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EFFECT OF SHEAR RATE ON THROMBUS GROWTH

Elham Tolouei
Division of Biological Engineering
Fluids Laboratory for Aeronautical and Industrial Research
Department of Mechanical and Aerospace Engineering
Monash University
Melbourne, Australia, 3800
Email: elham.tolouei@eng.monash.edu.au

Andreas Fouras
Division of Biological Engineering
Fluids Laboratory for Aeronautical and Industrial Research
Department of Mechanical and Aerospace Engineering
Monash University
Melbourne, Australia, 3800
Email: andreas.fouras@eng.monash.edu.au

Josie Carberry
Division of Biological Engineering
Fluids Laboratory for Aeronautical and Industrial Research
Department of Mechanical and Aerospace Engineering
Monash University
Melbourne, Australia, 3800
Email: josie.carberry@eng.monash.edu.au

ABSTRACT
In this study micro and nano scale measurement techniques are applied to platelet studies and determination of factors in platelet aggregation and thrombus formation. Conventionally it has been assumed that platelets are stimulated by blood clotting factors and platelet activators to aggregate and form a thrombus at sites of vascular injury. We have recently shown that a primary factor in initiating platelet aggregation is hemodynamic shear. This paper presents the effect of shear rate on the time evolution of thrombus formation and the final geometry of a mature thrombus. A relationship between maximum mature thrombus height and local shear rate is formulated. We have shown that the shear rate is not only an important factor in initiating platelet aggregation but is also one of the main inhibitors of platelet aggregation and thrombus formation. We propose that when the platelets reach a critical height, they encounter a specific local hemodynamic range, which prevents further thrombus growth.

INTRODUCTION
Developments in micro and nano scale techniques over the past decade are now being applied to aid our understanding of blood flow within the human body and treatment of blood diseases. Several important physiological and pathological phenomena occur in the microcirculation, the effect of flow behavior on blood cell function has become an important field of study in micro scale research. In platelet studies it is very important to understand the detailed flow fields near the wall as the high shear rates and changing hemodynamic forces in this region are believed to affect platelet activation, adhesion and thrombus formation.

A thrombus or blood clot is a platelet plug, which is an essential part of the response to vessel injury. However, pathological thrombus formation plays a central role in arterial diseases leading to heart attacks or stroke[1-3]. A number of key variables regulating platelet function have been recognized for more than a century including the endothelial layer, stimulation of thrombogenic factors and hemodynamic forces. The roles of the first two factors have been extensively investigated[4-6], however the effect of hemodynamic parameters on platelet aggregation has been not well defined. Conventional models assume that platelet activation and aggregation are promoted by secretion of chemical substances (soluble agonist) from the platelets or damaged vessel wall, leading to platelet shape change and activation[7-8]. In contrast, our recent experimental study examined flow around thrombi revealed that the initial growth of platelet aggregate is more reliant on the local hemodynamic conditions than previously anticipated[9].

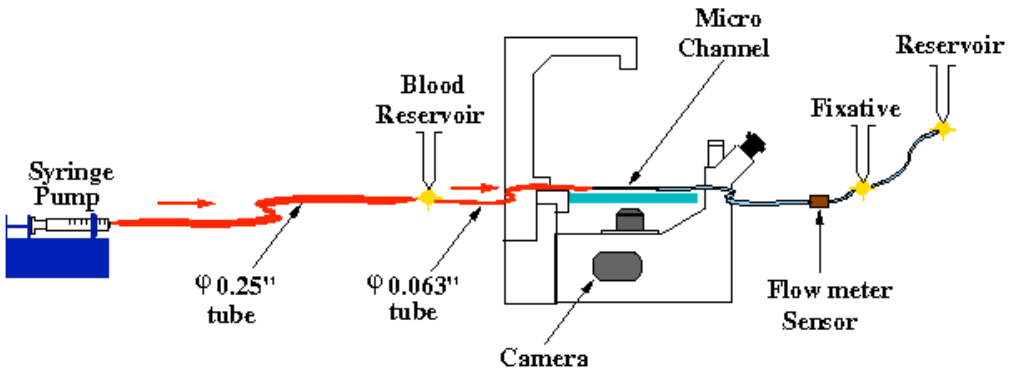

FIGURE 1. SCHEMATIC SHOWING EXPERIMENTAL SETUP FOR PREPARING THROMBI.

Platelets are exposed to a broad range of hemodynamic conditions *in vivo*, ranging from low mean shear rates downstream of existing thrombi or bifurcations to high mean shear rate at sites of abrupt reduction in vessel cross sectional area (or stenosis). Depending on the local shear rate thrombi can either continue to grow or diminish and fragment into the circulation. Thrombus formation is a dynamic process with cycles of aggregation and disaggregation of platelets depending on shear rate[10-11].

Platelet recruitment around an existing thrombus generally occurs at the rear of the thrombus, corresponding to the region where the local shear rate is low. Importantly, the platelets experience a high shear rate as they move around the side of the thrombus before reaching the lower shear environment at the rear of the thrombus. It has been observed that initial platelet recruitment to the surface occurs at the high shear region. These initial interactions are always unstable resulting in rapid platelet translocation to the trailing edge of the thrombi. As a consequence, more than 75% of stable discoid platelet aggregation occurs within the low shear region behind the thrombi. These factors provide favorable conditions for further platelet adhesion and aggregation in the region behind the thrombus[9,12-13].

Previous research has shown at least three distinct mechanisms of platelet aggregation at different shear rate zones[14-18]. These mechanisms are classified as biochemical stable aggregation (at low shear conditions: <1000 s^{-1}), discoid aggregation (at high shear conditions: 1000-10000 s^{-1}) and rolling aggregation (at pathological shear conditions: >10000 s^{-1}). Likewise, the local shear rate changes during thrombus formation play an important role in regulating platelet deposition or cessation of platelet aggregation. However, the precise mechanisms by which local flow properties affect platelet adhesion and aggregation are not well understood.

The objective of this work is to investigate the shear rate effects on the evolution of thrombus formation, specifically that thrombus growth is limited by a critical shear rate threshold. A number of thrombi were prepared at different bulk shear rates and exposure times to measure the effect of these parameters on the three-dimensional thrombus geometry. Exposure time is defined as the total amount of time that blood perfuses inside the micro channel where platelets are exposed to shear conditions and available to aggregate. Steady flow is assumed inside the micro channel with the bulk shear rate $\gamma_z = \partial u/\partial z$ calculated using a Poiseuille flow profile, $\gamma_z = 6Q/wh^2$, where Q is the flow rate and w and h are the channel width and height, respectively. The theoretical Poiseuille flow equation is used to estimate the shear rate at the wall and for all z-positions. We further estimate that the shear rate at the apex of thrombus is represented by the bulk shear rate at that height.

EXPERIMENTAL METHODOLOGY

Fig.1 illustrates a schematic of the experimental setup, which consisted of an inverted microscope (DM IRB, Leica microscope, Germany), a syringe pump (Harvard Apparatus, MA, USA), a camera and connecting tubes. The thrombi were prepared inside a glass micro channel by coating the micro channel with collagen type I (10 μg/ml; to promote platelet adhesion on glass) and washed out immediately with Tyrode's buffer. To generate steady flow in the micro channel blood infuses through a 69cm long, 6.35mm (0.25inches) diameter tygon tube then a 7cm section of 1.6mm (0.063inches) diameter of silicon tube before entering the micro channel. The flow rate was confirmed as constant by a flow meter just after the micro channel during perfusion. Micro channel cross-section is 2mm×0.2mm and 100mm long (Vitrotubes, VITRO COM, Mountain Lakes, NJ, USA) where the reference axes x, y and z are along length, width and height of the micro channel. The direction of the upstream flow is parallel to the unit vector x. Whole blood (anticoagulated with Hirudin) obtained from healthy human donors was perfused through the coated micro channel using the syringe pump at a constant flow rate to enable interaction of platelets with the coated matrix. Thrombus formation was monitored by the microscope equipped with a ×63 water immersion objective, NA 1.2, and the camera for video recording and online checking. Once the pump was running for a specified time the blood flow was stopped and the thrombi were fixed using formaldehyde solution.

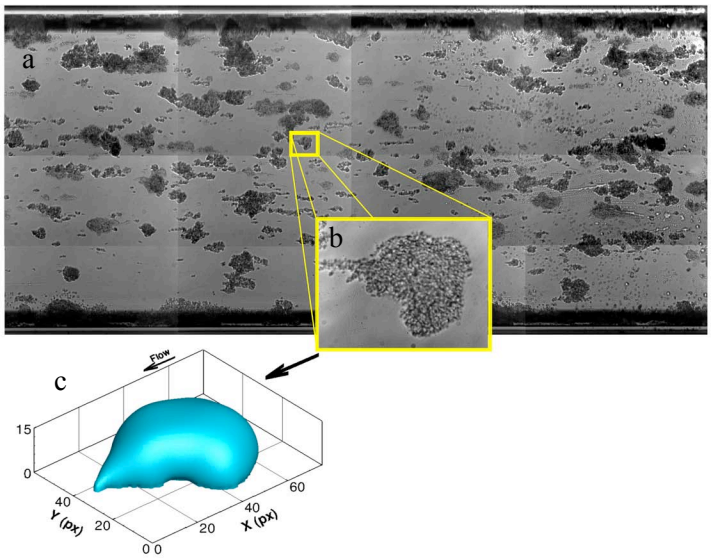

FIGURE 2. a) TOP VIEW OF FIXED THROMBI INSIDE A GLASS MICRO CHANNEL. b) ZOOMED VIEW OF A THROMBUS WITH 40× OBJECTIVE. c) THREE-DIMENSIONAL SHAPE OF THE MEASURED THROMBUS

The geometry of the prepared fixed thrombi was measured using a high spatial resolution bright field scanning microscopy technique developed in-house. The glass micro channel with fixed thrombi was placed horizontally on a microscope equipped with a vertical stage with resolution of 0.1 µm. A camera (MotionPro X3, Redlake Inc., USA) was connected to the outlet port of the microscope and a 2.5× adapter to allow full utilization of the camera sensor. The whole micro channel was mapped at low resolution to estimate the thrombi location inside the micro channel (×5 dry objective, NA 0.15; Fig. 2a). Thrombi were selected on the basis of their location (far from the micro channel wall) and the three-dimensional geometry of each thrombi measured. The number of selected thrombi were 20, 31 and 34 thrombi at shear rate 1200, 1600 and 1800 s^{-1}, respectively and 15, 20, 25, 23, 26 and 28 thrombi at exposure times between 2.5 to 10 min, respectively. To generate three-dimensional geometries two-dimensional bright field images were captured using a ×40 dry objective, NA 0.5 (Fig. 2b) at different parallel xy-planes from the bottom wall (z=0.0) up to the maximum thrombus height with scanning step of 1µm. All images were analyzed with a MATLAB code to develop an average three-dimensional geometry.

RESULTS AND DISCUSSIONS

The present technique offers the ability to measure thrombus geometry developments in response to known upstream flow conditions and exposure times. This allows us to study the effects of velocity changes on thrombus growth.

This report investigates platelets aggregation mechanisms through two approaches: one is to measure the maximum height of mature thrombus as a function of upstream bulk shear rate where the maximum height of mature thrombus is defined the height at a sufficiently long time (10 min) such that the thrombus has stopped growing. The other is to identify the evolution of thrombus growth by measuring the thrombus geometry at different exposure time.

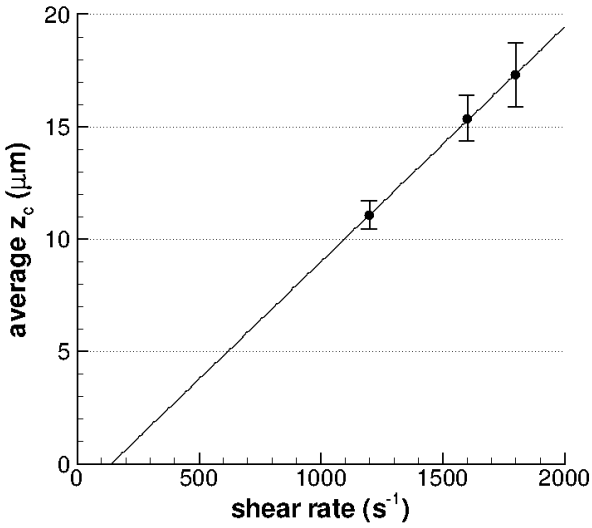

FIGURE 3. AVERAGE THROMBUS CENTROID HEIGHT AT THREE DIFFERENT SHEAR RATES. ERROR ANALYSIS WAS PERFORMED USING THE STUDENT T-TEST WITH 90% CONFIDENCE INTERVAL (N=20, 31, AND 34 FOR SHEAR RATE 1200, 1600 AND 1800 s^{-1}, RESPECTIVELY).

Fig. 3 shows the correlation between the mature thrombus centroid height (z_c) and shear rate. The centroid height is defined as the weighted-average location of all mass points within the thrombus ($z_c = \sum_{i=1}^{k} V_i z_{c_i} / \sum_{i=1}^{k} V_i$, where V_i is the volume of object i, z_{c_i} is the z-location of the centroid of the object i, and k is the total number of small volumes within the thrombus). The centroid height was measured at three bulk shear rates (1200, 1600 and 1800 s^{-1}) and grows linearly with increasing bulk shear rate. It was observed that not only the thrombus height decreased with decreasing the shear rate, but also the density of thrombi forming on the micro channel surface decreased with decreasing the shear rate (data not shown). The intersection of the linear growth with the x-axis calculated to be around 150 s^{-1}. Results within shear rates 1200 s^{-1} and 1800 s^{-1} are consistent with the centroid height decreasing linearly with decreasing shear rate. If this trend persists at lower shear rates then the minimum required shear rate for platelet aggregation will be approximately 150 s^{-1}. Further investigations are required to determine the minimum threshold value of shear rate, which can contribute to platelet activation and aggregation.

Previous results showed that platelets travelling around a growing thrombus experience a unique shear sequence[12]. First the platelets encounter a region of high shear rate at the top and

sides of the growing thrombus, followed by a region of low shear rate downstream of the thrombus. It was found that a high shear zone followed by a low shear zone is a requirement for a discoid platelet aggregation and thrombus formation[9]. Based on this finding it is expected that different bulk shear rates result in different local shear rate histories, which affect the platelet aggregation. It is anticipated that high upstream bulk shear rate may produce more effective local shear history and induce more platelet aggregation and higher thrombus height.

In the second part of the experiment, the time history of thrombus growth was studied at a bulk shear rate of 1800 s^{-1} by allowing platelets to aggregate for a range of exposure times ranging from 1.5 to 10 min. Measured centroid heights at different exposure times is presented in Fig. 4. At the first two exposure times (1.5 and 2 min) no thrombi were formed inside the micro channel and only a few monolayer platelet aggregations were observed. The centroid height increases with increasing the exposure time. Thrombus height rises more steeply at the beginning of the formation process and then remains stable (Fig. 4 & Fig. 6). The variation in thrombus centroid height, as described as a standard deviation increases with increasing the exposure time except for the first exposure time, which the error is more than next two exposure times. The increased error at the first exposure time can be explained by the fact that at this exposure time a few thrombi developed during the perfusion time.

To study the evolution of thrombus geometry, an average three-dimensional geometry was determined as a function of exposure time and shear rate. To find the average three-dimensional geometry an ellipse was fitted to all thrombi at 100 z positions using a least square methods. Major and minor axes of the fitted ellipse at each z position were averaged between all thrombi at each exposure time and shear rate. Major and minor axes were defined to be parallel and perpendicular to the upstream flow, respectively. The ratio of average major to minor axis, called the geometry ratio, was found to be constant in all cases. These geometry ratios were again averaged between thrombus base (z=0.0) and 80% of maximum height at each exposure time and shear rate (called average geometry ratio shown at Fig. 5). Above 80% of maximum height the thrombi become milled and erratic reducing the validity and accuracy of the fitting process.

At all shear rates the average geometry ratio of the mature thrombi (exposure time 10 min) is almost constant, around 1.45±0.45. However, the average geometry ratio grows gradually with increasing exposure time; from 1.2 at 2.5 min to 1.45±0.45 at 10 min.

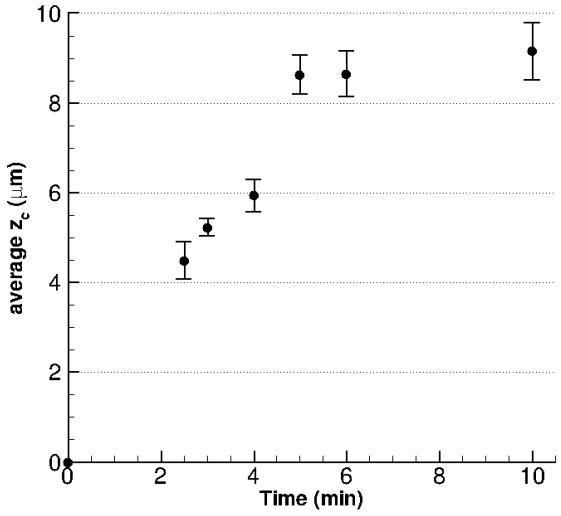

FIGURE 4. AVERAGE THROMBUS CENTROID HEIGHT AT DIFFERENT EXPOSURE TIME. ERROR BARS REPRESENT THE CONFIDENCR LIMIT WITH 90% CONFIDENCE INTERVAL (N=15, 20, 25, 23, 26, AND 28 FOR EXPOSURE TIMES FROM 2.5 MIN TO10 MIN, RESPECTIVELY).

Sample thrombi geometries are shown in Fig. 6 for a range of exposure times at a shear rate of 1800 s^{-1}. The results show that at early stages in thrombus growth, the thrombus cross section area is inclined to be more circular in shape and as the thrombus grows, the cross section area becomes more elliptical (Fig. 5 & 6). It is consistent with the previous findings that platelet recruitment occurs downstream of the thrombus where platelets pass through the high shear zone and enter the low shear zone.

Platelets flowing around a thrombus experience first a locally high shear followed by low shear in the region towards the back of the thrombus. It is believed that this sequence of shear variation is essential for platelet aggregation. However, the exact shear values required for platelet aggregation are not well understood. As the geometry of the growing thrombus changes the shear experienced by the platelets flowing over it will be altered. It appears that in this way the geometry of the thrombus plays a role in regulating platelet deposition and further thrombus growth. Table 1 summarizes a three-dimensional geometry of an average mature thrombus and shear rate magnitude at the apex of the thrombus for three different bulk shear rates. It is evident that the geometry of a mature thrombus depends on upstream bulk shear rate and the thrombus height increases with increasing the bulk shear rate.

The magnitude of the shear rate at the thrombus apex at all bulk shear rates is nearly constant, ranging between 900 to 1000 s^{-1}. This indicates that thrombi grow until reaching a critical height, which depends on the magnitude of local shear rate.

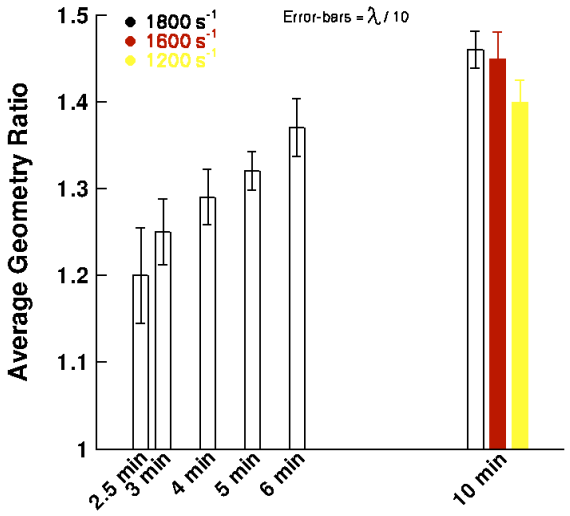

FIGURE 5. NORMALIZED GEOMETRY RATIO AT DIFFERENT BULK SHEAR RATES AND EXPOSURE TIME. λ IS THE CONFIDENCE LIMIT.

CONCLUSION

Advances in micro scale technology and novel interdisciplinary approaches have allowed us to analyze platelet aggregation processes and consider the hemodynamic forces, not only as a primary factor in platelet activation, but also a main inhibitor in thrombus formation process.

Investigation of thrombus geometry showed that the mature thrombus height grows linearly with increasing the bulk shear rate. The examination of the temporal analysis of thrombus evolution revealed that the thrombi grow from the rear part of the initial aggregation with the majority of aggregation occurring within the first 5 mins. The thrombus cross sectional shape shows a gradual adjustment of the initial circular cross-section to the elliptical cross section proving that platelets have a higher tendency to aggregate at the rear part of the existing thrombus where the shear rate is low. This finding is consistent with previous literature showing that platelet recruitment occurs at the back of the thrombus.

Our results predict that the local shear rate is not only an important factor in initiating platelet aggregation but is also one of the main inhibitors of platelet aggregation and thus controls the final height of mature thrombus. When the platelets reach a certain height, they encounter a critical local shear rate preventing further platelet aggregation.

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REFERENCES

1. Ross, Z., Glomset, J.A., 1976. "Pathogenesis of atherosclerosis". *New England Journal of Medicine*, **295(7)**, pp. 369-377.
2. Ruggeri, Z. M., 2002. "Platelets in atherothrombosis". *Nature Medicine*, **8**, pp. 1227-1234.
3. Huo, Y.Q., Ley, K.F., 2004. "Role of platelets in the development of athersclerosis". *Trends in Cardiovascular Medicine*, **14(1)**, pp. 18-22.
4. Ruggeri, Z. M., Mendolicchio, G. L., 2007. "Adhesion mechanism in platelet function". *Circ. Res.*, **100**, pp. 1673-1685.
5. Ruggeri, Z. M., 2007. "The role of von willebrand factor in thrombus formation". *Thromb. Res.*, **120**(1), pp. S5-9.
6. Kato, K., Kanaji, T., Russell, S., Kunicki, T. J., Furihata, K., Kanaji, S., Marchese, P., Reininger, A., Ruggeri, Z., M., Ware, J., 2003. "The contribution of glycoprotein VI to stable platelet adhesion and thrombus formation illustrated by targeted gene deletion". *Blood*, **102**, pp. 1701-1707.
7. Pallister, C., 1994. "Blood physiology and pathophysiology". *Butterworth-Heinemann Ltd*.
8. White, J. G., 1998. "Platelet membrane ultrastructure and its changes during platelet activation". *Prog Clin Biol Res*, **283**, pp. 1-32.
9. Nesbitt, W. S., Westein, E., Tvar-Lopez, F., J., Tolouei, E., Mitchell, A., Fu, J., Carberry, J., Fouras, A., Jackson, S., P., 2009. "A shear gradient-dependent platelet aggregation mechanism drives thrombus formation". *Nature Medicine*, **15**(6), pp. 665-673.
10. Falk, E., 1985. "Unstable ungina with fatal outcome: dynamic coronary thrombosis leading to infarction and/or sudden death. Autospy evidence of recurrent mural thrombosis with peripheral embolization culminating in total vascular occlusion". *Circulation*, **71**, pp. 699-708.
11. Jackson, S. P., Nesbitt, W. S., Westein, E., 2009. "Dynamics of platelet thrombus formation". *Journal of thrombosis and Haemostasis*, **7**, pp. 17-20.
12. Tolouei, E., Nesbitt, W.S., Fouras, A., Carberry, J., 2008. "A high spatial method to determine three-dimensional velocity gradient tensor using micro particle image velocimetry". *Proc 14th International Symposium on Applications of Laser Techniques to Fluid Mechanics*, Lisbon, Portugal, 7-10 July 2008.
13. Tolouei, E., Fouras, A., Carberry, J., 2009. "In vitro micro PIV measurements of velocity profiles near a wall". *The 8th international symposium on Particle Image Velocimetry*, Melbourne, Australua, August 2009.
14. Jackson, S. P., 2007. "The growing complexity of platelet aggregation". *Blood*, **109**, pp. 5087-95.
15. Ruggeri, Z. M., Orje, J. N., Habermann, R., Federici, A. B., Reininger, A. J., 2006. "Activation-independent platelet adhesion and aggregation under elevated shear stress". *Blood*, **108**, pp. 1903-10.
16. Kulkarni, S., Dopheide, S. M., Yap, C. L., Ravanat, C., Freund, M., Mangin, P., Heel, K. A., Street, A., Harper, I. S., Lanza, F., Jackson, S. P., 2000. "A

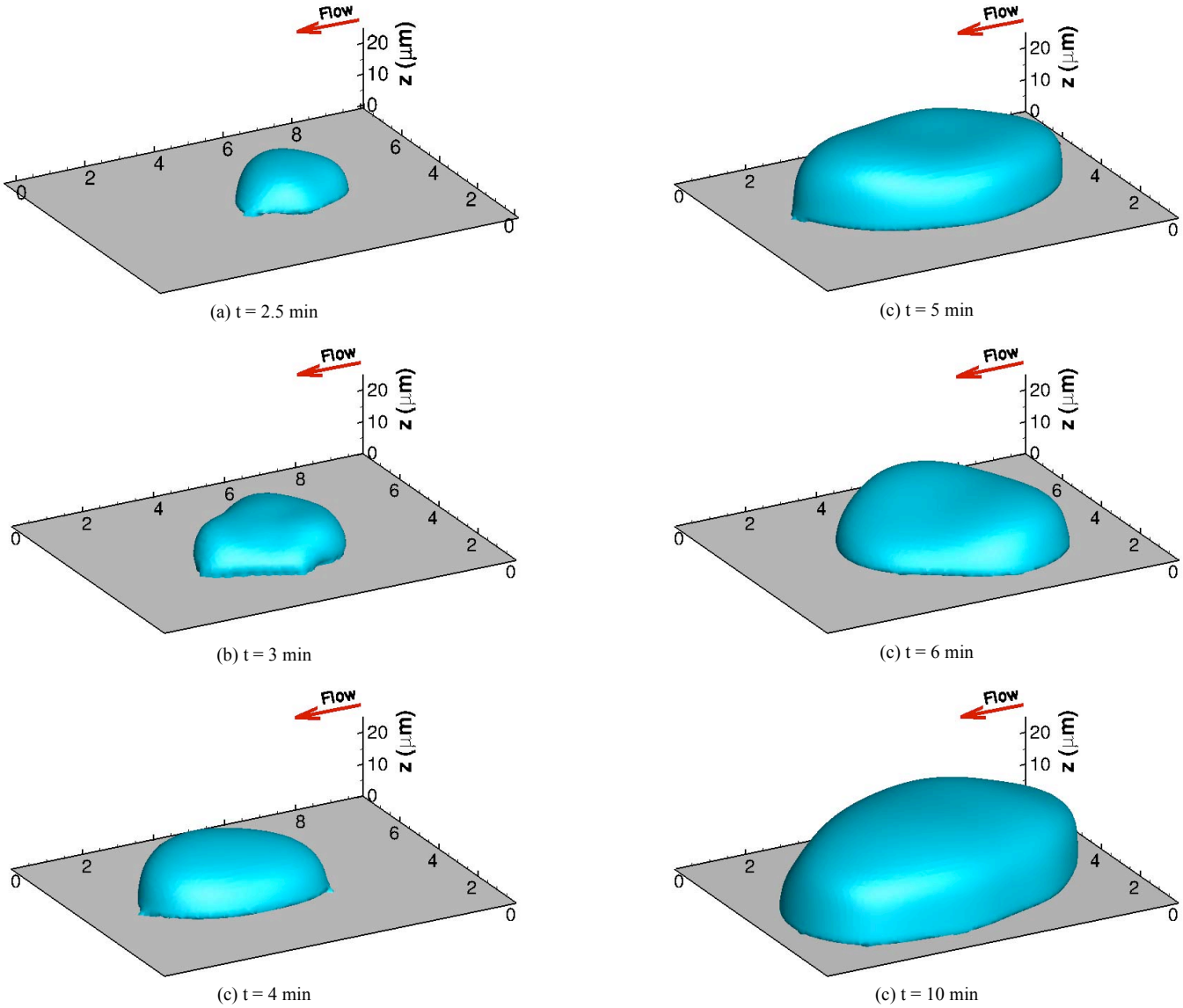
revised model of platelet aggregation". *J. Clin Invest*, **105**, pp. 783-91.

17. Maxwell, M. J., Westein, E., Nesbitt, W. S., Giuliano, S., Dopheide, S. M., Jackson, S. P., 2007. "Identification of a 2-stage platelet aggregation process mediating shear-dependent thrombus formation". *Blood*, **109**, pp. 566-76.

18. Ikeda, Y., Handa, M., Kawana, K., Kamata, T., Murata, M., Araki, Y., Anbo, H., Kawai, Y., Watanabe, K., Itagaki, I., 1991. "The role of von willebrand factor and fibrinogen in platelet aggregation under varying shear stress". *Blood*, **109**, pp. 566-76.

TABLE 1. AVERAGE GEOMETRY AND LOCAL SHEAR RATE AT THROMBUS APEX

MAX THEORETICAL WALL SHEAR RATE (s^{-1})	MAX AVERAGE THROMBUS HEIGHT (μm)	MAX SEMI MAJOR AXIS (μm)	MAX SEMI MINOR AXIS (μm)	SHEAR RATE AT THROMBUS APEX (s^{-1})
1200	27.11	3.42	2.44	900.00
1600	40.59	2.97	2.06	950.55
1800	45.63	2.65	1.78	978.66



(a) t = 2.5 min

(c) t = 5 min

(b) t = 3 min

(c) t = 6 min

(c) t = 4 min

(c) t = 10 min

FIGURE 6. TIME HISTORY OF THREE DIMENSIONAL MODEL OF SAMPLE THROMBI AT SHEAR RATE = 1800 S^{-1}.