Measurement of platelet responsiveness using antibody coated magnetic beads for lab-on-a-chip applications

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Abstract

We investigate novel methods for the quantification of platelet responsiveness that are suited for implementation in lab-on-a-chip devices. Magnetic beads are convenient carriers for rapid capture and manipulation of biological cells in a miniaturized system. In this paper we demonstrate that antibody-coated magnetic beads can be used to quantify platelet responsiveness. We use anti-CD62P coated beads to capture activated platelets from samples stimulated with a PAR-1 specific agonist SFLLRN, also known as Thrombin Receptor Activator Peptide (TRAP). The responsiveness of the platelets is analyzed via the remaining unbound platelets in solution and compared to a reference method in which the number of activated platelets is analyzed via fluorescent labeling. The effective concentrations for platelet activation are in agreement for the two assay types, proving that platelet responsiveness can be quantified using antibody-coated magnetic beads. We discuss the outlook for application in lab-on-a-chip devices.
Introduction

Platelets are highly reactive cells that are responsible for the maintenance of adequate blood circulation. In the normal circulation platelets show minimal interactions with other blood cells and with endothelial cells. When the vascular system is disturbed, for example after vessel wall damage, platelets undergo a series of changes. The platelets adhere to collagen in the extracellular matrix and get activated as a consequence. Upon activation a wide variety of proteins is secreted from the platelets’ alpha granules into the blood stream, specific membrane proteins are expressed on the cell surface and the shape of the platelet changes [1, 2, 3].

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

Platelet responsiveness can be measured via the platelet reaction to a stimulus. Current methods to evaluate platelet responsiveness include aggregometry, bleeding time and flow cytometry [1, 2, 4]. However, these techniques are complex and time consuming. Therefore we are interested in novel methods for the quantification of platelet responsiveness that are suited for implementation in a lab-on-a-chip format, as a basis for future easy to use advanced diagnostic tests. We focus on methods based on antibody recognition in order to be flexible in the biomarkers that are to be analyzed. Furthermore, we focus on the use of magnetic beads
because these are highly suited for the integration of assays into a lab-on-a-chip format [8, 9, 10].

Non-magnetic ligand-coated beads have been used for platelet function testing, with detection by flow cytometry[11, 12], by bioluminescence and electron microscopy [13], and by agglutination [1, 14]. Magnetic beads have been used to study platelet glycoprotein deficiency [15] and to extract platelet specific proteins from whole blood [16]. Our approach is to use magnetic beads which are functionalized with platelet-specific antibodies. Fig. 1A sketches a concept for a magnetically-controlled platelet assay in a lab-on-a-chip system. The assay has three magnetically-controlled phases: (i) the platelet sample is exposed to a well-defined biochemical or biophysical stimulus and is incubated with antibody-coated magnetic beads in a microchamber, (ii) the beads with specifically captured cells are concentrated at an antibody-coated sensing surface, and (iii) unbound and weakly bound beads are removed from the sensing surface by magnetic forces, followed by detection of the bound cells and beads. A full platelet responsiveness curve can be established in the lab-on-a-chip device by having microchambers with different degrees of stimulation.

The basis of the envisaged lab-on-a-chip platelet responsiveness assay is the binding of magnetic beads to responsiveness-specific biomarkers on the platelets. In this paper, we demonstrate in a model assay in which antibody-coated magnetic beads are used to quantify the responsiveness of platelets to stimulation, see Fig. 1B. We use anti-CD62P coated beads and biochemical stimulation by the PAR-1 agonist, Thrombin Receptor Activator Peptide (TRAP). Upon activation, CD62P is increasingly expressed at the platelets’ membrane [17, 18,19]. We study the binding of platelets to the functionalized beads by measuring the
remaining free platelets in solution and we compare our results to a reference method [20,21] in which platelet activation is measured via fluorescent labeling and flow cytometry analysis.

_Figure 1_

**Materials and Methods**

**Preparation of functionalized beads**

The procedure for the preparation of the functionalized beads was based on the two-step-coating procedure using N-hydroxysuccinimide (NHS) suggested by the manufacturer. Carboxylic acid beads (2.8 µm) from Dynal Biotech, were washed twice with 25 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer pH 5.0 prior to use. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and NHS were dissolved in the MES buffer just before use and added to the bead solution with a final concentration of 12.5 mg/ml for both chemicals. The solution was mixed for 30 minutes at room temperature. The tube was then placed on a magnet and the beads solution was washed three times with MES buffer. Bovine Serum Albumin (BSA, Sigma Scientific) or CD62P antibody (R&D Systems) was added to the activated bead solution with a final concentration of 500 µg/ml, the solution was mixed for 1 hour at room temperature. After incubation the supernatant was removed and the reaction was quenched with 50 mM ethanolamine in phosphate buffered saline (PBS) pH 8.0 for 1 hour at room temperature. Prior to use, the beads were washed five times with wash buffer (1% BSA and 0.1% Tween-20 in PBS).
Preparation of washed platelets (WP)

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

Dose-response curves

Washed platelets were stimulated by TRAP-6 (Bachem) with final concentrations of 0, 80 nM, 800 nM, 4 µM, 8 µM, 40 µM, 80 µM and 800 µM. 5 µl of washed platelets was incubated together with the agonist and 2 µl of each fluorescent detection label. Two
fluorescently labeled antibodies were used: The platelet specific marker Allophycocyanin (APC) conjugated mouse anti-Human CD42b (BD Pharmingen) was used for the detection of the platelets, Phycoerythrin (PE) conjugated mouse anti-human CD62P (BD Pharmingen) was used as activation marker. 5 µl of anti-CD62P-bead solution was added and the reaction volume was increased to 50 µl. The reagents were gently mixed for 20 minutes at room temperature. The ratio between the beads and the platelets during incubation was 1:1, the concentration of both particles during incubation was 1 $10^5$/µl. Control samples were incubated with BSA coated beads instead of the anti-CD62P coated beads, to evaluate the nonspecific adsorption of platelets onto the beads. Samples with only beads or platelets were used as additional controls. For the reference dose-response curve, in which the percentage of activated platelets was evaluated, the beads are omitted in this incubation step. To prevent further activation, all samples were fixed for 10 minutes at room temperature with 500 µl aqueous solution containing 0.2% formaldehyde in 0.9% NaCl.

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

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

**Signal selection: Discrimination between beads and platelets**

Reference samples containing BSA coated beads, washed platelets or both were used for a first signal selection on the basis of the forward and sideward scatter. BSA coated beads were used to avoid binding of the platelets to the beads. Due to the difference in scattering properties of the beads and the platelets, they appear as distinct populations in the forward versus sideward scatter plot of the flow cytometry analysis. Figure 2 shows the forward sideward scatter plot of the sample containing the BSA beads as well as the washed platelets. Two distinct groups can be observed, in which the beads appear in the higher sideward scatter region (M2) and the unbound platelets in the lower sideward scatter region (M1). The gates around these areas were set according to the control samples, containing only BSA beads or washed platelets.

*Figure 2*

**Signal selection: Couple formation bead with platelet**

A mixture of CD62P coated beads and washed platelets was incubated with the two fluorescently labeled antibodies, for additional signal selection. The platelet specific marker APC mouse anti-Human CD42b was used for the detection of the platelets, PE mouse anti-human CD62P was used as specific platelet activation marker. Unbound platelets are specifically measured as CD42b positive particles in the M1 gate. The CD42b-APC positive signals within the M2 gate, discriminates between beads with and without a platelet attached to it.
Two control samples were used to set the APC threshold within both gates. The first sample (Figure 3A) contains only beads including both fluorescent labels, anti-CD42b-APC and anti-CD62P-PE. From experiments in which beads were incubated with and without labels we know that the observed signal in figure 3A is caused by the autofluorescence of the beads and to a lower extent by non-specific adsorption of the fluorescent labels to the beads. The second control sample contains only labeled platelets (figure 3B). The APC threshold was set such that the amount of false positives for couples did not exceed 1%.

The fluorescent intensity recorded on the APC channel of a sample containing both anti-CD62P beads and platelets stimulated with TRAP, is shown in figure 3C. Two separate populations can be seen in the histogram. The low intensity signals are single beads, the high APC intensity signals registered in M2 represent platelets captured by an anti-CD62P bead. After the flow cytometry analysis the samples were inspected under the microscope, which confirmed the capture of the platelets by the anti-CD62P coated beads. The same settings of the APC threshold are used in gate M1, to discriminate between unbound platelets and debris.

**Figure 3**

**Signal selection: Activation status of the platelets**

Discrimination between resting and activated platelets was made by a threshold value for the CD62P density. This threshold was determined by the CD62P-PE intensity of a control sample from previous measurements containing 100 ng/ml cPGI inhibited washed platelets, such that the false positives did not reach 5%. Platelets with a CD62P density higher than this threshold were designated as “activated”, while platelets with a density below this threshold as “resting”.

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Figure 4 shows the CD62P density on the unstimulated (figure 4A) and 800 µM TRAP stimulated (figure 4B) control samples in the dose response series of washed platelets. A small fraction of the population of the unstimulated sample is above the threshold. The slight increase in activation of the platelets is probably caused by sample handling such as pipetting and mixing. The positive control shows a major intensity shift of the distribution indicating a high binding of the activation marker anti-CD62P-PE to the platelets. The error introduced by setting the threshold will be used as error margin in the data analysis of the reference dose response curves.

Figure 4

Results & Discussion

Specificity of the binding of activated platelets to anti-CD62P beads

The specificity of the capturing of the activated platelets by the anti-CD62P coated beads was analyzed. Washed platelets were incubated with BSA beads, with anti-CD62P beads or without any beads. The BSA coated beads serve to investigate the non-specific adsorption of platelets to the beads. In addition these samples were incubated with and without agonist. The sample containing unstimulated platelets without any beads was used as reference. The formation of clusters of anti-CD62P beads and platelets was observed for high agonist concentrations, as is sketched in figure 1B. The clusters sediment rapidly and are therefore not measured by the flow cytometer. The timescale of the sedimentation of the clusters is in the order of minutes, whereas the sedimentation of unbound platelets in the samples is in the order of hours. Therefore the number of unbound platelets in solution (M1 in figure 2) is
analyzed rather than the formation of the couples (anti-CD42b-APC positive particles in M2, figure 2). Figure 5 presents the average relative concentration of the unbound platelets in M1; the error bars indicate the spread in duplicates.

**Figure 5**

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

Figure 5 shows that about 54% of the unstimulated platelets are captured by the anti-CD62P beads this is caused by the increased CD62P expression of the platelets due to sample handling as discussed earlier. The specificity of the binding of the activated platelets to the anti-CD62P beads was confirmed by the difference observed in the unbound platelet concentration between the unstimulated and stimulated platelets incubated with anti-CD62P beads. The concentration of the platelets in the sample without agonist was about 46% whereas the relative concentration of platelets reduced to about 0.5% for the stimulated samples. In contrast, the platelet concentration in the sample with BSA beads decreased from 95% to about 53% upon activation.
Since the autofluorescence of the beads interferes with the PE-label, it is not possible to investigate the activation status of the platelets bound to the beads. Therefore we analyze the density of CD62P expressed on platelets that were not in complex (M1) in order to confirm that activated platelets were captured from the samples by the anti-CD62P beads. In the unstimulated sample containing BSA beads the percentage of activated platelets was 2.5% in M1, whereas the percentage activated platelets in the sample containing anti-CD62P beads was only 0.1% in the same gate. This indicates that from the population of unbound platelets, the activated platelets were captured by the anti-CD62P beads.

**Dose-response curves**

In order to compare the anti-CD62P bead assay with respect to the reference method, we measured dose response curves of platelet responsiveness using the same donor material. Samples with washed platelets were stimulated with TRAP concentrations in the range between 0 and 800 µM TRAP and both fluorescently labeled antibodies were added. In the bead assay, the anti-CD62P beads were also added with a 1:1 ratio of platelets to beads. After 20 minutes of incubation, the samples were fixed as described in the previous section. The number of unbound platelets in solution (M1) is analyzed rather than the formation of the couples (M2), since the couples form larger clusters for high agonist concentrations. For the reference method, the CD62P density was evaluated for all CD42b-APC positive cells as a function of the concentration of TRAP. Platelets were considered as activated when the CD62P density was higher than the threshold. Samples with eight different TRAP concentrations are prepared in duplicate and measured. A dose response was obtained from the relation between the percentage of activated platelets as a function of the TRAP concentration. The relative error in the data points is scaled with the square root of the total number of counted particles.
Figure 6 shows dose response curves of the two assays for platelets of the same donor. The percentage of activated platelets and the concentration of unbound platelets are plotted as a function of the agonist concentration. Both assays give an S-shaped dose response curve. The curve for the bead assay records the unbound platelets (APC-positive particles in M1), therefore the curve is inverted with respect to the curve of the reference assay. A sigmoidal function can be used to fit and quantitatively compare the data:

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

where $C$ the concentration of the stimulus, $S$ the slope of the curve and $P_1$ and $P_2$ are the plateau’s for the response at low and high concentrations respectively. $C_0$ is the effective concentration (EC$_{50}$) which is the concentration at which the reaction is half way between $P_1$ and $P_2$. The fit for the assay with anti-CD62P coated beads gives an effective concentration of 17.9 µM with a fitting error of 5.6 µM. The reference method with fluorescent labels gives an EC$_{50}$ value of 23.5±0.4 µM. The errors in the EC$_{50}$ values as determined by the fit, are an underestimate of the experimental error in the EC$_{50}$ values, since this accuracy is mainly determined by the setting of the threshold and the measured number of samples. The experiments were repeated using blood platelets of several healthy donors in buffer (n=4) as well as in 10% plasma (n=3). A representative selection of the curves is given in the Supplementary Material. For all donors assays with anti-CD62P coated beads reveal sigmoidal dose response curves. For every donor the effective concentrations have been determined for the bead assay and the reference assay showing comparable values.
Conclusion

We have demonstrated the measurement of platelet activation by TRAP using anti-CD62P coated magnetic beads. The curve of the amount of unbound platelets in solution as a function of the agonist concentration has a sigmoidal shape, with good correspondence to the percentage of activated platelets recorded by fluorescent labeling. The sigmoidal shape of the dose response curve is observed for assays in buffer as well as in 10% plasma. Our experiments demonstrate for the first time the use of antibody functionalized beads to measure a dose response curve for the expression of a platelet-specific activation marker. These results form a solid basis for further developments toward miniaturized lab-on-a-chip systems for platelet multifunctional biosensing. As presented in figure 1A, we envisage to stimulate platelets in reaction microchambers, label the platelets with magnetic beads, and use electromagnetic forces to move the activated platelets to a sensing surface. The amount of binding to the surface is a measure for the amount of activated platelets in the sample volume. In such an integrated magneto-microfluidic system, different reaction chambers can be operated in parallel, in order to screen for different concentrations of agonist and different types of stimulation, allowing for accurate measurements of platelets and their functional properties. An advantage of a miniaturized system is that only a very small amount of sample will be needed, much less than the few milliliters that is needed to evaluate platelet responsiveness in flow cytometry. In addition, the use of magnetic beads reduces the need for sample handling, since mixing and labeling can be integrated in one point of care system. Thus far, we have tested the bead assays in buffer and in plasma. In practical use, whole blood will be the input sample. The feasibility of a magnetic bead-based platelet responsiveness assay in whole blood still needs to be investigated. In case red and/or white blood cells appear to interfere with the assay, then one may consider to design the lab-on-a-
chip device with two consecutive modules: first a module wherein red and white blood cells are separated, followed by a module wherein the resulting platelet-rich plasma is analyzed by the integrated magnetic bead assay. In conclusion, the results of this paper represent a first step toward the realization of an integrated lab-on-a-chip system for multifunctional platelet testing using antibody coated magnetic beads.

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Declaration of interests

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References


Figure 1: A: Schematic representation of a lab-on-a-chip microchamber platelet assay based on antibody coated magnetic beads. i: Antibody coated beads are incubated with the sample and an agonist to trigger activation of the platelets. ii: The beads are moved through the sample fluid by applied magnetic fields, catching the activated platelets which express specific activation markers on their cell membrane, and transporting the cells to the antibody-coated sensor surface. iii. A magnetic force is applied in order to remove the unbound beads from the sensor surface. The amount of beads bound to the surface is a measure for the amount of activated platelets. By the use of multiple reaction microchambers with different concentrations of agonist, a responsiveness dose response can be established.

B. Schematic representation of the activation and binding process that is investigated in this paper. Platelets are activated by exposure to TRAP. Binding between anti-CD62P coated beads and platelets generates aggregation and sedimentation to the bottom of the incubation tube. An activation dose-response curve is determined by quantifying the platelets that remain in solution.
Figure 2: Sideward versus forward scatter plot of BSA coated beads incubated with washed platelets. BSA-beads were used to prevent platelet adhesion to the beads. The ratio of the beads to the platelets is 1:1. The data was collected after 20 minutes of incubation at room temperature. The unbound platelets fall in the M1 gate and the BSA beads in M2.

Figure 3: The control samples containing only anti-CD62P coated beads (A) or only platelets (B) are compared with the sample with anti-CD62P coated beads, incubated with 800nM TRAP-stimulated platelets (C). In contrast with the control sample containing only beads an additional population appears, indicating binding of CD42b-APC labeled platelets to the functionalized beads. All signals are measure within gate M2.
Figure 4: Control samples that were used to set the CD62P-PE-threshold. Signals having an intensity above this threshold were designated as activated platelets. A: washed platelets without agonist. B: washed platelets activated with 800 µM TRAP. Anti-CD42b-APC was used as a detection label for platelets, anti-CD62P-PE was used as a label for the activation status of the platelets.

Figure 5: The relative concentration changes in unbound platelets (figure 2, M1) in solution after 20 minutes of incubation at room temperature with or without functionalized beads. No stimulus was added for the control platelets (unstimulated WP). Platelet stimulation was performed with 800 µM TRAP (stimulated WP). Error bars represent the spread in relative platelet numbers of the duplicates.
Figure 6: The platelet responsiveness curves for both methods. Left axis: the responsiveness of the washed platelets obtained from the reference flow cytometry analysis with the use of fluorescent labels (•). The percentage of activated platelets is the amount of platelets positive for APC as well as PE in the M1 gate. The data points are the average values obtained from the duplicates. The measurement error is 5%, which is the maximum amount of false positives in the control sample. Right axis: the responsiveness of the washed platelets determined using the new method, based on incubation with anti-CD62P coated beads (□). The concentration of the unbound platelets after stimulation is recorded. The data points are average values obtained from the duplicates. The measurement error is scaled with the number of counted particles (\(\sqrt{n}/n\)). The dotted lines represent fits according to equation 1, with the error representing the accuracy of the fit.
Supplementary material

Supp.figure: Measurement of the platelet responsiveness for various healthy donors. Left axis: the responsiveness obtained using reference method i.e. flow cytometry analysis with the use of fluorescent labels (•). The percentage of activated platelets is the amount of platelets positive for APC as well as PE in the M1 gate. The data points are the average values obtained from the duplicates. The measurement error is 5%, which is the maximum amount of false positives in the control sample. Right axis: the responsiveness of the washed platelets determined using the new method, based on incubation with anti-CD62P coated beads (□). The concentration of unbound platelets after stimulation is recorded. The dotted lines represent fits according to equation 1, the depicted EC50 values are obtained from the sigmoidal fit, with the error representing the accuracy of the fit. The data points are average
values obtained from the duplicates. The measurement error is scaled with the number of counted particles (√n/n). Data presented in panels A and B are performed in buffer, data presented in panel C and D are recorded in 10% plasma.