

Study of methods for platelet function testing in the perspective of lab-on-chip applications

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Study of methods for platelet function testing in the perspective of lab-on-chip applications

PROEFSCHRIFT

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Summary

Study of methods for platelet function testing in the perspective of lab-on-chip applications

Research goals

Platelets are reactive cells with the main function to maintain blood vessel integrity. Altered platelet function can lead to cardiovascular diseases such as thrombosis or bleeding disorders and platelets can also play a role in atherosclerosis. Current methods to quantify platelet function are laboratory techniques such as flow cytometry, aggregometry or luminescent assays. However, these techniques are complex and time consuming. Therefore we are interested in novel methods suited for future application in a lab-on-a-chip format. In our research we have focused on the use of well-established biomarkers, such as membrane marker expression, cytosolic calcium signaling and secretion markers, to quantify platelet activation as well as responsiveness. We report studies on platelet function using these read-out parameters and we discuss the relevance of the results for lab-on-a-chip biosensor research.

Measurement of platelet membrane markers using antibody coated magnetic beads

Magnetic beads are convenient carriers for rapid capture and manipulation of biological cells in a miniaturized system. We have studied the use of antibody coated magnetic beads to measure platelet function via membrane markers expressed upon activation. We used anti-P-selectin coated beads to capture activated platelets from samples stimulated with Thrombin Receptor Activator Peptide (TRAP). The responsiveness of the platelets was analyzed via the remaining unbound platelets in solution and compared to a reference method in which the number of activated platelets is analyzed via fluorescent labeling.

The effective concentration for platelet responsiveness found with the bead capture assay ($17.9 \pm 4.9 \mu\text{M}$) was in good agreement with the effective concentration found with the reference assay ($23.5 \pm 0.4 \mu\text{M}$) in buffer. In 10% plasma the effective concentrations were $14.0 \pm 4.4 \mu\text{M}$ and $13.8 \pm 0.3 \mu\text{M}$ respectively, proving that platelet responsiveness can be quantified using antibody coated magnetic beads. In addition, we showed that we were able to discriminate between non-specific and specific interactions between functionalized beads and immobilized platelet with the use of magnetic actuation. The demonstrated read-out method is an interesting first step toward a lab-on-a-chip system for platelet function testing.

Measurement of calcium signaling to study platelet-surface interactions

Most lab-on-chip biosensor concepts are based on the use of a substrate for immobilization and subsequent detection of the biological system of interest. So for lab-on-chip platelet function testing it is important to understand the influence that a substrate can have on the platelets. We studied the interaction between platelets and various surfaces with calcium signaling in platelets. We used a calcium indicator and studied the response of individual platelets upon

adhesion to Bovine Serum Albumin (BSA), Poly-L-lysine (PLL), mouse immunoglobulin (IgG), anti-GPIb and collagen coated surfaces. We recorded the fraction of cells that increase their cytosolic calcium upon binding to these surfaces. Furthermore, we recorded the delay time between the moments of platelet adhesion or chemical stimulation and the increase of cytosolic calcium.

The experiments showed binding of platelets to PLL, IgG, anti-GPIb and collagen, but not to BSA coated surfaces. We found that under static conditions the number of cells that respond upon binding to an IgG coated surface ($40 \pm 7\%$ with Fc-receptor blocker; $41 \pm 6\%$ without Fc-receptor blocker) was the lowest, followed by adhesion on a PLL coated surface ($74 \pm 7\%$). On an anti-GPIb ($88 \pm 3\%$) or a collagen coated surface ($89 \pm 4\%$), the percentage of responding cells was similar. In addition, we found that the percentage of responding immobilized cells on a chemical stimulus was the lowest on anti-GPIb ($7 \pm 4\%$) or collagen ($6 \pm 4\%$), followed by an IgG coated surface with Fc-receptor blocker ($39 \pm 3\%$) or without Fc-receptor blocker ($35 \pm 2\%$). The percentage of cells responding to chemical stimulation was the highest on a BSA coated surface ($95 \pm 4\%$). These measurements showed that there is a negative correlation between the percentage of cells that respond upon binding and that respond to chemical stimulation after binding. The delay times found upon binding ranged from 11 ± 8 s for platelets on PLL, followed by an anti-GPIb (29 ± 19 s) or collagen (24 ± 21 s) coated surface, and IgG coated surfaces with Fc-receptor blocker (62 ± 19 s) and without Fc-receptor blocker (51 ± 26 s). The delay times recorded on chemical stimulation were probably not sensitive enough to measure the physiological processes in adhered platelets. From the data we conclude that BSA is the most quiescent surface for platelet immobilization, while an anti-GPIb coated surface showed to be as thrombogenic as a collagen coated surface.

Our experiments demonstrate that the analysis of the responding cells upon binding and chemical stimulation can discriminate between different types of platelet-surface interactions. We expect that the discrimination can be further improved e.g. by automated data processing. In conclusion, we find that the measurement of the responding fraction and the response delay time by calcium signaling are convenient methods to study the time-dependent interaction of platelets with surfaces relevant for lab-on-chip applications.

Measurement of exocytosis to study platelet-surface interactions

In our second approach to investigate the interaction between platelets and surfaces, we have studied the secretion process of dense granules of a cell ensemble, as well as single cell exocytosis. Adenosine triphosphate (ATP) secretion was quantified by the luminescent luciferin/luciferase reaction. Platelets were allowed to interact with BSA, PLL, mouse IgG and anti-GPIb coated surfaces, and after incubation the amount of secreted ATP was analyzed. The baseline signal was given by ATP secretion from resting platelets in suspension. ATP levels secreted from platelets immobilized on BSA were approximately 2 times as high as the baseline. The immobilization of platelets on PLL showed about 4 times baseline ATP concentrations. On IgG as well as anti-GPIb the maximum amount of ATP was secreted after 1 hour of platelet incubation. Again, we can conclude that the most quiescent surface for platelet immobilization is BSA, followed by PLL, mouse IgG and anti-GPIb.

We have also studied the immobilization of the enzyme luciferase on a surface, in order to enable the detection of ATP direct at a surface. We have demonstrated that luciferase adsorbed onto PLL is indeed active and generates luminescence in the presence of ATP. However, the assay still has a low sensitivity and needs further optimization.

Finally, we have investigated an exploratory method to resolve exocytosis in single platelet cells, using the fluorescent staining of the dense granules by the quinacrine dye. In our experiments we have observed that the exocytosis events are induced by the fluorescence excitation light. Very probably this is caused by the production of reactive oxygen, which disintegrates the vesicle membrane thereby releasing the vesicle content. Further studies may focus on the use of lower quinacrine concentrations, the use of lower light intensities, or on ways to scavenge reactive oxygen. It will be interesting to further develop the exocytosis assay, as a quantitative method for research on biosensor assays and surfaces suitable for biosensor applications.

Conclusions and outlook

The scope of the research presented in this thesis was to develop knowledge to support future technological developments, aiming at the measurement of platelet activation or responsiveness in a lab-on-chip device. We found that magnetic particles functionalized with specific antibodies can be used to measure platelet responsiveness. We have also studied the interaction of platelet with different surfaces, by an intracellular calcium signaling assay and by an ATP exocytosis assay using luciferin/luciferase. The most quiescent surface for platelets was BSA, but this surface has a low binding affinity for platelets. Anti-GPIb and collagen coated surfaces show stronger binding, but these surfaces change the activation status of the platelets. In view of these results, we have proposed a new concept to measure platelet function in a lab-on-chip device, namely by labeling platelet activation markers prior to platelet immobilization on a surface. We envision that this design will enable the sensitive labeling of platelets as well as the accurate readout of the platelets at a detection surface in the lab-on-chip device.

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Chapter 1

Introduction

Atherosclerosis is a systemic disease that affects almost all people in Western society. To identify patients at risk, the Framingham score is widely used, which is based on population characteristics such as age, gender and body mass index. The Circulating Cells project of CTMM (Center for Translational Molecular Medicine) has the hypothesis that circulating cells may become an additional biomarker for risk estimation in atherosclerosis. In this chapter, we will present the general scope of our research and discuss the role of platelets in the progression of atherosclerosis. The currently established methods to measure platelet function are discussed, as well as the trends toward point-of-care and lab-on-chip devices. At the end of the chapter, an envisioned lab-on-chip design for platelet function testing is sketched and our research goals are formulated.

1.1 Atherosclerosis and risk estimation

In the Western society atherosclerosis affects almost all individuals¹. In the Netherlands, 30% of the causes of death can be attributed to cardiovascular diseases, this means that every day 108 people die as a consequence of cardiovascular diseases²⁻⁵. Atherosclerosis is a progressive disease that develops over the years as a thickening of the intima, which is the inner layer of the artery (Figure 1.1). The thickening can progress slowly into lesions that remain stable, or may develop into so called vulnerable plaques which are prone to rupture. The rupture of a plaque can have major consequences; thrombus formation can occur, or the debris from the rupture can cut off blood supply to vital organs, such as the brains, resulting in a stroke, or to the heart, resulting in a myocardial infarction^{5,6}.

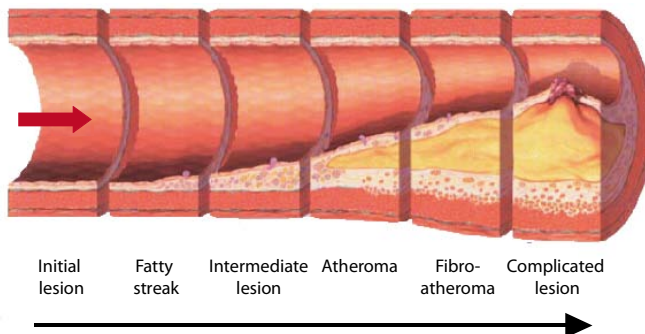


Figure 1.1: Development of atherosclerosis⁷⁻⁹. The development of atherosclerosis starts already in the early years. Initial lesions cause macrophage infiltration and the accumulation of foam cells in the vessel wall. In the fatty streak mainly intracellular lipids accumulate. From about the third decade of a human life the intermediate lesion starts to develop in which small lipid extracellular pools form. These develop into atheroma, in which intracellular lipids are present, as well as a lipid core. Multiple cores can develop over the years, including fibrotic/calcific layers, called a fibroatheroma. In the end, the thickening of the vessel wall can rupture, causing thrombus formation at the complicated lesion site. The figure is adapted from <http://en.wikipedia.org/wiki/Atherosclerosis>.

Due to the high number of people having a cardiovascular disease, there is a need for early detection of patients at risk, so that patients can be treated (e.g. with statins) and lifestyle advice can be given. These precautions have shown to significantly reduce the risk of death from cardiovascular diseases in people with high cardiovascular risk¹. The Framingham risk score is widely used to identify individuals at risk for cardiovascular events^{7,10}. The underlying cardiovascular risk factors were established over the past 60 years, and include age, body mass index, high blood pressure, diabetes, smoking, lipid profile and family history of premature coronary disease^{6,8,11}. However, the Framingham risk estimation is far from perfect and often leads to misclassification. In a large study on patients who had suffered an acute coronary infarction it was shown that only 25% of the patients were qualified for medical treatment based on the traditional risk factors¹². So patients that were classified as low or medium risk were in fact of high risk.

About ten years ago, the view on patient risk assessment has shifted to conditional risk factors⁶. The estimated risk varies with the condition of the vessel wall and the atherosclerotic plaque. The most commonly used technique to determine the condition of a vessel wall is with the use of angiography, in which a contrast agent is injected in the patient to image the blood vessels¹³. The use of angiography is time consuming, expensive and above all, invasive. Therefore new approaches, such as the use of biomarkers, are currently being investigated in order to improve the risk assessment of cardiovascular diseases^{14,15}.

1.2 Circulating cells as a biomarker for atherosclerosis

A biomarker is a substance that is indicative for a disease state. A biomarker can be a molecule of which the concentration changes in correlation with the development of the disease state of a patient. Currently there is no established biomarker that can identify high risk patients for cardiovascular diseases^{6,14,16}. Within the framework of the Circulating Cells project of the Center for Translational Molecular Medicine (CTMM)¹⁷, several research groups are investigating if altered cell function, in particular immune cells, may serve as a biomarker for the initiation and progression of atherosclerosis^{2,18}. Blood from patients suffering from cardiovascular diseases is collected in five major hospitals within the Netherlands. At the University Medical Center Utrecht (UMCU), circulating monocyte-platelet complexes, circulating activated platelets and platelet responsiveness are being investigated as biomarkers for predicting secondary events.

Cells are the most active components that circulate in the cardiovascular system. By contact of the circulating cells with a local injury or atherosclerotic plaque, cells can change their properties. Therefore it is the hypothesis that circulating cells can be used as messengers of the disease state of a patient and are thus potential biomarkers for atherosclerosis. In this thesis we will focus on circulating platelets. In this paragraph we will elaborate on the role of platelets in atherosclerosis and which potential biomarkers are of interest.

1.2.1 Role of platelets in atherosclerosis

Under normal conditions, platelets are small discoid cells that circulate in the cardiovascular system, having little interaction with other platelets or other cells. The main function of platelets is to maintain blood vessel integrity; this process is called hemostasis. Upon vascular damage, platelets become activated. During this activation process, platelets undergo a series of changes including shape change, expression of membrane markers and release of molecules from internal cell storages. These changes contribute to the formation of a platelet plug and later a thrombus, which seals the site of the lesion.

When an instable plaque ruptures, the same process in platelets is triggered as in case of damage of a healthy vessel wall; thrombus formation is initialized. Thus the role of platelets in the late stage of atherosclerosis is clear^{4,19,20}. However, the role of platelets in the onset of the atherosclerotic process much less clear^{4,11,20,21}. In this section, a short summary will be given of the current knowledge of the role of platelets in the early stage of atherosclerosis, as presented in literature^{4,8,11}. Figure 1.2 shows a schematic representation of the role of platelets and monocytes in the development of atherosclerosis.

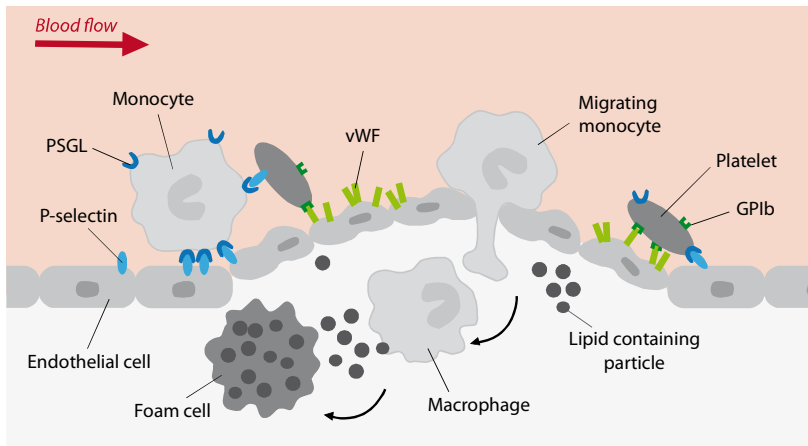


Figure 1.2: Role of platelets in the onset of atherosclerosis⁴. Activated endothelial cells express von Willebrand factor (vWF) as well as P-selectin, which leads to binding of platelets. Immobilized platelets capture circulating monocytes and initiate binding of monocytes to the endothelial cells. Due to the presence of secreted activation markers from the platelets, monocytes transform into macrophages and transmigrate through the layer of endothelial cells. Subsequently, in the diseased vessel wall, lipid containing vesicles are accumulated and these particles are invaginated by the migrating macrophages. The uptake of these oxidized low density lipoproteins transforms the macrophages into foam cells. More chemotactins are released, resulting in an increased number of foam cells. The accumulation of foam cells in the vessel wall can cause an occlusion of the blood flow or can lead to rupture of the plaque. GPIb=Glycoprotein Ib, vWF= von Willebrand Factor, PSGL=P-selectin glycoprotein ligand.

The cells coating the inside of a blood vessel, endothelial cells, are essential for maintaining the delicate balance between blood fluidity and rapid thrombus formation upon vascular damage. This hemostatic balance is maintained by the presence of prothrombotic agents such as Adenosine Diphosphate (ADP) and Thromboxane A₂ that are released by platelets, and anti-thrombotic agents such as Nitric Oxide (NO) and prostacyclin, released by the endothelial cells^{8,11,22}. In diseased arteries, the function of the endothelial cell may be altered, thus the hemostatic balance is changed. Two aspects are considered to be relevant for triggering an altered function of the endothelial cells: (i) the deposition of lipids on the vascular wall that are then metabolized and oxidized in the vascular wall and (ii) the local infiltration of white blood cells. Both events create an inflammatory environment, which affects the function of the endothelial cells. As a consequence, this leads to the interaction of endothelial cells with other circulating cells. For example an inflammatory response can lead to enhanced expression of von Willebrand factor (vWF) on the endothelial cells, which leads to the recruitment of platelets^{4,11}.

The initial interaction of platelets with the activated endothelial cells introduces a loose bond, which causes rolling of the platelets over the surface. This initial interaction is mediated by the expression of P-selectin and vWF from the activated endothelial cells. Their ligands are the P-selectin glycoprotein ligand (PSGL-1) and GPIb respectively, both present on the platelet

membrane^{8,23}. Thereafter, firm platelet adhesion to the endothelium is established via integrin binding, which introduces platelet activation; platelets undergo shape change and secrete different types of molecules from internal storages. These secreted molecules include inflammatory mediators, such as CD40L and interleukin-1 β , which are proteins that are able to further inflame the endothelium. As a consequence, monocytes are recruited to the site.

Via P-selectin, expressed on the cell membrane of the platelet after activation, immobilized platelets are able to facilitate binding of monocytes at the site of lesion. In addition, the monocytes can also interact directly with the endothelial cell that also express P-selectin. The release of platelet factor 4 (PF-4) from the activated platelets, leads to transmigration of the monocytes through the endothelial layers, thereby transforming into macrophages. By the uptake of oxidized low density lipoproteins (LDL), which are accumulated in the diseased vessel, the macrophages transform into foam cells. This introduces a positive feedback loop, thereby increasing the inflammatory response of the vessel wall, thus recruiting more inflammatory cells. The thickening of the vessel wall by the foam cells is called a plaque. In the end, this accumulation of foam cells in the vessel wall can lead to an occlusion of the blood stream, or in case of a vulnerable plaque, a rupture of the vessel wall^{4,8,11,13,19,20,24}.

1.2.2 Platelets as a biomarker for atherosclerosis

Since platelets play an important role in the different phases of atherosclerosis, they are a potential biomarker to identify high risk patients for cardiovascular diseases. Different types of read-out parameters of platelets as biomarker in cardiovascular diseases are suggested in literature, based on their physiologic role in the disease, including: the activation state of circulating platelets^{11,18,25,26}, the number of circulating platelet-monocyte complexes^{21,25,27} and platelet responsiveness^{25,26}.

As described in the previous section (section 1.2.1), platelets adhere to the activated endothelial cells in the initial phase of the development of the plaque. During this process platelets become activated and by the secretion of specific signaling molecules, additional platelets and monocytes are recruited to the site. Not only cells that attach to the site of lesion are activated, several studies have reported that the number of circulating activated platelets is higher in patients suffering cardiovascular diseases than in healthy individuals^{11,18,25,26}. In addition, the circulating activated platelets are able to form a complex with circulating monocytes^{8,11}. Currently, increasing evidence is presented which relates the increased number of circulating platelet-monocytes with cardiovascular diseases^{21,25,27}.

Since platelets play an important role in the primary hemostasis, it is obvious that an altered platelet function can lead to severe cardiovascular diseases. Low platelet responsiveness can cause bleeding disorders, while high platelet responsiveness can cause thrombotic disorders. Bath et al showed that the responsiveness of platelets is enhanced in the acute phase of most strokes²⁶. Also in patients with stable coronary artery diseases, increased platelet responsiveness is measured²⁵. It is hypothesized that the production of the platelets by the megakaryocytes is perturbed in some conditions, resulting in the production of hyperactive platelets²⁶.

The responsiveness of platelets can be measured by a dose response curve in which different concentrations of a stimulating chemical, a so called agonist, are added and the response of the platelets is quantified by measuring the amount of a specific activation marker such as a membrane marker or a secretion marker. Figure 1.3 sketches three typical dose response curves. The middle curve represents a dose response curve of a healthy individual. In patients suffering from thrombotic disorders (left curve), the platelets are hyperactive thus responding at lower agonist concentrations. In contrast, the right curve represents a typical dose response of a patient suffering a bleeding disorder, in which platelets react only on high concentrations of the agonist. With the evaluation of the dose response curve, the platelet function can be tested.

In this thesis, we focus on platelet activation (platelets that express high levels of activation markers such as P-selectin) and responsiveness (the effect that a stimulus has on the activation state of a platelet) as possible biomarkers for cardiovascular diseases.

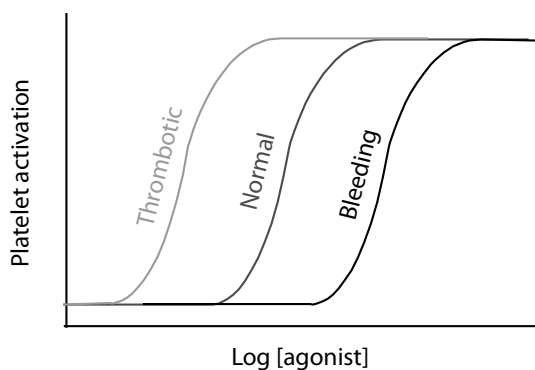


Figure 1.3: Dose response curves for platelet function. The reactivity or responsiveness of platelets is measured by stimulating platelets with different concentrations of a stimulating chemical (agonist). The response can be quantified by measuring specific activation markers such as membrane marker expression or the amount of secreted markers. The middle curve is the dose response of a healthy patient. Patients suffering thrombotic disorders (left curve), the platelets are hyperactive and will thus react already at lower concentrations of the stimulus. Patients suffering from bleeding disorders (right curve), the platelets are less active and thus need higher agonist concentrations before the activation is triggered.

1.3 Established techniques to measure platelet function

Platelet function can not only be evaluated via measurement of a dose response curve. Numerous other tests are available to measure platelet function. In the clinic, these tests are used for example to test the efficacy of antiplatelet drugs, to measure the quality of blood products or to identify patients with cardiovascular diseases^{8,28}. The most common techniques used in the clinic to measure platelet function are discussed in this section, namely bleeding time, aggregometry and flow cytometry.

1.3.1 Bleeding time

The bleeding time test is the oldest clinical tests to measure platelet function. The principle was first described by Milian²⁹ in 1901, but the paper of Duke³⁰ published ten years later had more influence upon medical thinking. Duke showed that patients suffering from a bleeding disorder had an increased bleeding time, which was related with low platelet counts in these patients. Nowadays, the test is done by making a small incision at the anterior surface of the forearm and by measuring the time it takes for the bleeding to stop. Normal bleeding times are between 2 to 10 minutes, whereas for patients suffering from bleeding disorders the bleeding time can increase up to 30 minutes.

The main advantages of the bleeding time test are that it is a direct read-out for the natural hemostasis of the patient, no variables are introduced due to blood sampling or anticoagulation and there is no need for expensive equipment. The major disadvantages of the test are that it is invasive, since a small incision has to be made in the forearm of the patient, the method is poorly reproducible and insensitive^{8,22,31}; in case of a normal bleeding time the test is not able to exclude von Willebrand disease, in case of abnormal bleeding times the clinician cannot predict the specific disorder with the use of this test⁸.

1.3.2 Aggregometry

Aggregometry is a technique to measure platelet function via the study of platelet-platelet interactions. Platelet rich plasma (PRP) is placed in a cuvette which is kept at 37°C and agitated with a stir bar. The light transmittance of the PRP is measured as a function of time, before and during the addition of a stimulus which introduces platelet activation and thus aggregation. Figure 1.4, shows a schematic overview of the working principle of an aggregometer, including examples of typical recorded light transmittance traces during aggregation.

The platelet response depends on the concentration and type of stimulus added. Figure 1.4b shows an example of aggregometry curves of platelets stimulated with different concentrations of adenosine diphosphate (ADP). The bottom curve shows the response of the PRP on a mild stimulation, which is sufficient to induce a conformational change of the platelets' membrane receptor GPIIb-IIIa, enabling platelets to form small aggregates via fibrinogen bridges. However, these aggregates are not stable and disintegrate over time, thereby reducing the light transmission again¹¹. This is called the reversible or primary aggregation. At a certain concentration of the stimulus, the so-called biphasic point is reached, at which the secondary aggregation starts. At this point, the positive feedback inside platelets is started, which enhances the activation processes (see chapter 2 for more details). For ADP this biphasic point is typically reached at a concentration of 2-3 μM ^{22,32}. Increasing the concentration of the stimulus further, increases the secondary aggregation response even further. In the end this leads to irreversible aggregation (top curve in Figure 1.4b), in which platelets are fully activated and form stable aggregates.

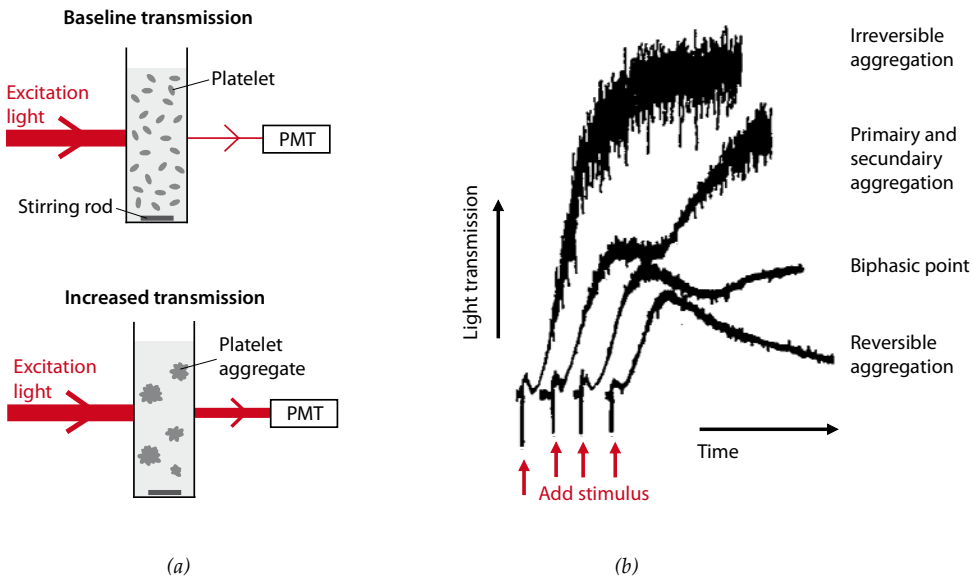


Figure 1.4: Schematic overview of aggregometry measurement. (a) A cuvette filled with platelet rich plasma (PRP) is placed in a light path of the light source and the photomultiplier tube (PMT). The light transmittance of the sample is measured as a function of time as a stimulus is added to the PRP. Platelets form aggregates and as a consequence the light transmittance of the sample increases⁸. (b) Different types of aggregation curves, in which the light transmittance is plotted as a function of time for various stimulus concentrations (in this case ADP). The initial increase in transmittance, which is visible in all curves, is caused by the shape change of the platelets. The bottom curve shows reversible aggregation, in which the PRP is stimulated with a low concentration of ADP. Platelet complexes are formed, however these aggregates are not stable and disintegrate after some time. For higher stimulus concentrations, the aggregation is biphasic, which means that initial stimulus is high enough to start the positive activation feedback loops in the platelets which includes the release of intracellular vesicles. As a consequence, the secondary irreversible aggregation is started. Increasing the stimulus concentration results in increasing secondary aggregation. Aggregometry curves are adapted from Hoffmann et al²².

The use of aggregometry to assess platelet function is highly dependent on the sample preparation and experimental procedure of the laboratory, thus making it sensitive for variations. The main sources of variations include the preparation protocol to obtain PRP, the type and concentration of the stimulus, and the platelet concentration^{8,33}. Compared to most other commercial devices for platelet function testing, the aggregometry test has the advantage that it can determine the specific type of thrombocytopathy or von Willebrand disease. Therefore aggregometry is still considered as gold standard for platelet function testing.

1.3.3 Flow cytometry

Figure 1.5a shows the working principle of a flow cytometer. A traditional flow cytometer uses a sheath flow to focus the cells in the sample volume in the middle of a detection channel. The individual cells pass a laser beam and the fluorophores attached to the cells are excited. By the use of band pass filters the specific emitted wavelength of the fluorophore can be detected in a

photo multiplier tube (PMT). The registered fluorescent intensity is a measure for the amount of fluorophores attached to the cell. The forward scattered light is also measured, which is a measure of the size of the cell that is passing the laser beam. The sideward scattered light is registered under an angle of 90° , which is a measure for the relative density of the cell.

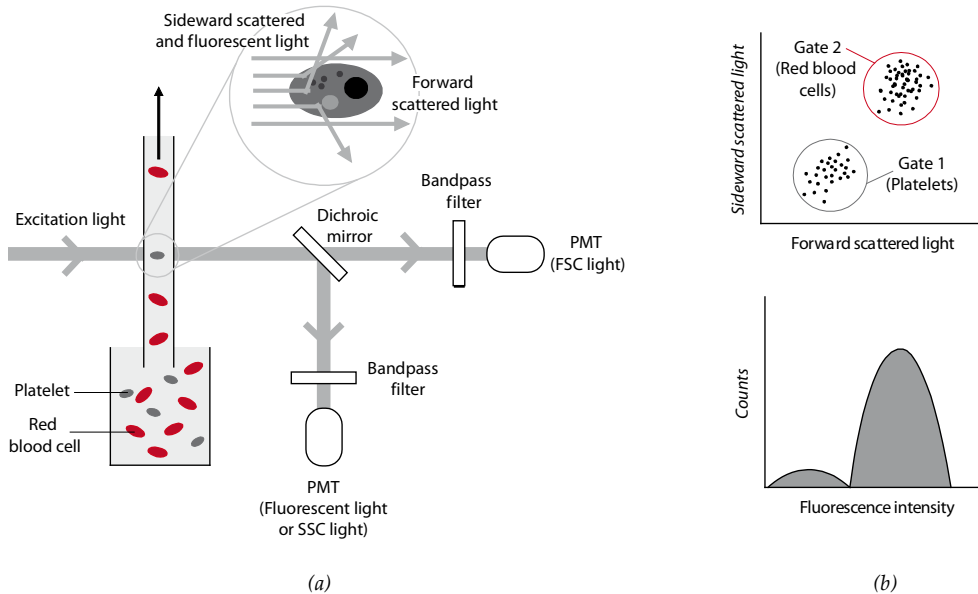


Figure 1.5: Schematic overview of the working principle of a flow cytometer. (a) Cells that are fluorescently labeled travel through a focused beam of the excitation laser. The fluorophores attached to the cells are thus excited and their emission is measured. Simultaneously, the forward scattered (FSC) and sideward scattered (SSC) light are measured. (b) Example of a scatter plot in which the sideward scattered light is plotted against forward scattered light: the sideward scattered light is a measure for the cell density, the forward scattered light is a measure of the cell size. Each dot in the plot represents one cell measured in the flow cytometer. In case of a sample containing red blood cells and platelets, two populations can be distinguished in the plot. Platelets are small cells, and thus appear in the lower region of the plot. Red blood cells are more abundant and bigger and appear in the higher region of the dot plot. Gates can be set around specific regions of interest, per selected subpopulation additional analysis can be done. In the bottom graph, an example is sketched of a fluorescent analysis of a cell population, labeled with a fluorescent antibody. The measured fluorescent intensity is directly proportional to the amount of antibodies bound to the cell. Typically, two populations can be distinguished in which the cells having a low fluorescent intensity express only minor amount of the specific membrane receptor and the population of cells having a high fluorescent intensity expressing high numbers of specific membrane receptor.

In the top graph of Figure 1.5b, a dot plot is visible in which the sideward versus forward scattered light is plotted of a sample containing red blood cells and platelets. Since platelets are smaller and less dense than red blood cells, they appear in the lower part of the plot. When a gate is applied, a subpopulation of the cell ensemble can be selected and analyzed. For example one can analyze the fluorescence intensity of the cells in gate 1 from the dot plot, in this

example, these are the platelets. Two populations can be distinguished in the fluorescent intensity graph: cells having low fluorescence intensity, and cells having high fluorescence intensity.

Since individual membrane markers can be targeted using fluorescently labeled antibodies, the use of flow cytometry in platelet research is versatile. Flow cytometry is for example used to measure the activation state of circulating platelets and their responsiveness to a stimulus or to study signal transduction in the cells. In the clinical laboratories, flow cytometry measurements are performed to diagnose specific platelet dysfunction, to monitor the effect of antiplatelet drugs, to perform quality controls of platelet concentrates in blood banks or to measure platelet counts.

Although the use of a flow cytometer is versatile, the disadvantages are that the instrument is expensive to purchase and maintain. In addition, sample preparation such as anticoagulation, labeling, diluting and fixation are often necessary prior to measurements. This complicates measurements and makes them labor intensive^{8,22,34,35}. Yet flow cytometry is very suitable for research and therefore used in the CTMM study.

1.4 Point-of-care systems to measure platelet function

As discussed in previous sections, the methods which are currently used to measure platelet function have major disadvantages. The bleeding time test is a fast screening test, but the reproducibility and sensitivity are poor. Both aggregometry and flow cytometry measurements need sample preparation, are time consuming and especially the latter technique is expensive. Therefore there is a need for small and simple systems that can measure platelet function.

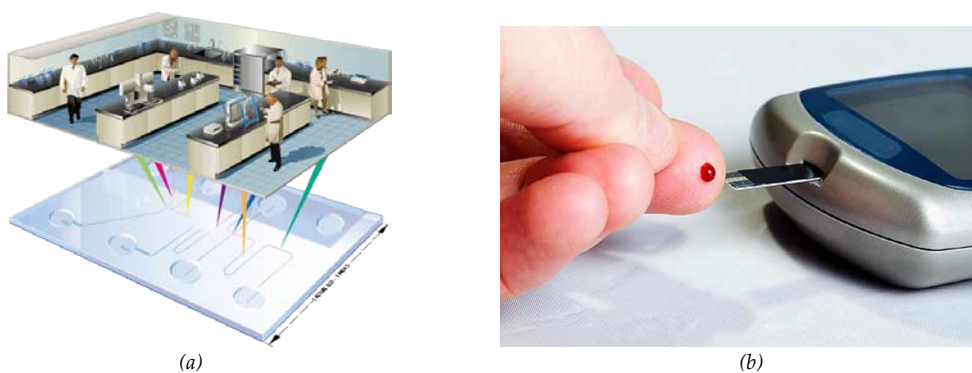


Figure 1.6: (a) Lab-on-chip concept, in which all processing steps, normally performed with large instruments in the lab, are now integrated on a chip. Picture adapted from <http://www.gene-quantification.de>. (b) Example of a glucose biosensor, which can measure the amount of glucose in a fingerstick of blood within a few minutes. Picture adapted from www.elements4health.com.

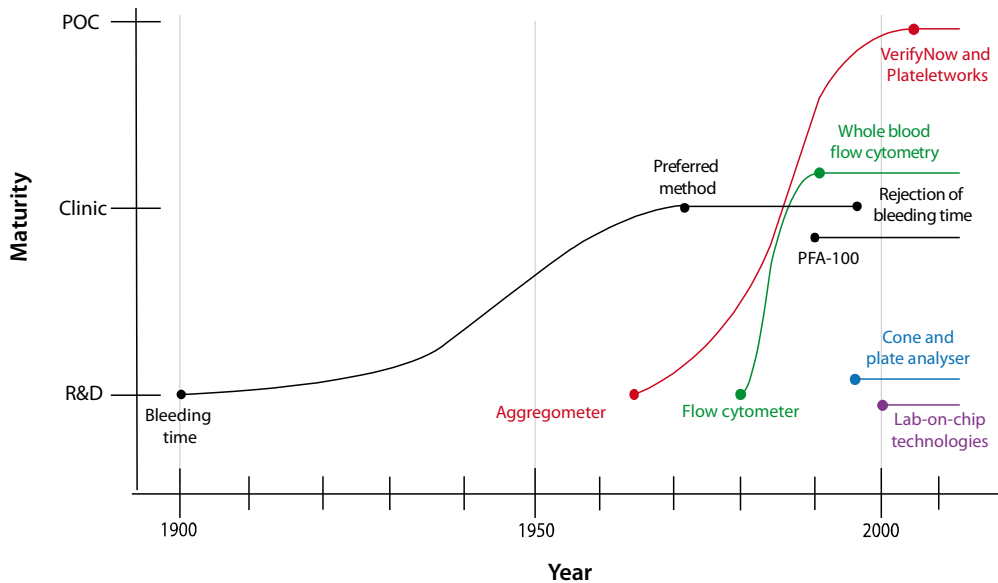


Figure 1.7: Overview of the development of platelet function tests. Bleeding time was the first test introduced in the beginning of the 20th century. Since there was still a lack of understanding about the biological processes in platelets, the time to the clinic was long. After some major publications in the early 70's the bleeding time became the preferred clinical method to screen for bleeding disorders. Meanwhile, the aggregometer and a decade later, the flow cytometer were developed and used in research environments and found their way quickly to the clinical laboratories. At the end of the 20th century, the bleeding time test was rejected after the publication of various papers that showed the poor reproducibility and predictive value of the test. The platelet function analyzer (PFA-100) was developed, based on the same principle as the bleeding time, but now the closure time of a gap in a membrane is measured. The cone and plate analyser was developed in that same period, in which platelet adhesion to a surface is measured as a function of time. The principle of the aggregometer is used in the point of care systems VerifyNow and Plateletworks, developed in the beginning of 2000. Increasing numbers of biosensor designs are presented in literature in the last decades. A few are inspired by established techniques such as flow cytometry or the cone and plate analyzer.

A device that can measure molecules or cells of interest in a body fluid such as blood, saliva or urine in the order of minutes, is called a biosensor. A biosensor is essentially a small lab, integrated on a chip and is therefore also often called a lab-on-chip (Figure 1.6a). Examples of commercially available biosensors are for example the pregnancy test, which measure the hCG hormone in urine, and the glucose sensor (Figure 1.6b), which uses an enzymatic reaction to measure the concentration of glucose in a drop of whole blood.

In the recent 5 years, a lot of progress has been made in the search for a simple and fast platelet function test. For example the VerifyNow system (Accumetrics, USA) and Plateletworks (Helena Laboratories, USA) are commercially available systems that measure platelet function via platelet aggregation^{8,36-39}. Both systems are a big step forward compared to the platelet function tests as presented earlier, since no complex sample handling or data analysis is

necessary to quantify the platelet function of the donor material. However, the VerifyNow system as well as the Plateletworks system only target very specific platelet dysfunctions and both systems still require milliliters of blood, collected from patients via a venapuncture. Therefore, the research in the field of biosensors for platelet function testing is receiving increasing interest in the last decade. The main purpose of the biosensor research is to minimize the amount of donor material needed and to simplify measurements even further.

An overview of the development of platelet function tests, from experimental research to the use in clinical laboratories, is sketched in Figure 1.7. Bleeding time was for a long time the preferred method in the clinic to screen for bleeding disorders. However, with the presentation of increasing evidence that the method was irreproducible and was unable to diagnose many bleeding disorders, the bleeding time test was rejected at the end of the 20th century. The design of the platelet function analyzer (PFA-100) was inspired by the bleeding time test: in the PFA-100, the closure time of a gap in a membrane is measured. The first report of the original aggregometer goes back to 1962, thereafter the technique became accepted in a clinical environment. The Verifynow and Plateletworks are point of care systems that are both based on the aggregometry principle⁸. The use of flow cytometry in research laboratories in the 80's, quickly led to the introduction of the apparatus to the clinical laboratories, especially when the whole blood flow cytometry tests were developed in 1987. Since the beginning of the present century, increasing interest is shown in the development of lab-on-chip designs to measure platelet function. A few of the designs are based upon established methods, such as flow cytometry or cone and plate analysis, but a wide variety of new approaches are also presented.

In table 1.1, we give an overview of the reported methods suitable for lab-on-chip devices for platelet function testing. We have divided the device technologies in four types; the first type is based on platelet adhesion to a surface. Herein, we distinguish different types of platelet binding to a surface namely: binding to a micropatterned surface, binding to a homogeneously coated surface, binding to antibody coated magnetic particles or shear controlled adhesion of platelets to a surface. The second device technology presented in the table is based on the same principle as flow cytometry; cells are fluorescently labeled and the fluorescence intensity of the platelets is measured. The third device technology uses an array of micro posts to measure platelet activation on the basis of platelet shape change. In the last presented device technology, platelets are mechanically activated via the use of shear stresses in a microfluidic device. The detection principles of these methods are shown per column in the table which we have divided into two types of detection: optical (fluorescent labeling and surface plasmon resonance) and piezoelectric resonance.

From Table 1.1, it is clear that nearly all technologies applied in miniaturized systems are optical detection, in particular, by the use of fluorescent labeling. The advantage of fluorescent labeling is that specific receptors or signaling pathways can be visualized. The disadvantage is that sample preparation is needed, prior to the measurements. The biosensor designs which are based on the detection of platelet adhesion by means of surface plasma resonance (SPR) or piezoelectric resonance, have the advantage to work without labeling and both techniques are able to measure real-time platelet adhesion and aggregation. However, both methods are very

sensitive to nonspecific absorption from proteins or other cells from the whole blood sample. In addition, it can be noted from Table 1.1 that many designs for platelet function testing are based on the interaction of platelets with a surface, including our own research on the specific capture of activated platelets with the use of antibody coated particles. The envisioned design which we used as an inspiration for the research of this thesis will be described in more detail in the next section.

Table 1.1: An overview of published device technologies to measure platelet function that are suitable for future application in lab-on-chip devices. The reported techniques are presented in a matrix in which the device technology is presented per row and the detection principle is presented per column. The main research group/university involved in the technology design is presented in the table. The literature includes biosensor technologies reported between 2000 and 2012.

Device technology		Detection		
		Optical		Piezoelectric resonance
		Fluorescent or particle labeling	Surface plasmon resonance	
Adhesion to surface	Microspot array	Sherbrooke Univ.(CAN) ²⁸ Dublin (IRE) ^{40,41}		
	Homogeneous coated surface			Toronto Univ.(CAN) ⁴² Drexel Univ.(USA) ⁴³
	Antibody coated magnetic particles	Eindhoven Univ.(NED) ⁴⁴		
	Shear controlled adhesion	Dublin (IRL) ^{41,45,46}	Linköping Univ.(SWE) ⁴⁷	
Lab-on-chip flow cytometer		Caliper Techn.(USA) ⁴⁸		
Separation by array of micro posts		Princeton Univ. (USA) ^{49,50}		
Shear activation		Melbourne/Victoria (AUS) ⁵¹		

1.5 Envisioned design of a biosensor to measure platelet function

The scope of this thesis is to develop knowledge for a technology to measure platelet function, suitable for application in a lab-on-chip device. Ideally, the device should use only micro liters of whole blood sample and give a read-out within a few minutes.

The possible processing steps of a future biosensor system are sketched in Figure 1.8. The design is inspired by the magnetic biosensor platform, as being developed by the CTMM project partner Philips Research. The Philips system is based on the use of magnetic particles that can concentrate, capture and detect specific molecules from a body fluid. A small amount of fluid is added to the disposable cartridge and by capillary forces the fluid is pulled into the

small reaction chamber. Magnetic particles are present in the reaction chamber and are actuated via external electromagnets. After a magnetic mixing and binding protocol, the biomarkers are sandwiched between the magnetic particles and the sensor surface. The amount of magnetic particles bound to the surface is optically detected and is a measure for the amount of biomarkers present in the sample^{52,53}.

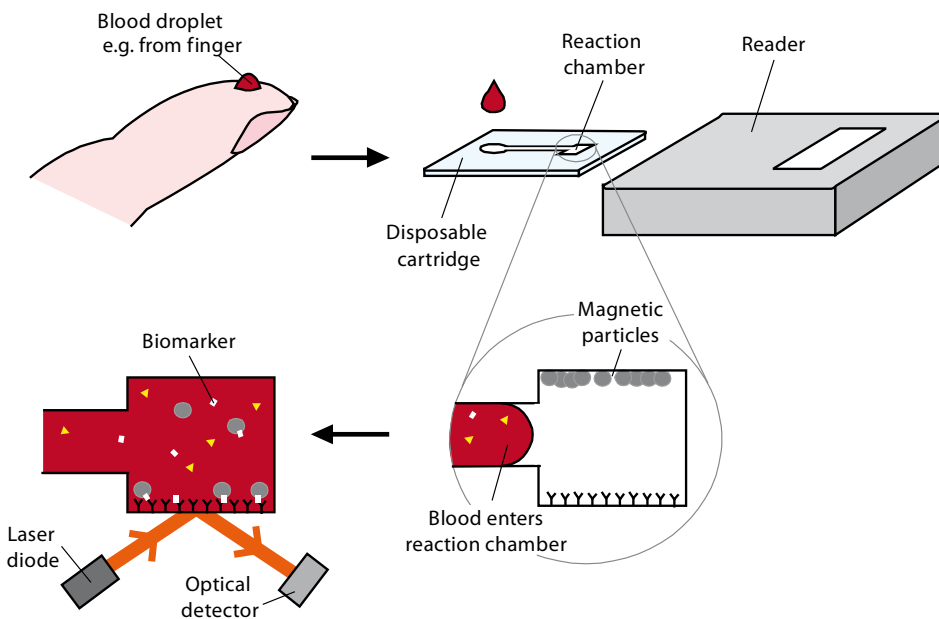


Figure 1.8: Sketch of possible process steps for a biosensor based on antibody-coated magnetic particles: a small blood sample is collected e.g. from the finger. The droplet is transferred to a disposable cartridge, into which the blood is pulled via capillary forces. Magnetic particles that are already present in the reaction chamber are immersed by the incoming fluid flow. The magnetic particles as well as the detection surface are coated with specific antibodies, which bind specifically to the biomarker molecules. After a mixing and binding procedure, the biomarkers are sandwiched at the surface between the bead and the sensor surface. Via optical detection, for example with the use of total internal reflectance principle, the number of bound magnetic particles is measured. This is a measure for the amount of biomarkers in the sample.

The use magnetic particles in a biosensor has the advantage that they can be used as a transport as well as a detection label for the biomarker of interest. Therefore, we have taken the principle of Figure 1.8 as an inspiration for the design of a biosensor to measure platelet function. The envisioned working principle of a platelet function biosensor with antibody-coated magnetic particles is sketched in Figure 1.9. The functionalized magnetic particles are incubated in a reaction chamber in order to capture and label the platelets, possibly accelerated by magnetic actuation (Figure 1.9a). Thereafter, the captured platelets are able to bind to a sensing surface (Figure 1.9b) and via an additional magnetic washing step, unbound magnetic particles can be removed (Figure 1.9c).

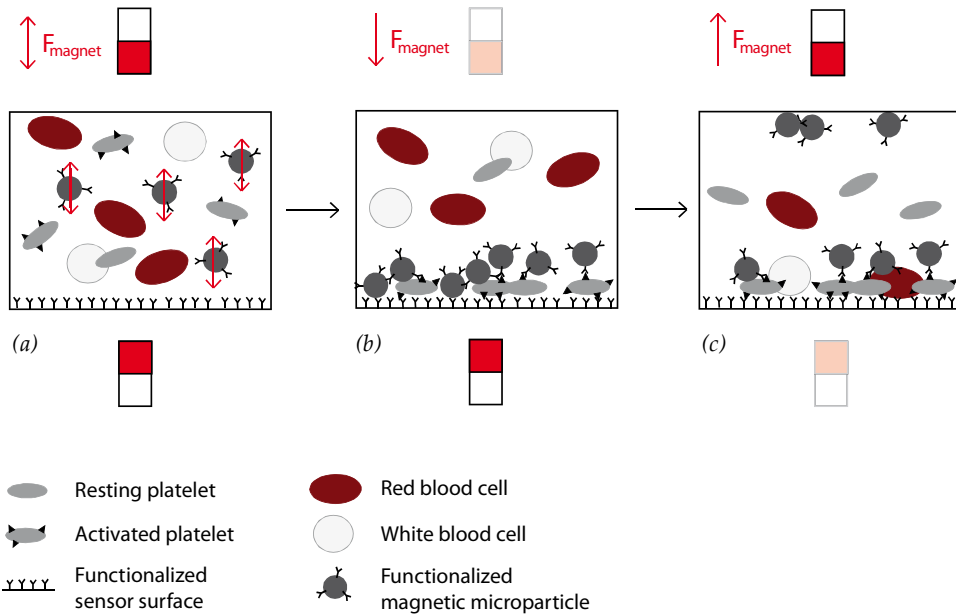


Figure 1.9: Schematic representation of a reaction chamber design for the measurement of platelet function based on the use of antibody-coated magnetic particles. (a) With the use of electromagnets, the magnetic particles are manipulated, to screen the sample for cells of interest, in our case, platelets. (b) The platelets are bound to the magnetic particles and are transported to the sensor surface. The sensor surface is functionalized with antibodies, to immobilize the platelets. (c) A magnetic force is applied by the top magnet, thus removing the unbound magnetic particles.

Like many of the device technologies as presented in Table 1.1, our design is also based on optical detection and makes use of the immobilization of platelets at a surface with the use of antibodies. The latter has the advantage that the system is flexible, since the type of capture antibody can be varied. However, unlike most of the other device technologies presented in Table 1.1, our design lacks externally driven microfluidics since the transport of the cells is based on capillary and magnetic forces. This has the advantage that no pumps or valves need to be integrated and thus the complexity of the final device technology may be reduced.

1.6 Research questions

At the Eindhoven University of Technology, our scope was to develop knowledge to support future technological developments, in order to measure platelet activation or responsiveness in a lab-on-chip device. In our research we used well-established biomarkers, such as membrane marker expression, calcium signaling and secretion markers, to quantify platelet activation as well as responsiveness.

In the first part of the research, we have investigated the use of magnetic particles to measure platelet function (Chapter 3). Therefore, magnetic particles were functionalized with antibodies

against specific membrane markers that are only expressed on activated platelets. Different amounts of stimulus were added to the sample containing functionalized magnetic particles and platelets, and the amount of captured platelets by the magnetic particles was quantified with the use of flow cytometry. The obtained dose response curves were compared with reference curves, in which the platelet activation was quantified by fluorescent labeling.

As pointed out in section 1.4, many biosensor designs for platelet function, including the design in Figure 1.9, are based on the adhesion of platelets to a sensor surface. We wanted to gain more knowledge of the interaction of platelets with different types of surfaces. We used two different approaches to study this interaction. First we used intracellular signaling in platelets to study the interaction of platelets with different types of surfaces (Chapter 4). A calcium indicator, which is a fluorescent molecule that changes its fluorescent properties in presence of calcium, was loaded into platelets and the real-time response upon binding was quantified. After the immobilization of platelets at the surface, the platelet functionality was tested by the addition of a chemical trigger. The response of the immobilized cells was measured and the results were compared with the earlier response upon binding of the cells. In our second approach, we studied the interaction of platelets with surfaces by measuring the amount of secretion markers which are released upon platelet activation (Chapter 5). We used a luminescent reaction with luciferin/luciferase to quantify the amount of secretion markers from an ensemble of immobilized platelets. In addition, we used the fluorescent label quinacrine in order to study single cell secretion events. Both studies were performed on the same sets of surfaces, such that the results of the secretion marker assay could be compared with the calcium signaling assay.

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Chapter 2

Platelets

Platelets are small discoid blood cells that are formed in the bone marrow in the human body. They are shed from megakaryocytes and are often called cell fragments, rather than cells, since platelets lack a nucleus. In this chapter, the most distinct features of platelets will be discussed. First a short overview is given of the formation, circulation and degradation of platelets. Then the morphology of platelets is discussed, which includes the membrane systems, cytoskeleton, granules and organelles. In addition, we will discuss the biochemistry of activation processes as well as the role of platelets in the primary hemostasis.

2.1 Introduction

Hundred fifty years ago when Osler discovered blood platelets¹, he was not sure whether these disc like structures were normal blood elements or exogenous “organisms”. Long it was believed that platelets were passive cell fragments circulating in the cardiovascular system. Nowadays we know that platelets are highly sophisticated cells, responsible for hemostasis and playing a prominent role in cardiovascular and inflammatory diseases.

Unlike most other cell types, platelets lack a nucleus and are therefore often designated as cell fragments, rather than cells. In this chapter we will highlight the most distinct features of platelets, which make them unique in their kind. A short overview of the platelet life cycle during circulation in the human body will be given. The morphology will be presented, in which we discuss the membrane system, cytoskeleton, granules and organelles. In addition, we will discuss the biochemistry of activation processes as well as the role of platelets in the primary hemostasis.

2.2 Platelet formation, circulation and degradation

Platelets are small discoid blood cells that are formed in the bone marrow in the human body. The earliest stage in the production process of platelets is the development of pluripotent stem cells, which have the ability to differentiate into any type of blood cell. The stem cells can differentiate in a lymphoid or myeloid cell, and under specific grow factors such as trombopoietin, the latter cell type differentiates to a megakaryoblast and matures into a megakaryocyte^{2,3}. A 100 years have passed since Wright published the paper which confirmed that platelets are derived from megakaryocytes⁴. Although this view is nowadays widely accepted, the mechanism behind the release process remains controversial. Three mechanisms have been proposed for platelet production:

- Platelet budding: in which platelets are thought to be shed from the periphery of the megakaryocytes cytoplasm⁵.
- Cytoplasmic fragmentation via the Demarcation Membrane System (DMS). The DMS is the precursor of the platelet membrane. In the DMS hypothesis it is proposed that there are preformed platelet-sized fields present within the megakaryocyte, and that release occurs via fragmentation of the megakaryocyte cytoplasm, along DMS fracture lines^{6,7}.
- Proplatelet formation: long and thin cytoplasmic extensions are formed from the megakaryocyte, and at the end of these extensions, platelet-sized swellings immerse. These endings are released, and platelets are formed^{2,8}.

Under normal conditions, the bone marrow in a healthy human produces $2 \cdot 10^{11}$ platelets per day, of which 30% is stored in the spleen and is exchanged with the circulating platelets population. The concentration of platelets in the blood circulation is about 150 to $450 \cdot 10^3$ platelets per μl , making them the second most abundant blood cell (normal red blood cell and

white blood cell counts are $5 \cdot 10^6$ and $7 \cdot 10^3$ per μl respectively). After a lifetime of 9 to 12 days, the platelets are decomposed in the spleen, liver or bone marrow.

2.3 Morphology

At the end of the 19th century, when platelets were first discovered, platelets were thought to be only passive bags of proteins, circulating in the blood stream. Nowadays we know that platelets are highly sophisticated cells, which play a crucial role in hemostasis but also in inflammatory diseases as well as cardiovascular diseases. In this section, we present a short overview of the basic morphological components of platelets including their main function. Figure 2.1 shows a schematic overview of platelet morphology with the components that will be discussed in this section.

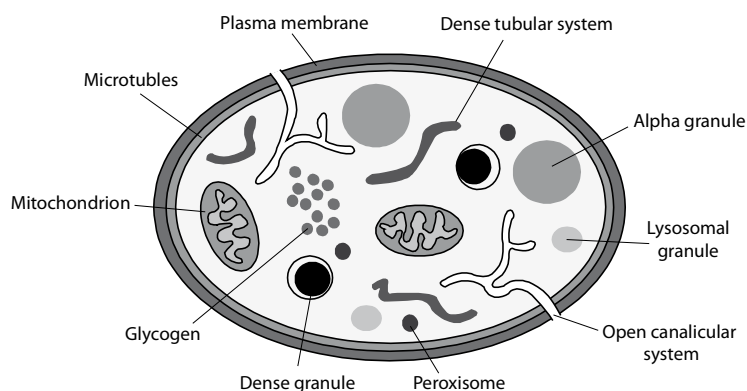


Figure 2.1: Schematic overview platelets morphology^{2,3,9}. Membrane systems include the plasma membrane, coating the outside of the cell, the open canalicular system (OCS), which is a tortuous system that provides additional cell membrane needed for platelets' shape change and the dense tubular system (DTS) that has as main function to store calcium. Different cytoskeleton structures are present inside platelets, mainly to maintain the platelet shape, only the microtubules structure just below the plasma membrane is shown in this schematic representation of the platelet structure. Other skeleton structures present in platelets are the spectrin network, present inside the plasma membrane and coating the OCS as well as the actin network inside the platelets' cytosol. Organelles which can secrete their content include alpha granules, dense granules and lysosomes. Other organelles present inside platelets are peroxisomes, mitochondria and glycosomes, of which the latter two organelles provide the power supply in platelets.

2.3.1 Membrane systems

The outermost part of the platelet is the plasma membrane, which separates the internal content of the cell from the external components. The plasma membrane is a lipid double layer, which is covered at the outside with a layer of absorbed proteins (e.g. glycocalyx). The structure of the platelet plasma membrane is similar to that of other cells. The membrane plays a crucial role in the acceleration of blood coagulation, which is a function not shared with other cells in the blood circulation^{2,3,9,10}.

The shape change observed in activated platelets is provided by internalized membrane for example from the open canalicular system (OCS). The OCS is a tortuous system, which also serves as a gate to transport molecules secreted from organelles into the extracellular space^{3,9}.

In contrast to the OCS, the dense tubular system (DTS) which is also a tortuous tunnel system in the platelet cytoplasm, is believed to be not connected with the plasma membrane of the platelet. The main function of the DTS is to store calcium and to maintain the calcium levels inside a resting platelet in the nanomolar range. In addition, it also serves as a site at which prostaglandins and thromboxane are synthesized. The OCS and DTS are two separate membrane systems, that are only found in platelets^{3,9}.

2.3.2 Cytoskeleton

A complex cytoskeletal system defines the typical discoid shape of a resting platelet and maintains the cell integrity at high fluid shear forces. The cytoskeleton, which is composed of different polymers, is able to rapidly change its shape to adjust to the environment^{3,9}. Figure 2.2 shows SEM images of the morphologic stages from a resting (a) to a fully spread platelet (d) at a surface. The critical components of the cytoskeleton are listed in this section, from the plasma membrane inward.

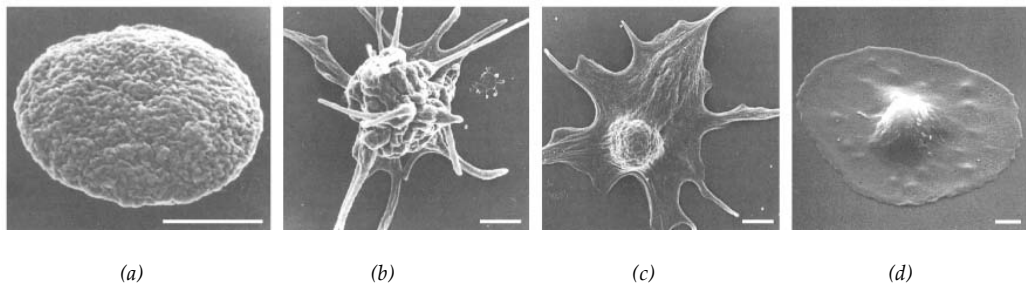


Figure 2.2: Scanning electron microscope images of the stages of platelet shape change*. (a) A resting platelet in its initial disc like shape with a diameter of approximately 2.5-4 μm and a thickness of 1 μm . (b) Dendritic platelet, having surface protrusions (pseudopods) extending in all directions. (c) Early stage of spreading of a platelet at a surface. The cytoplasm has filled in the spaces between the pseudopods, the central body of the plate is still clearly visible. (d) Spread platelet, which can extend to a diameter of about 2-3 times the initial diameter of a resting platelet. The white scale bar in the bottom right corner of the images indicate 1 μm .

The platelet plasma membrane as well as the open canalicular system are supported by a cytoskeletal membrane which coats the cytoplasmic surface of both systems. This system consists of spectrin strands, which are interconnected via actin filaments. The membrane glycoproteins are embedded in this cytoskeletal network^{3,9}.

* Reprinted from Platelets, 2nd edition, A.D. Michelson, Chapter 3: Platelet structure, p47-48., Copyright (2007), with permission from Elsevier

The microtubule coil, which is situated just beneath the plasma membrane, is the most distinguishing feature of a platelet. It is believed that only one single microtubule is present in a platelet, with a length of 100 μm . To fit this into the platelets' cytosol, the tubule is about 8 to 12 times wound into a coil. The main function of the microtubule coil, which consists of polymers assembled from tubulin dimers, is to maintain the platelet discoid shape in resting state^{3,9}.

Actin is the most abundant protein present inside platelets. About 2 million copies are present per platelet, of which about 40% assembles in actin polymers that form the cytoskeleton of the resting cell. The rest of the actin remains stored in the cytoplasm of the platelets and is also converted into fibers upon activation to drive the cell spreading. The major cross-linking proteins that form these actin filaments into a rigid cytoskeleton are filamin and α -actinin^{3,9}.

2.3.3 Granules and organelles

In platelets three types of secretory organelles exist, namely alpha granules, dense granules and lysosomes. Upon activation the content of these granules is released into the extracellular space, and this plays a crucial role in hemostasis, thrombosis and vascular remodeling. This release process will be described in more detail in Chapter 5.

The alpha granules are the most numerous organelles present in the cytosol of platelets; about 40-80 alpha granules can be present in a single platelet. Alpha granules have a diameter of 200 to 500 nm and contain von Willebrand factor, fibrinogen GPIIb-IIIa and many other proteins. Dense granules are less abundant in platelets; only 3 to 8 dense granules per platelet are present. The content of dense granules consists mainly of small molecules and comparatively few proteins. Ions such as calcium and magnesium are present in the dense granules, nucleotides adenosine diphosphate (ADP) and adenosine triphosphate (ATP) and a transmitter molecule serotonin¹¹. Even less abundant are the lysosomes in platelets; only a few lysosomes are present per platelet. They are slightly smaller than alpha granules and contain degradative enzymes such as cathepsin, aryl sulfatase and acid phosphatases. The primary function of the lysosomes is to break down material which was ingested into the platelet via phagocytosis, but the role of the platelet lysosomes in hemostasis remains unknown^{3,9,10}.

The energy supply of the platelet during their circulating in the blood stream is mainly provided by the mitochondria. Although there are only a few mitochondria present inside human platelets, they contribute significantly to the energy metabolism of the cell. Under an oxygen-rich environment the energy needed by the platelet is provided by the breakdown of glucose and lipids in the mitochondria. In addition, the glycosomes can supply energy in absence of glucose. The main organelle content of the glycosomes is the molecule glycogen, which is known to serve as long-term energy storage in cells^{2,3,9}.

Another platelet organelle, which is less well described, is the peroxisome. Inside peroxisomes the enzyme catalase is present which synthesizes phospholipids, such as platelet-activating factor.

2.4 Platelet activation

In this research project platelet activation plays an important role, since it is used to study platelet responsiveness as well as platelet-surface interactions and is thought to have a predictive value for cardiovascular diseases. In this section an overview will be given of the activation process of platelets, which is divided into two sections. In the first section, the biochemical process is described, followed by a physiological description of the *in vivo* activation processes which lead to the formation of a platelet plug.

2.4.1 Biochemistry

Although a wide variety of platelet stimulators exist, almost all stimuli act via coupling to a receptor on the platelets membrane. This first messenger cannot cross the plasma membrane and therefore transmits a signal to the inside of the cell via receptor clustering, receptor cleavage or electric charge to activate a second messenger. Figure 2.3 shows a schematic overview of the most important signaling cascades upon receptor activation in platelets.

Almost all platelet activators work via an elevation of cytoplasmic calcium levels, which is released from the intracellular calcium storages. The released calcium is needed to initiate other processes, for example to dissolve the tubuline such that the platelet changes shape, starts the conversion of soluble actin to fibrous actin which anchors glycoproteins in the membrane, induces translocation of phosphatidylserine (PS) to the outside of the plasma membrane to increase the procoagulant activity of the platelet and to activate of protein kinase C (PKC), which starts granular release and conformational changes in GPIIb-IIIa. The latter is also shown in Figure 2.3.

Strong platelet activators such as thrombin or collagen are able to complete the activation process in platelets via calcium and other signaling pathways. However, mild platelet activators such as adenosine diphosphate (ADP) or low concentrations of thrombin rely on two major feedback mechanisms that enhance the activation signaling: production of thromboxane A₂ (TxA₂) and release of ADP. These two feedback loops are marked with red arrows in Figure 2.3.

The cytosolic calcium content of the platelet is elevated via receptor coupling of the stimulus, this activates phospholipase A₂ which releases arachidonic acid from the phospholipids located at the inside of the platelets membrane. Arachidonic acid is converted by the enzyme cyclooxygenase into thromboxane A₂ which is a strong platelet activator as well as a vasoconstrictor. In addition, the increased cytosolic calcium and activation of protein kinase C leads to secretion of granule content. As a consequence ADP is released from the dense granules, which binds to one of the platelet ADP receptors (P₂Y₁ and P₂Y₁₂) and is able to induce activation^{2,3,9,12,13}.

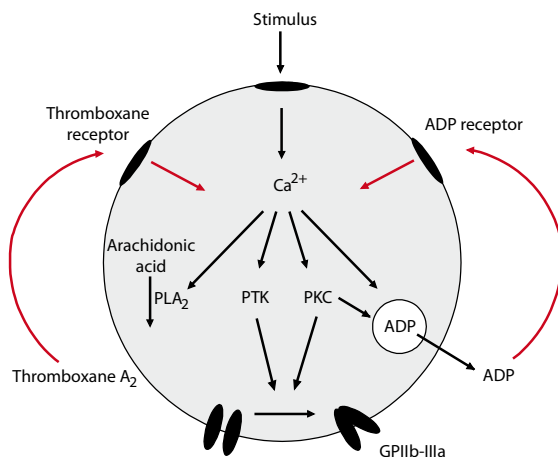


Figure 2.3: Positive feedback mechanisms in platelet activation^{2,12,13}. A stimulus binds to a specific membrane receptor; this receptor transmits a signal across the plasma membrane, which causes an elevation of the cytosolic calcium. As a consequence, a whole variety of enzymes is activated. For example, phospholipase A₂ (PLA₂) is activated, which releases arachidonic acid from the platelet membrane. The latter is then converted into thromboxane A₂. In addition, phosphotyrosine kinases (PTK) and protein kinase C (PKC) introduce a conformational change in the GPIIb-IIIa receptor, allowing binding to fibrinogen. The release of adenosine diphosphate (ADP) from dense granules is triggered by elevated cytosolic calcium levels. Both ADP as well as thromboxane A₂ are platelet stimuli themselves, introducing a positive feedback (indicated in the figure with red arrows), thus accelerating platelet activation. The figure was adapted from Hoffman et al² and modified.

2.4.2 In vivo platelet activation

Under normal conditions platelets circulate in the cardiovascular system without having interaction with other platelets or cells. In the presence of red blood cells, which are the most abundant cells in blood, the platelets are pushed toward the vessel wall. Due to secretion of nitric oxide as well as prostaglandins from the endothelial cells, a so-called thrombotic balance is present, which inhibits platelet activation and binding to healthy endothelial cells (Figure 2.4a). The main function of the platelets is to maintain the blood flow. Therefore they are involved in the primary hemostasis where they form a platelet plug upon vascular damage. The formation of the platelet plug can be divided in three overlapping stages: initiation, extension and perpetuation.

Initiation can occur in two ways; via collagen or thrombin exposure. Upon vascular damage, collagen is exposed to the blood stream. Von Willebrand Factor (vWF), which is present in blood, first attaches to the collagen. These molecules form long multimers that are able to bind to platelets via the GPIb receptor present at the membrane of a resting as well as activated platelets. This interaction causes rolling of platelets over the collagen. A more firm bond is established between the platelets and collagen, via the GPIV collagen receptor present on the platelet membrane¹⁴, which triggers conformational changes of integrins on the platelet surface.

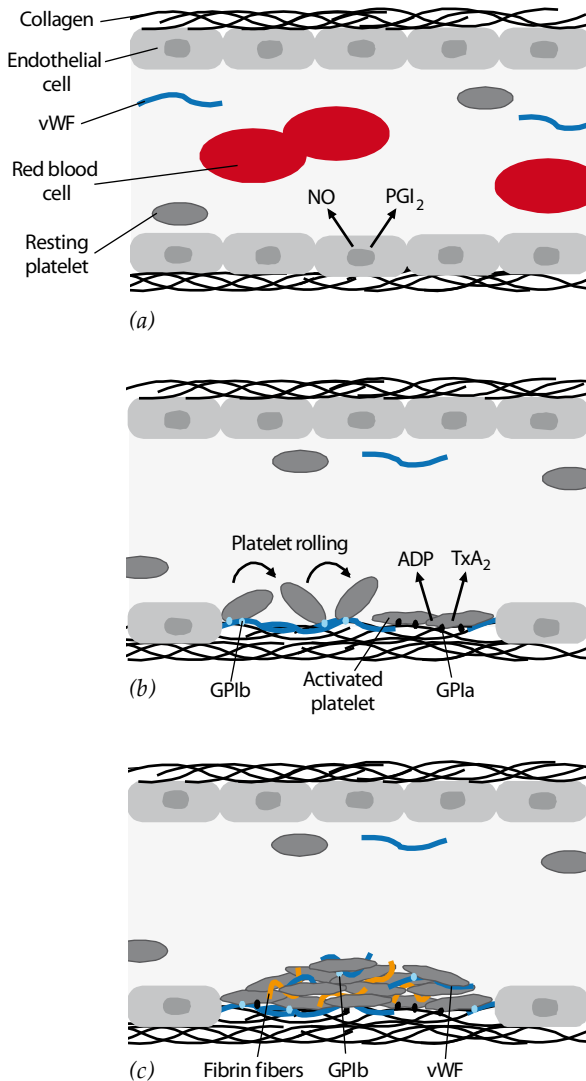


Figure 2.4: *In vivo* platelet activation^{2,3,9}. (a) Under normal conditions the platelets circulate in the cardiovascular system without having interaction with other platelets or cells. In presence of red blood cells, the platelets are pushed toward the endothelial layer. Due to the secretion of nitric oxide (NO) and prostaglandin (PGI₂) from the endothelial cells, platelet activation is suppressed. (b) Upon vascular damage, collagen is exposed to the blood stream. Initially, von Willebrand factor (vWF) present in the blood, attaches to the collagen. The vWF fibers slow down the platelets via the loose binding with the platelet GPIb membrane receptor for vWF. Binding of the platelet to collagen, via the GPIIb/IIIa receptor, initiates platelet activation; thereby thromboxane A₂ (TxA₂) and adenosine diphosphate (ADP) are secreted from the adhered platelets, which activates more platelets. (c) Activated platelets can bind to each other via fibronectin, fibrin or VWF and the platelets activated membrane GPIIb-IIIa receptor. The close contact between the platelets and the firm fibrin network in between the platelets, form a platelet plug, which closes the site of injury.

The role of collagen receptor GPIa, part of the integrin $\alpha_2\beta_1$ complex, is to anchor the platelets at the site of lesion^{14,15}. Under static conditions, the binding between platelets and collagen can occur in absence of vWF, however under arterial flow conditions, the vWF is essential in the initiation process for platelet plug formation.

The rolling, and more important, the binding to collagen via GPVI initiates platelet activation. As a consequence, the platelet changes shape, expresses additional membrane receptors and secretes adenosine diphosphate (ADP) and thromboxane A₂ (TxA₂). As described in the previous section (section 2.4.1), the latter two are platelet activators themselves. Figure 2.4b shows the process of rolling and binding over the vascular injury. The other route that can initiate the formation of a platelet plug is by the secretion of thrombin. This process particularly plays a role in thrombotic or inflammatory disorders^{3,9,13,16}.

The next step in the formation of a stable platelet plug is extension. When collagen is covered with a monolayer of platelets, the secretion of ADP and TxA₂ is essential, since this activates and recruits more platelets to the site of injury. The activated platelets at the site of lesion express the active form of integrin $\alpha_{IIb}\beta_3$, allowing platelet-platelet interactions. The contacts between the platelets are maintained by multivalent ligands that bind to GPIIb-IIIa, such as vWF, fibrinogen or fibrin. The process of platelets sticking to each other is commonly referred to as “cohesion” when studied *in vivo* and “aggregation” when studied *ex vivo*.

Perpetuation is the last step in the platelet plug formation (Figure 2.4c), in which the platelet plug is stabilized to prevent the plug from premature disintegration. This stabilization occurs via the close contacts of the platelets and outside-in signaling for example through the integrins. In addition a fibrin mesh is established through the platelet plug. In the final stage of the plug formation, the fibrin clot gradually pulls in on itself, making the platelet plug shrink to a smaller volume. From here the red blood cells take over and the secondary hemostasis continues the work which was started by the platelets^{2,3,9,13}.

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Chapter 3

Measurement of membrane markers using antibody coated beads

We investigate novel methods for the quantification of platelet responsiveness that are suited for implementation in lab-on-a-chip devices. Magnetic beads are convenient carriers for rapid capture and manipulation of biological cells in a miniaturized system. In this paper we demonstrate that antibody-coated magnetic beads can be used to quantify platelet responsiveness. We use anti-P-selectin coated beads to capture activated platelets from samples stimulated with a PAR-1 specific agonist SFLLRN, also known as Thrombin Receptor Activator Peptide (TRAP). The responsiveness of the platelets is analyzed via the remaining unbound platelets in solution and compared to a reference method in which the number of activated platelets is analyzed via fluorescent labeling. The effective concentrations for platelet activation are in agreement for the two assay types, proving that platelet responsiveness can be quantified using antibody-coated magnetic beads. In addition, research was performed in which beads were magnetically actuated with the use of magnetic tweezers. Results showed that we were able to discriminate between non-specific and specific interactions of functionalized beads with immobilized platelets. We discuss the outlook for application in lab-on-a-chip devices.

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3.1 Introduction

Platelets are reactive cells with the main function to maintain blood vessel integrity. In the normal circulation platelets show minimal interactions with other blood cells and with endothelial cells. When the vascular system is disturbed, for example after vessel wall damage, platelets undergo a series of changes. The platelets adhere to collagen in the extracellular matrix and get activated as a consequence. Upon activation a wide variety of proteins is secreted from the platelets' alpha granules into the blood stream, specific membrane proteins are expressed on the cell surface and the shape of the platelet changes¹⁻³.

The responsiveness of platelets is an indication for the thrombotic capacity of individuals. Subjects with low reactive platelets tend to bleeding disorders, while high reactive platelets lead to thrombotic risks. It has been demonstrated that the effectiveness of oral platelet inhibitors can be monitored with platelet activation tests in a patient^{4,5}. Furthermore, platelet granule release is thought to be related to inflammatory diseases and a wide variety of cardiovascular diseases such as atherosclerosis^{6,7}.

In this chapter the use of antibody coated magnetic beads to measure the platelets activation status will be discussed. In section 3.3, an assay principle and envisaged biosensor design will be presented. Details of the protocols and experimental settings will be discussed in section 3.4. Thereafter the results of our newly introduced assay are presented and discussed (section 3.5). In section 3.6, the results will be discussed of measurements in which we use magnetic tweezers to probe the interaction between functionalized beads and immobilized platelets. First some more background information is given about the membrane marker expression of platelets (section 3.2), relevant for this research.

3.2 Platelet membrane receptors

Platelets are one of the few cells types that lack a nucleus and hence are unable to adapt to different situations by protein synthesis. Therefore platelets are equipped with a wide variety of presynthesized molecules, which account for different functions in various conditions. The most important known classes of receptors on platelets are integrins, the leucine-rich repeat family (LRR), transmembrane receptors, the immunoglobulin superfamily and C-type lectin receptor family. In this section we will shortly introduce the GPIb-IX-V complex, part of the LRR class and P-selectin, part of the C-type lectin receptor family^{1,8}.

3.2.1 The GPIb-IX-V complex

The GPIb-IX-V complex is the second most abundant receptor, after integrin $\alpha\text{IIb}\beta\text{3}$, present on the platelet membrane. Approximately 20.000-50.000 copies of the complex are present on the platelet membrane in resting as well as activated state. The GPIb-IX-V complex is one of the major adhesive receptors on platelets and is composed of four different polypeptides GPIb α , GPIb β , GPIX and GPV, each having its own function. The interaction of the GPIb-IX-V complex with the subendothelial von Willebrand factor initiates platelet adhesion at sites of vascular injury, thus the receptor is crucial in primary hemostasis. Absence or deficiencies of the

receptor leads to one of the most common bleeding disorders linked to platelet receptors; the Bernard-Soulier syndrome^{1,8,9}.

3.2.2 P-selectin

P-selectin, also known as CD62P, PADGEM or GMP-140, is an adhesion molecule embedded in the membrane of platelets alpha granules but also in the membrane of endothelial Weibel-Palade bodies. During activation, platelets fuse their granule with the plasma membrane, as a consequence, the P-selectin appears at the surface of the platelet as indicated in Figure 3.1. In addition, a fraction of P-selectin is shed from the membrane upon activation and is thus released into the extracellular space as soluble P-selectin. Typical amounts of secreted soluble P-selectin from stimulated platelets from healthy individuals are 40 ng/ml; membrane bound P-selectin is about $1 \cdot 10^{-6}$ ng/platelet, which results in approximately 10.000 P-selectin molecules on an activated platelet, which translates into a density of approximately 350 sites/ μm^2 ^{1,10,11}.

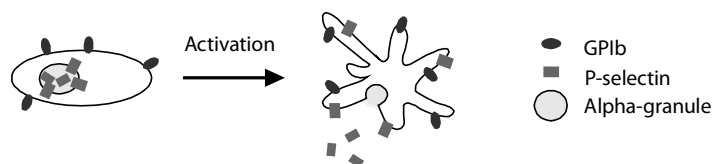


Figure 3.1: Platelet activation process. On the left side a resting platelet, on the right side an activated platelet. Upon activation, platelets change their shape, secrete specific markers from internal storages and express additional membrane proteins. In this research, GPIb (CD42b) is used as a platelet marker, which is present on the surface of resting and activated platelets. P-selectin (CD62P) is used as an activation marker, which is only present on the surface of activated platelets.

3.3 Biosensor design to measure membrane markers on platelets with the use of beads

Platelet responsiveness can be measured via the platelet reaction to a stimulus. Current methods to evaluate platelet responsiveness include aggregometry, bleeding time and flow cytometry^{1,2,4}. However, these techniques are complex and time consuming. Therefore we are interested in novel methods for the quantification of platelet responsiveness that are suited for implementation in a lab-on-chip format, as a basis for future easy to use advanced diagnostic tests. We focus on methods based on antibody recognition in order to be flexible in the biomarkers that are to be analyzed. Furthermore, we focus on the use of magnetic beads because these are highly suited for the integration of assays into a lab-on-chip format¹²⁻¹⁴.

Non-magnetic ligand-coated beads have been used for platelet function testing, with detection by flow cytometry^{15,16}, by bioluminescence and electron microscopy¹⁷, and by agglutination^{1,18}. Magnetic beads have been used to study platelet glycoprotein deficiency¹⁹ and to extract platelet specific proteins from whole blood²⁰. Our approach is to use magnetic beads which are functionalized with platelet-specific antibodies. Figure 3.2a sketches a concept for a magnetically-controlled platelet assay in a lab-on-chip system. The assay has three

magnetically-controlled phases: (i) the platelet sample is exposed to a well-defined biochemical or biophysical stimulus and is incubated with antibody-coated magnetic beads in a microchamber, (ii) the beads with specifically captured cells are concentrated at an antibody-coated sensing surface, and (iii) unbound and weakly bound beads are removed from the sensing surface by magnetic forces, followed by detection of the bound cells and beads. A full platelet responsiveness curve can be established in the lab-on-chip device by having microchambers with different degrees of stimulation.

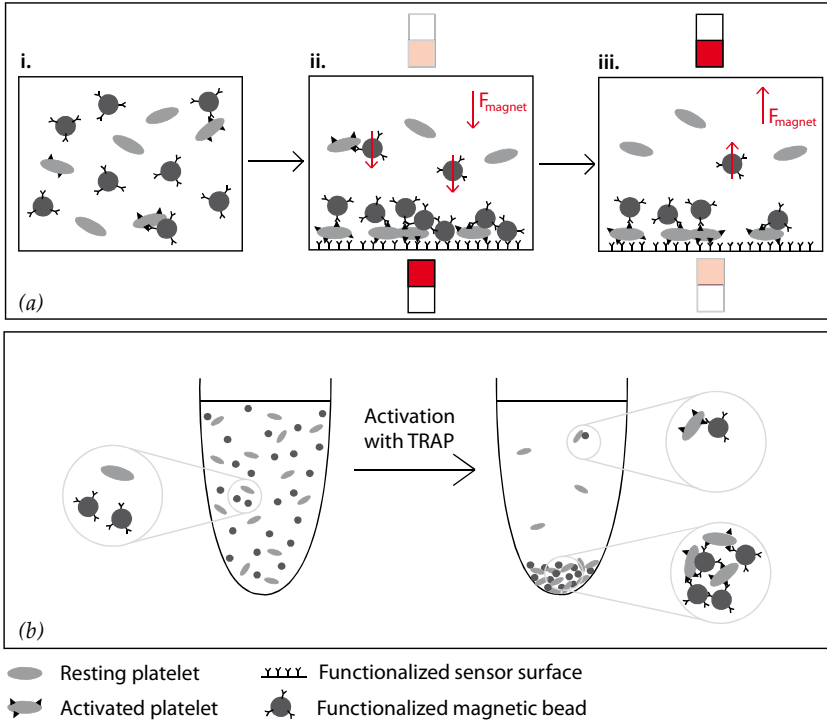


Figure 3.2: (a) Schematic representation of a lab-on-chip microchamber platelet assay based on antibody coated magnetic beads. i: Antibody coated beads are incubated with the sample and an agonist to trigger activation of the platelets. ii: The beads are transported through the sample fluid by applied magnetic fields, catching the activated platelets which express specific activation markers on their cell membrane, and transporting the cells to the antibody-coated sensor surface. iii: A magnetic force is applied in order to remove the unbound beads from the sensor surface. The amount of beads bound to the surface is a measure for the amount of activated platelets. By the use of multiple reaction microchambers with different concentrations of agonist, a dose response can be established. (b) Schematic representation of the activation and binding process that is investigated in this paper. Platelets are activated by exposure to TRAP. Binding between anti-P-selectin coated beads and platelets generates aggregation and sedimentation to the bottom of the incubation tube. An activation dose-response curve is obtained by quantifying the concentration of platelets that remain in solution.

The basis of the envisaged lab-on-chip platelet responsiveness assay is the binding of magnetic beads to responsiveness-specific biomarkers on the platelets. In this chapter, we demonstrate in a model assay in which antibody-coated magnetic beads are used to quantify the responsiveness of platelets to stimulation, see Figure 3.2b. We use anti-P-selectin coated beads and biochemical stimulation by the PAR-1 agonist, Thrombin Receptor Activator Peptide (TRAP). Upon activation, P-selectin is increasingly expressed at the platelets' membrane²¹⁻²³.

We studied the binding of platelets to the functionalized beads by measuring the remaining free platelets in solution and we compare our results to a reference method^{24,25} in which platelet activation is measured via fluorescent labeling and flow cytometry analysis. The results of this platelet capture assay will be described in section 3.5. In addition, we studied the specificity of different types of functionalized beads with immobilized platelets by magnetic actuation in a magnetic tweezers set up. These results will be discussed in section 3.6.

3.4 Materials and Methods

In this paragraph details of the experimental procedures will be discussed. In the first two sections, the general preparation protocol of functionalized beads (Section 3.4.1) and washed platelets (Section 3.4.2) can be found. The next section will describe the protocol used to obtain the dose response curve with and without functionalized beads (Section 3.4.3). The last part of this paragraph describes the signal selection in the flow cytometry experiments. Section 3.4.4 shows that beads can be discriminated from platelets by measuring the forward and sideward scattering. Next, it is shown how to select the beads which have captured a platelet (section 3.4.5) and finally how to discriminate resting from activated platelets (section 3.4.6).

3.4.1 Preparation of functionalized beads

The procedure for the preparation of the functionalized beads was based on the two-step-coating procedure using N-hydroxysuccinimide (NHS) suggested by the manufacturer. Carboxylic acid beads (2.8 μm) from Dynal Biotech, were washed twice with 25 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer pH 5.0 prior to use. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and NHS were dissolved in the MES buffer just before use and added to the bead solution with a final concentration of 12.5 mg/ml for both chemicals. The solution was mixed for 30 minutes at room temperature. The tube was then placed on a magnet and the beads solution was washed three times with MES buffer. Bovine Serum Albumin (BSA, Sigma Scientific), mouse immunoglobulin (mouse IgG, Calbiochem) or P-selectin antibody (R&D Systems) was added to the activated bead solution with a final concentration of 500 $\mu\text{g/ml}$, the solution was mixed for 1 hour at room temperature. After incubation the supernatant was removed and the reaction was quenched with 50 mM ethanolamine in phosphate buffered saline (PBS) pH 8.0 for 1 hour at room temperature. Prior to use, the beads were washed five times with wash buffer (1% BSA and 0.1% Tween-20 in PBS).

3.4.2 Preparation of washed platelets

Fresh whole blood samples were purchased from Sanquin, the Dutch organization for the supply of blood and blood products. Blood donors voluntarily donated blood for this research after being informed and signing an informed consent form. Venous blood was collected from healthy donors who reported to be free of aspirin for at least 10 days. Whole blood was anticoagulated with 3.2% sodium citrate. Platelet-rich plasma (PRP) was prepared from whole blood within 1 hour after collection by centrifugation at 250 g for 20 minutes at room temperature. The PRP was transferred to a clean tube and anticoagulated with Citrate Dextrose (ACD, 0.25% Citrate, 0.15% Citric Acid and 0.2% D-Glucose). The mixture was then centrifuged at 520 g for 20 minutes and the supernatant was discarded. Carbaprostacyclin (cPGI, Cayman Chemical Company) was added with a final concentration of 100 ng/ml to reduce the platelet activation due to sample handling. The platelets were resuspended in a HEPES buffer with pH 6.5 (containing 10 mM HEPES, 150 mM NaCl, 5 mM KCl, 0.3 mM NaH₂PO₄·H₂O, 10 mM MgSO₄·7H₂O and 50 mM D-glucose) and the volume was adjusted with this buffer to the original PRP volume, obtained after the first centrifugation step. The platelets were washed by a third centrifugation step at 520 g for 20 minutes. The supernatant was discarded and the platelets were resuspended in a HEPES buffer with a pH of 7.3, the volume was adjusted to the original volume of the donor material. Prior to the use of the washed platelets (WP), the suspension was kept for 30 minutes at room temperature to return to a resting state.

3.4.3 Dose-response curves

Washed platelets were stimulated by TRAP-6 (Bachem) with final concentrations of 0, 80 nM, 800 nM, 4 μM, 8 μM, 40 μM, 80 μM and 800 μM. 5 μl of washed platelets was incubated together with the agonist and 2 μl of each fluorescent detection label. Two fluorescently labeled antibodies were used: the platelet specific marker Allophycocyanin (APC) conjugated mouse anti-Human GPIb (BD Pharmingen) was used for the detection of the platelets, Phycoerythrin (PE) conjugated mouse anti-human P-selectin (BD Pharmingen) was used as activation marker. 5 μl of anti-P-selectin bead solution was added and the reaction volume was increased to 50 μl. The reagents were gently mixed for 20 minutes at room temperature. The ratio between the beads and the platelets during incubation was 1:1, the concentration of both particles during incubation was 1·10⁵ /μl. Control samples were incubated with BSA coated beads instead of the anti-P-selectin coated beads, to evaluate the nonspecific adsorption of platelets onto the beads. Samples with only beads or platelets were used as additional controls. For the reference dose-response curve, in which the percentage of activated platelets was evaluated, the beads are omitted in this incubation step. To prevent further activation, all samples were fixed for 10 minutes at room temperature with 500 μl aqueous solution containing 0.2% formaldehyde in 0.9% NaCl.

Experiments with PRP used plasma of the donor material that was stored after the second centrifugation step in the preparation of washed platelets. In the experiments for the PRP dose response curves, the samples had a final plasma content of 10%. All samples are prepared in duplicate.

Samples were transferred into a 96-microtiter plate, for evaluation in the Guava EasyCyte 8HT flow cytometry system (Millipore). In contrast to traditional flow cytometry systems in which sheath flow is used, the analyzed volume in the Guava system is known. As a consequence, the particle concentration of the sample can be measured. The system is validated for particles count and fluorescent intensity detection prior to each measurement. The observed coefficient of variations (CV) in particle count in the performed measurements was below 2%. The number of unbound platelets as a function of the concentration of TRAP is analyzed for these samples.

3.4.4 Signal selection in the flow cytometer: Discrimination between beads and platelets

Reference samples containing BSA coated beads, washed platelets or both were used for a first signal selection on the basis of the forward and sideward scatter. BSA coated beads were used to avoid binding of the platelets to the beads. Due to the difference in scattering properties of the beads and the platelets, they appear as distinct populations in the forward versus sideward scatter plot of the flow cytometry analysis. Figure 3.3 shows the forward-sideward scatter plot of the sample containing the BSA beads as well as the washed platelets. Two distinct groups can be observed, in which the beads appear in the higher sideward scatter region (M2) and the unbound platelets in the lower sideward scatter region (M1). The gates around these areas were set according to the control samples, containing only BSA beads or washed platelets.

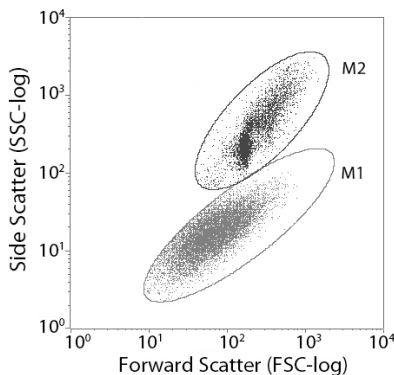


Figure 3.3: Sideward versus forward scatter plot of BSA coated beads incubated with washed platelets. BSA-beads were used to prevent platelet adhesion to the beads. The ratio of the beads to the platelets is 1:1. The data was collected after 20 minutes of incubation at room temperature. The unbound platelets fall in the M1 gate and the BSA beads in M2.

3.4.5 Signal selection in the flow cytometer: Couple formation between bead and platelet

A mixture of anti-P-selectin coated beads and washed platelets was incubated with the two fluorescently labeled antibodies, for additional signal selection. The platelet specific marker APC mouse anti-human GPIb was used for the detection of the platelets, PE mouse anti-human P-selectin was used as specific platelet activation marker. Unbound platelets are specifically measured as GPIb positive particles in the M1 gate. The GPIb-APC positive signals within the M2 gate, discriminates between beads with and without a platelet attached to it.

Two control samples were used to set the APC threshold within both gates. The first sample (Figure 3.4a) contains only beads including both fluorescent labels, anti-GPIb-APC and anti-P-selectin-PE. From experiments in which beads were incubated with and without labels we know that the observed signal is caused by the autofluorescence of the beads and to a lower extent by non-specific adsorption of the fluorescent labels to the beads. The second control sample contains only labeled platelets (Figure 3.4b). The APC threshold was set such that the amount of false positives for couples did not exceed 1%.

The fluorescent intensity recorded on the APC channel of a sample containing both anti-P-selectin coated beads and platelets stimulated with TRAP, is shown in Figure 3.4c. Two separate populations can be seen in the histogram. The low intensity signals are single beads, the high APC intensity signals registered in M2 represent platelets captured by an anti-P-selectin coated bead. After the flow cytometry analysis the samples were inspected under the microscope, which confirmed the capture of the platelets by the anti-P-selectin coated beads. The same settings of the APC threshold were used in gate M1, to discriminate between unbound platelets and debris.

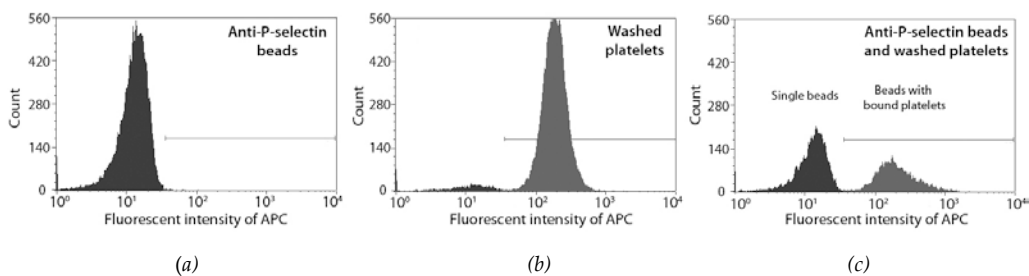


Figure 3.4: The control samples containing only anti-P-selectin coated beads (a) or only platelets (b) were compared with the sample with anti-P-selectin coated beads, incubated with 800 nM TRAP-stimulated platelets (c). In contrast with the control sample containing only beads an additional population appeared, indicating binding of anti-GPIb-APC labeled platelets to the functionalized beads. The signals in (b) were measured in gate M1, the signals in (a) and (c) were measured in gate M2.

3.4.6 Signal selection in the flow cytometer: Activation status of the platelets

Discrimination between resting and activated platelets was made by a threshold value for the P-selectin density. This threshold was determined by the anti-P-selectin-PE intensity of a control sample from previous measurements containing 100 ng/ml cPGI inhibited washed platelets, such that the false positives did not reach 5%. Platelets with a P-selectin density higher than this threshold were designated as “activated”, while platelets with a density below this threshold as “resting”.

Figure 3.5 shows the P-selectin density on the unstimulated (Figure 3.5a) and 800 μ M TRAP stimulated (Figure 3.5b) control samples in the dose response series of washed platelets. A small fraction of the population of the unstimulated sample is above the threshold. The slight

increase in activation of the platelets is probably caused by sample handling such a pipeting and mixing. The positive control shows a major intensity shift of the distribution indicating a high binding of the activation marker anti-P-selectin-PE to the platelets. The error introduced by setting the threshold will be used as error margin in the data analysis of the reference dose response curves.

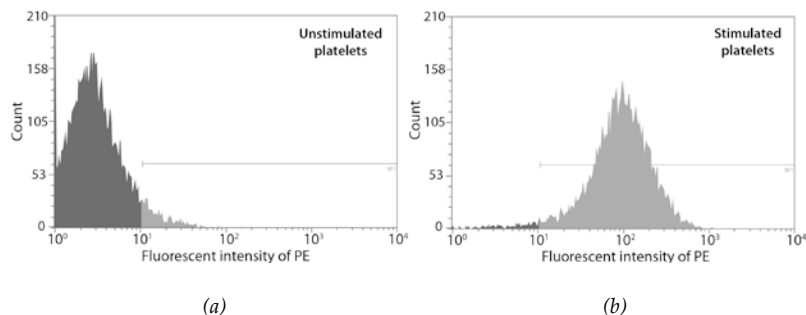


Figure 3.5: Control samples that were used to set the anti-P-selectin-PE-threshold. Signals having an intensity above this threshold were designated as activated platelets. (a) Washed platelets without agonist. (b) Washed platelets activated with 800 μ M TRAP. Anti-GPIIb-APC was used as a detection label for platelets, anti-P-selectin-PE was used as a label for the activation status of the platelets.

3.5 Bead assay without magnetic actuation

With the use of the flow cytometry settings as described in previous paragraph, the specificity of the anti-P-selectin coated beads for activated platelets will first be tested (section 3.5.1). Thereafter the use of anti-P-selectin coated beads to obtain a dose response curve will be tested and compared with the reference method in which platelets were fluorescently labeled (section 3.5.2).

3.5.1 Specificity of the binding of activated platelets to anti-P-selectin coated beads

The specificity of the capturing of the activated platelets by the anti-P-selectin coated beads was analyzed. Washed platelets were incubated with BSA beads, with anti-P-selectin coated beads or without any beads. The BSA coated beads serve to investigate the non-specific adsorption of platelets to the beads. In addition these samples were incubated with and without agonist. The sample containing unstimulated platelets without any beads was used as reference. The formation of clusters of anti-P-selectin coated beads and platelets was observed for high agonist concentrations, as is sketched in Figure 3.1b. The clusters sediment rapidly and are therefore not measured by the flow cytometer. The timescale of the sedimentation of the clusters is in the order of minutes, whereas the sedimentation of unbound platelets in the samples is in the order of hours. Therefore the number of unbound platelets in solution (M1 in Figure 3.3) is analyzed rather than the formation of the couples (anti-GPIIb-APC positive particles in M2, Figure 3.3). Figure 3.6 presents the average relative concentration of the unbound platelets in M1; the error bars indicate the spread in duplicates.

After 20 minutes of incubation with 800 μM TRAP the number of washed platelets decreased with 27%. This decrease is probably caused by the conformational changes of the platelet upon activation, in which the platelets are more prompt to adhere non-specifically to surfaces, such as the walls of the reaction tube. In the presence of BSA beads, the relative concentration of unbound platelets decreased to about 47%. The additional decrease was likely caused by nonspecific binding of the activated platelets onto the BSA beads; this was confirmed after inspection of the samples by microscopy.

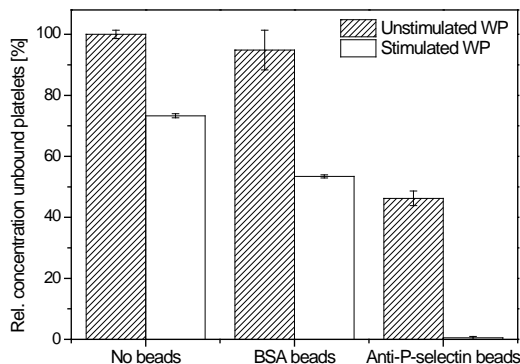


Figure 3.6: The relative concentration changes in unbound platelets (Figure 3.3, M1) in solution after 20 minutes of incubation at room temperature with or without functionalized beads. No stimulus was added for the control platelets (unstimulated WP). Platelet stimulation was performed with 800 μM TRAP (stimulated WP). Error bars represent the spread in relative platelet numbers of the duplicates.

Figure 3.6 shows that about 54% of the unstimulated platelets are captured by the anti-P-selectin coated beads this is caused by the increased P-selectin expression of the platelets due to sample handling as discussed earlier. The specificity of the binding of the activated platelets to the anti-P-selectin coated beads was confirmed by the difference observed in the unbound platelet concentration between the unstimulated and stimulated platelets incubated with anti-P-selectin coated beads. The concentration of the platelets in the sample without agonist was about 46% whereas the relative concentration of platelets reduced to about 0.5% for the stimulated samples. In contrast, the platelet concentration in the sample with BSA beads decreased from 95% to about 53% upon activation.

Since the autofluorescence of the beads interferes with the PE-label, it is not possible to investigate the activation status of the platelets bound to the beads. Therefore we analyze the density of P-selectin expressed on platelets that were not in complex (M1) in order to confirm that activated platelets were captured from the samples by the anti-P-selectin coated beads. In the unstimulated sample containing BSA beads the percentage of activated platelets was 2.5% in M1, whereas the percentage activated platelets in the sample containing anti-P-selectin

coated beads was only 0.1% in the same gate. This indicates that from the population of unbound platelets, the activated platelets were captured by the anti-P-coated beads.

3.5.2 Dose-response curves

In order to compare the anti-P-selectin coated bead assay with respect to the reference method, we measured platelets dose response curves using the same donor material. Samples with washed platelets were stimulated with TRAP concentrations in the range between 0 and 800 μM TRAP and both fluorescently labeled antibodies were added. In the bead assay, the anti-P-selectin coated beads were also added with a 1:1 ratio of platelets to beads. After 20 minutes of incubation, the samples were fixed as described in the previous section. The number of unbound platelets in solution (M1) is analyzed rather than the formation of the couples (M2), since the couples form larger clusters for high agonist concentrations. For the reference method, the P-selectin density was evaluated for all GPIb-APC positive cells as a function of the concentration of TRAP. Platelets were considered as activated when the P-selectin density was higher than the threshold. Samples with eight different TRAP concentrations are prepared in duplicate and measured. A dose response was obtained from the relation between the percentage of activated platelets as a function of the TRAP concentration. The relative error in the data points is scaled with one over the square root of the total number of counted particles.

Figure 3.7 shows dose response curves of the two assays for platelets in buffer (left panel) and 10% plasma (right panel). The percentage of activated platelets and the concentration of unbound platelets are plotted as a function of the agonist concentration. Both assays give a typical S-shaped dose response curve in buffer as well as 10% plasma. The curve for the bead assay records the unbound platelets (APC-positive particles in M1), therefore the curve is inverted with respect to the curve of the reference assay.

A sigmoidal function which is commonly used to describe a dose response relation²⁶, can be used to fit and quantitatively compare the data:

$$P(C) = P_2 - \frac{P_2 + P_1}{1 + \left(\frac{C}{C_0}\right)^S} \quad (3.1)$$

where C the concentration of the stimulus, S the slope of the curve and P1 and P2 are the plateau's for the response at low and high concentrations respectively. C_0 is the effective concentration (EC_{50}) which is the concentration at which the reaction is half way between P1 and P2.

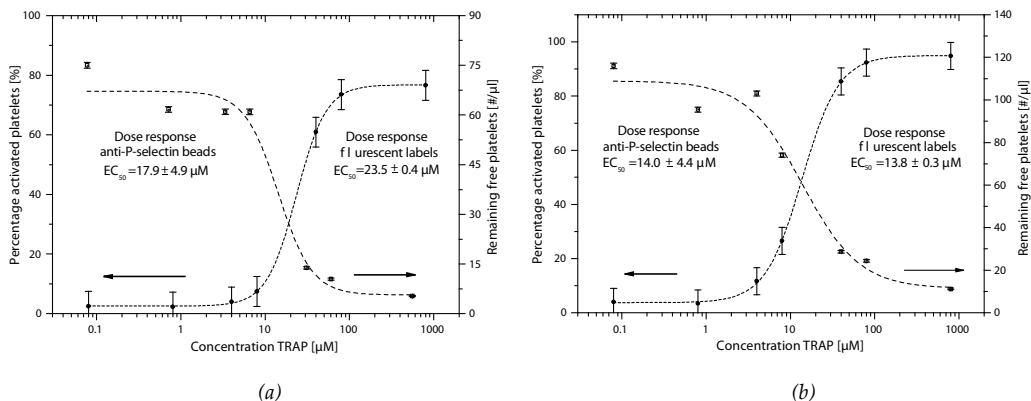


Figure 3.7: The platelet responsiveness curves for both methods in buffer (a) and 10% plasma (b). Left axis: the responsiveness of the washed platelets obtained from the reference flow cytometry analysis with the use of fluorescent labels (\bullet). The percentage of activated platelets is the amount of platelets positive for APC as well as PE in the M1 gate. The data points are the average values obtained from the duplicates. The measurement error is 5%, which is the maximum amount of false positives in the control sample. Right axis: the responsiveness of the washed platelets determined using the new method, based on incubation with anti-P-selectin coated beads (\square). The concentration of the unbound platelets after stimulation is recorded. The data points are average values obtained from the duplicates. The measurement error is scaled with the number of counted particles (N/n). The dotted lines represent fits according to equation 3.1, with the error representing the accuracy of the fit. The EC_{50} value found for the reference method was $23.4 \pm 0.4 \mu\text{M}$ and $17.9 \pm 4.9 \mu\text{M}$ for the bead assay in buffer. In 10% plasma the EC_{50} value found for the reference method was $13.8 \pm 0.3 \mu\text{M}$ and $14.0 \pm 4.4 \mu\text{M}$ for the bead assay

The fit for the assay with anti-P-selectin coated beads in buffer (Figure 3.7a) gives an effective concentration of $17.9 \mu\text{M}$ with a fitting error of $4.9 \mu\text{M}$. The reference method with fluorescent labels gives an EC_{50} value of $23.5 \pm 0.4 \mu\text{M}$. In 10% plasma the EC_{50} values were $14.0 \pm 4.4 \mu\text{M}$ and $13.8 \pm 0.3 \mu\text{M}$ respectively (Figure 3.7b). The errors in the EC_{50} values as determined by the fitting procedure are an underestimate of the experimental error in the EC_{50} values, since this accuracy is mainly determined by the setting of the threshold. The experiments were repeated using blood platelets of several healthy donors in buffer ($n=4$) as well as in 10% plasma ($n=3$). For all donors assays with anti-P-selectin coated beads reveal sigmoidal dose response curves. Each experiment shows good agreement in EC_{50} values for the bead assay with the reference assay.

3.6 Bead assay with magnetic actuation

In previous sections the results were presented of a platelet-capture assay with the use of anti-P-selectin coated beads in solution. We showed that we are able to bind anti-P-selectin coated beads specifically to activated platelets in solution, which is the first step in the biosensor design we envision (Figure 3.2). In addition, we want to make use of the magnetic properties of the beads to discriminate between resting and activated platelets after immobilization at a substrate.

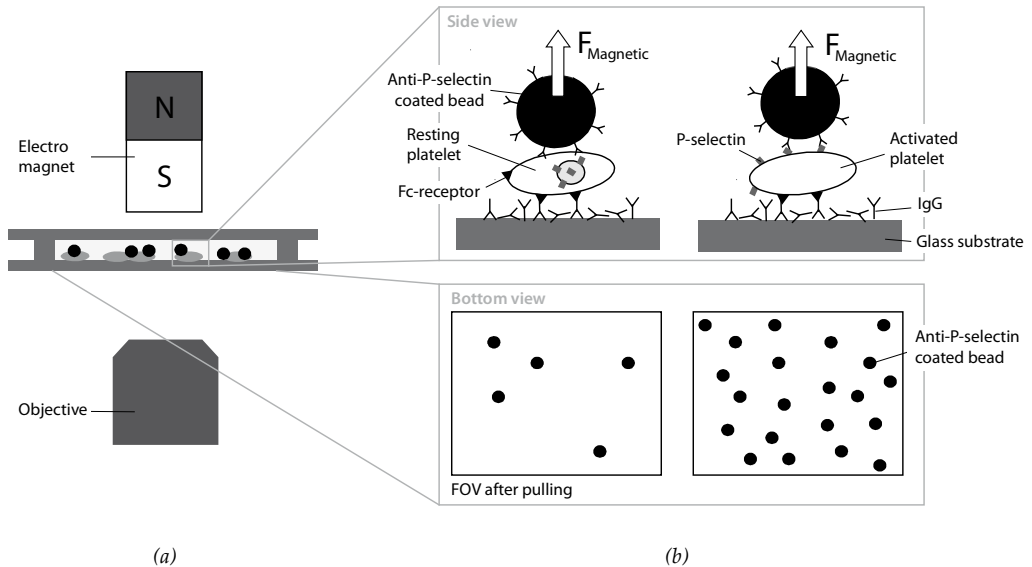


Figure 3.8: Experimental set-up of the magnetic tweezers experiments. (a) Platelets were adhered to an IgG coated surface for 30 minutes to which they bind via their Fc-receptor, then the unbound platelets were removed by gentle agitation in a beaker with HEPES buffer (pH 7.3). Functionalized beads were allowed to interact with the immobilized platelets for 2 ½ minutes, then a constant force of 50 pN was applied for 100 seconds. During this period images were recorded with a high speed camera. (b) A sketch of a resting (left) and activated platelet (right) immobilized on an IgG-coated surface, on which anti-P-selectin coated beads were bound. A more firm bond between the anti-P-selectin coated bead and activated platelet is expected compared to the resting platelet. Below an ensemble of anti-P-selectin coated beads on immobilized platelets is sketched. More beads stay attached to the activated platelets during the pulling experiment (right FOV), compared to the resting platelets (left FOV). Since platelets are highly transparent, they are barely visible in bright field imaging. The beads will appear as dark spots on the recorded images.

Figure 3.8a shows the experimental set up of the magnetic tweezers experiment, in which the electromagnet is placed above the sample positioned on an inverted microscope. Figure 3.8b shows a sketch of a resting and activated platelet and the field of view (FOV) after a magnetic force is applied on an ensemble of anti-P-selectin coated beads on resting or activated platelets. Since more P-selectin is expressed on activated platelets, it is expected that a more firm bond is established between activated platelets and the anti-P-selectin coated beads, compared to the resting platelets and the anti-P-selectin coated beads. As a consequence, the number of anti-P-selectin coated beads that detach from the surface during pulling will be higher for resting platelets than for activated platelets. This is visualized in the bottom planes of Figure 3.8b.

Before the binding between anti-P-selectin coated beads to resting or activated platelets was tested, the non-specific bonds between the surface and different types of beads were tested, as well as the specificity of the beads with the immobilized platelets. In these experiments mouse Immunoglobulin G (10 $\mu\text{g/ml}$ mouse IgG in PBS, Calbiochem) was covalently coupled to a

glass substrate via a linker (11-(Triethoxysilyl)undecanal, ABCR). Platelets were incubated for 15 minutes at the IgG coated surface at room temperature, thereafter unbound platelets were removed by immersing the samples in a beaker filled with Hepes buffer (pH 7.3). BSA, IgG or anti-P-selectin coated beads were allowed to sediment for 2 minutes on the substrate, then the sample was placed on the microscope set up and the magnetic tweezers were switched on generating a force of approximately 50 pN²⁷. Bright field images were recorded with a sample frequency of 30 Hz and per recorded frame the number of beads at the surface was counted²⁸. Figure 3.9 shows the number of remaining functionalized beads on an IgG coated surface in absence (panel a) or presence (panel b) of platelets as a function of time during the pulling experiment.

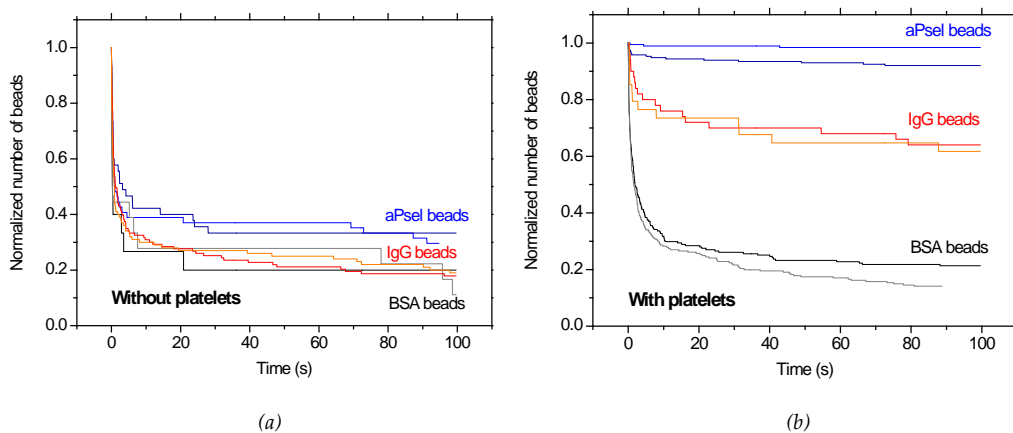


Figure 3.9: Results of magnetic tweezers experiments. After sample preparation with or without platelets, beads were allowed to interact with the sample for 2 ½ minute. A magnetic force of 50 pN was applied for 100 s and from the recorded images, the number of beads at the surface was counted. The number of beads at $t=0$ (the moment at which the magnet is turned on) is used for normalization. (a) Non-specific interaction between BSA (black curves), IgG (red curves) and anti-P-selectin (aPsel, blue curves) coated beads, with an IgG-coated surface, in absence of platelets. (b) Interaction between BSA (black curves), IgG (red curves) or anti-P-selectin (aPsel, blue curves) coated beads with platelets immobilized on an IgG-coated substrate.

Figure 3.9a shows that the number of beads that detach from the surface by applying a magnetic force for 100 s, is approximately the same for BSA, IgG or anti-P-selectin coated beads; about 60-80% of the beads detach during the experiment. This shows that the non-specific interaction between the functionalized beads and the IgG-coated surface is not much affected by the surface coating of the beads. Figure 3.9b shows that the bead coating does show differences in binding to immobilized platelets. Approximately 80% of the BSA coated beads detach from the surface in presence or absence of immobilized platelets. This leads to the conclusion that the interaction between platelets and BSA coated beads is minimal, which is in agreement with the control experiments showed in section 3.5.1. In case of IgG-coated beads can be seen that less beads detach from the surface in presence or absence of immobilized

platelets: 35 and 80% was found respectively. This interaction is probably caused by the presence of Fc-receptors at the platelet membrane, which are able to bind to the crystalline part of antibodies at the bead. The data shows that the number of anti-P-selectin coated beads that detach from the surface in presence of immobilized platelets during the pulling experiment is minimal (approximately 5%). We attribute this observation to the presence of P-selectin on the platelet membrane. Although the platelets were incubated in a resting state with the IgG-coated surface, probably the immobilization to the IgG-coated surface (section 5.5.1; The effect of surface coating and substrate on platelet activation) and washing step (Section 4.6.3; Reduced responsiveness after washing procedure) triggered a platelet response, resulting in P-selectin expression. As a consequence the anti-P-selectin coated beads form a firm bond with the immobilized platelets and were difficult to detach from the surface.

In follow-up experiments, we incubated resting and TRAP activated platelets at an IgG-coated surface and bound anti-P-selectin coated beads to them. No differences in anti-P-selectin bead detachment during pulling experiments could be observed between the stimulated and unstimulated platelets; most anti-P-selectin beads did not detach from the immobilized platelets at the surface. This supports our hypothesis that platelets were already activated due to the preparation protocol. Follow-up experiments may focus on preventing additional washing steps after platelet immobilization and the use of an Fc-receptor blocker, in order to reduce the P-selectin expression on the platelets.

Overall, we were able to show that we can specifically bind functionalized particles to immobilized platelets. With the use of magnetic tweezers we succeeded in discriminating between different types of bonds established between functionalized beads and immobilized platelets.

3.7 Conclusion

We have demonstrated the measurement of platelet activation by TRAP using anti-P-selectin coated magnetic beads. The curve of the amount of unbound platelets in solution as a function of the agonist concentration has a sigmoidal shape, with good correspondence to the percentage of activated platelets recorded by fluorescent labeling. The sigmoidal shape of the dose response curve is observed for assays in buffer as well as in 10% plasma. Our experiments demonstrate for the first time the use of antibody functionalized beads to measure a dose response curve for the expression of a platelet-specific activation marker. In addition, we showed that we were able to discriminate between non-specific and specific interactions between functionalized beads and immobilized platelet with the use of magnetic actuation.

These results form a basis for further studies toward miniaturized lab-on-chip systems for platelet multifunctional biosensing using magnetic beads. As presented in Figure 3.2a, we envisage to stimulate platelets in a reaction microchamber, label platelets with magnetic beads and use electromagnetic forces to transport activated platelets to a sensing surface. The amount of platelets and thus antibody coated beads attached to the surface is a measure for the amount of activated platelets in the sample volume. In such an integrated magneto-microfluidic system,

different reaction chambers can be operated in parallel, in order to screen for different concentrations of agonist and different types of stimulation, allowing for accurate measurements of platelets and their functional properties. An advantage of a miniaturized system is that only a very small amount of sample will be needed²⁹, much less than the few milliliters that is needed to evaluate platelet responsiveness in flow cytometry. In addition, the use of magnetic beads reduces the need for sample handling, since mixing and labeling can be integrated in one point of care system.

Thus far, we have tested the bead assays in buffer and in plasma. In practical use, whole blood will be the input sample. The feasibility of a magnetic bead-based platelet responsiveness assay in whole blood still needs to be investigated. The main challenges of the use of magnetic beads in whole blood to measure platelet function will probably be the formation of nonspecific bead clusters due to protein absorption on the beads and the nonspecific binding of other cells to the beads. In case red and/or white blood cells appear to interfere with the assay, then one may consider to design the lab-on-a-chip device with two consecutive modules: first a module wherein red and white blood cells are separated, followed by a module wherein the resulting platelet-rich plasma is analyzed by the integrated magnetic bead assay. In conclusion, the results of this chapter represent a first step toward the realization of an integrated lab-on-chip system for multifunctional platelet testing using antibody coated magnetic beads.

3.8 Acknowledgements

I would like to acknowledge Claudia Schot for performing additional measurements of the platelet capture assay in platelet rich plasma and whole blood. In addition, I would like to thank Bart Hoekstra, who performed magnetic tweezers experiments (Section 3.5) as a part of his Bachelor internship.

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Chapter 4

Measurement of calcium signaling to study platelet-surface interactions

Calcium plays a key role in platelet function, it is for example needed to trigger activation but also for platelet aggregation and shape change. Calcium indicators have been used to visualize intracellular calcium variations in platelets. In this work the calcium indicator Oregon Green BAPTA-1 AM was used to study the interaction between platelets and various surfaces coatings namely Bovine Serum Albumin (BSA), Poly-L-Lysine (PLL), mouse immunoglobulin (IgG), anti-GPIb and collagen. We introduce a new method to quantify the platelet response, in which the cells that increase their cytosolic calcium upon binding or upon a chemical trigger are designated as responding cells. The number of responding cells as well as the time-to-response were analyzed. When studying the chemical response sensitivity of cells bound to a surface, we found that the percentage of cells responding to a chemical trigger was low for surfaces that had already induced a cell response when the cells got bound to the surface. From the analyzed surfaces, we concluded that BSA is the most gentle for platelet immobilization, followed by IgG and PLL. On an anti-GPIb and collagen coated surface, the surface caused activation of almost all platelets.

4.1 Introduction

In many processes in platelets calcium plays an important role, for example, membrane bound calcium is essential for the integrity of the fibrinogen receptor and hence platelet aggregation. Also for the secretion process and shape change of platelets, the cytosolic calcium concentration plays an important role^{1,2}. Therefore calcium signaling in platelets is an important read-out for platelet function.

In many technologies available for future biosensor applications to measure platelet function, the designs are based upon the use of a substrate to immobilize platelets³⁻⁷. Therefore it is important to understand the influence such a substrate can have on the cells. In this chapter intracellular calcium signaling is used as a read-out parameter to study the interaction of platelets with different types of surfaces. We used the calcium indicator Oregon Green BAPTA-1 AM to visualize variations in cytosolic calcium concentrations in platelets. We introduce a new method to quantify the platelet response, in which the cells that increase their cytosolic calcium upon binding or upon a chemical trigger are designated as responding cells. The number of responding cells as well as the time-to-response were analyzed.

The surface coatings that were selected for platelet adhesion experiments were Bovine Serum Albumin (BSA), Poly-L-Lysine (PLL), mouse immunoglobulin (IgG), anti-GPIb and collagen. Since the protein BSA is well known for its ability to reduce nonspecific binding, a BSA coated surface serves as a negative control in the experiments. It was expected that platelets will have little or no interaction with a BSA coated surface⁸⁻¹¹. In addition, a PLL coated surface was used to study non-specific interactions of platelets with a substrate. The platelet cell membrane is negatively charged and the PLL coated surface positively charged, which results in binding based upon electrostatic attraction¹²⁻¹⁴. One of the pivotal glycoproteins on the platelet cell membrane is GPIb, which was already described in more detail in Chapter 3. GPIb is a part of the GPIb/IX/V complex, which is the receptor for the von Willebrand factor. Per platelet about 12.000-25.000 of these receptors are present at the surface of resting as well as activated platelets^{2,15}. Therefore we use an anti-GPIb coated surface to study the specific binding of platelets with a surface. Since platelets can bind to an antibody via the specific epitopes, situated at the Fab (fragment, antigen binding) region and via the Fc (fragment crystallizable) region, we used an IgG coated surface as a control to study the latter interaction. In addition, the adhesion of platelets to collagen was analyzed, since collagen is a well known platelet activator it will serve as a positive control in the experiments^{2,15}. Experiments in which platelets were incubated under static and dynamic conditions were performed and the effect of the surface, chemical stimulus or sample handling on cytosolic calcium levels were measured with the use of a calcium indicator. At the end of the chapter we will discuss our results in the perspective of future biosensor applications. First a short introduction to calcium signaling in platelets will be given.

4.2 Calcium signaling in platelets

In resting platelets the cytosolic concentration of calcium is maintained at approximately 100 nM, which is about 10,000-fold less than the concentration of free calcium in plasma^{1,16}. Two distinct processes are known to keep the intracellular calcium concentrations in platelets low: sacroplasmic/endoplasmic calcium ATPases (SERCAs) pump the calcium into the intracellular storage compartments (i.e. the Dense Tubular System, DTS, the platelet equivalent of the smooth endoplasmic reticulum¹⁷) and the plasma membrane calcium ATPases (PMCA) pump cytosolic calcium out of the cell. If these pumps fail to operate normally, the cytosolic calcium could rise and cause premature cell death^{16,18}.

Several storage sites for calcium exist within the platelet, including the dense tubular system (DTS, which primary function is to store calcium), dense granules, mitochondria and the extracellular calcium pool. Upon activation the cytosolic calcium level can exceed 1-10 μM , the release from the DTS and plasma membrane most likely account for these calcium levels in platelets^{1,2,16,19}. Figure 4.1, shows a schematic representation of the calcium release from the dense tubular system into the platelet cytosol.

However, it is important to realize that calcium is a secondary messenger in the platelet activation process; elevated levels of cytosolic calcium contributes to various steps of platelet activation^{1,18}. As a consequence, an increased calcium level does not indicate the irreversible activation process as measured with P-selectin expression (Chapter 3) or ATP secretion (Chapter 5).

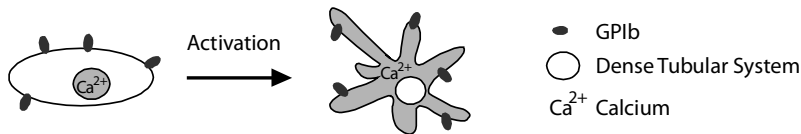


Figure 4.1: Schematic representation of the calcium release from the dense tubular system (DTS) into the cytosol upon platelet activation.

Thrombin Receptor Activating Peptide (TRAP), which triggers platelet activation via the same signaling cascade as the natural platelet agonist thrombin, was used as a positive control in the experiments. The platelet response to thrombin and thus TRAP is mediated by members of the protease-activated receptors (PAR) family of the G protein coupled receptors (GPCRs). Two receptors of this family (PAR1 and PAR4) are present on the membrane of human platelets and can be activated by thrombin. Receptor activation occurs when the thrombin cleaves the extended N-terminus of each of these receptors, exposing a new N-terminus (SFLLRN) that serves as a tethered ligand. In cell lines, thrombin receptors shut off soon after activation, at least partly as a result from receptor phosphorylation (which is the addition of a phosphorous-group to the protein, which deactivates it). In case of platelets, about 40% of thrombin receptors remain on the surface after activation^{2,20}. Synthetic variants like TRAP can mimic the binding of the tethered ligand of PAR1 and will activate the receptor without the cleavage of the receptor^{21,22}.

As a consequence of the coupling via the PAR receptor, phospholipase C β (PLC β) is activated which cleaves (via hydrolysis) membrane phosphatidylinositol 4,5-bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP₃). The latter binds to a receptor on the dense tubular system, which triggers the release of the stored calcium^{1,2}. The time scale of the calcium response of Fura-2 loaded platelets stimulated with thrombin was measured by stopped flow fluometry and was found to be about 1 second^{1,23}. A schematic representation of the above described signaling cascade is shown in Figure 4.2.

In vivo, collagen is exposed to the blood stream after vascular damage and will initiate the primary hemostasis, involving platelets. GPIa and GPVI are two well established collagen receptors on platelets. GPIa is present at the platelet membrane in a complex with GPIIa, this complex is a part of the integrin $\alpha_2\beta_1$ family which are involved in cell-cell and cell-matrix interactions. About 1.000 of these complexes are present on the membrane of the platelet^{2,15,24}. The binding to collagen is initiated by the coupling of GPIV to its ligand^{24,25}. Clustering of the GPVI receptor occurs upon binding to collagen and in the end will lead to activation of phospholipase-C γ_2 (PLC γ_2). PLC γ_2 just like other forms of PLC in platelets, follows the same cascade as thrombin activation, which in the end results in increasing cytosolic calcium concentrations^{2,24,26,27}. During this activation process, integrins change their conformation allowing for example the collagen receptor GPIa to anchor the platelets at the site of lesion.

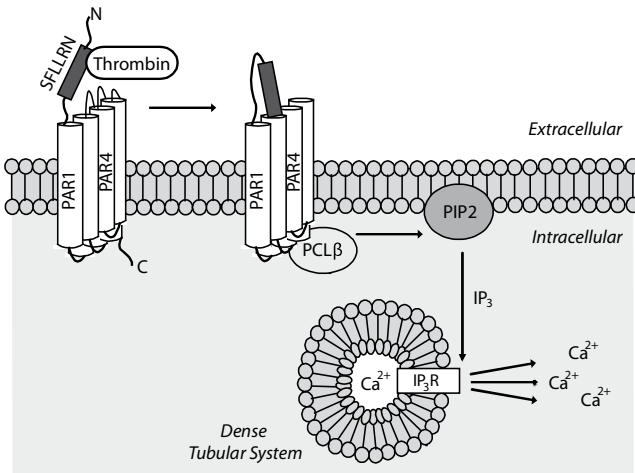


Figure 4.2: Schematic representation of the signaling cascade inside platelets upon thrombin stimulation. Thrombin cleaves the extended N-terminus of the PAR receptor exposing a new N-terminus (SFLLRN) that serves as a tethered ligand. As a consequence of the coupling via the PAR receptor, phospholipase C β (PLC β) is activated which cleaves (via hydrolysis) membrane phosphatidylinositol 4,5-bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP₃). The latter binds to a receptor on the dense tubular system, which triggers the release of the stored calcium. PAR=protease-activated receptor, PLC β =Phospholipase C β , PIP₂=Phosphatidylinositol 4,5-bisphosphate, IP₃=inositol 1,4,5-triphosphate. Figure was adapted from Michelson² and Coughlin²¹ and modified.

It is known that platelets can easily be activated, even without the presence of an agonist such as collagen or TRAP, for example by handling (e.g. pipetting or mixing) and/or surface contact. Glass is a well-known example of a surface that introduces platelet activation upon contact^{2,15,28,29}. An increase in cytosolic calcium can therefore already be observed as a consequence of the interaction of the platelet with a surface¹⁶.

4.3 Measure platelet-surface interactions with a calcium indicator

Since the early eighties, calcium indicators are widely used in the research of intracellular calcium signaling in cells. The use of calcium indicators in platelet research covers a wide variety of research topics, from revealing proteins involved in the specific signaling cascades^{10,30,31} to response times in platelet activation in bulk^{23,32,33} as well as single cell responses to contact or agonist activation²⁰. We will use the calcium indicator Oregon Green BAPTA-1 AM to study platelet-surface interactions under static (sedimentation) and dynamic (perfusion) conditions. In the perfusion experiments, an additional calcium indicator (Fura Red AM) was used to obtain dual color fluorescence signals. The properties of Fura Red will be described shortly in this section, but since the use of Fura Red AM was not optimized yet, we will restrict the data presented in the results section to the fluorescent indicator Oregon Green BAPTA-1 AM. In the outlook of this chapter (section 4.9.1), we will present some preliminary results on the use of Fura Red AM.

In the first section (section 4.3.1), the working principle of the calcium indicator is specified. Thereafter, our newly introduced methodology to quantify the platelet-surface interaction with the use of a calcium indicator will be discussed (section 4.3.2).

4.3.1 Specifications of the calcium indicators

A wide variety of calcium indicators exist, where Oregon Green BAPTA-1 AM (Invitrogen) is shown to work well in the research of calcium signaling in platelets^{30,34}. Upon binding to calcium the indicator increases its fluorescent intensity up to 14-fold, without significant shift in emission wavelength. Figure 4.3 shows the optical characteristics of the fluorophore as specified by the manufacturer.

Fura Red AM has an excitation range of 450-500 nm and an emission maximum at 660 nm. In contrast to Oregon Green BAPTA-1 AM, Fura Red AM decreases its fluorescent intensity once calcium binds to the indicator.

The acetoxymethyl (AM) derivative of the calcium indicator was used, since that probe is cell membrane permeable. The modifications of the carboxylic acids with AM groups results in an uncharged molecule which can penetrate through the cell membrane. The natural occurring esterases inside the cell, cleave the lipophilic blocking groups from the molecule, which results in a charged form of the indicator unable to permeate the cell membrane again (Figure 4.4)^{35,36}.

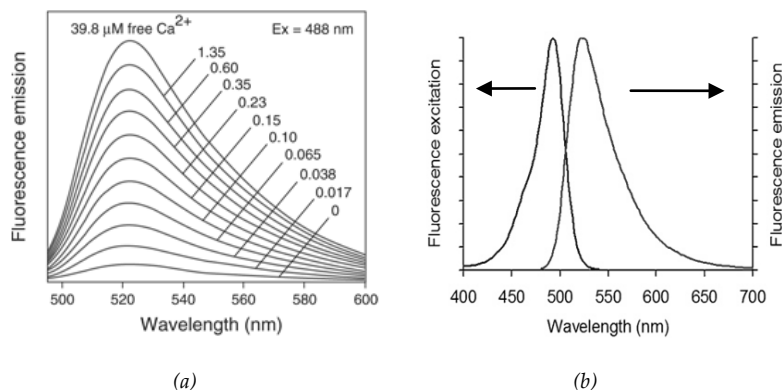


Figure 4.3: Optical characteristics of the Oregon Green BAPTA-1 AM. (a) Emission spectra of Oregon Green BAPTA-1 AM at various concentrations of Calcium. The cytosolic calcium concentration in platelets ranges from 100 nM in the resting state, to 1-10 μM in activated state. (b) The excitation and emission spectra of Oregon Green BAPTA-1 AM saturated with Calcium³⁷.

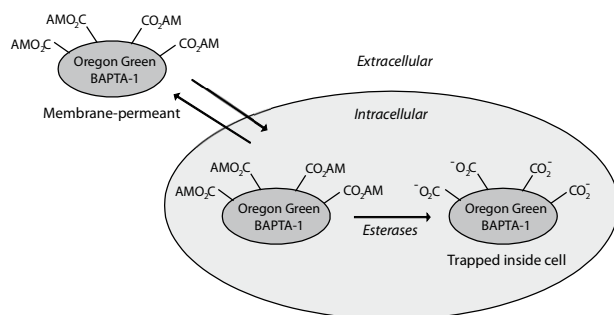


Figure 4.4: Principle of loading an acetoxymethyl (AM) ester into a cell. The additional AM-groups mask the negative charge of the carboxyl group, thereby creating an uncharged calcium indicator which is able to permeate the cell membrane. Once in the intracellular space, the natural occurring esterases cleave the lipophilic groups, resulting in a charged molecule which is unable to permeate the cell membrane³⁵.

4.3.2 New methodology: Quantify platelet-surface interactions with cytosolic calcium variations

Although calcium signaling in platelets has been intensively studied, not much literature can be found on single cell studies on platelet interaction with foreign surfaces with the use of cytosolic calcium as a read-out^{12,16}. Studies rather use morphology changes^{12,16,38}, membrane markers³⁹ or secretion markers²⁸ as a measure for platelet-surface interactions. We introduce a method to analyze the interaction between platelets and surfaces, which divides the cell population in responding and non-responding cells and quantifies the delay time between the applied trigger and response.

The fluorescent intensity of individual platelets, loaded with calcium indicator, was recorded as a function of time with a light-sensitive camera attached to an inverted microscope. Figure 4.5 shows in a schematic representation the typical stages that were observed during incubation of platelets onto a substrate. The intensity versus time plot, in the right panel was adapted from a measurement showing the typical behavior of a responding platelet upon binding. First, a low intensity spot can be observed while a platelet approaches the focal plane at t_0 . Then the platelet moves into the selected region of interest (ROI) thereby increasing the measured fluorescent intensity slightly. The moment at which the platelet stops its Brownian motion was determined by eye from the recorded movies. This time point was designated as the initial binding time point (t_2). When the immobilized platelet increased its fluorescent intensity after binding (i.e. t_3) the cell was designated as a responding cell. In case of the sedimentation experiments the time between platelet adhesion (t_2) and increased fluorescent light intensity (t_3) was called the delay time. When a chemical stimulus was applied to a substrate with immobilized platelets, the delay time was the time between the addition of the stimulus and the increased fluorescent intensity. More details of the experimental set-up can be found in section 4.4.3.

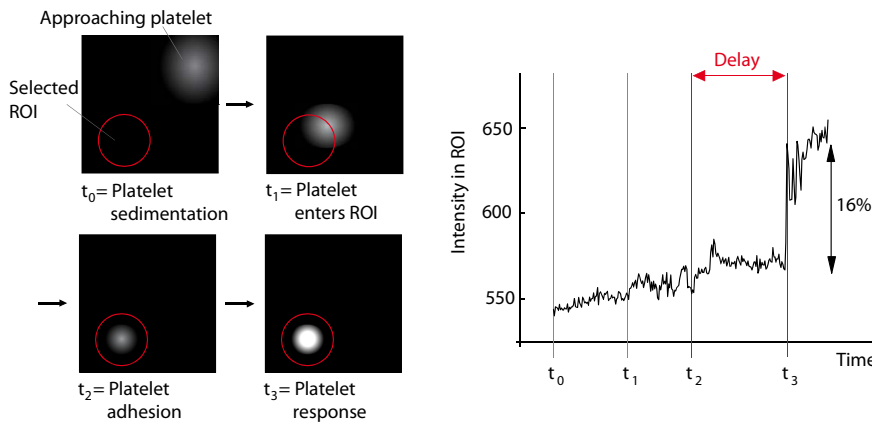


Figure 4.5: Schematic overview of various stages during the sedimentation experiment in which Oregon Green BAPTA-1 AM loaded platelets were allowed to settle on a substrate. In the right panel, a typical time trace of the fluorescence signal for a platelet binding to an anti-GPIIb coated surface is presented. For clarity, schematic representations of the fluorescent images are shown in the left panel. At t_0 unbound platelets moves in and out of the focal plane. At t_1 a platelet enters the selected region of interest (ROI) and therefore the measured light intensity will slightly increase. The moment at which the platelet stops its Brownian motion is denoted as the adhesion time, in this case indicated as t_2 . A responding cell increases the fluorescent intensity after binding, and is maximum at t_3 . In case of adhesion experiments, the time between adhesion (t_2) and maximum increased intensity (t_3) is designated as the delay time. In case of chemical stimulation, the delay time is the time between the addition of the chemical stimulus and the increased fluorescent intensity.

4.4 Materials and methods

Based on knowledge from the literature and in cooperation with the University Medical Center Utrecht (UMCU), we established protocols in order to study platelet-surface interactions via calcium monitoring. The experimental procedures used in our research such as sample preparation, experimental set up and data analysis, are described in this section.

4.4.1 Preparation of washed platelets loaded with calcium indicator

Fresh whole blood samples were purchased from Sanquin, the Dutch organization for the supply of blood and blood products. Venous blood was collected from healthy donors who reported to be free of aspirin for at least 10 days. Whole blood was anticoagulated with 3.2% sodium citrate. Platelet-rich plasma (PRP) was prepared from whole blood within 1 hour after collection by centrifugation at 250 g for 20 minutes at room temperature. The PRP was transferred to a clean tube and anticoagulated with Citrate Dextrose (ACD, 0.25% Citrate, 0.15% Citric Acid and 0.2% D-Glucose). The mixture was then centrifuged at 520 g for 20 minutes and the supernatant was discarded. Carbaprostacyclin (cPGI, Cayman Chemical Company) was added with a final concentration of 100 ng/ml to reduce the platelet activation due to sample handling. The platelets were resuspended in a platelet buffer with pH 6.5 (Hepes buffer containing 10 mM Hepes, 150 mM NaCl, 5 mM KCl, 0.3 mM $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$, 10 mM $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ and 50 mM D-glucose) and the volume was adjusted with this buffer to the original PRP volume, obtained after the first centrifugation step. The platelets were washed by a third centrifugation step at 520 g for 20 minutes. The supernatant was discarded and the platelets were resuspended in a platelet buffer with a pH of 7.3 (Hepes buffer containing 10 mM Hepes, 150 mM NaCl, 5 mM KCl, 0.3 mM $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$, 10 mM $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ and 50 mM D-glucose) and the volume was adjusted to the original volume of the donor material. When needed, Fc-receptor blocker (Miltenyi Biotec) was incubated for 10 minutes at room temperature, to block the Fc-receptor (CD32) at the platelet membrane.

1 μM calcium indicator Oregon Green BAPTA-1 AM (Invitrogen, dissolved in DMSO) was incubated with the prepared washed platelets for 30 minutes at 37°C. The excess label was removed by centrifugation at 520 g for 20 minutes. Calcium rich Hepes buffer (1 mM CaCl_2 , in Hepes buffer pH 7.3) was used to resuspend the washed platelets.

The perfusion experiments were carried out at the UMCU. Washed platelets were loaded with 1 μM Oregon Green BAPTA-1 AM and 1.25 μM Fura Red AM (Invitrogen, dissolved in DMSO), and incubated for 30 minutes at 37°C. After loading and washing of the platelet suspension, red blood cells were added to obtain the initial hematocrit level required for optimal platelet adhesion during perfusion over the collagen coated surface. The red blood cells were washed twice with the calcium rich Hepes buffer (1 mM CaCl_2 , in Hepes buffer pH 7.3) before reconstitution. The centrifugation speed after loading was 340 g for 15 minutes instead of 520 g for 20 minutes, as used in our protocol.

4.4.2 Surface preparation

Glass cover slips were cleaned with ethanol before incubation with the surface coatings of interest. Poly-L-Lysine (PLL, Sigma) in deionized water, mouse immunoglobulin (mouse IgG,

Calbiochem) or anti-GPIb (R&D systems) in phosphate buffered saline (PBS, Sigma) were added to the substrate and incubated overnight at room temperature. After incubation, the samples were rinsed with 1% Bovine Serum Albumin (BSA, Sigma) in PBS. In addition, all samples were blocked with 3% BSA (BSA, Sigma) in PBS, for 2 hours at room temperature.

Prior to the coating procedure of glass microscope slides with collagen, the surface was cleaned with chromic sulphuric acid and thereafter rinsed thoroughly with deionized water. 50 μl of 100 $\mu\text{g/ml}$ collagen in (Kollagenreagens Horm, Nycomed) in SKF solution (isotonic glucose solution, pH 2.7-2.9) was spread over the surface and incubated for 1 $\frac{1}{2}$ hour at room temperature. After incubation, samples were blocked with 1% Human Serum Albumin (HSA fraction V, Biomedicals) overnight at 4°C. All collagen samples used in the experiments were prepared in the UMCU by Arjan Barendrecht.

To obtain a BSA coated glass slide, only the blocking procedure was applied after the cleaning of the glass slide. All prepared samples were washed with platelet buffer (Hepes buffer with 1mM CaCl₂, pH 7.3), just before use.

4.4.3 Experimental set up for the static incubation and chemical activation of platelets

Platelets were allowed to sediment onto the prepared surface and images were recorded in fluorescent mode with a frequency of 2 Hz (Andor Luca camera). An external light source was used for sample excitation (Leica EL6000). The Leica L5 filter cube was used for the fluorescent recordings, including an excitation filter 480/40 nm, a dichroic mirror of 505 nm and a band pass filter of 527/30 nm. An objective lens with a magnification of 20x and a numerical aperture of 0.4 was used. Regions of interest (ROI) covering individual platelets were manually selected from these movies with the use of the program ImageJ.

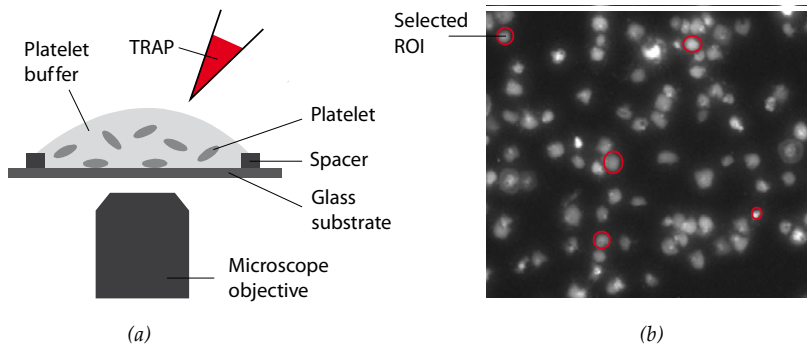


Figure 4.6: Experimental set up to study the interaction of platelets with a substrate or response on a chemical stimulus. (a) Platelets were allowed to sediment on the substrate on an inverted microscope, after platelet immobilization 800 μM TRAP was added to stimulate the platelets. (b) Images were recorded with a frame rate of 2 Hz. The regions of interest were manually selected from the recorded movie (b) per selected ROI the average intensity was measured.

During imaging of the cells at the surface, the amount of excitation light was minimized. Binning was used in most experiments to increase signal to noise, as a consequence the spatial resolution was reduced. Bleaching during a typical experiment of 5 minutes was about 1-5%. Figure 4.6 shows the experimental set up for the sedimentation and chemical stimulation experiments.

4.4.4 Experimental set up for the perfusion of platelets over collagen

At the University Medical Center Utrecht, a microfluidic device was designed to study the adhesion of platelets under physiologic conditions. The dimensions of the flow cell are 2 –0.125 –45 mm (width–height–length) on which different substrates can be clamped. Platelet adhesion to collagen was studied with a shear rate of 1600 s⁻¹ under fluorescent imaging on an inverted microscope (Zeiss observer Z1) with an external light source (Colibri). The Zeiss filterset 79HE w1 was used to obtain double excitation wavelengths at 470/27 nm and 556/25 nm. The emitted light of Oregon Green BAPTA-1 AM was evaluated at 512/30 nm and the emitted light of Fura Red AM at 630/98 nm. Images were recorded with an integration time of 125 ms (Zeiss, AxioCam MRm). The experimental set up is presented in Figure 4.7. Data from the binding experiments at the perfusion set up was acquired by Arjan Barendrecht.

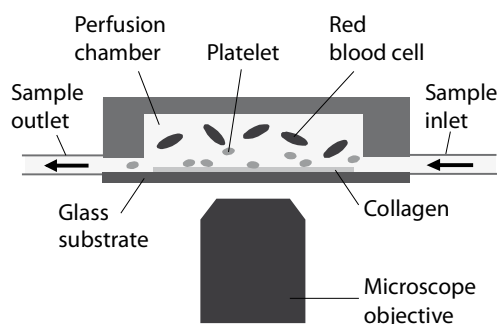


Figure 4.7: Experimental set up of the perfusion experiment. Washed platelets were loaded with Oregon Green BAPTA-1 AM and Fura Red AM and flowed over a collagen coated substrate in presence of red blood cells.

4.4.5 Data analysis

The fluorescence intensity of individual platelets was analyzed as a function of time to study the interaction between individual platelets and the surface. The typical stages that were observed during sedimentation experiments were already presented in

Figure 4.5. The time point of platelet binding to the surface was determined by eye. From calibrations of the optical set up, with the use of a Thor Labs calibration sample, it was found that the spatial resolution was 355 nm/px. Therefore platelet displacements smaller than this resolution remain undetected. From reproducibility tests was found that the temporal resolution to determine this time point was five frames, which results in a 2.5 seconds uncertainty.

Platelets that increase their fluorescence intensity after binding or after the addition of a chemical stimulus were designated as responding cells. The intensity increase recorded of platelets binding to the surface was typically in the range of 5-20 %. In contrast, the intensity variations observed in freely diffusing cells over a non-interacting BSA coated surface were typically 1-5 % and showed more gradual intensity changes as a function of time. The intensity changes which could be expected from focal shifts were analyzed by focusing through platelets adhered at the substrate. From these control measurements we found that the fluorescence intensity did not change more than 2 % for a focal shift of 2 μm .

The uncertainty in the number of responding cells was determined by analyzing the individual fluorescence intensity traces of the cells and counting the number of traces which were ambiguous, e.g. could be designated either as non-responding or responding cells. Typically these cells showed a more gradual increase in fluorescent intensity or an intensity change of <5 %. This resulted in a relative error in the percentage of responding cells between 3-7 %.

The time point at which the TRAP had reached the immobilized platelets at the surface, was determined from the response of unbound surrounding platelets. From reproducibility tests was found that the temporal resolution to determine this time point was five frames, which results in a 2.5 s uncertainty. In addition, in some movies there was a spatial gradient visible in platelets responses; platelets at one side of the field of view (FOV) seem to respond later than platelets at the other side of the FOV. The observed time was typically on the order of seconds over the field of view of about 200 μm .

4.5 Results: Calcium responses during platelet adhesion

In this section the interactions of Oregon Green BAPTA-1 AM loaded platelets with different types of surface coatings will be presented. The response of the platelets upon surface contact will be evaluated under static as well as flow conditions. Data will be analyzed with our newly introduced methodology in which the number of responding cells as well as their response delay time is measured.

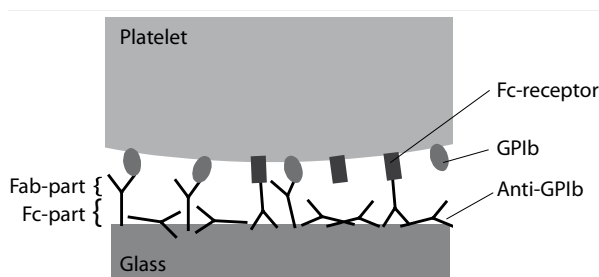


Figure 4.8: Binding of a platelet to an anti-GPIIb coated surface: the platelet can bind via the Fc-receptor present on the membrane of the platelet and the Fc-part of the antibody at the surface. The platelet can also bind via the specific epitopes of the antibody at the surface, present at the Fab-part of the antibody, and the GPIIb receptor present on the membrane of the platelet. Fab=Fragment, antigen binding, Fc=Fragment crystallizable

Platelets were allowed to sediment on a BSA, PLL, IgG, anti-GPIIb or collagen coated surface and their calcium response was studied upon the interaction with these surfaces. Since the protein BSA is well known for its ability to reduce nonspecific binding⁸⁻¹¹, it serves as a negative control. In addition, a PLL coated surface was used to study non-specific interactions of platelets with a substrate. The specific binding of platelets to a surface was studied with the use of an anti-GPIIb coated surface. An IgG coated surface was used as a control to study the interaction between the platelet Fc-receptor and the immobilized antibodies. Figure 4.8 shows the possible bonds between a platelet and an anti-GPIIb coated surface. Platelets incubated with and without Fc-receptor blocker were allowed to interact with the IgG coated surface. As a positive control we included a collagen coated surface, since collagen is well-known to induce platelet activation upon binding. The binding of platelets to collagen was not only studied under sedimentation (static conditions), but platelets were also adhered to collagen under flow (dynamic) conditions. The latter experiment was performed by Arjan Barendrecht, at the perfusion set-up in Utrecht University Medical Center Utrecht (Figure 4.7). Platelets were flowed over a collagen coated surface with a shear rate of 1600 s⁻¹ in presence of red blood cells.

The number of cells that showed an increase in fluorescent intensity upon binding was analyzed. The data of various samples, recorded with donor material from various patients was pooled and the percentage of responding cells upon binding to the different types of substrates is presented in Figure 4.9. The error bars represent the percentage of ambiguous cells counted in the various experiments. The number *n*, indicates the total number of cells analyzed for the different surface coatings. Results of platelets binding on the BSA coated surface cannot be found in the bar plot, since no real-time platelet binding events were recorded on a BSA coated surface.

The binding of antibodies to the platelet Fc-receptor (CD32) is known to induce platelet activation²⁸. Therefore we used the Fc-receptor blocker, which blocks the platelet receptor for the crystallizable part of an antibody, in order to prevent platelets from activation upon binding to an antibody coated surface. However, from Figure 4.9 it can be seen that the percentage of responding platelets treated with Fc-receptor blocker on an IgG coated surface was $40 \pm 7 \%$ and without Fc-receptor blocker was $41 \pm 6 \%$. Thus, no significant reduction in the percentage of responding cell, by the addition of the Fc-receptor blocker could be observed. This is in contrast with the results that will be presented in Chapter 5, in which the ATP secretion of immobilized platelets on IgG was measured. Platelets incubated with Fc-receptor blocker showed a decrease in amount of secreted ATP of 28 % compared to the untreated platelets (section 5.5.2). Two differences in experimental method can probably account for this difference. In the first place, the calcium signaling assay is a time-dependent measurement in which the real-time interaction of cells with a surface is measured. In contrast to the ATP secretion assay, which is an end-point analysis that measures the amount of secreted ATP after 1 hour of incubation of the platelets at an IgG coated surface. Secondly, the release of ATP is a specific marker for the dense granule release and thus for irreversible activation of platelets. In contrast, calcium is a secondary messenger in the platelet activation process. Elevated calcium levels are for example also visible during platelet morphology changes^{1,18}. As a consequence, different processes are monitored in the different experiments. These two differences, may have

led to the different result concerning the influence of the Fc-receptor blocker on the platelet response upon immobilization.

From Figure 4.9 it can also be noted that under static conditions the number of cells that respond upon binding to an IgG coated surface (40 ± 7 % with Fc-receptor blocker; 41 ± 6 % without Fc-receptor blocker) is the lowest, followed by adhesion on a PLL coated surface (74 ± 7 %). On an anti-GPIIb (88 ± 3 %) or collagen coated surface (89 ± 4 %), the percentage responding cells was similar. Collagen is a well-known platelet activator, it thus served as a positive control in our experiments and high response was expected. Platelet adhesion on an anti-GPIIb coated surface induces similar effects in intracellular calcium signaling as on a collagen coated surface. In Chapter 5, where we will present data on surface-induced activation of platelets via measurements of secretion markers, we will also show that anti-GPIIb induces high platelet response.

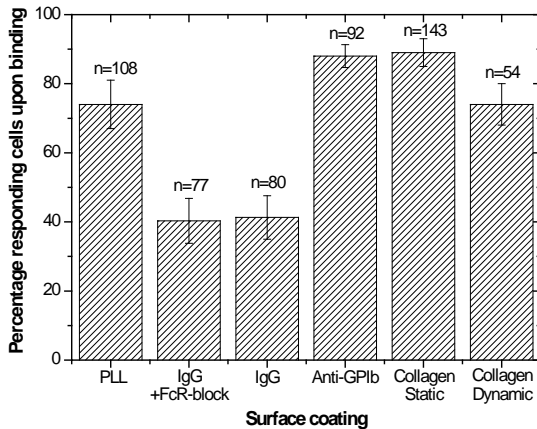


Figure 4.9: The percentage of platelets that showed an increased fluorescence intensity upon binding to a surface. Platelet adhesion to five different types of surface coating was tested under static conditions (via sedimentation), namely BSA, PLL, IgG, anti-GPIIb and collagen. Since no binding events were recorded on BSA, this surface is excluded from the bar plot. Platelets were incubated with and without Fc-receptor blocker on IgG coated surfaces. The number of responding cells to collagen was also measured in presence of a shear flow of 1600 s^{-1} (collagen dynamic). *n* is the number of responding cells analyzed, the error bars represent the standard deviation in the analyzed cell ensemble.

Platelets under high shear conditions are known to be more sensitive to external triggers, than without the presence of flow. It was therefore expected that the percentage of responding platelets upon binding to a collagen coated surface in the perfusion experiment (collagen dynamic), would be higher than platelets binding under sedimentation (collagen static). From Figure 4.9 it can be noted that the opposite is true. In case of static incubation the percentage of responding cells upon binding to collagen was 89 ± 4 %, for platelets under dynamic incubation at a collagen coated surface the number of responding cells was 74 ± 6 %. It should be noted

that the recording time of the adhesion was 23 seconds in this perfusion experiment, in contrast to the 150 seconds during the static experiments. Some cells that bind in the last frames of the movies are included in the analysis, but probably their increased intensity was not registered anymore. Therefore the 74 ± 6 % responding cells is probably an underestimate of the real number of responding cells under dynamic incubation on collagen.

In order to reduce the effect of the surface on the platelets calcium signaling, platelets were incubated with 100 ng/ml of the inhibitor carbaprostacyclin (cPGI, Calbiochem) which is the stable analog of the natural platelet inhibitor prostacyclin. Carbaprostacyclin increases intracellular cyclic adenosine monophosphate (cAMP) levels, which has the effect that it inhibits the release of intracellular calcium^{1,2,15}. Although similar concentrations of cPGI were used as during preparation protocol of washed platelets (Section 4.4.1), no inhibiting effect on the calcium response of the treated platelets was found. The percentage of cells that respond upon binding to the anti-GPIb surface was similar for treated and untreated platelets (data not shown). Apparently the inhibitor is not able to upregulate the amount of intracellular cAMP concentrations, which should reduce the amount of calcium that is released upon activation. As we will later conclude in this chapter, the binding of platelets to the anti-GPIb coated surface has the same effect as binding to the well-known thrombogenic surface collagen. Therefore it is our hypothesis, that the 100 ng/ml cPGI is effective during the preparation protocol of washed platelets to inhibit the mild distortions caused by sample handling, but is not able to inhibit the activation induced by strong agonists, such as the binding to thrombogenic surfaces.

In addition, the delay times of the cells that showed a response upon binding were analyzed. Figure 4.10 shows the histograms of the recorded delay times of responding platelets upon binding to the different types of surfaces. For comparison, the x-axes are presented on the same time scale, since the recorded delay time of platelets binding under dynamic conditions to a collagen coated surface were short, an additional zoom-in of the x-axis is presented in the inset of Figure 4.11, gives an overview of the average delay times and the standard deviation at the various types of surfaces. The number n indicates the total number of cells analyzed for the different surface coatings. The significant difference between the different types of surfaces was analyzed with a two-sample paired student t-test with a significance level of 0.05. The pairs that showed no significant difference based on this analysis are marked with n.s. (not significant) in the figure.

From Figure 4.11 it can be seen that under static incubation of platelets, the measured delay time on a PLL coated surface was the lowest (11 ± 8 s), followed by an anti-GPIb (29 ± 19 s) or collagen (24 ± 21 s) coated surface and IgG coated surfaces with Fc-receptor blocker (62 ± 19 s) and without Fc-receptor blocker (51 ± 26 s). From these surfaces, platelet adhesion to PLL is the only non-specific interaction, based on the positively charged PLL and negatively charged platelet membrane. Platelet binding to the IgG, anti-GPIb or collagen coated surface is a more complex bond, since the membrane receptor has to couple to its ligand or antibody at the surface. Our results show that the delay time upon nonspecific binding is lower than in case of specific binding, the cause of this difference remains open.

Measurement of calcium signaling to study platelet-surface interactions

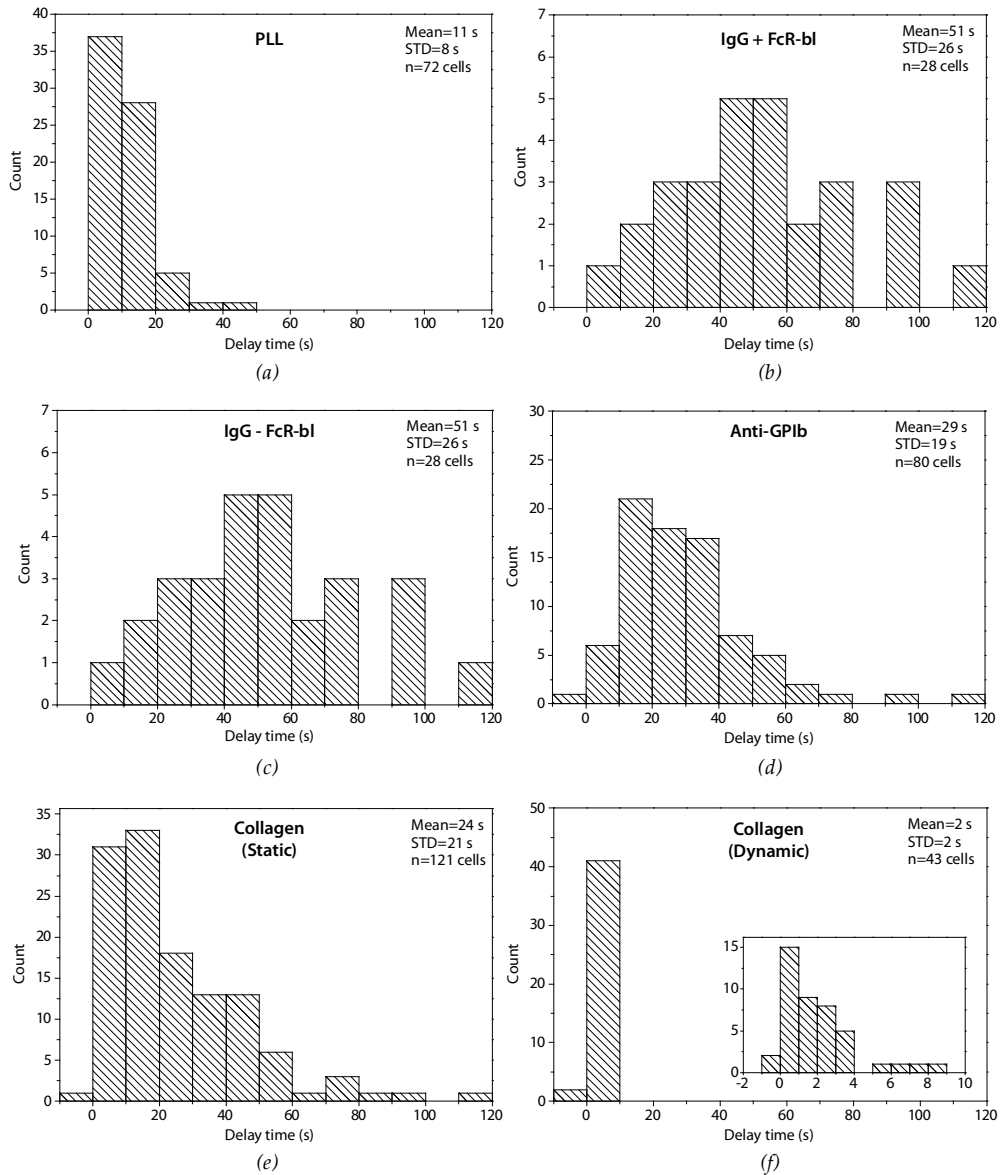


Figure 4.10: Histograms of the recorded delay times between platelet adhesion and increased fluorescence intensity. (a) Platelet adhesion to a PLL coated surface (number of analyzed cells-samples-donors: 72-6-2). (b) Adhesion of platelets treated with Fc-receptor blocker (FcR-bl) to an IgG coated surface (26-8-4). (c) Adhesion of platelets treated without Fc-receptor blocker (FcR-bl) to an IgG coated surface (28-8-4). (d) Platelet adhesion to an anti-GPIb coated surface (80-5-2). (e) Platelet adhesion to a collagen coated surface (121-6-2). (f) Delay times of platelet adhesion to a collagen coated surface under dynamic conditions (43-1-1). A shear rate of 1600 s^{-1} was applied and perfusion of platelets was done in presence of red blood cells. Measurements were performed at the UMCU and were analyzed at the TUI/e. The inset of the figure shows a zoom-in of the x-axis, to show more details of the distribution.

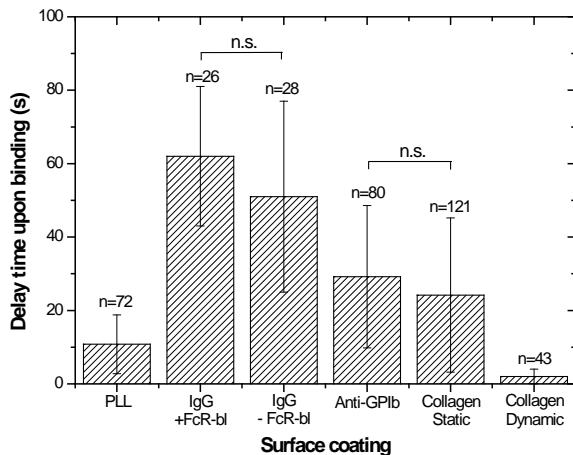


Figure 4.11: Average delay times found from platelets that respond upon binding to the different types of surfaces. n is the number of responding cells analyzed, the error bars represent the standard deviation in the analyzed cell ensemble. All samples showed a significant difference based on the pair-wise statistical analysis with the use of a two sample student t -test, with a significance level of 0.05, except the pairs indicated with n.s. (not significant).

A significant difference can be observed in the delay time of platelets binding under static (24 ± 21 s) and dynamic (2 ± 2 s) conditions. From literature it is known that integrin-mediated platelet adhesion under high shear rate conditions enhances the activation signaling in platelets^{24,25}. Thus the observed significant reduction in the delay times of platelets under high shear flow in our experiments is in agreement with literature.

The length of the recorded movies, and thus the observation window for platelet binding and response, was 150 seconds in the current experimental procedure. It should be noted that cells that bind at the end of the movie were included into the analysis, while their response can take place outside the recorded movie. For fast responding cells, the number of measured responding cells is probably a good estimate of the real percentage of responding cells. However, for increasing delay times, the measured percentage of responding cells becomes more underestimated. In our results we have shown that the delay times of the platelet response on an IgG coated surface were high (62 ± 19 s for platelets treated with Fc-receptor blocker and 51 ± 26 s for non-treated platelets). Since multiple movies were recorded in one experiment (typically at $t=5, 10$ and 15 minutes after the addition of platelets to the sample) we performed additional analyses of the binding platelets on IgG coated samples. We observed that additional platelet responses were visible in movies recorded at later time points. When these additional responses were included the percentage of responding cells for platelets treated with Fc-receptor blocker increased from $40 \pm 7\%$ to $59 \pm 8\%$ and from $41 \pm 6\%$ to $52 \pm 6\%$ for platelets without Fc-receptor blocker on an IgG coated surface. Thus time dependence should be kept in mind in this analysis. For future measurements, it is interesting to vary the time window of the recordings, to quantify the time dependence in these measurements in detail.

4.6 Results: Calcium responses in adhered platelets

From literature^{2,15} and flow cytometry measurements (Chapter 3) it is well known that TRAP is a strong platelet agonist. In this paragraph, the cytosolic calcium variations in adhered platelets are evaluated to test if the adhered cells are still able to respond to an agonist after immobilization. In the first section the percentage of responding cells upon chemical stimulation is analyzed and their delay times are presented (section 4.6.1). Next, the effect of sample handling on adhered platelets is analyzed (section 4.6.2).

Additional analyses were performed, in which the initial state of immobilized platelets on PLL and anti-GPIb were analyzed. The calcium variations in the immobilized cells was measured and the percentage of active cells was measured. Since the sample to sample variations in these experiments were high, the analysis method as well as the results can be found in the supplementary data at the end of this chapter.

4.6.1 Calcium response upon chemical stimulation of adhered platelets

As described in the introduction of this chapter, the cytosolic calcium concentration in platelets is increased after chemical stimulation with TRAP, which triggers the same signaling cascade as the natural occurring platelet agonist thrombin. From literature it is known that the time between the addition of the stimulus and the increase in intracellular calcium of platelets in solution is about 1 second^{1,23}. In our experiments we determined the calcium response time after the addition of TRAP for platelets adhered at BSA, PLL, IgG, anti-GPIb and collagen coated surfaces.

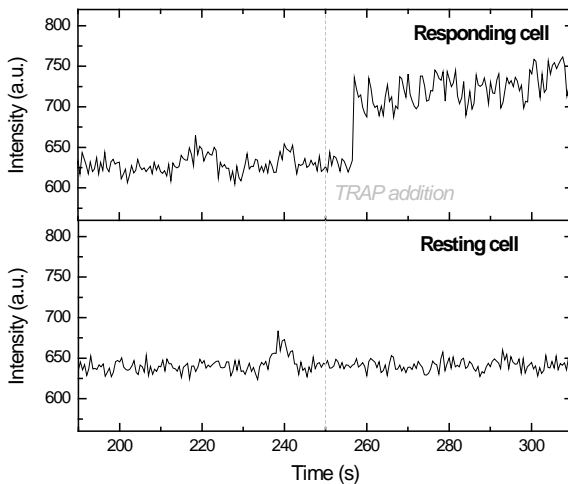


Figure 4.12: Cells adhered on a BSA coated surface for 1 hour were stimulated at $t=250$ seconds with $800 \mu\text{M}$ TRAP. The bottom curve shows a cell that does not respond on agonist stimulation. The top curve shows the typical behavior of an immobilized cell that showed a response 7 seconds after TRAP addition. The fluorescence intensity showed a stepwise increase of approximately 17 %.

Platelets were incubated for 15 minutes on the surface of interest (on a BSA coated surface the incubation time was extended to 1 hour). Thereafter TRAP was manually added with a pipette to obtain a final concentration of 800 μM . The fluorescence intensity of the adhered cells was measured before, during and after the addition of TRAP. The number of responding cells, as well as the delay times of the responding cells upon chemical stimulation was measured. Representative intensity curves of platelets adhered on a BSA coated surface are presented in Figure 4.12, the dotted vertical line indicates the moment of TRAP addition. The bottom curve in the figure shows a cell that did not respond on the chemical stimulation, the upper curve shows a cell that increased its intensity upon chemical stimulation.

Figure 4.13 shows the average percentage of responding cells at the different surfaces after chemical stimulation. The error bars represent the percentage of ambiguous cells, counted in the various experiments. The number n indicates the total number of cells analyzed for the different surface coatings. It should be kept in mind that this chemical stimulation experiment is an end-point analysis for the surface-induced activation, rather than a real-time platelet-surface analysis as in case of the recordings of the real-time binding events of platelets at a substrate.

Figure 4.13 shows three different groups in the response behavior of immobilized platelets on a chemical stimulus: high response (BSA: $95 \pm 4\%$), intermediate response (PLL: $34 \pm 6\%$, IgG with Fc-receptor blocker: $39 \pm 3\%$, and IgG without Fc-receptor blocker: $35 \pm 2\%$) and low response (anti-GPIIb: $7 \pm 4\%$ and collagen static: $6 \pm 4\%$).

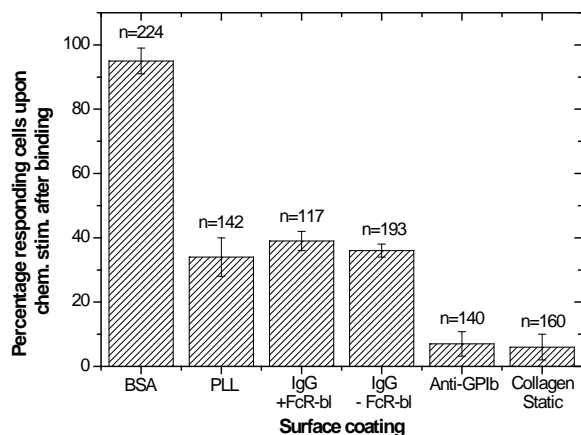


Figure 4.13: The percentage of responding immobilized platelets on a chemical stimulus. Platelets were allowed to bind to the surface for 15 minutes (in case of BSA, for 1 hour), then TRAP was added. Five types of surface coatings were tested, namely BSA, PLL, IgG, anti-GPIIb and collagen. On an IgG coated surface, platelets treated with and without Fc-receptor blocker (FcR-bl) were incubated. n is the number of responding cells analyzed (pooled from various samples), the error bars represent the standard deviation in the analyzed cell ensemble.

The histograms of the delay times upon chemical stimulation of platelets on BSA, PLL, and IgG (platelets treated with and without Fc-receptor blocker) coated surfaces can be found in Figure 4.14. Figure 4.15 shows the average delay times, including the standard deviation. The significant difference between the different types of surfaces was analyzed with a two-sample paired student t-test with a significance level of 0.05. The pairs marked with n.s. (not significant) showed no significant difference based on this analysis.

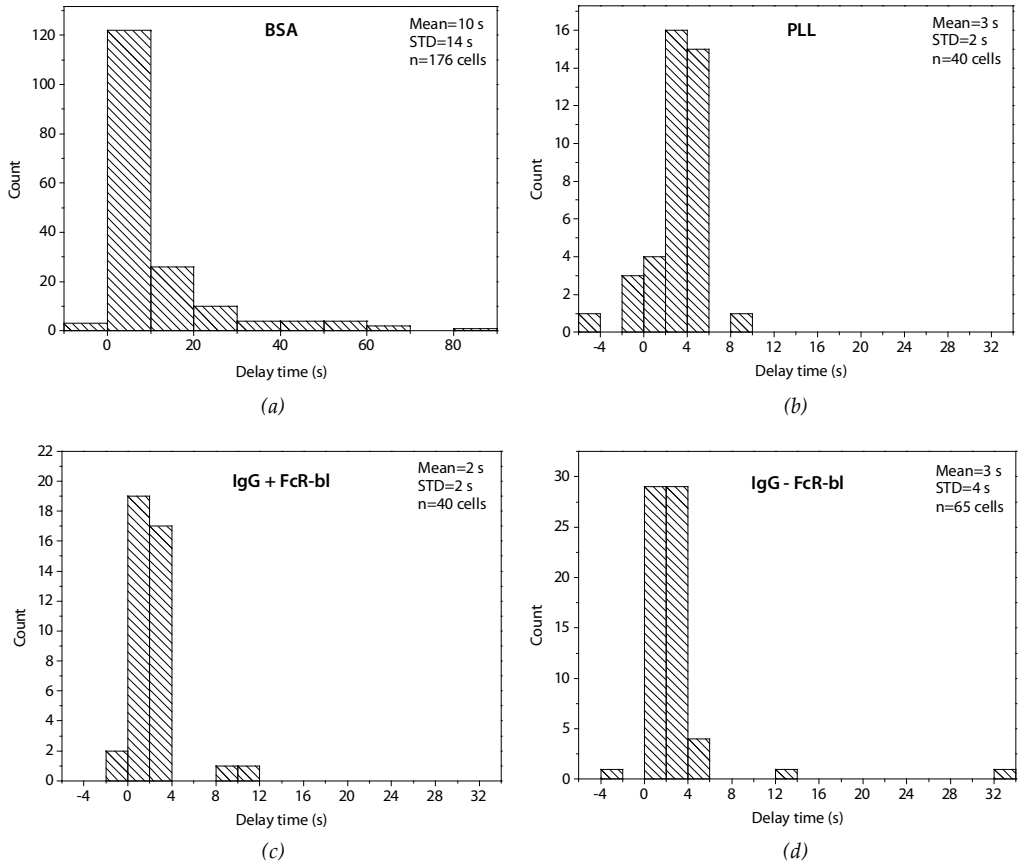


Figure 4.14: Histograms of the recorded delay times of immobilized platelets on different types of surfaces upon chemical stimulation. (a) Platelets immobilized on a BSA coated surface (number of analyzed cells-samples-donors: 176-7-3). (b) Platelets immobilized on a PLL coated surface (40-2-1). (c) Platelets treated with Fc-receptor blocker, immobilized on an IgG coated surface (40-7-3). (d) Platelets immobilized on an IgG coated surface (65-7-3).

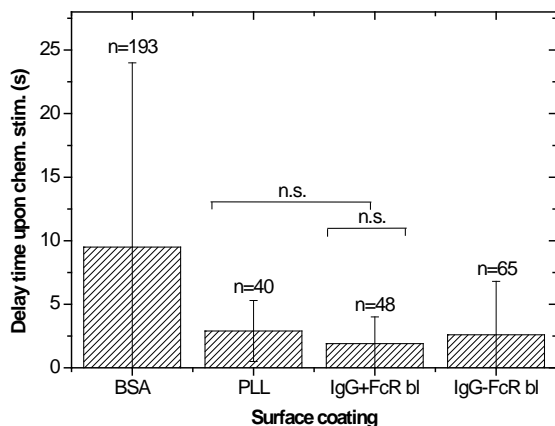


Figure 4.15: The Average response delay time measured on platelet bound to different surfaces, when the cells were exposed to a chemical trigger. n is the number of responding cells analyzed, the error bars represent the standard deviation in the analyzed cell ensemble. All samples showed a significant difference based on the pair-wise statistical analysis with the use of on a two sample student t -test, with a significance level of 0.05, except the pairs indicated with *n.s.* (not significant).

Since only 3 immobilized platelets on an anti-GPIb coated surface showed a response upon chemical stimulation, the data was omitted from Figure 4.14. On a BSA coated surface, the average delay time was the highest (10 ± 14 s). The delay times observed on a PLL or an IgG-coated surface were lower; 2.9 ± 1.4 s on PLL, 1.9 ± 2.1 s on IgG with Fc-receptor blocker and 2.6 ± 4.2 s without Fc-receptor blocker.

Since all immobilized platelets were stimulated with TRAP, which triggers the signaling cascade as presented in Figure 4.2, the delay time was expected to be independent from the substrate on which the platelets were immobilized. In literature, it has been reported that the time between the binding of the receptor binding and the increased cytosolic calcium is approximately 1 second²³. However, from our results presented above, we observe longer delay times on different types of surfaces upon chemical stimulation. In the Methods section (Section 4.4.5 Data analysis), we already discussed that the error in the determination in the time point of stimulation is approximately 2.5 seconds and that gradients in the time response can add up to this uncertainty. This probably results in an experimental method that is not able to resolve this fast platelet response upon chemical stimulation.

4.6.2 Reduced responsiveness after washing procedure

It is known that platelets are cells that can easily be activated by high shear rates⁵ or by air contact (e.g. air bubbles)¹⁵. In some samples, we have introduced an additional washing step, to remove unbound platelets after incubation. Therefore we wanted to test the responsiveness of the adhered platelets after the introduced washing step, to see if an additional washing has influence on the percentage of responding cells upon chemical stimulation.

After platelet incubation, the sample was washed to remove the unbound platelets. This was done by manually agitating the sample for about 10 seconds in a beaker filled with platelet buffer. Thereafter the fluorescence intensity of the remaining adhered platelets during TRAP addition was analyzed. As previously described in Section 4.3.2 platelets showing a response upon the addition of the chemical stimulus were designated as responding cells. From these responding cells, the delay time between TRAP addition and the increased cytosolic calcium was analyzed.

From this data it can be concluded that the percentage of cells that respond to the chemical stimulus TRAP is lower for the washed samples (<1 %) than for samples in which this washing step was omitted (4-50 %). Figure 4.16 shows the delay times of the responding cells on an anti-GPIb coated surface, which did or did not had a washing step in the procedure. The responding cells on the unwashed samples (n=3 cells) showed a response time within 3 s. While the platelets on the unwashed sample (n=40 cells) showed a response time of 6 ± 2 s.

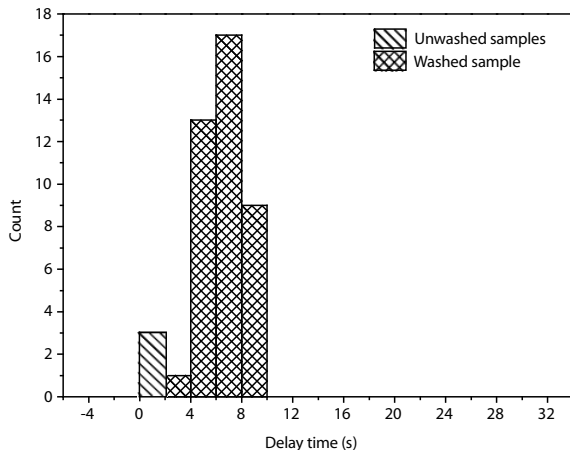


Figure 4.16: Histogram of the recorded delay times of immobilized platelets on an anti-GPIb coated surface upon chemical stimulation. Data was collected from unwashed samples (3 cells, on 2 different samples, with donor material obtained from 2 different donors) and a washed sample (40 cells, on 1 sample, with donor material obtained from 1 donor).

During the washing procedure a meniscus moves over the surface with adhered platelets. It is our hypothesis that the meniscus, which can apply a significant force on the adhered platelets, induces a platelet response. This could explain the reduced percentage of responding cells upon TRAP addition, after a washing procedure is applied on the sample. Although the statistics are low on the delay times measured from immobilized platelets on an unwashed sample, it seems that the average delay time of the immobilized platelets is increased after the washing procedure. Possibly, the heterogeneity of the cells to a response, in this case the applied shear force, causes this shift in the delay time distribution. We hypothesize that cells

that show a short delay time, were more sensitive for a trigger. Thus, responding on the applied washing step. For future experiments, it is advisable to avoid washing steps after platelet immobilization, or the washing should be done more gently.

4.7 Discussion

With the use of the calcium indicator Oregon Green BAPTA-1 AM, the interaction between platelets and a substrate were studied. In order to quantify platelet-surface interactions, a new methodology was introduced, in which the number of responding cells was analyzed and the delay time of the response was measured. We will discuss the combined results, including the interaction between platelets and surfaces (section 4.7.1), followed by a discussion on the responsiveness of platelets after immobilization on a surface (section 4.7.2). The current knowledge in literature about these topics is included in the various sections.

4.7.1 Surface-induced platelet activation: The percentage of responding cells

To quantify the interaction of platelets with different types of surfaces the number of responding cells upon binding to BSA, PLL, IgG, anti-GPIIb and collagen coated surfaces were compared. In addition, the percentage of responding immobilized cells after chemical stimulation was analyzed. Since platelet activation is an irreversible process, we expect that cells that were already activated upon binding to a surface, would not show any response anymore on a chemical trigger such as TRAP. To test our hypothesis, the combined results of the percentage of responding cells are presented Figure 4.17.

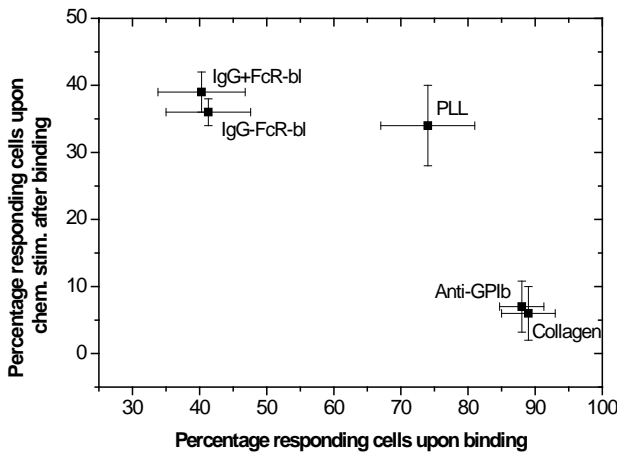


Figure 4.17: The percentage responding cells upon binding versus the percentage of responding cells upon chemical stimulation (chem. stim.) after binding. Since platelets can only be activated once, a relationship was expected from these parameters. This relationship is confirmed by the presented data. For example, on an IgG coated surface a mediate response is observed during binding and chemical stimulation. On a collagen coated surface, almost all cells respond upon binding and little cells respond on a chemical stimulus after binding.

Figure 4.17 shows that a general trend can be seen in the pooled data, in which a lower percentage of responding cells upon binding to a surface, results in a higher percentage of responding cells upon a chemical stimulation subsequent to binding, for example as observed on IgG coated surfaces. When cells tend to respond upon binding to a surface, then a lower percentage responds to the subsequent chemical stimulation, as was observed on anti-GPIb and on collagen coated surfaces. Thus these results confirm our hypothesis.

It should be kept in mind that the analysis of the real-time binding of platelets to a surface is a time-dependent measurement, while the chemical stimulation experiment is an end-point measurement. The latter means that a fixed incubation time for platelet immobilization was used and thereafter a chemical stimulus was added. The observation window in which the binding of the platelets was analyzed can play a role in the outcome of the percentage of responding platelets. We found that increasing the observation window of the analysis (e.g. on IgG coated surfaces) resulted in a higher number of responding cells. For longer delay times, this effect will become stronger, since observation window is then limiting the measurement. For future research it is interesting to include a time-dependent analysis, since it will give more information about the time dependency of the platelet-surface interaction.

In addition, calcium responses in platelets are not a specific marker for irreversible platelet activation. For example cytosolic calcium is also increased when platelets change shape². Since TRAP is a well-known strong platelet activator, it is evident that the introduced calcium response upon the addition of TRAP indicates an irreversible activation. Thus the analysis of the response of platelets on TRAP stimulation after binding holds information about the surface-induced activation of platelets. But the calcium responses induced upon surface contact hold information about the various processes during platelet adhesion which rely on intracellular calcium signaling. This should be kept in mind when using one or the other read-out parameter to study platelet-surface interactions.

From our results we conclude that the most quiescent surface for platelet immobilization is BSA. This is probably due to the repelling force between the negatively charged BSA and the negatively charged platelet membrane. As a consequence, the BSA surface has a low binding affinity for platelets.

Intermediate surface-induced activation was observed on IgG coated surfaces. We hypothesize that the binding to the platelet Fc-receptor initiates platelet activation during immobilization on an IgG-coated surface. The pretreatment of platelets with Fc-receptor blocker did not affect the percentage of responding platelets upon binding or chemical stimulation in the described calcium experiments, which seems to contradict the hypothesis. The effect of the addition of an Fc-receptor blocker will be further investigated in the next chapter, in which secretion markers are measured to investigate platelet-surface interactions.

From Figure 4.17 it can be observed that the nonspecific platelet immobilization to a PLL coated surface showed a high number of responding cells during binding (74 ± 7 %) and an intermediate percentage of cells that respond on chemical stimulation after binding (34 ± 6 %).

Since PLL is known for its high binding affinity for cells and allows cells to easily spread over the surface, it is our hypothesis that the high percentage of responding cells upon binding to PLL is influenced by the upregulation of calcium, which is necessary for shape change.

In our experiments we found that an anti-GPIb coated surface was as thrombogenic as the binding to our positive control; a collagen coated surface. Not only will the Fc-receptor binding contribute to this surface-induced activation process, but probably also the receptor binding to the specific paratopes of the antibody. The role of the GPIb receptor in initiation of the platelet activation process has been described in literature^{40,41}, in agreement with our interpretation.

It is interesting to compare our results to experiments of Ikeda et al.¹² on static sedimentation of platelets onto PLL, von Willebrand factor (vWF) or fibrinogen (Fg) coated surface. The initial intracellular calcium concentrations of platelets just after surface contact with the different types of surfaces were of similar magnitude (about 100 nM)¹². The calcium concentration increased from 94.0 ± 8.7 to 766.5 ± 155.2 nM by adsorption of platelets on a Fg-coated surface, and an increase from 101.3 ± 8.6 to 419.4 ± 50.0 nM was found on a vWF-coated substrate. It was suggested that the vWF, which is a ligand for GPIb, triggers a signal transduction to start morphology changes in which calcium also plays a key role¹².

In contrast to our data Ikeda et al.¹² found no increased calcium concentrations upon binding of platelets to PLL. Their coating procedures and buffer contents are slightly different from ours; yet we think that their findings may have been caused by a different data acquisition and data analysis. A sample frequency of 0.03 Hz was used in their measurements, while we used a sample frequency of 2 Hz. In addition, Ikeda et al. pooled the data of analyzed cells ($n=12$ cells), making no distinction between responding and non-responding cells. Interestingly, in one of the presented figures, the initial calcium concentration in a platelet bound to PLL was about 40 nM, while one minute after adhesion this concentration increased up to 500 nM. This does show a platelet response upon binding to PLL under static conditions; however this effect was not discussed in the paper.

4.7.2 Responsiveness of platelets upon chemical stimulation: Suspended versus immobilized platelets

TRAP is a well-known strong platelet activator. In our flow cytometry experiments that were described in Chapter 3, TRAP was used to stimulate platelets in suspension. In this section, we compare the results of these measurements with the results of the chemical stimulation experiments of immobilized platelets as presented in this chapter.

In the intracellular calcium experiments, platelets were allowed to bind the different types of surface coatings for 15 minutes, thereafter the immobilized platelets were chemically stimulated with 800 μ M TRAP. The number of responding immobilized cells ranged from $6 \pm 4\%$ on a collagen coated surface to $95 \pm 4\%$ on a BSA coated surface. In the flow cytometry measurements as presented in Chapter 3, platelets were stimulated with different concentrations of TRAP and the P-selectin expression was measured after 20 minutes of incubation with fluorescent labels and agonist with the suspended platelets. The recorded dose

response curves showed that almost all platelets responded to the chemical trigger (80-100 %). This is much higher than the percentage of responding platelets on a chemical trigger after immobilization on PLL (34 ± 6 %), IgG (39 ± 3 % with Fc-receptor blocker and 36 ± 2 % without Fc-receptor blocker), anti-GPIb (7 ± 4 %) or collagen (6 ± 4 %).

Possibly, differences in experimental procedures cause these different observations. In case of the flow cytometry analysis, platelets were stimulated with TRAP for 20 minutes and P-selectin expression was analyzed after fixation. In case of the activation of immobilized platelets at anti-GPIb the increase in intracellular calcium was used as a read-out within 3 minutes after stimulation. Since the response time of platelets on a chemical trigger is approximately 1 second, the different incubation times cannot account for this effect. However, maybe the platelet state before incubation to the surface was already altered due to the additional sample handling steps needed for Oregon Green BAPTA-1 AM loading into the platelets. In addition, we know from our flow cytometry analysis that platelets become less responsive on TRAP stimulation after approximately 2 hours after the washing protocol. After 4 hours in buffer, the number of responding cells is decreased by approximately 30 % compared to freshly prepared washed platelets⁴². But this does not yet fully explain the low number of only 7 ± 4 % responding cells found at the anti-GPIb coated surface. However, in Figure 4.17 we already showed that a higher percentage of responding cells upon binding results in a lower percentage of cells that respond on a subsequent chemical trigger. In case of an anti-GPIb coated surface, a high percentage (88 ± 3 %) of cells showed already a response upon binding, which can explain the difference between the percentage of responding cells upon chemical stimulation after immobilization (7 ± 4 %) and the percentage of responding cells in solution (80-100 %).

4.8 Conclusions

In the research presented in this chapter, we used the calcium indicator Oregon Green BAPTA-1 AM to measure changes in cytosolic calcium in platelets. The effect on platelet cytosolic calcium concentrations of immobilization on a surface and of chemical stimulation was quantified by counting the number of cells that showed an increase of fluorescence intensity. From these responding cells at BSA, PLL, IgG, anti-GPIb or collagen coated surfaces, the corresponding delay time (e.g. the time between trigger and response) was also measured.

From our experiments we conclude that BSA is the most quiescent surface for platelet immobilization, since the majority of the immobilized cells are still sensitive for a chemical trigger, followed by IgG and PLL. In case of anti-GPIb and collagen, the majority of the cells responded upon binding to these types of surfaces and a low percentage of immobilized cells responded to a chemical trigger. In conclusion, from these experimental data we confirmed our hypothesis that a relationship is present between the percentage of cells that respond upon binding and the percentage of cells that respond on chemical stimulation.

The method to measure the fraction of responding cells and delay times of the responding cells, was shown to be sensitive enough to distinguish between different types of surfaces. Since individual cell responses were evaluated, the analysis holds not only information about the cell

ensemble, but also about individual cells. The moment of binding as well as the cell response was determined manually in our presented data. To obtain more reproducible and fast analysis method, it is preferable to automate this analysis procedure. The main challenge in automating the analysis, will probably be the selection of the region of interest and the time point of binding.

The use of a calcium indicator to measure platelet function is a research tool than an applicable technique for future biosensors. In the first place, because the cytosolic calcium signaling in platelets is a secondary messenger which is involved in many processes during platelet activation. Secondly, loading of the calcium indicator in platelets is needed to measure cell response. In our current set-up, an incubation time of 30 minutes was used, which is a long time for a point-of-care system. The latter can possibly be solved by using the more sensitive total internal reflection fluorescence, which is a technique that probes fluorescent signals only emitted by fluorophores close by the substrate⁴³, the signal to noise can be enhanced. As a consequence, the amount of fluorophore can be reduced and therefore possibly the incubation time with the calcium indicator can be reduced.

4.9 Outlook

In this section we will address specific research questions related to optimizing the use of calcium indicators for measuring cytosolic calcium in platelets in response to different stimuli. First ratiometric methods are discussed, which can be used to correct for effects that introduce fluorescent intensity variations, which are not calcium related such as shape change or focal drift (section 4.9.1). Second, we discuss how to make the analysis method more qualitative (section 4.9.2). In the last part of the outlook, the use of magnetic tweezers for platelet research is discussed, a novel tool which can be applied to study platelet responses upon mechanical stimulation (section 4.9.3).

4.9.1 Ratiometric measurements

In our study, the Oregon Green BAPTA-1 calcium indicator was used to visualize variations in intracellular calcium concentrations. As an extension of the applied method, ratiometric calcium detection could be used. This can be implemented in two different ways: The use of multiple calcium indicators or by the use of a ratiometric calcium indicator³⁵.

A commonly used combination of calcium indicators for ratiometric studies in platelet research is Oregon Green BAPTA-1 AM with Fura Red AM^{9,20,30}. Both fluorophores can be excited with the same wavelength (488 nm), their emission wavelength is 523 and 670 nm respectively. Where Oregon Green BAPTA-1 AM increases its fluorescent intensity upon increasing calcium concentrations, Fura Red AM will decrease its intensity upon increasing calcium concentrations³⁷. In order to register both signals with one camera, fast switching is needed between two filter cubes. Alternatively, a dual camera set-up can be used in which two channels can record the fluorescent signals of the different fluorophores simultaneously.

In the perfusion experiments performed by Arjan Barendrecht, both calcium indicators were used. Figure 4.18 shows an example of the fluorescent traces in the individual channels. In

these preliminary results, the arrows indicate the opposite intensity changes for the two fluorophores, however this effect was not shown in all samples. To obtain reproducible results with the use of Fura Red AM, the experimental procedures should be optimized.

Oregon Green BAPTA-1 AM is a non-ratiometric calcium indicator, meaning that it will solely increase its fluorescent emission intensity upon calcium binding. In contrast, ratiometric calcium indicators exhibit not only intensity changes with changing calcium concentration, also their excitation and/or emission wavelength change. In platelets research, commonly used ratiometric calcium indicators are Fura-2^{16,20,33,44} or Fluo-3^{20,40}.

Figure 4.19 shows the excitation spectra of Fura-2, which changes upon the presence of calcium, while the emission spectra are not altered³⁵.

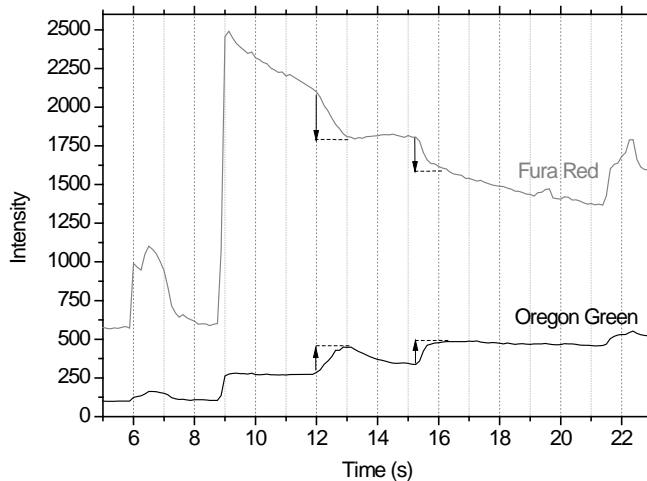


Figure 4.18: Recorded fluorescent intensities of Oregon Green BAPTA-1 AM (bottom curve) and Fura Red AM (top curve) of a platelet binding to a collagen substrate. Oregon Green BAPTA-1 AM ($\lambda_{ex}=470/27$ nm, $\lambda_{em}=512/30$ nm) will increase its fluorescent intensity and Fura Red AM ($\lambda_{ex}=556/25$ nm, $\lambda_{em}=630/98$ nm) will decrease its intensity upon increasing intracellular calcium concentrations. This is the case for example at $t=12$ s and $t=15.5$ s, which are marked with arrows in the plot. Data was acquired by Arjan Barendrecht at the perfusion set-up at the UMCU.

The advantage of ratiometric measurements over the method used in this chapter, is that fluorescent variations caused by other effects than calcium variations, such as cell morphology changes or leakage of the indicator out of the cell, can be filtered out. For example, an adhered platelet spreading out over a surface changes its thickness, which affects the intensities of both measured wavelengths equally. Thus the use of the ratio between the two wavelengths will equal out such a shape change artifact, since both measured intensities will decrease. In case of changes in intracellular calcium, the ratio between the two wavelengths will not equal out, since a shift the emission spectrum is responsible for the fluorescence intensity change.

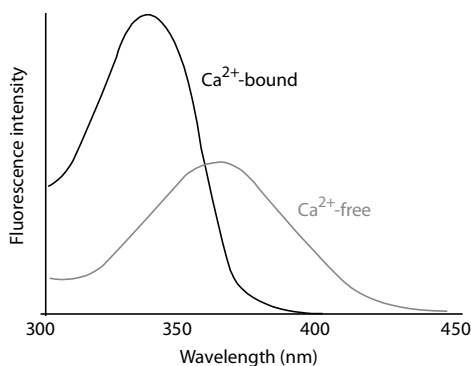


Figure 4.19: The excitation spectra of the ratiometric calcium indicator Fura-2 (measured at $\lambda_{\text{emission}}=505\text{nm}$). The black curve is the excitation spectrum of Fura-2 in presence of calcium and the grey curve is the excitation spectrum of Fura-2 in absence of calcium³⁵.

4.9.2 Calibration of calcium concentrations

To obtain absolute intracellular calcium concentrations with the use of calcium indicators, a calibration of the fluorophore can be done. By measuring the fluorescent intensities of Oregon Green BAPTA-1 AM and Fura Red AM at well-defined calcium concentrations, a calibration curve can be determined experimentally. But more frequently used is a calibration based upon the minimum and maximum fluorescent intensity of the loaded calcium indicators^{30,35,44}.

In practice, the maximum fluorescent signal can be obtained by the use of digitonin, which makes the cell permeable thus allowing free entrance of extracellular calcium¹². Ionophores, which are molecules that are able to transport calcium or other divalent ions through the cell membrane, can also be used to obtain maximum fluorescent signal. Commonly used ionophores in cell biology are calcimycin or ionomycin^{30,40,44}. The minimum fluorescent intensity is obtained by addition of ethylene glycol tetraacetic acid (EGTA) or ethylene diamine tetraacetic acid (EDTA); chelating agents with high selectivity for calcium^{29,30,35,44}.

As a proof of principle for this procedure with the use of Oregon Green BAPTA-1 AM, measurements were conducted by Arjan Barendrecht (UMCU) in a spectrophotometer to illustrate the effect of digitonin as well as EDTA in a platelet suspension (Figure 4.20). Upon the addition of digitonin at $t=50\text{ s}$ and after closing the cover of the apparatus, the fluorescent intensity increases; when EDTA was added at $t=135\text{ s}$ the free calcium concentration drops and thereby the fluorescent intensity.

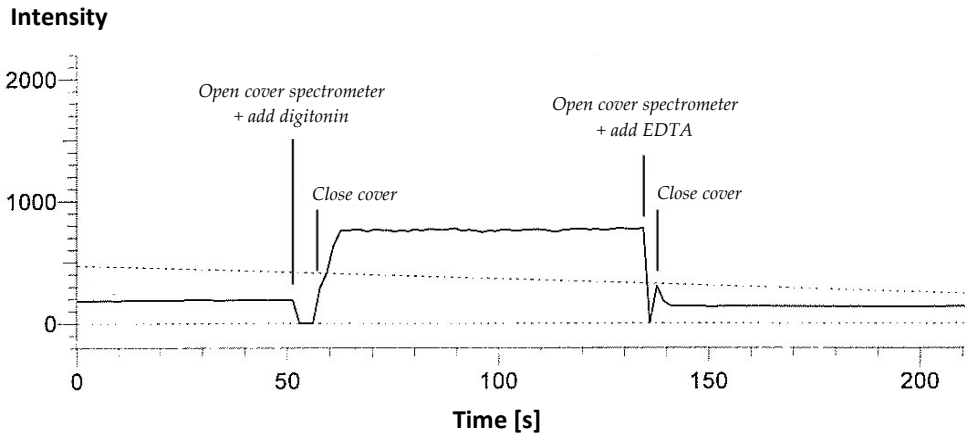


Figure 4.20: Increase and decrease the fluorescent intensity of Oregon Green BAPTA-1 AM loaded platelets in suspension, recorded with $\lambda_{ex}=480\text{nm}$ and $\lambda_{em}=530\text{ nm}$. Digitonin makes the cell membrane permeable therefore increasing the calcium concentration inside the cell thus increasing the fluorescent emission of the fluorophore. EDTA binds free calcium with a high affinity, thereby reducing the calcium concentration and thus the fluorescent intensity in the sample. Data was acquired by Arjan Barendrecht (UMCU).

4.9.3 Mechanical stimulation

Under physiological conditions platelets can be triggered by agonists for example by thrombin or collagen, but also mechanically via shear stress. In the previous sections, stimulation of platelets was studied by adhesion to a surface or chemical stimulation. In addition platelets were also mechanically stimulated via anti-GPIb coated superparamagnetic beads in a magnetic tweezers.

Platelets were allowed to interact with a surface as described in paragraph 4.4.3 and unbound platelets were removed after 15 minutes. Anti-GPIb coated beads were sedimented for 2 minutes on the immobilized platelets, the sample was turned and the magnetic tweezers were switched on. Images in bright field were recorded to locate the bead positions and movies were recorded in fluorescent mode, to measure the calcium fluxes before and during the magnetic actuation.

Figure 4.21 shows a schematic representation of the experiment. On the left side the set up is shown and on the right side the overlay of the bright field image of the fluorescent image of the platelets and the bead outlines (marked in red). Since beads and platelets have the same size, the larger outlines which are visible in the overlay represent bead clusters which are caused by the nonspecific interactions between individual anti-GPIb coated beads.

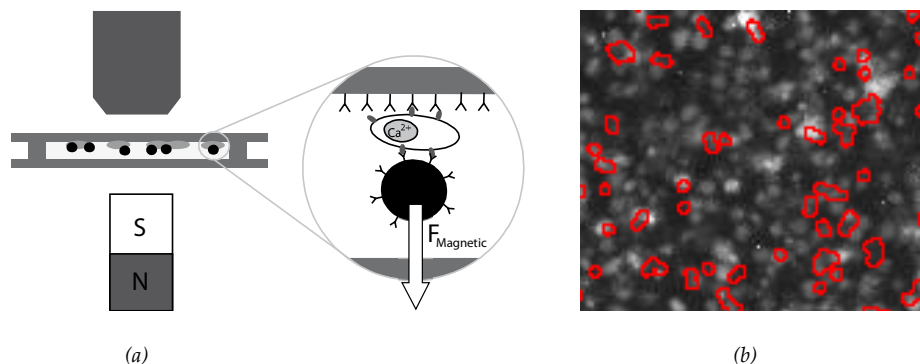


Figure 4.21: Experimental set up for mechanical stimulation of platelets with the use of beads. (a) The set up of the magnetic tweezers implemented on an upright microscope. (b) Overlay of the fluorescent image in which the Oregon Green BAPTA-1 AM loaded platelets are visible and an overlay in which the locations of the beads are marked red. Since beads and platelets have the same size, larger outlines represent clusters of beads which are caused by the nonspecific interaction between individual anti-GPIb coated beads.

In total, more than twenty different samples were tested in the magnetic tweezers set up, but none of the cells showed any response on mechanical stimulation. In section 4.6.2, it was already concluded that the additional washing step after platelet adhesion introduces a reduced responsiveness of the cells at the surface. It is our hypothesis that this is also the cause that the cells do not respond on a mechanical trigger. The forces used in the experiment are about 50 pN⁴⁵, which is about three orders of magnitude lower than the forces experienced by the cells by the meniscus⁴⁶. For future pulling experiments, it is therefore necessary to exclude this washing step, for example by pre-incubation of the platelets together with beads as done in the platelet capture assay described in Chapter 3. By this procedure, platelets are allowed to bind to an anti-GPIb coated bead first and can then be immobilized at the surface, where the magnetic force can be applied.

4.10 Acknowledgements

I would like to acknowledge Arjan Barendrecht from University Medical Center Utrecht for the introduction to the Oregon Green BAPTA-1 AM loading procedure for platelets, performing perfusion experiments on a collagen coated surface, spectrophotometry measurements (Figure 4.20) and providing collagen coated substrates. I would like to thank Jarno Riegman, who performed additional real-time adhesion and activation experiments on BSA and IgG coated surfaces, as a part of his Bachelor internship. In addition I would like to thank Harry Heijnen from the University of Strasbourg for reviewing our work and sharing his expertise on calcium signaling in platelets.

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Supplementary data chapter 4

Calcium variations in adhered platelets on PLL and anti-GPIb coated surfaces

On PLL and anti-GPIb coated substrates additional analyses were performed to evaluate the intracellular calcium signaling in immobilized platelets. Platelets were loaded with the fluorescent dye Oregon Green BAPTA-1 as described in Section 4.4.1 and were allowed to interact with the surface for 20 minutes, thereafter the cytosolic calcium variations in the immobilized cells was analyzed.

The fluorescent intensity changes in the adhered platelets were recorded as a function of time with the same settings as described in Section 4.4.5. To reduce experimental artifacts, the data was filtered in Matlab. To reduce the pixel noise, a five point smoothing filter was used. In order to correct for bleaching or other slow intensity changes in the recorded series, a 5th order high pass Butterworth filter was used. The cut off frequency of the filter was determined by the length of the movie ($f_{\text{cutoff}} = \text{Movielen}gth/4$).

In order to analyze the activity of the platelets immobilized at the surface, the adhered cells were categorized in two populations: active and resting cells. To discriminate between these groups, the fluorescence standard deviation was analyzed for all adhered cells at the substrate. The five lowest standard deviations recorded in the cell ensemble were used for setting a threshold to discriminate between the resting and active cells. The threshold was five times the average standard deviation of these five most silent cells. Figure 4.22 shows typical intensity curves after data filtering of a resting and an active cell.

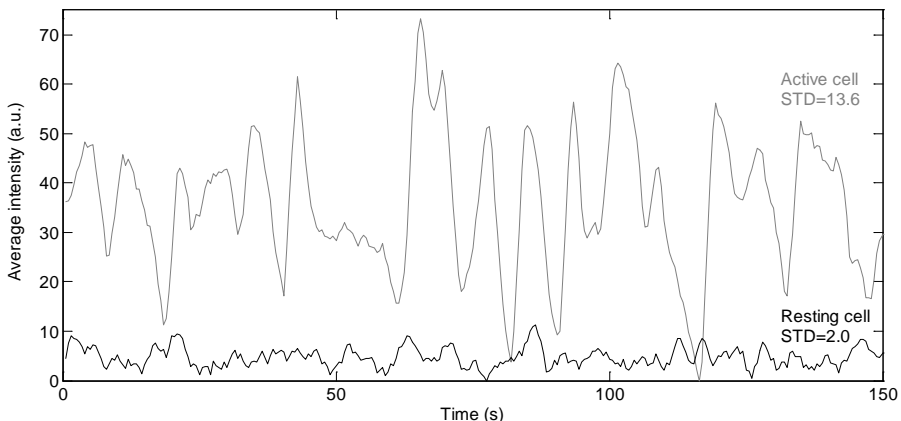


Figure 4.22: Example of a resting and active cell adhered at a PLL coated surface. Typically, an active cell shows a much higher standard deviation in the fluorescence intensity trace than a resting cell. Low pass and high pass filtering was applied on the data.

From the analyzed samples (10 samples coated with PLL, 31 samples coated with anti-GPIb), it can be concluded that the percentage of active cells at the surface differs a lot from sample to sample. The fraction of active cells at the surface ranges from 1% to 80% on PLL and anti-GPIb. The cause of the obtained sample to sample differences remains unknown. The calcium variations of platelets adhered on PLL as well as anti-GPIb was sometimes sustained throughout the whole time scale of the recording, which is about 20 minutes. Such time scales have also been reported in literature for platelets adhered at BSA and fibrinogen coated substrates^{10,47}.

Jackson et al. have studied platelet adhesion to vWF under flow and found sustained calcium variations in relation to platelet translocation³⁰. They used their data to find a relation between the two major platelet adhesion receptors GPIb/V/IX and integrin $\alpha_{IIb}\beta_3$. From their experiments it was concluded that only a transient calcium increase was visible from platelets without translocation and that sustained calcium variations were present in platelets that undergo translocation under flow. In addition, blocking of the integrin $\alpha_{IIb}\beta_3$ receptor with aggrastat resulted in reduced calcium variations in adhered platelets. All together this suggests that the integrin $\alpha_{IIb}\beta_3$ which is required for firm platelet adhesion, is probably also playing a crucial role in our measurements where sustained calcium variations are visible throughout the whole experiment. This hypothesis could be tested with the use of aggrastat-treated platelets, which should prevent the interaction of integrin $\alpha_{IIb}\beta_3$ with the surface.

Chapter 5

Measurement of exocytosis to study platelet-surface interactions

We have used the secretion process of dense granules to study the interaction between platelets and surfaces, in a cell ensemble as well as single cells. ATP secretion was quantified using the luminescent luciferin/luciferase reaction. Platelets were allowed to interact with BSA, PLL, mouse IgG and anti-GPIIb coated surfaces, and after incubation the amount of secreted ATP was analyzed. The baseline signal was given by ATP secretion from resting platelets in suspension. ATP levels secreted from platelets immobilized on BSA were approximately 2 times as large as the baseline. The immobilization of platelets on PLL resulted in ATP levels 4 times as large as the baseline ATP concentrations. On IgG as well as anti-GPIIb the maximum amount of ATP was secreted, which was as much as the ATP released from our positive control consisting of a chemically stimulated platelet suspension. We can conclude that the most quiescent surface for platelet immobilization was BSA, followed by PLL, mouse IgG and anti-GPIIb.

We have also studied the immobilization of the enzyme luciferase on a surface, in order to bring the enzyme in close proximity of the ATP, released from adhered platelets. We have demonstrated that luciferase adsorbed onto PLL is active and generates luminescence in the presence of ATP and luciferin. However, the assay still has a low sensitivity and needs further optimization.

Finally, we have investigated an exploratory method to resolve exocytosis in single platelets, using fluorescent staining of the dense granules by the dye quinacrine. In our experiments we have observed that the exocytosis events are induced by the fluorescence excitation light. We hypothesize that this is caused by the production of reactive oxygen, which disintegrates the vesicle membrane thereby releasing the vesicle content. Further studies may focus on the use of lower quinacrine concentrations, the use of lower light intensities, or on ways to scavenge reactive oxygen. Further development of the exocytosis assay might lead to a quantitative method for biosensor applications.

5.1 Introduction

Platelets contain three types of secretion organelles, namely the alpha granules, dense granules and lysosomes. Each of these granules has a unique content, which is released into the blood stream upon platelet activation and allows the platelet to activate other cells or facilitate cellular adhesion. Diseases which involve altered function or content of one of these organelles are designated as storage pool diseases^{1,2}.

The process of release of cell granule content is referred to as exocytosis or secretion. Laboratory techniques which are currently used to measure altered function of the secretion process in platelets are flow cytometry, aggregometry, bleeding time and luminescent or fluorescent assays. However, these techniques are complex and time consuming. Therefore, we are interested in novel principles for future lab-on-chip integration of platelet function measurements. Lab-on-chip concepts are generally based on the use of a surface to immobilize the platelets³⁻⁷. In our research it is thus important to be able to quantify the influence that a binding surface can have on the cells. In this chapter we will focus on an assay to measure the secretion of adenosine triphosphate (ATP) from the dense bodies in platelets (Figure 5.1), with the use of the luminescent luciferin/luciferase reaction and we will use the assay to study the effect that surface immobilization has on platelets.

The luminescent assay gives information about the average platelet function of a cell ensemble, but cell to cell variations are not revealed, since the total signal of a whole cell population is measured. To gain information about individual cells, the spatial resolution of the luciferin/luciferase assay should be enhanced. In this chapter we will explore the possibility of immobilization of the enzyme luciferase on the surface, thereby measuring the ATP release in close proximity of the adhered cells. In addition an exploratory study was performed to study single cell release events in real-time. The fluorescent molecule quinacrine was used, which has a high affinity for adenine nucleotides and thereby stains the dense bodies in platelets⁸⁻¹⁰. By loading quinacrine inside the dense granules of platelets, individual secretion events were studied. Details of these luminescent and fluorescent assays will be discussed in paragraph 5.3, after a short introduction on the secretion process in platelets in paragraph 5.2.

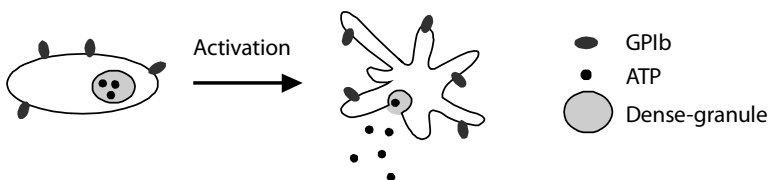


Figure 5.1: Schematic overview of the secretion process in platelets. Dense granules, which contain ATP and other small molecules, fuse with the platelets membrane upon activation and release their content into the extracellular space.

5.2 Physiology of vesicle release in platelets

Exocytosis is the process in which vesicle content is released in the extracellular space via fusion with the membrane. In this section, the characteristics of ATP containing vesicles in platelets (dense granules) and their secretion process will be described.

5.2.1 Dense granules

Normal human platelets contain three to eight dense granules per platelet, with an average diameter of 40-80 nm^{2,11}. Since they are highly electron-opaque, the dense bodies appear as dark spots when viewed with transmission electron microscopy (Figure 5.2a). The content of dense granules consists mainly of small molecules and comparatively few proteins. Ions such as calcium and magnesium are present in the dense granules, nucleotides adenosine diphosphate (ADP) and adenosine triphosphate (ATP) and a transmitter molecule serotonin. Recent studies showed that dense granules are not homogeneous bodies, instead they consist of a dense core with a surrounding (arrow head in Figure 5.2a)^{1,2}.

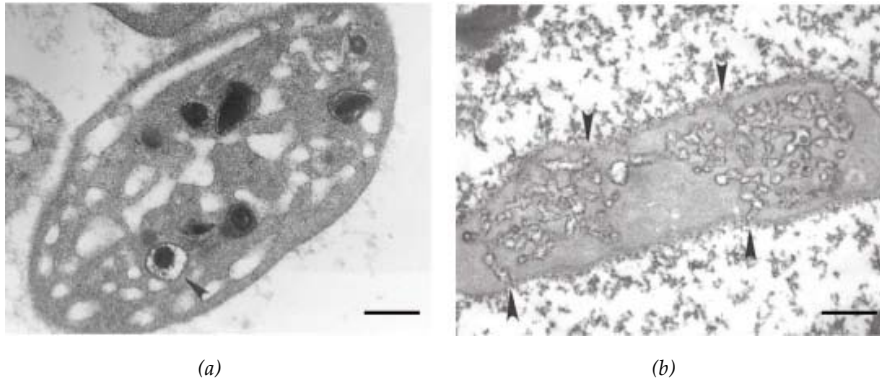


Figure 5.2: Scanning electron microscope images of resting platelet*. (a) Dense granules inside a human platelet are visible as dark regions at the image. The arrowhead indicates a dense granule with a surrounding halo. (b) Platelet with stained open canalicular system and membrane surface. The arrowheads indicate surface invaginations. The black scale bars in the right corner of the images indicate 0.5 μm .

5.2.2 Exocytosis

Exocytosis of platelet granules is triggered by increased cytosolic calcium, which is elevated by platelet activation for example via an external chemical stimulus. This process has already been described in more detail in Chapter 4. In response to strong agonists such as the Thrombin Receptor Activating Peptide (TRAP), the secretion can occur after about 1.5 seconds and is almost complete after 5 seconds¹².

* Reprinted from Platelets, 2nd edition, A.D. Michelson, Chapter 3: Platelet structure, p61 and p67, Copyright (2007), with permission from Elsevier

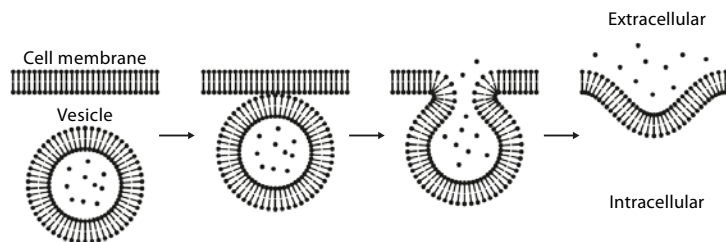


Figure 5.3: Illustration of exocytosis: Motor proteins transport the vesicle to the cell membrane, allowing docking. The vesicle membrane fuses with the membrane of the open canalicular system (OCS), creating a small pore. As a consequence a small portion of the granule content will be released. Finally the vesicle membrane is completely incorporated in the cell membrane and the content is secreted into the extracellular matrix. Figure adapted from Goudsmit¹³.

Upon activation platelets release their granule content into the extracellular space, which *in vivo* is the blood stream. The secretion of the molecules occurs via the open canalicular system (OCS), which is a tortuous network of narrow channels of membrane invaginations inside the platelet (Figure 5.2b)^{2,14}. The release process consists of a series of steps as illustrated in Figure 5.3. The vesicle is first transported to the membrane by motor proteins and then it docks to the membrane of the OCS via SNARE-proteins. After docking, both membranes fuse and a small pore is created, allowing an initial outflux of the granule content. In the end the vesicle membrane is completely incorporated in the membrane, thus creating additional cell membrane which is needed for platelet adhesion and spreading^{1,2,14}.

5.3 Methods to measure exocytosis in platelets

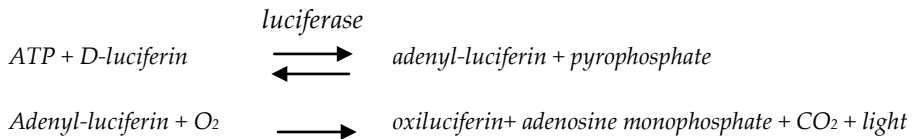
The release process of the dense granules content of platelets can be studied in various ways. In this section we will give an overview of techniques that are commonly used to measure platelet secretion markers. We will divide the discussion in platelet ensemble measurements (section 5.3.1) and single cell measurements (section 5.3.2). From the presented literature, the advantages as well as the disadvantages of current techniques available for ensemble as well as single platelet measurements will be discussed and our research questions will be addressed.

5.3.1 Measurement of secretion markers from platelet ensembles

Secretion markers are often used as a read-out for platelet function measurements. An example of a current laboratory technique to measure exocytosis in platelets is with the use of radioisotope labeling. For example isotopically labeled serotonin can be loaded into the dense bodies of platelets and thereafter the radioisotope concentration after stimulation is measured. An advantage of the technique is that it is a relative simple laboratory test. However, a disadvantage of the technique is that no baseline can be recorded, since the isotopically labeled serotonin first has to be loaded into the cells, before the secretion can be measured^{15,16}. Another method is to measure the secretion of thromboxane A₂ from platelets. However, the use of this

assay is limited since aspirin (acetylsalicylic acid) inhibits the upregulation of thromboxane A₂ and thus the secretion of thromboxane A₂ does not represent the natural platelet function of the donor^{15,16}.

The most common secretion marker measured in platelet function assays is ATP, which is secreted from the dense granules (Figure 5.1). The luminescent reaction of firefly luciferase in combination with D-luciferin is widely used to quantify ATP concentrations in biological samples^{17,18}. The reaction has a high sensitivity and specificity and it covers a broad detection range (pM to mM). ATP is consumed during oxidation of D-luciferin, and luciferase catalyzes the reaction. During this process a photon is released, resulting in light emission (560 nm):



We have used the luciferin/luciferase luminescent assay to measure the interaction of platelets with different types of surfaces. Platelets were allowed to interact with a surface and after 1 hour the supernatant was transferred to a well plate. The amount of ATP released by the platelets into the supernatant was quantified with the use of the luminescent reaction Figure 5.4). With the use of this method, the effect of various surface coatings on platelet activation was studied and experimental adjustments were made in order to reduce the surface induced activation.

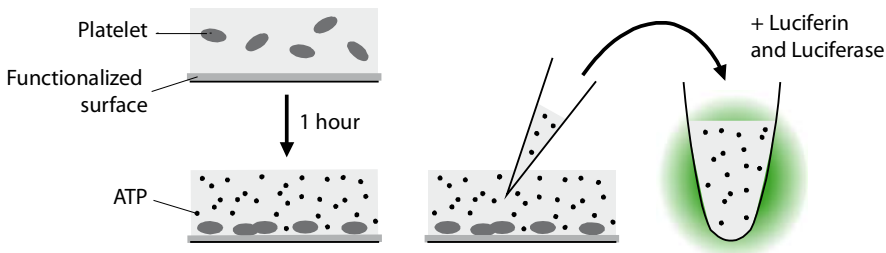


Figure 5.4: Experimental set-up used to study platelet-surface interaction. Platelets were incubated for 1 hour at the surface of interest; thereafter the supernatant was transferred to a well plate holding luciferin and luciferase. A luminescent signal is measured due to the secreted ATP from the dense granules of activated platelets.

5.3.2 Visualization of exocytosis in single platelets

The luminescent luciferin/luciferase assay, as presented in previous section (section 5.3.1), probes the interaction of an ensemble of platelets and the surface. Since cells behave differently within a cell population, it is interesting to also look at individual cell responses on the different types of surfaces.

One of the recent and interesting developments in the use of secretion markers to study exocytosis in individual platelets is from Ge et al.^{11,19-21}, who have been using a small electrode to measure the release of serotonin from the dense granules. The microneedle is brought in close proximity of a cell of interest and measures the electrochemically induced oxidation of the secreted molecule. However, since in these experiments a single microneedle is positioned close to a single cell, the throughput of this method is low.

We explored another approach to measure dense granule release from platelets, which has the potential to resolve single cell exocytosis over a large cell population. For this purpose, we studied the use of fluorescent labeling of platelet dense bodies with the drug quinacrine, which was used as an antimalaria drug. Irvin et al.⁸ reported in 1954 already the interaction of quinacrine (mepacrine) with adenine nucleotides. The dense granules of platelets contain high concentrations of ADP (0.7 μM) and ATP (0.4 μM) compared to other organelles present inside the cell¹. As a consequence, quinacrine accumulates in, and thereby stains, the dense granules^{9,10,22}. Using fluorescent microscopy, the dense granules appear as bright spots on the image^{23,24}. Our experiments are based on the assumption that secretion of ATP and thus quinacrine from the dense granules results in a decrease of the fluorescent intensity of the dense granules.

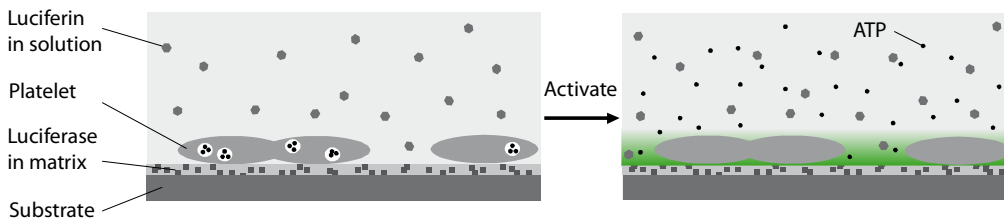


Figure 5.5: Immobilization of luciferase at a substrate for biosensor applications. By immobilization of luciferase, the enzyme is closer to platelets and thus the ATP released upon activation. This leads to lower amount of reaction products needed and possibly single cell resolution since the luminescence reaction is now localized.

In addition, we explored the possibilities to use the luciferin/luciferase reaction to obtain single cell resolution in the measurement of ATP secretion from dense granules. Methods have been reported in literature that probe the ATP concentration directly after release from cells by the use of firefly luciferase immobilized to a substrate²⁵⁻²⁸ or attached to the membrane of the cell²⁹⁻³¹. From a biosensor point of view, we prefer to immobilize the enzyme at a substrate rather than on the cell surface, since the latter is difficult to integrate in a lab-on-chip device. Therefore we explored the possibilities of immobilized luciferase on a substrate (Figure 5.5) for the use in future biosensor designs to measure exocytosis in platelets.

5.4 Materials and methods

In this paragraph details of the experimental procedures will be discussed. In the first two sections, the preparation protocol of washed platelets (section 5.4.1) and functionalized surfaces (section 5.4.2) can be found. Then the set up of the luminescent luciferin/luciferase assay will be discussed in section 5.4.3, including a dose response curve measured with the assay showing that the released ATP can be used as a read-out for platelet activation. In the last two sections, the experimental set up of the fluorescent measurements to visualize single cell exocytosis with the use of quinacrine will be discussed. In section 5.4.4 the characteristics of quinacrine will first be presented, followed by the specifications of the optical system as used for the imaging of the quinacrine loaded dense granules in section 5.4.5.

5.4.1 Preparation of washed platelets

Fresh whole blood samples were purchased from Sanquin, the Dutch organization for the supply of blood and blood products. Venous blood was collected from healthy donors who reported to be free of aspirin for at least 10 days. Whole blood was anticoagulated with 3.2% sodium citrate. Platelet-rich plasma (PRP) was prepared from whole blood within 1 hour after collection by centrifugation at 250 g for 20 minutes at room temperature. The PRP was transferred to a clean tube and anticoagulated with Citrate Dextrose (ACD, 0.25% Citrate, 0.15% Citric Acid and 0.2% D-Glucose). The mixture was then centrifuged at 520 g for 20 minutes and the supernatant was discarded. Carbaprostacyclin (cPGI, Cayman Chemical Company) was added with a final concentration of 100 ng/ml to reduce the platelet activation due to sample handling. The platelets were resuspended in a platelet buffer with pH 6.5 (Hepes buffer containing 10 mM Hepes, 150 mM NaCl, 5 mM KCl, 0.3 mM $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$, 10 mM $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ and 50 mM D-glucose) and the volume was adjusted with this buffer to the original PRP volume, obtained after the first centrifugation step. The platelets were washed by a third centrifugation step at 520 g for 20 minutes. The supernatant was discarded and the platelets were resuspended in a platelet buffer with a pH of 7.3 (Hepes buffer containing 10 mM Hepes, 150 mM NaCl, 5 mM KCl, 0.3 mM $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$, 10 mM $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ and 50 mM D-glucose), the volume was adjusted to the original volume of the donor material. When needed, FcR blocker (Miltenyi Biotec) was incubated for 10 minutes at room temperature, to block the Fc-receptor (CD32) at the platelet membrane.

5.4.2 Surface preparation

Glass cover slides were cleaned with ethanol before incubation with the surface coatings of interest. Poly-L-Lysine (PLL, Sigma) in deionized water, mouse immunoglobulin (mouse IgG, Calbiochem) or anti-GPIIb (R&D systems) in phosphate buffered saline (PBS, Sigma) were added to the substrate and incubated overnight at room temperature. After incubation, the samples were rinsed with 1% Bovine Serum Albumin (BSA, Sigma) in PBS. In addition, all samples were blocked with 3% BSA in PBS, for 2 hours at room temperature.

5.4.3 Measurement of ATP concentrations with a luciferin/luciferase luminescent assay

Quantitative analysis of ATP concentrations were performed in a well plate reader (Fluoroskan Ascent FL, Thermo electron corporation). Protocols were developed by Thijs van Holten (University Medical Center Utrecht) and we optimized them for our measurements. 50 to 100

μl of platelet suspension or supernatant with unknown ATP concentration were transferred into a 96-well plate, which was pretreated with 3% BSA. 10 μl firefly luciferase (Sigma-Aldrich) and D-luciferin (Tebu-Bio) with final concentrations of 20 $\mu\text{g}/\text{ml}$ and 50 $\mu\text{g}/\text{ml}$ respectively, were added just before dynamic luminescence measurement in the well plate reader. An integration time of 1 second was used per measurement point per well and the luminescent signal was measured for about 1 hour. The maximum intensity during the measurement was used for analysis. Samples with known concentrations of ATP were used for calibration of the assay.

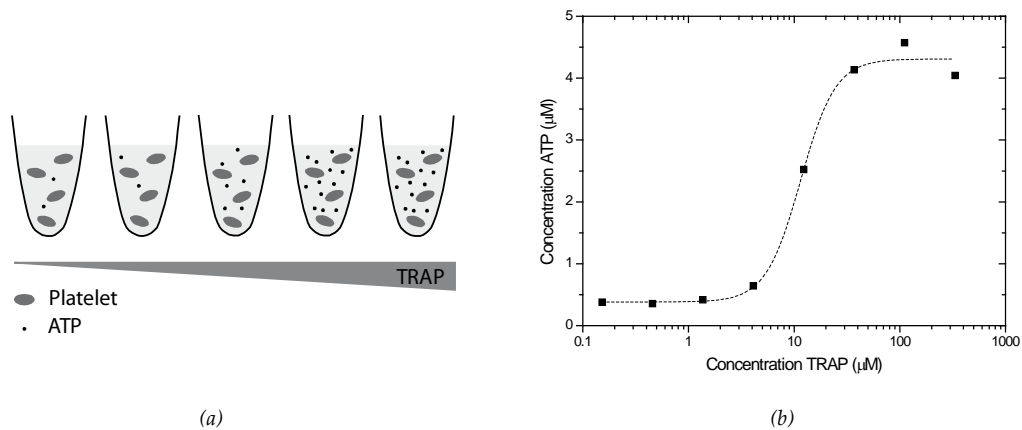


Figure 5.6: Experimental set up for dose response curve measurements of platelets with the luminescent luciferin/luciferase assay. (a) Platelets were stimulated with different concentrations of TRAP for 5 minutes, resulting in different amounts of secreted ATP. Then luciferin and luciferase were added and the light output was measured. (b) Dose response of washed platelets. Quantitative ATP concentrations were obtained with the use of calibration samples. From the fitting of the data points with formula 3.1 an EC_{50} of $11.5 \pm 1.2 \mu\text{M}$ TRAP was found.

In order to validate the luminescent luciferin/luciferase assay, a dose response curve was recorded of platelets stimulated with different concentrations of TRAP (Figure 5.6a). Measurements in the well plate reader give a sigmoidal dose response behavior (Figure 5.6b). Platelet responsiveness can be described with the use of the EC_{50} value, which represents the concentration at which a half maximum response is observed. An EC_{50} of $11.5 \pm 1.2 \mu\text{M}$ TRAP was found from the dose response curve. This EC_{50} was in the expected range (1-30 μM) for healthy donors, which we previously determined by flow cytometry measurements as described in Chapter 3.

The ATP concentration found for maximum stimulation (TRAP concentration $> 100 \mu\text{M}$) was about 4.3 μM ATP. A platelet concentration of 89200 platelets/ μl was determined by flow cytometry analysis of the used samples, resulting in an approximate amount of ATP of 5 nmol/ 10^8 platelets. This ATP concentration is comparable to concentrations reported by Bayer et al (4-10 nmol/ 10^8 platelets)³² and Born et al (2-8 nmol/ 10^8 platelets)³³.

The above data has shown that the EC_{50} value as well as the amount of secreted ATP are in the expected range, indicating that with the use of the luminescent assay with luciferin/luciferase, we can measure secreted ATP concentrations from a platelet ensemble.

The luminescent assay with luciferin/luciferase was used to measure the interaction between platelets and different types of surfaces. To confirm that most platelets interact with the surface and do not stay in the supernatant, control measurements were performed in which a stimulus was added to the supernatant. If the ATP concentration increases after stimulation, this is indicative for the presence of platelets in the supernatant. Platelets were incubated for 1 hour at room temperature on a BSA coated surface. Then the supernatant was removed, one sample was stimulated with 200 μ M TRAP and one sample remained untouched. The levels of ATP in both supernatant samples were compared and normalized with the use of a positive control (200 μ M TRAP activated platelet suspension). ATP concentrations in the samples without and with TRAP relative to the positive control were 0.25 and 0.38 respectively. This results shows that the supernatant of a surface experiment contains only a low number of platelets. In addition, it is known from sedimentation experiments, that after 1 hour of incubation most of the platelets are sedimented onto the substrate. These two controls demonstrate that most of the platelets interact with the surface.

5.4.4 Quinacrine characteristics

Quinacrine excitation wavelengths range from 350 to 475 nm, and the emission wavelengths range from 440 nm to 600 nm²³. To quantify the fluorescent intensity of quinacrine at different concentrations, bulk measurements were performed in which different concentrations of quinacrine were placed in a closed fluid cell and the fluorescent intensity was measured at three different spots (Figure 5.7a). The measured calibration curve is presented in Figure 5.7b, in which each data point represents the average fluorescent intensity including the spread in the three different spots.

From the calibration curve (Figure 5.7b) we see that a maximum intensity is reached at approximately 5 mM quinacrine. The insets in the figure show the expected intensity time traces upon granule release for concentrations lower (left inset) and higher (right inset) than 5 mM. For quinacrine concentrations lower than 5 mM the fluorescent intensity of the dense granule is expected to decrease upon exocytosis. In contrast, for quinacrine concentrations higher than 5 mM the release event is expected to show an increased light intensity due to the dequenching of the quinacrine, followed by a decrease in light intensity. The average spacing between the quinacrine molecules at a concentration of 5 mM is about 10 nm. At such a small distance, energy transfer is possible between individual molecules when sufficient spectral overlap in excitation and emission spectra is present. This energy transfer between quinacrine molecules inside the dense granule, could introduce the quenching effect at these high concentrations^{34,35}.

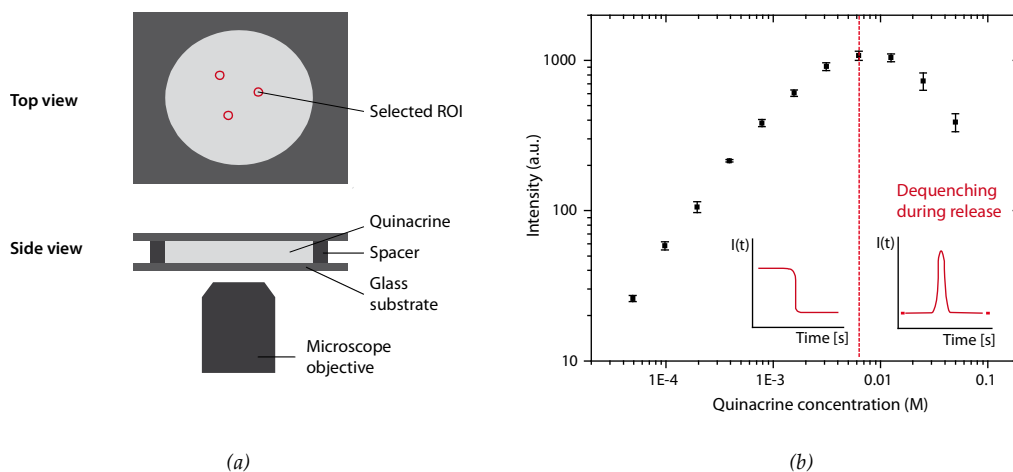


Figure 5.7: (a) Experimental set up for recording the calibration curve of quinacrine. Different dye concentrations were placed in a closed fluid cell, and the fluorescent intensity was measured at three different spots with the inverted microscope. (b) The measured fluorescent light intensity (\pm spread between the different measurement points). For quinacrine concentrations higher than 5 mM, a strong quenching effect (decreased light output) is visible. The inset shows two expected curves of the fluorescent intensity of a dense granule upon exocytosis as a function of time. For quinacrine concentrations higher than 5 mM the release event will show an increased light intensity due to the dequenching of the quinacrine, followed by a decrease in light intensity. In contrast, for quinacrine concentrations lower than 5 mM the fluorescent intensity of the dense granule will decrease upon exocytosis.

We tested the effect of the quinacrine loading on the responsiveness of the platelets. Platelets were loaded with and without quinacrine and their responsiveness was measured with a dose response curve as previously described in section 5.4.3. Again, we used the EC_{50} values as a measure for the responsiveness of platelets (Chapter 3). Only a small deviation in the EC_{50} values was found between the two series; $11.5 \pm 1.2 \mu\text{M}$ TRAP for platelets without quinacrine and $15.8 \pm 1.7 \mu\text{M}$ TRAP for platelets loaded with quinacrine. The concentration of released ATP for maximum stimulation ($300 \mu\text{M}$ TRAP) from platelets loaded with quinacrine was lowered by about 18% compared to unloaded platelets. These results indicate that the loading of quinacrine had a small effect on the status of activated platelets. Since more quinacrine is released at higher TRAP concentrations, it is also possible that the reduced light output is caused by interference of the released quinacrine from the platelets with the luminescent assay, thereby reducing the light output when higher quinacrine concentrations are present during the reaction.

5.4.5 Imaging of the dense granule release

Fluorescent images of quinacrine loaded platelets were recorded with a high sensitivity camera (Hamamatsu C1060010B, ORCA-R2) attached to an inverted microscope (Leica DMI6000B). A Leica lens (HCX PL FLUOTAR L) with a magnification of 40x and numerical aperture of 0.60 was used; for sample excitation an external light source (Leica EL6000) was used. The Leica L5

filter cube was used consisting of an excitation filter of 480/40 nm, a dichroic mirror of 505 nm and a band pass filter of 527/30 nm. Fluorescent images were recorded at a sampling rate of 2 to 5 Hz. In addition, bright field images were recorded before and after fluorescent imaging to locate the platelets' positions. With the use of a calibration sample and the above settings, this led to an area of $0.25 \times 0.25 \mu\text{m}^2$ per pixel in the image plane. Positions of the dense granules were determined by visual inspection. A 5×5 pixel region of interest (ROI) was placed over the selected dense granule and the average intensity of the selected ROI was analyzed.

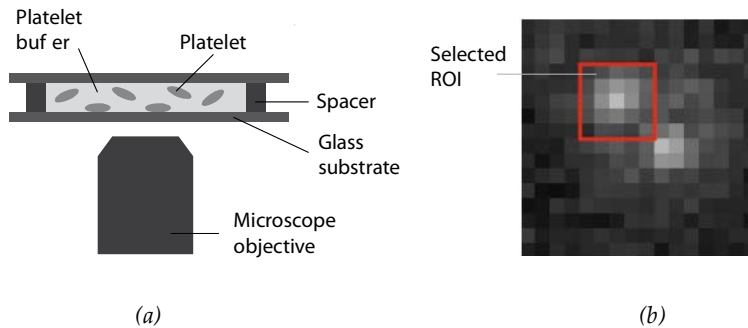


Figure 5.8: Experimental set up to study the granule release of quinacrine stained platelets. (a) Platelets were incubated for 30 minutes at room temperature, allowing the platelets to bind to the surface. Then the unbound platelets were removed by a washing step with Hepes buffer (pH 7.3), thereafter the fluid cell was closed. Images were recorded with a high sensitivity camera on an inverted microscope. (b) Example of a fluorescent image of a platelet in which two granules are visible. The red square marks the selected region of interest (ROI). The resolution of the recorded fluorescent image lead to an area of $0.25 \times 0.25 \mu\text{m}^2$ per pixel in the image plane, which was determined with the use of a calibration sample.

5.5 Results of exocytosis measurements in platelets

The results of two types of experiments will be presented in this paragraph; in the first part of the paragraph, results obtained with the use of the luminescent luciferin/luciferase assay will be described. This covers the results of the effect of the surface coating and substrate on platelet activation (section 5.5.1), the reduction of the surface induced activation on platelets by the use of lower concentrations of surface coating or Fc-receptor blocking (section 5.5.2) and the immobilization of the luciferase (section 5.5.3). In the second part of this paragraph, the results of the real-time visualization of dense granule secretion with the use of quinacrine will be presented (section 5.5.4).

5.5.1 Effect of surface coating and substrate on platelet activation

In the reference measurements in section 5.4.3 we showed that the amount of secreted ATP from platelets can be measured with the luciferin/luciferase luminescent assay and represents the activation status of the platelets. In this section we apply the method to study the interaction of platelets with different types of surface coatings. The amount of secreted ATP is used as a read-out for the surface induced activation of the immobilized platelets.

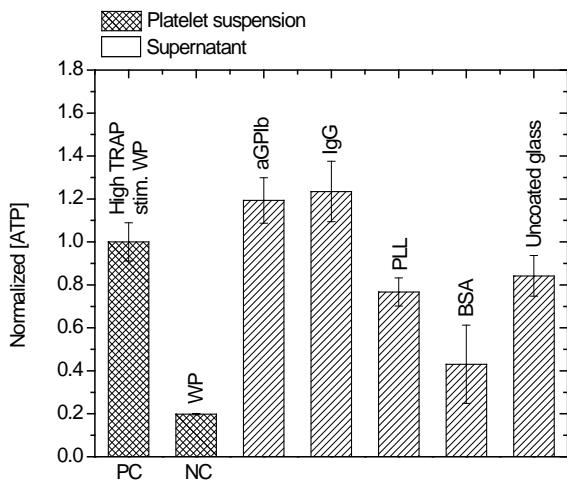


Figure 5.9: Platelet interactions with different surface coatings. Washed platelets were allowed to interact with the surface for 1 hour, then the supernatant was removed and ATP concentrations were measured with the luminescent luciferin/luciferase assay. In addition, measurements were performed in platelet suspension; baseline levels of ATP concentration were recorded from unstimulated platelets in suspension (negative control, WP) and from 200 μM TRAP stimulated platelet samples (positive control: high TRAP stim. WP). The latter was used for normalization of the data. Averages of three samples are presented; error bars indicate the spread in the samples. Washed platelets from the same donor were used for all samples. The amount of secreted ATP from platelets adhered on anti-GPIIb (aGPIIb) and IgG is the highest; on BSA is the lowest. PC=positive control, NC=negative control.

Anti-GPIIb, mouse IgG and PLL coated surfaces were prepared as described in section 5.4.2. In addition, a BSA blocked surface (3% BSA in PBS) and uncoated glass slides were included. Platelets were allowed to interact for 1 hour at room temperature with the surface, thereafter the supernatant was transferred to a well plate and the amount of ATP in the supernatant was analyzed. Two reference samples were added to the series: a platelet suspension to measure baseline ATP concentrations (negative control) and a sample with 200 μM TRAP-stimulated platelets to obtain maximum ATP secretion (positive control). The latter sample was used for normalization of the data. Per sample type, triplicates were prepared with washed platelets obtained from one donor; the results are shown in Figure 5.9, the error bars indicate the spread in the samples.

From Figure 5.9 it can be noted that the platelet immobilization on a BSA coated surface introduces the least amount of secreted ATP (0.4 ± 0.2). The relative concentration of ATP secreted from immobilized platelets on BSA is about two times as high as baseline levels (negative control = unstimulated washed platelets solution, 0.2 ± 0.01). BSA is often used in experiments to prevent protein or cell adhesion to a surface, probably because its high negative charge introduces a repelling force. Therefore only a mild interaction was expected for platelet binding onto a BSA coated surface. Possibly the removal of the supernatant, which introduces

shear flow on the sample surface, causes platelet activation and ATP release from the immobilized platelets. Intermediate surface-induced ATP secretion was observed on PLL (0.8 ± 0.1) and uncoated glass (0.8 ± 0.1) and high amounts of ATP were measured on anti-GPIb (1.2 ± 0.2) and IgG (1.2 ± 0.1). The latter is probably caused by the activation induced by the binding between the Fc-receptors present at the platelet membrane and the crystalline region of the immobilized antibodies at the substrate. This interaction is known to trigger platelet activation. The effect of the use of a Fc-receptor blocker prior to platelet immobilization will be discussed in more detail in Section 5.5.2.

From Figure 5.9 it can also be noted that the measured amount of secreted ATP on IgG and anti-GPIb coated surfaces is higher than the positive control, which was a platelet suspension stimulated with TRAP. This is probably caused by evaporation of the supernatant during platelet incubation of 1 hour at room temperature, which increases the concentration of the ATP. Since the platelet suspensions are kept in a closed tube, this does not affect the reference values.

Measurements of secretion markers released after platelet-surface interactions were also described in literature; Ill et al. studied the surface induced activation of platelets on laminin, fibronectin and BSA by radioisotope labeling³⁶. Platelets were loaded with radioactive serotonin ($[^{14}\text{C}]$ -serotonin) and allowed to interact with the various substrates. After incubation, the supernatant was removed and the amount of secreted label was measured for the population of bound and unbound platelets. Our conclusion is in agreement with their experimental observations; the effect of a BSA coated surface on the activation status of platelets is minor. In addition, Ill et al. showed that a laminin coated surface was a suitable surface for platelet immobilization since it did not introduce platelet activation.

To see if the underlying substrate used for adhesion of proteins has an effect on the surface induced activation of platelets, polystyrene and glass surfaces were prepared according to the protocol presented in section 5.4.2. Anti-GPIb, mouse IgG and PLL coatings were prepared on polystyrene and glass. In addition, a BSA blocked surface (3% BSA in PBS) and uncoated substrates were included. Platelets were allowed to interact with the surfaces for 1 hour at room temperature; thereafter the amount of secreted ATP was measured with the use of the luciferin/luciferase reaction and normalized. Figure 5.10 shows the results, in which the left columns present the glass slides and the right columns the polystyrene slides. Since the individual series were recorded with different donor material, four reference measurements are shown at the left side in the figure; baseline levels of ATP concentration were recorded from unstimulated platelets in suspension (WP) and maximum ATP levels were recorded from 200 μM TRAP stimulated platelets in suspension (High TRAP stim. WP). The latter samples were used for normalization of the individual series.

From the data presented in Figure 5.10, similar trends are visible for both glass and polystyrene substrates coated with different proteins: BSA gives the lowest amount of secreted ATP (0.4 ± 0.2 and 0.43 ± 0.03), followed by PLL (0.76 ± 0.06 and 0.7 ± 0.1), mouse IgG (1.2 ± 0.2 and 1.1 ± 0.1) and anti-GPIb (1.2 ± 0.1 and 1.1 ± 0.1). This leads to the conclusion that the protein coating

(BSA, PLL, IgG or anti-GPIIb) rather than the substrate (glass or polystyrene) determines the effect on the platelet status reflected by the ATP secretion. Although the hydrophobicity of the substrates differs and thus probably the interaction of the proteins with the surface differs, this did not result in significant surface-induced platelet activation. Clearly, the reactive groups of the surface coating molecules are available for platelet binding, independent of the underlying substrate.

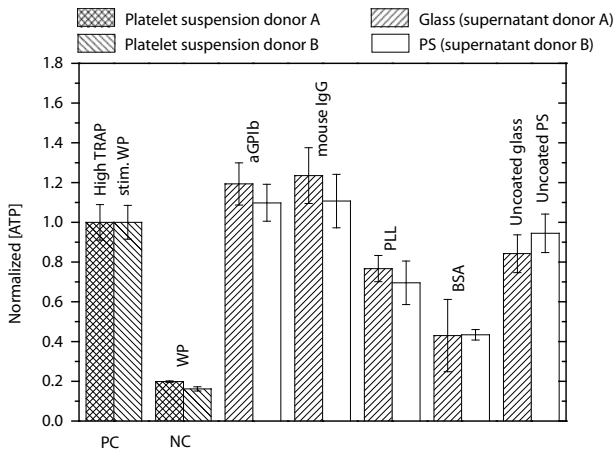


Figure 5.10: Surface induced activation tested on glass (left bars) and polystyrene (right bars) surfaces, with and without protein coating. Washed platelets were allowed to interact with the surface for 1 hour, then the supernatant was removed and ATP concentrations were measured with the luminescent luciferin/luciferase assay. In addition, measurements were performed in platelet suspension; Baseline levels of ATP concentration were recorded from unstimulated platelets in suspension (negative control: WP) and 200 μM TRAP stimulated platelet samples (positive control: high TRAP stim. WP) were used as reference. Averages of three samples are presented; error bars indicate the spread in the samples. Different donor material was used for the two series: glass (donor A), polystyrene (donor B). PC=positive control, NC=negative control, PS=polystyrene.

5.5.2 Reduction of the surface induced activation

In order to reduce the surface induced platelet activation, the influence of the surface coverage of the proteins was tested for mouse IgG. High (IgG high= 200 $\mu\text{g}/\text{ml}$) and low (IgG low=10 $\mu\text{g}/\text{ml}$) mouse IgG concentrations were incubated on a glass surface. In literature it was already reported that the use of an Fc-receptor blocker could reduce platelet activation³⁷. Therefore, platelets were incubated with and without Fc-receptor blocker (FcR b1) prior to surface adhesion to investigate the effect of the Fc-receptor blocker. Platelets were allowed to interact with these substrates for 1 hour at room temperature. Then the supernatant was transferred to a well plate and the amount of ATP secreted from the platelets was measured with the luminescent luciferin/luciferase assay.

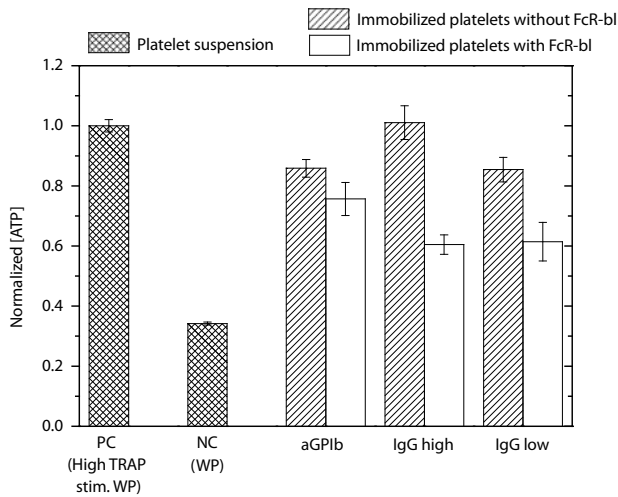


Figure 5.11: Reduce surface induced platelet activation, by reducing protein coating concentrations or adding Fc-receptor blocker. Platelets were incubated with and without Fc-receptor blocker (FcR bl) prior to adhesion. ATP concentrations were measured with luminescent assay after 1 hour of incubation of the platelets. Different types of surface coating were tested: anti-GPIIb (aGPIIb), 200 $\mu\text{g/ml}$ mouse IgG (IgG high) and 10 $\mu\text{g/ml}$ mouse IgG (IgG low). Baseline levels of ATP concentration were recorded from unstimulated platelets in suspension (WP) and 200 μM TRAP stimulated platelet samples (High TRAP stim. WP) were used as reference. Each bar represents the average ATP concentration ($n=3$), the error bars indicate the spread in the samples. The amount of secreted ATP was reduced by the use of an FcR blocker as well as by reducing the concentration of IgG at the surface. PC=positive control, NC=negative control.

Figure 5.11 shows that reducing the concentration of IgG during the coating procedure from 200 $\mu\text{g/ml}$ (IgG high) to 10 $\mu\text{g/ml}$ (IgG low), the amount of secreted ATP from immobilized platelets was reduced with 16 %. In addition, the use of an Fc-receptor blocker, which blocks the platelet receptor for the crystallizable part of an antibody, also reduced the amount secreted ATP from the dense granules during platelet immobilization. On the 10 $\mu\text{g/ml}$ IgG coated surface, the addition of the Fc-receptor blocker reduced the ATP concentration by 28 %. On the 200 $\mu\text{g/ml}$ IgG coated surface, the addition of the Fc-receptor blocker reduced the ATP concentration even by 41 %. The Fc-receptor blocker reduced the ATP secretion from platelet sedimented onto anti-GPIIb only by 11 %. This can be explained by the fact that two types of specific bonds can be established for the immobilization of platelets on anti-GPIIb. In the first place, the binding can occur via the specific paratopes of the anti-GPIIb with the membrane protein GPIIb. In addition, also the Fc-receptor present in the platelet membrane, can bind the Fc-part of the anti-GPIIb at the surface. In case of the IgG coated surface the latter bond is the only specific bond that will occur. Assuming a random orientation of the antibodies at the surface, the effect of blocking the Fc-receptor at the platelets' membrane has less effect on platelets binding to anti-GPIIb than on IgG coated surfaces. Figure 5.12 gives an overview of the specific bonds on anti-GPIIb and IgG coated surfaces, without and with the presence of a Fc-receptor blocker.

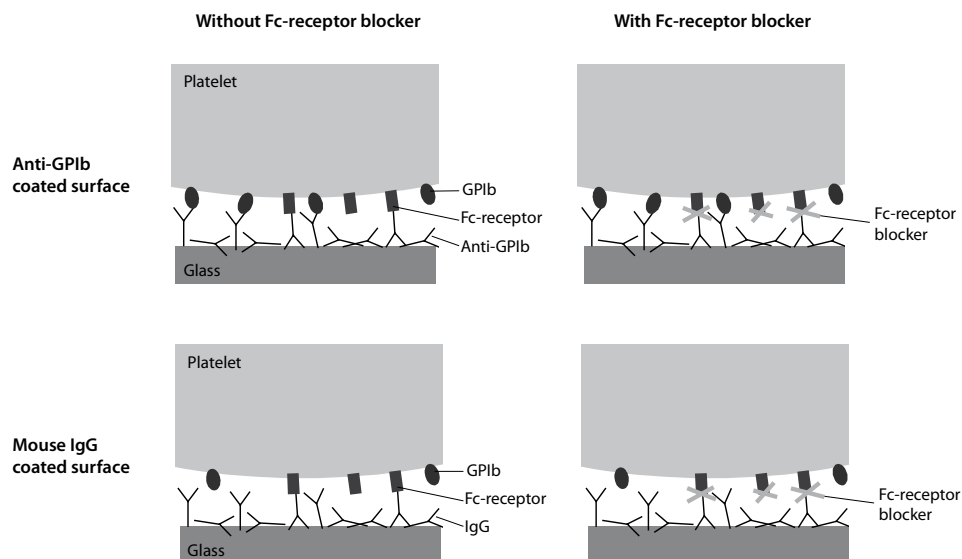


Figure 5.12: The specific bonds which can occur between a platelet and an antibody coated surface. Top row shows a platelet immobilized on an anti-GPIb coated surface without (left) and with (right) Fc-receptor blocker. Bottom row shows a platelet immobilized on an IgG coated surface without (left) and with (right) Fc-receptor blocker. In case of the anti-GPIb coated surface, the platelet can attach via GPIb or Fc membrane receptors. In case of attachment to an IgG coated surface, only the latter specific bond can occur. As a consequence, it is expected that the use of an Fc-receptor blocker will have more effect on platelets immobilized on IgG than on anti-GPIb, which was confirmed by our measurements.

Both the reduction of the concentration of the coating protein as well as the addition of the Fc-receptor blocker, have the effect to reduce the number of specific bonds between the platelet and the surface. In case of the reduction of coating protein, the number of ligand molecules at the surface is reduced. When the Fc-receptor blocker is used, the number of bonds between the platelet Fc-receptor and the immobilized antibodies is reduced. We hypothesize that, the number of bonds used for the immobilization of platelets to a surface determines the effect the surface has on the activation of platelets.

5.5.3 Immobilization of luciferase to localize the luminescent reaction

In the previous sections we already showed that the luciferin/luciferase assay can be used to quantify the amount of secreted ATP from platelets, such that the activation status of the platelets can be measured. To obtain single cell resolution, we want to localize the luminescent reaction such that the luciferase is in close proximity of the released ATP. Therefore we took the approach to immobilize the enzyme luciferase onto the substrate.

In our envisioned biosensor design, as presented in Figure 1.9 in the introduction of this thesis, both the sensing surface as well as the surface of the beads can be used for the enzyme

immobilization. In this way, the luciferase is in close proximity to the cell where the ATP is secreted. A schematic representation of the immobilized luciferase at the surface was already presented in the introduction of this chapter (Figure 5.5). As a proof of principle, the prepared samples were measured with solutions spiked with ATP, instead of ATP secreted from washed platelets.

Luciferase was immobilized onto beads via the EDC/NHS reaction (see Chapter 3, Section 3.4.1 for technical details). This reaction establishes a covalent bond between the carboxyl beads and the amine groups of luciferase. A negative control was prepared with the same chemical procedure, only luciferase was omitted from the protocol. We observed no difference in luminescent signal between the beads incubated with and without luciferase. This indicates that no coupling occurred between the carboxyl beads and the luciferase or that the coupling procedure inactivates the enzyme. The latter effect has been reported in literature; the covalent coupling of the enzyme is often avoided, since it is known to reduce the activity after the coupling procedure²⁸.

In addition, we have immobilized luciferase on a substrate without covalent coupling of the enzyme. Glass cover slides were cleaned with ethanol, thereafter a mixture of PLL with luciferase was prepared according to Ribeiro et al.³⁸ and incubated overnight at a glass surface at room temperature. However, no significant luminescent signal could be detected in the well plate reader during incubation with ATP and luciferin. Also visual inspection with fluorescence microscopy did not show any increased light intensity compared to blank controls. Ribeiro et al. showed that excellent biofunctionality was observed with the coating procedure, even after storage of 3 months at -80 °C, but we did not observe any signal in the well platelet reader nor the microscope. Probably slight variations of the protocols, such as different chemicals or incubation circumstances, have caused the different results.

A more direct approach was tested for the immobilization of luciferase to a substrate. PLL was first incubated for 2 hours at the glass surfaces, followed by an overnight incubation of 2 mg/ml luciferase. Signals obtained after the addition of ATP and luciferin were too low to be observed in a microscope, but measurements in the well plate reader did show a significant signal increase compared to the blank surface. Therefore we concluded that the immobilization of luciferase on PLL is feasible, however for the use in a point-of-care system more steps have to be taken. For example, the coating procedure can be further optimized and the stability and reproducibility of the immobilized luciferase should be tested.

5.5.4 Single cell fluorescence measurements of quinacrine loaded platelets

Fluorescent labeling with quinacrine was used to visualize exocytosis in platelets, as described in Section 5.4.5. Washed platelets were incubated with 20 μM quinacrine (Sigma Aldrich) for 30 minutes at room temperature and then sedimented on a BSA coated substrate to minimize surface induced activation. Under fluorescent imaging, bright spots inside platelets were designated as dense granules and were manually selected. The average light intensity per selected region of interest (ROI) was measured as a function of time; two example traces are shown in Figure 5.13a. Two types of release events were visible during the experiments: bright

spots decreasing the fluorescent intensity in 5 to 10 seconds upon ATP and thus quinacrine release (top curve) and bright spots that were increasing the fluorescent intensity with a factor of 2 to 10 within a few seconds upon (bottom curve). The first type of release events were assigned to granules having a low quinacrine concentration, thus releasing their granule content will result in a decrease of fluorescent intensity. The second type of events is attributed to high concentrations of quinacrine present in the dense granules, thereby dequenching the fluorescence upon the release of granule content. Both type of release events were already sketched in Figure 5.7b, and are both counted as events in our analysis.

To test whether the granule release events are induced by surface induced activation or by light or heat induced activation, two different field of views (FOV) with immobilized platelets were selected at the sample surface and per FOV the cumulative release events were plotted as a function of time (Figure 5.13b). Both types of release events as plotted in Figure 5.13a are incorporated in the cumulative release events. At the first FOV the light intensity was increased with a factor four after 1 minute (black curve), at the second FOV the excitation light intensity was kept constant during the whole recording (grey curve).

From the curves in Figure 5.13b it can be concluded that the current set up is sensitive for changes in the excitation light: increased light intensity results in an increased number of release events, indicating that a significant part of the release events is photo induced rather than surface or receptor induced.

Additional proof of photo-induced vesicle release was observed, when platelets were incubated with the fixative formaldehyde (0.2% in physiological salt) which shuts down all physiological processes inside the cell. After inspection of the platelets loaded with quinacrine, events were still visible, showing that the excitation light triggers this event rather than the interaction of the platelet with the surface.

Photo-induced vesicle release was already reported in literature for another commonly used fluorescent probe, acridine orange³⁹⁻⁴¹. The cause of the photo-induced activation is the production of reactive oxygens under the excitation of blue light of the acridine orange. These reactive oxygens cause peroxidation of the membrane lipids, eventually leading to disruption of the membrane. As found by Hiruma et al.⁴⁰, the removal of oxygen from the cell environment reduced the vesicle disruption significantly. In addition, the vesicle disruption was also inhibited in presence of singlet oxygen scavengers, such as sodium azide, ascorbic acid and L-histidine⁴⁰.

Jaiswal et al.³⁹ showed that the photo-induced release events that were recorded in acridine orange loaded astrocytes were depended on the dye concentration as well as the intensity of the illumination light. The number of release events due to excitation light can be reduced by lowering the quinacrine concentrations or intensity of excitation light; as a consequence the signal to noise of the measurement will also decrease. By decreasing the background signal, for example by the use of total internal reflection microscopy¹⁹ or additional washing steps to remove the excess quinacrine, the signal to noise ratio can be enhanced.

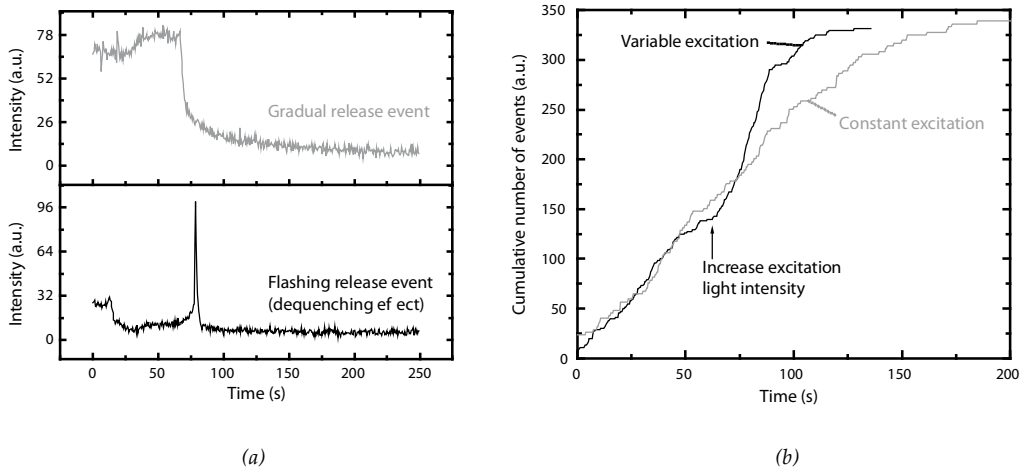


Figure 5.13: Single cell fluorescent measurements with quinacrine loaded platelets (a) Examples of two typical fluorescent intensity traces as a function of time, recorded from quinacrine labeled platelet at a BSA coated surface. The bottom curve shows a release event in which the selected vesicle increases its intensity upon exocytosis. This is probably caused by the high concentration of quinacrine present inside the granule, which is dequenched upon its release into the extracellular space. The top curve shows a decreasing intensity upon exocytosis. (b) Cumulative number of events counted in two different field of views (FOV) spots on the same sample, at the first spot the excitation light intensity was increased after 1 minute (black curve); at the second spot, the illumination light intensity was kept constant (grey curve).

We can conclude that in our current experimental set up the observed release events of quinacrine loaded dense granules are photo-induced rather than surface-induced. This was shown by observing that the number of recorded release events was dependent on the excitation light intensity and that release events were still recorded after fixation of the samples. Possibly, the removal of reactive oxygen, reduction of the excitation light intensity and quinacrine concentration can reduce the amount of photo-induced release events in future experiments.

5.6 Conclusion

We analyzed the exocytosis in platelet ensembles and we have attempted to visualize exocytosis in single cells. With the use of the luminescent luciferin/luciferase assay, the amount of secreted ATP from immobilized platelets was used as marker for platelet activation. We showed that the amount of secreted ATP found after platelet immobilization on BSA was the least, followed by PLL, anti-GPIb and IgG coated surface. This is consistent with the results found from analysis of calcium responses in platelets with the use of the calcium indicator Oregon Green BAPTA-1, as presented in Chapter 4. In that chapter we showed that the number of responding cells on PLL ($74 \pm 7\%$) was lower than on anti-GPIb ($85 \pm 4\%$). In the calcium indicator experiments, on a BSA coated surface no binding and thus no response was observed.

This is possibly caused by the shorter incubation time used for the calcium experiments (15 minutes) compared to the luciferin/luciferase ATP secretion assay (1 hour).

Since we want to explore technologies suitable for a biosensor design to measure platelet function, we are interested in a surface which is able to immobilize platelets but does not alter their function. From the tested surfaces we conclude that a BSA coated surface is the most quiescent surface for platelets. However, from various experiments we know that immobilization of platelets to BSA takes about 60 minutes, which is not an appropriate time scale for a point-of-care system. The next most quiescent surface for platelet immobilization found from experiments was PLL, to which the binding of platelets occurs within 10 minutes. However, the adsorption of negatively charged platelets to the positively charged PLL surface is a nonspecific interaction. Keeping in mind the envisioned biosensor design as presented in the introduction of this chapter (Figure 1.9), additional sample filtering will be needed to separate the platelet rich plasma from the whole blood sample, to prevent other cells from binding to this adhesive surface.

The use of a different substrate (glass or polystyrene in our experiments) for protein immobilization did not have any effect on the amount of secreted ATP molecules from the adhered platelets. Apparently the type of coating, rather than the substrate, dominates the release reaction from immobilized platelets. Reducing the amount of bonds between platelet and immobilized antibodies by using a lower amount of coating proteins at the surface, or with the use of an Fc-receptor blocker, reduced the surface induced activation of the platelets. The latter observation is consistent with literature³⁷.

For the integration of the luminescent luciferin/luciferase assay in a point-of-care system, we have attempted to immobilize the enzyme luciferase to a substrate. We showed that by the use of a simple adsorption procedure to PLL, the luminescent assay was still able to detect ATP in solution. Covalent coupling of luciferase to beads or luciferase embedded in a PLL matrix, showed no working luminescent assay. Although we showed a first proof of principle of the immobilized luciferase assay, for the implementation in a point-of-care system, more steps have to be taken. For example the immobilization procedure should be optimized, by changing incubation times or concentrations of the immobilized proteins. When the protocol is optimized, the stability of the immobilized enzyme should be tested and experiments with platelets instead of spiked ATP solutions should be performed.

The research in which quinacrine was used to visualize real-time exocytosis in platelets, showed that in the current experimental set up the observed secretion events of the dense granules were photo-induced rather than physiological. The cause of the photo-induced activation is probably the production of reactive oxygens under the excitation of quinacrine by blue light. These reactive oxygens can cause peroxidation of the membrane lipids, eventually leading to disruption of the membrane. Removal of the reactive oxygen by the use of scavengers or creating oxygen free environment is expected to reduce the number of release events caused by vesicle ruption. The use of quinacrine labeling for the visualization of platelet exocytosis is a research tool rather than applicable for a point-of-care system. The main

limitation of using quinacrine or other fluorescent labels for cell function measurements is that a significant time, typically 10-30 minutes, is needed for appropriate cell labeling. By using the more sensitive total internal reflection fluorescence, which is a technique that probes fluorescent signals only emitted by fluorophores close by the substrate¹⁹, the signal to noise can be enhanced. As a consequence, the amount of fluorophore can be reduced and therefore possibly the incubation time with the fluorophore can be reduced. However, since the incubation time of quinacrine with platelets is 30 minutes in our current set up, optimization is needed to reduce this incubation time to make it suitable for biosensor applications.

5.7 Acknowledgements

Data presented in this chapter was mainly the result of the master research project of Joris Goudsmits, I would like to acknowledge him for his contribution to the research on visualizing exocytosis in platelets. In addition, I would like to thank Mark Roest and Thijs van Holten of University Medical Center Utrecht, for sharing their expertise on the luminescent luciferin/luciferase reaction which was used as a basis for the research on the platelet-surface interactions.

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Chapter 6

Conclusions and outlook

In the introduction of this thesis we have presented an envisioned lab-on-chip design to measure platelet function. In this final chapter of the thesis, we will give an overview of our results obtained in the research to gain more knowledge about lab-on-chip technologies and place them in a broader perspective. In the outlook we will consider some technical challenges considering the blood collection and whole blood assay. We will also give our view on possible device designs in which our gained knowledge about platelet-surface interactions is incorporated. In addition, we will give our view on the application of the gained knowledge in a clinical environment and discuss the challenges for the integration of the device in such an environment.

6.1 Conclusions

The scope of the research presented in this thesis was to develop knowledge to support future technological developments, aiming at the measurement of platelet activation or responsiveness in a lab-on-chip device. We have presented one possible design in the first chapter of this thesis (Figure 1.9), inspired by the magnetic biosensor platform as is being developed by the CTMM project partner Philips Research. In the design we propose to use magnetic particles (beads) as capture and detection label to measure platelet function in a lab-on-chip application. With the envisioned design in mind, we have addressed two research questions in this thesis: can we use beads to measure platelet function? And secondly, what is the effect of a surface on platelet function? In this paragraph we will give an overview of our findings.

6.1.1 Magnetic particles can be used to measure platelet function

To test whether beads could be used to measure platelet function, beads were functionalized with different surface coatings. Nonspecific interactions with platelets were tested with Bovine Serum Albumin (BSA)-coated beads and specific interactions were tested with anti-P-selectin coated beads. The latter should bind to the P-selectin membrane markers, which are translocated to the platelet membrane upon activation. We used anti-P-selectin coated beads to capture activated platelets from samples stimulated with Thrombin Receptor Activator Peptide (TRAP). The responsiveness of the platelets was analyzed via the measurement of remaining unbound platelets in solution and compared to a reference method in which the percentage of activated platelets was analyzed via fluorescent labeling. We found low binding of activated platelets to BSA-coated beads and high binding of activated platelets to anti-P-selectin coated beads. In addition, we quantified the platelet responsiveness from the dose response curves recorded with anti-P-selectin coated beads and a reference curve with fluorescent labels by means of the effective concentration. We found that the effective concentration for platelet responsiveness with our bead capture assay in buffer ($17.9 \pm 4.9 \mu\text{M}$) was in good agreement with the effective concentration found with the reference assay ($23.5 \pm 0.4 \mu\text{M}$). In 10 % plasma we found effective concentrations of $14.0 \pm 4.4 \mu\text{M}$ and $13.8 \pm 0.3 \mu\text{M}$ respectively. Our experiments demonstrate for the first time the use of antibody functionalized beads to measure a dose response curve for the expression of a platelet-specific activation marker.

In addition, we wanted to make use of the magnetic properties of the functionalized beads to discriminate between resting and activated platelets after immobilization at a substrate. Therefore, we used BSA, mouse immunoglobulin G (IgG) and anti-P-selectin coated beads and incubated them with and without immobilized platelets in a magnetic tweezers set-up. In absence of platelets, we showed that the majority of the beads detached from the IgG coated glass surface. From this result we can conclude that non-specific binding of the BSA, IgG and anti-P-selectin coated beads with the IgG coated surface was low. Also, we were able to show that we can bind IgG and anti-P-selectin coated beads specifically to immobilized platelets. In contrast, the majority of the BSA coated beads detached from the immobilized platelets, when a force of 50 pN was applied. With the use of magnetic tweezers we succeeded in discriminating between specific bonds between IgG or anti-P-selectin coated beads and immobilized platelets, and non-specific bonds of BSA coated beads and immobilized platelets.

6.1.2 Surface-induced activation plays an important role in platelet immobilization

Many proposed biosensor technologies to measure platelet function are based on the immobilization of platelets to a surface. It is thus important to gain knowledge on the interaction of platelets with such a surface, in particular the influence of surface binding on platelet activation. We have used two different approaches to study this interaction.

First we used intracellular signaling in platelets to study the platelet-surface interactions. A calcium indicator, which is a fluorescent molecule that changes its fluorescent properties in presence of calcium, was loaded into platelets and the real-time response upon binding to five different surface coatings was quantified. The prepared surfaces included: BSA, poly-L-Lysine (PLL), IgG, anti-GPIb and collagen. After the immobilization of platelets at the surface, the platelet functionality was further tested by monitoring the calcium indicator signal before and after the addition of a chemical trigger.

We found that the percentage of responding cells upon binding to the different types of surfaces showed not much variation; typically the percentage of responding cells ranged between 70-90 %. Except on an IgG coated surface, where a percentage of about 40% responding cells was observed. However, a clear difference in response behavior was found upon chemical stimulation of the platelets that were immobilized on the different types of surfaces. The majority of the immobilized platelets on BSA showed a calcium response (about 90%) upon chemical stimulation. On a PLL or IgG coated surface, the number of responding cells upon chemical stimulation was lower (typically about 40 %). On an anti-GPIb or collagen coated surface, only a few percent (about 5 %) of the immobilized platelets responded to a chemical stimulus.

In our second approach to study the effect of a surface on the platelet activation state, we measured the amount of secreted ATP from the platelets dense granules. The luminescent luciferin/luciferase reaction was used to measure the amount of secreted ATP after incubation of platelets on BSA, PLL, IgG or anti-GPIb coated surfaces. We showed that the amount of secreted ATP found after platelet immobilization on BSA was the least, followed by PLL, anti-GPIb and IgG coated surfaces. We showed that the substrate onto which the proteins were coated (glass or polystyrene) did not affect the amount of secreted ATP from the immobilized platelets. The addition of an Fc-receptor blocker or the reduction of the amount of antibodies at the surface reduces the amount of secreted ATP significantly.

In Figure 6.1 we combine the results obtained by the two approaches to quantify platelet-surface interactions: on the x-axis the amount of secreted ATP measured with the luciferin/luciferase assay is plotted, on the y-axis the percentage of responding cells on chemical stimulation measured with a calcium indicator is plotted. The release of ATP from the dense granules, used as an activation marker in the luminescent assay, is an irreversible activation process^{1,2}. The same holds for the stimulation of immobilized platelets with high TRAP concentrations^{1,2}, as used in the fluorescent calcium indicator experiments. This means that we can be sure that each platelet can only be activated once. As a consequence we expect an inverse relationship between the amount of secreted ATP and responding cells upon

chemical stimulation. In Figure 6.1, we present the combined results of the luminescent secretion assay as well as the calcium signaling assay.

The figure indeed shows the hypothesized inverse relation between the ATP release and responding cells. For a BSA coated surface, the relative amount of secreted ATP from immobilized platelets is low, while the majority of the immobilized platelets on a BSA coated surface still show a response upon TRAP stimulation. In contrast, on an anti-GPIIb coated surface the amount of secreted ATP from the immobilized platelets is high and the number of responding cells upon a chemical stimulus is low. In the outlook we will discuss how we can incorporate this knowledge in a lab-on-chip design for platelet function testing.

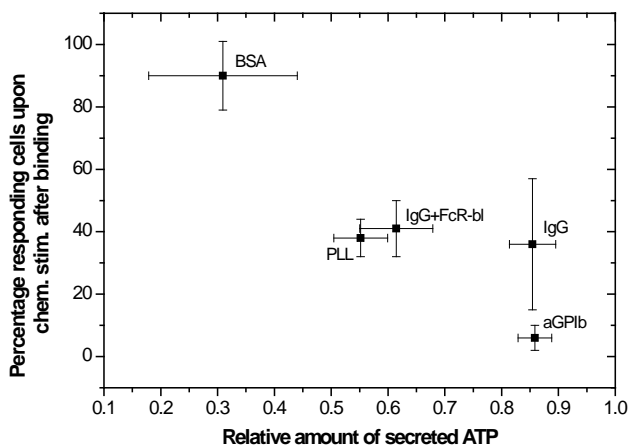


Figure 6.1: The relation between the results obtained from two different methods to quantify the surface induced activation of platelets. On the x-axis the relative amount of secreted ATP from platelets immobilized at different types of surfaces is presented. The ATP concentration was quantified with the use of the luminescent luciferin/luciferase reaction as described in Chapter 5. Each value is the average of three samples; the error bars represent the standard deviation. Since not all samples were measured in one experiment, the relative amount of secreted ATP is presented, scaled to the common sample in the series (an anti-GPIIb-coated surface). On the y-axis the percentage of platelets showing a calcium response upon chemical stimulation (chem. stim.) with TRAP is presented, recorded after immobilization. This percentage was quantified by the measurement of the fluorescent intensity of the platelets that were loaded with a calcium indicator as described in Chapter 4. Each value represents the average of multiple samples with donor material obtained from multiple donors. Since both the ATP secretion and TRAP stimulation introduce irreversible activation in platelets, it is expected that an inverse relation would be found for ATP release versus response on a trigger. This means that for platelets coupled to a mild surface such as BSA, a low amount of ATP is secreted, and a high percentage of cells respond to TRAP activation. FcR-b1=Fc-receptor blocker, aGPIIb=anti-GPIIb.

6.2 Outlook: Technical challenges

Now we want to bring the results of our research in the perspective of the envisioned biosensor design for platelet function testing as presented in the introduction of this thesis. We will discuss some important reconsiderations about the use of whole blood in a biosensor (Section

6.2.1) we will discuss the consequences of the results found on surface-induced platelet activation and the possible designs which incorporate our gathered knowledge (Section 6.2.2).

6.2.1 Whole blood assay

The concentration of platelets in normal whole blood samples is high (~200.000 platelets/ μ l), but since the platelets are small (~3 μ m in diameter) they make up only about 0.1% of the volume of whole blood. Therefore it is important to think about possible techniques to generate platelet rich plasma (PRP) from whole blood in a lab-on-chip device, in order to avoid interfering effects of white or red blood cells with the platelet function testing. Two approaches can be used to obtain PRP from a whole blood sample: specific lysis of red and white blood cells, or filtration.

Two types of cell lysis can be used: physical lysis (such as sonication, manual grinding, or freeze and thaw) or chemical lysis. Since the physical lysis methods are not cell specific, we will not discuss them here. A large variety of chemical lysis procedures exists and they are already applied in lab-on-chip designs^{3,4}. However, the major drawback of the use of specific chemicals (such as detergents) or enzymes is that they possibly interfere with the assay. In addition, an even greater disadvantage of using lysis to generate PRP from whole blood is that the debris resulting from the lysis will also be present in the reaction chamber and will probably hinder the assay. Also it is well-known that each cell contains ADP, which is a platelet agonist. So lysis of red and white blood cells causes the release of ADP that can introduce platelet activation and thus affect the measurement of platelet function. Therefore we now discuss the use of filtration methods for lab-on-chip applications.

Platelets are a factor 2 to 6 times smaller than red blood cells and 3 to 11 times smaller than white blood cells. Most filtration principles that separate PRP from whole blood make use of these size differences. Clogging has been the main disadvantage of traditional filters, therefore new lab-on-chip filtering technologies are presented in literature. For example Inglis et al⁵ demonstrated that with the use of micro posts platelets could be separated from whole blood. The device as presented by Dimov et al.⁶ is an elegant design in which a trench is used to capture the red and white blood cells. From experimental data and simulations it was shown that with the use of this passive structure, 99.9 % to 100 % red and white blood cell retention was achieved. Other state of the art design for lab-on-chip cell separation are based on the use of centrifugal forces⁷, cross-flows⁸, or the Zweifach-Fung effect⁹.

Thus for future development of the envisioned lab-on-chip design, one of the above separation techniques should be kept in mind if the white or red blood cells appear to hinder the analysis of platelet function. Although all of the designs are suitable for lab-on-chip applications, we think that the simplicity of the trench capture design⁶ would be very suited as a prefiltering step before the platelet function is analyzed in a reaction chamber.

6.2.2 Surface effects

As concluded in Section 6.1.2, the surface can have a major effect on the platelet status. This should be considered in the design of a lab-on-chip technology to measure platelet function. To

minimize surface-induced activation, one can target membrane markers for activation such as P-selectin. In our bead capture assay (Chapter 3) we have already shown, that we can measure platelet function with the use of anti-P-selectin coated beads. In Figure 6.2, the possible process steps of such an assay are sketched.

The first process step is to split the collected donor material into separate incubation chambers, in which different concentrations of agonist are present together with anti-P-selectin coated beads. After a magnetic mixing procedure, the platelets expressing P-selectin are captured by the functionalized beads and transported to the sensor surface that is also coated with anti-P-selectin. The amount of beads bound to the surface, is indicative for the activation of the platelets in the donor material. By measuring at different agonist concentrations, a dose response curve could be obtained and the platelet function could be evaluated.

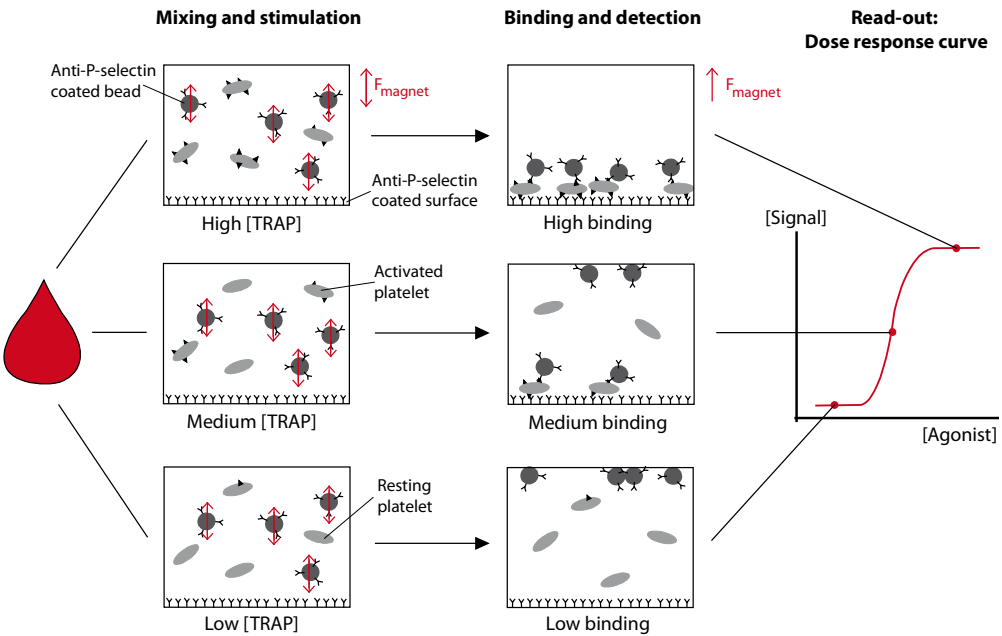


Figure 6.2: Possible process steps in a biosensor design in which a specific platelet membrane activation marker (such as P-selectin) is targeted with the use of magnetic beads. For simplicity, we have assumed a filtration step in which the red and white blood cells are separated from the platelet rich plasma (PRP). Blood is guided to different reaction chambers, which contain functionalized beads and various concentrations of a chemical stimulus (e.g. TRAP). With the use of external magnets, the beads can be actuated through the PRP. The sensor surface is coated with specific antibodies, such that activated platelets bind to the surface. In the reaction chamber with low TRAP concentrations, the P-selectin expression on platelets and thus the binding of platelets and beads is low. This results in the baseline level in the dose response curve. For increasing agonist concentrations, increasing number of activated platelets and thus anti-P-selectin coated beads bind to the sensor surface which results in a dose response curve. Platelet function can be determined from this dose response curve.

Platelet binding to a surface with a specific antibody such as anti-GPIb, of which we have shown that it activates the platelets upon binding, may also be possible in a biosensor system. Our experiments showed that a fraction of the immobilized platelets on anti-GPIb respond to a chemical trigger. If this fraction is representative for the whole platelet population in the donor material, we may still use these responding cells to measure the platelet function of immobilized platelets on an anti-GPIb coated surface. The process steps would look the same as in Figure 6.2, only the sensor surface would then be coated with anti-GPIb instead of anti-P-selectin.

An essential future step to test the feasibility of the assay for platelet function is to investigate whether the amount of functionalized beads that bind to the sensor surface is a measure for the activation status of the platelets in the reaction chamber. Since one magnetic bead is able to bind to multiple platelets and one platelet is able to bind to multiple beads, bead-platelet clusters can possibly complicate this assay. Different platelet to beads ratios and different antibody densities will need to be studied.

6.2.3 Membrane markers, secretion markers or calcium signaling as a biosensor read-out

In this thesis we have focused on three different read-out parameters to measure platelet responsiveness namely, membrane marker expression (Chapter 3), intracellular calcium signal (Chapter 4) and secretion markers (Chapter 5). While all three read-out parameters are well-established markers for the measurement of platelet responsiveness, it is important to view the advantages and disadvantages of these parameters in the perspective of a lab-on-chip design.

We showed that the expression of the P-selectin membrane marker on platelets can be measured with the use of antibody coated magnetic beads. The advantage of magnetic beads is that they can be used both as label and as carrier for transport. Since any antibody can be coated onto the bead, the labeling is not restricted to the use of P-selectin, but also other membrane receptors could be targeted. The detection of the amount of beads bound to the platelets and the surface can for example be detected optically as in the Philips system (Figure 1.8), but we can also think of a system in which we combine the use of magnetic particle with secretion markers or calcium signaling. For example by using the beads first as carriers to capture and transport platelets to the surface and by then applying a force and thus mechanically stimulating the platelets. As a read-out for the platelet status, the amount of secreted ATP could be measured with the use of the luminescent luciferin/luciferase assay, or with the use of a fluorescent calcium indicator inside the platelets. The advantage of such a system may be that the time point and length of the applied stimulation is well defined. During stimulation, the platelet status can be monitored. One may even be able to probe the responses of individual cells as a function of time. A technical challenge will be to develop a system which is sensitive enough to measure the release of ATP from the dense granules of platelets or the intracellular calcium signaling in platelets. The efficiency of the detection labels and/or the sensitivity of the detection system should be increased. We think that such assays with single-cell resolution and multiparameter readout can be useful tools to study biophysical questions and may in the future also carry diagnostic information.

6.3 Outlook: Clinical challenges

Not only technical challenges need to be overcome, there are also clinical challenges that need to be reconsidered for future lab-on-chip applications. In this outlook, we will discuss our view on the future perspective of the use of a biosensor for platelet function in a clinical setting (Section 6.3.1) and the challenges we are facing in the field of sample collection (Section 6.3.2).

6.3.1 Biomarkers for atherosclerosis

In the research described in this thesis, we have focused on measuring platelet responsiveness on a trigger. As a read-out for the platelet activation status we used well-established markers, such as P-selectin expression, intracellular calcium signaling or dense granule secretion. The patient cohort study of CTMM to see if platelets may serve as a predictive biomarker for the initiation and progression of atherosclerosis is still in an early phase, thus the validity of the biomarker is still to be established. However, it is well-known that altered platelet function can lead to severe cardiovascular diseases. Low platelet responsiveness can cause bleeding disorders, while high platelet responsiveness can cause thrombotic disorders. Therefore it could already be helpful if a rapid biosensor is developed which can measure platelet function. For example, to screen patients brought to the emergency room for bleeding disorders or to test the efficiency of medicines that inhibit platelet function, so that the treatment or medication can be adjusted when needed.

6.3.2 Sample collection: fingerstick versus venapuncture

In an ideal device design for platelet function testing, we envision an easy to use handheld device, which is able to measure platelet function from blood collected with a fingerstick. However, it is important to realize that a fingerstick damages blood vessels and thus triggers primary hemostasis. As a consequence, platelet agonists are released by damaged endothelial cells, which trigger platelet activation. In addition, the blood sample collected via a fingerstick contains not only blood, but also undetermined portions of interstitial and intracellular fluids.

The literature on platelet function in blood collected from a fingerstick is very limited. The paper of Johnson et al.¹⁰ describes the use of blood collected from a fingerstick to analyze platelet function. In the collection procedure, the fingertip was first cleaned with alcohol, followed by warming of the hand during 4 minutes. The first drop of blood was discarded and the second was transferred to the cartridge of the device. The blood droplet is pulled into a microchannel in which a small coil of $0.25 \times 0.25 \mu\text{m}$ is placed, having a gap size of 0.09 mm. The sample volume is pumped in alternating directions through the coil, and by this process the shear stress is sufficient to induce platelet activation. Aggregates will form and cover the coil, this induces an increase in pressure in the channel. The test is stopped at 6 Pa, this time is designated as the platelet reactivity time (PRT), which is a measure for platelet function. The presented results showed a close relationship between platelet function measured from the fingerstick blood in their biosensor design, and a reference method in which platelet function of blood collected via venapuncture was measured with aggregometry.

In addition, Wall et al.¹¹ compared the quinacrine uptake of platelets collected from a fingerstick with platelets collected via a venapuncture with the use of flow cytometry analysis. A small incision in the finger was made with a Tenderlett lancet (ITC Edison) and the blood was collected with a Unopette (Becton-Dickinson). Flow cytometry analysis showed that the quinacrine uptake as well as the expression of activation marker P-selectin on platelets in fingerstick blood was similar to platelets in venous blood. This indicates that the platelets collected via a fingerstick were not activated.

These two references suggest that the collection of blood via a fingerstick can be done without affecting the state of the platelets. Since the literature on this research topic is very limited, we suggest that additional experiments should be carried out in which a comparison is made between the responsiveness of platelets in blood collected from a fingerstick or via venapuncture. For example a dose response could be made, in which platelets are stimulated with different amounts of stimulus and their P-selectin expression is measured with a flow cytometer.

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List of abbreviations

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List of abbreviations

aGPIb	Anti-GPIb
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
aPsel	Anti-P-selectin
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumine
cAMP	Cyclic Adenosine monophosphate
cPGI	Carbaprostacyclin
CTMM	Center for Translational Molecular Medicine
DAG	Diacylglycerol
DLD	Deterministic lateral displacement
DTS	Dense Tubular System
Fg	Fibrinogen
FSC	Forward scattered (light)
GP	Glycoprotein
GPCR	G Protein Coupled Receptor
hCG	Human Chorionic Gonadotropin
IgG	Immunoglobulin G
IP ₃	Inositol 1,4,5-triphosphate
iPC	Interfacial platelet cytometry
LDL	Low density lipoproteins
OCS	Open canalicular system
PAR	Protease-activated receptor
PBS	Phosphate Buffered Saline
PF-4	Platelet factor-4
PIP ₂	Phosphatidylinositol 4,5-bisphosphate
PLC β	Phospholipase C β
PLL	Poly-L-Lysine
PMCA	Plasma Membrane Calcium ATPase
PMT	Photon multiplier
PRP	Platelet Rich Plasma
PSGL	P-selectin glycoprotein ligand
ROI	Region of interest
SERCA	Sarcoplasmic/endoplasmic calcium ATPase
SSC	Sideward scattered (light)
TIRFM	Total internal reflection fluorescent microscopy
TRAP	Thrombin Receptor Activating Peptide
vWF	von Willebrand Factor
WP	Washed platelets

Scientific output

2012

Manuscript in preparation:

Intracellular calcium signaling in platelets: A new method to quantify platelet-surface interactions real-time, H.M. van Zijp, A. Barendrecht, J. Riegman, A.M. de Jong, H. Kress, M.W.J. Prins

Research paper:

Measurement of platelet responsiveness using antibody-coated magnetic beads for lab-on-chip applications, H.M. van Zijp, C.C.M.M. Schot, A.M. de Jong, N. Jongmans, T.C. van Holten, M. Roest, M.W.J. Prins, *Platelets* (2012), p1-8, DOI: 10.3109/09537104.2011.651516

Poster presentation:

Time-dependent calcium signaling in blood platelets: A new method to quantify platelet-surface interactions, H.M. van Zijp, A. Barendrecht, J. Riegman, H. Kress, A.M. de Jong, M.W.J. Prins
MicroNano conference 2012 – Ede – December 2012 and FOM Biophysics 2012 – Veldhoven – October 2012

Poster presentation:

Real-time activation of blood platelets studied with magnetic microparticles, H.M. van Zijp, M. Roest, H. Kress, A.M. de Jong, M.W.J. Prins, Biophysical Society Annual meeting – San Diego – February 2012

Popular scientific article:

Van biosensoren tot matjes disco – De museumnacht in NEMO, H.M. van Zijp, N! – Faculteitsblad Technische Natuurkunde, TU/e – April 2012

2011

Poster presentation:

Measurement of platelet activation with anti-P-selectin coated magnetic microparticles, H.M. van Zijp, T.C. van Holten, M. Roest, A.M. de Jong, M.W.J. Prins, Biophysical Society Annual meeting – Baltimore – March 2011 and Biomedica – Eindhoven – April 2011

Oral presentation:

Platelet catching with magnetic particles, H.M. van Zijp, T.C. van Holten, M. Roest, A.M. de Jong, M.W.J. Prins, Annual meeting Circulating Cells CTMM – Utrecht – March 2011

Invited talk:

Platelet catching with magnetic particles, H.M. van Zijp, T.C. van Holten, M. Roest, A.M. de Jong, M.W.J. Prins, CTMM Annual meeting – Utrecht – September 2011

Poster presentation:

Real-time visualization of exocytosis in blood platelets, H.M. van Zijp, J.M.G. Goudsmits, T.C. van Holten, M. Roest, H. Kress, M.W.J. Prins, FOM Biophysics 2011 – Veldhoven – October 2011, Cell Mechanics Conference AMOLF – Amsterdam – October 2011 and CTMM Annual meeting – Utrecht – September 2011

Invited talk:

Smart materials @ MBx: Magnetische beads in biosensoren, H.M. van Zijp, Fontys lectures – Eindhoven – October 2011

Invited talk:

Atherosclerose ontmaskerd: Het opsporen en detecteren van biomarkers, H.M. van Zijp and J.K van Keulen, NEMO museumnight – Amsterdam – November 2011

Oral presentation:

Platelet catching with magnetic particles, H.M. van Zijp, T.C. van Holten, M. Roest, A.M. de Jong, M.W.J. Prins, MicroNano Conference – Ede – November 2011

2010

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Platelet catching with magnetic particles, H.M. van Zijp, T.C. van Holten, M. Roest, A.M. de Jong, M.W.J. Prins, Annual meeting Circulating Cells CTMM – Utrecht – March 2010

2009

Poster presentation:

Biosensor technology for platelet function testing, H.M. van Zijp, A.M. de Jong, L.J. van IJzendoorn, M.W.J. Prins, European Biophysics Conference – Genova – June 2009 and CTMM Annual meeting – Utrecht – October 2009

Curriculum Vitae

Helena Maria (Loes) van Zijp

Born the 4th of August 1981 in Prinsenbeek, the Netherlands

Work

- 2008-2012 PhD student at Eindhoven University of Technology, in the group Molecular Biosensors for Medical Diagnostics. Thesis title: *Study of methods for platelet function testing in the perspective of lab-on-chip applications*
- 2007-2008 Océ Technologies, Research and Development, Print head group
Increasing print head performance by the use of real-time acoustic measurements

Education

- 2003-2007 MSc in Applied Physics, Eindhoven University of Technology, the Netherlands.

Master project in the group Molecular Biosensors for Medical Diagnostics (MBx) at the Eindhoven University of Technology. Thesis title: *Preparation and characterization of on activated Gold surface for bond force measurements with Streptavidin coated beads*
- 1999-2003 BSc in Applied Physics, Fontys Hogescholen Eindhoven, the Netherlands

Bachelor project at Philips Lighting, New Business Creations Backlights Group. Thesis title: *Life time testing of backlight systems for tiled-LCD*

Internship at University Medical Center Utrecht, Clinical Physics: *Shining a light on bladder cancer*
- 1993-1999 HAVO, Markenhage College Breda, the Netherlands

Activities

- 2009-2012 Editorial staff of the magazine of the student association and the alumni association of the Department of Applied Physics at Eindhoven University of Technology
- 2009-2010 Board member of the alumni association of Applied Physics (VENI)
- 2003-2005 Member of various committees of the student association, including the Exchange Committee 2003 and Symposium Committee 2005

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