In vitro investigation of the effect of platelet aggregation ability on the adhesion of platelets to micro-structures

Akiko Oota-Ishigaki¹, Osamu Maruyama¹, Toru Masuzawa², Masahiro Nishida¹,*

¹National Institute of Advanced Industrial Science and Technology, 1-2-1 Namiki, Tsukuba-city, Ibaraki, Japan
²Ibaraki University, 4-12-1 Nakamaru-sawa, Hitachi-city, Ibaraki, Japan

Received: 6 June 2021/Accepted: 8 September 2021
© Japanese Society of Biorheology 2021

Abstract  Thrombus formation on rough surfaces in cardiovascular devices implanted into patients with heart failure is a significant problem. It is, therefore, necessary to investigate the thrombus formation mechanism on rough surfaces. We have proposed a hypothesis that micro-secondary flows which are proportional to the size of micro-structure composing the rough surfaces contribute the platelet adhesion around micro-structure, that is, enhance possibility of thrombus formation. In this study, blood perfusion tests were conducted, using bovine blood with different platelet aggregation abilities, to evaluate platelet adhesion on a test piece with micro-cylinders on its surface. It was found that the platelet adhesion was sufficiently enhanced and the platelets exhibited high aggregation ability. This phenomenon depends on shear flow, and the platelets may selectively adhere to the perimeter of larger structures in the presence of micro-structures of different sizes. Our results indicate that the platelet adhesion increases in proportion to size of micro-structures when there is an average platelet aggregation per unit platelet (NL-PATI/PLT) ratio of over 0.002. These results can aid in designing the surface roughness of the blood contact material to help reduce the risk of thrombus formation.

Keywords  platelet adhesion, platelet aggregation, platelet aggregation threshold index, micro-structure, antithrombic properties, surface roughness

1. Introduction

Thrombus formation is a serious challenge in the application of cardiovascular devices to patients with heart failure because detached thrombi from material surfaces can clog cerebral blood vessels and cause neurological symptoms [1–4]. Cardiovascular devices are designed to have low surface roughness to suppress thrombi formation [5–7]. However, the surface roughness threshold on a material surface has not yet been established thoroughly in consideration of blood compatibility.

Thrombus formation on rough surfaces is initiated by protein adsorption and platelet adhesion on the material surface, and the relationship between morphological design and platelet adhesion has been investigated in previous studies [8–15]. Linneweber et al. [8] reported that platelet adhesion increases with increasing average surface roughness of the blood pump. When roughness is produced by normal surface processing, the surface is composed of microstructures of various shapes and sizes. In order to set an appropriate threshold for surface roughness, it is necessary to investigate the cause of thrombus formation on rough surfaces.

When considering which aspect of the surface causes an increase in platelet adhesion, we hypothesized that the micro-flow, generated around micro-structures that constitute rough surfaces, enhances the physical adhesion phenomenon of platelets. Based on this hypothesis, we previously used albumin micro-beads to morphologically mimic surface roughness and the physical adhesion phenomenon of platelets. Based on this hypothesis, we previously used albumin micro-beads to morphologically mimic surface roughness and the physical adhesion phenomenon of platelets. Our results indicate that the adhesion rate was affected not only by average shear rate but also by the micro-secondary flow around the micro-column. The size of micro-secondary flow depends on the size and shape of the micro-structures. Therefore, the presence of one large micro-structure increases the probability of platelet adhesion and thrombus formation even though the overall surface roughness value of the material surface is small.

Our previous studies [16, 17] have focused only on “physical phenomena” such as the effect of shear rate and micro-secondary flow. It is unknown whether other effects, such as chemical and physiological phenomena, affect
platelet adhesion. For example, Chan et al. [18] reported a relationship between the size of platelet aggregation and shear loading with human blood. Their results have shown that the average size of platelet aggregates formed under physiological shear rates of 360–3000 s⁻¹ was significantly larger compared to shear rates of >6000 s⁻¹. When blood is exposed to varying shear stress, such as in a blood pump, it is predicted that a change in the magnitude of platelet aggregation affects the level of platelet activation. Therefore, the platelet adhesion rate to microstructures, which constitute rough surfaces, may affect the degree of platelet activation and platelet aggregation.

It is necessary to investigate the extent to which platelets adhere around micro-structures using real blood with not only physical flow effects but also chemical and biochemical reactions. This study focused on blood properties, especially the ability of platelet aggregation after shear loading. As a basic study to assist in establishing the surface roughness threshold on a material surface, this study aimed to investigate platelet adhesion affected by micro-flows generated around micro-columns with perfusion tests in vitro using blood samples with and without pre-sheared flow. As a basic study, bovine blood, instead of human blood, was selected as the target blood assuming that animal experiments are used for the development of cardiovascular devices.

2. Materials and Methods

2.1 Experimental equipment for in vitro perfusion test

A smooth surface, which is defined as a surface with an average surface roughness (Ra) <0.2 μm and a maximum Ra <1.6 μm, is empirically adopted into the design of material surfaces for cardiovascular devices, such as a blood pump. Referring to these values, a polydimethylsiloxane (PDMS) test piece with four types of micro-columns was constructed using a microprinting technique, as shown in Fig. 1(a). The micro-columns were arranged in eight rows and five columns, and the sizes of the micro-columns were the same as in those in our previous study [17]. Forty micro-columns were categorized into four types, which are D30H10, D10H30, D10H10, D30H30 (D and H refers to the diameter and height, respectively and the unit is μm). Therefore, ten micro-columns of each type were constructed on the right side of the test piece (the blood flowed from left to right) with a wide separation to ensure that the flows around the micro-columns do not interfere with each other. The micro-columns were arranged in the following order: D30H10 in the 1st row, 2nd row, D10H30 in the 3rd and 4th rows, D10H10 in the 5th and 6th rows, and D30H30 in the 7th and 8th rows. (See Fig. 1(a) for details)
The calculated $Ra$ on the surface of the test piece is approximately 0.45 μm, which differs from that normal surfaces in cardiovascular devices because the test piece is designed to investigate the effects of micro-secondary flow and platelet aggregation on platelet adhesion, and there was limit of technique to construct micro-structures on a PDMS test piece. The surfaces of the test pieces were modified to introduce hydrophilicity to promote easy platelet adhesion through plasma shower coating (MSP-10, Vacuum Device Co., Ltd., Japan) at a power current of 20 mA under a pressure of 10 Pa for 1 min.

Fig. 1(f)–(i) show the manufactured acrylic perfusion chamber, which has an 11-mm square pocket at the center of the test piece, for the adhesion tests. The fluid in the perfusion chamber exhibited a plane Poiseuille flow [15]. Fig. 2 shows the perfusion chamber for the in vitro adhesion test.

2.2 Blood preparation

Commercial bovine blood was used in the blood perfusion tests because bovine blood samples are usually employed in animal experiments for blood pumps. One liter of bovine blood obtained from a slaughterhouse was initially anticoagulated with 10 ml of heparin (10,000 units of heparin) and refrigerated at 4 °C. The concentration of heparin was higher than the normal concentration of anticoagulant that is usually mixed with human blood to ensure the maintenance of anticoagulation during transport. The experiment was started within 24 h of blood collection. The activated clotting time (ACT) was measured in advance using an ACT measuring device (HEMOCHRON Response; Accriva Diagnostics Holdings, Inc., USA) when the test was started, and the measured ACT was >1500 s.

Before the perfusion test, the bovine blood was uniformly shear loaded for 30 s at a shear rate of 10000 s⁻¹ using a developed double-cylindrical shear stressor to investigate the difference in platelet adhesion due to the addition of shear loading. The blood sample was loaded with a shear stress of approximately 30 Pa. As a control, the same batch of blood with no shear loading was prepared. Four different bovine blood batches were prepared in the same manner for the four perfusion tests.

2.3 In vitro blood perfusion test

In vitro blood perfusion tests were performed using a perfusion circuit. The prepared blood was perfused in the circuit at a flow rate of 100 mL/min through a peristaltic tube pump for 120 min. The temperature of the perfusion circuit was maintained at 20 °C. After perfusion was complete, the test piece was removed, carefully rinsed with saline twice, and then soaked in 2% glutaraldehyde phosphate buffered saline (PBS) solution for 2 h to fix the adhered blood cells on the surface of the test piece. Two hours later, the test piece was sequentially dehydrated with 50%, 70%, 90%, and 99.5% ethanol, dried naturally, and coated with platinum ions via ion shower coating (MSP-10, Vacuum Device Co., Ltd., Japan) at a power current of 50 mA under a pressure of 10 Pa for 1 min for scanning electron microscopy (SEM; TM1000, Hitachi High-Technologies Co. Ltd, Japan) observation. The adhered platelets around the micro-cylinders on the test piece were observed and scanned after platinum ion coating using SEM. The perfusion tests were performed four times under two blood conditions: (A) non-pre-shear loading and (B) 10000 s⁻¹ of pre-shear loading (the same batch of bovine blood was used in both cases).

The adhesion rates of the platelets on the test pieces were obtained using ImageJ image processing software. As shown in Fig. 3(a), the $150 \times 150 \text{μm}^2$ area (twenty plane area) around the micro-cylinder was considered as the observation area because, as per a CFD analysis in our previous study [17], the flow in this area is slower by approxi-

![Perfusion circuit](image-url)
approximately 20% compared to that in the remaining area. The image was converted to 8-bit grayscale from 0 to 255. The column area was cut from the observation area, and the edges of blood cells were found (Fig. 3(b)). The white and black colors were then inverted (Fig. 3(c)), and the image was binarized at a threshold of 200 (Fig. 3(d)). Platelets were counted using the Particle Analysis function in Image J. At this time, areas <9 pixels were excluded to prevent the background points (substrate surface of the test piece) at a gray scale of 200 or more from being counted as blood cells. The twenty plane area (area without micro-cylinders) was selected as a control and the adhered platelets were counted in the same manner as above.

To investigate the change in platelet properties, the platelet numbers before and after the perfusion experiment were measured using a fully automated hemocytometer (Celltac α MEK-6450; Nihon Kohden Co., Ltd., Japan). The platelet aggregatory threshold index (PATI) value, which indicates the ability of platelet aggregation in whole blood, was measured using a platelet agglutination analyzer (Hematracer ZEN; LMS Co., Ltd., Japan). The PATI value was recalculated as NL-PATI = –log(PATI), which is the original index, and the NL-PATI value per unit platelet was calculated to quantitatively express the change in platelet properties in this study [19]. The average change in the NL-PATI value per unit platelet before and after perfusion was measured in four perfusion tests.

2.4 Data analysis

The average number of platelet adhesions per unit area (cell/μm²) around the micro-cylinder area and plane area (control) were calculated and compared. Data were expressed as means ± SE and analyzed using the Student’s t-test. Four experiments were conducted using blood from different bovine.
3. Results

3.1 Properties of platelet aggregation of perfusion blood

Fig. 4 shows the average platelet aggregation per unit platelet (NL-PATI/PLT) for the four perfusion tests. The NL-PATI/PLT value shown in Fig. 4 indicates the ability of platelet aggregation in whole blood per unit platelet for the two types of blood that are pre-shear loaded at 0 s\(^{-1}\) and 10000 s\(^{-1}\). In other words, this value indicates the ability of platelet aggregation of the two types of blood used in the perfusion tests.

The NL-PATI/PLT immediately before perfusion was 0 at shear rates of 0 s\(^{-1}\) and 10000 s\(^{-1}\). The platelet aggregation measuring device indicated that the platelet aggregation level at the pre-shear loading of 10000 s\(^{-1}\) was \(-1.5\) and higher than that at the pre-shear loading of 0 s\(^{-1}\), which was \(-1.75\). On the other hand, the NL-PATI/PLT values of each type of blood immediately after perfusion for 2 h were different, and the NL-PATI/PLT at a pre-shear loading of 10000 s\(^{-1}\) was more than twice that at 0 s\(^{-1}\).

3.2 Platelet adhesion around micro-cylinder area

Fig. 5(a) and (b) show the average number of adhered platelets (ANAP) around each micro-cylinder for the four tests. When the pre-shear loading is 0 s\(^{-1}\), the number of adhered platelets at the fifth column, which is one of the last columns that the blood flow arrives at, is larger than that at the first column. When the pre-shear loading is 10000 s\(^{-1}\), the number of adhered platelets in the center rows tended to be low. Fig. 6 shows the average number of platelet adhesions per unit area, ANAPA (cell/μm\(^2\)) around the micro-cylinder area for each micro-cylinder size, and the plane area. When the pre-shear loading was 0 s\(^{-1}\), the ANAPA did not differ significantly among the micro-cylinders. On the other hand, when the pre-shear loading was 10000 s\(^{-1}\), the ANAPA of the D30H30 micro-cylinder model was significantly higher than that of the other micro-cylinder models. In addition, the ANAPA was the lowest for the small cylindrical model of D10H10 and was significantly lower than that of the other models. There was no significant difference in the ANAPA value between the D10H30 and D30H10 models.

A comparison of the ANAPA value between the two types of platelet properties (0 s\(^{-1}\) and 10000 s\(^{-1}\) of pre-shear loading) indicated that the ANAPA under 10000 s\(^{-1}\) of pre-shear loading was significantly higher than that under 0 s\(^{-1}\) of pre-shear loading in the D30H30 model. In contrast, in the D10H10 model, the ANAPA under a pre-shear loading of 10000 s\(^{-1}\) was significantly lower than that under no pre-shear loading. Furthermore, there was no significant difference in platelet properties between the D10H30 and D30H10 models.

When comparing the ANAPA around the micro-cylinders and the plane area, the D10H10 model had a significantly higher value than the plane area at 0 s\(^{-1}\) of pre-shear loading. At 10000 s\(^{-1}\) of pre-shear loading, the D30H30 model,
the D10H30 model and the D30H10 model had a significantly higher value than the plane area, and the D10H10 model had a significantly lower value than the plane area.

4. Discussion

4.1 Platelet adhesion around micro-cylinders

From the results of NL-PATI/PLT before and after perfusion, it was found that the blood with a pre-shear loading of 10000 s$^{-1}$ for 30 s exhibited approximately twice the platelet aggregation ability as compared to blood with no pre-shear loading. These results mean that the blood with a pre-shear loading of 10000 s$^{-1}$ for 30 s have the ability to trigger high platelet activation. Chen et al. reported nonphysiological shear stress caused significantly more platelets to become activated with increasing shear stress level [20]. It was predicted that the blood with shear loading would have increased platelet activity that was high enough to enable adhesion around micro-cylinders on the material surface.

Furthermore, the results of the adhesion test showed that the change in the adhesion rate with respect to the size of the micro-cylinder was larger in the blood with shear loading at 10000 s$^{-1}$ compared to the blood with no shear loading. The results of our previous study indicated that the physical adhesion rate increased in response to the magnitude of the micro-secondary flow [17]. From the results of the adhesion test, it is predicted that the adhesion phenomenon of platelets is sufficiently activated, and the platelets have a high aggregation ability, with an NL-PATI/PLT ratio of over 0.002; this phenomenon tends to depend on physical effects such as micro-secondary flows.

However, the adhesion of platelets to the smallest micro-cylinder was less in the blood with shear loading at 10000 s$^{-1}$. Activated platelets may selectively adhere to the perimeter of larger structures when micro-structures of different sizes are present. Ruggeri et al. [21] have reported platelets interacting with immobilized von Willebrand factor (VWF) aggregate independently of activation when soluble VWF is present, and the shear rate exceeds 10000 s$^{-1}$. Furthermore, Chan et al. [18] have reported that the size of platelet aggregation in human blood after shear loading was significantly smaller compared to that at a shear loading >6000 s$^{-1}$. In the blood used in our tests, pre-shear loading of 10000 s$^{-1}$ caused not only platelet activation but also a change in platelet aggregate size and the magnitude of platelet aggregation. It is considered that residual platelets after pre-shear loading change their properties and acted as a particle with physical effect than a particle with biochemical effect, compared to platelets with no pre-shear loading. For these reasons, our results with pre-shear loading of 10000 s$^{-1}$ show the same trend as the previous experiment using micro-particles [16, 17]. Micro-particles adhere in response to the size of the micro-cylinder, which is in proportion to the size of the micro-secondary flow.

When blood is exposed to varying shear stress, such as in a blood pump, even if the arithmetic average surface roughness ($Ra$) is the same, the risk of platelet adhesion and thrombus formation on the material surface is higher in the case of a surface with one large micro-structure compared to a surface with micro-structures of the same size on average. Our results indicate that the platelet adhesion increases in proportion to size of micro-structures that is averaged num-
ber of platelet adhesion per unit area of D30H30 is threefold increase compare to that of D10H10, when there is an average platelet aggregation per unit platelet (NL-PATI/PLT) ratio of over 0.002. These results are useful when designing the surfaces of blood contacting materials. The risk of thrombus formation can be reduced by considering the size of the micro-structure that constitutes the surface roughness.

4.2 Study limitations

In this study, bovine blood obtained from a slaughterhouse was pre-shear loaded for 30 s and perfused for 2 h to evaluate platelet adhesion around micro-cylinders. As the next research step, the experimental time of pre-shear loading and perfusion, which affect the ability of platelet aggregation and adhesion around micro-columns, should be considered. In addition, this study only considered micro-cylinder diameters of 10 and 30 μm and heights of 10 and 30 μm. Different sizes, distances between micro-structures, and micro-structure shapes are expected to result in different platelet adhesion performances. The use of blood from different animal species should also be investigated in a future work. Furthermore, in this study, the results were obtained based on in vitro experiments. Investigation of platelet adhesion in vivo may facilitate identification of the threshold of surface roughness.

5. Conclusion

To investigate the influence of surface roughness on antithrombotic properties on blood contacting surfaces, the adhesion phenomenon of platelets to test plates comprising specific micro-structures was investigated. Blood with different platelet properties was used, with a focus on the platelet aggregation ability. The experimental results of the adhesion test indicate that the platelet adhesion is sufficiently enhanced, and the platelets have high aggregation ability, with an NL-PATI/PLT value greater than 0.002. Furthermore, we found that this phenomenon depends on physical effects such as micro-secondary flows, and the platelets may selectively adhere to the perimeter of larger structures when there are micro-structures of different sizes. Our results indicate that the platelet adhesion increases in proportion to size of micro-structures that is averaged number of platelet adhesion per unit area of D30H30 is three-fold increase compare to that of D10H10, when there is an average platelet aggregation per unit platelet (NL-PATI/PLT) ratio of over 0.002. These results are useful when designing the surfaces of blood contacting materials. The risk of thrombus formation can be reduced by considering the size of the micro-structure that constitutes the surface roughness.

Declaration of Conflicting Interests The authors declare that there is no conflict of interest.

Acknowledgements The authors disclose receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by Grants-in-Aid for Scientific Research of Japan [grant number 18J40257].

References

1. Baldwin JT, Borovetz HS, Duncan BW, et al. The national, heart, lung, and blood institute pediatric circulatory support program: A summary of the 5-year experience. Circulation. 2011; 123: 1233–40.
2. Cevik C, Izgi C, Dechyprioum W, Nugent K. Treatment of prosthetic valve thrombosis: rationale for a prospective randomized clinical trial. J Heart Valve Dis. 2010; 19: 161–70.
3. Potapov EV, Stepanenko A, Krabatsch T, Hetzer R. Managing long-term complications of left ventricular assist device therapy. Curr Opin Cardiol. 2011; 26: 237–44.
4. Ogawa S, Richardson JE, Sakai T, Ide M, Tanaka KA. High mortality associated with intracardiac and intrapulmonary thromboses after cardiopulmonary bypass. J Anesth. 2011; 26: 9–19.
5. Zingg W, Neumann AW, Strong AB, Hum OS, Absolom DR. Effect of surface roughness on platelet adhesion under static and under flow conditions. Can J Surg. 1982; 25: 16–9.
6. Chepurov AK, Mertsalova NN, Dubovich TI, Ifashkin GV, Chekanova VD. Effect of roughness of polymer surfaces on thrombus formation. Biomed Eng. 1977; 11: 312–5.
7. Imachi K. Surface modification in the artificial heart. J Surface Finishing Soc Japan. 1995; 46: 907–11.
8. Linneweber J, Dohmen PM, Kertzschker U, Affeld K, Nosé Y, Konertz W. The effect of surface roughness on activation of the coagulation system and platelet adhesion in rotary blood pumps. Artif Organs. 2007; 31: 345–51.
9. Fujisawa N, Poole-Warren LA, Woodard JC, Bertram CD, Schindhelm K. A novel textured surface for blood-contact. Biomaterials. 1999; 20: 955–62.
10. Rose EA, Levin HR, Oz MC, Frazier OH, Macmanus Q, Burton NA, et al. Artificial circulatory support with textured interior surfaces. A counterintuitive approach to minimizing thromboembolism. Circulation. 1994; 90: 1187–91.
11. Chen L, Han D, Jiang L. On improving blood compatibility-From biospired to synthetic design and fabrication for biointerfacial topography at micronano scale. Colloids Surf Biointerfaces. 2011; 85: 2–7.
12. Yamada Y, Nishinaka T, Mizuno T, Taenaka Y, Tatsumi E, Yamazaki K. Neointima-inducing inflow cannula with titanium mesh for left ventricular assist device. J Artif Organs. 2011; 14: 269–75.
13. Miyamoto T, Nishinaka T, Mizuno T, Tatsumi E, Yamazaki K. LVAD inflow cannula covered with a titanium mesh induces neointimal tissue with neovessels. Int J Artif Organs. 2015; 38: 316–24.
14. Ye X, Shaoa YL, Zhoua M, Li J, Cai L. Research on microstructure and hemo-compatibility of the artificial heart valve surface. Appl Surf Sci. 2009; 255: 6686–90.
15. Watanabe N, Affeld K, Schaller J, Schmitteimer S, Reininger AJ, Goubargrits L, et al. Investigation of human platelet adhesion under low shear conditions in a rotational flow chamber. J Biorheol. 2011; 25: 64–70.
16. Oota-Ishigaki A, Masuzawa T, Shibata T. Effect of flow around three-dimensional micro-geometric structures on adhesion phenomena. Lifesupport. 2017; 29: 49–55.
17. Oota-Ishigaki A, Masuzawa T, Nagayama K. Analysis of the effect of the size of three-dimensional micro-geometric structures.
on physical adhesion phenomena using microprint technique. Int J Artif Organs. 2018; 41: 277–83.

18. Chan CHH, Inoue M, Ki KK, Murashige T, Fraser JF, Simmonds MJ, et al. Shear-dependent platelet aggregation size. Artif Organs. 2020; 44: 1286–95.

19. Oota-Ishigaki A, Maruyama O, Sakota D, et al. Quantitative investigation of platelet aggregation under high shear force for anti-platelet aggregation in vitro tests. Int J Artif Organs. 2021; e391398211020765.

20. Chen Z, Mondal NK, Ding J, et al. Paradoxical effect of nonphysiological shear stress on platelets and von Willebrand factor. Artif Organs. 2016; 40: 659–68.

21. Ruggeri ZM, Orje JN, Habermann R, et al. Activation-independent platelet adhesion and aggregation under elevated shear stress. Blood. 2006; 108: 1903–10.