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Platelet-surface interactions
calcium fluxes inside platelets

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Abstract

In this thesis, the results of our study, the interaction of platelets on different surfaces are presented. The interaction was studied, by measuring the intracellular calcium signaling upon immobilization on a surface and upon chemical stimulation. In our study we used the calcium-indicator, Oregon Green BAPTA-1 AM, to visualize changes in cytosolic calcium. Once platelets get activated, its intracellular calcium concentration increases which in turns increases the intensity of the Oregon Green BAPTA-1 AM. Platelets, which showed this fluorescent intensity increase, were marked as responding cells. The delay time (the time between trigger and response) was measured for platelets on BSA (Bovine Serum Albumin) and IgG (Immunoglobulin G) coated surfaces.

Due to this low binding probability we were unable to record real-time bindings on a BSA coated surface. After most platelets had immobilized on the surface, the platelets were chemically stimulated with Thrombin Receptor Activating Peptide (TRAP). 87±4% of the immobilized platelets on BSA could be chemically triggered with an average delay time of 18±39 s.

On IgG coated surfaces 65±4% of the adhered platelets responded upon binding and only 35±1% of the immobilized platelets could be chemically activated. In attempt to reduce the calcium response of platelets upon binding to an IgG coated surface, platelets were treated with Fc-receptor blocker. 64±4% of the analyzed platelets, treated with Fc-receptor blocker, activated upon binding to IgG. When activated with TRAP, 38±3% of the immobilized platelets with Fc-receptor blocker gave a calcium response. This shows that blocking the Fc-receptor does not reduce the percentage of activating platelets upon binding to an IgG-coated surface.

The purpose of this research was to study the interaction of platelets on different types of surfaces, to understand the interactions that could play a role in future biosensor designs that measure platelet function. It is shown that platelets immobilized on BSA are still sensitive for chemical stimulation, however it takes long (±80 minutes) for platelets to adhere to BSA coated surfaces. On IgG coated surfaces most platelets get activated upon binding to it. This means that it is more difficult to measure the functionality of the platelets after binding. For these reasons, the investigated surfaces are not suitable for biosensors, but thanks to this research a better understanding of the processes occurring during platelet-surface interaction is gained.
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1 Introduction

Cardiovascular diseases are number one cause of death in western society nowadays\textsuperscript{1,2}. It is believed that platelets play an important part in many cardiovascular diseases. These diseases can alter the platelet function. Any thrombotic tendency should be noticeable in platelet (and other circulating cell) function before any physical symptoms are noticeable. These circulating cells can therefore be used as messengers, to give an indication of the patient’s disease status.

1.1 Blood platelets

Blood contains three main cell types. The most numerous cells are the red blood cells. They make up about 40\% of our total blood volume, which corresponds to about 5.000.000 red blood cells per micro liter. The task of the red blood cells is to transport oxygen from the lungs to the rest of the tissues.

Besides the large amount of red blood cells, blood also contains white blood cells. They are the largest cells in the blood (10-12 $\mu$m in diameter), but are also the fewest cells in the blood. One micro liter contains only 5.000 to 10.000 white blood cells. Their task is fight infections.

The last cell type that can be found in the blood, are blood platelets. Blood platelets are spherical cells with a diameter of 2-4 $\mu$m (figure 1.1a), which is only about 20\% of the diameter of red blood cells\textsuperscript{1}. In healthy humans the platelet count is 15.000 to 350.000 per micro liter blood\textsuperscript{1}, but since they are so small, they are responsible for only a tiny fraction of the blood volume. Platelets, as well as the red cells and most white blood cells, are produced in the bone marrow. The platelets are produced from large bone marrow cells called megakaryocytes. When these megakaryocytes become giant cells, they fragmentize and release over 1.000 platelets a time\textsuperscript{1}. This is equivalent to $10^{11}$ new platelets each day.

The outside of the platelets contains a wide variety of proteins that allow them to interact with proteins, surfaces, other platelets and cells. Under normal conditions the blood vessel walls are lined with cells called endothelium cells, which prevent the platelets from sticking to the blood vessel wall. However when the blood vessel ruptures, the endothelial layer gets damaged and collagen is exposed to the bloodstream. When the platelets come in contact with the collagen, they get activated. As a consequence specific receptors are exposed on the platelet membrane, which support adhesion of platelets onto the damaged blood vessel.

In addition, platelet will secrete molecules into the surrounding blood. Inside the platelets several storage sides are located. Each of these storage sides contains its own component. The most important storage sides are the alpha and dense granules, which help to accelerate the activation processes. When platelets get activated, the storage sides excrete their contents into
the platelet interior and the surrounding blood. The excretion of this contents helps with the adhesion, aggregation and repairing of the damaged blood vessel.

Activation also causes the platelets to change their shape and spread over the surface. Several stages of a spreading platelet are shown in figure 1.1. First pseudopods extend in every direction (figure 1.1b), after which the cytoplasm spreads and fills the space between the pseudopods (figure 1.1c). Once fully spread, the platelet cannot be unbound from the surface anymore.

In vivo, this activation process occurs naturally when blood vessels rupture and platelets are exposed to their natural stimulus, collagen or thrombin. It is difficult to examine this process in the human body; therefore this is mainly studied in a lab. For example by using Thrombin Receptor Activating peptide (TRAP) to initiate the activation process in vitro. TRAP triggers platelets activation in the same way as the natural platelet agonist thrombin. With the use of TRAP it is possible to measure in the platelet function, due to diseases.

![Figure 1.1: Stages of platelet spreading](image)

*Figure 1.1: Stages of platelet spreading*. a: Unactivated platelet, LVHR-SEM (low voltage high resolution scanning electron microscope), magnification 30.000x. b: pseudopods are extended in every direction, LVHR-SEM, magnification 13.000x. c: Cytoplasm spreads and fills the spaces between the pseudopods, magnification 11.000x. d: Spread platelet, conventional SEM, magnification 9.000x.*
1.2 Platelet function
In normal healthy humans the platelets work as explained in the previous section, but cardiovascular diseases can alter the platelet function. For example, when platelets respond too slowly to activation triggers, a patient suffers from a bleeding disease. When platelets are too easily activated, a patient suffers from a thrombotic disease. To identify these diseases, it is important to know, how to measure the platelet function. One way of measuring the platelet function is by analyzing the cytosolic calcium changes, after the addition of a stimulus.

In normal resting condition blood platelets have a calcium concentration of approximately 100 nM, which is 10,000-fold less than the calcium concentration in blood plasma. This concentration inside platelets is maintained by pumping Ca\(^{2+}\) from the plasma in or out of the cytosol (the fluid inside a platelet) or by pumping the Ca\(^{2+}\) into intracellular storage compartments. If these pumps fail to operate normally, the cytosolic calcium levels could rise and causes premature death of the platelet.

Several storage sites for calcium exist within the platelet, including the dense tubular system (DTS), which has the function to store calcium. When activated, the DTS releases its calcium and increases the cytosolic calcium level to 1-10 μM. This is schematically represented in figure 1.2. If it is possible to monitor the calcium change caused by activation, it could be used in a biosensor to measure the platelet function.

1.3 Biosensors
Biosensors are devices that use specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals. And can be used to perform reliable, quick and accurate analyses of tissue. A well known example of a biosensor is the blood glucose meter. It is used by diabetic patients to measure their glucose levels. A small drop of blood is placed on a test strip. The glucose in the blood reacts with chemicals inside the device. This reaction generates a charge, which is measured for a period of time. The charge generated is a measurement for the concentration of glucose in the blood sample.
The purpose of this research is to lay the foundation for a biosensor which is able to detect the platelet functionality. Most biosensors are based on immobilization of the cells of interest on a substrate. Therefore it is important to understand the influence of the substrate on the cells.

1.4 Research questions
For the development of a biosensor to measure platelet, it is thus important to know what the influence of such surfaces is on platelets. In this report we will investigate the platelet-surface interaction in two ways;

- What happens with platelets upon binding to a surface?
- Can immobilized platelets still be triggered with a chemical stimulus?

In previous research, Loes van Zijp has studied the interaction between platelets and Bovine Serum Albumin (BSA), Poly-L-Lysine and anti-GPIb coated surfaces. We will extend this research by looking more thorough at BSA coated surfaces and at IgG coated surfaces. Previous studies showed that BSA coated surfaces have little or no interaction with platelets. The reason is that both the platelet and the BSA are negatively charged, so on a macroscopic scale they repel each other. Probably due to small potential differences in the BSA coating on molecular scale, platelets are sometimes able to bind to BSA after sufficient long incubation times. Therefore BSA is used to study nonspecific interaction of platelets on a coated surface (figure 1.3a).

Figure 1.3b shows the interaction between platelets and an IgG coated surface. It shows that platelets can interact with IgG though the Fc-receptors on the blood platelets. The crystalline part (Fc-part) of the antibody is the part that interacts with the Fc-receptor. From literature it is known that binding through the Fc-receptor, induces platelet activation. By treating platelets with a Fc-receptor blocker, it is expected that the calcium response, of platelets upon binding to IgG coated surfaces, is reduced.
Figure 1.3: The bonds which can occur between a platelet and a coated surface. (a) shows a platelet immobilized on a BSA coated surface. Both the platelet and BSA are negatively charged, so they repel each other. For this reason why platelets do not bind easily to BSA coated surfaces. The other figures show a platelet immobilized on an IgG coated surface without (b) and with (c) Fc-receptor blocker. In case of the IgG coated surface, the platelet can attach via Fc membrane receptors. As a consequence it is expected that the use of an Fc-receptor blocker will have effect on platelets immobilized on IgG.
2 Materials & Methods
For this research the experimental protocols were adopted from Loes van Zijp, PhD-student, who studies new technologies for platelet function testing in biosensor designs, at Eindhoven University of Technology. In this chapter we will explain the used protocols in more detail. First we will discuss the preparation and labeling of the platelets (section 2.1). After which the surface preparation will be explained (section 2.2). Then the specifications of the used calcium indicator will be presented (section 2.3). Next we will discuss the used experimental setup (section 2.4) and the used data analyzing techniques (section 2.5). After which the performed control experiment is explained in section 2.6, to validate our used protocol. And finally some typical calcium response curves are shown in section 2.7.

2.1 Preparation of washed platelets and labeling with calcium indicator
For the experiments, 6 ml fresh whole blood samples were purchased from Sanquin, the Dutch organization for providing blood. The blood donors reported to be free of aspirin for at least 10 days. The blood samples were anticoagulated with 3.2% sodium citrate. Within one hour after collecting the whole blood, the platelets rich plasma (PRP) was separated using centrifugation at 250 g for 20 minutes at room temperature. The PRP was then transferred to a clean tube, after which anticoagulation Citrate Dextrose (ACD, 0.25% Citrate, 0.15% Citric Acid and 0.2% D-Glucose) was added in the ratio of 1:10. To separate the platelets from the plasma, the mixture of PRP and ACD was centrifuged for 15 minutes at 520 g at room temperature. The plasma was discarded and replaced by a buffer with pH 6.5 (Hepes buffer containing 10 mM Hepes, 150 mM NaCl, 5 mM KCl, 0.3 mM NaH₂PO₄·H₂O, 10 mM MgSO₄·7H₂O and 50 mM D-glucose), the total added volume was equal to the original PRP volume, obtained after the first centrifugation step. Before the platelets are suspended in the buffer, Carbaprostacyclin (cPGI, Cayman Chemical Company) was added in the concentration of 100 ng/ml, to prevent the pre-activation of the blood platelets due to the sample handling. The obtained solution was centrifuged again at 520 g for 15 minutes. The supernatant was discarded and replaced by a platelet buffer with pH 7.5 (Hepes buffer containing 10 mM Hepes, 150 mM NaCl, 5 mM KCl, 0.3 mM NaH₂PO₄·H₂O, 10 mM MgSO₄·7H₂O and 50 mM D-glucose) to restore the original volume of the donor material.

1 μM calcium indicator Oregon Green BAPTA-1 AM (Invitrogen, dissolved in DMSO) was incubated with 2 ml of the prepared washed platelets for 30 minutes at 37°C. The excess label was removed using another centrifugation step at 520 g for 15 minutes. After this last centrifugation step, the platelets were suspended in a new buffer. This buffer is a Calcium rich Hepes buffer (1 mM CaCl₂, in Hepes buffer pH 7.3).
For preparing the Fc-receptor blocked platelets additional steps were needed. After resuspending the platelets in the Calcium rich Hepes buffer, 200 μl of the washed platelets was transferred to two LowBind eppendorf cups. One cup was used for the FcR blocked platelets and the other one was used as reference. Fc-receptor blocker (Miltenyi Biotech, prodnr 130-059-901) was added in a dilution of 1:9 to the washed platelets in one LowBind eppendorf cup. This solution was than incubated for 10 minutes at room temperature. In the reference sample Calcium-buffer was added 1:9 to the washed platelets instead of Fc-receptor blocker, so that both platelet concentrations are the same.

2.2 Surface preparation
Before incubating the surface with the protein of interest, the glass cover slides were cleaned. This was done by submerging the cover slides in ethanol, after which they were blown dry with air. For preparing the IgG coated surfaces, the next step was to place a fluid cell on the cover slides. These fluid cells were then covered with 50 μl of 10 μg/ml IgG solution and placed in a humid environment to incubate overnight at room temperature. The next day, the cover slides were submerged in Phosphate Buffered Saline (PBS) to remove the excess IgG. After this washing step cover slides were covered with 80 μl solution of 3% Bovine Serum Albumin (BSA, Sigma) in PBS and incubated for 2 hours at room temperature. This was done to block possible empty spots at the surface.

Just before the use of the cover slides, the slides were washed with the calcium buffer (Hepes buffer with 1mM CaCl₂, pH 7.3). After which 50 μl of platelet-solution is placed on the cover slides.

BSA coated cover slides are prepared according to the same protocol as used for the preparation of the IgG coated cover slides. The only difference is that the cover slides are not covered with the IgG solution and therefore not incubated overnight. For the rest, the same steps were followed for the BSA coated surface preparation.

2.3 Oregon Green BAPTA-1 AM specifications
In this research Oregon Green BAPTA-1 AM (Invitrogen) is used as calcium indicator, because it is shown in previous studies that this works well. When Oregon Green BAPTA-1 AM binds to calcium it increases its fluorescent intensity up to 14 times, without shifting in emission wavelength. This can be seen in figure 2.1.

The acetoxymethyl (AM) derivative of Oregon Green was used, because this derivative is able to penetrate the cell membrane. Normally the carboxylic acids with AM groups are uncharged molecules, which can penetrate the cell membrane. Once in the cell, the molecule is charged by
a natural process. The change prevents the molecule to penetrate the cell membrane again. So the Oregon Green BAPTA-1 AM is trapped inside the platelet\textsuperscript{5}.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure2_1.png}
\caption{Optical characteristics of the Oregon Green BAPTA-1 AM\textsuperscript{10}. Emission spectra of Oregon Green BAPTA1 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.}
\end{figure}

\subsection*{2.4 Experimental setup for the incubation and chemical activation of platelets}

We want to study the real-time interaction of platelets with BSA and IgG coated surfaces. Therefore, 50 μl of platelet-solution was placed upon the coated surface. The platelets in the solution were allowed to sediment onto the prepared surfaces in order to capture the binding of platelets to the prepared surfaces. This sedimentation process was recorded with a frequency of 2 Hz with an Andor Luca camera in fluorescent mode. The platelets were excited by an external light source (Leica EL6000). An objective lens with a magnification of 20x and a numerical aperture of 0.4 was used. During imaging of the cells at the surface, the amount of excitation light was minimized. Binning was used in the experiments to increase signal to noise, as a consequence the spatial resolution was reduced. The used experimental setup is shown in figure 2.2a. A typical image recorded after 20 minutes of incubation on IgG is shown in figure 2.2b.

Besides knowing how platelets interact with different surfaces, we also want to know what happens when the immobilized platelets are chemically stimulated. This gives an idea how many platelets are still functional. After most platelets had sedimented on the coated surface (on BSA ±80 minutes and on IgG ± 20 minutes), 4 μl of 5 mM TRAP was added to 50μl platelet buffer using a pipette. During this experiment we recorded 325 seconds with the same settings as used in the previous experiment. At frame $t=150$ seconds the TRAP was added. From $t=150$ seconds till $t=325$ seconds the chemical stimulation process was recorded. By adding the TRAP
11 seconds, we could first distinguish bound platelets from unbound platelets, in the same movie the chemical stimulation process was recorded.

![Figure 2.2](image)

**Figure 2.2: 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 4 μl of 5 mM TRAP TRAP solution 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.

### 2.5 Data analysis

The acquired images were processed using ImageJ. The regions of interest (ROI) were manually selected and per selected ROI the average intensity was measured. For the sedimentation experiments the ROI was chosen as shown in figure 2.3. First the platelets drift around in the solution through Brownian motion. After some time \((t_0)\) the platelet approaches to the coated surface and also the focus of the microscope, after which it binds to the surface \((t_1)\), this place is chosen as the ROI. Within a certain time the platelet gets activated and releases calcium from the Dense Tubular System. This increases the intracellular calcium concentration. The intracellular calcium reacts with the Oregon Green BAPTA-1 AM, which was loaded into the platelet, and increases its intensity. This can be seen at \((t_2)\) in figure 2.3. The time between the binding to the surface \((t_1)\) and the increased intensity \((t_2)\) is called the delay time.

Using ImageJ the moment of binding was manually determined. Sometimes the platelet binds partly to the coated surface, for example via its pseudopods. Then the platelet is still able to move around, tethered around the binding point. Platelets that move less than their own radius around one point are designated as bound platelets. In Origin the measured average intensity versus time plots were analyzed. From these individual curves, the moment of activation was also manually selected. The moment that a clear intensity increase was observed in the images and in the graphs was designated as the response moment \((t_2)\). The uncertainty in the determination of the binding time is 3 seconds, due to the manual determination of the binding moment.
Figure 2.3: 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. First an unbound platelet moves around in the buffer. At $t_0$, platelets 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 $t_1$. A responding cell increases the fluorescent intensity after binding, and is maximum at $t_2$. In case of adhesion experiments, the time between adhesion ($t_1$) and maximum increased intensity ($t_2$) 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.\(^6\)

The error in the percentage of activating platelets is determent by dividing the measured platelets in three groups. The first group contains the non responding platelets. The second one contains the platelets which give a clear responding curve. Example curves will be presented in section 2.7. The third group contains the ambiguous platelets. These platelets show an intensity increase, but this intensity increase is not clear enough to mark them as responding cells. Some platelets show a couple of short activation flashes after another. This intensity increase could be caused by activation of the platelet or by a platelet that rotates while bound to the surface. The percentage of platelets that are ambiguous determines the uncertainty in the number of responding cells.

For the chemical response experiments, similar analyzing methods were used; first the recorded images were analyzed using ImageJ. In this experiment the ROI is located around the immobilized platelet. This ROI was manually selected. For every ROI the average intensity was measured. The average intensity was plotted as a function of the time and analyzed using Origin. The delay time in these chemical stimulation experiments was the time between the TRAP addition and the calcium response of the immobilized platelets. Typical response curves, observed in the experiments, will be discussed in section 2.7.

### 2.6 Control experiment

During the experiment the TRAP was manually added to the washed platelets in the sample volume, using a pipette. 4 μl of TRAP was added to the sample, which introduces a flow in the sample. This flow could in turn cause shear stress on the platelets, which promotes the activation of platelets\(^1\). During the experiments with TRAP, we want to be sure that the
Platelets are activated by the TRAP and not the flow caused by the addition of TRAP. To examine if this is the case, a control experiment was conducted.

Platelets were allowed to settle on BSA for 80 minutes, this allowed enough platelets to immobilize on the BSA coated surface. BSA was used in the control experiments, because it was expected to induce the fewest platelet activations when binding to the surface. During the control experiment we recorded 325 seconds. At $t=150$ seconds $4 \mu l$ of Calcium buffer was added to the sample using a pipette. This causes the same flow disturbance as the experiments with TRAP. The recorded images were analyzed using ImageJ. The ROI were manually selected and per selected ROI the average intensity was measured. The ROI were chosen around platelets, which were immobilized on the BSA coated surface. In total 44 platelets were analyzed. None of the platelets showed an increase in fluorescence intensity. This indicates that the platelets were not activated by the introduced flow, thus we can conclude that TRAP causes the platelets to activate during the chemical stimulation experiments and not the introduced flow.

2.7 Typical calcium responses

During our experiments, different types of responses were recorded. Since TRAP is a well known stimulus, in this section we will present responses obtained upon chemical stimulation of TRAP. Two common response curves to TRAP are shown in figure 2.4.

After chemical stimulation, most of the responding platelets (70%) show a step response in their average intensity (figure 2.4a). In this example $4 \mu l$ of 5 mM TRAP was added after 152 seconds to the sample. After which it takes 68 seconds (delay time) before the intensity increases with 17%. This indicates the calcium response of the platelet. In this case the dense tubular system (DTS) releases its calcium after chemical stimulation. This increases the calcium concentration in the platelet which in turns increases the intensity. The intensity stays high, which means that the calcium does not escape out of the platelet.

In other cases (30%) the platelet has only a short activation flash after which it damps out (figure 2.4b). During this flash the intensity increases with 18%. The chemical stimulation causes the DTS to release its calcium also this time. But the increase of calcium is just for a few seconds ($\pm30$ seconds for figure 2.4b), after which the calcium concentration decreases again. An explanation for this pulse response could be that the system that releases the calcium from the DTS can also be reversed. The calcium is pumped back again into the DTS. Another reason for the short calcium response could be that the platelet ruptures and releases all its calcium indicator into the buffer.
Figure 2.4: Different responses of immobilized platelets on BSA surfaces upon chemical stimulation with TRAP. (a) A typical calcium response (70%) on adding a chemical stimulus to the platelet solution. This graph represents the intensity versus the time of platelets which have adhered on a BSA surface for 63 minutes. The platelet was stimulated at t=152 s with TRAP. When activated with TRAP the intensity of the blood platelet increases stepwise within 2 seconds. The activation of the platelet occurs on t=220 s. Resulting in a delay time of 68 seconds. (b) A short response flash of chemical stimulated platelets. This response curve occurs 30% of the time. This graph represents the intensity versus the time of platelets which have adhered on a BSA surface for 80 minutes. At t=149 s the platelets are stimulated with TRAP. When activated with TRAP the intensity of the platelet increases within 2 seconds (t=155.5 s). Which results in a delay time of 6.5 seconds. After about 30 seconds the intensity decreases again.

During the platelet adhesion experiment, the same response curve types were observed. 78% of the responding platelets show a step response as figure 2.4a. The other 22% show a flash response like figure 2.4b.

During the platelet adhesion experiments platelets enter the ROI, which causes an increase in intensity. This increase in intensity is about 4%. In comparison, the intensity increase due to activating platelets is 15%. This shows that intensity increase due to activation can be distinguished from intensity increase due to platelets entering the focus area.
3 Results & discussion

In this section the interaction of platelets with BSA and IgG coated surfaces will be presented. The response of the platelets upon surface contact will be evaluated (section 3.1) as well as the response of the platelets on a chemical stimulus (section 3.2). The data was analyzed according to the method presented in the previous chapter.

3.1 Results: Calcium responses during platelet adhesion

Blood platelets were allowed to sediment on BSA and IgG coated surfaces, to study the interaction of platelets on different surfaces. From literature it is known that BSA prevents specific bindings of platelets on a surface\(^7,11,12\). For this reason it is expected that most platelets will not react on contact with a BSA coated surface. This is why it is used as a negative control.

Besides BSA we also looked at the reaction upon binding to an IgG coated surface. As seen in chapter 1, platelets can bind to IgG using Fc-receptors. Binding through the Fc-receptor induces platelet activation\(^13\). Therefore it is thought that by blocking the Fc-receptor using the Fc-receptor blocker less platelets will bind to the IgG coated surface and that it will cause less activation of the platelets. In order to prevent possible donor-to-donor variation, from every donor the same amount of samples were analyzed without and with Fc-receptor blocker. In total 8 different samples from 4 different donors were used for platelets without and with Fc-receptor blocker. These samples were prepared as described in the last part of section 2.1.

The surfaces were prepared as described in section 2.2. Then 50 μl of platelet-solution was placed on the cover slides, after which movies were recorded and analyze as described in section 2.5. The fraction of responding platelets upon binding to a coated surface was analyzed. And the delay time of the platelets upon binding to a coated surface was also analyzed. In this experimental setup the delay time is the time between the moment that the platelet binds to the surface and the platelet’s calcium response.

On BSA coated surfaces binding events did occur. But because it takes a long time for the binding events to occur, no real-time binding events were recorded with the used setup. Typically about 20 platelets bind in 80 minutes in the Field of view. In contrast on specific surfaces, such as IgG, the same amount of platelets bound within 20 minutes. These results are consistent with the results found by Joris Goudsmits\(^14\).
Figure 3.1 shows the histogram of the recorded delay times, between binding of the platelet to an IgG coated surface and the increased fluorescent intensity. Results of washed platelets treated without Fc-Receptor blocker and with Fc-Receptor blocker are shown in panel a and b respectively. Table 3.1 shows the overall results of the Calcium responses during platelet adhesion.

Table 3.1: Interaction of platelets at different types of surfaces. The number of responding cells is determined from the fluorescent intensity plots as a function of time, including the counting error; n is the total number of curves analyzed. The depicted delay times are mean values (± standard deviation) of the cells that showed a clear intensity response; n is the number of cells used for this calculation.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Responding cells [%]</th>
<th>Delay times [s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IgG without FcR-blocker</td>
<td>65±4 (n=73)</td>
<td>45±8 (n=37)</td>
</tr>
<tr>
<td>IgG with FcR-blocker</td>
<td>64±4 (n=62)</td>
<td>56±7 (n=35)</td>
</tr>
</tbody>
</table>

Table 3.1 shows the mean delay time on the two coated surfaces. We have chosen for the geometric mean, because this is often used in cell counting in Europe\textsuperscript{15,16,17}. The advantage of using the geometric mean is that, a single high count in a data set has less effect upon calculating the mean.
It was expected that less platelets treated with Fc-receptor blocker would respond upon binding to IgG than platelets without Fc-receptor blocker. As shown in table 3.1 this is not the case. 65±4% of the non treated platelets activate upon binding to an IgG coated surface in comparison to 64±4% for platelets treated with Fc-receptor blocker.

When looking at the geometric mean, it can be seen that there is a difference in delay time; platelets without Fc-receptor blocker show a response 45±8 seconds after binding to the surface. In comparison Fc-receptor blocked platelets have a delay time of 56±7 seconds. This shows that the Fc-receptor blocker does not prevent platelets from activating, but it delays the activation time with about 24%.

3.2 Results: Calcium responses in adhered platelets

After the platelets have adhered to the surface, it is important to know if the platelets are still functional. This was done by measuring the calcium response of immobilized platelets to a chemical stimulus (TRAP). The percentage of responsive platelets and the delay time of the platelets were measured. In this case the delay time is the time between the TRAP addition and the calcium response of the immobilized platelets. In this paragraph the results of adhered platelets to a chemical stimulus (TRAP), on the two different surfaces (BSA and IgG), will be presented. In addition it was tested whether there is a different response for platelets treated without and with Fc-Receptor blocker on an IgG coated surface. On BSA coated surfaces the platelets were able to settle for approximately 80 minutes before adding TRAP to the prepared samples. This incubation time was chosen to obtain sufficient platelet immobilization. On an IgG coated surface the platelets were allowed to settle for 20 minutes.

Figure 3.2 shows a histogram of the delay time upon stimulating the platelets with TRAP on a BSA coated surface. This graph shows that most platelets (80%) respond within the first 40 seconds, but that there is also a population (20%) that takes longer to respond on a chemical stimulation. The different populations will be discussed in further detail in section 3.3.

As shown in previous section, platelets bound to BSA, but these real-time binding events were not recorded. It is expected that platelets do not respond upon binding to a BSA coated surface, because of the gentle nature of BSA. In section 1.1 it is also shown that, once platelets are fully activated they cannot activate once more. Therefore it is expected that almost all platelets will react to a chemical stimulation. This is the case as can be seen in table 3.2. 87±4% of all examined immobilized platelets respond when TRAP was added. The geometric mean delay time for chemical stimulation on a BSA coated surface is 18±39 seconds. Because of the wide distribution of delay time the error of the calculated delay time is larger than the delay time itself. In section 3.3 the error analysis in the experiments will be discussed in more detail.
Figure 3.2: Histogram of the recorded delay times between adding TRAP and increased fluorescence intensity. Platelet delay times upon adhesion to BSA coated substrate. In total 252 cells were analyzed on 13 different samples, with donor material obtained from 5 different donors. Of these 252 cells 226 cells responded to TRAP.

Table 3.2: Response of adhered platelets upon TRAP stimulation. The number of responding cells is determined from the fluorescent intensity plots as a function of time including the counting error; n is the total number of curves analyzed. The depicted delay times are mean values (± standard deviation) of the cells that showed a clear intensity response upon chemical stimulation; n is the number of cells used for this calculation.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Responding cells [%]</th>
<th>Delay times [s]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>BSA</td>
<td>87±4 (n=252)</td>
<td>33±40 (n=226)</td>
</tr>
<tr>
<td>IgG without FcR-blocker</td>
<td>35±1 (n=213)</td>
<td>12±8 (n=77)</td>
</tr>
<tr>
<td>IgG with FcR-blocker</td>
<td>38±3 (n=117)</td>
<td>13±11 (n=48)</td>
</tr>
</tbody>
</table>

Figure 3.3 shows the histograms of the delay time upon chemical stimulation on an IgG coated surface, without (figure 3.3a) and with (figure 3.3b) Fc-receptor blocker. Both histograms show that the majority of the responding platelets (94%) respond within 20 seconds after TRAP is added. The other 6% consists of extreme values which disturb the arithmetic mean that is why the geometric mean is used to calculate the average delay time.
Figure 3.3: Histograms of the recorded delay times between adding TRAP and increased fluorescence intensity. (a) Platelet delay times upon adhesion to an IgG coated substrate with Fc-receptor blocker coated platelets. In total 213 cells were analyzed on 7 different samples, with donor material obtained from 3 different donors. Of these 213 cells 77 cells response to TRAP. (b) Platelet delay times upon chemical stimulation on an IgG coated substrate without Fc-receptor blocker coated platelets. In total 117 cells were analyzed, on 7 different samples with donor material obtained from 3 different donors. Of these 117 cells 48 cells response to TRAP.

It is expected that, platelets once activated, will not respond for a second time. And as can be seen in section 3.1 most platelets (65%) respond upon binding on an IgG coated surface. Therefore it is expected that most platelets will not respond to TRAP, because they have already been activated before. This is the case as shown in table 3.2. This table also shows that there was no significant difference found between the delay time without (10±5 s) and with (9±12 s) Fc-receptor blocker. These delay times correspond to the delay times L. v. Zijp\(^5\) found for Poly-L-Lysine and anti-GPIb coated surfaces. There was also no significant difference found between the percentage of responding cells without and with Fc-receptor blocker; these values are respectively 35±1% and 38±3%. It is not known if this percentage, of responding cells, corresponds to the 35% of the platelets that did not respond upon binding to IgG. More research is required to investigate this.

When comparing the delay time upon chemical stimulation of BSA with the delay time found for IgG. It can be seen that there is no significant difference in chemical delay time between surfaces coated with BSA (18±39 seconds) and IgG (9±12 seconds without and 10±5 seconds with Fc-receptor blocker).

### 3.3 Reproducibility of the BSA calcium responses in adhered platelets

In previous paragraph it was shown that the delay time between adding TRAP and the calcium response of the platelets consists of two populations. What is the reason for the occurrence of these two platelet populations? To examine this, every BSA sample was examined individually. This is shown in table 3.3. From this table we can see that the different populations correspond to different samples. All samples with a delay time of 40 seconds or less (80%) are considered as the first population. This population consists of 10 samples and has an average delay time of
12±9 seconds. The other three samples represent the second population with a delay time of 102±13 seconds. The histograms of these two populations are shown in figure 3.4.

Table 3.3: Response of adhered platelets on BSA upon TRAP stimulation. This table shows the percentage of responding cells as well as the delay time of each BSA sample. The number of responding cells is determined from the fluorescent intensity plots as a function of time including the counting error; the second column represents the number of curves analyzed of each sample. The depicted delay times are geometric mean values (± standard deviation) of the cells that showed a clear step wise intensity response upon chemical stimulation; n is the number of cells used for this calculation.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Number of platelets</th>
<th>Responding cells [%]</th>
<th>Delay time [s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>50</td>
<td>23±14</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>75</td>
<td>40±8</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>94</td>
<td>11±3</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>100</td>
<td>116±0</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>83</td>
<td>10±5</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>75</td>
<td>4±3</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>75</td>
<td>36±40</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>95</td>
<td>118±19</td>
</tr>
<tr>
<td>9</td>
<td>21</td>
<td>95</td>
<td>8±1</td>
</tr>
<tr>
<td>10</td>
<td>23</td>
<td>100</td>
<td>19±6</td>
</tr>
<tr>
<td>11</td>
<td>31</td>
<td>84</td>
<td>94±21</td>
</tr>
<tr>
<td>12</td>
<td>67</td>
<td>100</td>
<td>7±2</td>
</tr>
<tr>
<td>13</td>
<td>47</td>
<td>79</td>
<td>28±19</td>
</tr>
</tbody>
</table>

There are multiple possibilities for the occurrence of two populations. First it was though that the different delay times corresponded with different donors. When looked closer to the samples individually, it shows that this is not the case. The different samples from one donor have both fast and slow reacting platelets.

Another explanation for the two populations may relate to the sensitive nature of the blood platelets. In section 2.1 the protocol for preparing the platelets is explained. The platelets are extracted from their natural environment (blood) and suspended in a calcium hepes buffer. The longer the platelets are suspended in the buffer, the less sensitive they are for chemical triggers. For this reason it was thought that the delay time of 102±13 seconds corresponded to measurements performed later on the day. This means that the platelets are longer suspended in a calcium hepes buffer. However two samples from the slow population were measured early on the day, and the other one was measured at the end of the day. This excludes the time-effect as a cause for the presence of these two platelet populations. The
actual reason for these two different populations remains unknown, but it is not time or donor dependent.

The same phenomenon can be observed when chemically activating platelets on IgG coated surfaces, as shown in figure 3.3. One of the seven analyzed samples show a longer average delay time than the other six samples. This corresponds with 6% of the total analyzed platelet on IgG.
4 Conclusion

In the experiments presented in this report, the interaction of platelets on different surfaces was studied. Using calcium indicator Oregon Green BAPTA-1 AM the intracellular calcium concentration change was analyzed upon binding to a surface and upon chemical stimulation. Once platelets get activated, its intracellular calcium concentration increases which in turns increases the intensity of the Oregon Green BAPTA-1 AM. Platelets, which showed this fluorescent intensity increase, were marked as responding cells. The delay time (the time between trigger and response) was measured for platelets on BSA and IgG coated surfaces.

On BSA it takes about 80 minutes for 20 platelets to bind to the surface. In contrast on an IgG coated surface, the same amount of platelets bound within 20 minutes. Due to this low binding probability we were unable to record real-time bindings on a BSA coated surface. When looking at the chemical stimulation results, it shows that almost all platelets (87±4%) can be chemically stimulated on BSA coated surfaces.

In addition the interaction of platelets with an IgG coated surface was studied. Platelets can only bind to IgG through Fc-receptors. By blocking these receptors it is expected that it reduces the calcium response of platelets upon binding to an IgG coated surface. 65±4% of all platelets without Fc-receptor blocker activated upon binding to the surface. The average time between binding and activation was 45±8 seconds. For Fc-receptor blocked platelets 64±4% of the analyzed platelets got activated upon binding. The average recorded time between binding and activation was 56±7 seconds. Looking at the chemical stimulation experiments, it shows that without Fc-receptor blocker 35±1% of the platelets respond to a chemical stimulus (TRAP), with an average time between adding TRAP and activation of 10±5 seconds. In comparison 38±3% of the platelets with Fc-receptor blocker respond to a chemical stimulus, with an average time between adding TRAP and activation of 9±12 seconds.

It was thought that by blocking the Fc-receptor on platelets the percentage of responding cells upon binding to IgG coated surfaces could be reduced. From the collected data it can be concluded that blocking the Fc-receptor on platelets has no effect upon binding to an IgG coated surface. There is also no significant difference between the percentage of responding cells without and with Fc-receptor blocker. So Fc-receptor blocker does not influence the percentage of activating platelets on IgG, but does increase the delay time upon binding with 24%. A reason for this could be that not all Fc-receptors are blocked. First the platelet binds to the IgG through the blocked Fc-receptor. After which a not blocked Fc-receptor binds to IgG. This activates the platelet. Due to the time between binding through the blocked Fc-receptor...
and de not blocked Fc-receptor, the delay time increases. This increases the delay time, but not
the percentage of responding platelets.

To make a good biosensor, it has to perform quick and accurate analyses of the platelets.
Looking at the results we see that BSA is not suitable as a surface, because it takes a long time
(±80 minutes) for bindings to occur on the coated surface. On IgG binding to the surface occur 4
times faster, but most platelets already react upon binding to IgG. So the functioning of the
platelets cannot be determent after platelets are immobilized on the surface. The researched
surfaces are not suitable for biosensors, but thanks to this research a better understanding of
the processes occurring during platelet-surface interaction is gained.
5 Literature


17. Fried, J. Mean, geometric mean, or median grain count in cell cycle studies. (2004)