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Polydiacetylene Liposome Microarray toward Facile Measurement of Platelet Activation in Whole Blood

Deokwon Seo, Terry C. Major, Do Hyun Kang, Sungbaek Seo, Kangwon Lee, Robert H. Bartlett, and Jinsang Kim*

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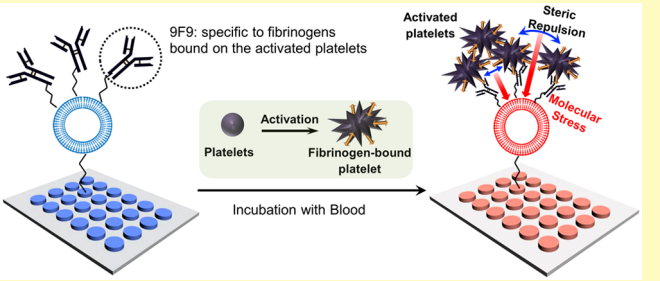
ABSTRACT: The necessity of a simple measurement of platelet activation has been increasing in clinical medicine to regulate the proper dose of the antiplatelet drugs for patients having clinical outcomes in acute situations such as angina pectoris, stroke, or peripheral vascular disease or procedures involving angioplasty or coronary thrombolysis. We developed a self-signaling polydiacetylene (PDA) liposome microarray to detect activated platelets from whole blood samples in a single step. A specific antibody, 9F9 antibody, to platelet-bound fibrinogen was selected and conjugated to the PDA liposome microarray to quantify the fibrinogen-bound platelets. The developed PDA liposome−9F9 microarray generated an intense fluorescence signal when activated platelets in whole blood were introduced and also successfully distinguished the reduced platelet activation in the presence of Tirofiban, a model antiplatelet drug. The results of this single-step benchtop assay incorporates simple, sensitive, and rapid attributes that can detect the extent of platelet activation prior to needed clinical procedures.



KEYWORDS: *polydiacetylene liposome, microarray, platelet activation, fluorescence signal, 9F9 antibody, self-signaling*

Heart disease and its related symptoms affect hundreds of thousands of Americans each year. According to the Center for Disease Control and Prevention, heart disease remains the leading cause of death in the United States. For many patients, taking antiplatelet medication, aspirin, Plavix, and Tirofiban, has been a commonly recommended practice to help prevent heart attacks and strokes. This family of antiplatelet drugs is commonly used to prevent blood clotting in patients with cardiovascular conditions such as myocardial infarction, stroke, and prosthetic heart valve issues. However, medicating antiplatelet drugs is often hazardous, since it can lead to haphazard bleeding upon accident or surgery, which may require an emergency operation. In addition, many medical symptoms such as sepsis, malignancies, extracorporeal circulation, and dialysis may cause abnormal platelet activity. Continuous monitoring of platelet function from patients with cardiac records is critical, as many patients with the record may adjust dosage arbitrarily or take multiple medications from other diseases. However, monitoring of platelet function has been crudely implemented by either measuring bleeding time from a wound or by analyzing blood samples using complicated methods such as aggregometry,[1] cytometry,[2] or atomic force microscopy (AFM).[3] Although the latter provides accurate results, it complicates the monitoring too much for the patients since it requires access to a laboratory, excessive examination time, and costs. Consequently, patients are often simply advised to take the standard dose without accurate and vital monitoring of platelets, a practice that may endanger the lives of the patients.

Polydiacetylene (PDA) is a unique sensory material which changes its optical properties in response to molecular stress produced by steric repulsion between the captured analytes on adjacent receptors, providing useful self-signaling optical detection as illustrated in Scheme 1A.[4−6] Upon exposure to various molecular stresses, the color of PDA is changed from blue to red through the distortion of its conjugated *yne-ene* main chain and a consequential bandgap change. Furthermore, the converted red-phase PDA also emits red fluorescence while the original blue-phase PDA has no fluorescence emission. Through such a dual colorimetric and fluorometric self-signaling property, the PDA has been used to conveniently and sensitively detect various molecular stresses generated by heat,[7−9] humidity,[10,11] and binding of metal ions,[12−14] chemicals,[15−21] or biomolecules.[22−28] We previously reported a highly sensitive and selective, and generic biosensor platform

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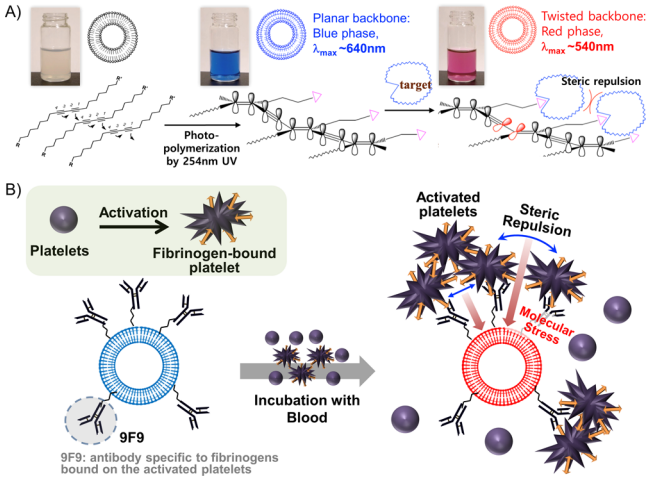
Scheme 1. . Schematic Illustration of (A) PDA Liposome Self-Assembly, Photopolymerization, and Stimuli-Responsive Property; (B) Platelet Activation Assay Based on Sensory PDA Liposome−9F9 Antibody



using PDA materials.[10,12−14,17,22,24,29] We have developed an amphiphilic PDA monomer having an epoxy headgroup and self-assembled them into the liposome form.[14] Then, the resulting PDA−epoxy liposomes were efficiently tethered to amine-functionalized substrates by amine−epoxy chemistry, rendering practical a microarray-type of PDA liposome biosensors. Subsequently, various amine-containing bioreceptors (i.e., DNA aptamers, peptides, antibodies) can be bound to the epoxy head groups of the immobilized PDA liposome surface followed by photopolymerization with a 254 nm UV light to form blue PDA liposomes, which are sensitive to molecular stress produced by various external stimuli. The prepared sensory PDA microarray can be readily used for selective recognition of target molecules such as mercury ion,[14] bovine viral diarrhea virus antibody,[22] and influenza viruses,[24] which successfully produce the intense optical signals based on the recognition-induced molecular stress. Moreover, the signal intensity increases gradually with increasing amounts of target analytes and saturates as the receptors are occupied by analytes, allowing quantitative analysis provided that a correlation curve is established.[12,14,16−18,22,24,29]

We rationally applied this sensory PDA platform to the development of an activated platelet detection system by employing a specific antibody, 9F9 antibody, to provide selectivity since the 9F9 antibody is known to have specific interaction with platelet-bound fibrinogen (Scheme 1B).[30,31] The developed PDA liposome−9F9 microarray produced an intense fluorescence signal when the platelets in whole blood were activated and also could distinguish reduced platelet activation when varying amounts of an antiplatelet drug, Tirofiban, were added to blood samples by the signal intensity change. The presented results of this single-step benchtop assay suggest a potentially practical test that processes a large number of samples economically and can derive test results in a time frame meaningful for clinical outcomes in acute situations such as angina pectoris, stroke, or peripheral vascular disease or procedures involving angioplasty or coronary thrombolysis. The assay incorporates simple, sensitive, and rapid attributes that can detect the extent of platelet activation. The developed PDA liposome assay allows (1) a rapid and simple measurement using whole blood samples without the need for expensive and complicated blood cell separation equipment as well as the need for additional in vitro platelet stimulation with exogenous agonists such as ADP or collagen and (2) a sensitive assessment of various degrees of platelet activation, especially the determination of platelet function of patients on low-dose aspirin or of patients on antiplatelet drugs.

EXPERIMENTAL SECTION

Materials. A PDA monomer, 10,12-pentacosadiynoic acid (PCDA)-epoxy, was synthesized as described in our previously published results.[14] A phospholipid, 1,2-dimyristoyl-sn-glycero-3-phosphate (DMPA), was ordered from Avanti Polar Lipids. Unlabeled FITC-9F9 Antibody, Collagen, and Tirofiban were purchased from Biocytex, Chrono-log, and Sigma-Aldrich, respectively. The chemicals such as solvents, buffers, and blocking agents were also purchased from Sigma-Aldrich.

PCDA−Epoxy Liposome Assembly. The PDA liposomes consisting of PCDA-epoxy and DMPA lipids were assembled by the following injection method.[14] PCDA-epoxy and DMPA were codissolved (4:1 molar ratio) in the 0.1 mL of tetrahydrofuran/water mixture (9:1 v/v), and the lipid solution was injected to 20 mL of 5 mM HEPES buffer at pH 8. The total lipid concentration in the final aqueous solution was 0.5 mM. The liposome solution was probe-sonicated at 120 W for 10 min and was filtrated through a 0.8 μM cellulose acetate syringe filter. The filtrate was stored at 5 °C before use.

PDA Liposome Microarray Fabrication. The surface of the glass was treated with 3-aminopropyltriethoxysilan to provide amine functionality following the literature process.[24] A slight modification was made when immobilizing PDA liposomes on the resulting amine-modified glass substrate, and the details are as follows. Glass slides were washed with chloroform, acetone, and 2-propanol for 15 min each. The cleaned glass slides were then sonicated in sulfuric acid containing NOCHROMIX for 2 h. After a thorough rinse with deionized water and air-dry, the glass slides were placed in a UV/Ozone cleaning apparatus and treated for 30 min. The glass slides were then stirred in a 2 wt % 3-aminopropyltriethoxysilane in anhydrous toluene solution on an orbital shaker for 6 h in a glovebox at 70 °C and afterward baked at 115 °C for 30 min. The glass slides were sonicated in toluene, toluene:methanol (1:1), and methanol for 15 min each to remove any unbound silane reagent.

To fabricate the PDA liposome microarray, the liposomes were covalently immobilized on amine-modified glass slides through the amine−epoxy chemistry. 0.5 mM PCDA−epoxy liposome solution was spotted on amine-coated glass slides with a manual microarrayer (VP 475, V&P Scientific, Inc.) and was incubated at 5 °C for 24 h to prevent fast drying of the spotted solution. After removing the unbound liposomes by rinsing with 5 mM of pH 8 HEPES buffer, the PDA liposome-spotted slides were incubated with 0.06 mg/mL of 9F9 antibody in 5 mM of pH 8 HEPES buffer for 1 h. After removing the unbound antibodies by rinsing with 5 mM of pH 8 HEPES buffer, the polymerization of the immobilized PCDA−epoxy liposomes on the slides was carried out by illuminating 254 nm UV (1 mW/cm^2) for 1 min. The resulting PDA liposome microarray was used for the subsequent detection analysis.

Detection of Activated Platelet with PDA Liposome Microarray. Samples were taken from healthy blood donors who had abstained from taking aspirin for 2 weeks before sampling. All donor consent was obtained according to GT IRB H15258. Blood samples were taken by median venipuncture into a sodium citrate tube (BD Vacutainer) and were used immediately. Platelet-free plasma was obtained from the supernatant after 15 min centrifugation of the blood at 1500 G and was used immediately. For the subsequent experiment described in this paper, 4 μL of collagen solution (1 mg/mL) and 2 μL of various concentration of Tirofiban solution were added to the 94 μL of blood samples, and the mixture was loaded immediately on the PDA liposome microarray. After 20 min of incubation, fluorescence microscope images were obtained on an

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Olympus BX 71 microscope equipped with a mercury lamp and a cutoff filter of 540 nm excitation and 600 nm emission. For fluorescence signal intensity measurement, the fabricated PDA liposome−9F9 microarray was placed on top of a 96-well plate, and the combined substrates were inserted into a plate reader. Fluorescence emission intensities at 634 nm of total 120 microarray spots from three different devices were individually measured by using the excitation wavelength of 548 nm.

Fluorescence-Activated Cell Sorting Analysis. Platelets diluted from whole blood (1:100) were treated with Tirofiban (0−10 nM) and GPIIb/IIIa fibrinogen receptor inhibitor and incubated for 20 min at room temperature. In a separate tube, 1 μL 10% DMSO was added to serve as a vehicle control (i.e., 0 nM Tirofiban). Collagen (40 μg/mL) was then added (4 μL) and incubated for 2 min at room temperature in all tubes. Platelet activation was measured by using 20 μL of the anti-9F9 antibody-FITC and 10 μL of the constitutive platelet marker, anti-CD61-PE (Phycierythrin). As a control for the anti-9F9 antibody, 10 μL of the isotype control, anti-mouse IgG1-FITC, was used with 10 μL of anti-CD61-PE in a separate tube. The antibodies were incubated at room temperature in the dark for 15 min. After incubation, 700 μL of 1% formalin/dPBS was added to each tube and stored at 4 °C for up to 24 h post-fixing until ready to run the fluorescence-activated cell sorting (FACS) analysis on a FACSCalibur flow cytometer (Beckton Dickenson San Jose, CA). CellQuest software (Beckton Dickenson San Jose, CA) was employed for the data analysis.

Cell populations were identified for data collection by their log forward scatter (FSC) and log side scatter (SSC) light profiles. For each sample, 5000 total events were collected in a region (R1) gated on platelets within the FSC versus SSC profile (Figure 4A,C,E). Mean fluorescence intensity (MFI) of the anti-9F9 immunostaining was quantitated by a FITC (FL1) versus PE (FL2) log plot analysis. The platelets from region R1 were plotted, and a gated region R2 was drawn to separate the platelets from debris on the FL2 axis (Figure 4B,D,F). At this point, all tubes were run on the cytometer noting the MFI change in the platelet cloud in region R2. The adjusted MFI was expressed as the geometric mean channel fluorescence minus the appropriate isotype control. The platelet reactivity index (PRI) was calculated by the following equation:

$$\mathrm{PRI} = [(\mathrm{MFI}_{c(T1)} - \mathrm{MFI}_{C(T2)})/\mathrm{MFI}_{C(T1)}] \times 100 \quad (1)$$

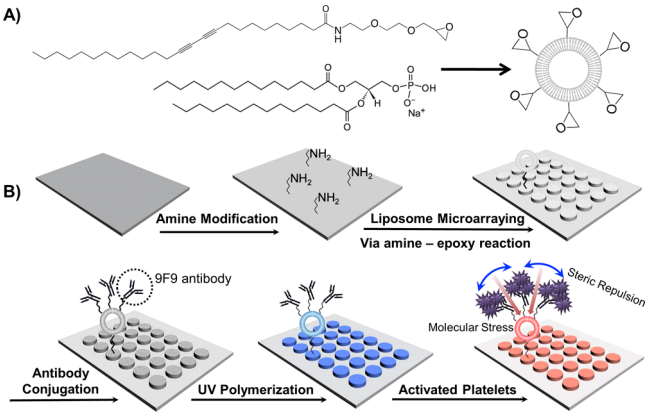
where $\mathrm{MFI}_{c(T1)}$ is the adjusted MFI for the collagen plus vehicle control tube, and the $\mathrm{MFI}_{c(T2)}$ is the adjusted MFI for the collagen plus each concentration of Tirofiban tubes.

RESULTS AND DISCUSSION

The overall fabrication procedure of the developed PDA liposome−9F9 antibody microarray is schematically illustrated in Scheme 2. We specifically aimed for solid-state biosensors to directly use a whole blood sample without pretreatment or preseparation. The predominant reason for pretreating blood samples lies in the fact that whole blood comprises nonspecific components such as red blood cells, white blood cells, and inactivated platelets that may interfere with the optical detection through colorimetric change. To circumvent this problem, a separation process is usually required—centrifugation or filtration, especially as seem in the solution-type detection system. However, this makes the sensor cumbersome to use and significantly lengthens the time for detection or data acquisition.

We constructed PDA liposomes by using (10,12-pentacosadiynoic acid)-epoxy (PCDA-epoxy) and 1,2-dimyristoyl-*sn*-glycero-3-phosphate (DMPA) as shown in Scheme 2A. PCDA-epoxy monomer was rationally designed for the following two reasons. First, the epoxy group allows the epoxy−amine chemistry between the PDA liposome and the amine-modified glass substrate that enables stable and covalent immobilization

Scheme 2. (A) Chemical Structure of PCDA−Epoxy and DMPA. (B) Schematic Illustration of the PDA Liposome Microarray Fabrication Procedure for Detecting Platelet Activation



of PDA liposome onto the glass substrate.[14,24] This was further beneficial for binding of 9F9 antibodies to the PDA liposome surface, as we can use the same epoxy−amine chemistry between the epoxy group on the PCDA−epoxy and the amine groups of the antibody. A phospholipid DMPA, another constituent for self-assembling PDA liposome, was used to alter the self-assembly of the PDA monomer and to prevent high-order packing. Such insertion of phospholipid increased the flexibility of the liposome bilayer, which led to increased device sensitivity.[17,22,24]

We immobilized PDA liposome on the glass substrate to develop the solid-type sensor as described in the Experimental Section. To confirm successful immobilization of PDA−epoxy liposomes onto the amine-modified glass substrate, we incubated a PCDA−epoxy liposome solution on the amine substrate, photopolymerized, and applied heat, the most straightforward external stimulus, to the immobilized PDA liposomes at 150 °C for 3 min, which would effectively distort the resulting conjugated PDA backbone and produce red fluorescence. We could observe the saturated fluorescence intensity of red-phase PDA by using a fluorescence microscope (Figure 1A). There was only 3.45% of the average difference in fluorescence intensity across 36 devices, indicating the robust

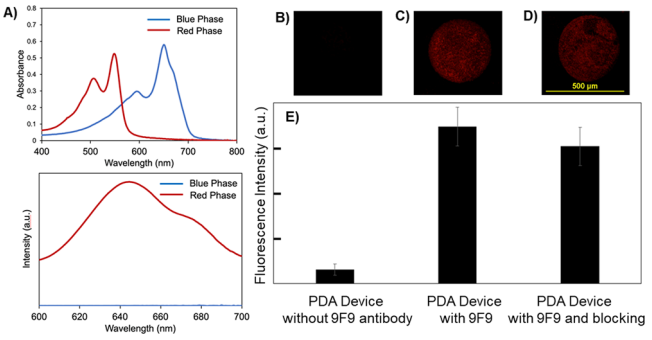


Figure 1. (A) Absorption and emission spectra of the blue and red phases of PCDA-epoxy liposome tethered on an amine-functionalized glass substrate. Fluorescence microscopy images of (B) a negative control PDA device without antibody incubation, a PDA microarray device having 9F9 antibodies without (C) and with (D) ethanolamine blocking, (E) fluorescence emission intensity of the PDA liposome of each sample arrays.

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and homogeneous immobilization of the PDA liposome onto the glass substrate as well as the reproducibility of our developed devices.

9F9 antibodies were subsequently tethered to the immobilized PDA liposome layer on the amine substrates followed by blocking any unreacted epoxy groups of the PDA layer with ethanolamine. Further testing was implemented to confirm that this blocking does not affect the efficacy of the antibody before applying whole blood samples on the developed PDA liposome−9F9 microarray. As shown in Figure 1B, upon incubating with the whole blood samples, no signal was detected from the PDA microarray device without having 9F9 antibodies but simply blocked with ethanolamine. On the contrary, as shown in the Figure 1D, antibody-incubated and ethanolamine-blocked device emitted red fluorescence signal from the samples. The device having 9F9 antibodies without the ethanolamine blocking (Figure 1C) showed 13.72% higher fluorescence signal intensity than the ethanolamine-blocked device (Figure 1E). We believe that nonspecific binding of nontargeted blood components to unreacted epoxy groups resulted in additional random background signal.

The two major receptors on the platelet surface, integrin $\alpha_2\beta_1$ and GPVI, are used for interaction with collagen, which activates platelets. Additionally, adenosine diphosphate (ADP) and epinephrine activate platelets which are mediated by three purinergic receptors (P2Y1, P2Y12, and P2X1) and the alpha2 adrenergic receptor, respectively. Platelet activation then leads to the increased affinity of the GPIIb/IIIa to plasma fibrinogen molecule leading to platelet aggregation.[32,33] Our PDA microarray device was developed to selectively measure activated platelets, since the 9F9 antibody tethered on the PDA surface selectively interacts with fibrinogen bound to the activated platelets. In order to ensure specific measurement for activated platelets, we conducted a control experiment comparing whole blood samples with and without 40 μg/mL of collagen. As seen in Figure 2, the PDA device with added

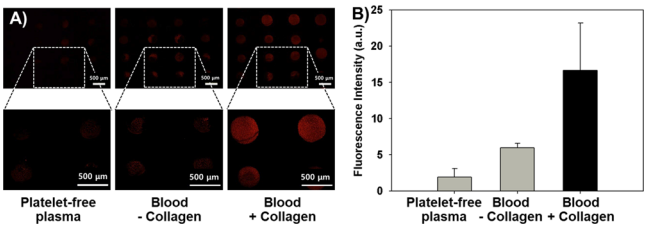

Figure 2. (A) Fluorescence microscope images and (B) fluorescence emission intensity of PDA liposome arrays after 20 min incubation at room temperature. Scale bars are 500 μm.

collagen displayed a red fluorescence intensity 2.8 times greater than the device without it. The significant increase in the fluorescence signal of the sample with collagen confirmed that our PDA liposome−9F9 microarray device detected specifically for activated platelets and was unaffected by the number of total platelets present in the samples.

As shown from the results above, the devised PDA microarray device is specific to the activated platelets. Due to its specificity, we further investigated whether our device can be used to determine the appropriate dosage of antiplatelet drugs for individual patients and their personal healthcare providers. Tirofiban is an inhibitor of platelet-activated coagulation which acts by inhibiting GPIIb/IIIa. It was incubated in the blood samples for 20 min. The results showed that the Tirofiban concentration was inversely related to the fluorescence signal intensity of the PDA microarray device (Figure 3). As the concentration of incubated Tirofiban increases in the sample, the signal decreases. The limit of detection of Tirofiban in the sample was measured to be 0.16 μM.

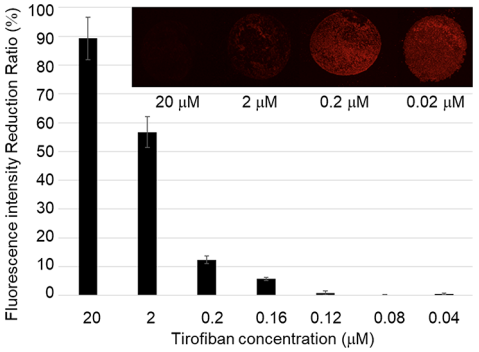

Figure 3. Fluorescence intensity reduction ratio of PDA liposome−9F9 microarray devices after 20 min incubation with Tirofiban and whole blood. (inset) Fluorescence microscopy images.

The 20 min incubation time of the anticoagulation drug was selected to obtain the optimal signal. We incubated 100 μL of the blood samples with various Tirofiban concentrations to 9-mm-diameter silicon isolator of the PDA microarray device and could find that the red fluorescence signal for all samples significantly increased when Tirofiban was incubated more than 30 min. After 50 min, even the blood sample having 20 μL of Tirofiban showed a similar signal intensity to samples without Tirofiban. Altogether, we realized that in the open-air condition with longer than 30 min incubation time, signal intensities from both the Tirofiban-treated group and the nontreated group showed similar levels of coagulation from activated platelets. Therefore, we concluded that the anticoagulation drug should be incubated for 20 min for the best reliable and reproducible result to be presented in the PDA microarray device.

To confirm the results from the PDA liposome−9F9 microarray, a modified flow cytometric assay was conducted using the same anti-human fibrinogen antibody, 9F9, as in the PDA liposome−9F9 complex as described in the Experimental Section.[34] Figure 4 shows the flow cytometric analysis (A−F) of the inhibition of collagen and epinephrine stimulated human platelets by Tirofiban, collagen concentration responses on 9F9 antibody expression (G), and the Tirofiban's inhibition of 40 mg/mL collagen and 10 mM epinephrine-stimulated human platelets (H). This stimulation of platelets elicited a maximum platelet activation response.

The results of the modified flow cytometric assay confirmed the results of the PDA liposome−9F9 microarray. The level of Tirofiban inhibition directly affects the measured fluorescence intensity of FACS analysis, since the fluorescence intensity is determined by the level of bound F9F antibody−FITC conjugate to platelets. Less bound F9F conjugate to platelets means an increase in the inhibition of platelet activation. Flow cytometry analysis data shown in Figure 4G demonstrates that 9F9 antibody expression increases as the concentration of collagen increases, supporting the results shown in Figure 2. As shown in Figure 4H, the increase in Tirofiban concentration resulted in an increase in the % inhibition of platelet activation

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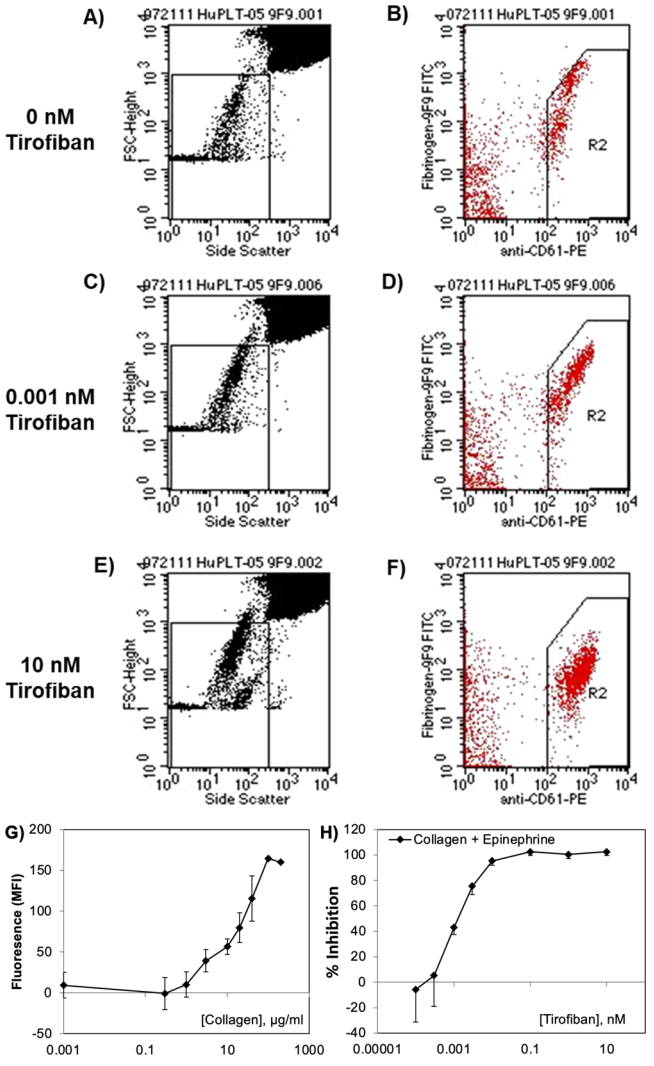

Figure 4. (A–F) FACS analysis of the FITC-9F9 antibody in human whole blood. (G) Flow cytometric analysis of collagen concentration response on 9F9 antibody expression. MFI = maximum fluorescence intensity. (H) concentration response of GPIIb/IIIa inhibitor, Tirofiban, on collagen and epinephrine stimulated human platelets.

via collagen stimulation. The Tirofiban inhibited collagen with IC_{50} values of about 0.0015 nM. Therefore, direct detection of platelet activation using the specific antibody, 9F9, to platelet-bound fibrinogen allows the determination of the extent of platelet activation under most conditions including contact activation in extracorporeal circulations, antiplatelet drug effects and hereditary platelet diseases. Similarly, as shown in Figure 3, the PDA liposome–9F9 complex produced weaker fluorescence when the concentration of Tirofiban was increased. Thus, the PDA liposome–9F9 assay provides a simple single-step measurement procedure compared to the complex flow cytometric method to assess the real-time functional state of circulating platelets.

CONCLUSION

We devised a PDA-based microarray sensor device as an effective tool to specifically determine the level of activated platelets in whole blood samples. 9F9 antibodies that are specific for fibrinogen bound to the activated platelets are tethered at the PDA liposome surface to provide selective recognition of activated platelets. The fluorescence signal intensity is inversely related to the amount of Tirofiban, an inhibitor of platelet activated coagulation, added to whole blood samples, implying possible determination of appropriate dosage of antiplatelet drugs. Without any special separation process or pretreatment of the whole sample, patients as well as their healthcare providers can conveniently monitor the level of activated platelets as a reference for appropriate antiplatelet drug dosage.

AUTHOR INFORMATION

Corresponding Author

Jinsang Kim − *Department of Materials Science and Engineering, Macromolecular Science and Engineering, Department of Chemical Engineering, Department of Chemistry, and Biointerfaces Institute, University of Michigan, Ann Arbor, Michigan 48109, United States;* orcid.org/0000-0002-1235-3327; Email: jinsang@umich.edu

Authors

Deokwon Seo − *Program in Nanoscience and Technology, Graduate School of Convergence Science and Technology, Seoul National University, Seoul 08826, Republic of Korea; Department of Materials Science and Engineering, University of Michigan, Ann Arbor, Michigan 48109, United States*

Terry C. Major − *Department of Surgery, University of Michigan Medical School, Ann Arbor, Michigan 48109, United States*

Do Hyun Kang − *Department of Materials Science and Engineering, University of Michigan, Ann Arbor, Michigan 48109, United States;* Present Address: Department of Nanomanufacturing Technology, Korea Institute of Machinery and Materials, Daejeon, 34103, Republic of Korea; orcid.org/0000-0001-6588-0000

Sungbaek Seo − *Macromolecular Science and Engineering, University of Michigan, Ann Arbor, Michigan 48109, United States;* Present Address: Department of Biomaterials Science, College of Natural Resources and Life Science, Life and Industry Convergence Research Institute, Pusan National University, Miryang, 50463, Republic of Korea

Kangwon Lee − *Program in Nanoscience and Technology, Graduate School of Convergence Science and Technology, Seoul National University, Seoul 08826, Republic of Korea;* orcid.org/0000-0001-5745-313X

Robert H. Bartlett − *Department of Surgery, University of Michigan Medical School, Ann Arbor, Michigan 48109, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acssensors.1c01167

Author Contributions

T.C.M. and J.K. conceived and designed the research. D.S., D.H.K., and S.S. designed and synthesized materials and prepared devices. D.S. and T.C.M. carried out the measurements with whole blood samples. D.S. and T.C.M. wrote the manuscript with input from all authors. J.K. supervised the project and revised the manuscript.

Notes

The authors declare the following competing financial interest(s): A patent application has been filed based on the results presented in this publication.

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