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Effect of Cyclodextrin on the Gelation of Fibrinogen

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Fibrinogen, which is the central factor of blood clotting, gels by thrombin. It has been known that the gelation of mixture of fibrinogen and thrombin is enhanced by the addition of dextran. In the gelling kinetics of fibrinogen by thrombin, it was observed that a remarkable lag in gelation time appears or no gelation occurs by the addition of cyclodextrin (CD) even at 1 wt% revealed by the dynamic light scattering (DLS) and turbidity measurements. In DLS measurement, when thrombin was added to the fibrinogen solution, scattered light intensity increased rapidly resulting the gelation. On the contrary, when β-CD (7 units of glucopyranose) was added to the fibrinogen-thrombin system, the mixture did not gel, although any visible change did not occur. Temporal variation of turbidity on the fibrinogen-thrombin system with the addition of β-CD revealed a qualitative difference from that without β-CD. When β-CD was added, it was found that the growth of loosely assembled fibrin fibers up to the state with high density was inhibited substantially and only a very weak and imperfect network is formed.
Key words: Cyclodextrin, Fibrinogen, Gelation, Dynamic Light Scattering, Turbidity

1. INTRODUCTION

Fibrinogen is a rod shaped protein with the molecular weight of 3.4×10^5, and assembles into a three-dimensional gel network by the action of thrombin (removal of fibrinopeptide A and B). Formation of the fibrin gel proceeds in a stepwise manner: activation of fibrinogen in order to originate the fibrin monomer, end-to-end staggered polymerization of fibrin monomers (protofibril formation), and side-by-side association of protofibrils (fiber growth) resulting the gel formation [1,2].

The growth kinetics of fibrin gel has been studied by the light scattering [3,4,5], turbidity methods [2,6,7] and electron microscopy [1]. On the other hand, in fibrinogen to fibrin gel conversion by thrombin, it was reported that appreciable effects appear of the pH change [8] or addition of divalent cations [9,10], sphingosin [11] and dextran [6,7]. Dextran interacts both with fibrinogen, reducing solubility [12,13], and with fibrin, accelerating its polymerization [14]. Moreover, it was recently found that the gelation is enhanced with the increase of the molecular weight of added dextran.

Cyclodextrin has a doughnut-like structure and the central hole provides hydrophobic-environment. Thereby, the residue and the molecule can be taken into this hole. Because of these characteristics, various interesting inclusion phenomena have been observed.

In the present work, the effect of addition of β-CD on the gelation kinetics of fibrinogen was investigated using DLS and turbidity measurement.

2. EXPERIMENTAL

Bovine fibrinogen (Sigma-Aldrich Co.) was dissolved in a physiological saline solution (pH 7.4 ; 136.9 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.10 mM Na_2HPO_4) and this solution was dialyzed against the physiological saline solution overnight. Concentration of fibrinogen solution was determined from the absorbance at 280 nm, using an extinction coefficient of 1.51 ml/mg·cm.

Thrombin (Mochida Co., Japan) solution was prepared in the physiological saline solution at the concentration of 1 NIH units/ml. Cyclodextrin (Tokyo Kasei Co., Japan) solution was prepared by dissolving CD in the physiological saline solution to the final concentration of 1 wt% to a fibrinogen solution.

Before mixing the fibrinogen and thrombin solutions to start the reaction, fibrinogen solution with CD was prepared to make a final concentration of 2.2 mg/ml.

Just before the measurements, the both solutions of fibrinogen and thrombin was mixed at the volume ratio of 100:1, and this time of mixing was denoted as the elapsed time $t = 0$ in the time course of gelation process.

Turbidity measurements were made using HITACHI U-2000A spectrophotometer. Wavelength dependence of turbidity was investigated scanning over 800 to 400 nm. For the light scattering measurement, the mixed solution was immediately put into a cylindrical cell of the optical path length of 6 mm by passing it through a membrane filter of 0.2 μm pore size. The preparation was carried out in a clean dry box in order to prevent contamination of impurities.

Dynamic light scattering measurements were carried out using a homemade spectrometer and an ALV-5000 multiple-tau digital correlator to obtain the correlation function of scattered light $g^{(2)}(\tau)$ and the averaged scattered light intensity $< I >$ simultaneously. The decay time distribution function $G(\tau^*)$ was obtained from $g^{(2)}(\tau)$ by using CONTIN program. Light source was Ar ion laser with the wavelength $\lambda = 488$ nm and the details of the apparatus was described elsewhere [15]. The measurement of $g^{(2)}(\tau)$ in the time course of gelation were carried out at the scattering angle $\theta = 30^\circ$ and were obtained by the homodyne mode.

All the measurements of turbidity and light scattering were performed at the temperature of 37 °C.

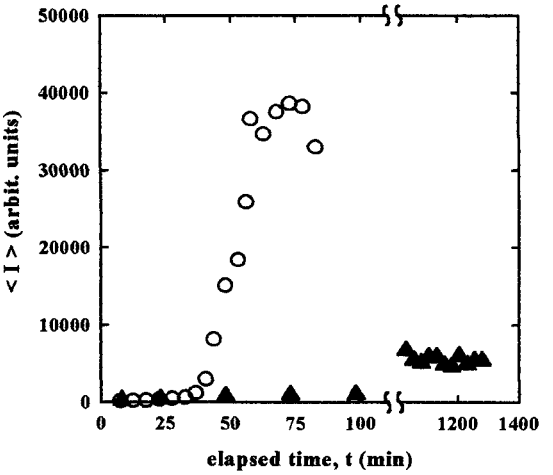

Fig. 1. Time evolution of the scattered light intensity at θ =30 ° for the fibrinogen-thrombin system (○) and for the system in the presence of 1 wt% β-CD (▲)

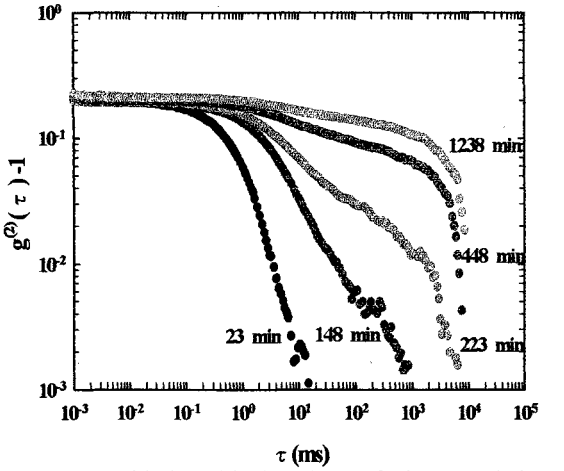

Fig. 2. Double-logarithmic plots of the correlation function $g^{(2)}(\tau)$ - 1 as a function of the delay time τ in the presence of 1 wt% β-CD. Numbers in the figure mean the elapsed time, t (min).

3. RESULTS AND DISCUSSION

Figure 1 shows the scattered light intensity < I > as a function of the elapsed time t. In the fibrinogen-thrombin system, the scattered light intensity showed a low value for t < 40 min, and rapidly increase at t > 40 [16]. However, in case of the sample with 1 wt % β-CD, such a rapid increase of the scattered intensity was not observed. Even if the elapsed time passed over 20 hours, the intensity did not become about 1/5 of the fibrinogen-thrombin system (without β-CD). This very slow and weak increase of the scattered light intensity in the system with β-CD suggests that appreciable contrast change in the concentration (density) fluctuation was hindered compared to fibrinogen-thrombin system.

Figure 2 shows double-logarithmic plots of the correlation function $g^{(2)}(\tau)$ - 1 as a function of delay time τ in the solution of 1 wt% β-CD. The $g^{(2)}(\tau)$ - 1 shows a characteristic decay behavior similar to the usual polymer solution with a broad decay time distribution for the first time. With the increase of elapsed time, $g^{(2)}(\tau)$ - 1 became to show slowing down of the decay. However, a long time tail in the range of long delay time was now observed, but a behavior suggesting the existence of cut-off of the longest decay time was observed. The initial value corresponding to the coherence of detection did hardly change, and any substantial nonergodicity (or inhomogeneity) was not detected corresponding to the nonappearance of oscillating behavior of the scattered light intensity. Especially, any power-law type behavior of $g^{(2)}(\tau)$ – 1 was not observed.

Figure 3 shows the time evolution of the decay time distribution function G(τ*), τ* being the decay time. In the initial stage, G(τ*) showed a unimodal distribution in the short decay time region. G(τ*) of fibrinogen solution without thrombin is almost the same as that at the initial stage, expect for having a narrower distribution. The peak of G(τ*) in an initial state sifted to a longer decay time with the increase of elapsed time.

These results of DLS measurements suggest that a uniform and very loose assemblies, where the respective fibrinogen molecules or protofibrils might be able to

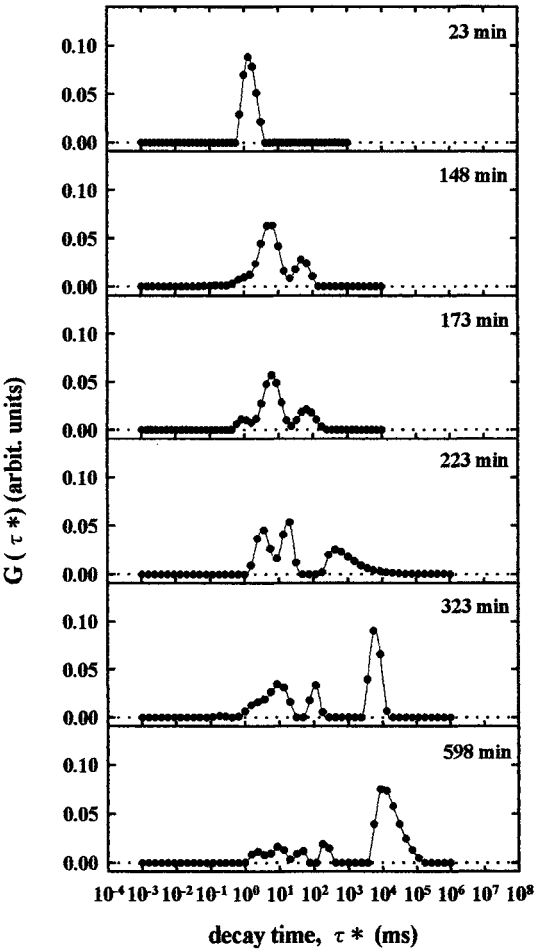

Fig. 3. Typical result of the time evolution of decay time distribution function G(τ*) in the presence of 1 wt% β-CD to the fibrinogen-thrombin solution.

achieve a Brownian motion almost freely in the very viscous media, were formed at large elapsed time region

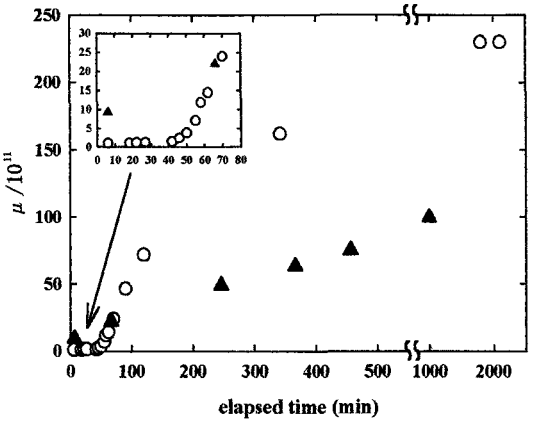

Fig. 4. Variation of mass/length ratio as a function of elapsed time. Fibrinogen-thrombin system (○) and in the presence of 1 wt% β-CD (▲).

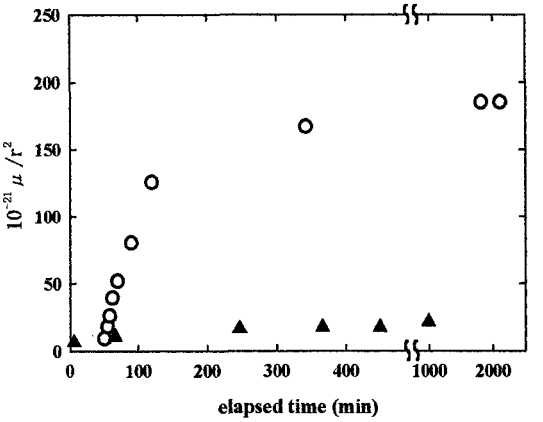

Fig. 5. Density variation of fibrin fiber assembly as a function of elapsed time. Fibrinogen-thrombin system (○) and in the presence of 1 wt% β-CD (▲).

when β-CD was added.

In order to characterize this assembled state in more details, turbidity measurements were carried out with accompanying the elapsed time in the wavelength range of 800 to 400 nm. Because fibrinogen molecules and protofibrils can be regarded as long rodlike particles, turbidity relates to global nature of the rod. The following function [7] was used for analysis.

$$(C / \tau \lambda^3) = A / \mu + B (r^2 / \mu) \lambda^{-2} \qquad (1)$$

where c is the fibrinogen concentration, τ is turbidity, λ is wavelength of the incident light, μ is the mass/length ratio of the assembled fibers, r is the radius, and A and B are the constants. Equation (1) implies that a plot of $c/\tau\lambda^3$ vs. λ^{-2} should give a straight line whose intercept is proportional to the reciprocal of the mass/length ratio. In addition, the ratio of the slope to the intercept is proportional to the square of the radius of the rods.

Figure 4 shows a growth of mass/length ratio μ with the elapse time. In fibrinogen-thrombin system, it was shown that μ increased almost at the same time as that the scattered light intensity increased drastically (Fig. 1). However, when β-CD was added, μ became a large value from immediately after the mixing, and only a gradual and less increase was observed. Figure 5 shows the variation of μ/r^2 with the elapsed time. μ/r^2 means the density of assembled fiber. Because protofibril is too thin to be analyzed by eq. (1), the data before about 50 minutes of fibrinogen-thrombin system (protofibrils were already formed and gelation occurred at about 50 min) are not shown. Just after the gelation without β-CD, the fiber density is quite low and fiber should be very loose. Thereafter, fibers grow and became denser, that is, strong network formation proceeds. Therefore, the contrast of spatial density variation was strengthened, and the scattered intensity increased. On the other hand, when β-CD was added, the variation of density was hardly observed within the experimental elapsed time, although the weak growth (increase) of fiber assembly occurred. This observation means that very loose assembly of fibrinogen (or protofibrils) was formed from immediately after the mixing, but the strengthening of the network structure and the lateral fiber aggregation was hindered very much. The self-associating tendency of β-CD presumably causes the condensation of β-CD molecules around fibrinogen and/or protofibrils, which results in the large μ value at the early stage, and inhibits the firm lateral aggregation for the compact fiber formation. Therefore, fairly uniform and incomplete network structure is formed in the presence of β-CD. Because of the incompleteness of network, the diffusional motion of respective assemblies is possible.

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