THERMAL CHARACTERISTICS OF FIBRIN CLOT FORMATION IN VENOUS THROMBUS

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Abstract. Temperature effects on the fibrin based thrombus formation measured in cuvette, micro-glass tube and microchannel flows are evaluated in this paper. Coagulation time, which is the time required for the thrombus and fibrin clot to form, were evaluated through these measurements by measuring the variations in the transmitted light intensity of the sample and flow rate. Visualization of platelets, plasma and fibrin were performed in the microchannel measurement applying fluorescence labeling techniques. Further, time distribution of the fibrin concentration in the cuvette was evaluated by measuring the time profile of the transmitted light intensity. A first order reaction model was applied to curve fit with the obtained distribution to derive the reaction rate of the fibrin generation. The results showed that the fibrin network growing from the activated platelets plays a major role in thrombus formation under the flow condition of the vein. Temperature affected the fibrin formation characteristics and the coagulation time showed the minimum value at 37°C in the cases of with and without flows. The reaction coefficient increased as the temperature increased in the range of $24 \leq T \leq 37$°C showing that the decrease of the coagulation time of the fibrin clot is attributed to faster fibrin generation reaction.

Keywords: Fibrin Clot, Temperature effect, Reaction rate, Coagulation, Microchannel, Venous Thrombus

1. INTRODUCTION

Venous thromboembolism is one significant issue in blood flow in terms of the fact that it can lead to serious symptoms as 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 (Cohen *et al.*, 2007), and in Japan 7,864/year (Sakuma *et al.*, 2002). The cause of the difference in these numbers and rate among the regions are still under discussion: trying to find if the cause is related to genetic thrombolytic factors, treatment and dosing, diet and habits, or lack of statistical data from the hospitals (Klatsky *et al.*, 2000). Nevertheless, these numbers are increasing especially in the Asian countries, therefore, the cause of venous thrombus and the major factors and conditions which affects its formation must be studied and understood to apply an appropriate medical treatment.

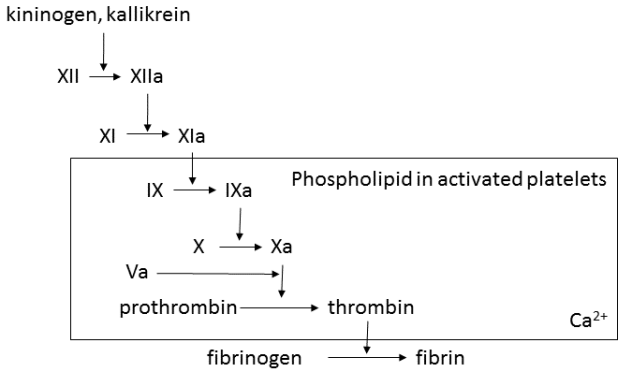


Figure 1. Example of the reaction pathway for fibrin generation in blood. Various pathways exists in relation to the factors affecting the reaction.

Thrombus formation and fibrinolysis are affected by various external and internal factors (Weiss *et al.*, 1986, Sarvepalli *et al.*, 2009). The internal basically follows 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. Namely, measurement and analysis of the diffusion coefficient, reaction coefficient, and the release and attachment of thrombolysis and fibrinolysis factors at the cell surfaces need to be established.

Among the external effects, temperature effects needs a systematic and quantitative analysis and discussion. 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 yet.

One may say that the temperature in human body is constant and uniform. However, this is not the case in the hospital and surgery. 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 (Sessler, 2000). 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 (Etulain *et al*., 2011). The temperature difference can either increase the probability of thrombus generation or impair the hemostasis (Wolberg *et at*., 2004). Therefore, it is important to understand the thermal characteristics of the thrombosis and thrombolysis processes.

In this study, measurement of thrombus formation in cuvette system, micro-capillary flow and microchannel flow are performed to understand the effects of the temperature on the thrombus formation. 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. The thrombus formation in the flow accompanied case is measured by using the micro-capillary and evaluating the change in the flow rate under constant pressure gradient flow condition. Further, 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. In these three types of measurements, temperature conditions are varied and the effects of temperature on the coagulation time of the thrombus and the time distribution of the fibrin concentrations are measured. Thermal characteristics and reaction rate coefficient of thrombus formation are evaluated and discussed from these measurements.

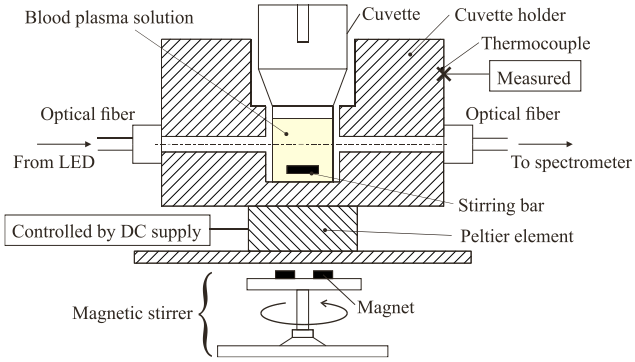
2. SAMPLE PREPARATION AND EXPERIMENTAL METHODS

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

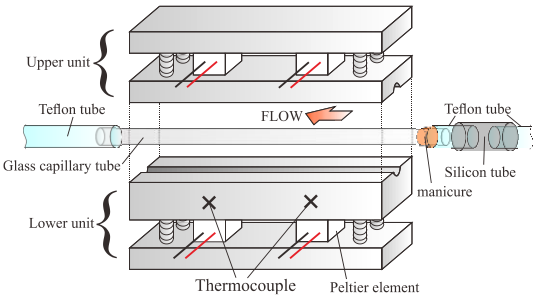
Figure 2 shows the schematics of the experimental apparatuses. Figure 2 (a) shows the cuvette-type system designed for the fibrin coagulation (clot formation) measurement. The cuvette is placed in a temperature control unit and the magnetic stirrer mixes the fluid in the cuvette by rotating the stirring bar immersed in fluid. Light of wavelength 417nm is irradiated to the sample and the transmitted light intensity I is measured from the other side by a spectrometer (OceanOptics Co.: FLMS01061).

Plasma (PPP) of 70μL is put in the cuvette and is first incubated for 1min at 37°C with the stirring bar rotated at 300rpm. Thrombin (Sysmex Co.: Multifibrin U) is added after the incubation with the stirring bar kept rotating. The time between the addition of thrombin and generation of fibrin clot is measured by detecting the change of I appearing in each process. This is defined as the coagulation time t_c.

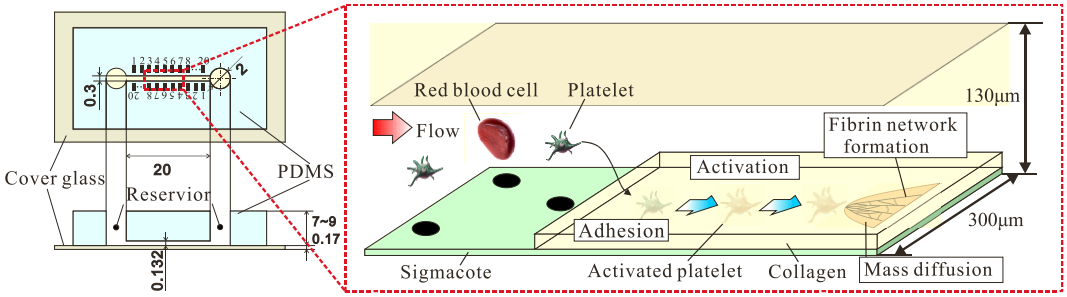
Measurement of I was also made without rotating the stirring bar (stationary fluid condition) to measure the reaction rate coefficient of the thrombus reaction (fibrin generation reaction). The stationary fluid case was chosen in order to remove the stirring effect on the thrombin generation. In this case, the exact time required for the clot to be formed in the cuvette is less clear compared to the stirring case, however, the time dependent variation of I could be measured accurately. This made it possible to measure the concentration of fibrin monomer and polymers. Curve fitting to the measured time distribution of I was performed by applying the equation presenting the relationship between reaction rate coefficient and fibrin concentration to the curve fitting. The process will be explain in detail in Section 3.



(a) Time distribution of transmitted light intensity



(b) Fibrin clot generation time, t_c



(c) Microchannel and the schematic of fibrin network formation at the channel wall

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

Glass capillary tube measurement was conducted to evaluate the flow effects on the relationship between temperature and thrombus formation. Figure 2(b) shows the schematic of the micro-glass capillary tube measurement. The glass capillary tube (Fujirika Co.: glass capillary) has inner diameter of 200μm and length of 120mm. It was placed in a groove machined on the temperature-controlled block. The capillary tube was connected and bonded with the Teflon and silicon tubes. The tube was connected to a pump supplying the fluid to the capillary tube with pressurized air. Fluid was driven under constant pressure gradient, and flow rate was measured using micro flow sensor (Sensirion Co.: LG16-0025). The pressure was set so as the initial flow rate was 1.4μL/min. Tubes were attached to the outlet of the capillary tube to observe the location of fluid outlet interface and to prevent the surface tension effects on flow rate.

Figure 3(c) shows the schematic of the microchannel and the initial stage of thrombus formation. The width and height of the microchannel were 300μm and 132μm, respectively. The microchannel was fabricated with PDMS using the standard photolithography and soft lithography. The PDMS was first degassed by decompression and then casted using SU-8 (MicroChem Inc.) mold fabricated by the photolithography process. Holes were cored from the PDMS at the inlet and outlet of the channel with a biopsy puncher. The PDMS was then attached to the cover glass after both surfaces were treated by O_2 plasma using etcher (Samco Co.: FA-1). The channel walls 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 area located downstream of the channel. This area represents the damaged blood vessel in which the collagen layer is exposed to the blood.

The channel was placed on a temperature-controlled stage and channel temperature was monitored by thermocouples attached to the channel. Blood flow was supplied to the channel through Teflon tubes in the same way applied to the capillary tube measurement. Pressure gradient was set to make the initial flow rate be 1.3μL/min. Fluorescence labeling was conducted to visualize the platelet attached to the wall and the formation of fibrin fibers. Fibrinogen-Alexa Fluor R546 (ThermoFisher Co.) was used to label the fibrin, and Fluorescein Quinacrine dihydrochloride(Santa Cruz Biotechnology Inc., SC-204222A) and Fluorescein isothiocyanate-dextran were used to label the platelets and plasma. Epi-fluorescence microscope, fluorescence filters, objective lens of ×100 magnification and sCMOS camera (Andor Co.: Zyla 4.2 plus) were used to measure the fluorescence.

Table 1. Composition of blood sample used in microchannel measurements.

Blood samples and reagents	RCC	PPP	WP	Ca^{2+} (1M/L)	alexa Four546 (1.5mg/mL)	Quinacrine (5mM/mL)	Fluorescein isothiocyanate-dextran
volume [μL]	112.5	100	25.0	4.0	2.0	5.0	1.0

Table 2. Composition of blood sample used in micro-glass capillary measurements.

Blood samples and reagents	RCC [μL]	PPP [μL]	WP [μL]	Ca^{2+} (1M/L) [μL]
RBC+PPP+WP case	56.25	50.0	12.5	2.0
PPP+WP case	–	100.0	12.5	2.0

3. RESULTS AND DISCUSSION

3.1. Microchannel flow measurement

Figure 3 shows the results of visualizations of red blood cells, platelets and fibrin fibers conducted at the channel bottom wall. Measurement was made at the location 1mm downstream from the upstream 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 activate the platelets and enhance the thrombus reaction and formation. The composition of blood is shown in Table 1. Further, to prevent the platelet activating in the Eppendorf tube, the blood was supplied to the microchannel immediately after Ca^{2+} was added to blood. Figure 3(c) shows the fluorescence image of the fibrin and platelets at t=941s measured at the same location of Figs. (a) and (b) for the same test sample.

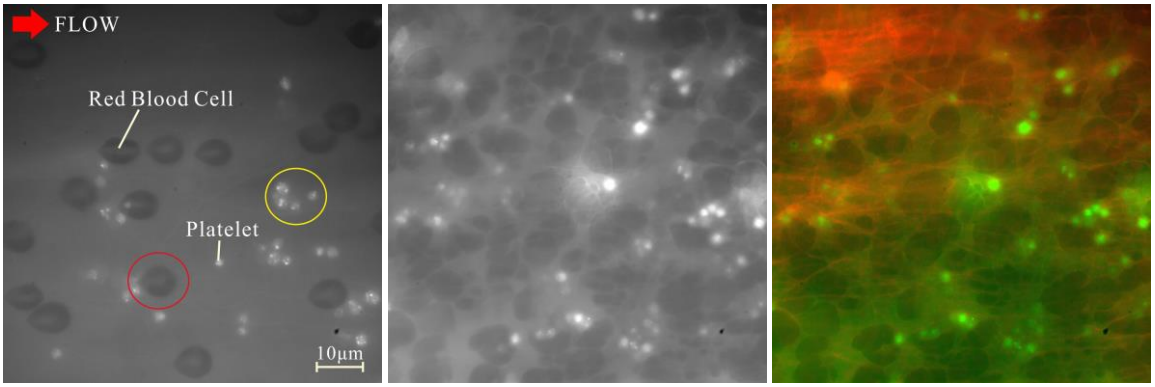
In Fig. 3(a) platelets starts to attach to the channel bottom wall. This is the first step of the formation of the thrombus formation as shown in Fig. 2(c). As the time elapses, the number of platelets adhering to the wall increases. By the time of the period t=943.5s, red blood cells are observed to adhere to the channel bottom wall as shown in Fig. 3(b). In this case, the fibrin fibers are generated and grows from the platelets and form a fibrin network like the schematic shown in Fig. 2(c). Indeed, fibrin fibers are observed in Fig. 3(c) which covers the overall region and trap the red blood cells. This is the reason why red blood cells were observed like adhering to the channel bottom wall. Although, not shown here, this fibrin network and blood cells tangled with the fibers were observed also in the channel height direction. This is the basic structure and process of the thrombus formation under the low flow rate condition similar to those of the vein.

As shown in Fig. 3, thrombus is formed in the channel with platelets activated, fibrin fibers growing and red blood cells captured and tangled by the fibrin network. Such structures are observed in the overall area of the channel, and increases their volume as time elapse. The thrombus will interfere and block the flow which leads to decrease of the flow rate under constant pressure gradient flow conditions. Figure 4 (a) shows the time profile of the flow rate Q. Fluorescence intensity of fibrin spatially averaged over the measured area I_f is superposed in the figure. I_f is normalized by the value of $I_{f,90}$ which represents the value at t=90s. Q remains constant at the initial flow rate 1.3μL/min and suddenly decrease at approximately t=800s. This is considered to be the moment when the thrombus starts to form in the channel and block the flow. Indeed, $I_f/I_{f,90}$ starts to increase at the same period when Q decreases and shows that the fibrin fibers are rapidly growing and forming the thrombus.

To evaluate the effects of temperature on the thrombus formation, the temperature of the microchannel was changed and flow rate was measured. The time between the blood supplied to the microchannel and decrease of flow rate was measured and defied as coagulation time of the thrombus (clot) t_c. Figure 4(b) shows the relation between the temperature T and t_c. Although the conditions of temperature examined in this case is limited, one can see the trend that t_c increases as the temperature decreases from T=37°C. The venous thrombus formation rate is believed to decrease as temperature decreases even when flow is flowing the channel (vein), which influences the thrombin formation.

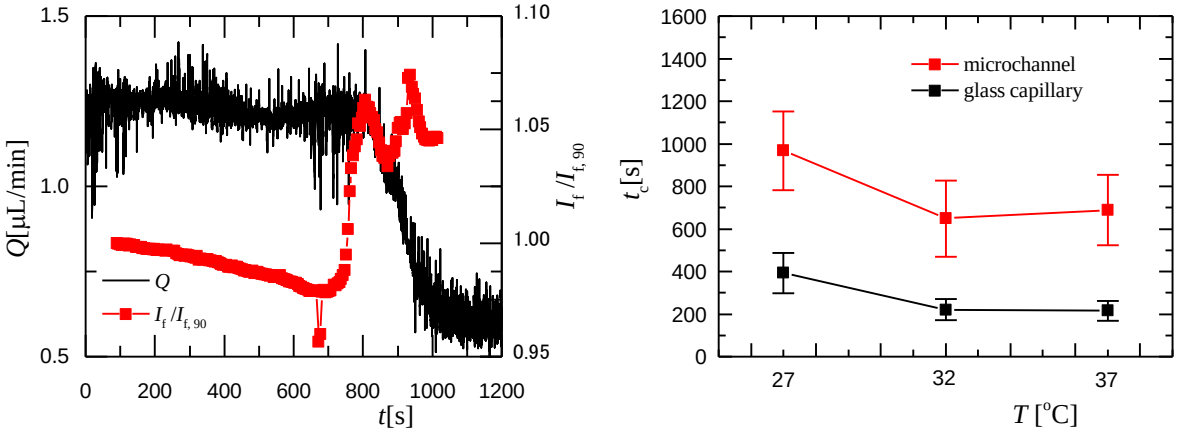
3.2. Micro-glass capillary flow measurement

Temperature effects on the coagulation time of the thrombus appeared in the microchannel flow measurement. The number of temperature conditions was, however, limited to discuss the temperature characteristics of the thrombus formation. This was due to the limitation of the numbers of microchannel that could be arranged. To simplify the system and increase the number of measurements, micro-glass capillary tube with the diameter of 200μm which is close to the



(a) t=11.5s (bright light) (b) t=943.5s (bright light) (c) t=941s (fluorescence)

Figure 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 for fibrin and, respectively.



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

Figure 4. (a) Time distributions of the flow rate of the microchannel and fluorescence intensity of the fibrin. (b) temperature effects on the time required for the thrombus to be generated in the channel, t_c. Q is the flow rate of the microchannel decreasing significantly at approximately t=800 owing to thrombus formation in the channel. I_f is the spatially averaged fluorescence intensity of fibrins and $I_{f,90}$ is the value of t=90s. t_c is the time t when Q decreases and T is the channel temperature.

dimension of microchannel was used as an alternative method. The components of blood used in the measurement in this case is summarized in Table 2. Blood samples with different components were prepared: one is the blood with WP, PPP and RCC and the other is WP and PPP. The latter condition was examined to evaluate the temperature effects on the fibrin formation. As described in Sections 1 and 3.1 and shown in Fig. 2(c), thrombus formation in vein is believed to be influenced more by fibrin network formation rather than the coagulation of platelets. This is considered to be owing to the low flow velocity and shear rate. Therefore, it is important to evaluate the thermal characteristics of the fibrin (clot) formation by subtracting the red blood cells from the blood. Thus the two blood samples were tested in the measurement.

Flow rate Q was monitored in the same way as the microchannel flow measurement. Q distribution showed a similar tendency with that of the microchannel: Q remains constant and starts to decrease at a certain time. Coagulation time t_c was defined as was in Section 3.1. Figure 5 shows the relationship between capillary tube temperature T and t_c. In the case of RBC+PPP+WP, the temperature that shows the minimum t_c is 37°C. t_c increases as temperature increase or decreases from 37°C. The same distribution is plotted in Fig. 3(b) to compare with the results of microchannel measurements. In spite that the absolute values show some difference, the distribution pattern agrees well indicating the validity of the measurements.

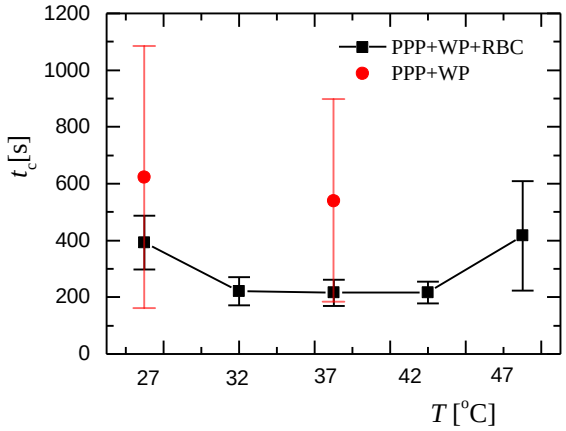
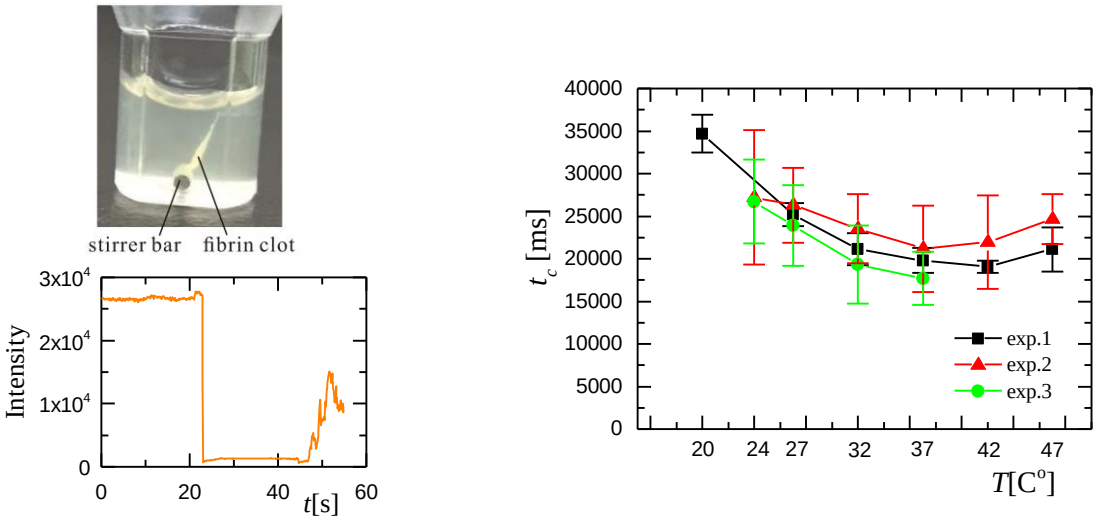


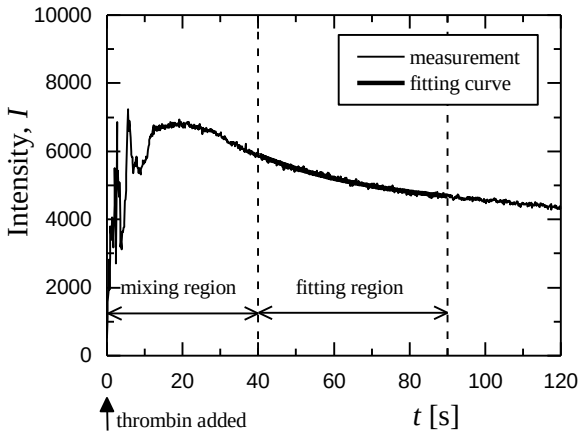
Figure 5. Relationship between temperature and coagulation time measured in micro glass capillary tube flow. Coagulation time t_c is measured by monitoring the change in the flow rate of the blood flow driven to the capillary under constant pressure condition. Black symbol represents the case of blood of the mixture of poor platelet plasma (PPP), washed platelets (WP) and red blood cells, and the red symbol shows the case of blood of PPP and WP (without red blood cells).



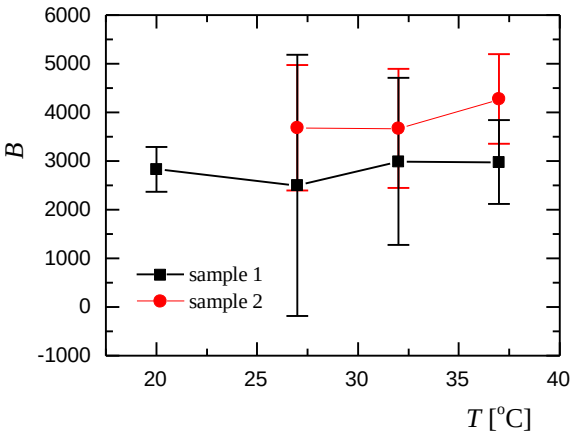
(a) Fibrin clot and time profile of transmitted light intensity (b) Temperature effects on coagulation time

Figure 6. (a) above is a photograph of the fibrin clot formed in the cuvette after mixing the thrombin and stirring the stirrer bar. Bellow is the graph showing the time distribution of the intensity of the light transmitted through the cuvette. Coagulation time t_c is measured as the time between the moment when the intensity suddenly drops and the moment when the intensity increases and starts to fluctuates. (b) shows the relation between T and t_c. The three symbols represent cases with different blood samples.

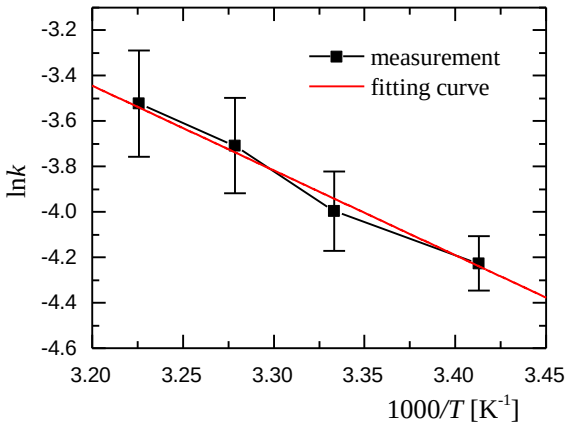
Comparing the results of RBC+PPP+WP and PPP+WP cases, t_c does also increase as the temperature increase in the PPP+WP case. However, the error bar is much larger compared to the RBC+PPP+WP case. Fundametally, the fibrin reaction is based on fibrinogen and thrombin as shown in Fig. 1. These enzymes are mainly produced from platelets and plasma and not directly from red blood cells. It is, however, observed in the microchannel experiments that the existence of red blood cells plays an important role for thrombus formation not only within the meaning of tangled by the fibrin network. It appears that the rotational motion or adhesion of red blood cells affects the activation of platelets and fibrin production. Detail of the functions of the red blood cells has not been understood yet, but it may be related to the enhancements of the mass transfer in the wall near region, mechanical stress applied to platelets, or biological reaction at the surface of the red blood cells. Thus, as shown in Fig. 5, the existence of red blood cells enhanced the thrombus formation and reduced t_c in the case of RBC+PPP+WP. Further, enhancement and stimulation functions of the red blood cells helped triggering the thrombus formation reaction and eventually increased the repeatability and reliability of the measurement which leaded to a smaller error bar.



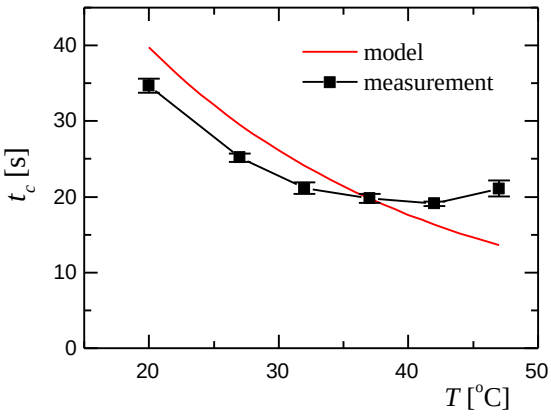
(a) Time distribution of transmitted light intensity



(b) Temperature effects on coefficient *B*



(c) Arrhenius plot to temperature of reaction coefficient



(d) Comparison with stirred case

Figure 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*. (d) shows the comparison of the t_c-*T* distribution shown in Fig. 6(b) and the results calculated by the model using the reaction coefficient *k* for temperatures lower than 37°C.

3.3. Cuvette measurement and reaction rate coefficients

Measurements using microchannel and micro-glass capillary tube showed that fibrin network formation is one key factor of venous thrombus formation, and that temperature affects the thrombus coagulation time. The reason of the decrease of the coagulation time when temperature decrease can be the decrease of reaction rate coefficient. Since the fibrin fiber plays an important role in the thrombus formation, the temperature effects on this thrombus formation should be strongly influenced by the fibrin production reaction and its thermal characteristics. In this section, the fibrin reaction coefficient is measured using a cuvette. As mentioned in Section 2, this equipment can measure the coagulation time of large number of samples with high accuracy. In addition to this, the concentration of the components in the blood sample – in this case the concentration of the fibrin monomer and polymers – can be measured. These measurements will be conducted using blood samples with components of plasma (PPP) and platelets (WP) to remove the effects of other blood cells and understand the thermal characteristics of fibrin production and clot formation.

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

Coagulation time measured by using the microchannel and capillary tube include these three processes and the reaction rate coefficient cannot be evaluated form their results. Therefore, it is necessary to measure the fibrin monomer and fibrin polymer production process only, which is performed by measuring the concentrations of fibrin monomer and polymer. Concentrations of these components are obtained by measuring the transmitted light intensity.

Before evaluating the reaction rate coefficient, the temperature effects on the coagulation time is measured using the cuvette type measurement system to compare with the microchannel and capillary tube measurements and validate the system. Figures 6(a) and (b) show 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 blood sample in the cuvette. I remains constant and starts to increase and fluctuate after a while. The time between the decrease of I and starting point of increase of I is defined as coagulation time t_c.

To understand the behavior of I distribution, fibrin clot shown in Fig. 6(a) is obtained after the period of the increase and fluctuation of I. After thrombin is added to the plasma, fibrin monomer is produced. In this measurement, however, the mixing and reaction is enhanced when the stirring bar is rotating. Thus, reaction of fibrin monomer production proceeds in a very short time and the influences of fibrin monomer concentration on I could not be observed clearly in Fig. 6(a) below. As the fibrin polymer concentration increases, bonding and tangle of polymers occurs in the solution. After polymer tangles and fluid viscosity increase, thread type fibrin clot shown in Fig. 6(a) above is formed owing to the swirl generated in the cuvette.

Figure 6(c) shows the relation between t_c and temperature T. The three symbols (lines) represent the results of different blood samples. t_c shows the minimum value approximately at T=37°C. t_c increases gradually as T decreases or increase from this value. This agrees well with the measurements of microchannel and capillary tube. Thus, the temperature effects on the fibrin clot formation (coagulation) of plasma show the same characteristics with blood under flowing condition. Furthermore, the validity of the cuvette type measurement system is confirmed from these results. In the low temperature region, larger t_c is believed to be 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 could be attributed to thermal denaturation of fibrinogen and thrombin.

Transmission coefficient of the fibrin differs with those of fibrinogen and plasma. Therefore, the transmission coefficient decreases as fibrin monomer and polymer are produced and their concentration increases. The reaction rate coefficients, thus, can be evaluated by measuring the time distributions of the transmitted light intensity.

The influence of fibrinogen and fibrin concentrations c on the transmitted light intensity I can be presented by the Lambert-Beer equation shown in Eq. (1).

$$\ln \frac{I}{I_0} = -\alpha L = -\varepsilon c L \tag{1}$$

I_0 is the intensity of the incident light. α and ε are the absorption coefficient and molar absorption coefficient, respectively. L is thickness of the solution through which the light transmits.

On the other hand, thrombin shows scattering feature against light and the relationship with the thrombin concentration can be described as Eq. (2).

$$\ln \frac{I}{I_0} = -\tau L = -NCL \tag{2}$$

τ and N are the turbidity and number density of the dissolved substance. C is scattering cross section.

We then assume that thrombin concentration is kept constant through all measurements and it does not change much compared with fibrin during the reaction. In this case, I/I_0 can be expressed as Eq. (3).

$$\frac{I}{I_0} \propto e^{-\left(\varepsilon_{mon} c_{fbg,0} + \left(\varepsilon_{fbg} - \varepsilon_{mon}\right) c_{fbg}\right) L} \tag{3}$$

Subscripts *fbg*, *fbg,0* and *mon* represent the values of fibrinogen, fibrinogen at t=0 and fibrin monomer. Further, we applied the relationship $c_{fbg,0} = c_{fbg} + c_{mon}$ to subtract c_{mon} from the equation. Equation (3) can, then, be expressed in a simple form as Eq. (4).

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

Here, D is related to the physical properties of fibrinogen and fibrin monomer, and B relies only on $c_{fbg,0}$ 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. (5).

$$c_{fbg} = c_{fbg,0} e^{-kt} \tag{5}$$

By combining Eqs.(4) and (5), the relationship between I, c_{fbg} and k can be driven as Eq. (6).

$$I = Be^{Dc_{fbg}} = Be^{Ee^{-kt}} \tag{6}$$

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.

Figure 7(a) shows the time distribution of I for one 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. As mentioned in Section 2, the stirrer bar is not rotated after thrombin is added to blood plasma. The fluctuation is caused by the convection generated in the cuvette by the flow from the pipette when thrombin is added. After I shows the maximum value, the flow becomes steady and I gradually decreases owing 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. (6). The result is shown in Fig. 7(a) represented by the thick solid line which shows a good agreement. Considering then that E relies on $c_{\mathrm{fbg},0}$ and that $c_{\mathrm{fbg},0}$ does not vary with blood sample, E can be set as constant. Thus, E was temporally obtained from the results changing the temperature condition. Averaged value of E for all data including those of different temperature was, then, calculated. The averaged value was substituted to Eq. (6) as constant and the curve fitting was again applied to the results. This process reduces the degree of freedom in the curve fitting and increase the accuracy in obtaining B and k.

Figures 7(b) shows the relationship between B and T by the curve fitting for two blood samples. Figure 7(c) is the Arrhenius plot of k for T. In Fig. 7(b), B is not affected by T and is constant for both blood samples. This is reasonable as B is depended only on $c_{\mathrm{fbg},0}$ and I_0, and these values do not vary much for the same blood sample. On the other hand, B shows different values between the two blood samples. Although the condition of I_0 was controlled to be the same for each measurement, I_0 might have change when the system was restarted to measure different samples. Further, variation in $c_{\mathrm{fbg},0}$ can exist for different blood samples for a certain level large enough to affect B. As mentioned above, E and B are both dependent on $c_{\mathrm{fbg},0}$. However, while E is proportional to $c_{\mathrm{fbg},0}$, B varies exponentially with $c_{\mathrm{fbg},0}$ and is more sensitive to its variation.

In Fig. 7(c), k shows a strong linear correlation in the Arrhenius plot in which k increases as $1/T$ increases. The validities of the model proposed in the present work and the measurement method are confirmed from these results. Furthermore, the reaction between fibrinogen and fibrin monomer and polymer can be expressed by a simple first order reaction model.

As reaction rate coefficient of fibrin production is obtained, we will try to calculate the thrombus (clot) coagulation time t_{c} from this value. Obviously, t_{c} shown in Fig. 6 includes the process of fibrin monomers and polymers forming 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. (6) and the relation $c_{\mathrm{fbg},0} = c_{\mathrm{fbg}}+c_{\mathrm{mon}}$. Thus t_{c} can be expressed as Eq. (7).

$$t_c = \frac{\ln c_{fbg,0} - \ln\left(c_{fbg,0} - c_{th}\right)}{k} \tag{7}$$

To obtain the values $c_{\mathrm{fbg},0}$ and c_{th}, Eq. (7) 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. Figure 7(d) shows the relationship between T and t_{c} of the results obtained from the model and measurement (same as that shown in Fig. 6(b)). In the region of temperature higher than 37°C, the distribution shows completely different pattern which is attributed to the fact that the effects of the thermal denaturation of the thrombin and fibrinogen is not considered in the model. On the other hand, the distribution for temperature lower than 37°C shows a reasonable agreement. This shows that the thermal characteristics of fibrin formation reaction plays an important role in temperature effects on fibrin thrombus formation. The model can be expected to be the fundamental one for thrombus formation and can be expanded to cases measured in microchannel and capillary tube flows to which additional effects of convection and red blood cell motions should be considered and applied to the models.

4. CONCLUSION

Temperature effects on venous thrombus formation was measured using microchannel flow, micro-glass capillary tube flow and cuvette. Fluorescence observation of platelets, plasma and fibrin showed that venous thrombus formation is initiated by adhesion of the platelets to the wall and their activation, and growth of fibrin fibers and networks from platelets. Formation of thrombus and them plugging the flow were influence by temperature: namely, the time required

for thrombus formation (coagulation time t_c) increased as the temperature decreased. This tendency was observed in the micro-capillary flow measurement, in which t_c showed minimum value at 37°C. In the temperature region lower than 37°C, the decrease of the reaction rate is considered to be the mail reason for the increase of t_c while in the region higher than 37°C, increase of t_c is due to thermal denaturation of thrombin and fibrinogen. Further in this measurement, existence of red blood cell in the blood decreased t_c indicating that red blood cell enhances the reaction of the thrombus formation. Temperature effects on fibrin clot (thrombus) formation and reaction rate coefficient k were evaluated in the cuvette measurement. The relationship between t_c and temperature showed good agreement with the results of microchannel and capillary tube measurements. In the evaluation of k, a strong linear relation in the Arrhenius plot of k and $1/T$ was obtained. Further, by applying the model and obtained k to calculate t_c, a reasonable agreement between the predicted value and measurement was achieved.

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ETHICAL APPROVAL

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

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