Published in final edited form as:

Cell Mol Bioeng. 2014 June 1; 7(2): 285–290. doi:10.1007/s12195-014-0328-9.

Development of a method to quantify platelet adhesion and aggregation under static conditions

Sandra M. Baker-Groberg[†], Flor A. Cianchetti[†], Kevin G. Phillips[†], and Owen J.T. McCarty[†]

[†]Department of Biomedical Engineering, School of Medicine, Oregon Health & Science University, 3303 SW Bond Ave, Portland, OR 97239, USA

Abstract

Platelets are important players in hemostasis and thrombosis. Thus, accurate assessment of platelet function is crucial for identifying platelet function disorders and measuring the efficacy of antiplatelet therapies. We have developed a novel platelet aggregation technique that utilizes the physical parameter of platelet concentration in conjunction with volume and mass measurements to evaluate platelet adhesion and aggregation. Platelet aggregates were formed by incubating purified platelets on fibrinogen- or fibrillar collagen-coated surfaces at platelet concentrations ranging from 20,000 to 500,000 platelets/ L. Platelets formed aggregates under static conditions in a platelet concentration-dependent manner, with significantly greater mean volume and mass at higher platelet concentrations (400,000 platelets/ L). We show that a platelet glycoprotein IIb/IIIa inhibitor abrogated platelet-platelet aggregation, which significantly reduced the volume and mass of the platelets on the collagen surface. This static platelet aggregation technique is amenable to standardization and represents a useful tool to investigate the mechanism of platelet activation and aggregation under static conditions.

Keywords

Platelets; Aggregation; Blood; Microscopy

Platelets are anucleate blood cells that are critical to the process of hemostasis and thrombosis. During hemostasis, the endothelium produces inhibitory factors that keep platelets in a resting state. However, during vascular injury, the extracellular matrix is exposed to blood, resulting in local platelet adhesion and activation to initiate platelet aggregation and thrombus formation.⁸ Platelets bind the exposed extracellular matrix proteins collagen and von Willebrand factor through integrin $\alpha_2\beta_1$ and glycoprotein (GP) Ib, respectively, allowing for rapid activation via GPVI.^{12,14} Upon platelet activation, GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$) changes conformation to its active form on the platelet surface and

Correspondence: Sandra Baker-Groberg, Department of Biomedical Engineering, School of Medicine, Oregon Health & Science University, 3303 SW Bond Ave, Portland, OR 97239 Tel: 503-418-9307; Fax: 503-418-9311; bakersa@ohsu.edu.

CONFLICTS OF INTEREST

S.M.B., F.A.C., K.G.P., and O.J.T.M. declare that they have no conflicts of interest.

ETHICAL STANDARDS

All human subjects research was carried out in accordance with institutional guidelines approved by the Oregon Health & Science University Institutional Review Board. No animal studies were carried out by the authors for this article.

binds the blood plasma protein fibrinogen to help mediate platelet-platelet adhesion. Activated platelets release platelet agonists (e.g., ADP and thromboxane A₂) that activate other platelets in the blood stream, further augmenting the platelet aggregation process.^{6,8} Vessel injury also exposes tissue factor to the blood, which activates the coagulation cascade to generate thrombin. Thrombin converts the platelet-bound fibrinogen into fibrin to create a fibrin meshwork that solidifies around the platelet aggregate to form a thrombus. However, in the conditions of disease, normal platelet hemostatic function is often disrupted, resulting in bleeding and/or thrombotic complications.^{8,13}

We introduce a platelet function technique that utilizes the physical parameter of platelet concentration in conjunction with volume and mass quantification to assess platelet adhesion and aggregation. Purified platelets are incubated on protein coated glass coverslips under static conditions at physiologically low, normal, or high platelet concentrations to form platelet aggregates. Platelet-substrate and platelet-platelet interactions are visualized using a basic laboratory microscope, and platelet aggregate mass and volume are measured using the HTDIC/NIQPM imaging technique. We have previously used the HTDIC/NIQPM imaging technique to quantify the volume and mass of red blood cells, platelet aggregates, and thrombi.^{3,4,9–11} Combining HTDIC/NIQPM imaging with static platelet aggregation provides a quantitative platelet aggregation technique that can be used to study platelet function and evaluate the efficacy of antiplatelet therapies.

Human venous blood was collected from healthy volunteers into sodium citrate and acid/citrate/dextrose as previously described.^{2,7} Written informed consent was obtained from study participants, and the Oregon Health & Science University Institutional Review Board approved the protocol. Platelets were purified from collected blood as previously described.¹ Glass coverslips (32 mm) were placed in 24 well-plates and coated with 50 μ l of fibrinogen (50 μ g/mL) or fibrillar collagen (100 μ g/mL) for 1 hr at 25°C, followed by washing with PBS and blocking with BSA (5 mg/mL, 1 hr at 25°C). Purified platelets were incubated with the fibrinogen-or collagen-coated coverslips for 45 min at 37°C at the physiologically low (20,000 platelets/L), normal (100,000 to 400,000 platelets/L), or high (500,000 platelets/L) platelet concentrations.⁵ The coverslips were washed with modified Hepes/Tyrode buffer (136 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Hepes, 2 mmol/L MgCl₂, 2 mmol/L CaCl₂, 5.6 mmol/L glucose, 0.1% BSA; pH 7.45) and fixed with 4% paraformaldehyde. The samples were mounted onto glass microscope slides with Fluoromount-G (SouthernBiotech, Birmingham, AL). Experiments were repeated using blood from three different donors.

The samples were imaged using a 63 oil-coupled, 1.4 numerical aperture (NA) objective and an upright Zeiss Axiovert 200M microscope (Carl Zeiss MicroImaging GmbH, Germany). Through-focus transverse differential interference contrast (DIC; illumination condenser NA of 0.9) and bright field images (illumination condenser NA of 0.1) of the samples were separated by a 0.1 μ m axial increment. A green filter ($\lambda = 540 \pm 20$ nm; Chroma Technology Corp., Bellows Falls, VT, USA) was used during bright field image acquisition. The microscope was operated under the control of SlideBook 5.5 (Intelligent Imaging Innovations, Denver, CO). Volume and mass measurements were obtained using the custom HTDIC/NIQPM program written in MATLAB® (The MathWorks, Inc., USA), as previously described.^{3,11} DIC and bright field Z-stack images were processed for each

region (32 μm by 32 μm ; 12 regions per field of view; three fields of view per sample) and were taken from the surface of the slide to 5 μm above the platelet aggregates. We have established the noise floor and dynamic range of our system with polystyrene spheres. At and below the diffraction limit of our system, we observe that our noise cutoff is $\sim 0.14 \text{ pg}/\text{m}^2$.

It was observed that under static conditions, platelets aggregated at the higher platelet concentrations of 400,000 and 500,000 platelets/ L, while platelets only formed microaggregates or single platelet monolayers at the lower concentrations of 20,000 and 100,000 platelets/ L (Figure 1 and 2). The mean region volume and mass of platelet aggregates increased with platelet concentration on both the fibrinogen and collagen surfaces (Figure 3). To determine whether our technique could be used to evaluate antiplatelet therapies, platelets were incubated with the GPIIb/IIIa inhibitor, eptifibatide (20 $\mu\text{g}/\text{mL}$; Millennium Pharmaceuticals, USA) for 10 min at 25°C prior to platelet incubation on collagen-coated glass coverslips at platelet concentrations of 400,000 and 500,000 platelets/ L. We found that inhibition of GPIIb/IIIa abrogated platelet aggregation, resulting in only a monolayer of platelets remaining, accompanied by a significant decrease in the mean region volume and mass as compared to vehicle treatment (Figure 4). For both the conditions utilizing 400,000 and 500,000 platelets/ L, it is noteworthy that the mean volume and mass of platelets on collagen in the presence of the GPIIb/IIIa inhibitor were comparable to the mean volume and mass of platelets on collagen for 100,000 platelets/ L, where only a confluent monolayer of platelets was observed.

This study describes the use of a novel static platelet aggregation method to quantify platelet aggregation on the single cell level. To the best of our knowledge, this is the first report of the role of platelet count in the induction of platelet-platelet aggregation under static conditions. This method could be used to investigate the mechanisms of platelet-platelet interactions as a function of platelet count in a closed system, where the ADP and thromboxane A_2 feedback pathways may dominate platelet activation. Utilization of our method could provide a quantitative assay to evaluate the efficacy of platelet antagonists that target the ADP and thromboxane A_2 pathways.

Acknowledgments

This work was supported by the National Institutes of Health (R01HL101972, O.J.T.M.; R01HL101972-S1, F.A.C.) and a Medical Research Foundation Early Clinical Investigator Award (K.G.P.). S.M.B. is a Whitaker International Fellow. O.J.T.M. is an American Heart Association Established Investigator (13EIA12630000).

ABBREVIATIONS

BSA	bovine serum albumin
DIC	differential interference contrast
NA	numerical aperture
PBS	phosphate buffered saline
GP	glycoprotein

HTDIC	Hilbert-transform differential interference contrast
NIQPM	non-interferometric quantitative phase microscopy

REFERENCES

1. Aslan JE, et al. The PAK system links Rho GTPase signaling to thrombin-mediated platelet activation. *Am. J. Physiol. Cell Physiol.* 2013; 305:C519–528. [PubMed: 23784547]
2. Aslan JE, Tormoen GW, Loren CP, Pang J, McCarty OJT. S6K1 and mTOR regulate Rac1-driven platelet activation and aggregation. *Blood.* 2011; 118:3129–3136. [PubMed: 21757621]
3. Baker SM, Phillips KG, McCarty OJT. Development of a label-free imaging technique for the quantification of thrombus formation. *Cell. Mol. Bioeng.* 2012; 5:488–492. [PubMed: 23585817]
4. Baker-Groberg SM, Phillips KG, McCarty OJT. Quantification of volume, mass, and density of thrombus formation using brightfield and differential interference contrast microscopy. *J. Biomed. Opt.* 2013; 18:16014. [PubMed: 23348747]
5. Hanke AA, et al. Impact of platelet count on results obtained from multiple electrode platelet aggregometry (Multiplate™). *Eur. J. Med. Res.* 2010; 15:214. [PubMed: 20562061]
6. Maloney SF, Brass LF, Diamond SL. P2Y12 or P2Y1 inhibitors reduce platelet deposition in a microfluidic model of thrombosis while apyrase lacks efficacy under flow conditions. *Integr. Biol. Quant. Biosci. Nano Macro.* 2010; 2:183–192.
7. McCarty OJT, et al. Rac1 is essential for platelet lamellipodia formation and aggregate stability under flow. *J. Biol. Chem.* 2005; 280:39474–39484. [PubMed: 16195235]
8. Michelson AD. Platelet function testing in cardiovascular diseases. *Circulation.* 2004; 110:e489–e493. [PubMed: 15533872]
9. Phillips KG, et al. Quantification of cellular volume and sub-cellular density fluctuations: comparison of normal peripheral blood cells and circulating tumor cells identified in a breast cancer patient. *Front. Cancer Mol. Targets Ther.* 2012; 2:96.
10. Phillips KG, Jacques SL, McCarty OJT. Measurement of single cell refractive index, dry mass, volume, and density using a transillumination microscope. *Phys. Rev. Lett.* 2012; 109:118105. [PubMed: 23005682]
11. Phillips KG, McCarty OJT. Optical quantification of cellular mass, volume, and density of circulating tumor cells identified in an ovarian cancer patient. *Front. Cancer Mol. Targets Ther.* 2012; 2:72.
12. Roberts DE, McNicol A, Bose R. Mechanism of collagen activation in human platelets. *J. Biol. Chem.* 2004; 279:19421–19430. [PubMed: 14981087]
13. Ruggeri ZM. Platelets in atherothrombosis. *Nat. Med.* 2002; 8:1227–1234. [PubMed: 12411949]
14. Shattil SJ, Kashiwagi H, Pampori N. Integrin signaling: The platelet paradigm. *Blood.* 1998; 91:2645–2657. [PubMed: 9531572]

FIGURE 1. Platelet aggregation on fibrinogen-coated surfaces

(a,d,g,j) XY and XZ DIC projections of platelet aggregates formed on fibrinogen-coated coverslips at 20,000, 100,000, 400,000, or 500,000 platelets/ L, respectively. Representative image from 3 independent experiments. (b,e,h,k) Magnified image of region within the white box in (a,d,g,j). (c,f,i,l) Projected density of the white box in (a,d,g,j) determined with the NIQPM technique.

FIGURE 2. Platelet aggregation on collagen-coated surfaces

(a,d,g,j) XY and XZ DIC projections of platelet aggregates formed on fibrillar collagen-coated coverslips at 20,000, 100,000, 400,000, or 500,000 platelets/L. Representative image from 3 independent experiments. (b,e,h,k) Magnified image of region within the white box in (a,d,g,j). (c,f,i,l) Projected density of the white box in (a,d,g,j) determined with the NIQPM technique.

FIGURE 3. Platelet aggregate formation under static conditions as a function of platelet count Mean platelet aggregate (a) volume and (b) mass for a 32 μm by 32 μm region collected over three trials. Error bars are ± standard error of the mean.

FIGURE 4. Platelets treated with a GPIIb/IIIa inhibitor form platelet monolayers on collagen-coated surfaces

Platelets were incubated with the GPIIb/IIIa inhibitor, eptifibatide (20 μg/mL; 10 min at 25°C), followed by incubation on fibrillar collagen-coated glass coverslips at platelet concentrations of (a,b,c) 400,000 or (d,e,f) 500,000 platelets/ L. (a,d) XY and XZ DIC projections of GPIIb/IIIa-treated platelet aggregates. Representative image from 3 independent experiments. (b,e) Magnified image of region within the white box in (a,d). (c,f) Projected density of the white box in (a,d) determined with the NIQPM technique. Mean platelet aggregate (g) volume and (h) mass for a 32 μm by 32 μm region collected over three trials. Error bars are ± standard error of the mean.