THERMAL CHARACTERISTICS MEASUREMENT OF FIBRIN REACTION AND CLOT FORMATION IN VENOUS THROMBUS USING MICROCHANNEL FLOW

**K. Tatsumi[1*], Y. Yamamoto[1], A. Yanamoto[1],
H. Hirakata[2], N. Sugita[3], K. Inoue[4], R. Kuriyama[1], K. Nakabe[1]**

[1]Kyoto University, Kyotodaigaku-katsura, Nishikyo-ku, Kyoto 615-8540, Japan
[2]Kyoto City Hospital, Mibuhigashitakadacho 1-2, Nakagyo-ku, Kyoto 604-8845, Japan
[3] Psychiatry, Kyoto University, Yoshida-konoemachi, Sakyo-ku, Kyoto 606-8501, Japan
[4] Primary Care & Emergency, Kyoto University, Yoshida-konoemachi, Sakyo-ku, Kyoto 606-8501, Japan

ABSTRACT

Temperature effects on the coagulation time and reaction rate coefficient of fibrin based thrombus formation were measured in this study. Coagulation time, which is the time required for the thrombus and fibrin clot to form, were evaluated using microchannel flow, micro glass capillary flow, and cuvette measurements. Fluorescence labeling technique based visualization of platelets, plasma and fibrin performed in the microchannel measurement showed that the fibrin fibers growing from the activated platelets play a major role in the initial stage of thrombus formation for low shear rate flow conditions of vein. Thermal characteristics of the fibrin generation reaction, therefore, becomes one of the key factors for thrombus formation rate in blood flow in this case. Temperature affected the fibrin formation characteristics and the coagulation time showed the minimum value at temperature of 32-42°C in the cases of with and without flows. A first order reaction model was applied to curve fitting with the time distribution of fibrin concentration obtained by the cuvette measurements to derive the reaction rate of the fibrin generation. Reaction coefficient increased as the temperature increased in the range of 24°C-37°C showing that the decrease of the coagulation time of the fibrin clot in this temperature range was attributed to faster fibrin generation reaction.

KEY WORDS: Thrombus formation, Fibrin, Fibrinogen, Temperature effects, Microchannel flow, Reaction rate

1. INTRODUCTION

Thrombus formation and fibrinolysis are affected by various external and internal factors [1-2]. The internal factors basically follow the biological reaction cascade shown in Fig. 1. The external factors are related to damage or scleriasis of the blood vessel wall, flow shear stress particularly generated at the wall, flow stagnation, infections, constituent concentration of blood, and temperature. The effects of these external factors on the thrombus formation itself has been carefully studied in vivo and vitro, however, mostly by measuring the bulk properties and not in scale of single cells. Further, the effects of these factors on the thrombus formation and detachment from the wall under flow condition are not clear yet [3]. Therefore, it is necessary to perform measurement and analysis of the diffusion coefficient, reaction coefficient, and the release and attachment of thrombolysis and fibrinolysis factors at the cell surfaces. Among the external effects, temperature effect is one important factor to be systematically and quantitatively evaluated. However, although empirical numbers and conditions are provided and carefully examined by referring the clinical cases particularly in blood tests, the fundamental physics is not clear.

*Corresponding Author: tatsumi@me.kyoto-u.ac.jp

One may say that the temperature in human body is constant and uniform. However, this is not the case for patients in the hospital and having surgical operation. For example, during the cardiac operation, the blood is circulated using the artificial heart and lung (pumps and filters). The temperature of the blood supplied from these equipment are set as 33~35°C which is lower than human average body temperature in order to prevent damages in the brain. Further, the blood temperature can decrease to 25°C owing to the hypothermia which is attributed to the body heat loss and the anesthesia of the surgery [4]. The temperature can easily drop by 1°C even by the avascularization since the blood temperature of the certain part with treatment of blood stanching can decrease close to room temperature. Hyperthermia can also increase the temperature of the surrounding organs, blood vessels and cells [5]. The temperature difference can either increase the probability of thrombus generation or impair the hemostasis [6].

Thrombus formation in artery and vein can lead to serious symptoms and complications. For example, venous thromboembolism (VTE) is one significant issue in blood flow in terms of the fact that it can lead to deep venous thrombosis and pulmonary embolism (PE). Sudden death is the first symptom appearing in about one-quarter (25%) of people who have a PE. Death rate is considerably high, and the number of mortality related to PE in United States is estimated to be 60,000-100,000/year (U.S. Center for Diseases), in European Union 126,145/year [7, 8], and in Japan 7,864/year [9]. The condition at which VTE and PE are caused is still not clear yet particularly in the blood flow. In order to prevent these issues, we need to know how the external factors will affect the thrombus generation in addition to medication. Evaluating the thermal characteristic of thrombus formation, therefore, can contribute to establish guidelines for what treatment and under which conditions the patient should be served.

In this study, measurement of thrombus formation in microchannel flow, micro-capillary flow, and cuvette system are performed to understand the effects of the temperature on the thrombus formation. Measurement is carried out using microchannel flow to understand the mechanism of the venous thrombus formation in cellular size level. Platelets and fibrin fiber patterns in the thrombus are visualized by fluorescence labeling techniques. Further measurement with the flow accompanied case is done by using the micro-capillary and evaluating the change in the flow rate under constant pressure gradient flow condition. The cuvette system is a coagulation measurement equipment in which the time required for the fibrin clot formation in the plasma is measured by measuring the transmitted light intensity under stirring and stationary fluid conditions. We will discuss the temperature effects on the thrombus – especially the fibrin clot – coagulation time and fibrin reaction rate under the low shear rate condition close to that of flow in vein.

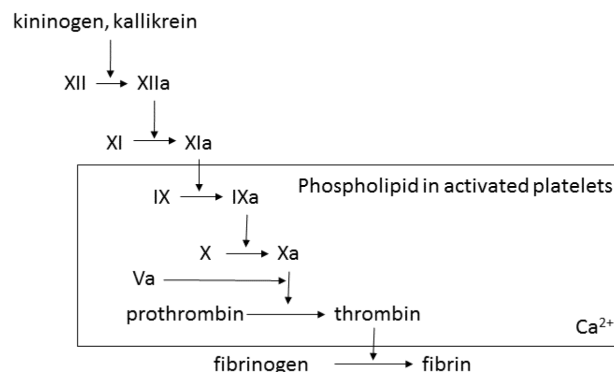


Fig. 1 Reaction pathway for fibrin generation in blood. Various pathways exist in relation to the factors affecting the reaction.

2. SAMPLE PREPARATION AND EXPERIMENTAL METHODS

Blood were collected from healthy informed volunteers. Blood was anticoagulated by mixing 3.8wt% sodium citrate with the volume ratio of 10%. The sample was then separated to platelet rich plasma (PRP), platelet poor plasma (PPP), and highly concentrated red blood cells (red cell concentrate: RCC). These samples (PRP, PPP and RCC) were cooled with ice until they were mixed just before carrying out the

experiments using microchannel, micro-capillary and cuvette-type systems. The blood was, thus, separated and mixed to control and keep the hematocrit of the red blood cells constant for the measurement.

Figure 2 shows the schematics of the experimental apparatuses. Microchannel measurement was first conducted to evaluate the thrombus formation mechanism in the near wall region under the flow condition similar to that of blood flow in vein. Fluorescence labeling measurement was performed in the case to understand the role of the platelets and fibrin fibers in the thrombus formation. Further, thrombus formation time (coagulation time) t_c was measured by evaluating the variation in the time distribution of the flow rate as the flow was supplied under constant pressure difference. Temperature effects on t_c was then evaluated. In addition to the microchannel flow measurement, glass capillary tube measurement was carried out to further evaluate the temperature effects on t_c. As will be discussed in Section 3.1, we made the capillary tube measurement in order to increase the number of measurements and conditions. In this case, the coagulate time t_c was measured in the same way as in the microchannel measurement by evaluating the flow rate.

Figures 2 (a) and (b) show the schematic of apparatus for microchannel and capillary tube type measurements. The width and height of the microchannel were 300μm and 132μm, respectively. The microchannel was fabricated with PDMS using the photolithography and soft lithography processes. The microchannel was placed on a temperature controlled stage and thermocouples were embedded in the PDMS part of the microchannel on both sides of the channel with the distance of 1.5mm from the channel centerline. The temperature variation among the stage and these thermocouples were less than ±0.17°C. The average value of these temperatures were referred to as the temperature of the microchannel.

The inner diameter and length of the glass capillary tube (Fujirika Co.: glass capillary) were 200μm and 120mm, respectively. The glass tube was placed in a groove machined on the temperature-controlled block. The temperature of the heated block was measured by the thermocouples inserted in the holes drilled in the block at the two locations depicted in Fig. 2 (b). The temperature difference between these two positions were less than ±0.2°C, and the average value of these temperatures was referred to as the tube temperature.

The wall of the capillary tube and microchannel were first coated by Sigmacote (Sigma-Aldrich Co.) to prevent the adhesion of the blood cells to the wall. Collagen was then coated on the walls in the downstream region of the channel. This area represents the damaged blood vessel in which the collagen layer is exposed to the blood and enhance the thrombus formation.

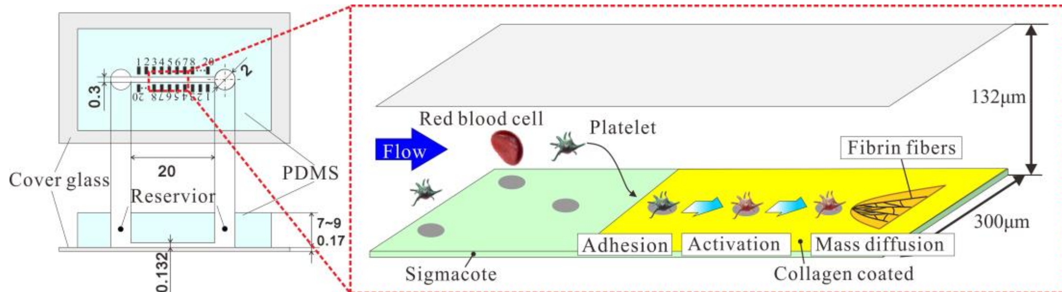
Fluid (blood sample) were supplied to the capillary tube and microchannel by a pressure controlled flow pump with flow rate of 1.4μL/min and 1.3μL/min, respectively. Fluid was driven under constant pressure gradient, and the flow rate was measured using the micro flow sensor (Sensirion Co.: LG16-0025).

Fluorescence labeling was conducted for the microchannel measurements to visualize the platelets attached to the wall and the formation of fibrin fibers. Fluorescein Quinacrine dihydrochloride(Santa Cruz Biotechnology Inc., SC-204222A) and Fluorescein isothiocyanate-dextran were used to label the platelets and plasma, and fibrinogen-Alexa Fluor R546 (ThermoFisher Co.) was used to label the fibrin, respectively. The composition of blood sample supplied to the microchannel, thus, was RCC 112.5μL, PPP 100μL, WP 25μL, Ca(1M/L) 4.0μL, alexa Four546(1.5mg/mL) 2.0μL, Quinacrine(5mM/mL) 5.0μL, Fluorescein isothiocyanate-dextran 1.0μL. The composition of blood sample in capillary tube case was RCC 56.25μL, PPP 50μL, WP 12.5μL, Ca(1M/L) 2.0μL.

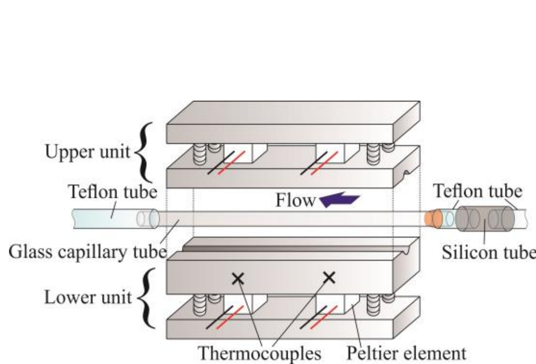
As will be indicated in Section 3.1, fibrin generation showed an important role in the thrombus formation in the microchannel flow. To discuss the temperature effects on the thrombus formation in more detail, we measured the coagulation time t_c and the reaction rate of fibrin generation using the cuvette-type system shown in Fig. 2 (c). The cuvette was placed in a temperature control unit and the magnetic stirrer mixed the fluid in the cuvette by rotating the stirring bar immersed in the fluid. Light of 417nm wavelength generated by the LED was irradiated to the sample. We measured the intensity I of the light transmitted to the other side of the cuvette by a spectrometer (OceanOptics Co.: FLMS01061).

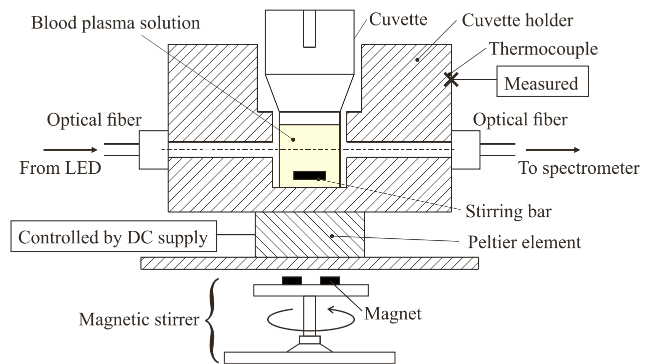
To measure the temperature characteristics of fibrin and fibrin clot formation in plasma and excluding the effects of other blood cells, PPP of 70μL only was measured by the cuvette system. The sample was first incubated for 1min at 37°C with the stirring bar rotated at 300rpm. Thrombin (Sysmex Co.: Multifibrin U)

was added after the incubation while the stirring bar was kept rotating. We measured the moment when the thrombin was added and the fibrin clot was generated by detecting the change of *I*. The time between these moments was defined as the coagulation time t_c. In addition to this, time distribution of *I* under steady state flow condition (without stirring process) was measured and evaluated to obtain the reaction coefficient of fibrin generation. Detail of the measurement procedure and principle for the reaction coefficient will be shown in Section 3.2.



(a) Microchannel and the schematic of fibrin fiber formation at the channel wall





(b) Glass-capillary-tube-type measurement system (c) Cuvette-type measurement system

Fig. 2 Measurement apparatus of the microchannel, micro-glass capillary tube, and cuvette-type fibrin coagulation measurement system. (a) Microchannel measurement visualizes the fibrin fiber pattern by applying fluorescence labeling, and detects the clot formation by measuring the flow rate. (b) Micro-glass capillary measures the change in flow rate of the blood flow in the capillary driven under constant pressure. (c) Cuvette-type system detects the fibrin clot formation and measures the fibrin concentration by measuring the transmitted light intensity.

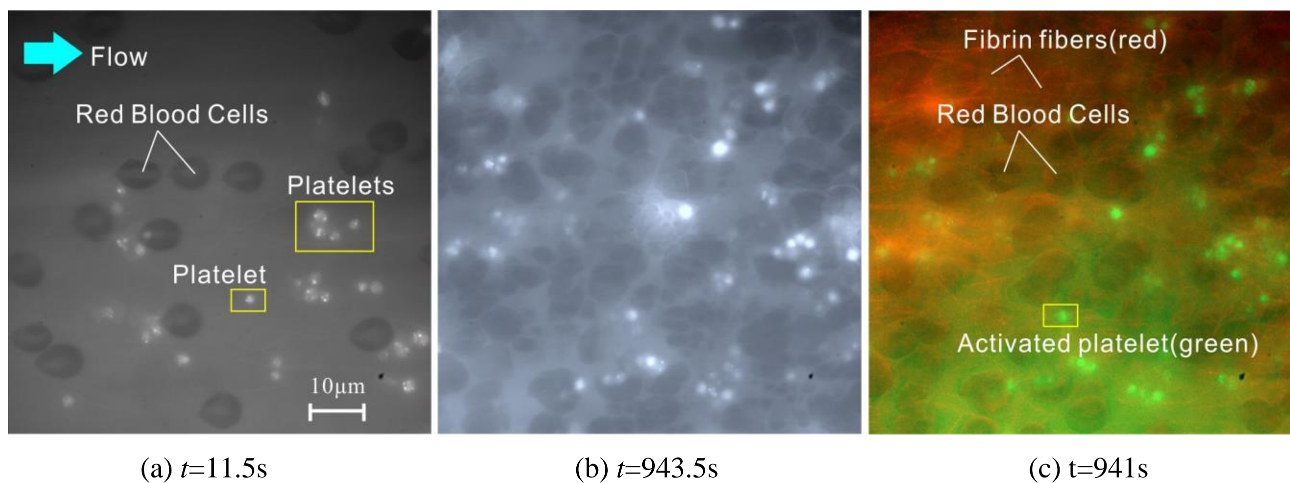
3. RESULTS AND DISCUSSION

3.1 Coagulation time in microchannel and micro-glass capillary flows

To understand the mechanics and process of thrombus formation in flow under the conditions of the shear rate similar to that in vein, visualization of platelets and fibrins were performed using fluorescence labeling techniques in microchannel flows. Figure 3 shows the images of the red blood cells, platelets and fibrins measured at the channel bottom wall. Measurement was made at the location 1mm downstream from the leading edge of the collagen-coated region. Figures 3(a) and (b) show the results of fluorescence visualization of the platelets and blood plasma at the time period of t=11.5 and 943.5s, respectively. t is the time after the blood was supplied to the microchannel. It should be noted that blood with the components of WP (washed platelets), PPP and RCC was used and Ca^{2+} was added to blood to help the activation of the platelets and enhance the thrombus reaction and formation. Further, the blood was supplied to the microchannel immediately after Ca^{2+} was added to blood to prevent the platelet activating in the Eppendorf tube. Figure 3(c) shows the fluorescence image of the fibrin and platelets at t=941s measured at the same location and for the same test sample shown in Figs. (a) and (b).

In Fig. 3(a) platelets start to attach to the channel bottom wall. This is the first step of the formation of the thrombus formation as presented in the schematic shown in Fig. 2(a) [10]. As the time elapses, the number of platelets adhering to the wall increases. By the period of t=943.5s, red blood cells are observed to adhere to the channel bottom wall as shown in Fig. 3(b). In this case, the fibrins are generated and fibrin fibers grow from the platelets and form a fibrin fiber network. These fibrin fibers cover the overall region of the channel bottom wall and trap the red blood cells. Although not shown here, this fibrin fiber network and blood cells tangled with the fibers were observed also in the area at higher positon of the channel.

The phenomena shown above are the basic structure and process of the thrombus formation under the low flow rate condition similar to those of vein. We can see that thrombus formation initially starts by the fibrin network formation at the channel wall which is triggered by the adhered and activated platelets. This differs from the phenomenon observed in the case of thrombus formation in artery where platelet aggregation is more important for such high shear rate condition.



(a) t=11.5s (b) t=943.5s (c) t=941s

Fig. 3 Platelets and red blood cells attached to the channel bottom wall and fibrin fibers distribution visualized by fluorescence measurement. (a) and (b) are fluorescence images of platelets and plasma using Quinacrine dihydrochloride and Fluorescein isothiocyanate-dextran measured at time t=11.5 and 943.5s after the flow is driven. (c) shows fluorescence measurement of fibrin fiber and platelets using Fibrinogen-alexa flour R546, respectively.

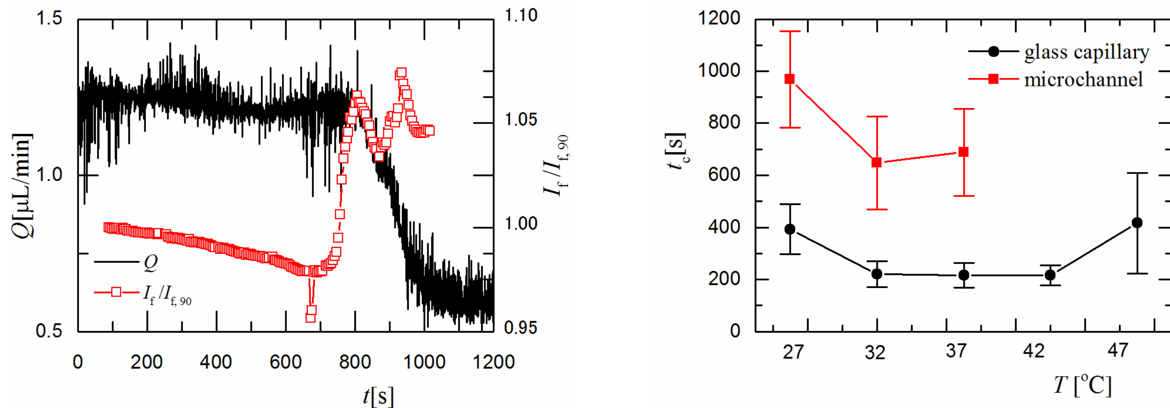
To evaluate the time required for the thrombus to form in the channel, the flow rate was monitored and the time when the flow rate decreased was defined as the thrombus formation time (coagulation time) t_c. Figure 4(a) shows the time distributions of the flow rate Q. For comparison, the average fluorescence intensity of fluorescence labeled fibrins is shown in the figure. The fluorescence intensity significantly increases at the period of approximately t=700-800s. The time when the Q decreases corresponds reasonably well with this time showing that the t_c can be evaluated by monitoring the flow rate. There is some delay in the decrease of Q compared with the increase of fluorescence intensity. This is attributed to the fact that the fluorescence is measured for the wall near region, and time is required for the thrombus to grow in the height direction and clog the channel to decrease the flow rate.

Figure 4 (b) shows the temperature effects on the coagulation time t_c for the microchannel case. Although the conditions of temperature T examined in this case is limited, one can see the trend that t_c shows larger value at T=27°C compared with the other two higher temperature conditions.

To increase the number of samples for wider range of temperature conditions and evaluate the effects in detail, micro-glass capillary flow measurement were conducted under similar flow conditions with the microchannel case. The relationship between T and t_c is shown in Fig. 4 (b). In this case, the temperature effects appear more clearly: t_c shows minimum value in the area of T=32-42°C and shows larger value at 42°C. Although the absolute value of t_c shows some difference between the microchannel and glass-capillary tube flows, some similarity between the two cases can be observed qualitatively. The reason for the

difference in t_c for the two cases is considered to be related to the shape difference between the microchannel and tube. The hydraulic diameter of the tube is larger compared to the microchannel and, thus, the shear rate at the wall is small. In the case of venous thrombus generation case, production of fibrin fibers increases for low shear rate conditions which is believed to be attributed to the local concentration of thrombin released from the platelets [10]. Similar characteristics could be observed in the present experiment, where we found more fibrin fibers generated at the channel corners of the microchannel with rectangular cross-section. Axisymmetric flow with low shear rate in micro-tube could have enhanced the fibrin fiber production and thrombus formation compared to the microchannel flow.

The results shown in above may present two important facts. One is that temperature affects the thrombus formation in convective fluid flow, and for lower and higher temperatures the formation speed decreases. The other is that the fibrin fiber generation shows an important contribution on the thrombus formation from the initial stage under the conditions of low flow rate (shear rate) close to the one in vein. Therefore, it is important to understand the temperature effects on the fibrin generation and fibrin clot formation to evaluate the thermal characteristics of the thrombus formation under such conditions.



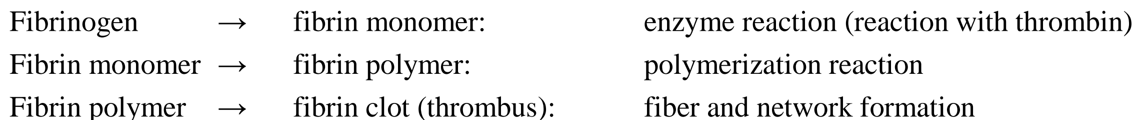
(a) Time distribution of flow rate and fluorescence intensity (b) Temperature effects on thrombus formation

Fig. 4 Results of microchannel and glass capillary measurements. (a) Relationship between the time distributions for the fluorescence intensity of fibrin fibers and flow rate. (b) Effects of temperature T on coagulation time t_c.

3.2 Coagulation time and reaction rate coefficient measurements using cuvette

Temperature effects on the thrombus formation observed in Section 3.1 can be attributed to the decrease of the reaction rate coefficient of the bio-chemical chain reaction shown in Fig. 1. Since the fibrin fiber plays an important role in the thrombus formation, the temperature effects on the thrombus formation should be strongly influenced by the fibrin production reaction and its thermal characteristics. In this section, the reaction coefficient of fibrin formation and its temperature characteristics are evaluated by the measurement using the cuvette-type system shown in Fig. 2 (c). We will measure the coagulation time t_c and the concentration of the fibrin monomer and polymers by analyzing the transmitted light intensity I.

The reaction process of the fibrin clot formation can be divided into three terms: (1) fibrinogen to fibrin monomer (enzyme reaction), (2) fibrin monomer to fibrin polymer (polymerization reaction), and (3) fibrin clot formation from fibrin polymer and fibers.



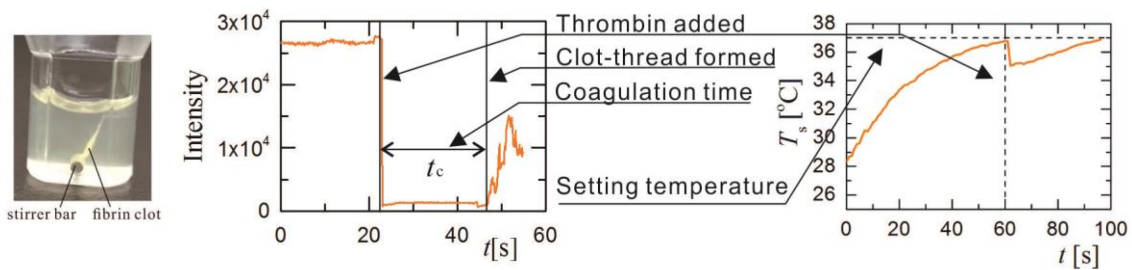
Fibrinogen	→	fibrin monomer:	enzyme reaction (reaction with thrombin)
Fibrin monomer	→	fibrin polymer:	polymerization reaction
Fibrin polymer	→	fibrin clot (thrombus):	fiber and network formation

Concentrations of fibrin monomer and polymer are measured by measuring the transmitted light intensity of the solution in the cuvette which will be described and discussed shortly.

Before evaluating the reaction rate coefficient, the temperature effects on the coagulation time t_c is measured using the cuvette type measurement system to compare with the microchannel and capillary tube measurements. Figure 5(a) shows the fibrin clot (thrombus) formed in the cuvette and the time distribution of

transmitted light intensity I. The period when I markedly decreases is the moment when thrombin is added to the plasma in the cuvette. I remains constant for a certain time and then starts to increase and fluctuate. The time between the decrease of I and increase of I is defined here as the coagulation time t_c. It should be noted here that fibrin monomer is produced after adding the thrombin to the plasma. In the present measurement, the mixing and reaction are enhanced by the stirring. Therefore, the reaction for fibrin monomer production proceeds in a very short time and the influence of fibrin monomer concentration on I could not be observed clearly in Fig. 5(a).

Figure 5(b) shows the time variation of the fluid temperature in the cuvette measured by inserting the thermocouple. After adding the sample fluid in the cuvette at t=0, the temperature gradually increases toward the setting temperature of the cuvette. The temperature drops when the thrombin is added at t~60s and then starts to re-approach to the setting temperature. Therefore, the fluid temperature in the cuvette shows some variation in the range of 2°C after thrombin is added during the measurement.



(a) Fibrin clot and light intensity distribution (b) Temperature profile in the cuvette

Fig. 5 (a) Photograph of the fibrin clot formed in the cuvette after mixing the thrombin and stirring the stirrer bar, and the graph for the time distribution of the intensity of the light transmitted through the cuvette. Coagulation time t_c is defined as the time between the moments when the light intensity suddenly drops and when the intensity starts to increase and fluctuate. (b) Temperature profile of the fluid in the cuvette when the sample fluid and thrombin are added to the cuvette, respectively.

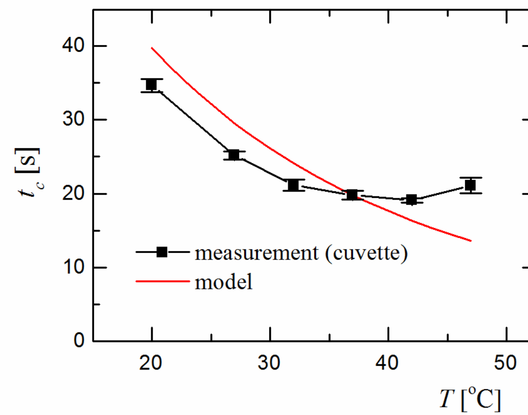


Fig. 6 Effects of temperature T on coagulation time t_c in the cases of cuvette measurement. The model in the graph is the value calculated by Eq. (5) using the reaction coefficient k calculated from the data for temperatures lower than 37°C.

Figure 6 shows the relation between t_c and the cuvette temperature T. t_c shows the minimum value approximately at T=42°C. t_c increases gradually as T decreases or increase from this value. Larger t_c in the low temperature region is owing to the decrease of the reaction rate coefficient which will be discussed shortly. On the other hand, increase of t_c in the high temperature region is believed to be attributed to thermal denaturation of fibrinogen and thrombin.

To evaluate the temperature effect on the reaction rate of fibrin production, we measured the time distribution of the transmission light intensity. Transmission coefficient of the fibrin shows smaller value compared with those of fibrinogen and plasma. Thus, the transmission coefficient decreases as the fibrin monomer and polymer are produced and their concentration increases. As previously mentioned, the fibrin

production is enhanced by the stirring and occurs in a very short time. Therefore, the stirring was stopped after thrombin was added and the fluid remained steady to increase the measurement accuracy in this measurement.

The influences of fibrinogen and fibrin concentrations on the transmitted light intensity I can be presented by the Lambert-Beer equation. Thrombin, on the other hand, shows scattering feature against light.

We assume that thrombin concentration is kept constant through all measurements and it does not change much compared with the fibrin concentration c during the reaction. In this case, I/I_0 can be expressed as Eq. (1).

$$\frac{I}{I_0} \propto e^{-\left(\varepsilon_{mon} c_{fbg,0} + \left(\varepsilon_{fbg} - \varepsilon_{mon}\right) c_{fbg}\right) L} \tag{1}$$

Subscripts *fbg*, *fbg,0* and *mon* represent the values of fibrinogen, fibrinogen at t=0, and fibrin monomer, respectively. Further, we applied the relationship $c_{\mathrm{fbg},0} = c_{\mathrm{fbg}} + c_{\mathrm{mon}}$ to subtract c_{mon} from the equation. Equation (1) can, then, be expressed in a simple form as Eq. (2).

$$I = Be^{Dc_{fbg}} \tag{2}$$

Here, D is related to the physical properties of fibrinogen and fibrin monomer, and B relies only on $c_{\mathrm{fbg},0}$ and I_0, which are the initial conditions of the measurements.

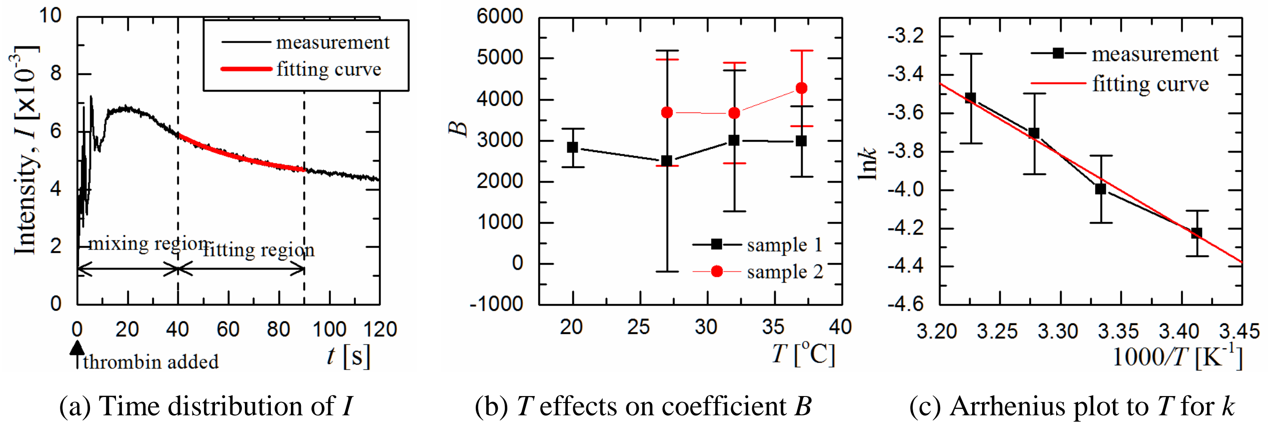
First order reaction model on the basis of the reaction rate constant k is applied to the reaction between fibrinogen and fibrin monomer. Thus, the relationship between c_{fbg} and k can be expressed as Eq. (3).

$$c_{fbg} = c_{fbg,0} e^{-kt} \tag{3}$$

By combining Eqs. (2) and (3), the relationship between I, c_{fbg} and k can be driven as Eq. (4).

$$I = Be^{Dc_{fbg}} = Be^{Ee^{-kt}} \tag{4}$$

Here, E is $E=Dc_{\mathrm{fbg},0}$ and is related to optical properties of the solution and cuvette, and the initial condition of the light and concentrations. This simplified model of k and I is applied to the measurement to obtain the value of k.



(a) Time distribution of I (b) T effects on coefficient B (c) Arrhenius plot to T for k

Fig. 7 Measurement results using the cuvette system to obtain the reaction coefficient of the thrombus (fibrin clot) formation and the effects of temperature. (a) shows the time distribution of the transmitted light intensity after adding thrombin to the blood sample (plasma). Stirrer is not rotated in this case. (b) shows the temperature effects on coefficient B of Eq. (4). (c) is the Arrhenius plot to the temperature for reaction coefficient k.

Figure 7(a) shows the time distribution of I for one of the cases of the measured data. t=0 is the time when the thrombin is added to blood plasma in the cuvette. I increases and shows a maximum value at around t=20s and fluctuates during this period. The fluctuation is caused by the convection generated in the cuvette by the flow from the pipette when thrombin is added. After I showing the maximum value, the flow becomes

steady and I gradually decreases attributed to the increase of fibrin concentration. Curve fitting was applied to the values in the region of $40<t<90$s to obtain constants B, E and k in Eq. (4). The result is shown in Fig. 7(a) represented by the thick solid line and agrees well with the measurement curve. Considering then the facts that E relies on $c_{\text{fbg},0}$ and that $c_{\text{fbg},0}$ does not vary with blood sample, E can be assumed to be constant in the present measurement. We, therefore, first obtained E from the results of changing the temperature and then averaged the values of E. The average value was substituted to Eq. (4) as constant and the curve fitting was re-apply to the distribution to obtain B and k.

Figure 7(b) shows the relationship between B and T. Figure 7(c) is the Arrhenius plot of k for T. In Fig. 7(b), B is not affected by T and is constant. This is reasonable as B is dependent only on $c_{\text{fbg},0}$ and I_0, and these values do not vary much for the same blood sample. B shows different values between the two blood samples. This may be attributed to the change of I_0 when the system was restarted for measurements of different samples. In addition to this, variation in $c_{\text{fbg},0}$ can exist for different blood samples for a certain level which is large enough to affect B as B varies exponentially with $c_{\text{fbg},0}$ and is more sensitive to its variation.

In Fig. 7(c), k shows a linear correlation in the Arrhenius plot in which k increases as $1/T$ increases. This shows that the reaction between fibrinogen and fibrin monomer and polymer can be expressed by a simple first order reaction model.

As the reaction rate coefficient of fibrin production is obtained, we will now try to calculate the thrombus (clot) coagulation time t_c from this results. It is expected that t_c shown in Fig. 6 includes the time for the fibrin monomers and polymers to form the thread type clot with the stirrer rotating in the cuvette. Therefore, estimating t_c from k only is beyond the scope of the paper while the discussion may provide some insights on the effects of the fibrin monomer production reaction on the thermal characteristics of thrombus formation.

t_c was calculated by assuming that the fibrin clot will form if fibrin concentration c_{mon} increases and exceeds a certain threshold c_{th}. c_{mon} can be obtained from Eq. (3) and the relation $c_{\text{fbg},0} = c_{\text{fbg}}+c_{\text{mon}}$. Thus t_c can be expressed as Eq. (5).

$$t_c = \frac{\ln c_{fbg,0} - \ln\left(c_{fbg,0} - c_{th}\right)}{k} \tag{5}$$

To obtain the values $c_{\text{fbg},0}$ and c_{th}, Eq. (5) is first adjusted to t_c of the measurement obtained at T=37°C. t_c of other temperatures is, then, calculated from the equation. The relationship between T and t_c of the results obtained from the model is shown in Fig. 6. As the model considers the characteristics of the reaction rate increasing as T increases, t_c shows the same correlation with T, and the distribution for temperature lower than 37°C shows a reasonable agreement. On the other hand, the distribution shows completely different pattern in the temperature region higher than 42°C where the effects of the thermal denaturation of the thrombin and fibrinogen is considered to take place. Nevertheless, the results show that the thermal characteristics of fibrin formation reaction plays an important role in temperature effects on fibrin thrombus formation at least in the region of T<42°C.

The model is expected to represent the fundamental phenomena for venous thrombus formation. It can be expanded to the cases of microchannel and capillary tube flows as the temperature characteristics of the coagulation time showed similar characteristics with the cuvette case. However, additional effects produced by the flow convection and red blood cell motions should be considered. In the flow accompanied case, we have shown in our previous work that the patterns of the fibrin fibers generated at the channel wall could be affected by the flow in terms of the concentration distribution of the coagulation factor released from the platelets being affected by the flow convection [10, 11]. As the reaction rate affects the generation of the fibrin fibers, the local temperature of the fluid due to the convective heat transfer may affect the thrombus formation in a similar way.

4. CONCLUSION

Temperature effects on venous thrombus formation was measured using microchannel flow, micro-glass capillary tube flow, and cuvette. The time required for the thrombus formed in the flow was affected by the temperature: the coagulation time t_c showed minimum value in the region of approximately 32-42°C. Based

on the reaction coefficient characteristics of fibrin generation using the transmitted light intensity measurement for the fluid sample in cuvette, temperature effects on fibrinogen to fibrin and fibrin polymer reactions were one of the dominant factors to characterize this temperature effects of thrombus formation. This is understandable as the fibrin fibers are initially formed in the area adjacent to the channel wall and helps the red blood cells and platelets to concentrate in that area under the condition of which flow shear rate and shear stress are small and the platelets do not aggregate. Further, as t_c showed minimum values for the temperature range similar to that of cuvette in the flow accompanied cases, we believe that temperature effect on fibrin generation is not influenced much by the flow shear rate and shear stress. On the other hand, t_c differed among the three measurements. This is believed to be attributed to flow convective effects on blood cell motion and concentration distribution of micro-particles and bio-chemical component released from the activated platelets. It is necessary to specify and model these effects to validate the application of the model for temperature effects on local fibrin formation reaction in flow. Temperature effects on fluid viscosity is another important factor to be evaluated in the future work.

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ETHICAL APPROVAL

This work has been approved by the Kyoto University Graduate School and Faculty of Medicine, Ethics Committee.

REFERENCES

[1] Weiss, H. J., Turitto, V. T., Baumgartner, H. R., "Role of Shear Rate and Platelets in Promoting Fibrin Formation on Rabbit Subendothelium", *J. Clinical Investigation*, Vol. 78, pp. 1072-1082, (1986).

[2] Sarvepalli, D. P., Shmidtke, D. W., Nollert, M. U., "Design Considerations for a Microfluidic Device to Quantify the Platelet Adhesion to Collagen at Physiological Shear Rates", *Annals Biomedical Engineering*, Vol. 37, pp. 1331-1341, (2009).

[3] Savage, B., Saldivar, E., Ruggeri, Z. M., "Initiation of Platelet Adhesion by Arrest onto Fibrinogen or Translocation on von Willebrand Factor", Cell, Vol. 84, pp. 289-297, (1996).

[4] Sessler, D., "Perioperative Heat Balance", *Anesthesiology*, Vol. 92, pp. 578-596, (2000).

[5] Etulain, J., Lapponi, M.J., Patrucchi, S.J., Romaniuk, M.A., Benzadón, R., Klement, G.L., Negrotto, S., Schattner, M., "Hyperthermia Inhibits Platelet Hemostatic Functions and Selectively Regulates the Release of Alpha-granule Proteins", *J. Thrombus haemostasis*, Vol. 9, pp. 1562-1571, (2011).

[6] Wolberg, A. S., Meng, Z. H., Monroe, D.M., Hoffman, M., "A systematic evaluation of the effect of temperature on coagulation enzyme activity and platelet function", *J. Trauma*, Vol. 56, pp. 1221-1228, (2004).

[7] Cohen, A. T., Agnelli, G., Anderson, F. A., Arcelus, J. I., Bergqvist, D., Brecht, J. G., Greer, I. A., Heit, J. A., Hutchinson, J. L., Kakkar, A. K., Mottier, D., Oger, E., Samama, M. M., Spannagl, M., "Venous Thromboembolism (VTE) in Europe", *J. Thrombus haemostasis*, Vol. 98, pp. 756-764, (2007).

[8] Klatsky, A., Armstrong, M. A., Poggi, J., "Risk of Pulmonary Embolism and/or Deep Venous Thrombosis in Asian-Americans", *The American J. of Cardiology*, Vol. 85, pp. 1334-1337, (2000).

[9] Sakuma, M., Konno, Y., Shirato, K., "Increasing Mortality from Pulmonary Embolism in Japan, 1951-2000", *Circulation J.*, Vol. 66, pp. 1144-1149, (2002).

[10] Y. Yamamoto, K. Tatsumi, H. Shirouzu, H. Hirakata, N. Sugita, K. Inoue and K. Nakabe, "Measurement of Venous Thrombosis Formation and Mass Transfer Effects in the Initial Stage Using Microchannel Flow", *Proc. 20th Int. Conference on Miniaturized Systems for Chemistry and Life Sciences (microTAS-2016)*, pp. M099e, (2016).

[11] N. Sugita, H. Hirakata, K. Inoue, K. Koike, K. Tatsumi, S. Ii, H. Niioka, S. Wada, "The Blood Components Strongly Affect Thrombus Structure Formed in a Novel Microfluidic Device", *International Society on Thrombosis and Haemostasis 2017 Congress*, (2017).