Master

Quinacrine as a fluorescent label to study real-time exocytosis of single platelet dense granules

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Quinacrine As a Fluorescent Label
to Study Real-Time Exocytosis of
Single Platelet Dense Granules

J.M.H. Goudsmits
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Master’s Thesis

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Abstract

Platelets play a critical role in haemostasis. Their activation and aggregation is facilitated by exocytosis of proteins and other small messenger molecules from granules. The release dynamics of this process are rather unknown. In this study, quinacrine, a fluorescent label for platelet dense granules, is explored as a tool to measure real-time exocytosis of ATP stored in single dense granules.

A luciferase assay was used to assess quinacrine influence on platelet activation in bulk. No significant effect of quinacrine on platelet functioning was found. The same assay was also used to investigate substrate-induced activation. These studies revealed that BSA coated glass is the most suitable surface for platelet immobilisation, which is needed for microscopy experiments. Real-time fluorescence microscopy studies on single platelets showed that quinacrine photoinduces granule lysis, before exocytotic events can take place. Suggestions for an analytical approach to distinguish lysis from exocytosis are made, as well as proposals to continue research on visualisation of single platelet activation.
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Chapter 1

Introduction

Platelets, or thrombocytes, play an important role in haemostasis, the process that stops bleedings caused by injured blood vessels. Blood loss should be stopped quickly. Blood flow in intact vessels however should not be obstructed by unwanted clot formation. The correct functioning of platelets is thus of vital importance.

1.1 Platelets

1.1.1 Role in Haemostasis

Haemostasis is a complex process that involves interactions between platelets, blood vessels, numerous blood plasma proteins and several small molecules. This process has a few steps. First, immediately following damage, vessels constrict, therefore lowering the blood flow. Second, platelets adhere to collagen that is exposed at the damaged site. By adhesion, platelets are activated and release proteins and other molecules that activate nearby platelets. As a result, platelets interact and form a loose plug; this is called primary haemostasis. At the same time, secondary haemostasis occurs: fibrin proteins are made that reinforce the platelet plug by forming a fibre mesh. Finally, the vessel is repaired and the blood clot is slowly dissolved by enzymes.

1.1.2 Structure

Human platelets, when in an unactivated resting state, are discoid-shaped cells lacking a nucleus. The cells are 2 to 5 µm in diameter, 0.5 µm thick and have a volume of 6 to 10 fl. In healthy subjects, 150,000 to 300,000 platelets/µl are present. Precursor cells called megakaryocytes form platelets and release them into the bloodstream by fragmentation. Platelets contain organelles found in most eukaryotes: endoplasmic reticulum, Golgi apparatus, mitochondria, glycosomes and lysosomes. Two important types of secretory organelles that play a key role in the function of platelets are present: alpha granules and dense granules (delta granules).

Alpha granules are the most numerous of platelet organelles. Depending on the platelet size, there are 40 to 80 granules. The round shaped organelles are 200
1.1. Platelets

to 500 nm in diameter and contain many coagulation proteins. Dense granules are smaller (100 nm diameter), fewer in number (4 to 8 per platelet), and are irregular in form (Figure 1.1a). They contain small molecules: serotonin (65 mM), adenosine diphosphate (ADP, 653 mM), adenosine triphosphate (ATP, 436 mM) – both adenine nucleotide are non-metabolic, i.e. they are not involved in energy pathways –, calcium-ions (2.2 M) and pyrophosphate (326 mM) [3]. As observed from scanning electron microscope (SEM) images, in dense granules there exist a so called halo region between the dense core and granular membrane (Figure 1.1a). There is evidence that the halo contains serotonin taken up from cytoplasm [4], and that the core consists of high molecular weight aggregates of ATP and other molecules [5, 6].

1.1.3 Activation: Exocytosis and Shape Change

Upon activation, platelets degranulate. That is, they release the contents of both types of granules into the extracellular space, normally the bloodstream, through exocytosis. Exocytosis is a process that transports contents of vesicles out of the cell membrane by fusion of the cell and vesicle membranes (lipid bilayers) (Figure 1.2). This process involves a few steps. First, the vesicle is transported towards the cell membrane by motor proteins. Next, mediated by so-called SNARE proteins [7], the vesicle docks to the cell membrane and both membranes fuse. A small fusion pore is created through which diffusion of vesicle contents already starts. Finally, the vesicle membrane is completely incorporated into the cell membrane and its contents are completely released. Any proteins that were present in the vesicle membrane itself are now incorporated in the cell membrane.

In human platelets, the products stored in granules are not discharged into the extracellular space directly, but into the open canalicular system (OCS). The

Figure 1.1: Scanning electron microscope (SEM) image of resting platelet. From [2]. (a) Dense granules (visible as electron dense (black) regions) are irregular in form, 22,000x. The arrowhead indicates a dense granule with a clear halo region. (b) Discoid platelet with stained OCS and membrane surface, 22,000x. Arrowheads indicate surface invaginations.

1Not all platelets have an OCS, such as bovine and equine platelets.
1.1. Platelets

Figure 1.2: Illustration of exocytosis (simplified). A vesicle approaches the cell membrane (left image). Both membranes (vesicle and cell) are lipid bilayers. So-called SNARE proteins (not displayed) mediate the docking of the vesicle to the membrane and the membrane fusion (middle images). Finally, the vesicle membrane is completely incorporated into the cell membrane and its contents are released (right image).

OCS is a tortuous network of membrane invaginations tunnelling through the cytoplasm (Figure 1.1b). This tubular system increases the total surface area exposed to plasma, providing a way for substances to quickly reach the innermost of the cell. Alpha en dense granules fuse with the OCS membrane, which is cell surface membrane. The OCS membrane also supplies the additional membrane that is needed for spreading (see next paragraph).

Besides organelles described earlier, cell structures exist that shape the platelet. Microtubules, situated as a circumferential coil, form the cytoskeletal support. Microfilaments, or actomyosin filaments (contractile proteins also found in muscle cells), are responsible for internal transformations and overall shape change upon activation: platelets form pseudopods and spread, increasing surface area and thus bind more strongly. The central body of the cell, incorporating most of the organelles, remains visible as a bulge (Figure 1.3).

Platelet activation is a complex process involving many intracellular pathways. As mentioned earlier, platelets can be activated by collagen in damaged vessel walls. Other platelet activators (agonists) are: ADP, ATP (both present in dense
1.2. Platelet Function Testing

Platelet function testing evaluates platelets' ability to aggregate and form fibrin clots. This is achieved through the release of granules, epinephrine, thrombin (protein that cleaves fibrinogen into insoluble fibrin that leads to the formation of clots) and thrombin receptor activating peptide (TRAP, synthetic peptide with strong activation response). Shear stress and low temperatures can also trigger activation [8]. Both activation processes, degranulation through exocytosis and shape change, are two separate processes that describe the activation status. In other words, a shape change does not necessarily mean that degranulation also takes place. Small structural deformations are reversible [2], degranulation however marks irreversible platelet activation.

1.2 Platelet Function Testing

The clinical importance of diagnostic tests for abnormal platelet functioning is evident. Moreover, research from the last years indicate that platelets are also involved in inflammatory functions [9] and are critically involved in the onset of atherosclerosis [10], therefore making platelet function analysis important in preventive healthcare.

Apart from clinical studies, research is needed to understand fundamental properties of platelet activation and granule release dynamics. In the following sections, existing techniques for platelet function testing are summarised [11, 12].

1.2.1 Bulk Measurements

In general, the (bulk) responsiveness of platelets can be quantified by their steady state activation as a function of agonist, presented in a dose-response curve (Figure 1.4). Deviations in reactivity, such as high-responsive platelets indicating thrombotic risks or low-responsive platelets indicating bleeding disorders, result in a curve change. Another method to describe responsiveness is to study the temporal behaviour after stimulation.

![Figure 1.4: Sketch of dose-response curves displaying platelet responsiveness for healthy subjects and subjects with thrombotic and bleeding disorders.](image)

Apart from clinical studies, research is needed to understand fundamental properties of platelet activation and granule release dynamics. In the following sections, existing techniques for platelet function testing are summarised [11, 12].

The oldest in vivo technique to assess platelet functionality is bleeding time, in which a cut with standardised width and depth is made. The time it takes for the bleeding to stop is a quantitative measure for platelet functioning.
1.2. Platelet Function Testing

Aggregometry is the most widely used platelet function test. Upon in vitro stimulation of a blood sample with agonist, platelets start to aggregate and the sample’s light transmission is increased. The light that is scattered, i.e. not transmitted, as a function of agonist is a measure for platelet aggregation.

Another way to quantify activation is by measuring granule secretion. The most used analytical assay to determine the dynamic (bulk) release of ATP from dense granules is a luciferase bioluminescence assay [13, 14]. Another recent study presents an electrochemical assay to measure real-time serotonin release from platelets [15].

1.2.2 Single Cell Measurements

Whilst bulk techniques have benefits like speed and simplicity, they also manifest shortcoming. Some rare events might be obscured and it is difficult to obtain statistical distributions. Therefore, single cell measurements provide better understanding of fundamental properties.

Flow cytometry is an analytical technique to examine single cell functions, such as platelet function. Before analysis, platelets are labelled with fluorescent antibodies that bind specific membrane glycoproteins (GPs). For example, GP1b is a platelet specific membrane marker and P-selectin (CD62P) is a protein that is present on the surface of activated endothelial cells and activated platelets. By measuring fluorescence and scattered light characteristics of single platelets passing a laser beam, their level of activation can be quantified. Although flow cytometry yields statistical distributions from a very low whole blood sample volume, measurements require sample preparation, are slow and no dynamic information can be obtained.

Other bioanalytical tools exist to study single platelet exocytosis [16]. One is an electrochemical measurement of real-time serotonin (from dense granules) release from activated platelets by placing a microelectrode above a single platelet. With this method, quantitative serotonin detection on a sub-millisecond resolution can be achieved [4, 17]. This technique lacks the ability to obtain spatial information and to multiplex, i.e. address multiple platelets at once, though.

1.2.3 New Approach

In this research project, a new technique to visualise real-time single platelet activation was examined. By fluorescent labelling of dense granule contents with quinacrine, exocytosis is associated with loss of fluorescent intensity. As a result, spatial as well as temporal information can be obtained in parallel for tens of platelets by fluorescent imaging. This multiplexed and spatiotemporal approach gives new insights in the release dynamics of single platelets. The advantage of using a fluorescent label is, that it can be combined with other techniques to measure or locally stimulate single platelets. For example, magnetic particles can be attached to platelets [18]. An external magnetic field can then be used to apply forces to the particles, activating the platelets.

\[ ^2 \text{P-selectin is present on the } \text{alpha granule membrane. Upon degranulation, the protein is expressed on the outer platelet surface.}\]

\[ ^3 \text{Endothelial cells form a thin layer of cells lining the interior surface of blood vessels.}\]
1.3 Thesis Overview

Fluorescent imaging of dense granules labelled with quinacrine is investigated. First, bulk measurements were performed to assess the effect of quinacrine on platelet activation by means of a dose-response curve. Next, substrate induced activation of immobilised (needed for microscopy experiments) platelets was studied. With this method, the most suitable substrate for imaging platelet activation was determined. Then, surface immobilised platelets with stained granules were imaged to visualise real-time degranulation. The effect of light and fluorescent dye was studied.
Chapter 2

Methods

Traditionally, bulk techniques like bleeding time and aggregometry are used to evaluate platelet responsiveness. Another technique utilises a luciferase assay, which measures ATP concentrations, to quantify platelet degranulation in bulk. The latter method was first used to prove validity of the single cell measurements using quinacrine. Finally, quinacrine was explored as a tool to visualise real-time exocytosis of fluorescently labelled dense granules.

In this chapter, first the global principles of the assays are discussed together with important characteristics of used substances. Next, the technical details of the procedures are treated.

2.1 Luciferase Assay for Bulk Activation

Platelet reactivity was quantified by measuring the ATP release from dense granules with firefly luciferase. Firefly luciferase in combination with D-luciferin is commonly used as a bioluminescence assay to measure ATP [19, 20]. The assay has high sensitivity, broad range (pM to mM) and high specificity. D-luciferin is oxidised while consuming ATP and producing light. The reaction, yielding one photon per ATP molecule, is catalysed by luciferase, a 63 kDa protein:

\[
\text{ATP} + \text{D-luciferin} \xrightleftharpoons{\text{Luciferase, Mg}^{2+}} \text{adenyl-luciferin} + \text{PP}_i,
\]

\[
\text{adenyl-luciferin} + \text{O}_2 \rightarrow \text{oxyluciferin} + \text{AMP} + \text{CO}_2 + \text{light (560 nm)}.
\]

ATP released from an activated platelet suspension was measured for a range of TRAP concentrations (sub-µM to mM), i.e. a dose-response curve was measured. This curve was determined for both untreated platelets and platelets incubated with quinacrine to assess quinacrine influence on platelet activation.

2.2 Substrate-Induced Activation Assay

In order to determine a suitable substrate for fluorescent imaging of the platelets, platelet activation induced by differently coated glass coverslips was measured. For this purpose, glass coverslips were coated with the desired substance first. The
2.3 Single Platelet Activation Assay: Visualising Dense Granule Exocytosis

Substances tested were anti glycoprotein 1b (aGP1b, antibody that binds to GP1b, a platelet surface membrane glycoprotein), mouse immunoglobulin G (IgG, general antibody), poly-L-lysine (PLL, commonly used for cell adhesion) and bovine serum albumin (BSA, blocking agent). After that, platelet suspension was added onto the coated glass and incubated for 60 minutes: platelets sediment and interact with the surface. Upon a strong enough stimulus, platelets activate and release ATP (Figure 2.1). Next, supernatant was transferred into a well and the ATP concentration was subsequently analysed with the luciferase assay.

![Figure 2.1: Substrate-induced activation assay. A glass coverslip is coated with the desired substance. After incubating platelets for 60 minutes, supernatant is transferred and analysed with the luciferase assay.]

2.3 Single Platelet Activation Assay: Visualising Dense Granule Exocytosis

Single platelet activation was quantified by measuring real-time exocytosis of dense granules. Exocytosis was visualised by labelling the granules with quinacrine. Quinacrine (aka Mepacrine, Atabrine), an old antimalarial drug, is a fluorescent label that binds adenine nucleotides like ATP [21]. Dense granules contain 436 mM ATP [3], which is high compared to other organelles. As a result, only dense granules are stained with quinacrine [22]. Dense granule staining was used before in flow cytometry measurements [23], as bulk fluorescence assay [24], or it was used to obtain the number of dense granules by counting flashes on prolonged irradiation [25, 26]. As already shown in mast cells [27], pancreatic B-cells [28] and astrocytes [29], the release of secretory granule contents is associated with the decrease or loss of quinacrine fluorescence. Accordingly, platelet degranulation can be visualised real-time by observing changes in quinacrine fluorescence.

Quinacrine Characteristics

Quinacrine fluorescence excitation (absorbance) ranges roughly from 350 to 475 nm, emission ranges from 440 to 600 nm (Figure 2.2a) [30]. The dye is a base with different absorption spectra for multiply charged cations (Figure 2.2b) [31]. Around the physiological pH of 7, there is an equilibrium mainly between singly and doubly charged cations. For lower pH, the equilibrium shifts towards the doubly charged cation, conversely for higher pH the equilibrium shifts towards the singly charged cation and neutral molecule. Giving the fact that quinacrine is...

---

1 The degree of protonation depends on pH.
2.3. Single Platelet Activation Assay: Visualising Dense Granule Exocytosis

excited between 450 and 490 nm (cf. Section 2.4.4), the absorption (and hence fluorescence) is stronger at lower pH.

Quantitative bulk measurements of quinacrine in a closed fluid cell (cf. Section 2.4.4) were performed to determine the pH dependent fluorescence (Figure 2.3a). Phosphate buffer was used because of its simplicity to fix pH. As the reference point shows, phosphate buffer has no significant influence compared to Hepes buffer, which is normally used. From pH 5.5, the results show a decreasing fluorescent intensity for increasing pH, up to a factor of 2 at pH 8.0. These results are in agreement with what is expected from absorbance graphs (Figure 2.2b). Dense granules are slightly acidic at pH 6.1 [32]. When granules secrete their contents in the extragranular space at a pH around 7 - 7.4, intensity drops by 15 to 20% (only considering the pH effect).

Other bulk measurements of quinacrine in Hepes buffer pH 7.3 show that quinacrine fluorescence depends on dye concentration (Figure 2.3b). Fluorescent intensity is strongly non-linear for high concentrations. At concentrations above 10 mM a strong quenching effect is visible. The implication is that when quinacrine concentration drops (e.g. by exocytosis), dequenching can occur for initially high quinacrine concentrations. This will result in an increase in fluorescent intensity. The exact nature of quinacrine quenching remains unclear, however it strongly depends on its environment, such as calcium concentration [33]. The quenching strength, i.e. the ratio between maximum intensity and minimum intensity at saturating dye concentration, might be of the order of one to ten or even more [34, 35].

---

Extragranular space can be platelet cytoplasm or platelet exterior.
2.4 Materials and Setup

2.4.1 Preparation of Washed Human Platelets

Blood was drawn from healthy donors (free of aspirin for 10 days) and anticoagulated with 3.2% sodium citrate. Within one hour from collection, the whole blood sample was centrifuged at 250g for 20 minutes at room temperature. Platelet rich plasma (PRP) with volume $V_{PRP}$ was transferred and anticoagulated with citrate dextrose (ACD, 0.25% citrate, 0.15% citric acid and 0.2% D-glucose). Next, the PRP was centrifuged at 520g for 16 minutes at room temperature to pellet the platelets. The pellet (supernatant was disposed) was then resuspended in Hepes buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 0.3 mM NaH$_2$PO$_4$5H$_2$O, 10 mM MgSO$_4$.7H$_2$O, 50 mM D-glucose, pH 6.5) with 100 ng/ml carbaprostacyclin (cPGI$_2$, Cayman Chemical) to a final volume of $V_{PRP}$.

Carbaprostacyclin prevents platelet activation caused by e.g. resuspending. It is a stable analog of prostacyclin (PGI$_2$, having a lifetime of 3 minutes), which is an inhibitor for platelet secretion and aggregation. PGI$_2$ is released by healthy endothelial cells.

---

Figure 2.3: Quinacrine fluorescence depends on pH and dye concentration. In both plots, error bars represent the spread between different positions in the closed fluid cell. (a) Increasing pH (from 5.5) decreases fluorescent intensity up to a factor of 2 at pH 8.0. (b) At concentrations above 1 mM fluorescence starts to level off. At concentrations above 10 mM a strong quenching effect is visible. The red line indicates a linear behaviour.

Certain dyes (e.g. acridine orange) are strong at photo-induced production of reactive oxygen, others (e.g. FITC-dextran) are poor. This reactive oxygen can cause oxidation of co-factors, proteins and desaturated fatty acids in lipids (lipid peroxidation). When a fluorescent dye, that is loaded into vesicles, produces reactive oxygen, lipid peroxidation causes membrane degradation. This can eventually result in vesicle lysis, disintegration by rupture of the membrane. For vesicles loaded with fluorescent dye, both lysis (a physical effect) and real exocytosis (a biological effect) result in a decrease of dye concentration. Hence, lysis can lead to false positive exocytotic events, which is highly undesirable. From literature it is unknown if quinacrine stained dense granules induce granular membrane degradation or granule lysis under fluorescent excitation.
2.4. Materials and Setup

Again, the platelets were pelleted by centrifuging at 520g for 16 minutes at room temperature. The pellet was finally resuspended in Hepes buffer, pH 7.3 to the desired volume – about the original whole blood volume. Before using the washed platelets (WP), the WP suspension was kept at room temperature for 30 minutes. This allows platelets that are in a reversible activation state to return to their resting state.

2.4.2 Measuring ATP with Luciferase

A 96-well plate was coated with bovine serum albumin (BSA) to minimise substrate-induced platelet activation. Therefore, 3% BSA in phosphate buffered saline (PBS, 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4) was incubated in the wells for 1 hour and then washed with PBS. 50 to 100 µl of solution (based on Hepes buffer pH 7.3) with unknown ATP concentration was added to the wells. In case TRAP (Bachem) was used to activate platelet suspension in the well, it was added from a 10x solution approximately 5 minutes prior to the next step. Firefly luciferase (Sigma-Aldrich, 20 µg/ml final concentration) and D-luciferin (Tebu-Bio, 50 µg/ml final concentration) were pre-mixed in Hepes buffer pH 7.3 and then added as 10 µl to the wells just before the measurement. Dynamic luminescence was measured by a well plate reader using 1-second integration time per point per well for at least 1 hour. A few samples of ATP with known concentration were used for calibration in each measurement series. Luminescent signal intensity typically showed a slight increase in the first 30 minutes, followed by a slight decrease. The maximum intensity was used for data analyses.

2.4.3 Substrate-Induced Activation

Glass coverslips (24 mm x 36 mm) were thoroughly washed in detergent (solved in deionised water), deionised water and isopropanol respectively and blow-dried with air. The desired primary coating was applied by incubation at room temperature. After washing with 1% BSA in PBS, a secondary BSA coating (3% BSA in PBS, 2 hours incubation at room temperature) was applied in order to block the surface. Then, coverslips were washed with Hepes buffer pH 7.3 and gently blow-dried. 100 µl of WP was added on top of the coverslip and incubated for one hour at room temperature in a sealed petri dish. After incubation, 50 to 75 µl of supernatant was transferred into a well to determine the ATP concentration with the luciferase reaction (Section 2.4.2). Although incubation took place in a sealed petri dish, results were corrected for evaporation, which is of the order of 10%.

Results were compared with ATP concentrations in untreated WP and activated WP (activated with 400 µM TRAP for at least 5 minutes prior to the measurement).

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4Protocol adapted from Van Holten, Department of Clinical Chemistry and Haematology, University Medical Centre Utrecht
5From microscope images it was concluded that in one hour most of the platelets sediment and interact with the substrate.
2.4. Materials and Setup

2.4.4 Imaging Labelled Granules

Glass coverslips were thoroughly washed (Section 2.4.3). The surface was coated with BSA (3% BSA in PBS, 2 hours at room temperature). After incubation, the coverslips were washed with Hepes buffer pH 7.3. 0.5 µl of WP was added per 1 mm² of surface area, followed by 20 µM quinacrine (final concentration, added from 10x stock). WP and quinacrine were incubated for 30 minutes at room temperature, allowing the platelets to sediment onto the coated surface. Unbound platelets and excess quinacrine were washed out with Hepes buffer. Unless stated otherwise, biocompatible imaging spacers (9 mm well diameter, 0.12 mm height) were used to create a closed fluid cell filled with Hepes buffer (Figure 2.4).

![BSA-coated coverslip incubated with WP](image)

Figure 2.4: Fluid cell construction, exploded view. Biocompatible double-sided tape (9 mm well diameter, 0.12 mm height) is sandwiched between a BSA-coated coverslip incubated with washed platelets and a clear coverslip.

Labelled platelets on the bottom of a fluid cell were imaged with an inverted microscope (Leica DMI5000 B) and a 40x objective lens. The fluorescence light, provided by a metal halide bulb (Leica EL6000), excites fluorophores in a ∼ 500 x 500 µm² area. A filter cube with excitation filter (bandpass, 450 - 490 nm), dichroic mirror (510 nm) and suppression filter (lowpass 515 nm) (Leica I3) was used.

Stained granules were imaged by a high sensitivity camera (Hamamatsu C10600-10B (ORCA-R2)) at 2 or 5 Hz. At 40x one pixel covers 0.25 x 0.25 µm², the total imaging area is approximately 330 x 250 µm². Bright field images were taken prior and/or posterior to fluorescence movies to identify the position of the platelets.

2.4.5 Data Analysis

Movie files were analysed by home-brew software. Granules were pinpointed manually. The software analyses a 5 x 5 pixel region (which is approximately the size of the point spread function of the microscope objective) around the centre, i.e. the brightest pixel, of the granule (Figure 2.5a). The average intensity, corrected for photobleaching and other fluctuations with background intensity from a nearby platelet-free area, is plotted over time. From the curve, parameters such as release delay, release duration, initial intensity and final or remaining intensity can be extracted (Figure 2.5b). Intensity parameters were fitted by horizontal lines, the release duration is approximated by a straight best-fit line. The time $t = 0$

An automated granule recognition algorithm needs careful approach, due to the irregular nature of granules and the high density in some regions.
2.4. Materials and Setup

can be defined by a special event, such as the addition of agonist or the onset of fluorescent light.

Figure 2.5: Data analysis. (a) Fluorescence image of platelet showing two granules. The marked area indicates a 5 x 5 pixel (= 1.25 x 1.25 µm²) region around the centre of the granule used for analysis. (b) Granule intensity over time. Release delay, release duration, initial intensity and final or remaining intensity can be extracted.
Chapter 3

Results

Platelet activation in bulk and substrate-induced activation was first investigated with the luciferase assay. Those results were used for an optimal single platelet activation assay using quinacrine.

3.1 Bulk Activation

3.1.1 Quinacrine Does Not Influence TRAP-induced Platelet Activation Significantly

A dose-response of TRAP-stimulated platelets was measured with the luciferase assay for both unlabelled washed platelets (WP) and WP (same donor) incubated with 20 µM quinacrine (Figure 3.1). ATP concentrations represent the degree of degranulation, i.e. the level of activation. A sigmoid curve fit is applied to the data (Table 3.1):

\[ s(c) = s_0 + \frac{s_1 - s_0}{1 + (c_0/c)^p}. \]  

(3.1)

Here, \( c \) denotes the concentration of TRAP, \( c_0 \) the concentration which induces a response \( s \) halfway between the baseline \( s_0 \) and maximum response \( s_1 \) and \( p \) is a measure for the slope at \( c_0 \). The response (ATP concentration) was calibrated with samples of known ATP concentrations. Measurements without WP, but with fixed concentrations of ATP (not shown) indicate that quinacrine and TRAP have no significant effect on the luciferase reaction for the ATP range typical in experiments with platelets (10 µM and below).

With a platelet concentration of 89200 platelets/µl (counted with a flow cytometer), fully activated WP release 4.83 nmol ATP/10^8 platelets, which is in agreement with values found in literature [39].

It can be concluded that quinacrine does not effect platelet activation by TRAP significantly: there is no notable difference in \( c_0 \). A decreased level of maximum activation of about 15% for WP incubated with quinacrine is observed, however platelets can still be activated with TRAP. The effect of quinacrine on processes other than dense granule release through TRAP remains unknown.
3.1. Bulk Activation

Figure 3.1: Dose-response curve of TRAP-stimulated washed platelets (WP), either incubated with 20 µM quinacrine or without. Each point was only sampled once.

Table 3.1: Fitting parameters for dose response curve of unlabelled washed platelets (WP) and WP incubated with 20 µM quinacrine.

<table>
<thead>
<tr>
<th></th>
<th>WP</th>
<th>WP + Quinacrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>$s_0$ (µM ATP)</td>
<td>0.38 ± 0.14</td>
<td>0.33 ± 0.09</td>
</tr>
<tr>
<td>$s_1$ (µM ATP)</td>
<td>4.31 ± 0.16</td>
<td>3.55 ± 0.13</td>
</tr>
<tr>
<td>$c_0$</td>
<td>11.5 ± 1.2</td>
<td>15.8 ± 1.7</td>
</tr>
</tbody>
</table>

3.1.2 Platelet Responsiveness Changes over Time

In order to evaluate platelet activation over the course of time, a flow cytometry study is performed by Loes van Zijp (for technical details, see [18]). In this experiment, P-selectin expression on the outer membrane, a quantity for platelet activation, is measured over time (Figure 3.2). Two batches of WP from the same donor were analysed to investigate the best storage method. Batch 1 was prepared approximately 30 minutes after blood was collected from a donor, batch 2 was prepared ∼350 minutes later (whole blood was kept at room temperature). Baseline and maximum response (cf. Section 3.1.1) were measured. The time $t = 0$ is 30 minutes after the last washing step (cf. Section 2.4.1). Actual measurements took place 30 minutes after the indicated time: samples were incubated for 20 minutes with 800 µM TRAP and fixed with 0.2 % formaldehyde solution for 10 minutes.

Neglecting outliers, results show a clear trend of decreasing responsiveness (maximum response). In four hours, responsiveness is reduced by 35%. The data points for batch 1 and batch 2 overlap remarkably well. Therefore it can be concluded that storing platelets in whole blood does not result in higher responsiveness compared to storing WP in buffer. The decrease in maximum response is not compensated by an increase in baseline response (non-activated WP). The decrease in maximum response is not compensated by an increase in baseline response (non-activated WP). The decrease in maximum response is not compensated by an increase in baseline response (non-activated WP).

1Fixation, i.e. termination of any ongoing biochemical reactions, is needed for flow cytometry measurements. Formaldehyde does so by cross-linking proteins.

2Outliers from the trend currently cannot be explained since procedural errors cannot be excluded completely.
3.2 Substrate-Induced Activation

exact nature of this behaviour and its implications for fluorescent labelling are unknown.

In conclusion, older platelets are less sensitive to activation by TRAP and there is no increase in pre-activation.

3.2 Substrate-Induced Activation

3.2.1 BSA Coated Surface Shows Least Induced Activation

Different surface coatings were applied to glass coverslips (Table 3.2) to immobilise platelets. Washed platelets (WP, same donor) were incubated on the coated glass for one hour. Next, the ATP concentration of supernatant was analysed with the luciferase assay (Figure 3.3a). As a reference, ATP concentrations of both untreated WP and WP incubated with 200 μM TRAP, i.e. baseline en maximum response (cf. Section 3.1.1), are shown. BSA clearly induces the least activation. The analysed supernatant only contained few platelets demonstrating that most of the platelets interacted with the surface.

It is unclear from these results if the interactions between substances and platelet (receptors) are responsible for activation. For example, from flow cytometry studies it is known that platelets labelled with aGP1b in solution are not activated completely. Moreover, there is no evidence in literature that BSA induces direct platelet activation. From these observations, it is deduced that binding to a surface leads to activation, rather than the interactions themselves. The strength of the (nonspecific) surface bonds might determine the degree of activation.

In a different measurement, supernatant from a BSA coated surface was incubated with 200 μM TRAP, i.e. any remaining platelets were activated. Results were compared to pure (i.e. non-treated) supernatant. ATP concentrations relative to bulk activated WP were respectively 0.38 and 0.25. This indicates supernatant contained only few platelets.
3.2. Substrate-Induced Activation

Table 3.2: Different glass coating procedures.

<table>
<thead>
<tr>
<th>coating (primary)</th>
<th>wash</th>
<th>coating (secondary)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aGP1b (10 µg/ml in PBS, ~20h)</td>
<td>1% BSA in PBS</td>
<td>3% BSA in PBS (~2h)</td>
</tr>
<tr>
<td>mouse IgG (200 µg/ml in PBS, ~20h)</td>
<td>1% BSA in PBS</td>
<td>3% BSA in PBS (~2h)</td>
</tr>
<tr>
<td>PLL (1 mg/ml in H2O, ~20h)</td>
<td>PBS</td>
<td>3% BSA in PBS (~2h)</td>
</tr>
<tr>
<td>BSA (3% in PBS, ~2h)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Figure 3.3: Substrate-Induced Activation. Measurements in (a) and (b) are from two different donors. In both figures, ATP concentrations are normalised to the ATP concentration found in activated washed platelet suspension. Each value is the average of three samples, error bars represent the spread. (a) Different surface coatings induce dissimilar platelet activation. BSA induces the least activation. (b) Platelets incubated with Fc receptor blocker (FcR bl) show reduced substrate-induced activation. Two concentrations were used for mouse IgG surface incubation: 200 µg/ml (high) and 10 µg/ml (low).]

On the outer surface of platelets, Fc receptors (FcR) are present. These proteins bind to the Fc region of antibodies. Experiments were conducted where WP were incubated with Fc receptor (FcR) blocker (Miltenyi Biotech, 10 times diluted from stock) for ten minutes at room temperature preceding incubation on coated glass. FcR blocker prevents binding of platelets with antibodies via the Fc region. The results show that substrate-induced platelet activation is lowered when WP were incubated with FcR blocker (Figure 3.3b). This demonstrates that when no blocker is used, Fc bonds that induce surface activation are present.

The difference in relative activation by antibody between the two measurements series can be attributed to donor variance. The latter is clear from the ratio baseline to maximum for bulk WP, i.e. the ratio of green bars (Figure 3.3).

To sum up: among other coatings, BSA is the most suitable coating for platelet immobilisation. The binding of platelets to the surface rather than the interaction with BSA causes activation.

---

It is unknown how the antibodies like aGP1b and mouse IgG are oriented on the surface. It is assumed that part of the antibodies are oriented such that the Fc region is available to platelets.
3.2. Substrate-Induced Activation

3.2.2 Platelets Can Still Be Activated with TRAP After Incubation on Substrate

Previous results show that platelets immobilised on a BSA substrate are activated by approximately 30%. Next, experiments were carried out to prove that those bound platelets can still reach a higher level of activation by adding TRAP.

As before, primary supernatant was removed from the surface, following a 60 or 120 minutes platelet incubation (Figure 3.4). Next, coverslips were gently washed in Hepes buffer pH 7.3. After that, Hepes buffer with or without 200 µM TRAP was added and incubated for 15 minutes. In this way, immobilised platelets can be completely activated. Then, secondary supernatant was removed and analysed with the luciferase assay.

Secondary supernatant with TRAP shows that adhered platelets can still be activated, even when platelets were incubated for two hours on the substrate (Figure 3.5). A negative control series with merely Hepes buffer added after the washing step shows that platelets were barely activated by this buffer. This observation confirms that TRAP causes the activation and not the procedure or the buffer itself. Summing the relative ATP concentrations of primary and secondary supernatant does not yield 1. The washing step, removing some (non-activated) platelets and remaining supernatant, is most likely the reason.

![Figure 3.4](image1.png)

**Figure 3.4:** Two-step substrate-induced activation assay. After primary supernatant is transferred and analysed with the luciferase assay, the coverslip is gently washed and incubated for 15 minutes with Hepes buffer pH 7.3 with or without TRAP. Then, secondary supernatant is removed and analysed.

![Figure 3.5](image2.png)

**Figure 3.5:** Two-step substrate-induced activation. Two series for 60 and 120 minute platelet incubation are displayed. Secondary supernatant with TRAP shows that adhered platelets can still be activated.
3.3 Fluorescent Imaging of Single Platelet Activation

Previous measurements show that platelets immobilised on a BSA coated glass substrate are suitable for fluorescence microscopy with quinacrine labelled dense granules. The BSA surface already induces degranulation, however, adding TRAP should result in additional release events visible as decrease or loss of fluorescence intensity.

3.3.1 Types of Release Events

Without discussing the origin yet, several exocytotic-like events were visible when imaging washed platelets incubated with 20 \( \mu \)M quinacrine on a BSA substrate. Events were analysed by plotting granular fluorescent intensity versus time. Some events show a monotonically decreasing intensity (Figure 2.5b), whereas others show a spike prior to decrease (Figure 3.6a) which is visible as a flash in the movie. Movements along the z-direction, i.e. movements in or out of the focal plane, contribute to an intensity change of at most 10% for shifts of \( \sim 1 \mu \)m.

As a result, flashing phenomena are not caused by drifts in or out of the focal plane, but are attributed to quenching (or actually dequenching) of quinacrine (Section 2.3). Due to high background levels of the order of 100 light intensity units, the actual increase in intensity is a factor of two. Although exact quenching interactions are unknown, this factor matches the expected factor from quinacrine quenching solely based on concentration (Figure 2.3b). The high background intensity combined with strong quenching also obscures some granules; only flashes (dequenching) signal the presence of granules (Figure 3.6b). The background signal is mainly caused by bulk fluorescence of the fluid with low concentrations of leaked quinacrine. This was measured by varying fluid cell height: background intensity was proportional to cell height.

Figure 3.6: Some release events from a single measurement. Background intensity is of the order of 100 light intensity units. (a) A spike, or flash, prior to decrease in signal intensity is attributed to quenching of quinacrine. (b) High background intensity combined with strong quenching obscures some granules; only flashes (dequenching) signal the presence of granules. (c) A visually apparent single granule shows well-separated events in time, indicating the presence of two granules. The two first peaks (at 5 and 25 seconds) originate from neighbour granules.
3.3. Fluorescent Imaging of Single Platelet Activation

The point spread function of $\sim 1 \, \mu \text{m}$ leads to visual overlap in granules. With the current hardware, it is impossible to visually separate granules distanced less than 500 nm. Moreover, the irregular shape of dense granules makes it even harder to discriminate between two granules. However, if a visually apparent single granule shows well-separated (release) events in time (Figure 3.6c), it can be concluded that more granules contribute to the intensity graph.\(^5\)

Heuristic observations indicate a strong sample to sample variation from the same donor. Within one fluid cell, there is even a notable spot to spot difference, making it inherently challenging to compare results. Also a time dependence needs to be taken into account, as already measured before (Section 3.1.2).

3.3.2 Granule Lysis Is Induced by Excitation Light

To investigate if excitation light in combination with quinacrine can lead to vesicle lysis, the number of events were analysed as a function of time (Figure 3.7). Washed platelets were incubated with 20 $\mu \text{M}$ quinacrine on a BSA substrate. No agonist was used, which means that any observed event is either caused by substrate-induced activation or by granule lysis. Two measurements on the same sample were performed. First one with low excitation intensity during one minute, after which intensity is increased by a factor of four. A second spot was analysed with constant low light intensity (Figure 3.7a). With intenser excitation, an increased number of events per time unit is clearly visible, indicating light induced events. Another sample (same preparation) was analysed, now $t = 0$ is defined as the onset of excitation light (first exposure) (Figure 3.7b). The first event was recorded at 16 seconds and a reduced event rate is observed in the first minute. Again, this indicates light induced events: if merely substrate induced events were recorded, a concave graph is expected (i.e. the slope of the graph would not increase in time).

In order to evaluate if the photo-induced observed events are truly lysed granules – and not photo-induced exocytosis (i.e. photo activation of platelets) – proteins were disabled by fixation with formaldehyde (cf. Section 3.1.2). As a consequence, platelets are unable to deform and release granules, both processes mediated by proteins (cf. Section 1.1.3).

After platelet incubation on the substrate and washing, a closed fluid cell was filled with 0.2% formaldehyde instead of Hepes buffer. Samples were incubated at room temperature for 1 hour prior to fluorescent imaging. Recordings clearly show events (decrease or loss of fluorescence) (Section 3.3.3). This indicates that the observed events are the result of physical rather than biological processes. Ergo, excitation light induces granule lysis. The next section deals with characteristics of the observed events.

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\(^5\)It is assumed granular fusion is an all-or-none event.
3.3. Fluorescent Imaging of Single Platelet Activation

Figure 3.7: Granule lysis induced by excitation light. The vertical axis denotes the cumulative count of events. Increasing excitation light intensity clearly results in an increased number of events per time unit. Both graphs are normalised, such that for $0 < t < 60$ seconds they overlap. The onset of light is at $t = 0$. A reduced rate of events during the first minute after the onset of excitation light ($t = 0$, first exposure) indicates light induced events.

3.3.3 Discriminating Granule Lysis from Exocytosis

From bulk measurements it is known that platelets can still be activated with TRAP after incubation on a BSA substrate. Although excitation light induces false positive exocytotic events, platelets should show degranulation upon activation with TRAP. Non-activated platelets were compared with activated platelets to investigate differences in events. An attempt is made to discriminate granule lysis from exocytosis.

Washed platelets (WP) were incubated on a BSA coated glass substrate as before. No cover glass is used to close the cell, the spacer remains in place. The open well was then filled with 20 µl of Hepes buffer and positioned on the microscope stage. 2.5 µl of 10 mM TRAP (yielding a final concentration of $\sim 1$ mM) was carefully pipetted into the open fluid cell. Prior to addition and immediately following addition, data was recorded at different spots for two minutes. The average number of exocytotic-like events per platelet is equal prior to and following addition of TRAP for two samples (Figure 3.8). Thus, addition of TRAP does not result in extra events. From these results, it is unclear whether real exocytotic events are present or absent in the pool of all events. Hence, the features of the events were analysed, such as duration and intensity. Measurements on formaldehyde-fixed platelets were compared to post-TRAP events. Fixed platelets were chosen to be absolutely positive that all events are identified as vesicle lysis.
3.3. Fluorescent Imaging of Single Platelet Activation

![Graph showing fluorescent imaging of single platelet activation](image)

Figure 3.8: Average number of (release) events per platelet prior to and immediately following addition of TRAP are equal for two samples. Events were recorded during approximately one minute. The difference between samples, both incubated at the same time and measured only 15 minutes apart, might be the result of time.

![Histograms of event durations](image)

Figure 3.9: Histogram of event durations for events posterior to addition of TRAP (a) and when platelets were fixed with formaldehyde (b). Counts are normalised to the second and highest bin between 2 and 4 seconds. Each data set is composed of two samples from the same donor. Different donor material is used in TRAP and formaldehyde experiments.

Calculations and simulations demonstrate that exocytosis of a granule through the OCS takes well below 1 second, more specifically of the order of 10 ms (Appendix B). In these calculations, it is assumed that quinacrine and ATP diffuse freely through the OCS and that both substances are non-bound in the granule, i.e. they can freely diffuse out of the granule. Events in measurements on formaldehyde-fixed platelets manifest a typical time scale of a few seconds (Figure 3.9b). This confirms that these events are not exocytotic. Measured time scales match time scales as measured for photo-induced vesicle lysis in astrocyte cells [36].

Event durations of fixed platelets were compared to event durations from experiments with addition of TRAP (Figure 3.9a). A small increase in short duration events is visible for measurements with TRAP. This difference can be caused by real exocytotic events or by natural sample variations. Therefore, those relatively fast events are analysed further by investigation of cytosolic fluorescence.
3.3. Fluorescent Imaging of Single Platelet Activation

When granules lyse, their contents diffuse through the entire cell cytosol. Seemingly, quinacrine diffuses slowly through the outer cell membrane, and an increased cytosolic fluorescence might be visible for several seconds (Figure 3.10a). In fact, this cytosolic fluorescence sometimes causes an apparent event duration of several seconds, while the actual event (lysis) might take shorter. The cytosolic light is clearly not from light scattered (scattering is a linear effect) from the vesicle. Data (not shown) indicate that granule intensity sometimes does not increase, while cytosolic intensity does in those cases. It is highly unlikely that cytosolic fluorescence can be attributed to quinacrine fluorescence inside the OCS, since calculations show that once inside the OCS, quinacrine diffuses out rapidly (of the order of a few milliseconds).

(a) Bright field image and fluorescence movie showing cytosolic fluorescence

(b) Cytosolic vs. granule intensity

Figure 3.10: (a) Left image: bright field image of platelet, outlined with a red dotted line. Other five images: fluorescence movie of release event. Cytosolic fluorescence can be observed, indicating the contents of lysed granules diffuses slowly out of the platelet. Images are shown for every 0.5 second, increasing in time from left to right. Image dimension are $10 \mu m \times 10 \mu m$. (b) Cytosolic intensity (black line, averaged per pixel, area in between the two squares (15 x 15 pixels and 5 x 5 pixels)) and granule intensity (red line, averaged per pixel, small square (5 x 5 pixels)) are plotted versus time. In the graph that is shown, the ratio of peak cytosolic intensity and peak granule intensity equals 0.72.

The cytosolic fluorescence can be used as a parameter to identify vesicle lysis. In order to make a quantitative analysis, the ratio of peak cytosolic intensity and peak granule intensity is examined (Figure 3.10b). The granule intensity is as defined before: the average (per pixel) intensity of a 5 x 5 pixels area around the granule centre. The cytosolic intensity is defined as the average intensity of the area between a 15 x 15 pixels square centred around the granule and the 5 x 5 pixels square (Figure 3.10a). The distribution of ratios for formaldehyde-fixed platelets is plotted to characterise vesicle lysis (Figure 3.11). All events, including those that do not show a clearly visible cytosolic fluorescence, are included. Ratios
3.3. Fluorescent Imaging of Single Platelet Activation

are typically 0.7 and higher. For exocytotic events, ratios are expected below 0.6 (this number is limited by the background intensity).

The three fastest events, around 0.6 seconds or 3 frames, from the post-TRAP measurement (Figure 3.9a) are candidates to be identified as exocytosis. For these events, the ratios peak cytosolic intensity to granule intensity lie between 0.67 and 0.78. Comparing with the distribution for formaldehyde-fixed platelets, the selected events cannot be identified as exocytosis.

It can be concluded that for experiments performed in this work, no physiological events can be recognised with analysis based on event duration and fluorescent intensity.

Figure 3.11: Distribution of the ratio peak cytosolic intensity and granule intensity (cf. Figure 3.10b) for formaldehyde-fixed platelets. This distribution characterises vesicle lysis. Background intensity typically limits the ratio to around 0.6.
Platelets play a crucial role in haemostasis. Upon activation, platelets release small messenger molecules from their granules to facilitate platelet aggregation. Single cell techniques are required to measure fundamental properties of this release.

Prior to the study presented in this work, the luciferase assay was reviewed for measuring single platelet activation. This assay can be used to visualise exogenous ATP secreted from dense granules in bulk (cf. Section 2.1). Previous studies show that luciferase immobilised on beads [40] or on the platelet surface [41] can be used to measure local ATP concentrations at the platelet surface. However, these immobilisation methods were only used in bulk. Estimations based on diffusion theory show that detection of ATP released by a single platelet is highly challenging (Appendix A). From experiments (not shown in this work), it can be concluded that using luciferase for a single platelet assay is not feasible with the current hardware.

In this study, fluorescent labelling of dense granules with quinacrine, which binds ATP, was explored to obtain spatial and temporal characteristics of the dense granule release.

Bulk measurements showed that quinacrine clearly has an effect on platelets. Activation by TRAP, studied by means of ATP release from dense granules, is approximately 15% less for platelets incubated with quinacrine. However, since platelets can still be activated and the halfway response of the dose response curve does not change for quinacrine, it is assumed that the fluorescent label does not influence release characteristics considerably. The effect of quinacrine on other platelet activation processes, such as membrane receptor sensitivity for other molecules than TRAP, alpha granule release or shape change, remains unknown.

Platelet immobilisation needed for fluorescence microscopy is a challenging objective. Any surface other than endothelial cells (cells that line the inner blood vessel wall) will activate platelets. Endothelial cells release prostacyclin, an inhibitor for platelet secretion and aggregation, making them not suitable as substrate. BSA coated glass was found suitable for immobilisation. Although the surface induces partial activation – from fluorescence images with quinacrine it is believed that part of the dense granules in a single platelet is released, however further studies need to be conducted – adding TRAP will activate platelets more. Exact time scales on which the agonist activates platelets are unknown. The lu-
The ciferase assay is rather slow to investigate this, so a better procedure needs to be explored. This can be done by e.g. electrochemical measurements [15]. However, TRAP is known to be a strong agonist [2], and other agonists like thrombin have an activation delay of the order of seconds [15]. Therefore it is assumed that TRAP is appropriate for real-time single cell microscopy, which requires a fast-acting agonist.

Quinacrine is not very suitable for studying real-time granule release. Events were undoubtedly related to photo-induced processes. Experiments with formaldehyde-fixed platelets confirm that the events do not result from light-induced activation, but are merely a physical process. Like with other dyes as acridine orange [38], it is thought that under excitation light it produces reactive oxygen which causes peroxidation of (granular) membrane lipids eventually leading to lysis. A lower concentration of quinacrine would reduce the amount of lysis events and would also increase the time delay before initiation of events, as found for acridine orange [38]. In order to decrease the quinacrine concentration or light intensity and still have sufficient signal, the fluorescent background should be reduced. This can be achieved for example by reducing the height of the fluid cell or using a flow cell with continuous supply of non-fluorescent buffer. Another option is to use total internal reflection fluorescence microscopy (TIRFM), that has an intrinsic high signal to background ratio. Low dye concentration (which is also present in the cell cytosol, but at lower concentration than in granules) or reduction of excitation light intensity will still lead to production of reactive oxygen. Though possibly not resulting in granule lysis, the radicals might still alter cell behaviour. Scavengers for (reactive) oxygen might be used to prevent granule lysis. However, such agents may alter cell physiology. The same bulk techniques that were used to study quinacrine influence can be used to address the effect of these oxygen scavengers.

An attempt was made to resolve granule lysis from exocytosis. When compared to non-treated resting platelets, addition of TRAP did not change the average number of events per platelet. Therefore, the duration of events was analysed. Calculations show that granules fusing with the membrane release their contents in a period of the order of 10 ms, which matches release times of serotonin (also a dense granule substance) [4]. Events, both for formaldehyde-fixed platelets and for platelets treated with TRAP, last for several seconds, suggesting non-exocytotic events. The long duration events can be explained in two ways. First, membrane degradation may results in a perforated membrane, leading to slow diffusion of quinacrine. Second, some platelets show long duration cytosolic fluorescence, which may mask the true (lysis) event. Some short-duration events (around 0.6 seconds) were recorded for TRAP-treated platelets, while not present in formaldehyde-fixed platelets. This difference is present due to sample variations: the events still showed a cytosolic fluorescence, marking them as lysis. No exocytotic events can be recognised for TRAP-treated platelets with analysis based on event duration and fluorescent intensity. This can be caused by the effect of reactive oxygen, which might alter the TRAP-induced response. Bulk measurements in combination with quinacrine and excitation light can be used to evaluate this effect.
In conclusion, quinacrine is a marker that induces unwanted effects such as granule lysis. Current measurements make it impossible to discriminate vesicle lysis from exocytosis. Future work can focus on the reduction of unwanted effects by reducing quinacrine concentration and excitation power after increasing the signal to noise ratio or on the use of oxygen scavengers. Another option is to abandon quinacrine and use other endogenous dyes, such as Ca dyes, to visualise platelet activation. Also exogenous markers can be used, though losing direct and spatial information.
Chapter 5

Acknowledgements

The author would like to thank Loes van Zijp and Arthur de Jong for supervising this research project. They and also Holger Kress are acknowledged for helpful discussions. I would like to thank all fellow students in the student room for their company and discussions, and again Loes for providing some 2D fishes to make the view in the room more bearable. Finally, my thanks go to all other members of the MBx group for the help in the labs, (lunch) talks, board game nights, and many other things I do not mention here.
Appendix A

Using Luciferase for a Single Platelet Assay

Here, the feasibility of using luciferase for visualising ATP released from a single granule will be discussed. For that purpose, diffusion theory is used to estimate the number of ATP molecules available for the luciferase reaction (cf. Section 2.1). This number is compared to the number of photons that are eventually collected and detected by the camera.

For simplicity, the shape of a platelet is modelled as a sphere with radius $R$. In first approximation, the ATP is released homogeneously distributed over the entire cell surface. The solution of the steady state diffusion equation, $\nabla^2 c = 0$, where $c$ denotes the (molar) concentration, is for spherical symmetry

$$c(r) = c_R \cdot \frac{R}{r}, \quad (A.1)$$

with $c_R$ the concentration at the surface. From Fick’s first law of diffusion, $j = -D \cdot \nabla c$, the ATP diffusion flux at the surface $j_R$ can now be acquired:

$$c_R = j_R \cdot \frac{R}{D}, \quad (A.2)$$

Here, $D$ is the diffusion coefficient of the ATP molecules. The flux can be estimated by

$$j_R = \frac{n_{ATP}}{\Delta t \cdot A}, \quad (A.3)$$

where $n_{ATP}$ denotes the number of moles ATP in a single granule, $\Delta t$ the release duration and $A$ the platelet surface area.

As a next step, the number of ATP molecules $N$ present in the focal volume of a single pixel, $V_{pix}$, can be estimated using Avagadro’s number $N_A$:

$$N = \frac{1}{\alpha} \int c \cdot N_A \, dV. \quad (A.4)$$

1ATP from dense granules is released through the OCS which has surface invaginations spread over the outer membrane. A correction for this is applied later.

2Again for the sake of simplicity, steady state is assumed and consumption of ATP molecules by the luciferase reaction is neglected. Therefore, the obtained result should rather be interpreted as an upper limit than an expected value.
The factor $\frac{1}{\alpha} (\alpha < 1)$ is present to correct for the fact that ATP is only released from surface invaginations of the OCS. As a consequence, the effective concentration becomes higher. It is assumed that the focal area of a single pixel $A_{pix} (0.25 \times 0.25 \mu m^2$, parallel to the membrane) is much larger than the opening of the OCS. Furthermore, it is assumed that $V_{pix}$ only covers zero or one openings. Then, $\alpha$ is defined as the ratio of number of pixels that cover a surface invagination to the total number of pixels that cover the platelet. That is, $\alpha = \frac{N_{opening}}{(A/A_{pix})}$, with $N_{opening}$ the total number of surface invaginations.

Combining equations (A.1) through (A.4) and filling in the corresponding numbers (Table A.1), the expected number of ATP molecules in a focal volume is $N = 1 \cdot 10^5$. The value is comparable to the number of ATP molecules available per opening: $n_{ATP} \cdot \frac{N_{A}}{N_{opening}} \approx 5 \cdot 10^5$

<table>
<thead>
<tr>
<th>Table A.1: Typical values for quantities involved in granule release.</th>
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<tbody>
<tr>
<td>Quantity</td>
</tr>
<tr>
<td>$R$</td>
</tr>
<tr>
<td>$D$</td>
</tr>
<tr>
<td>$n_{ATP}$</td>
</tr>
<tr>
<td>$\Delta t$</td>
</tr>
<tr>
<td>$N_{opening}$</td>
</tr>
<tr>
<td>$V_{pix}$</td>
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</table>

To arrive at the number of collected photons on the camera, one needs to take a few factors into consideration. First, the concentration of luciferase is limiting the number of produced photons, since the molar ratio of luciferase [3] to ATP is 2 to 1. This effect is estimated to reduce efficiency by a factor of ten. Second, collection efficiency, that is the relative number of photons that enter the objective, reduces the photon count be a factor of ten [4]. Third, the camera quantum efficiency around the 500 nm wavelength is about 50%, lowering the number of counts even more. In the end, the number of detected photons is around 500. The pixel noise of the camera is of the same order of magnitude.

In conclusion, it is highly challenging to observe light, produced by the luciferase reaction, originating from a single granule.

---

3Luciferase and luciferin where used at (near) maximum soluble concentrations of 0.3 mM and 10 mM respectively.

4Calculated from the numerical aperture and refractive index of the objective.
Appendix B

Granule-OCS Exocytosis: Calculations and Simulations

Platelet granules (both alpha and dense) fuse with the OCS to release their contents through exocytosis [2]. In the following calculations and simulations, typical release times are estimated for different geometries. Therefor, solutions of the diffusion equation \( \frac{\partial c}{\partial t} - D \nabla^2 c(\vec{r}, t) \) – where \( c \) denotes the concentration, \( D \) the diffusion coefficient, \( \vec{r} \) position and \( t \) time – are studied. Any convective effects are neglected.

B.1 Diffusion of Gaussian Profile in Free Space

For spherical symmetry, the solution of the diffusion equation with an initial gaussian profile is

\[
    c(r, t) = c_0 \left(\frac{t'}{t + t'}\right)^\frac{3}{2} \exp\left(-\frac{r^2}{4D(t + t')}\right).
\]

(B.1)

Here, \( r \) is the radial position, and the following relation holds between the full width at half maximum \( w \) and \( t' \):

\[
    w = 4\sqrt{\ln(2)} \sqrt{D(t + t')}. \tag{B.2}
\]

In other words, \( t' \) can be defined by the initial (at \( t = 0 \)) full width at half maximum \( w_0 \): \( t' \approx \frac{w_0^2}{11D} \).

Filling in numbers gives an idea about the time it takes for a granule-sized profile to diffuse in free space. For \( w_0 \sim 0.1 \mu m \) (granule diameter) and \( D \sim 100 \mu m^2/s \) (Appendix A), the centre concentration, i.e. \( c(0, t) \), drops by a factor of 1000 in 1 millisecond.

B.2 Granular Content Diffusing Through Channel

Imagine a granule, with constant volume \( V \) and spatially constant concentration of a molecule \( c_0 \), connected to a channel of length \( L \) and cross section \( A(x) \) as a function of the position inside the channel \( x \) (Figure B.1). For small and smooth
changes in \( A(x) \), diffusion is only in the \( x \)-direction. As a result, the molecular concentration inside the channel \( c(x, t) \) is independent of the radial position.

Next, the discharge \( Q \) is independent of the position:

\[
- \frac{d(c_0 V)}{dt} = Q = A(x) j(x) = A(x) D \frac{\partial c}{\partial x}.
\] (B.3)

Note however that the equation only holds when the concentration profile has fully developed. This assumption will be validated later on. The concentration in the channel can be described by:

\[
c_0(t) - \int_0^L \frac{\partial c}{\partial x} dx = \alpha c_0(t),
\] (B.4)

where \( \alpha c_0 \) is the concentration outside the channel and can be approximated by 0 (cf. Section B.1). Combining equation (B.3) and (B.4) yields:

\[
\frac{dc_0}{dt} = -\frac{c_0}{\tau}, \quad \tau = \frac{V}{(1-\alpha)D} \int_0^L \frac{dx}{A(x)}.
\] (B.5)

Table B.1 shows two approximated timescales \( \tau \): one for a granule docked to the membrane (diffusion through a small pore) (Figure 1.2) and one for ATP passing a long channel mimicking the OCS. Note that the transport of ATP is completely driven by diffusion, no shape or volume change is taken into account. A vesicle fusing with the cell membrane (or OCS) however decreases in size (cf. Section 1.1.3), thus reducing release times.

Table B.1: Estimated order of magnitude of release times for diffusion through a small pore in a membrane-docked granule and diffusion through a long channel, mimicking the OCS.

<table>
<thead>
<tr>
<th>( V ) (( \mu )m(^3))</th>
<th>( D ) (( \mu )m(^2)/s)</th>
<th>( L ) (( \mu )m)</th>
<th>( A ) (( \mu )m(^2)) (avg.)</th>
<th>( \tau ); ( \tau' ) (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1(^3)</td>
<td>100</td>
<td>0.010 (membrane)</td>
<td>1 \cdot 10^{-5}</td>
<td>10; 2.5e-4</td>
</tr>
<tr>
<td>1 (platelet dim.)</td>
<td>1 \cdot 10^{-3} (from [2])</td>
<td>10; 2.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Typical approximated release time scales appear to be much larger than time scales of free diffusion in open space.
Finally, the assumption of a fully developed concentration profile needs to be addressed. Therefore, the diffusion equation is solved with boundaries conditions $c(0, t) = c_0$ (kept constant in time) for all $t$ and $c(x, 0) = 0$ for $x > 0$, i.e. the initial profile is a step function. The solution of the diffusion equations is:

$$c(x, t) = c_0 \text{erfc} \left(-\frac{x}{2\sqrt{Dt}}\right).$$ (B.6)

The quantity $2\sqrt{Dt}$ is the length over which to profile has developed. The time it takes for the front to reach the channel end, that is the settling time $\tau'$ ($2\sqrt{Dt'} = L$) is also displayed in Table B.1. It can be concluded that the settling time does not play a major role.

In the above calculations, the volume ratio of the channel versus the granule is not taken into consideration. That is, the effect of a channel that is approximately equal or larger in volume than the granule is neglected. This could have implications for the settling time. Though, simulations in the following section show that this effect only plays a minor role.

### B.3 Granular Content Diffusing Through OCS

The OCS is a complex tortuous system that can be modelled as a network with long channels and dead-end branches (Figure B.2). In order to investigate the diffusion properties of such a system, the diffusion equation will be solved numerically. Furthermore, the estimations on which equation (B.5) is based will be verified.

The code, plain $c$, to solve the 2D (planar) or 3D (cylindrical symmetry) partial differential equation uses the straightforward finite forward difference method. As a grid size and time resolution, respectively 5 nm and 0.01 ms is taken. Above the opening of the channel, i.e. the cell exterior, an open (initially zero concentration) space is simulated that is the same height $L$ as the channel.

For a single channel, the simulated release time (solving the diffusion equation in 3D) matches the time obtained from estimations (Section B.2) (Figure B.3a).
B.3. Granular Content Diffusing Through OCS

The initial stronger drop in the simulation is due to the (fast) development of the concentration profile. Simulations comparing (in 2D) a single channel with an OCS-like geometry (Figure B.2) – \( L = L' = 1 \mu m, A = 0.03^2 \mu m^2, V = 0.13 \mu m^3 \) \( n = 10 \) – show that there is no significant difference in release time (Figure B.3b). The geometry with 10 dead-end branches, which introduces an increased effective channel volume, leads to a larger initial slope.

\[ 2 \text{D is used because of simplicity and speed.} \]

\[ 3 \text{Since the simulation is in 2D, actual parameters are: granule dimension 0.175 x 0.175 \mu m^2, channel width 0.030 \mu m. In that way, the ratio granule size to channel size is equal for 2D and 3D parameters.} \]
Glossary

**agonist**  Substance that initiates a physiological response when combined with a receptor. [5] [6] [15]

**alpha granule**  Secretory organelle that contains growth factors and other proteins involved in coagulation. [8] [29]

**coagulation**  Process of blood clotting. [4]

**dense granule**  (aka δ-granule or dense body) Secretory organelle that contains small molecules (ADP, ATP, serotonin, ionized calcium. [3] [5] [7] [8] [10] [22] [23] [29] [35]

**exocytosis**  Process that transports contents of vesicles out of the cell membrane by membrane fusion. [4] [10] [37]

**Fc receptor**  Protein on the cell surface that binds the Fc region – a constant region, in contrast to two variable Fab regions that bind specific antigens – of antibodies. [20]

**glycoprotein**  Protein that is covalently linked as a side-chain to a polysaccharide chain. [7]

**haemostasis**  Process that stops bleedings caused by injured blood vessels. [3] [29]

**lysis**  Disintegration of a cell or vesicle by rupture of the membrane. [12] [23] [24]

**OCS**  Open Canalicular System, tortuous network of membrane invaginations tunnelling through the cytoplasm. [4] [25] [26] [35] [40]

**photobleaching**  Phenomenon that a fluorophore loses the ability to fluoresce due to photon-induced chemical damage. [14]

**quenching**  Process such as energy transfer or complex formation that decreases fluorescence intensity. [11] [22]

**TRAP**  Thrombin Receptor Activation Peptide, synthetic peptide that evokes a very strong activation response, without the complications of clot formation. [9] [13] [17] [19] [21] [22] [24] [29]
Bibliography


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