact angle goniometry, scanning electron microscopy (SEM), ellipsometry, fourier transform infrared spectroscopy (FT-IR), fluorescence recovery after photobleaching (FRAP) and quartz crystal microbalance with dissipation (QCM-D). However, describing these techniques in detail would fall outside the scope of the thesis.

Chapter 5

5. Summary of Papers

Presented in the following section are short summaries of the methodology, results and main conclusions from the included papers.

5.1 Paper I

Imaging of blood plasma coagulation and its propagation at surfaces

Lars Faxälv, Pentti Tengvall, Tomas L. Lindahl

The purpose of this paper was to present the novel method for imaging of coagulation. We also wanted to evaluate its performance and reproducibility in both platelet-rich plasma (PRP) and platelet-free plasma (PFP). To achieve this, we used the method to analyze two well-characterized surfaces; hydrophilic and hydrophobic glass.

Analytic procedure

Hydrophilic glass surfaces were prepared by incubating cut microscopy slides in 70% ethanol with 0.35 M HCl. Hydrophobic glass surfaces were prepared by incubating cleaned hydrophilic glass in a solution of 1% dichloro dimethylsilane ($(CH_3)_2SiCl_2$) in xylene for 5 minutes. The coagulation process at the hydrophilic and hydrophobic glass was thereafter captured in the imaging of coagulation setup. Four replicates were simultaneously run for both PRP and PFP from a single donor. In total, plasma from four different donors was used. The coagulation process in each cuvette was analyzed with regard to surface coagulation time and propagation rate of coagulation.

Figure 18. Hydrophilic glass was obtained by incubating microscopy slides in a solution of 70% ethanol with 0.35 M HCl overnight. This rendered the glass surface highly hydrophilic with an advancing contact angle of < 10°. The hydrophobic surfaces were prepared by incubating hydrophilic glass in a 1% solution of dichloro dimethylsilane in xylene for 5 minutes. The glass surfaces were after subsequent washing in ethanol and water extremely hydrophobic with an advancing contact angle of ~100°.

Results

The hydrophilic surfaces activated coagulation rapidly and the coagulation propagated in a well-defined manner from the surfaces. Hydrophobic surfaces were extremely inert, resulting in very long surface coagulation times that in addition were subject to large variations. Furthermore, the propagation phase at the hydrophobic surfaces could not be analyzed successfully since the long initiation time resulted in spontaneous coagulation throughout the cuvette.

Conclusions

Imaging of coagulation is a method well suited for measuring the onset of coagulation at any type of surface, although, surfaces that are poor activators of coagulation will generate large variations in the detected surface coagulation time. However, these large variations are most certainly not related to this specific method. Analysis regarding propagation of coagulation is only suitable for surfaces that activate coagulation rapidly.

5.2 Paper II

Glycerol Monooleate - Blood Interactions

Emma M. Ericsson, Lars Faxälv, Anna Weissenrieder, Agneta Askendal, Tomas L. Lindahl and Pentti Tengvall

In this work we tested the haemocompatibility of glycerol monooleate (GMO), a proposed substance for use in biomaterial applications.

Analytical procedure

GMO is in room temperature a greasy substance and was therefore coated on a matrix composed of cross-linked human serum albumin (HSA) for analysis of protein adsorption, stability under shear, and surface initiated coagulation. Hemolytic activity and effects on whole blood coagulation with free-oscillation rheometry was performed by solving GMO in dimethyl sulfoxide (DMSO).

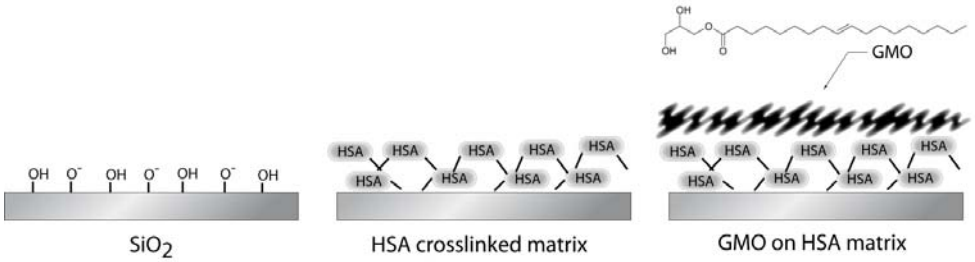


Figure 19. Glycerol monooleate (GMO) was coated onto a matrix of crosslinked human serum albumin (HSA). The crosslinked HSA matrix was attached to a silicon substrate. The silicon substrate, the HSA matrix and the GMO coating were all tested in the imaging of coagulation and cone-and-plate setup.

Results

It was found that GMO did not activate coagulation to any great extent either in plasma or in whole blood. GMO did however induce some hemolysis in whole blood. When GMO coated surfaces were exposed to phosphate buffer or whole blood a considerable amount of the GMO was removed. However, 75 Å of GMO coating seemed to be firmly attached to the HSA matrix and could not be removed in neither stagnant nor shear conditions.

Conclusions

GMO is a more or less haemocompatible substance. However, thick coatings seem to dissolve in contact with blood and the substance can therefore only be recommended for extravascular biomaterial applications.

5.3 Paper III

Blood compatibility of photografted hydrogel coatings

Lars Faxälv, Tobias Ekblad, Bo Liedberg, Tomas L. Lindahl

PEG-containing hydrogels have demonstrated excellent protein and cell resistant properties (125, 161, 162) and have therefore been widely proposed for biomaterial applications. In this paper we investigated the haemocompatible properties of PEG-containing hydrogels and compared them with hydrogels constructed from three different non-PEG-containing monomers.

Analytical procedure

Thin hydrogel coatings were produced on polystyrene (PS) substrates by self-initiated photografting and photopolymerization (SIPGP) (163). The monomers 2-hydroxyethyl methacrylate (HEMA), poly(ethylene glycol) methacrylate (PEGMA), ethylene glycol methyl ether methacrylate (EGMEMA), 2-carboxyethyl acrylate (CEA) or a mixture of HEMA and PEGMA were used for hydrogel grafting. A hydrophilic radio frequency plasma treated PS [PS(RF)]surface was also included in the test. The PS surfaces and the hydrogel coatings were thoroughly characterized by water contact angle goniometry, infrared spectroscopy (FT-IR) and ellipsometry. The haemocompatible properties were determined by protein adsorption, platelet adhesion in stagnant and shear conditions (utilizing the cone-and-plate setup) and coagulation assay by the use of the imaging of coagulation setup.

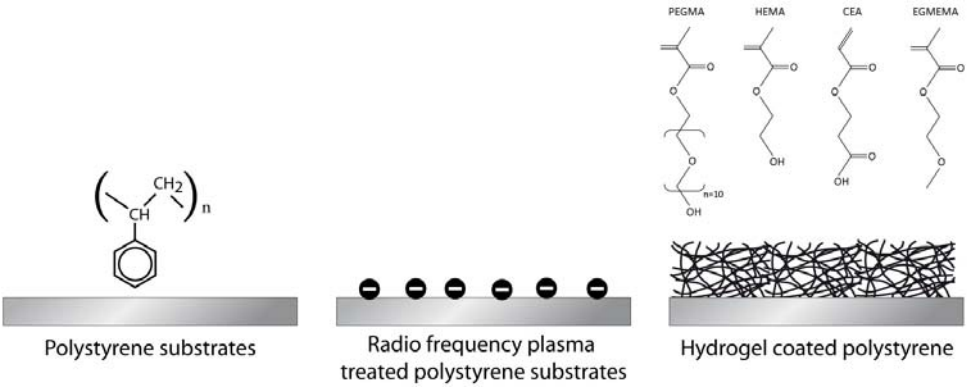


Figure 20. Hydrogel coatings for biomaterial applications were produced by UV-photografting on polystyrene substrates. The hydrogels used in this work were grafted from the monomers 2-hydroxyethyl methacrylate (HEMA), poly(ethylene glycol) methacrylate (PEGMA), ethylene glycol methyl ether methacrylate (EGMEMA), 2-carboxyethyl acrylate (CEA) or a mixture of HEMA and PEGMA. A hydrophilic radio frequency plasma treated PS [PS(RF)]surface was also included.

Results

Protein adsorption measurements revealed that large amounts of fibrinogen adsorbed on the PS and PS(RF) surfaces. High molecular weight kininogen was specifically adsorbed on the PS(RF) surface. The hydrogels produced from EGMEMA and CEA also adsorbed detectable amounts of protein, but the specific protein type could not be determined. Hydrogels containing PEGMA and/or HEMA did not facilitate any detectable protein adsorption. Platelets adhered readily to PS and PS(RF) surfaces but only in minute quantities to EGMEMA and CEA. Hydrogels containing PEGMA and/or HEMA were highly resistant towards platelet adhesion. Neither the PS substrate nor the hydrogel coatings activated coagulation to any great extent. There was an indication that the negatively charged hydrogels produced from CEA had a reduced surface coagulation time. The hydrophilic PS(RF) surface was found to be a strong activator of coagulation.

Conclusions

It can be concluded that all the grafted hydrogels demonstrated good haemocompatibility and particularly those produced from the monomers PEGMA and/or HEMA. Interestingly, hydrogels produced from HEMA has in previous studies facilitated platelet adhesion (164, 165), and we therefore hypothesize that part of the haemocompatibility can be attributed to the SIPGP grating technique. In summary, we can recommend the use of PEGMA and HEMA containing hydrogels grafted by the SIPGP method for blood contacting biomaterial applications.

5.4 Paper IV

Activation of blood coagulation at charged supported lipid membranes

Lars Faxälv, Jasmin Hume, Tomas L. Lindahl, Bengt Kasemo, Sofia Svedhem

The purpose of this work was to investigate the relationship between surface charge of phospholipid membranes and initiation and propagation of coagulation. Also, we wanted to demonstrate that coagulation at phospholipid membranes could successfully be studied in the method for imaging of coagulation.

Analytical procedure

Supported phospholipid membranes were formed from palmitoyl-oleoyl-glycero-3-ethyl-phosphocholine (POEPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS) on silicon substrates. The surface charge of the phospholipid membranes was controlled by using different compositions of POPS (negative net charge), POPC (~ zero net charge) and POEPC (positive net charge). Imaging of coagulation experiments were performed on all phospholipid membrane coated surfaces as well as the clean silicon substrate. The experiments were performed in platelet-free plasma (PFP) diluted 1:1 with phosphate buffered saline (PBS).

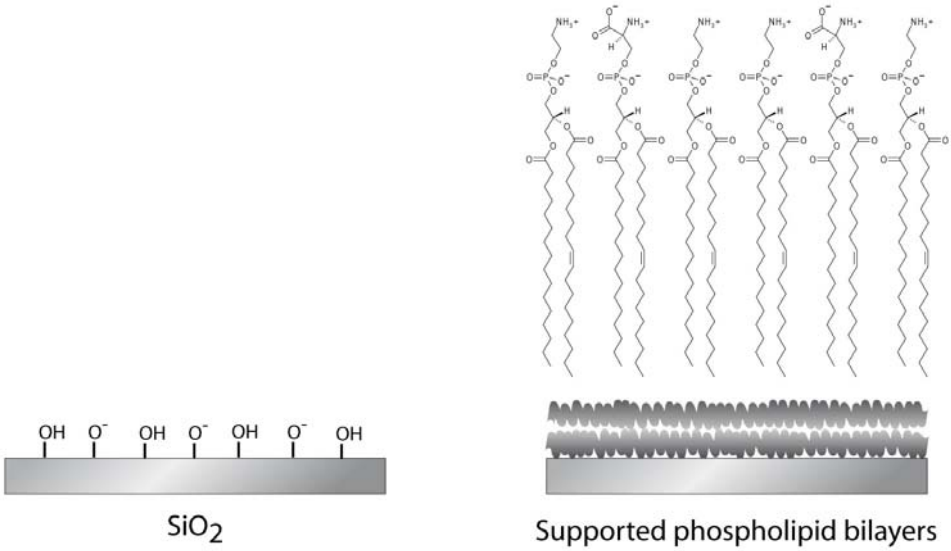


Figure 21. Silicon substrates were coated with phospholipid membranes. The membranes were produced with different compositions of palmitoyl-oleoyl-glycero-3-ethyl-phosphocholine (POEPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS). The surfaces above demonstrates silicon substrate (left) and a POPS(30%)/POPC(70%) (right) supported bilayer.

Results

In the comparison of the negatively charged SiO_2 surface with the negatively charged POPS (30%)/POPC(70%) we found an interesting difference. Although both surfaces activated coagulation rapidly, the POPS surface facilitated a faster propagation of coagulation from the surface than the SiO_2 surface. It was also found that in order for the phospholipid membranes to exert procoagulant properties, the POPS content in the membrane had to exceed ~6 %. It was also found that positively charged phospholipid membranes did not induce activation of coagulation.

Conclusions

The work in this paper demonstrated that the coagulation process at phospholipid membranes can be studied in a straightforward manner using the imaging of coagulation setup. Furthermore, we speculate that the negatively charged phospholipid membranes but not the SiO_2 surface can support the binding of coagulation factor complexes, thus facilitating a faster propagation of coagulation. The fact that the POPS content must exceed ~ 6% to fully exert procoagulant properties was also a very interesting result, especially since platelets, when activated, become procoagulant by increasing their negatively charged phosphatidylserine exposure from ~0 % to maximally ~10 % (101-104).

5.5 Paper V

The role of coagulation factor XII in propagation of coagulation

Lars Faxälv, Sofia Ramström, Kristina Soutukorva, Pentti Tengvall, Tomas L. Lindahl

The aim of this paper was to elucidate the physiological role (if any) of factor XII in human haemostasis and thrombosis. The role of factor XII has for a long time been perused since, intriguingly, individuals lacking factor XII can lead seemingly healthy lives without any bleeding problems (64, 72). The topic has recently gained renewed interest after Renne et al. found that mice deficient in factor XII do not have normal thrombus formation (82).

Analytical procedure

Flow cytometry was used to detect factor XI and factor XII on the surface of resting and activated platelets. To determine if any endogenous surface associated with ongoing thrombus formation could activate factor XII, platelets as well as clots produced from platelet-free plasma, platelet-rich plasma and whole blood were tested for potential factor XII activation. Imaging of coagulation and *in vitro* thrombus formation experiments were performed in order to determine if factor XII was involved in the initiation or propagation phases of normal haemostasis. In these experiments coagulation and thrombus formation were initiated by surfaces with thrombogenic coatings; tissue factor/phospholipid coating for imaging of coagulation and tissue factor/phospholipid, fibrinogen and von Willebrand factor coating for *in vitro* thrombus formation. The effect of factor XII activation in the different experiments was determined through inhibition of factor XIIa by corn trypsin inhibitor (CTI) (166).

Figure 22. Thrombogenic coating of surfaces intended for use in the imaging of coagulation setup consisted of a tissue factor (TF)/phospholipids coating on top of hydrophobic glass. The thrombogenic coating for the cone-and-plate based thrombus formation model consisted of tissue factor (TF)/phospholipids and the adhesive molecules fibrinogen and von Willebrand factor.

Results

It was clear that both factor XI and XII bound to the surface of activated platelets, however, neither activated platelets nor the surface of any coagulum could activate factor XII to any great extent. However, analysis of propagation rates from imaging of coagulation experiments revealed that inhibition of factor XII resulted in a platelet dependent decrease in propagation rate. Also, in presence of a strong contact activating agent (in this case the hydrophilic SiO_2 surface), the propagation of coagulation at a tissue factor coated surface was greatly enhanced. Inhibition of factor XII had a moderate attenuating effect on fibrin network formation in the thrombus formation model, but only during low shear conditions.

Conclusions

We found that factor XI and XII both bind to activated platelets and that this binding most probably enhances the propagation of the intrinsic pathway of coagulation. Furthermore, we also found that tissue factor initiated coagulation could be greatly accelerated by the presence of contact activating agents. This could be of importance in biomaterial applications as contact activation on the biomaterial can, not only induce thrombus formation on the actual biomaterial surface, but also boost ongoing tissue factor initiated thrombus formation at other locations in the vascular system.

Chapter 6

6. Concluding remarks

During the course of this work we developed the method for imaging of coagulation. The development process was at first proceeding very fast, rapidly generating a number of interesting results that we planned to investigate in the near future. However, the results also disclosed several problems with the initial setup that needed to be corrected, and this work took a substantial amount of time and effort. In addition, several software functions were also written to efficiently handle and analyze the large amount of image data that was generated by the method. When finally all these improvements had been adopted, the method performed very well and the data could be analyzed and presented in a number of different ways.

The main advantage with the imaging of coagulation setup is that the coagulation process can be followed with both time and spatial distribution, whereas most conventional method are restricted to measuring coagulation as a bulk process. With this feature it is possible to determine both where and when coagulation is initiated, as well as analyzing the kinetics of the propagation phase. The coagulation event can thus be followed all the way from initial activation to the final clotting of the whole sample.

After the completed development of the imaging of coagulation setup we learned that a system with the same principle of detection had previously been constructed in the group of F.I. Ataullakhanov (167-169). Their setup did however differ significantly from ours allowing only one single plasma sample experiment to be performed at a time. Also, they used polystyrene (PS) for their experimental chamber, which induce more contact activation than our PMMA cuvettes (which was also the reason why we chose PMMA cuvettes instead of PS cuvettes).

It should be pointed out that although the imaging of coagulation setup can detect coagulation directly at surfaces, it is not a surface sensitive technique per se, unlike surface plasmon resonance (SPR) or quartz crystal microbalance with dissipation (QCM-D) which previously have been applied to coagulation studies (124, 170). It is reasonable to assume that such surface sensitive methods have a greater sensitivity for the initial fibrin deposition on the surface. However, such methods have other drawbacks like a relatively short sensing distance and limitations in the type of experimental surfaces that can be

used. Imaging of coagulation is in this regard a superior method since propagation can be measured over long distances and the setup is very flexible, allowing almost any type of material or experimental surface to be studied, providing it will fit in a standard spectrophotometry cuvette. These properties make the imaging of coagulation setup a perfect tool for evaluating the blood compatibility of biomaterials.

Tack

Det är många som förtjänar ett stort tack för att ha stöttat och hjälpt mig i arbetet som har lett fram till denna avhandling.

Först och främst vill jag tacka min huvudhandledare ***Tomas Lindahl*** för ett stort stöd och engagemang under hela doktorandtiden. ***Tomas*** har hjälp mig förverkliga många idéer på labbet och har alltid tagit sig tid att dela med sig av sin stora kunskap om hemostasens alla funktioner och mekanismer.

Även ett stort tack till min biträdande handledare ***Pentti Tengvall*** för att ha bidragit med ett stort kunnande om blodets interaktioner med ytor. Vi har under vägen haft många givande diskussioner där ämnet ofta glidit in på faktor XII, jag hoppas det fortsätter så.

Jag vill tacka alla trevliga och hjälpsamma människor i hemostasgruppen som jag trängts med på labbet: ***Kerstin Gustafsson, Nahreen Tynngård, Majid Osman, Karin Vretenbrant-Öberg, Martina Nylander, Knut Fälker, Roza Chaireti*** och ***Sofia Ramström***. Samt ***Kerstin Arbring*** (som jag trängts med på rummet) och ***Cecilia Jennersjö***.

Ett speciellt tack till ***Kerstin Gustafsson*** som sprider glädje på labbet och sticker bättre än bäst.

Ett stort tack till ***Ulf Hannestad*** för att ha bistått med gedigen teknisk expertis och många verktyg. De givande diskussionerna om sortimentet på Clas Ohlson och Jula-huset är inte heller att förglömma.

Jag vill tacka ***Tobias Ekblad***, för många långa samarbeten och en underbar humor.

Agneta Askendal för trevliga diskussioner om katter och för att du alltid har ställt upp med ytor av både det ena och andra slaget.

Fredrik Peterson, Kristina Soutukorva och ***Nanny Wallmark*** för att ha bidragit till min forskning genom väl genomförda examensarbeten.

Emma Ericsson, för ett bra och intressant samarbete om GMO.

Anders Sellborn och ***Helen Fink*** för ett väldigt trevligt och givande samarbete med bakteriecellulosan.

Sofia Svedhem och **Jasmin Hume** för ett spännande samarbete med fosfolipidytor.

Alexandra Krettek, ***Sara Sjöberg*** och ***Anders Sellborn*** för utforskningen av CD44.

Andreas Eriksson, ***Maria Bolin*** och ***Olle Andersson*** för angenäma samarbeten och intressanta diskussioner.

Även ett stort tack till ***all personal och medlemmarna i de andra forskargrupperna på Klinisk Kemi*** i Linköping för trevliga pratstunder i fikarummet och många fartfyllda fester.

Alla som har tagit sig tid att sticka… och självklart alla som har bidragit till min forskning genom att bli stuckna.

Stefan Klintström, för ett bra arbete och stort engagemang i Forum Scientium.

Mina vänner ***Tobias, Olle, Daniel, Fredrik*** och ***Magnus***, för många trevliga stunder och roliga upptåg under åren.

Mina trevliga nyvunna släktingar i familjerna ***Andersson*** och ***Lindell***.

Familjen Faxälv, ni har hela tiden funnits där och jag ser alltid fram emot nästa gång vi kommer att träffas.

Till dig ***mormor*** som alltid visat ett stort intresse för mina studier och för att du ser efter vår sommarstuga i både ur och skur.

Min blivande fru ***Sara*** ♥. Ett stort tack till dig min kära, för ett otroligt stöd under denna tid, speciellt de senaste veckorna då du fått sköta både hushåll, trädgård och kontakt med omvärlden. Jag ser fram emot att få mer tid tillsammans, för det är då jag trivs bäst.

Anja ♥, du förgyller mina dagar och trots din ringa ålder är du redan min idol.

Chapter 7

7. References

1. S.P. Jackson. 2007. The growing complexity of platelet aggregation. *Blood* 109:5087-5095.
2. D.M. Monroe, and M. Hoffman. 2006. What Does It Take to Make the Perfect Clot? *Arterioscler Thromb Vasc Biol* 26:41-48.
3. G. Cesarman-Maus, and K.A. Hajjar. 2005. Molecular mechanisms of fibrinolysis. *British Journal of Haematology* 129:307-321.
4. D.C. Rijken, and H.R. Lijnen. 2009. New insights into the molecular mechanisms of the fibrinolytic system. *Journal of Thrombosis and Haemostasis* 7:4-13.
5. J.E. Italiano, and J.H. Hartwig. 2007. Megakaryocyte development and platelet formation. In: Platelets. A.D. Michelson, editor Academic Press/Elsevier, Amsterdam ; Boston. 23-44.
6. J.N. George, and R.W. Colman. 2006. Overview of platelet structute and function. In: Hemostasis and thrombosis : basic principles and clinical practice. R.W. Colman, editor Lippincott Williams & Wilkins, Philadelphia, PA. 437-442.
7. A.P. Heyns. 1994. Thrombopoiesis and platelet kinetics. In: Haemostasis and thrombosis. A.L. Bloom, editor Churchill Livingstone, Edinburgh. 31-48.
8. G.L. Reed. 2007. Platelet secretion. In: Platelets. A.D. Michelson, editor Academic Press/Elsevier, Amsterdam ; Boston. xlii, 1343 p.
9. J.E. Italiano, Jr., J.L. Richardson, S. Patel-Hett, E. Battinelli, A. Zaslavsky, S. Short, S. Ryeom, J. Folkman, and G.L. Klement. 2008. Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. *Blood* 111:1227-1233.
10. D. Blockmans, H. Deckmyn, and J. Vermylen. 1995. Platelet activation. *Blood Rev* 9:143-156.
11. B. Nieswandt, and S.P. Watson. 2003. Platelet-collagen interaction: is GPVI the central receptor? *Blood* 102:449-461.
12. S.G. Eskin, and L.V. McIntire. 2006. Rheology of thrombosis. In: Hemostasis and thrombosis : basic principles and clinical practice. R.W. Colman, editor Lippincott Williams & Wilkins, Philadelphia, PA. 737-750.
13. V.T. Turitto, and H.J. Weiss. 1980. Red Blood Cells: Their Dual Role in Thrombus Formation. *Science* 207:541-543.
14. S.P. Jackson, N. Mistry, and Y. Yuan. 2000. Platelets and the injured vessel wall-- "rolling into action": focus on glycoprotein Ib/V/IX and the platelet cytoskeleton. *Trends Cardiovasc Med* 10:192-197.
15. R.K. Andrews, E.E. Gardiner, Y. Shen, J.C. Whisstock, and M.C. Berndt. 2003. Glycoprotein Ib-IX-V. *Int J Biochem Cell Biol* 35:1170-1174.
16. M. Schmugge, M.L. Rand, and J. Freedman. 2003. Platelets and von Willebrand factor. *Transfus Apher Sci* 28:269-277.
17. M. Broberg, and H. Nygren. 2001. Von Willebrand factor, a key protein in the exposure of CD62P on platelets. *Biomaterials* 22:2403-2409.
18. C.G. Knight, L.F. Morton, D.J. Onley, A.R. Peachey, T. Ichinohe, M. Okuma, R.W. Farndale, and M.J. Barnes. 1999. Collagen-platelet interaction: Gly-Pro-Hyp is uniquely specific for platelet Gp VI and mediates platelet activation by collagen. *Cardiovasc Res* 41:450-457.
19. P.A. Smethurst, D.J. Onley, G.E. Jarvis, M.N. O'Connor, C.G. Knight, A.B. Herr, W.H. Ouwehand, and R.W. Farndale. 2007. Structural Basis for the Platelet-Collagen Interaction: The smallest motif within collagen that recognizes and activates platelet glycoprotein vi contains two glycine-proline-hydroxyproline triplets. *J. Biol. Chem.* 282:1296-1304.
20. M. Tsuji, Y. Ezumi, M. Arai, and H. Takayama. 1997. A Novel Association of Fc Receptor gamma -Chain with Glycoprotein VI and Their Co-expression as a Collagen Receptor in Human Platelets. *J. Biol. Chem.* 272:23528-23531.

21. M. Moroi, and S.M. Jung. 2004. Platelet glycoprotein VI: its structure and function. *Thromb Res* 114:221-233.
22. B. Savage, and Z.M. Ruggeri. 2007. Platelet thrombus formation in flowing blood. In: Platelets. A.D. Michelson, editor Academic Press/Elsevier, Amsterdam ; Boston. 359-376.
23. E. Monnet, P. Sizaret, B. Arbeille, and F. Fauvel-Lafeve. 2000. Different role of platelet glycoprotein GP Ia/IIa in platelet contact and activation induced by type I and type III collagens. *Thromb Res* 98:423-433.
24. S.M. Jung, and M. Moroi. 2000. Activation of the platelet collagen receptor integrin alpha(2)beta(1): its mechanism and participation in the physiological functions of platelets. *Trends Cardiovasc Med* 10:285-292.
25. J. Chen, and J.A. Lopez. 2005. Interactions of platelets with subendothelium and endothelium. *Microcirculation* 12:235-246.
26. E.F. Plow, M.M. Pesho, and Y.-Q. Ma. 2007. Integrin alpha IIb beta 3. In: Platelets. A.D. Michelson, editor Academic Press/Elsevier, Amsterdam ; Boston. 165-178.
27. T.E. O'Toole, D. Mandelman, J. Forsyth, S.J. Shattil, E.F. Plow, and M.H. Ginsberg. 1991. Modulation of the Affinity of Integrin alpha IIb beta (GPIIb-IIIa) by the Cytoplasmic Domain of α_{IIb}. *Science* 254:845-847.
28. D.A. Law, L. Nannizzi-Alaimo, and D.R. Phillips. 1996. Outside-in integrin signal transduction. Alpha IIb beta 3-(GP IIb IIIa) tyrosine phosphorylation induced by platelet aggregation. *J Biol Chem* 271:10811-10815.
29. C. Gachet. 2001. ADP receptors of platelets and their inhibition. *Thromb Haemost* 86:222-232.
30. H.M. Jantzen, L. Gousset, V. Bhaskar, D. Vincent, A. Tai, E.E. Reynolds, and P.B. Conley. 1999. Evidence for two distinct G-protein-coupled ADP receptors mediating platelet activation. *Thromb Haemost* 81:111-117.
31. M. Cattaneo. 2007. The platelet P2 receptors. In: Platelets. A.D. Michelson, editor Academic Press/Elsevier, Amsterdam ; Boston. 201-220.
32. M.P. Mahaut-Smith, S.J. Ennion, M.G. Rolf, and R.J. Evans. 2000. ADP is not an agonist at P2X1 receptors: evidence for separate receptors stimulated by ATP and ADP on human platelets. *Br J Pharmacol* 131:108-114.
33. E.W. Davie, and O.D. Ratnoff. 1964. Waterfall Sequence for Intrinsic Blood Clotting. *Science* 145:1310-1312.
34. T. Grosser, S. Fries, and G.A. Fitzgerald. 2007. Thromboxane generation. In: Platelets. A.D. Michelson, editor Academic Press/Elsevier, Amsterdam ; Boston. 565-574.
35. F.A. Ofosu. 2003. Protease activated receptors 1 and 4 govern the responses of human platelets to thrombin. *Transfus Apheresis Sci* 28:265-268.
36. L.F. Brass. 2003. Thrombin and platelet activation. *Chest* 124:18S-25S.
37. L. Covic, A.L. Gresser, and A. Kuliopulos. 2000. Biphasic kinetics of activation and signaling for PAR1 and PAR4 thrombin receptors in platelets. *Biochemistry* 39:5458-5467.
38. M.L. Kahn, M. Nakanishi-Matsui, M.J. Shapiro, H. Ishihara, and S.R. Coughlin. 1999. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J Clin Invest* 103:879-887.
39. K. Vretenbrant, S. Ramstrom, M. Bjerke, and T.L. Lindahl. 2007. Platelet activation via PAR4 is involved in the initiation of thrombin generation and in clot elasticity development. *Thromb Haemost* 97:417-424.
40. E. De Candia, S.W. Hall, S. Rutella, R. Landolfi, R.K. Andrews, and R. De Cristofaro. 2001. Binding of Thrombin to Glycoprotein Ib Accelerates the Hydrolysis of Par-1 on Intact Platelets. *J. Biol. Chem.* 276:4692-4698.
41. S. Offermanns. 2006. Activation of platelet function through G protein-coupled receptors. *Circ Res* 99:1293-1304.
42. I. Ramasamy. 2004. Inherited bleeding disorders: disorders of platelet adhesion and aggregation. *Crit Rev Oncol Hematol* 49:1-35.
43. R.G. Macfarlane. 1964. An Enzyme Cascade in the Blood Clotting Mechanism, and its Function as a Biochemical Amplifier. *Nature* 202:498-499.
44. K.G. Mann, M.E. Nesheim, W.R. Church, P. Haley, and S. Krishnaswamy. 1990. Surface-dependent reactions of the vitamin K-dependent enzyme complexes. *Blood* 76:1-16.

45. S.J. Freedman, M.D. Blostein, J.D. Baleja, M. Jacobs, B.C. Furie, and B. Furie. 1996. Identification of the Phospholipid Binding Site in the Vitamin K-dependent Blood Coagulation Protein Factor IX. *J. Biol. Chem.* 271:16227-16236.
46. J. Hirsh, J.E. Dalen, D.R. Anderson, L. Poller, H. Bussey, J. Ansell, and D. Deykin. 2001. Oral Anticoagulants: Mechanism of Action, Clinical Effectiveness, and Optimal Therapeutic Range. *Chest* 119:8S-21S.
47. R.W. Colman, A.W. Clowes, J.N. George, S.Z. Goldhaber, and V.J. Marder. 2006. Overview of hemostasis. In: Hemostasis and thrombosis : basic principles and clinical practice. R.W. Colman, editor Lippincott Williams & Wilkins, Philadelphia, PA. 3-16.
48. E. Bächli. 2000. History of tissue factor. *British Journal of Haematology* 110:248-255.
49. N. Mackman. 2004. Role of Tissue Factor in Hemostasis, Thrombosis, and Vascular Development. *Arterioscler Thromb Vasc Biol* 24:1015-1022.
50. Y. Nemerson. 1988. Tissue factor and hemostasis. *Blood* 71:1-8.
51. Y. Nemerson, and D. Repke. 1985. Tissue factor accelerates the activation of coagulation factor VII: The role of a bifunctional coagulation cofactor. *Thrombosis Research* 40:351-358.
52. L.V. Rao, S.P. Bajaj, and S.I. Rapaport. 1985. Activation of human factor VII during clotting in vitro. *Blood* 65:218-226.
53. L.V. Rao, S.I. Rapaport, and S.P. Bajaj. 1986. Activation of human factor VII in the initiation of tissue factor- dependent coagulation. *Blood* 68:685-691.
54. J. Hathcock, E. Rusinova, H. Vaananen, and Y. Nemerson. 2007. Lipid-Bound Factor Xa Regulates Tissue Factor Activity. *Biochemistry* 46:6134-6140.
55. P.J. Fay. 1999. Regulation of factor VIIIa in the intrinsic factor Xase. *Thromb Haemost* 82:193-200.
56. K. Mertens, P.H. Celie, J.A. Kolkman, and P.J. Lenting. 1999. Factor VIII-factor IX interactions: molecular sites involved in enzyme-cofactor complex assembly. *Thromb Haemost* 82:209-217.
57. K.G. Mann, S. Butenas, and K. Brummel. 2003. The dynamics of thrombin formation. *Arterioscler Thromb Vasc Biol* 23:17-25.
58. J. Rosing, G. Tans, J.W. Govers-Riemslag, R.F. Zwaal, and H.C. Hemker. 1980. The role of phospholipids and factor Va in the prothrombinase complex. *J. Biol. Chem.* 255:274-283.
59. D. Gailani, and G.J. Broze, Jr. 1991. Factor XI Activation in a Revised Model of Blood Coagulation. *Science* 253:909-912.
60. I.M. Keularts, A. Zivelin, U. Seligsohn, H.C. Hemker, and S. Beguin. 2001. The role of factor XI in thrombin generation induced by low concentrations of tissue factor. *Thromb Haemost* 85:1060-1065.
61. S.J. Wielders, S. Beguin, H.C. Hemker, and T. Lindhout. 2004. Factor XI-dependent reciprocal thrombin generation consolidates blood coagulation when tissue factor is not available. *Arterioscler Thromb Vasc Biol* 24:1138-1142.
62. F.A. Baglia, C.N. Shrimpton, J. Emsley, K. Kitagawa, Z.M. Ruggeri, J.A. Lopez, and P.N. Walsh. 2004. Factor XI interacts with the leucine-rich repeats of glycoprotein Ibalpha on the activated platelet. *J Biol Chem* 279:49323-49329.
63. M.D. Rand, J.B. Lock, C. van't Veer, D.P. Gaffney, and K.G. Mann. 1996. Blood clotting in minimally altered whole blood. *Blood* 88:3432-3445.
64. O.D. Ratnoff, and J.E. Colopy. 1955. A familial hemorrhagic trait associated with a deficiency of a clot-promoting fraction of plasma. *J Clin Invest* 34:602-613.
65. D.E. Cool, C.J. Edgell, G.V. Louie, M.J. Zoller, G.D. Brayer, and R.T. MacGillivray. 1985. Characterization of human blood coagulation factor XII cDNA. Prediction of the primary structure of factor XII and the tertiary structure of beta-factor XIIa. *J. Biol. Chem.* 260:13666-13676.
66. F. Citarella, D.M. Ravon, B. Pascucci, A. Felici, A. Fantoni, and C.E. Hack. 1996. Structure/Function Analysis of Human Factor XII Using Recombinant Deletion Mutants. *European Journal of Biochemistry* 238:240-249.
67. B.J. Clarke, H.C. Cote, D.E. Cool, I. Clark-Lewis, H. Saito, R.A. Pixley, R.W. Colman, and R.T. MacGillivray. 1989. Mapping of a putative surface-binding site of human coagulation factor XII. *J. Biol. Chem.* 264:11497-11502.
68. R.A. Pixley, L.G. Stumpo, K. Birkmeyer, L. Silver, and R.W. Colman. 1987. A monoclonal antibody recognizing an icosapeptide sequence in the heavy chain of human factor XII inhibits surface-catalyzed activation. *J. Biol. Chem.* 262:10140-10145.

69. E.A. Vogler, and C.A. Siedlecki. 2009. Contact activation of blood-plasma coagulation. *Biomaterials* 30:1857-1869.
70. K. Naito, and K. Fujikawa. 1991. Activation of human blood coagulation factor XI independent of factor XII. Factor XI is activated by thrombin and factor XIa in the presence of negatively charged surfaces. *J. Biol. Chem.* 266:7353-7358.
71. H. Leiba, B. Ramot, and A. Many. 1965. Heredity and Coagulation Studies in Ten Families with Factor XI (Plasma Thromboplastin Antecedent) Deficiency. *British Journal of Haematology* 11:654-665.
72. K.G. Mann. 1999. Biochemistry and physiology of blood coagulation. *Thromb Haemost* 82:165-174.
73. T.R. Baird, and P.N. Walsh. 2002. Activated Platelets but Not Endothelial Cells Participate in the Initiation of the Consolidation Phase of Blood Coagulation. *J. Biol. Chem.* 277:28498-28503.
74. F.A. Baglia, K.O. Badellino, C.Q. Li, J.A. Lopez, and P.N. Walsh. 2002. Factor XI Binding to the Platelet Glycoprotein Ib-IX-V Complex Promotes Factor XI Activation by Thrombin. *J. Biol. Chem.* 277:1662-1668.
75. A.H. Schmaier, D. Schutsky, A. Farber, L.D. Silver, H.N. Bradford, and R.W. Colman. 1987. Determination of the bifunctional properties of high molecular weight kininogen by studies with monoclonal antibodies directed to each of its chains. *J. Biol. Chem.* 262:1405-1411.
76. D.M. Kerbiriou, and J.H. Griffin. 1979. Human high molecular weight kininogen. Studies of structure-function relationships and of proteolysis of the molecule occurring during contact activation of plasma. *J. Biol. Chem.* 254:12020-12027.
77. R.W. Colman, and A.H. Schmaier. 1997. Contact system: a vascular biology modulator with anticoagulant, profibrinolytic, antiadhesive, and proinflammatory attributes. *Blood* 90:3819-3843.
78. D. Sinha, F.S. Seaman, and P.N. Walsh. 1987. Role of calcium ions and the heavy chain of factor XIa in the activation of human coagulation factor IX. *Biochemistry* 26:3768-3775.
79. P.J. Fay. 2004. Activation of factor VIII and mechanisms of cofactor action. *Blood Rev* 18:1-15.
80. G.A. Nicolaes, and B. Dahlback. 2002. Factor V and thrombotic disease: description of a janus-faced protein. *Arterioscler Thromb Vasc Biol* 22:530-538.
81. H.R. Roberts, and A.D. Ma. 2006. Overview of inherited hemorrhagic disorders In: Hemostasis and thrombosis : basic principles and clinical practice. R.W. Colman, editor Lippincott Williams & Wilkins, Philadelphia, PA. 877-886.
82. T. Renne, M. Pozgajova, S. Gruner, K. Schuh, H.-U. Pauer, P. Burfeind, D. Gailani, and B. Nieswandt. 2005. Defective thrombus formation in mice lacking coagulation factor XII. *J. Exp. Med.* jem.20050664.
83. S.A. Smith, N.J. Mutch, D. Baskar, P. Rohloff, R. Docampo, and J.H. Morrissey. 2006. Polyphosphate modulates blood coagulation and fibrinolysis. *Proceedings of the National Academy of Sciences of the United States of America* 103:903-908.
84. H. Herwald, M. Morgelin, A. Olsen, M. Rhen, B. Dahlback, W. Muller-Esterl, and L. Bjorck. 1998. Activation of the contact-phase system on bacterial surfaces[mdash]a clue to serious complications in infectious diseases. *Nat Med* 4:298-302.
85. D.C. Morrison, and C.G. Cochrane. 1974. DIRECT EVIDENCE FOR HAGEMAN FACTOR (FACTOR XII) ACTIVATION BY BACTERIAL LIPOPOLYSACCHARIDES (ENDOTOXINS). *J. Exp. Med.* 140:797-811.
86. Y. Kawamoto, and M. Kaibara. 1990. Procoagulant activity of collagen. Effect of difference in type and structure of collagen. *Biochim Biophys Acta* 1035:361-368.
87. J.M. Koenig, A. Chahine, and O.D. Ratnoff. 1991. Inhibition of the activation of Hageman factor (factor XII) by soluble human placental collagens types III, IV, and V. *J Lab Clin Med* 117:523-527.
88. B. Blomback. 1996. Fibrinogen and fibrin--proteins with complex roles in hemostasis and thrombosis. *Thromb Res* 83:1-75.
89. B. Blomback, D. Banerjee, K. Carlsson, A. Hamsten, B. Hessel, R. Procyk, A. Silveira, and L. Zacharski. 1990. Native fibrin gel networks and factors influencing their formation in health and disease. *Adv Exp Med Biol* 281:1-23.
90. B. Blomback, K. Carlsson, B. Hessel, A. Liljeborg, R. Procyk, and N. Aslund. 1989. Native fibrin gel networks observed by 3D microscopy, permeation and turbidity. *Biochim Biophys Acta* 997:96-110.

91. L. Muszbek, V.C. Yee, and Z. Hevessy. 1999. Blood coagulation factor XIII: structure and function. *Thromb Res* 94:271-305.
92. J.J. Pisano, J.S. Finlayson, and M.P. Peyton. 1968. Cross-link in Fibrin Polymerized by Factor XIII: Îµ-(Î³ -Glutamyl)lysine. *Science* 160:892-893.
93. P.G. Board, M.S. Lososky, and K.J.A. Miloszewski. 1993. Factor XIII: Inherited and acquired deficiency. *Blood Reviews* 7:229-242.
94. M.E. Carr, Jr., D.A. Gabriel, and J. McDonagh. 1987. Influence of factor XIII and fibronectin on fiber size and density in thrombin-induced fibrin gels. *J Lab Clin Med* 110:747-752.
95. C.T. Esmon. 1989. The roles of protein C and thrombomodulin in the regulation of blood coagulation. *J. Biol. Chem.* 264:4743-4746.
96. E. Di Cera. 2003. Thrombin Interactions. *Chest* 124:11S-17S.
97. M.S. Bajaj, J.J. Birktoft, S.A. Steer, and S.P. Bajaj. 2001. Structure and biology of tissue factor pathway inhibitor. *Thromb Haemost* 86:959-972.
98. W. Li, D.J.D. Johnson, C.T. Esmon, and J.A. Huntington. 2004. Structure of the antithrombin-thrombin-heparin ternary complex reveals the antithrombotic mechanism of heparin. *Nat Struct Mol Biol* 11:857-862.
99. J.C. Rau, L.M. Beaulieu, J.A. Huntington, and F.C. Church. 2007. Serpins in thrombosis, hemostasis and fibrinolysis. *Journal of Thrombosis and Haemostasis* 5:102-115.
100. S.T. Olson, P.E. Bock, J. Kvassman, J.D. Shore, D.A. Lawrence, D. Ginsburg, and I. Björk. 1995. Role of the Catalytic Serine in the Interactions of Serine Proteinases with Protein Inhibitors of the Serpin Family. *J. Biol. Chem.* 270:30007-30017.
101. P.K. Schick, and T.S. Panetti. 2006. Platelet and megakaryocyte lipids. In: Hemostasis and thrombosis : basic principles and clinical practice. R.W. Colman, editor Lippincott Williams & Wilkins, Philadelphia, PA. 591-604.
102. E.M. Bevers, R.H.J. Tilly, J.M.G. Senden, P. Comfurius, and R.F.A. Zwaal. 1989. Exposure of endogenous phosphatidylserine at the outer surface of stimulated platelets is reversed by restoration of aminophospholipid translocase activity. *Biochemistry* 28:2382-2387.
103. J.L. Wolfs, P. Comfurius, J.T. Rasmussen, J.F. Keuren, T. Lindhout, R.F. Zwaal, and E.M. Bevers. 2005. Activated scramblase and inhibited aminophospholipid translocase cause phosphatidylserine exposure in a distinct platelet fraction. *Cell Mol Life Sci* 62:1514-1525.
104. R.F. Zwaal, P. Comfurius, and E.M. Bevers. 1998. Lipid-protein interactions in blood coagulation. *Biochim Biophys Acta* 1376:433-453.
105. S. Ramstrom, M. Ranby, and T.L. Lindahl. 2003. Platelet phosphatidylserine exposure and procoagulant activity in clotting whole blood--different effects of collagen, TRAP and calcium ionophore A23187. *Thromb Haemost* 89:132-141.
106. G.L. Reed. 2007. Platelet secretion. In: Platelets. A.D. Michelson, editor Academic Press/Elsevier, Amsterdam ; Boston. 309-318.
107. A.T. Nurden. 1994. Human platelet membrane glycoproteins. In: Haemostasis and thrombosis. A.L. Bloom, editor Churchill Livingstone, Edinburgh. 115-166.
108. S. Ramstrom, M. Ranby, and T.L. Lindahl. 2002. The role of platelets in blood coagulation--effects of platelet agonists and GPIIb/IIIa inhibitors studied by free oscillation rheometry. *Thromb Res* 105:165-172.
109. D. Seiffert, D.L. Pedicord, C.J. Kieras, B. He, A.M. Stern, and J.T. Billheimer. 2002. Regulation of clot retraction by glycoprotein IIb/IIIa antagonists. *Thromb Res* 108:181-189.
110. O.E. Olorundare, S.R. Simmons, and R.M. Albrecht. 1992. Cytochalasin D and E: effects on fibrinogen receptor movement and cytoskeletal reorganization in fully spread, surface-activated platelets: a correlative light and electron microscopic investigation. *Blood* 79:99-109.
111. S. Osdoit, and J.-P. Rosa. 2001. Fibrin Clot Retraction by Human Platelets Correlates with alpha IIbbeta 3 Integrin-dependent Protein Tyrosine Dephosphorylation. *J. Biol. Chem.* 276:6703-6710.
112. I. Yahara, F. Harada, S. Sekita, K. Yoshihira, and S. Natori. 1982. Correlation between effects of 24 different cytochalasins on cellular structures and cellular events and those on actin in vitro. *J. Cell Biol.* 92:69-78.
113. N.A. Booth, and F. Bachmann. 2006. Plasminogen - Plasmin system. In: Hemostasis and thrombosis : basic principles and clinical practice. R.W. Colman, editor Lippincott Williams & Wilkins, Philadelphia, PA. 335-364.

114. D.A. Gabriel, K. Muga, and E.M. Boothroyd. 1992. The effect of fibrin structure on fibrinolysis. *J. Biol. Chem.* 267:24259-24263.
115. M. Yepes, D.J. Loskutoff, and D.A. Lawrence. 2006. Plasminogen activator inhibitor - 1. In: Hemostasis and thrombosis : basic principles and clinical practice. R.W. Colman, editor Lippincott Williams & Wilkins, Philadelphia, PA. 365-380.
116. H.S. Cummings, and F.J. Castellino. 1984. Interaction of human plasmin with human .alpha.2-macroglobulin. *Biochemistry* 23:105-111.
117. A.E. Davis Iii, P. Mejia, and F. Lu. 2008. Biological activities of C1 inhibitor. *Molecular Immunology* 45:4057-4063.
118. L.O. Mosnier, and B.N. Bouma. 2006. Regulation of Fibrinolysis by Thrombin Activatable Fibrinolysis Inhibitor, an Unstable Carboxypeptidase B That Unites the Pathways of Coagulation and Fibrinolysis. *Arterioscler Thromb Vasc Biol* 26:2445-2453.
119. A. Koster, T. Fischer, M. Praus, H. Haberzettl, W.M. Kuebler, R. Hetzer, and H. Kuppe. 2002. Hemostatic Activation and Inflammatory Response during Cardiopulmonary Bypass: Impact of Heparin Management. *Anesthesiology* 97:837-841.
120. C. Marc. 2009. Antiplatelet therapy in percutaneous coronary intervention: A critical review of the 2007 AHA/ACC/SCAI guidelines and beyond. *Catheterization and Cardiovascular Interventions* 9999:NA.
121. P.D.V.M.P. Pibarot, and J.G.M.D.F. Dumesnil. 2009. Prosthetic Heart Valves: Selection of the Optimal Prosthesis and Long-Term Management. *Circulation* 119:1034-1048.
122. M.B. Gorbet, and M.V.M.V. Sefton. 2004. Biomaterial-associated thrombosis: roles of coagulation factors, complement, platelets and leukocytes. *Biomaterials* 25:5681-5703.
123. B.D. Ratner. 2000. Blood compatibility - a perspective. *Journal of Biomaterials Science, Polymer Edition* 11:1107-1119.
124. M. Andersson, J. Andersson, A. Sellborn, M. Berglin, B. Nilsson, and H. Elwing. 2005. Quartz crystal microbalance-with dissipation monitoring (QCM-D) for real time measurements of blood coagulation density and immune complement activation on artificial surfaces. *Biosens Bioelectron* 21:79-86.
125. K.M. Hansson, S. Tosatti, J. Isaksson, J. Wetterö, M. Textor, T.L. Lindahl, and P. Tengvall. 2005. Whole blood coagulation on protein adsorption-resistant PEG and peptide functionalised PEG-coated titanium surfaces. *Biomaterials* 26:861-872.
126. H. Nygren. 1996. Initial reactions of whole blood with hydrophilic and hydrophobic titanium surfaces. *Colloids and Surfaces B: Biointerfaces* 6:329-333.
127. H. Nygren, P. Tengvall, and I. Lundström. 1997. The initial reactions of TiO2 with blood. *Journal of Biomedical Materials Research* 34:487-492.
128. N.L. Anderson, and N.G. Anderson. 2002. The Human Plasma Proteome: History, Character, and Diagnostic Prospects. *Mol Cell Proteomics* 1:845-867.
129. C.A. Haynes, and W. Norde. 1994. Globular proteins at solid/liquid interfaces. *Colloids and Surfaces B: Biointerfaces* 2:517-566.
130. P. Tengvall, I. Lundstrom, and B. Liedberg. 1998. Protein adsorption studies on model organic surfaces: an ellipsometric and infrared spectroscopic approach. *Biomaterials* 19:407-422.
131. C.J. Wilson, R.E. Clegg, D.I. Leavesley, and M.J. Pearcy. 2005. Mediation of biomaterial-cell interactions by adsorbed proteins: a review. *Tissue Eng* 11:1-18.
132. P. Turbill, T. Beugeling, and A.A. Poot. 1996. Proteins involved in the Vroman effect during exposure of human blood plasma to glass and polyethylene. *Biomaterials* 17:1279-1287.
133. E.A. Vogler. 1998. Structure and reactivity of water at biomaterial surfaces. *Advances in Colloid and Interface Science* 74:69-117.
134. J.L. Ortega-Vinuesa, P. Tengvall, B. Walivaara, and I. Lundstrom. 1998. Stagnant versus dynamic conditions: a comparative adsorption study of blood proteins. *Biomaterials* 19:251-262.
135. J.M.G.C.D.M.T.A.H. Wei-Bor Tsai. 2002. Platelet adhesion to polystyrene-based surfaces preadsorbed with plasmas selectively depleted in fibrinogen, fibronectin, vitronectin, or von Willebrand's factor. *Journal of Biomedical Materials Research* 60:348-359.
136. J.M.G.T.A.H. Wei-Bor Tsai. 1999. Human plasma fibrinogen adsorption and platelet adhesion to polystyrene. *Journal of Biomedical Materials Research* 44:130-139.
137. J. Wetterö, P. Tengvall, and T. Bengtsson. 2003. Platelets stimulated by IgG-coated surfaces bind and activate neutrophils through a selectin-dependent pathway. *Biomaterials* 24:1559-1573.

138. J.M. Grunkemeier, W.B. Tsai, C.D. McFarland, and T.A. Horbett. 2000. The effect of adsorbed fibrinogen, fibronectin, von Willebrand factor and vitronectin on the procoagulant state of adherent platelets. *Biomaterials* 21:2243-2252.
139. T.E. O'Toole, Y. Katagiri, R.J. Faull, K. Peter, R. Tamura, V. Quaranta, J.C. Loftus, S.J. Shattil, and M.H. Ginsberg. 1994. Integrin cytoplasmic domains mediate inside-out signal transduction. *J. Cell Biol.* 124:1047-1059.
140. B. Savage, and Z.M. Ruggeri. 1991. Selective recognition of adhesive sites in surface-bound fibrinogen by glycoprotein IIb-IIIa on nonactivated platelets. *J. Biol. Chem.* 266:11227-11233.
141. N. Yayapour, and H. Nygren. 1999. Interactions between whole blood and hydrophilic or hydrophobic glass surfaces: kinetics of cell adhesion. *Colloids and Surfaces B: Biointerfaces* 15:127-138.
142. J.H. Lee, and H.B. Lee. 1998. Platelet adhesion onto wettability gradient surfaces in the absence and presence of plasma proteins. *J Biomed Mater Res* 41:304-311.
143. R. Zhuo, C.A. Siedlecki, and E.A. Vogler. 2006. Autoactivation of blood factor XII at hydrophilic and hydrophobic surfaces. *Biomaterials* 27:4325-4332.
144. R. Zhuo, C.A. Siedlecki, and E.A. Vogler. 2007. Competitive-protein adsorption in contact activation of blood factor XII. *Biomaterials* 28:4355-4369.
145. C.R. McMillin, H. Saito, O.D. Ratnoff, and A.G. Walton. 1974. The secondary structure of human Hageman factor (factor XII) and its alteration by activating agents. *J Clin Invest* 54:1312-1322.
146. M. Samuel, R.A. Pixley, M.A. Villanueva, R.W. Colman, and G.B. Villanueva. 1992. Human factor XII (Hageman factor) autoactivation by dextran sulfate. Circular dichroism, fluorescence, and ultraviolet difference spectroscopic studies. *J. Biol. Chem.* 267:19691-19697.
147. R.W. Colman. 1984. Surface-mediated defense reactions. The plasma contact activation system. *J Clin Invest* 73:1249-1253.
148. D.E. Chenoweth. 1987. Complement activation in extracorporeal circuits. *Ann N Y Acad Sci* 516:306-313.
149. J. Wettero, A. Askendal, T. Bengtsson, and P. Tengvall. 2002. On the binding of complement to solid artificial surfaces in vitro. *Biomaterials* 23:981-991.
150. S. Arvidsson, A. Askendal, and P. Tengvall. 2007. Blood plasma contact activation on silicon, titanium and aluminium. *Biomaterials* 28:1346-1354.
151. A. Sagripanti, and A. Carpi. 2000. Antithrombotic and prothrombotic activities of the vascular endothelium. *Biomed Pharmacother* 54:107-111.
152. V.W. van Hinsbergh. 2001. The endothelium: vascular control of haemostasis. *Eur J Obstet Gynecol Reprod Biol* 95:198-201.
153. S. Ramstrom. 2005. Clotting time analysis of citrated blood samples is strongly affected by the tube used for blood sampling. *Blood Coagul Fibrinolysis* 16:447-452.
154. M. Ranby, S. Ramstrom, P.O. Svensson, and T.L. Lindahl. 2003. Clotting time by free oscillation rheometry and visual inspection and a viscoelastic description of the clotting phenomenon. *Scand J Clin Lab Invest* 63:397-406.
155. P.A. Holme, U. Orvim, M.J.A.G. Hamers, N.O. Solum, F.R. Brosstad, R.M. Barstad, and K.S. Sakariassen. 1997. Shear-Induced Platelet Activation and Platelet Microparticle Formation at Blood Flow Conditions as in Arteries With a Severe Stenosis. *Arterioscler Thromb Vasc Biol* 17:646-653.
156. H.A. Barnes, J.F. Hutton, and K. Walters. 1989. An introduction to rheology. Elsevier, Amsterdam. 199 pp.
157. M. Zimmerman, B. Ashe, E.C. Yurewicz, and G. Patel. 1977. Sensitive assays for trypsin, elastase, and chymotrypsin using new fluorogenic substrates. *Analytical Biochemistry* 78:47-51.
158. M. Zimmerman, E. Yurewicz, and G. Patel. 1976. A new fluorogenic substrate for chymotrypsin. *Anal Biochem* 70:258-262.
159. S. Kawabata, T. Miura, T. Morita, H. Kato, K. Fujikawa, S. Iwanaga, K. Takada, T. Kimura, and S. Sakakibara. 1988. Highly sensitive peptide-4-methylcoumaryl-7-amide substrates for blood-clotting proteases and trypsin. *Eur J Biochem* 172:17-25.
160. H.C. Hemker, P. Giesen, R. Al Dieri, V. Regnault, E. de Smedt, R. Wagenvoord, T. Lecompte, and S. Beguin. 2003. Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol Haemost Thromb* 33:4-15.

161. B. Balakrishnan, D.S. Kumar, Y. Yoshida, and A. Jayakrishnan. 2005. Chemical modification of poly(vinyl chloride) resin using poly(ethylene glycol) to improve blood compatibility. *Biomaterials* 26:3495-3502.
162. J. Wang, C.J. Pan, N. Huang, H. Sun, P. Yang, Y.X. Leng, J.Y. Chen, G.J. Wan, and P.K. Chu. 2005. Surface characterization and blood compatibility of poly(ethylene terephthalate) modified by plasma surface grafting. *Surface and Coatings Technology* 196:307-311.
163. A. Larsson, T. Ekblad, O. Andersson, and B. Liedberg. 2007. Photografted poly(ethylene glycol) matrix for affinity interaction studies. *Biomacromolecules* 8:287-295.
164. M. Tanaka, A. Mochizuki, N. Ishii, T. Motomura, and T. Hatakeyama. 2002. Study of blood compatibility with poly(2-methoxyethyl acrylate). Relationship between water structure and platelet compatibility in poly(2-methoxyethylacrylate-co-2-hydroxyethylmethacrylate). *Biomacromolecules* 3:36-41.
165. M. Tanaka, T. Motomura, M. Kawada, T. Anzai, Y. Kasori, T. Shiroya, K. Shimura, M. Onishi, and A. Mochizuki. 2000. Blood compatible aspects of poly(2-methoxyethylacrylate) (PMEA)--relationship between protein adsorption and platelet adhesion on PMEA surface. *Biomaterials* 21:1471-1481.
166. A. de Agostini, H.R. Lijnen, R.A. Pixley, R.W. Colman, and M. Schapira. 1984. Inactivation of factor XII active fragment in normal plasma. Predominant role of C-1-inhibitor. *J Clin Invest* 73:1542-1549.
167. M.V. Ovanesov, J.V. Krasotkina, L.I. Ul'yanova, K.V. Abushinova, O.P. Plyushch, S.P. Domogatskii, A.I. Vorob'ev, and F.I. Ataullakhanov. 2002. Hemophilia A and B are associated with abnormal spatial dynamics of clot growth. *Biochimica et Biophysica Acta (BBA) - General Subjects* 1572:45-57.
168. Y.V. Krasotkina, E.I. Sinauridze, and F.I. Ataullakhanov. 2000. Spatiotemporal dynamics of fibrin formation and spreading of active thrombin entering non-recalcified plasma by diffusion. *Biochimica et Biophysica Acta (BBA) - General Subjects* 1474:337-345.
169. M.A. Panteleev, M.V. Ovanesov, D.A. Kireev, A.M. Shibeko, E.I. Sinauridze, N.M. Ananyeva, A.A. Butylin, E.L. Saenko, and F.I. Ataullakhanov. 2006. Spatial propagation and localization of blood coagulation are regulated by intrinsic and protein C pathways, respectively. *Biophys J* 90:1489-1500.
170. T.P. Vikinge, K.M. Hansson, P. Sandstrom, B. Liedberg, T.L. Lindahl, I. Lundstrom, P. Tengvall, and F. Hook. 2000. Comparison of surface plasmon resonance and quartz crystal microbalance in the study of whole blood and plasma coagulation. *Biosens Bioelectron* 15:605-613.