Simultaneous measurements of dynamic modulus and turbidity and effects of calcium ions on the process of thrombin-induced fibrin gel formation

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Abstract Aqueous solution of fibrinogen, one of blood clotting factors, forms a fibrin gel by the action of enzyme thrombin. Calcium ions are known to affect not only the gelation kinetics but the structure of fibrin gel network. Dynamic modulus and turbidity have been used to monitor the gelation, however, there are few reports to compare the two data sets. In this study, we have carried out simultaneous measurements of the dynamic modulus and transmitted light intensity during fibrin gelation using a newly developed coaxial-cylinder rotational rheometer with transparent inner and outer cylinders. The measurements for dynamic modulus were carried out at an oscillation frequency of 1 Hz and an oscillation amplitude of 1 mrad in the presence of calcium ions (1–50 mM). After the addition of thrombin to fibrinogen solution, an opaque fibrin gel was formed. The decrease in the transmitted light intensity occurred faster than the increase in the elastic modulus due to the stepwise fibrin polymerization. The time courses of the dynamic moduli and the transmitted light intensity were well expressed using empirical exponential-type equations. The parameters obtained by fitting the data to the equations closely related to the gelation kinetics and the network structure of fibrin. We discuss the effect of calcium ions on the parameters.

Keywords fibrinogen, calcium ion, fibrin gel, dynamic modulus, turbidity, bioadhesive materials

1. Introduction

Fibrinogen, one of blood clotting factors, is a dimer composed of three polypeptide chains, A\textalpha, B\beta and \gamma chains, as shown in Fig. 1. Enzyme thrombin cleaves Fibrinopeptides A (FpA: 1–16 residues) and B (FpB: 1–14 residues) at the N-termini of the A\textalpha and the B\beta chains, converting fibrinogen to fibrin. Knobs ‘A’ with GPRV and ‘B’ with GHRP amino acid sequence exposed by thrombin cleavage of FpA and FpB interact with holes ‘a’ and ‘b’ existing in the \gamma and the \beta- chains, respectively. Fibrin polymerization is a final step of the complex cascade of blood coagulation and proceeds in a stepwise manner [1]. The exposed knob ‘A’ : hole ‘a’ interactions assemble fibrin monomers in a half-staggered manner into two-stranded protofibrils. The protofibrils aggregate laterally to form fibers that branch into a three-dimensional gel network.

Fibrinogen has two Ca\textsuperscript{2+}-binding sites at the \gamma-nodules (\gamma\textsubscript{1} and \gamma\textsubscript{2}) and the \beta-nodules (\beta\textsubscript{1} and \beta\textsubscript{2}), respectively [2, 3]. Especially, high-affinity Ca\textsuperscript{2+}-binding sites (\gamma\textsubscript{1}) located near the hole ‘a’ are necessary for the protofibril formation [4] and affect the mechanical properties of both fibrin monomers and fibrin polymer network [5]. Thus, calcium ions have various effects on fibrin gel such as the stabilization of fibrinogen against thermal denaturation [6], the acceleration of fibrin polymerization [7–10] and the change in the rheological properties of fibrin gel [11].

Fibrin gel also has been utilized in many surgical fields as fibrin glues/sealants which are surgical bioadhesive materials composed of fibrinogen, thrombin, calcium chloride and factor XIII. The optimal conditions in surgical procedures have been examined for parameters such as the clotting time, concentrations of constituents and mechanical properties of the gel. Goessl et al. reported that calcium ions affected the clotting time, microstructure and tensile strength of a fibrin sealant [12]. However, clotting dynamics has not been clarified yet. To improve the fibrin-based glues/sealants, it is hoped to study the clotting behavior analytically.

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Sol-gel transformation are frequently accompanied by an increase in turbidity because of the appearance of spatial optical inhomogeneities in gelation. Thus, the transformation of fibrinogen (sol) to fibrin (gel) is monitored by turbidity measurements. The details of fiber structure composing the fibrin gel network such as mass/length ratio, radius and density were investigated from the wavelength dependence of turbidity [13–16]. One of the most widely used methods to monitor sol-gel transformation directly is storage and loss modulus measurements. The gel point is generally defined as the point at which storage modulus ($G'$) becomes larger than loss modulus ($G''$). As mentioned above, the two methods turbidity and dynamic modulus measurements monitoring different physical changes of the solution/gel associated by gelation have been widely used to study the gelation process. However, there are few reports to compare the data obtained by the two methods.

This research study was motivated by recent growing interest in the fibrin sealant for treatment of the skin after operation. Both enough elastic modulus and porous microstructure are necessary to prepare usable sealants. The former and latter have been evaluated by $G'$ and turbidity or transmittance ($I/I_0$), respectively [11, 12]. Furthermore, good performance can be obtained in a time frame that allows perfect manipulation. Therefore, the information on the time courses of $G'$ and ($I/I_0$) are requisite for the proper use. The sealant is now applying also in ophthalmology for treating leaking blebs, corneal perforations and ulcers as well as conjunctival grafts, lamellar keratoplasty and as a suture substitute [17]. The time frame needed depends on each application. Though a few kinds of sealant kits are available [12], they cannot be applied for all various uses. It is hoped to control the characteristics of equilibrium values of $G'$ and ($I/I_0$) and time constant for setting easily by varying preparation condition. The behavior of $G'$ and ($I/I_0$) is known to depend on $[\text{Ca}^{2+}]$. To find the best preparation condition, the range of $[\text{Ca}^{2+}]$ need not be limited to physiological one but could be extended up to a higher limit that causes inhibition of thrombin reaction due to the large ionic strength. The first and essential stage of the study for further development of fibrin sealants could be a measurement of time course of $G'$ and ($I/I_0$) in a wide range of $[\text{Ca}^{2+}]$ and an

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**Fig. 1** Schematic diagram of steps of thrombin-induced fibrin polymerization. (a) Three chains of $\alpha$, $\beta$ and $\gamma$ are shown as blue, red and green lines respectively. Stars indicate the calcium ion binding sites. (b) FpA and FpB are released by thrombin, and nobs ‘A’ and ‘B’ are exposed. (c) fibrin monomers are polymerized in a half-staggered manner via nob-hole interactions.
expression of the behaviors using a reasonably authorized empirical equation (double exponential equation) with a small number of parameters with a high precision. The symmetrical double exponential equation used for studies yet often make the fitting of the observed data to be quite difficult, and the resultant parameters depend on the initial values of fitting parameters [18, 19]. In this paper we obtained parameters which are determined independently of initial values for the fitting by dividing the gelation process into two stages. The analysis method could enable us to choose appropriate preparation condition for each application. According to this current situation, an experimental apparatus for simultaneous measurements of $G'$ and $(I/I_0)$ was designed.

2. Experimental

Fibrinogen (Bovine plasma, type I-S) was purchased from Sigma-Aldrich Co. and thrombin (Bovine plasma, 10000 units) and other chemicals (regent grade) were purchased from FUJIFILM Wako Chemicals Co. Fibrinogen was dissolved into Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.4, 150 mM NaCl). To remove citrate salts contained in the original sample, dialysis of the fibrinogen solution against TBS was carried out over-night. Fibrinogen concentration was determined by measuring the absorbance at 280 nm using an absorption coefficient of 1.5 mL mg$^{-1}$ cm$^{-1}$ and adjusted to 3.0 mg/mL. Fibrin gelation was initiated by adding 0.2 U/mL thrombin to the 3.0 mg/mL fibrinogen-TBS solution. For studying the effects of calcium ions, CaCl$_2$ was added to the fibrinogen-TBS solution at final concentrations 0, 1, 2, 10, 20, 30 and 50 mM.

The measurements of dynamic moduli and turbidity of the fibrinogen-TBS solution were carried out using a coaxial cylinder rotational rheometer with transparent inner and outer cylinders made of acrylic resin (ONRH-1, Ohna Tec. Inc., Japan) as shown in Fig. 2. The dynamic moduli ($G'$ and $G''$) were measured at an oscillation frequency of 1 Hz and an oscillation amplitude of 1 mrad. He-Ne laser beam was split by a beam splitter into two beams, one of which was directly detected by a photodiode to monitor the incident light intensity and the other passed through the transparent coaxial cylinder containing a sample solution. The turbidity was monitored by measuring the ratio $(I/I_0)$ of the intensity of the transmitted laser light through the transparent coaxial cylinder ($I$) and the incident laser light ($I_0$). Turbidity ($T$) is linearly proportional to $I/I_0$ as $T \propto I_0/I$. All the measurements were performed at the temperature of 25°C controlled by circulating water through a water jacket. The data were recorded by a PC-connected data logger every 400 seconds.

3. Results and discussion

Figure 3 magnifies the initial process of the time course of $G'$ and $G''$ after an addition of thrombin. The values of $G'$ and $G''$ increased with time ($t$) after an addition of thrombin. The time course of $G'$ showed three distinguishable phases: (i) lag phase, (ii) linear growth phase and (iii) exponential growth phase. The gelation time ($t_g$) was determined as the time when $G'$ become detectable ($G' > 0.2$ Pa). The lag time ($t_1$) was determined as the time for the intersection of the line for the phase (ii) and the horizontal axis, as shown in Fig. 3.
Figure 4 magnifies the initial process of the time course of $I/I_0$ after an addition of thrombin. The values of $I/I_0$ decreased with time. The time course of $I/I_0$ was composed of three phases, similarly to $G'$. The time for solution cloudiness ($t_c$) was determined as the time when $I/I_0$ becomes less than 0.98 and the lag time ($t_l$) was determined as the time for the intersection of the line for the phase (ii) and the line for $I/I_0 = 1$, as shown in Fig. 4.

Figure 5 shows the entire time courses of $G'$, $G''$ and $I/I_0$ in the presence of CaCl$_2$ at the various concentrations (0, 1, 2, 10, 20, 30 and 50 mM). Table 1 summarizes the values of $t_c$ and $t_l$ determined from the time courses of $G'$ and $I/I_0$ in the manner described above. With increasing the concentration of calcium ion ([Ca$^{2+}$]), the values of $t_c$, $t_l$, and $t_c$ decreased with [Ca$^{2+}$] up to [Ca$^{2+}$] = 20 mM, and above this concentration, these values increased. The decrease in $G'$ and $G''$ as the time for the intersection of the line for the phase (ii) and the line for the phase (iii) were less than one at all [Ca$^{2+}$] level in the experimental range.

Fibrin polymerization proceeds in a stepwise manner as follows [1]. In the first step, fibrin converted from fibrinogen by thrombin are assembled into half-straggled two-stranded protofibrils. In the second step, the protofibrils aggregate laterally to form fibrin fibers that branch into a three-dimensional gel network. The decrease in $I/I_0$ of fibrinogen solution can be ascribed to the formation of protofibrils in the first step. On the other hand, the increase in $G'$ can be ascribed to the formation of three-dimensional gel network in the second step. For this reason, the value of $t_c$ becomes smaller than that of $t_l$.

In the absence of Ca$^{2+}$, the time course of $G'$ was well expressed by

$$G' = G'_S \left[ 1 - \exp \left( \frac{t - t_1}{\tau_G} \right) \right],$$

where $G'_S$ is the saturated value of $G'$ at $t \rightarrow \infty$, $t_1$ is the time constant. In the presence of Ca$^{2+}$, a systematic error was observed in fitting by Eq. (1). Therefore, the following double-exponential-type equation of Eq. (2) was used to fit the data:

$$G' = G'_S \left[ 1 - \exp \left( \frac{t - t_1}{\tau_G} \right) \right] + \Delta G'_S \left[ 1 - \exp \left( \frac{t - t_1}{\tau_{\Delta G'} G'} \right) \right],$$

where $G'_S + \Delta G'_S$ is the saturated values of $G'$ at $t \rightarrow \infty$, $t_1$ is the time constant, $t_1$ (s) is the time when the increasing rate of adjacent $G'$ data recorded every 400 seconds is less than 0.02: $G' (t_1 + 400) - G' (t_1) < 0.02$. The time course of $G'$ was divided into the early stage at $t < t_1$ and the late stage at $t > t_1$. Kaibara et al. reported that the change of $G'$ for fibrinogen solution associated with gelation were represented by superposition of two first-order reaction processes with different reaction rate constants and expressed by a double-exponential-type equation [18, 19]. They concluded that the change of $G'$ in the first process was related to the number of cross-linking between fibers and the change of $G'$ in the second process was caused by stretching of the fibrin fibers between the crosslinks. The latter process gradually proceeded by the lateral aggregation of fibrin fibers after the initial network formation. The time course of $G'$ in the absence of Ca$^{2+}$ was well expressed by a single exponential function, i.e., only by the first term in Eq. (2), indicating that the stretching of fibrin strands
between cross-links hardly takes place. The parameters in Eqs. (1) and (2) are summarized in Table 2.

Time courses of \( G'' \) were well expressed by the following Eq. (3) both absence and presence of Ca\(^{2+}\):

\[
G'' = G''_S \left[ 1 - \exp \left( -\frac{t - t_1}{\tau_{G''}} \right) \right], \tag{3}
\]

where \( G''_S \) is the saturated value of \( G'' \) at \( t \rightarrow \infty \), and \( \tau_{G''} \) is the time constant. The parameters in Eq. (3) were determined by a least-squares method and are summarized in Table 3. The Ca\(^{2+}\) concentration dependences of \( G''_S \) and \( \tau_{G''} \) were similar to those of \( G'_S \) and \( \tau_{G'} \), respectively. Since the observed values of \( G'' \) are very small compared with those of \( G' \) and not much larger than the experimental error,

![Fig. 5 Time courses of \( G' \) (○), \( G'' \) (△) and \( I/I_0 \) (□) observed for 0.2 U/mL thrombin-induced gelation of 0.3 mg/mL fibrinogen solutions in the presence of CaCl\(_2\) at various concentrations of 0 (a), 1 (b), 2 (c), 10 (d), 20 (e), 30 (f) and 50 mM (g). The solid lines were the best fit to Eqs. (1)–(4) using a least-squares method.](image)

| \([\text{Ca}^{2+}] \) (mM) | \( t_g \) (s) | \( t_1 \) (s) | \( t_c \) (s) | \( t_2 \) (s) | \( t_g/t_1 \) | \( t_2/t_1 \) |
|----------------|-------------|-------------|-------------|-------------|-------------|-------------|
| 0              | 500         | 850         | 360         | 400         | 0.72        | 0.47        |
| 1              | 370         | 850         | 200         | 250         | 0.54        | 0.29        |
| 2              | 320         | 700         | 240         | 250         | 0.75        | 0.36        |
| 10             | 260         | 450         | 160         | 170         | 0.62        | 0.38        |
| 20             | 260         | 400         | 160         | 170         | 0.62        | 0.43        |
| 30             | 390         | 600         | 290         | 360         | 0.74        | 0.60        |
| 50             | 890         | 1100        | 650         | 750         | 0.73        | 0.68        |

These values were determined from the time courses of \( G' \) and \( I/I_0 \) in Fig. 3, as shown in Figs. 1 and 2.
we refrain further detailed analysis on $G''$. Time courses of ($I/I_0$) were well expressed by the following Eq. (4) and Eq. (5) in the absence and presence of Ca$^{2+}$, respectively:

$$\frac{I}{I_0} = \frac{I}{I_0}_S + \left[1 - \left(\frac{I}{I_0}_S\right)^{\frac{1-t_{\Delta I}}{\tau_{\Delta I}}}\right] \exp\left(-\frac{t-t_2}{\tau_I}\right),$$ (4)

$$\frac{I}{I_0} = \frac{I}{I_0}_S + \left[1 - \left(\frac{I}{I_0}_S\right)^{\frac{1-t_{\Delta I}}{\tau_{\Delta I}}}\right] \exp\left(-\frac{t-t_2}{\tau_I}\right) - \Delta\left[\frac{I}{I_0}_S \left[1 - \exp\left(-\frac{t-t_2}{\tau_{\Delta I}}\right)\right]\right],$$ (5)

where ($I/I_0)_S - \Delta(I/I_0)_S$ is the saturated values of ($I/I_0$) at $t\rightarrow\infty$, $\tau_I$ and $\tau_{\Delta I}$ are the time constants, $t_2$ (s) is the time when the increasing rate of adjacent ($I/I_0$) data recorded every 400 seconds is less than 0.01: [($I/I_0$) ($t_2 + 400$) - $I/I_0$ ($t_2$)]/[$I/I_0$ ($t_2$) < 0.01. The time course of ($I/I_0$) was divided into the early stage at $t < t_2$ and the late stage at $t > t_2$. The time course of ($I/I_0$) in the absence of Ca$^{2+}$ was expressed by a single exponential equation, i.e., only by the first term in Eq. (5) as is the case for $G'$. This result supports the hypothesis that Ca$^{2+}$ affects the proceeding of the lateral aggregation of fibrin fibers after the initial network formation [18]. The parameters in Eq. (4) and (5) were determined by a least squares method and are summarized in Table 4.

The [Ca$^{2+}$] dependences of $t_g$, $t_1$ and $G'$ were shown in Fig. 6 and 7, respectively. At low [Ca$^{2+}$] [< 2–3 mM (physiological concentration), the [Ca$^{2+}$] dependence of the parameters for both $G'$ and ($I/I_0)_S$ is quite complicated. This complexity could be attributed to the sensitivity

### Table 2 Parameters of Eqs. (1) and (2)

| [Ca$^{2+}$] (mM) | $G'$ (Pa) | $\tau_{G'}$ ($\times 10^3$ s) | $\Delta G'$ (Pa) | $\tau_{\Delta G'}$ ($\times 10^4$ s) | $t_1$ ($\times 10^3$ s) |
|----------------|-----------|----------------|----------------|----------------|----------------|
| 0 | 34.0 | 1.01 | — | — | — |
| 1 | 111 | 3.59 | $1.27 \times 10^4$ | 742 | 9.10 |
| 2 | 77.6 | 2.25 | 109 | 5.19 | 6.37 |
| 10 | 58.0 | 1.17 | 28.2 | 1.08 | 4.55 |
| 20 | 53.5 | 1.13 | 23.9 | 0.865 | 4.33 |
| 30 | 53.3 | 1.31 | 31.5 | 1.35 | 4.53 |
| 50 | 76.7 | 1.98 | 72.1 | 2.73 | 6.37 |

$G'$, $\tau_{G'}$, $\Delta G'$ and $\tau_{\Delta G'}$ were calculated using a least-squares method.

### Table 3 Parameters of Eq. (3)

| [Ca$^{2+}$] (mM) | $G''$ (Pa) | $\tau_{G''}$ ($\times 10^3$ s) |
|----------------|-----------|----------------|
| 0 | 3.51 | 0.645 |
| 1 | 4.66 | 2.43 |
| 2 | 4.71 | 1.96 |
| 10 | 3.47 | 0.969 |
| 20 | 3.28 | 0.696 |
| 30 | 3.22 | 0.956 |
| 50 | 3.93 | 1.40 |

$G''$, $\tau_{G''}$ were calculated using a least-squares method.

### Table 4 Parameters of Eqs. (4) and (5) in the absence and presence of Ca$^{2+}$

| [Ca$^{2+}$] (mM) | ($I/I_0)_S | $t_1$ (s) | $\Delta (I/I_0)_S | $\tau_{\Delta I}$ ($\times 10^3$ s) | $t_2$ ($\times 10^3$ s) |
|----------------|-----------|-----------|----------------|----------------|----------------|
| 0 | 0.38 | 651 | — | — | — |
| 1 | 0.33 | 622 | 0.056 | 3.19 | 2.78 |
| 2 | 0.32 | 555 | 0.048 | 3.20 | 3.05 |
| 10 | 0.31 | 352 | 0.037 | 3.88 | 2.17 |
| 20 | 0.29 | 278 | 0.039 | 6.43 | 1.80 |
| 30 | 0.28 | 289 | 0.047 | 5.20 | 1.96 |
| 50 | 0.44 | 585 | 0.057 | 6.67 | 3.15 |

($I/I_0)_S$, $t_1$, $\Delta (I/I_0)_S$ and $\tau_{\Delta I}$ were calculated using a least-squares method.

### Table 5 Parameters of Eqs. (4) and (5) in the absence and presence of Ca$^{2+}$

| [Ca$^{2+}$] (mM) | ($I/I_0)_S | $t_1$ (s) | $\Delta (I/I_0)_S | $\tau_{\Delta I}$ ($\times 10^3$ s) | $t_2$ ($\times 10^3$ s) |
|----------------|-----------|-----------|----------------|----------------|----------------|
| 0 | 0.38 | 651 | — | — | — |
| 1 | 0.33 | 622 | 0.056 | 3.19 | 2.78 |
| 2 | 0.32 | 555 | 0.048 | 3.20 | 3.05 |
| 10 | 0.31 | 352 | 0.037 | 3.88 | 2.17 |
| 20 | 0.29 | 278 | 0.039 | 6.43 | 1.80 |
| 30 | 0.28 | 289 | 0.047 | 5.20 | 1.96 |
| 50 | 0.44 | 585 | 0.057 | 6.67 | 3.15 |

($I/I_0)_S$, $t_1$, $\Delta (I/I_0)_S$ and $\tau_{\Delta I}$ were calculated using a least-squares method.

### Fig. 6 Ca$^{2+}$ concentration dependence of $t_g$, $t_1$ and $G'$. Panel (a): $t_g$ (●) and $t_1$ (○), panel (b): $G'$. 

| [Ca$^{2+}$] (mM) | $G'(s)$ | $\tau_{G'}$ ($\times 10^3$ s) |
|----------------|-----------|----------------|
| 0 | 3.51 | 0.645 |
| 1 | 4.66 | 2.43 |
| 2 | 4.71 | 1.96 |
| 10 | 3.47 | 0.969 |
| 20 | 3.28 | 0.696 |
| 30 | 3.22 | 0.956 |
| 50 | 3.93 | 1.40 |

$G'$, $\tau_{G'}$ were calculated using a least-squares method.
The \([\text{Ca}^{2+}]\) dependence of \((\text{I/I}_0)_S\) is roughly parallel to that of \(G'_S\) as shown in Figs. 6 and 7, since thickening of fibrin fiber (decrease of \((\text{I/I}_0)_S\)) induces lower number of the branching point (decrease of \(G'_S\)). Furthermore, since branching (networks forming) begins earlier when thickening is faster, \([\text{Ca}^{2+}]\) dependences of the time constants \(t_g, t_c, t_5\) and \(t_6\) are also roughly parallel to that of \(G'_S\) except for at low \([\text{Ca}^{2+}]\).

4. Conclusions

To control the mechanical and structural behavior of fibrin sealants, the time course of \(G'\) and \((\text{I/I}_0)\) of fibrin-thrombin systems was examined by a laboratory-made apparatus with varying \([\text{Ca}^{2+}]\) up to 50 mM. Both time courses were divided into two stages and well expressed by a single exponential function in the early stage and a double exponential function in the late stage. The equilibrium values of \(G'\) and \((\text{I/I}_0)\) match the requirement for enough rigidity and porous microstructure in the entire experimental range of \([\text{Ca}^{2+}]\). In the range of \([\text{Ca}^{2+}]\) less than physiological concentration ~ 2 mM, the values of \(G'\) and \((\text{I/I}_0)\) are very sensitive to change in \([\text{Ca}^{2+}]\) and exhibit a complicated \([\text{Ca}^{2+}]\) dependence. As \([\text{Ca}^{2+}]\) increases, \(G'\) and \((\text{I/I}_0)\) decrease and reach a plateau around 20–30 mM and then increase due to the inhibition of thrombin reaction by high ionic strength in the range more than 30 mM. The time constants for beginning of increase in elastic modulus and decrease in transmittance varies with \([\text{Ca}^{2+}]\) in parallel to \(G'\) and \((\text{I/I}_0)\). Therefore, we cannot change \(G'\), \((\text{I/I}_0)\) and time constants \(t_g\) and \(t_c\) for setting independently by controlling \([\text{Ca}^{2+}]\). We may still take advantage of the difference in the time constants \(t_g\) and \(t_c\) at plateau and those at \([\text{Ca}^{2+}] = 50\) mM. The former \((t_g = 260\) s = 4.3 min and \(t_c = 160\) s = 2.7 min) are 3–4 times smaller than the latter \((t_g = 890\) s = 14.8 min and \(t_c = 650\) s = 10.8 min). These results will provide an optimal \([\text{Ca}^{2+}]\) corresponding to various surgical operations. Future studies including XIII factor should substantiate the above essential behavior for practical use.

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