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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Blood Coagulation Studies on modified Surfaces using Quartz Crystal Microbalance

Promotor :
Prof. dr. Patrick WAGNER

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Yannick Eurlings

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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Preface

To conclude my senior practical training, I would like to thank some people and instances. Truly, these eight months at the University of Vienna have been a wonderful, instructive and inspiring time. First of all I would like to thank Prof. dr. P. Lieberzeit and by extension the whole research group Sensors and Rapid Analysis for giving me the opportunity to do my internship and thesis under their supervision. Special thanks go to Agnieszka Strallhofer for her daily supervision and guidance. Also, I would like to express my gratitude towards Andrea Bileck for taking the blood samples. I am especially thankful towards the University of Hasselt for providing me with the proper education throughout the bachelor program and more recently the master program. I would like to thank Prof. dr. P. Wagner, who has been very important in arranging this internship abroad. Furthermore, I am grateful towards Wim Cuypers and Cédric Libert, who have supported me with their friendship. Finally, I want to thank the Erasmus+ Program for providing the financial means for this exciting endeavor.

Abstract

Introduction: During blood transfusion or situations where blood comes into contact with exogenous materials (*e.g.* dialysis), unwanted platelet activation and subsequent aggregation poses a serious threat to patients. However, differentiating between activated and non-activated platelets is a challenge for current platelet function tests. For this reason, a new platelet function test is being developed to monitor blood coagulation. In this first stage of the project, we hypothesized that both blood coagulation and platelet aggregation can be induced on a gold electrode of quartz crystal microbalances (QCM) using immobilized thrombin and calcium.

Materials & Methods: QCM crystals were incubated first in mercaptopropionic acid (5% (v/v) in water) and subsequently in ethyl(dimethylaminopropyl) carbodiimide (100 mM in water). QCM was used to investigate the interactions during the different experiments. First of all, the binding of thrombin (140 U/ml in PBS) to the surface was investigated. Next, the effect of adding plasma and platelet-rich plasma (PRP) to immobilized thrombin was examined. Further, the effect of adding various calcium chloride ($CaCl_2$) concentrations (50, 75, 100 mM in water) to platelet rich plasma (PRP) (diluted 1:20 in PBS) was investigated. After adding $CaCl_2$, the flow cell was flushed to check for reversibility. Finally, $CaCl_2$ was added to plasma to evaluate the interaction between the two.

Results: First of all, thrombin was successfully bound to the functionalized surface. By washing multiple times with buffer, it was found that this covalent bond is highly stable. Adding plasma onto the immobilized thrombin resulted in an irreversible frequency shift. Adding 50 mM of $CaCl_2$ to the PRP did not result in a frequency drop. Upon adding higher concentrations of $CaCl_2$ (75 and 100 mM), frequency shifts (1000-4000 Hz) were observed, which after washing with buffer proved to be partly irreversible. Injecting $CaCl_2$ after plasma results in a frequency drop, which is also partly irreversible.

Discussion & Conclusion: These results indicate that coagulation occurs on the surface upon adding calcium to PRP. The irreversible frequency drop suggests that the blood clot is attached to the immobilized thrombin on the surface. Also plasma interacts with calcium and this interaction should be accounted for when interpreting the responses seen after adding calcium to PRP. Conclusions about platelet aggregation could not be drawn yet. QCM results remain inconsistent, possibly due to variance in surface roughness of the electrodes of the QCM crystals. Further experiments are needed to determine the factors that would improve reproducibility.

Samenvatting

Inleiding: Bij bloedtransfusie of situaties waarbij het bloed in contact komt met exogene stoffen (bijvoorbeeld dialyse), vormt ongewenste activering van bloedplaatjes en hun daaropvolgende aggregatie een ernstige bedreiging voor patiënten. Echter, het onderscheid tussen geactiveerde en niet-geactiveerde bloedplaatjes is een uitdaging voor de huidige platelet function tests. Daarom wordt een nieuwe test ontwikkeld om het bloedstollingsproces op te volgen. Onze hypothese was dat bloedstolling en aggregatie van thrombocyten kan worden geïnduceerd op de gouden elektrode van quartz crystal microbalances (QCM) gebruikmakend van geïmmobiliseerd thrombine en calcium.

Materiaal en Methoden: QCM kristallen werden eerst geïncubeerd in mercaptopropionzuur (5% (v/v) in water) en vervolgens in ethyl (dimethylaminopropyl) carbodiimide (100 mM in water). QCM werd gebruikt om de interacties tijdens de experimenten te meten. Eerst werd de binding van thrombine (140 U/ml in PBS) aan het oppervlak onderzocht. Vervolgens ook het effect van het toevoegen van verschillende calcium chlorideconcentraties ($CaCl_2$) (50, 75, 100 mM in water) aan platelet-rich plasma (PRP) (verdunning 1:20 in PBS). Na het toevoegen van $CaCl_2$ werd de meetcel gespoeld om de reversibiliteit te evalueren. Ten laatste werd ook $CaCl_2$ toegevoegd aan plasma om de interactie tussen beide te onderzoeken.

Resultaten: Ten eerste werd thrombine met succes gebonden aan het gefunctionaliseerde oppervlak. Door meerdere keren te wassen met buffer, werd vastgesteld dat deze covalente binding zeer stabiel is. Plasma blootgesteld aan geimmobiliseerd thrombine resulteerde in een irreversibele frequentiedaling. Het toevoegen van 50 mM $CaCl_2$ aan PRP leidde niet tot een frequentiedaling. Na toevoegen van hogere concentraties van calcium (75 en 100 mM) aan PRP werden wel frequentiedalingen (1000-4000 Hz) waargenomen. Na wassen met PBS buffer bleken deze deels irreversibel. $CaCl_2$ toegevoegd aan plasma induceerde een frequentiedaling, die evenees deels irreversibel was.

Discussie en Conclusie: Deze resultaten tonen aan dat coagulatie optreedt op het oppervlak na toevoegen van $CaCl_2$ aan PRP. De irreversibele frequentiedaling bewijst dat de bloedklonter via de geïmmobiliseerde thrombine bevestigd is aan het oppervlak. Ook plasma interageert met calcium en met deze interactie moet er rekening worden gehouden wanneer men de responsen, observeerbaar na het toevoegen van calcium aan PRP, interpreteert. Conclusies over het aggregeren van bloedplaatjes kunnen hier nog niet genomen worden. Echter, QCM resultaten blijven inconsistent, mogelijk als gevolg van variantie in de oppervlakteruwheid van de elektroden van de QCM kristallen. Verdere experimenten zijn vereist om de factoren te bepalen die reproduceerbaarheid zullen verbeteren.

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List of Abbreviations

(3-Aminopropyl)triethoxysilane	APTES
3-Mercaptopropionic Acid	MPA
5-Hydroxytryptamine	5HT
Activated Partial Thromboplastin Time	APTT
Adenosine Diphosphate	ADP
Adenosine Triphosphate	ATP
Cyclic Adenosine Monophosphate	cAMP
Cyclic Guanosine Monophosphate	cGMP
Cyclooxygenase	COX
Diacylglycerol	DAG
Extracellular Matrix	ECM
Glycoprotein	Gp
Inositol Triphosphate	IP_3
Junctional Adhesion Molecules	JAMs
N-Ethyl-N'-(3-Dimethylaminopropyl) Carbodiimide hydrochloride	EDC
N-HydroxySuccinimide	NHS
Phosphate Buffered Saline	PBS
PhosphatidylInositol Phosphate	PIP
PhosphoLipase C	PLC
Platelet-Rich Plasma	PRP
Prostacyclin	PGI_2
Protease-Activated Receptors	PARs
Prothrombin Time	PT
Quartz	SiO_2
Quartz Crystal Microbalance	QCM
Thromboxane A_2	TXA_2
Tissue Factor	TF
Thromboxane Receptor	TP
Von Willebrand Factor	vWF

1 Introduction

Without a doubt, blood is essential for humans as well as for other vertebrates. Besides delivering oxygen to all tissues and cells in the body, it is the most important transport medium for nutrients, metabolic waste, the immune system, the endocrine system and other essential compounds. Furthermore, blood ensures our constant body temperature. It consists of cells suspended in plasma, which contains water, hormones, electrolytes, lipids and proteins, such as albumin, globulins, coagulation factors and fibrinogen. In blood, mainly three different cell types are present: erythrocytes (red blood cells), leukocytes (white blood cells) and thrombocytes (platelets). While erythrocytes transport oxygen from the lungs to tissues, leukocytes play a major role in the immune system. Finally, thrombocytes act together with various coagulation factors to ensure the cessation of blood loss during bleeding (1).

Being produced out of megakaryocytes in bone marrow (1), normal blood contains 150000-450000 platelets per microliter (2). Platelets have a life span of 7-10 days (3). With a diameter of 2-5 µm and a thickness of 0,5 µm, they are the smallest cells present in blood (4). Scanning electron microscope images of both normal and activated platelets are shown in Figure 1. Their specific disk-like shape originates from a circumferential coil of microtubules, actin and myosin. Together with lacking a nucleus, their main characteristic is the open canalicular system. This system comprises a network of plasma membrane invaginations to facilitate endocytosis and exocytosis of granules (5). Characteristic organelles are α-granules and the less abundant dense-core granules, both containing molecules and proteins involved in the blood coagulation process. While proteins, such as von Willebrand factor (vWF), fibrinogen and various clotting factors are present within α-granules (6), dense-core granules contain smaller molecules, including adenosine triphosphate (ATP), adenosine diphosphate (ADP), serotonin, phosphates and high amounts of calcium ions (7). In case of vessel damage, platelets release their granule content, resulting in platelet aggregation. Eventually, aggregated platelets will form a plug clogging the damaged vessel and stopping blood leakage.

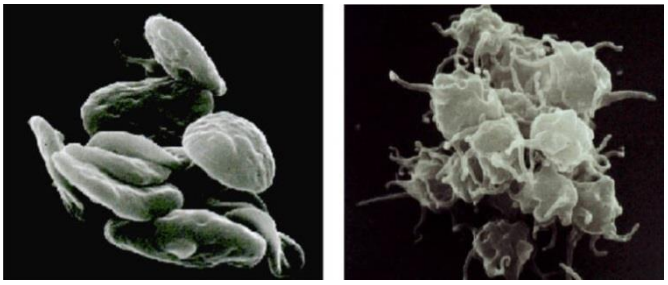


Figure 1. *Scanning electron microscope image of normal (left) and activated thrombocytes (right). Activation leads to major morphological changes of thrombocytes, such as the formation of filopodia. Source: IFM, Linköping University*

The process of the blood clot formation, which prevents hemorrhage, is called hemostasis. Undamaged endothelial cells produce prostacyclin (PGI_2) and nitrogen oxide (NO), preventing any unwanted platelet adhesion, activation and aggregation (8, 9). However, when the endothelium is

damaged, platelets and circulating coagulation factors come into contact with tissue factor (TF), activating the extrinsic pathway (10). Besides the extrinsic pathway coagulation can be trigged via the intrinsic pathway, which involves interaction of platelets with proteins bearing negative charges, such as collagen or with the membrane of other, previously activated platelets. The intrinsic pathway is especially suitable for in vitro initiation of blood coagulation. Both the intrinsic and extrinsic pathways comprise a cascade of zymogen conversions into active clotting factors. Eventually these pathways both converge into the common thrombolytic pathway with thrombin and fibrin as its main regulators (1, 10). Recent studies however show that separating the initiation of coagulation into an intrinsic and extrinsic pathway is arbitrary and that there is actually interplay occurring between these two processes during coagulation.(11)

Blood coagulation occurs in several stages. When a blood vessel is damaged, blood and thus platelets come into contact with various proteins present in the interstitium, but not in the blood vessel itself. Also, because the vessel is compromised, the platelets that leak through the breach are exposed to high shearing forces. Both contact with aforementioned proteins and exposure to high shearing forces induce the first phase of blood coagulation called the initiation or adhesion phase. Interaction between TF (in case of the external pathway) or extracellular matrix proteins (in case of the internal pathway) and the platelets is carried out by platelet receptors (10-12). These integral membrane glycoproteins, classified as integrines, consist of heterodimers, which are linked through a disulfide bond. Ligands of these receptors include vWF, collagen and fibronectin, which bind to glycoprotein (Gp) Ib/Ia, Gp Ia/IIa, and to Gp Ic/IIa respectively (13, 14). Interaction of ligands with these receptors results in partial activation of platelets, which in turn leads to exocytosis of α-granules. Figure 2 provides a schematic overview of the most important signaling pathways involved in platelet activation and aggregation.

The second phase, also labeled the amplification or activation phase, is characterized by platelet activation, which is mainly mediated by thrombin. Binding of thrombin and other previously mentioned proteins to their respective receptors on the platelets induces a conformational change of these receptors. After activation of phospholipase C (15), intracellular calcium concentration is increased through the production of second messengers diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG mediates calcium influx, while IP_3 effects intracellular release of calcium from granules (16). Besides these second messengers, ATP also induces calcium influx by binding to its receptor, the ligand gated ion channel P_2X_1. This increased intracellular calcium concentration induces various cellular changes. First, degranulation and exocytosis of α- and dense granules occur. Next, a conformational change in Gp IIb/IIIa allows binding of fibrinogen (17, 18). Furthermore, platelets undergo morphological changes, such as changes of the cytoskeleton, resulting in the formation of filopodia. Finally, changes in membrane phospholipid distribution can be observed (19).

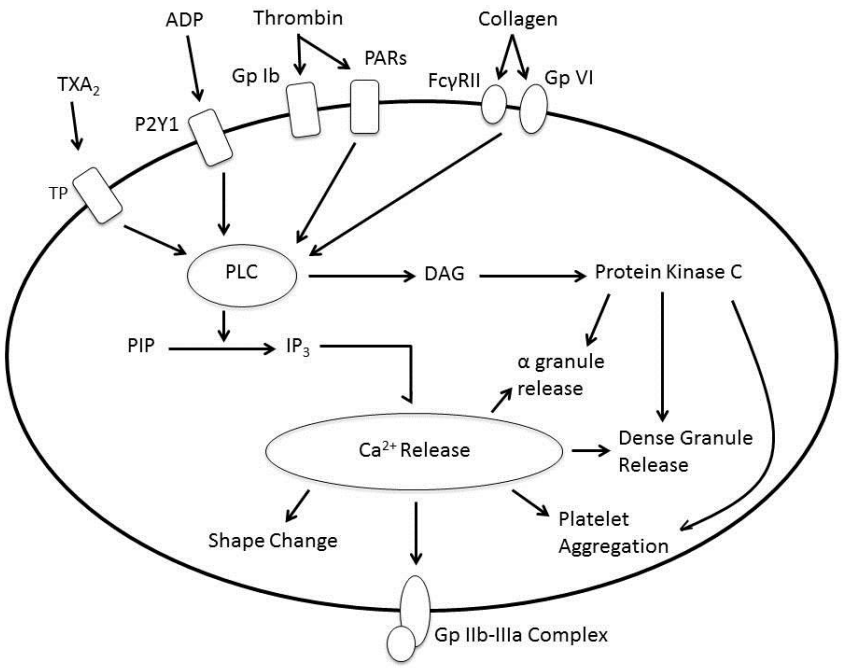


Figure 2. *Schematic overview of important platelet membrane receptors and their respective signaling pathways. Various proteins and molecules activate signaling pathways, ultimately leading to platelet activation and aggregation. 5HT: 5-hydroxytryptamine or serotonin; ADP: adenosine diphosphate; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; DAG: diacylglycerol; Gp: glycoprotein; IP_3: inositol triphosphate; NO: nitric oxide; PAR: protease-activated receptor; PIP: phosphatidylinositol phosphate; PLC: phospholipase C; TP: thromboxane Receptor; TXA_2: thromboxane A_2.*

Lastly, the coagulation process reaches the propagation or aggregation phase, which is characterized by platelet-to-platelet adhesion. Aggregation is facilitated by the filopodia, formed after activation of the platelet. As discussed earlier, the membrane integrin receptor Gp IIb/IIIa binds fibrinogen, which thus serves as the connection between two adjacent platelets (15). vWF can function as a bridge in the same way (20). Apart from fibrinogen and vWF are also junctional adhesion molecules (JAMs), ligand of the CD 40 marker (21, 22), mediators of platelet aggregation. Released signaling molecules, such as ADP, vWF, serotonin and thromboxane A_2 (TXA_2) provide a positive feedback loop by activating other platelets. Platelets will aggregate, ultimately forming a platelet plug, which can cover the rupture in the blood vessel. Additionally, in this phase, fibrinogen monomers are polymerized into fibrin. In vivo, fibrin forms a network into which platelets aggregate, creating a semisolid mass, known as a blood clot or thrombus (23, 24). Though interplay exists, the process of thrombogenesis and that of platelet plug formation are independent processes.

As can be seen, thrombin is one of the central and most important factors during blood coagulation. Also called fibrinogenase, Factor IIa or α-thrombin, thrombin consists of a light chain (MW ~ 6 kDa) and a heavy chain (MW ~31 kDa) (25). As a serine protease, it cleaves amide bonds of other polypeptides using a nucleophilic serine residue (26). Thrombin generation is regulated by factor Xa and factor Va, who together cleave the zymogen prothrombin into thrombin (27). First, thrombin

generation is increased by the conversion of prothrombin by thrombin itself. In a later stage, a negative feedback system ensures the termination of thrombin generation to avoid excessive coagulation.

Extensive research on the interactions of thrombin shows that it is one of the most important platelet activators in vivo, as well as ex vivo (28). When arriving at the platelet surface it binds with high affinity to the Gp Ib receptor (13, 29). This interaction induces a conformational change of thrombin, allowing improved proteolytic capabilities on protease activated receptors (PARs) (29). Thrombin activates platelets by hydrolyzing PAR-1 and to a lesser extent PAR-4, both transmembrane G protein-coupled receptors (30). After cleavage of the N-terminal exodomain, the new tethered N-terminus becomes a ligand, which binds intramolecularly to a recognition site on the PAR itself (31). This activates a transmembrane signaling pathway (32), resulting in a calcium influx that mediates the aforementioned changes in platelet morphology and surface functionalization (33), such as the release of TXA_2, expression of CD 40 on the platelet surface and the activation of the Gp IIb receptor (34). Termination of PAR activation occurs by internalization and subsequent lysosomal degradation (35). Besides PARs, thrombin activates many precursors into activated factors, such as the activation of factor XI (36) and factor VIII (37), with the latter ensuring a positive feedback on thrombin generation. Finally, another important action of thrombin is the proteolysis of the arginine-glycine bond of fibrinogen, creating fibrin monomers (27). These fibrin monomers can then polymerize, stabilizing the platelet plug into a thrombus.

Due to the complexity and high number of factors and agents involved in the process of blood coagulation and more specifically platelet plug formation, a wide range of disorders is classified, in which normal function of this cascade is distorted leading to either hemorrhage or thrombogenesis. Among others, hemophilia and von Willebrand disease are the most known and prevalent diseases and are characterized by patients suffering from excessive internal or external bleeding (11). Besides these diseases, in which coagulation factors are dysfunctional, disorders involving platelet abnormalities are also common. A distinction can be made between disorders whereby platelets are abnormal in function (qualitative defects) and disorders characterized by an abnormal number of platelets present in the patient's blood (quantitative defects). Qualitative defects may be due to abnormalities in receptors, granules or proteins involved in one of the signaling pathways. Often these abnormalities are caused by mutations in the respective genes or aberrations in the post-translational modification processes. Within the class of quantitative defects, two cases are described: thrombocytopenia and thrombocytosis. In case of the former, patients suffer from an abnormally low amount of platelets present in the blood. This condition may be either inherited or induced. Most often, the platelet production in the bone marrow is altered due to nutritional deficiencies, drug associated marrow suppression, excessive alcohol consumption, leukemia or certain viruses (38). Thrombocytosis, a disorder whereby the blood contains an abnormally high amount of platelets, can be inherited or caused by infection, excessive hemorrhage or inflammation (39, 40).

Nowadays, a wide range of blood coagulation tests exists to examine different parts of the coagulation pathway (11). Most important are the prothrombin time (PT) assay, the activated partial thromboplastin time (APTT) and thrombin time. In case of the prothrombin time assay, TF and calcium are added to blood plasma. This test triggers the external pathway and is used to investigate factors involved in the initiation phase. Developed to diagnose and monitor hemophilia, the activated partial thromboplastin time assay is performed by adding phospholipids to blood plasma, thus triggering the intrinsic pathway. This assay provides information about coagulation factors involved in the propagation phase. Finally, with the thrombin time test, the conversion time of fibrinogen to fibrin can be measured.

Alongside blood coagulation tests, which provide information about blood coagulation factors or fibrinogen, platelet function tests assess aggregation or granule content and release of platelets. The most conventional platelet function test is the bleeding time assay. In this in vivo screening test, a constant blood pressure is applied on the arm. Next, a standard cut is made in the arm using a springloaded template device and blood is removed at fixed time intervals. Using this technique, the time for cessation of bleeding is recorded (41). Platelet aggregometry is a test used to measure platelet aggregation (41). To induce aggregation, a platelet activator is added to citrated platelet-rich plasma (PRP). Subsequent aggregation can be monitored with techniques including impedance spectroscopy and luminescence photometry. Platelet aggregometry remains the most widely used test today. More recently, automated technologies have been developed to speed up and facilitate platelet function analysis. The platelet function analyzer: PFA-100® is an example of such an automated device (42). Here, citrated whole blood is exposed to high shear forces by flowing it through a membrane. Coagulation is induced by collagen, present on the membrane. The decrease in flow rate through the coated membrane is monitored as the coagulating blood increasingly clogs the membrane over time. Another automated technique is the Impact® Cone and Platelet Analyzer (41). Here, whole blood is also exposed to high shear and to a plate, coated with extracellular matrix (ECM) proteins. Then, platelet adhesion and aggregation to this plate is monitored. Measuring is done by staining the cells, followed by automated image analysis by the instrument itself. Finally, for research purposes flow cytometry can be used in this context as well (43). Flow cytometry allows quantification of glycoprotein density, granules and granule content.

Although many different blood coagulation and platelet function tests were developed over the last decades, detrimental issues keep research in this area going (41). One issue with the classical, non-automated tests is that they are labor intensive, since the samples need preparation or, in case of the bleeding test, a nurse or physician removing the blood at fixed intervals. For this reason, automated instruments have been developed. Unfortunately, these machines are very expensive regarding purchase as well as maintenance. Furthermore, standardization remains problematic, even though advance has been made by automation. The biggest problem, however, is that none of the previously described tests or techniques delivers a definitive result. Diagnosis requires multiple testing, even further increasing the costs (44). Thus, additional research is needed on the development of a new

platelet function test, which delivers definitive results, is automated and yet inexpensive. Most common platelet function tests are used when blood comes into contact with exogenous materials, for example in case of dialysis or blood transfusion. A risk of blood transfusion is rejection of the donor's blood by the recipient's blood, resulting in platelet activation and subsequent aggregation. Thus, a key feature of a new, automated and cheap platelet function test would be the capability to discriminate between activated and non-activated platelets, in order to monitor the status of the hemostatic equilibrium. The enhanced monitoring capabilities, made possible with this new test, should allow a better and faster intervention during precarious situations, such as blood transfusion or dialysis.

To achieve the previously mentioned criteria for a platelet function test that distinguishes activated from non-activated platelets, a biosensor could be developed. Over the last decade, tremendous advancements in this research area have shown that biosensors could enable cheap, fast and easy detection of a range of analytes, whose detection used to be expensive, difficult or even impossible. IUPAC defines a biosensor as *'a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element'* (45). A more general and less confining definition is given by Mohanty et al.: *'A chemical sensing device in which a biologically derived recognition entity is coupled to a transducer, to allow quantitative development of some complex biochemical parameter'* (46). A biosensor is expected to be cheap in production and robust, meaning that it remains functional, even in extreme environments (i.e. acidic or basic media). Furthermore, measurements of high selectivity and sensitivity are expected. Lastly, these measurements should have a relevant and interpretable output.

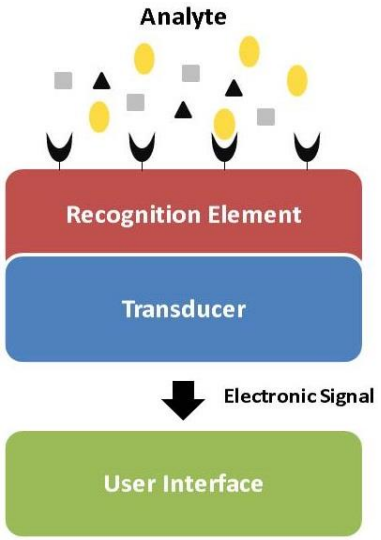


Figure 3. *General scheme of a biosensor. The analyte interacts with the recognition element. This interaction is converted into an electronic signal and processed by a computer.*

The general structure of a biosensor consists of a recognition element, a transducer and a computer. A schematic overview of a biosensor is depicted in Figure 3. The recognition element is a biological entity that binds or converts the analyte (47, 48). Examples of catalytic recognition elements that convert the analyte are enzymes, whole cells or tissues. In this case the consumption of analyte in the solution is monitored. With other recognition elements, it is possible to monitor interactions without consumption of the analyte. In this way, antibody-antigen interactions can be monitored using antibodies. Ionic currents through ion channels can be investigated when employing a receptor as recognition element. Also, nucleic acids can be used to monitor the hybridization process to test their complementarity. It is this component of the biosensor that ensures the selectivity of the sensor.

A transducer converts the interaction of the biological entity with the analyte into an electronic signal, which is suitable for processing (49). Electrochemical transducers do this by measuring the potential difference between reference and indicator electrodes (potentiometric transducers) or by measuring a current (conductometric transducers). Examples of the latter include impedimetric, voltammetric and amperometric transducers. Optical transducers detect photons and include techniques such as surface plasmon resonance, fluorimetry and UV/Vis spectroscopy. Next, magnetic transducers usually employ superparamagnetic nanoparticles onto which receptor molecules are bound. Finally, with microgravimetric transducers, mass loading on a vibrating crystal can be converted into a voltage, since loading decreases the resonance frequency of the crystal. Especially these microgravimetric transducers are of interest for various reasons when developing a biosensor for platelet function testing. First of all, the equipment and materials needed for measurements are inexpensive. Measurements using this type of transducer do not require labeling of the analyte, which facilitates measurements and lowers their costs. Further, these transducers depend on a highly reliable phenomenon and have small power requirements. Both construction and operation of this type of transducer is simple. Finally, high sensitivity is possible using this technique.

Microgravimetric transducers are based on the piezoelectric effect. This effect, first observed in 1880, implies that in certain solid materials, upon exposure to mechanical stress, a voltage proportional to this stress is produced (50). Moreover, the inverse is also true; a voltage applied to such a material results in its deformation. Examples of such materials are crystals, such as quartz, certain ceramics, bone and DNA. Quartz (SiO_2) is used most often because it is non-toxic, not dissolvable and abundantly present in nature as well as because of its high piezoelectric coefficient. Piezoelectric materials have in common their ionic, non-centrosymmetric crystal structure. When the crystal structure is compressed, a net polarization of the crystal arises, leading to a voltage.

When the crystal is connected to an alternating power source, it vibrates at a certain frequency. Piezoelectric materials can oscillate in different ways. In case of a surface acoustic wave, the amplitude is highest at the surface, but dies out when moving towards the bulk of the crystal. Bulk acoustic waves are characterized by a deformation of the entire crystal. Here, the wave propagates in the bulk of the material, with the same amplitude everywhere. Though bulk acoustic waves can be

present in different modalities, the thickness shear mode is the most common mode used for microgravimetric measurements. The amplitude of the vibration is highest when the crystal oscillates at its resonance frequency. The resonance frequency depends on the thickness of the crystal following the relationship:

$$f_0 = \frac{v_q}{2 \cdot d}$$

Equation 1

with $v_{quartz} = 3.34 \cdot 10^3$ m·s^{-1} the speed of sound in quartz, d the thickness of the quartz crystal and f_0 the resonance frequency of the unloaded crystal. It follows that the resonance frequency decreases when the crystal is thickened by for example adding a thin film. Thus mass loading onto the resonating crystal decreases the resonance frequency. Using this phenomenon, small changes in mass on the crystal, can be detected. This idea was first postulated by Sauerbrey in 1959 (51):

$$\Delta f = \frac{-2f_0^2}{A\rho_{quartz}v_{quartz}} \cdot \Delta m = -\frac{\Delta m}{m_0} \cdot f_0$$

Equation 2

with A the active surface area of the balance, $\rho_{quartz} = 2.65 \cdot 10^3 \frac{kg}{m^3}$ the density of quartz and m_0 the mass of the unloaded crystal. This equation provides a quantitative relationship between a frequency drop and mass loading onto the quartz crystal. It is the foundation for a quartz crystal microbalance (QCM). As the name suggests, this balance employs quartz as a resonator to detect small mass changes on the crystal surface. The first measurements with QCM were done in gas phase. However, more recently, also measurements in liquids became possible. Measuring in liquids phase complicates the relationship, as now viscosity and density of the liquid also play a role.

This work is part of a project, which aims at developing a biosensor that assays platelet function. This biosensor should be able to discriminate between activated and non-activated platelets. In the first stage of the project, in which this work is situated, the goal is to induce platelet aggregation on QCM crystals. For reasons discussed earlier, QCM was chosen as a transducer for this biosensor. Due to its significant role in the coagulation cascade, thrombin is chosen as a recognition element and is covalently immobilized on a gold electrode of a QCM crystal. Consequently, platelets passing over the surface are expected to bind to thrombin resulting in their activation. Calcium ions will then be used to enhance platelet activation leading to platelet aggregation.

2 Experimental

2.1 Materials

(3-Aminopropyl)triethoxysilane (APTES) (≥98%) was purchased from Sigma-Aldrich. Calcium chloride ($CaCl_2$) was obtained from VWR. Glutaraldehyde (50%) was supplied by Amresco. Bare gold SPR sensor chips (12 x 12 x 0.3 mm) were purchased from XanTec Bioanalytics. Brilliant gold paste GGP 2093 (12% gold content) was obtained from Heraeus. Quartz crystals (10 MHz, AT-cut, 14 mm diameter) were purchased from Great Microtama Electronics. 3-Mercaptopropionic acid (MPA) (≥98%), N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) were used as received from Merck. Components of phosphate buffered saline (PBS) were purchased from Applichem (sodium chloride), VWR (potassium chloride) and Merck (sodium dihydrogen phosphate dihydrate and potassium dihydrogen phosphate). The pH of PBS was adjusted to 7.2. Bovine thrombin was purchased from Merck. Blood was extracted in Vacuette® tubes, obtained from Greiner Bio-One and containing 3.2% sodium citrate.

2.2 Blood

Whole blood samples were extracted from healthy volunteers at the University of Vienna. Platelet-rich plasma (PRP) was obtained by centrifugation (Eppendorf Centrifuge 5702) of whole blood samples for 20 minutes at 14 x g. The supernatant constitutes the PRP and was separated from the pellet and subsequently diluted 1:20 in PBS (10 mM). Blood samples were centrifuged for ten minutes at 1898 x g to obtain plasma. Here again, supernatant was separated and diluted 1:20 in PBS (10 mM). These diluted PRP and plasma samples were stored in a fridge at 4°C for a maximum of three days. In the remainder of this work, the terms plasma and PRP refer to diluted plasma and PRP (1:20 each).

2.3 Quartz Crystal Microbalance

Dual electrode crystals were made by screen printing gold paste. The electrodes in the liquid phase were grounded and had twice the diameter of the electrodes on the other side of the crystal. Next, the crystals were placed in an oven at 400°C for five hours. The resonance frequency and damping of the crystals were checked on a network analyzer (HP 8572C). For the measurements, crystals were placed in a homemade measuring cell with a volume of 250 µL. A home-built oscillator circuit was used and the oscillator frequency was measured with an Agilent 53131A Frequency Counter. Frequency readout and processing was done using self-programmed software based on LabView. In Figure 4, pictures of the QCM setup used here are shown. The entire setup, displayed in Panel A comprises of a power source, frequency counter, oscillator circuit, flow cell and computer. A close-up of the flow cell is shown in Panel C, while Panel B depicts the dual electrode QCM crystals used here.

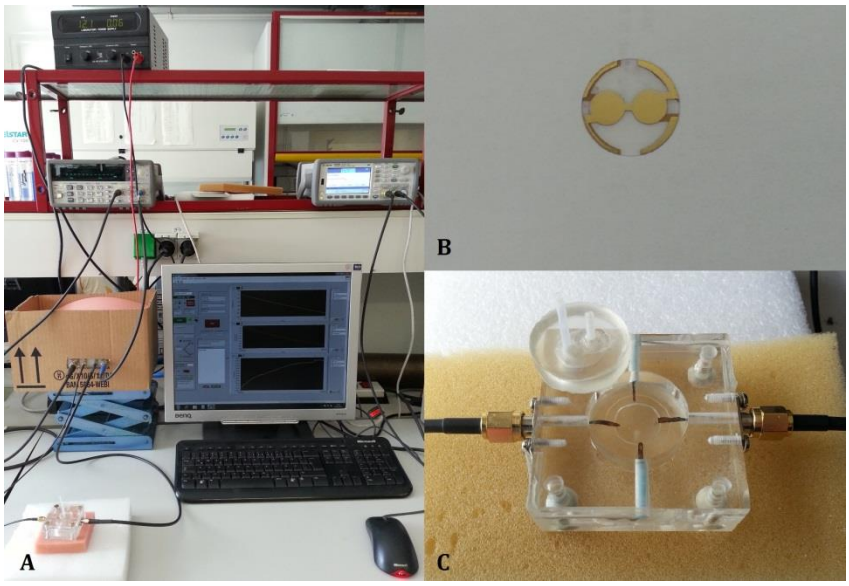


Figure 4. *Pictures of the QCM setup used for measurements.* ***Panel A*** *shows the entire setup containing a power source, frequency counter, oscillator circuit, flow cell and computer with software. A close-up of the flow cell is shown in* ***Panel C****. A dual electrode QCM crystal is displayed in* ***Panel B****.*

2.3.1 Functionalization of Quartz Crystal Microbalance Crystals

First, bare crystals are functionalized with carboxyl groups by immersing them in 5% (v/v) MPA in water for two hours at room temperature. Subsequently, crystals are rinsed with water and dried. To mediate the binding of the carboxylic acid of the MPA monolayer with the amine groups of thrombin, EDC and NHS are used. The crystals are incubated in an EDC (100 mM)/NHS (50 mM) solution for 30 minutes at room temperature. Then, the crystal were rinsed with water and dried. The surface is now functionalized with dry-stable NHS-esters, which will react with the amines of proteins, such as thrombin. A more detailed description of EDC coupling can be found in the appendix.

2.3.2 Quartz Crystal Microbalance Measurements

All fluids used for QCM measurements were heated to 37°C before injection into the flow cell. All measurements were done in stop-flow conditions. First, the resonance frequency was allowed to stabilize. To analyze the response and its reversibility of each of the different fluids, a series of experiments was conducted in which the specific component was added to the flow cell and the cell was subsequently rinsed. First of all, binding of thrombin was investigated by injecting thrombin (140 U/ml in water) in PBS (10 mM) and thereafter rinsing with PBS (10 mM). The same experiment was done on a crystal, which was not functionalized with EDC/NHS. To evaluate the response of plasma, thrombin and then plasma were added and the cell was subsequently rinsed with PBS (10 mM). Finally, thrombin (140 U/ml), plasma and PRP were injected one after the other with followed by rinsing with plasma and PBS (10 mM).

To investigate blood coagulation and platelet aggregation on the surface, first, thrombin (140 U/ml) in PBS (10 mM) was injected into the flow cell. Next, plasma was added and thereafter PRP. Calcium was added in the form of $CaCl_2$ solutions, which were filtered prior to injection. Different $CaCl_2$ concentrations (50 mM, 75 mM and 100 mM in water) were used to evaluate the influence of calcium concentrations on coagulation. To check for reversibility, washing steps were carried out using plasma and PBS (10 mM).

Control experiments were conducted to elucidate whether coagulation and platelet aggregation were occurring in the volume of the flow cell or on the surface of the QCM crystal. For this purpose, the protocol described in the previous paragraph was carried out on a crystal only functionalized with MPA and not with EDC/NHS. Furthermore, an experiment was done, in which thrombin and PRP were added together in the flow cell. After adding $CaCl_2$ (75 mM), the cell was flushed with PBS (10 mM). To probe the effect of $CaCl_2$ on plasma, a last control experiment was performed on a crystal functionalized with MPA and EDC/NHS. Here, thrombin (140 U/ml) and plasma were added consecutively. Thereafter, $CaCl_2$ (75 mM) was added and the cell was subsequently washed with PBS (10 mM).

2.4 Light Microscopy

Samples were investigated with a Nikon Eclipse LV100 Microscope, using different magnifications (10x, 50x, 100x). Images were processed and analyzed with NIS-Elements D 2.30 Imaging Software.

2.4.1 Functionalization Microscope Slides

To oxidize the surface, microscope slides (10 x 10 x 1 mm) were immersed in a piranha solution (3:1 H_2SO_4:H_2O_2) for 15 minutes at room temperature. After rinsing with water and drying, the slides were placed in an oven to dry at 110°C for one hour. Subsequently, the samples were functionalized with amine groups by incubating in a 6% APTES solution in water-free toluene for twelve hours. After rinsing first with toluene and then with tetrahydrofuran, the slides were placed in an oven at 100°C for one hour to maximize the number of ethoxy groups bound to the surface. Next, the slides were incubated in a 2.5% (v/v) solution of glutaraldehyde in PBS (3.8 mM) for 30 minutes in order to functionalize the amino groups and hence generate carbonyl functionalities on the surface. After rinsing with PBS and drying, the slides were put in a thrombin solution (140 U/ml) for two hours. Rinsing with water and PBS (10 mM) again, the next step included depositing a drop of PRP on the glass slides. Different PRP concentrations were used: pure, 1:10, 1:20 in PBS (10 mM). After one hour, the slides were spin coated for 25 seconds at 1000 rpm. One slide was not functionalized with glutaraldehyde, so thrombin is not able to immobilize on this slide. Before applying PRP onto the slide, thrombin was added to PRP, so that the latter had a final dilution of 1:15 in PBS. A droplet of this mixture was then deposited on this particular microscope slide.

3 Results & Discussion

3.1 Quartz Crystal Microbalance

In a first series of experiments, the response of QCM towards each fluid was investigated by adding the particular fluid and washing with either plasma or PBS (10 mM). First, the interaction of thrombin with the functionalized surface was evaluated. After the frequency had stabilized in PBS (10 mM), thrombin (140 U/ml) was added onto either a crystal functionalized with MPA or with both MPA and EDC/NHS. To check if thrombin was covalently bound, washing steps with PBS (10 mM) were carried out. Figure 5 shows the frequency shift as a function of time during this protocol.

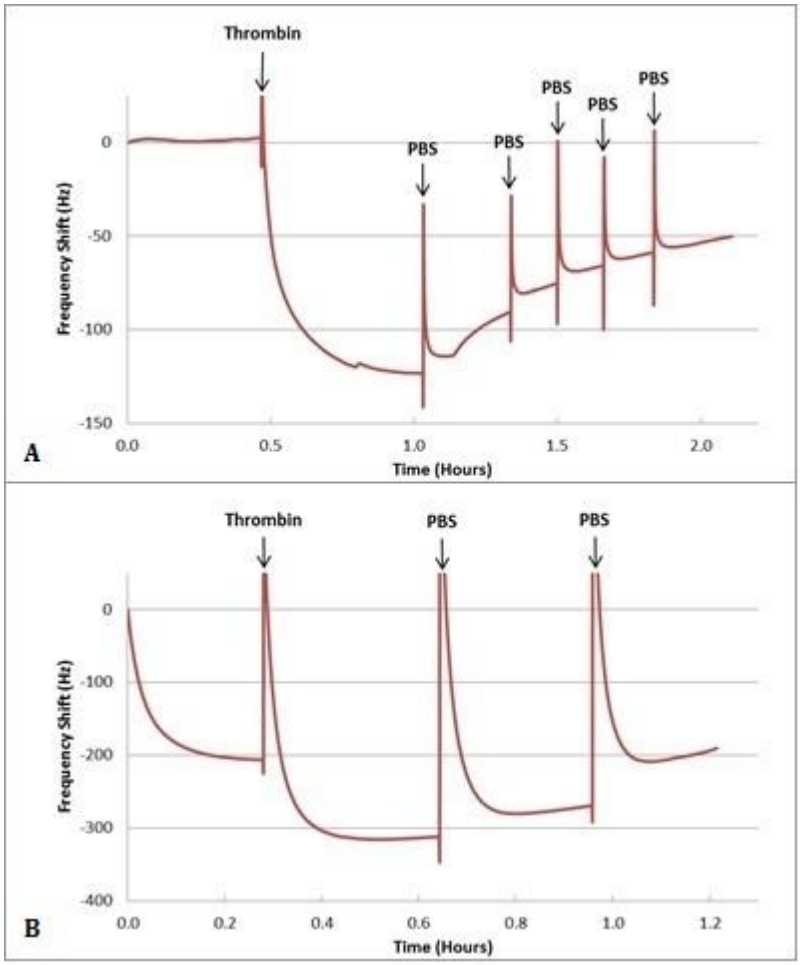


Figure 5.*Frequency response as a function of time after adding thrombin. Washing steps were carried out with PBS (10 mM). The crystal used for the measurement shown in* ***Panel A*** *was functionalized with MPA and EDC/NHS. Thrombin induces a frequency drop of ~ -125 Hz. After washing with PBS, the frequency recovered to ~ -50 Hz below the initial baseline. In* ***Panel B****, the measurement is shown for which the crystal was functionalized with only MPA. Here, thrombin causes a drop of ~ -100 Hz and two washing steps recover the frequency to the initial baseline.*

For the measurement in Panel A, a crystal was used, which was functionalized with MPA and EDC/NHS. Due to the presence of the NHS-ester, stable covalent bonds should be formed between thrombin and the surface. It can be seen in this measurement that thrombin induces a frequency drop of ~ -125 Hz. After washing five times with PBS, the frequency stabilizes at ~ -50 Hz below the initial frequency. This indicates that unbound thrombin was washed away and the remaining thrombin was covalently bound via an amide bond to the modified crystal. This immobilized thrombin will later serve to bind platelets in PRP. In case of Panel B, a crystal was used, which had only been functionalized with MPA and thus should not allow covalent bonds with thrombin. After adding thrombin, a frequency decrease of ~ -100 Hz can be observed, which is ~ -25 Hz less than the measurement in Panel A. Only two washing step are needed to bring the frequency back to the initial baseline. Even though thrombin adheres to the surface here, the interaction is non-covalent, resulting in thrombin being washed away by PBS. These results show that by functionalizing the gold electrodes of the QCM crystal with both MPA and EDC/NHS, thrombin can be covalently bound through an amide bond to the surface.

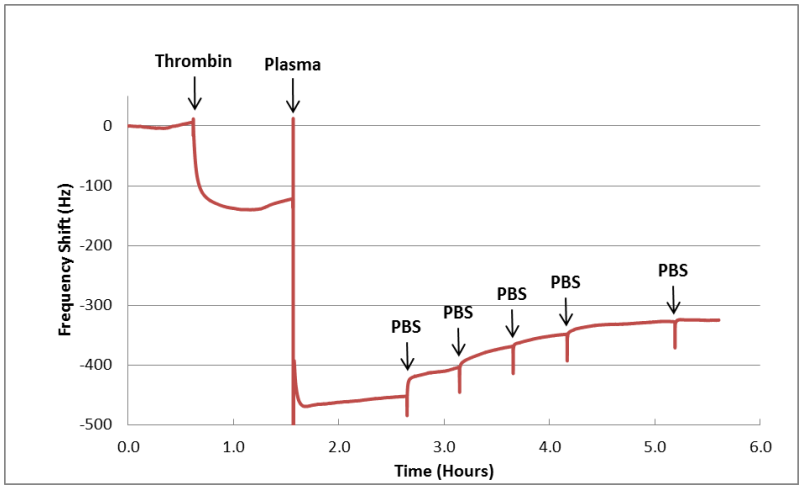


Figure 6. *Frequency response as a function of time after adding thrombin and plasma. Washing steps were carried out with PBS. Plasma induces a frequency drop of ~ -350 Hz. Subsequent washing shows that plasma interacts irreversibly with the surface, since the frequency does not recover to the baseline.*

The interaction of plasma with immobilized thrombin and the functionalized crystal was evaluated by adding thrombin and thereafter plasma. Here, also five washing steps were carried out with PBS (10 mM). By adding thrombin, the frequency drops by ~ -125 Hz, as was shown previously. Figure 6 depicts the frequency shift in function of time upon injecting thrombin, plasma and PBS. A frequency drop of ~ -350 Hz can be observed when plasma is injected. After washing, the frequency retained a value of -325 Hz relative to the initial frequency. This indicates that some plasma proteins adhere irreversibly to either thrombin or the crystals themselves. Most plausible is that fibrinogen, which is converted to fibrin by thrombin, is the protein that constitutes this irreversible frequency drop. However, this hypothesis should be tested by adding plasma on a crystal without thrombin being present and subsequently washing the cell with PBS.

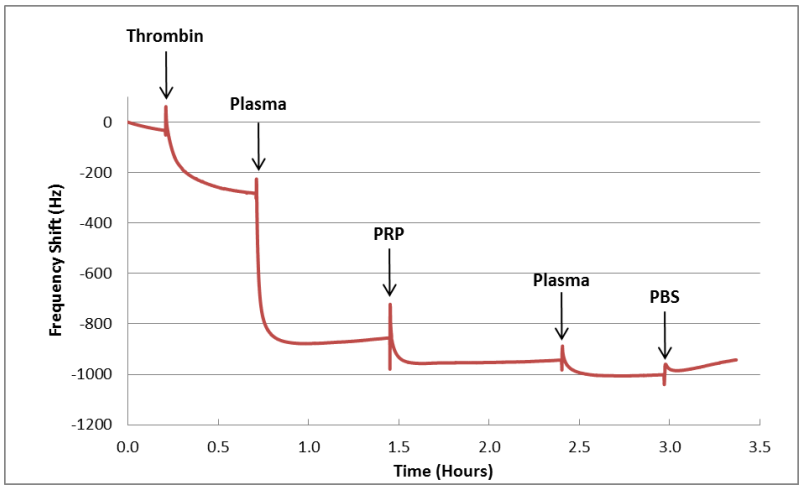


Figure 7. *Frequency response as a function of time after injecting thrombin, plasma and PRP. Thrombin and plasma cause similar drops as seen earlier. PRP causes a drop of ~ -100 Hz. Upon washing with plasma the frequency drops by ~ -50 Hz. Finally, washing with PBS increases the frequency.*

Finally, the effect of PRP was evaluated. Here, thrombin, plasma and PRP were added consecutively. The cell was washed afterwards with plasma and PBS (10 mM). By injecting plasma before and after PRP, only the interaction of platelets with thrombin or with the crystal can be observed, since the presence of platelets is the only difference between plasma and PRP. Figure 7 shows the frequency change as a function of time during this sequence. Thrombin and plasma give rise to a frequency drop of ~ -250 Hz and ~ -550 Hz respectively. These values are larger than the values observed in the two previous figures, which may be due to variance in surface properties of the gold electrodes between different crystals. When adding PRP, the frequency decreases with another ~ -100 Hz, meaning that either platelets or more plasma proteins bind to the surface. Upon washing with plasma, the frequency drops by ~ -50 Hz. Lastly, washing with PBS slightly increases the frequency. However, the frequency remains under the baseline established after adding PRP, indicating the irreversible binding of plasma proteins or platelets onto the surface.

The main goal of this work was to induce blood coagulation and platelet aggregation on QCM surfaces. For this purpose, the flushing protocol discussed in the experimental section was used. Thrombin, plasma, PRP and $CaCl_2$ are injected consecutively. Calcium is expected to induce platelet aggregation together with thrombin. Here, three different $CaCl_2$ concentrations were used: 50, 75 and 100 mM. To probe reversibility of the interactions, plasma and PBS (10 mM) were used to wash out unbound proteins and platelets. The frequency responses in function of time of these three protocols are indicated in Figure 8. Administering thrombin and subsequently plasma results in frequency drops as previously described. PRP induces a decrease in frequency of ~ -100 Hz. When $CaCl_2$ is added in the lowest concentration (Panel A), no drop occurs and the frequency stays at a constant value. After washing with plasma and PBS, the frequency decreases with ~ -175 Hz and ~ -80 Hz respectively. A $CaCl_2$ concentration of 50 mM in water is apparently not sufficient to enhance platelet activation and aggregation. However, injecting a higher $CaCl_2$ concentration (75 mM) does result in a frequency drop

of ~ -550 Hz (Panel B). The interaction occurring at the surface is irreversible, since washing with plasma does not change the frequency and washing with PBS (10 mM) increases the frequency with only ~ -150 Hz. Upon adding a $CaCl_2$ solution with a concentration of 100 mM the frequency decreases with ~ -4000 Hz. Washing with plasma results in an increase of ~ -1500 Hz and with PBS an increase of ~ -1000 Hz. Again here, interactions are partly irreversible since the frequency remains at a frequency of ~ -2000 Hz.

Increasing $CaCl_2$ concentration from 75 mM to 100 mM, increases the frequency shift with ~ -3450 Hz. The frequency shift between the initial baseline and the frequency after washing for the three different $CaCl_2$ concentrations (50 mM, 75 mM and 100 mM) are ~ -875 Hz, ~ -1100 Hz and ~ -1950 Hz respectively. The difference in this final shift between 50 mM and 75 mM is only 225 Hz, while the difference between 75 mM and 100 mM is 850 Hz. Thus, increasing the concentration from 50 mM to 75 mM increases the frequency shift with a factor 1.26. Increasing the concentration from 75 mM to 100 mM increases the frequency shift with a factor 1.77. In order to predict which $CaCl_2$ concentrations will result in which frequency drops, future experiments should be addressed at using different concentrations.

In all three cases, coagulation takes place on the surface. While a $CaCl_2$ concentration of 50 mM has no effect, using concentrations of 75 mM and 100 mM enhances coagulation. These results suggest that the $CaCl_2$ concentration administered is correlated to the amount of coagulation occurring at the surface. However, at this point it cannot be concluded with certainty yet that platelet aggregation occurs on the surface. The observed drops might be due to either plasma protein interactions, such as fibrin polymerization, or platelet aggregation.

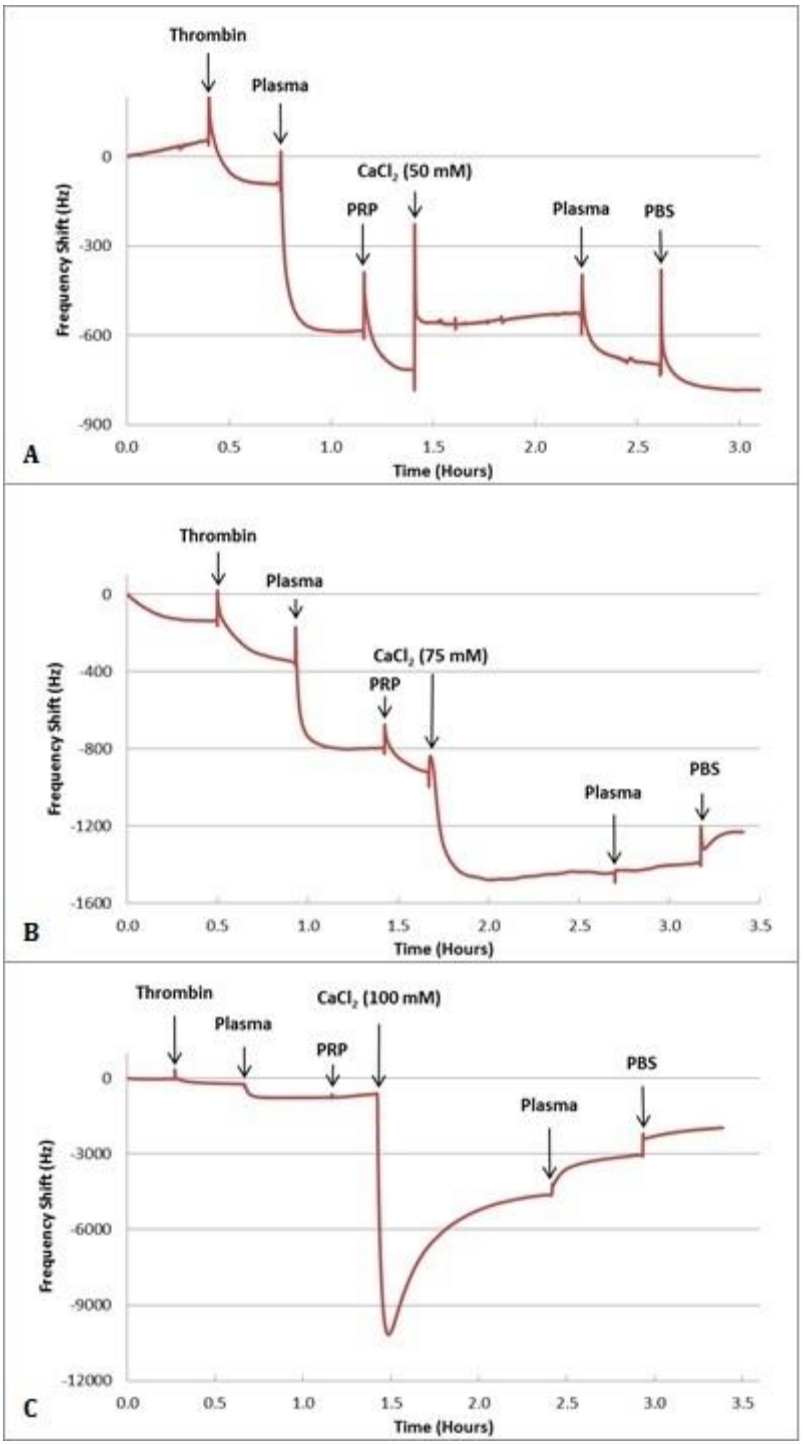


Figure 8. *Frequency response as a function of time after administering thrombin, plasma, PRP and $CaCl_2$.The cell was washed with plasma and PBS. Thrombin, plasma and PRP show similar frequency drops as seen earlier. Different $CaCl_2$ concentrations were added: 50 mM (**panel A**), 75 mM (**panel B**) and 100 mM (**panel C**). In case of a 50 mM solution, no frequency drop can be observed, while a 75 mM solution induces a frequency drop of ~ -550 Hz. Adding a 100 mM solution of $CaCl_2$ results in a drop of ~ -4000 Hz. Washing with plasma and PBS shows that the interactions in all three cases are irreversible.*

In order to draw accurate conclusions from the measurements displayed in Figure 8, control experiments are needed to elucidate what is happening on the surface during the respective experiments. A first control experiment was carried out with crystals that were only functionalized with MPA. Due to the absence of NHS-esters on these crystals, no covalent bonds between thrombin and the surface should be formed. The protocol was the same as during the measurements in Figure 8. The frequency change plotted as a function of time is shown in Figure 9. Thrombin causes a frequency drop of ~ -200 Hz, which is similar to drops seen on crystals that are functionalized with EDC/NHS. Plasma induces a smaller frequency decrease (~ -400 Hz) while adding PRP results in a larger drop (~ -350 Hz). Injecting $CaCl_2$ (75 mM) leads to a decrease of ~ -750 Hz. Compared to the drop on the crystal with thrombin covalently bound (~ -550 Hz), adding the same concentration (75 mM) results in a larger drop when thrombin is not covalently bound. However, washing with plasma and PBS (10 mM) shows that the interactions observed are partly reversible, since the frequency returns to a value of ~ -500 Hz compared to the initial baseline. During this measurement, coagulation took place, but rather in the volume of the measuring cell than on the surface. This shows the necessity of functionalizing the crystals with EDC/NHS to ensure covalent binding of thrombin to the surface. Only in this manner can coagulation take place on the surface.

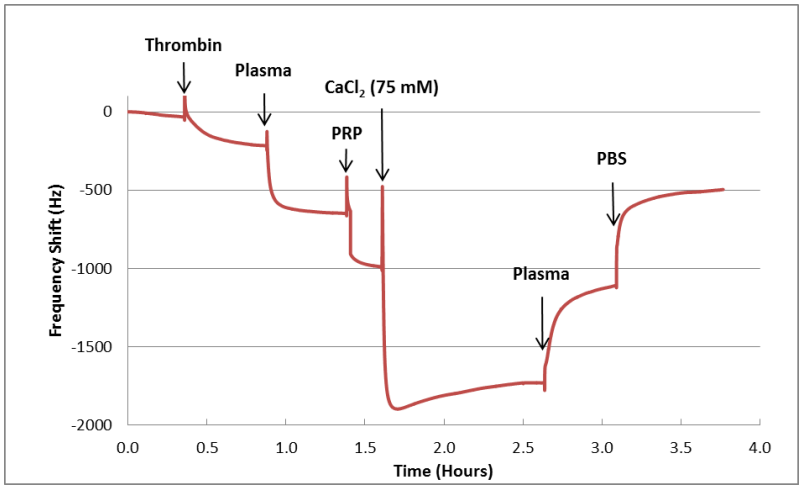


Figure 9. *Frequency response as a function of time after injecting thrombin, plasma, PRP and $CaCl_2$ (75 mM) into the cell containing a crystal, which has not been functionalized with EDC/NHS. Adding $CaCl_2$ results in a frequency drop of ~ -750 Hz. After washing with plasma and PBS the frequency returns to ~ 500 Hz under the initial baseline, indicating that the coagulation occurs not on the surface.*

In another control experiment, with which coagulation in the volume of the flow cell was examined, thrombin and PRP were injected together in the flow cell. Also in this case, the crystal was only functionalized with MPA and not with EDC/NHS. By adding platelets together with thrombin, platelets would already be partially activated upon entering the cell. After adding $CaCl_2$ (75 mM), the cell was flushed with PBS (10 mM). The relative frequency shift plotted against time is shown in Figure 10. Plasma induces a frequency drop of ~ -300 Hz, similar to drops induced by plasma as seen earlier. Thrombin and PRP cause a drop of ~ -325 Hz. When PRP would be injected after plasma in earlier experiments this would result in a drop between ~ -25-125 Hz, while in the previous measurement thrombin added onto a MPA-functionalized crystal resulted in a drop of ~ -175 Hz. The drop seen here is larger than the combination of an average PRP induced drop and a drop induced by thrombin. This indicates that there is already some coagulation occurring here. Adding $CaCl_2$ to the thrombin and PRP results in a drop of ~ -400 Hz, which is considerably smaller than the drops seen in Figure 8 (panel B) (~ -550 Hz) and Figure 9 (~ -750 Hz). The effect caused by $CaCl_2$ is reversible, since washing with PBS restores the frequency back to the baseline which was present after adding the thrombin-PRP mixture. Thereafter, the frequency remains constant, even after washing. Although coagulation was triggered in the volume of the measuring cell here, either plasma components or platelets interact irreversibly with the surface. For future experiments, adding another flushing step after injecting thrombin could reduce the coagulation triggered in the volume of the measuring cell.

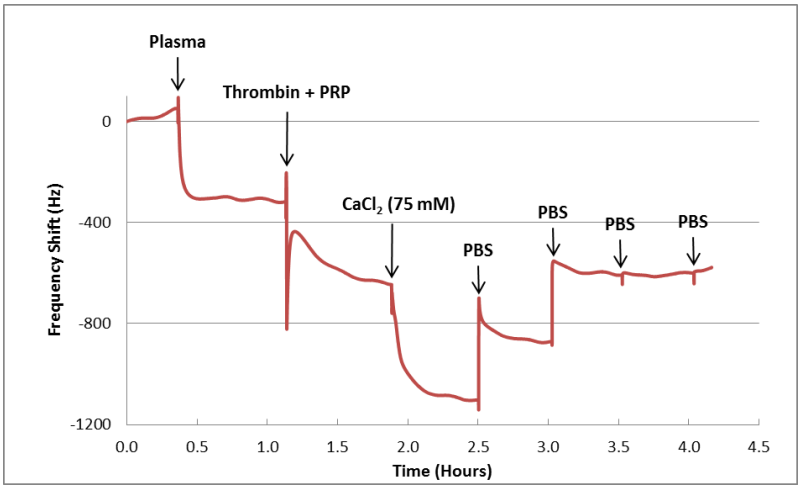


Figure 10. *Frequency response as a function of time after adding plasma; thrombin and PRP; and $CaCl_2$ on a crystal only functionalized with MPA. Adding thrombin and PRP together results in a drop (~ -325 Hz), which is larger than a combination of the drops when thrombin or PRP are added separately, indicating that coagulation is triggered here in the volume of the measuring cell.*

A last control experiment was conducted to probe the effect of $CaCl_2$ on plasma and - more specifically - fibrinogen. Binding to fibrinogen on a specific calcium binding site (52), calcium is an important mediator in fibrin polymerization (53). Therefore, the frequency drops caused by $CaCl_2$ seen in Figure 8 might be due in part to fibrin polymerization either in the volume of the flow cell or on the surface. In contrast with the last two control experiments, for this measurement a crystal functionalized with both MPA and EDC/NHS was used. Thrombin (140 U/ml) and plasma were added subsequently. Instead of injecting PRP, $CaCl_2$ (75 mM) was now added directly. PBS (10 mM) was used as a washing buffer. The frequency as a function of time during this measurement is shown in Figure 11. In this measurement, thrombin causes a drop (~ -75 Hz), which is smaller than the one seen in the previous experiments. Similar as before, adding plasma results in a drop of ~ -400 Hz. $CaCl_2$ induces a drop of ~ -725 Hz. When this is compared to the drop induced by $CaCl_2$ in Figure 8 (Panel B)(~ -550 Hz), the drop observed here is ~ -175 Hz larger. After washing with PBS, the frequency remains at a value of ~ -500 Hz below the initial baseline.

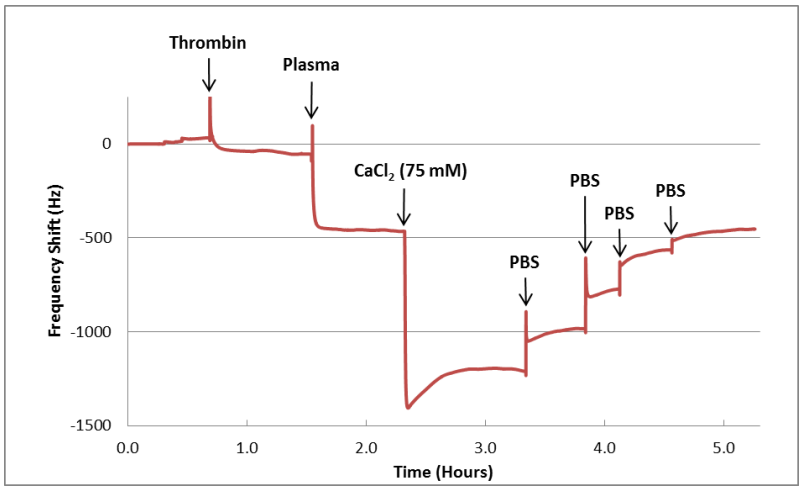


Figure 11. *Frequency response as a function of time after adding thrombin, plasma and $CaCl_2$ (75 mM). Subsequently, PBS was used to wash the flow cell. $CaCl_2$ induces a drop of ~ -725 Hz. After washing, the frequency remains at a value of ~ -500 Hz below the initial baseline.*

In the measurement shown in Figure 2, the frequency stayed at ~ -325 Hz below the initial baseline, probably due to thrombin-mediated conversion of fibrinogen into fibrin, which then adheres to the surface. This hypothesis is here further supported by the fact that the frequency in this measurement stabilizes at a value ~ -175 Hz lower than in the measurement in Figure 2, due to the presence of $CaCl_2$. This confirms that $CaCl_2$ indeed enhances fibrin polymerization. This hypothesis could also explain the behavior of the frequency in the control experiments done on crystals only functionalized with MPA: washing after the measurements displayed in Figure 9 and Figure 10 results in stabilization of the frequency at similar values (~ -575 and 500 Hz respectively) as in the case of the measurement in Figure 11. This suggests an enhancement of fibrin polymerization and subsequent adherence of fibrin to the surface in all cases. This would mean that fibrin adheres to the surface regardless of immobilized thrombin. However, further experiments will have to confirm whether this is truly so.

Anyway, knowledge of the interaction between $CaCl_2$ and plasma is important in evaluating the measurements in Figure 8. While it is clear that coagulation occurs, it remains yet unclear to what extent platelets or plasma proteins contribute to the frequency drop observed after adding $CaCl_2$ to PRP. Irreversibly bound mass in the measurement in panel B of Figure 8 constitutes a frequency drop of 1100 Hz (this is the difference between initial baseline and stable frequency after washing with PBS). If fibrin polymerization and its adherence to the surface would indeed contribute to the drop by 500 Hz in case of a $CaCl_2$ concentration of 75 mM, then this would mean that, taking into account a contribution of 50 Hz of thrombin, the aggregated platelets contribute 550 Hz to the total drop. Entered in the Sauerbrey equation (Equation 2) and solving for Δm, this equals a mass change of $4.89 \cdot 10^{-7}$ g. Taking the average mass of a platelet into consideration (10 pg), approximately 49000 platelets would be aggregated on the surface. Of course, previous assumptions and calculations are only speculations and further experiments and research is need to investigate the contribution of plasma proteins as well as platelets in the frequency drop.

When comparing frequency drops caused by thrombin, plasma and PRP, it is clear that there are differences in magnitude of the shifts, even though the exact same experimental conditions have been applied. Thrombin-induced drops range from ~ -100-250 Hz with an average of 160 Hz. In case of plasma, the frequency drops of the measurement presented in this work range from ~ -325-550 Hz with an average of 475 Hz. The drops caused by PRP show less variance: ~ -25-150 Hz with an average of 95 Hz. Moreover, beside measurements shown here in this work, many other measurements failed or were not suitable for interpretations due to too large deviations. Future experiments should search for factors that could give rise to this reproducibility problem. One such an important factor may be surface roughness of the gold electrodes on the crystals. The screen printing process by which gold is deposited on the crystal is prone to slight alteration and increased surface roughness. In a first attempt to tackle this problem bare crystals were placed in a chloroform atmosphere for 30 minutes of one hour. This treatment should decrease the roughness. However, preliminary experiments do not show any significant difference between crystals exposed to the chloroform atmosphere or crystals not exposed to chloroform. A new strategy to improve smoothness may be to apply the gold electrode on the crystal by gold sputtering.

3.2 Light Microscopy

Light microscopy was used to determine the feasibility of the approach and examine coagulation on a planar surface and thus provide independent information complementing QCM curves. Glass was cleaned and thereafter functionalized with APTES and glutaraldehyde. Thrombin was immobilized and the slides were subsequently exposed to different PRP dilutions. Figure 12 comprises four light microscopy images (magnification of 50x), each resulting from use of different PRP concentrations. Arrows in each image indicate clusters of platelets. In image A, PRP diluted 1:10 in PBS (10 mM) was used, whereas in image B, PRP was diluted 1:20 in PBS. Undiluted PRP was applied on the slide in image C. In image D, thrombin was not immobilized, but mixed with PRP. Platelets are clearly coagulated into clusters and even some filopodia are visible in image D. Thrombin added to the PRP has activated the platelets, with aggregation as a consequence. In case of immobilized thrombin, also coagulation occurs. When using undiluted PRP, large clusters of platelets are visible on the slide. Besides these clusters, fibrin polymers can be observed. In both cases where PRP was diluted, platelet clusters are visible, indicating that also here immobilized thrombin induces platelet activation and aggregation. It is clear here that thrombin maintains its activity towards platelets and fibrinogen, even though immobilized to the surface. Furthermore, this shows that platelet aggregation is feasible on planar surface triggered by immobilized thrombin.

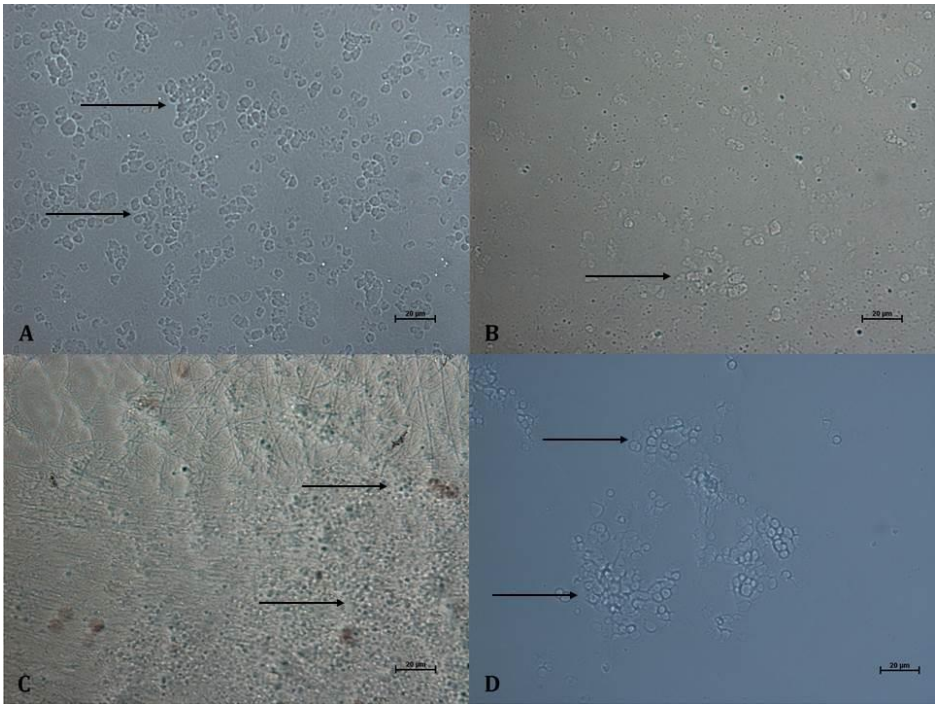


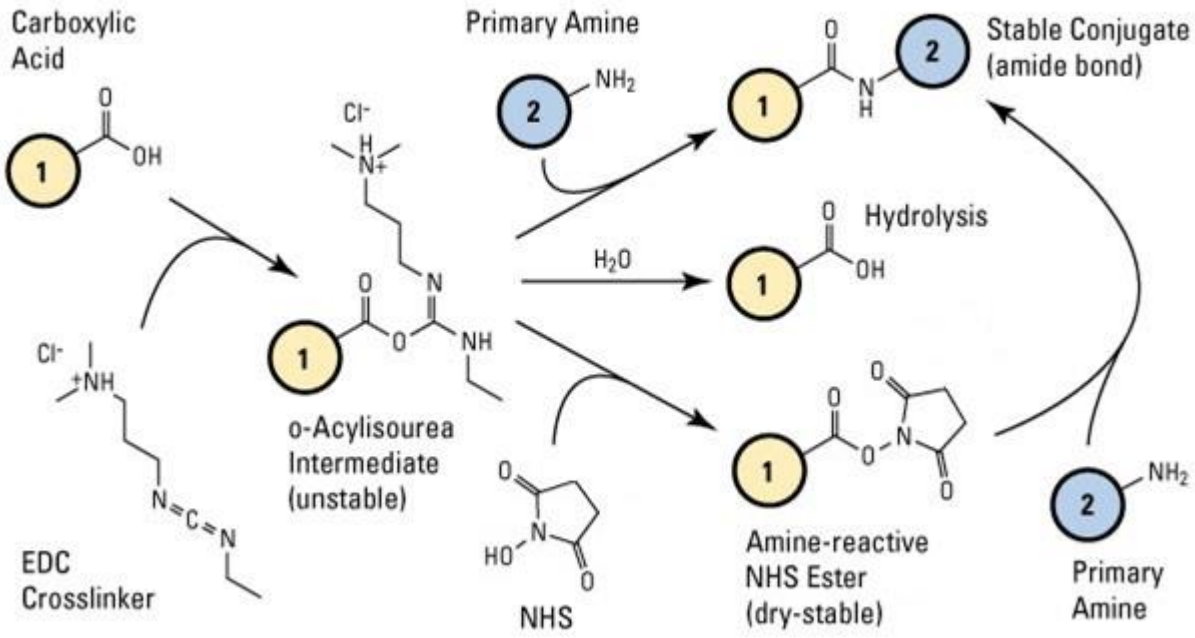
Figure 12. *Light microscopy images of functionalized glass slides onto which PRP has been deposited. All images were taken with a magnification of 50x. Thrombin has been immobilized on the glass slides in image* ***A-C****. In image* ***D****, thrombin was added to PRP before applying it on the slide. PRP was diluted 1:10, 1:15 and 1:20 in PBS (10 mM) in image* ***A****,* ***D*** *and* ***B*** *respectively. Undiluted PRP was used in image* ***C****. Platelet aggregation can be seen in all images. Thus, the capabilities of thrombin as a blood coagulation factor are not affected by immobilization of the protein.*

4 Conclusion

Even though nowadays a wide range of blood coagulation tests and platelet function tests exist, these are often expensive and their results not definitive. Nonetheless, in situations, such as dialysis or surgery, the assessment of platelet function is crucial. For this reason, a new platelet function test is being developed, which will be able to distinguish between activated and non-activated platelets. Since QCM is an inexpensive, simple and highly sensitive technique, it will be used as transducer for the new platelet function test. This work is situated within the first phase of development of such a test and focused on inducing platelet aggregation on the surface of QCM crystals. To trigger platelet activation and subsequently platelet aggregation, thrombin was immobilized on the crystals. QCM crystals were first functionalized with MPA and thereafter with EDC/NHS. Plasma and PRP were obtained from whole blood samples and diluted before use. First, the interactions of thrombin with the functionalized surface were investigated. Results showed that a stable covalent bond was formed between the NHS-ester and thrombin, which is necessary for the following experiments. Plasma induces a frequency drop which is partly irreversible. Adding $CaCl_2$ (75 mM and 100 mM) strongly enhances coagulation, as can be seen in the frequency drops these concentrations induce. Injecting a 100 mM $CaCl_2$ solution elicits more coagulation than when adding the 75 mM solution. By using other $CaCl_2$ concentrations in future experiments, a better understanding of this correlation can be formed. Control experiments show that coagulation also occurs in the volume of the flow cell. Since only platelet aggregation on the surface is desired, an additional flushing step could be added after injecting thrombin to avoid coagulation in the volume. A last control experiment assessed the effect of $CaCl_2$ on plasma. The measurement showed that administering $CaCl_2$ while plasma is present in the cell, results in an irreversible frequency shift, possibly due to the fibrin, which was converted by thrombin and subsequently adhered to the surface. If this would be true, than this means that a portion of the irreversible shift, seen after adding $CaCl_2$ to PRP, can be attributed to fibrin. This means that the portion of the shift caused by platelet aggregation can be calculated and the amount of platelets can be deduced. However, further experiments are needed to support this hypothesis.

In general, results in this work show that coagulation can be triggered on the surface by using immobilized thrombin and calcium. Even though the results here suggest that platelets are aggregating to the surface, this cannot be concluded with certainty yet. More research is needed to definitively prove that platelets are indeed aggregating during measurements with PRP and $CaCl_2$. Further defining the role plasma plays during these measurements is necessary. Finally, improving reproducibility of measurements is needed to allow more definitive results.

5 Appendix



Appendix. Mediation of EDC and NHS in the formation of a stable amide bond. EDC will react with a carboxylic group to form an unstable, amine reactive o-acylisourea intermediate. Subsequently, an amide bond is formed directly when an amine is present. The intermediate might also undergo hydrolysis back to the carboxylic group or the unstable intermediate might react with NHS to form a dry-stable amine reactive NHS-ester. The NHS-ester can then form a stable amide bond.Source: lifetechnologies.com

6 References

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