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Effect of urea for gelation dynamics and gel structure of fibrin gel observed by DLS and CLSM

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We studied gelation dynamics and the network structure of fibrin gel, which is the primary structural component of blood clots. The thrombin induced fibrin gel formation was investigated with time evolution measurements of dynamic light scattering (DLS). The 3-dimensional network structure of fibrin gel was obtained by confocal laser scanning microscopy (CLSM). The addition of urea, which is known to promote or destroy hydrogen bondings, showed significant contributions for gel properties. For low urea concentrations the stepwise gelation process was primarily the same with the solution of the absence of urea except for the proceeding time, whereas the gelation was suppressed at a sufficient amount of urea concentration, where the denaturation of fibrinogen was not taken place. The structural properties of the network are discussed with 3-d network structure images of CLSM and the time correlation function of DLS.

Key words: fibrinogen, gel, urea, dynamic light scattering, real space observation

1. INTRODUCTION

Gelation dynamics and gel structure of biopolymer gels are greatly influenced by their environment, such as pH, ionic strength, temperature and so on. There are many types of interactions in the gelling systems, where hydrogen bonding is one of the most important factors in order to understand the functions of biopolymer gels. The addition of highly polar molecules, such as urea or guanidine hydrochloride, modifies the hydrogen bonding and the local structures of water. Therefore properties of biopolymer gels are expected to be influenced in a great extent by the addition of polar molecules.

Recently, we reported gelation dynamics of a fibrin gel in PBS buffer solution by means of turbidity measurements, static light scattering (SLS) and dynamic light scattering (DLS) [1-4]. The stepwise gelation of fibrin gel was clearly observed by analyzing the time evolution of the correlation function of DLS. The three-dimensional (3-d) network structure of the fibrin gel in a hydrated state was also studied by means of confocal laser scanning microscope (CLSM). The structural feature of 3-d network was characterized by the real space images [3,4]. These studies showed that fibrinogen - thrombin solution is a good model system to study the gelling process and gel structure simultaneously, since the dynamic process of gelation can be characterized by DLS and the network structure at the molecular level, keeping the sample in the hydrated state, can be directly observed by CLSM.

In this study, we study the effect of urea on gelation dynamics and gel structure of fibrin gel by means of DLS and CLSM. Here, the urea concentration was sufficiently below the denaturation of fibrinogen [5]. The effects of both urea and thrombin concentration are examined systematically.

2. EXPERIMENT

2.1. Sample Preparation.

Bovine fibrinogen in 0.15 M phosphate buffer solution at pH 7.4 was used in this study. Details of the sample preparation have been described elsewhere [1]. The final concentration of the fibrinogen solution was adjusted to a physiological concentration of 0.22 g/100 ml. Urea was added to the fibrinogen solution in the range from 0 M to 1.0 M as the final concentrations. Thrombin was used for initiation of gelling process. Just before the measurements, solutions of fibrinogen and thrombin were mixed and the time of mixing was denoted as the elapsed time $t_e = 0$ in the time course of gelation. The final concentrations of thrombin were set as mentioned in each experimental result. Relatively low concentrations of thrombin were used to ensure the accurate measurements.

2.2. DLS Experiment.

DLS measurements were carried out using a homemade spectrometer with ALV-5000 correlator to obtain the correlation function $g^{(2)}(t)$, defined as $g^{(2)}(t) = \langle I(0)I(t)\rangle/\langle I(0)\rangle^2$ where $I(t)$ is the scattered light

intensity. The light source, a He-Ne laser with the wavelength λ = 632.8 nm was used. Details of the apparatus have been described elsewhere [6]. The measurements of $g^{(2)}(t)$ in the time course of gelation were carried out at a scattering angle θ = 30°. The temperature of the sample cell was controlled at 37.00 ± 0.01 °C.

The normalized electric field correlation function $g^{(1)}(t)$ is expressed by Siegert relation as

$$g^{(2)}(t) - 1 = \beta |g^{(1)}(t)|^2, \quad (1)$$

where β is a constant relating to the coherence of detection. $g^{(1)}(t)$ was analyzed by the constrained regularized method (CONTIN) to yield the distribution function $G(\tau)$ as a function of the characteristic decay time τ as

$$g^{(1)}(t) = \int G(\tau) \exp(-t/\tau)\, d\tau$$

$$= \int \tau\, G(\tau) \exp(-t/\tau)\, d(\ln \tau). \quad (2)$$

At the gelation point, $g^{(1)}(t)$ has no characteristic time and shows a power law behavior, as has been observed in many gelling systems,

$$g^{(1)}(t) \sim t^{-\Phi}. \quad (3)$$

The $g^{(2)}(t)$ is practically expressed as a sum of a cooperative diffusional mode relating to the entangled (crossbridged) structure and a power law behavior, which was first reported by Martin et al. [7] as

$$g^{(2)}(t) - 1 \sim [A \exp(-t/\tau_f) + (1 - A)(1 + t/\tau')^{-\Phi}]^2. \quad (4)$$

Here, A is an amplitude factor of the relative scattered intensity, and τ_f and τ' are the characteristic decay time of the fast (cooperative) mode and the lower cutoff time of the power law behavior, respectively. On the other hand, $g^{(2)}(t)$ of the pregel solution is well described similarly by the stretched exponential form as

$$g^{(2)}(t) - 1 \sim \{A \exp(-t/\tau_f) + (1 - A) \exp[-(t/\tau_s)^{\beta}]\}^2. \quad (5)$$

Here, τ_s is the characteristic decay time of the slow stretched mode. Approaching the sol-gel transition point, the correlation function $g^{(2)}(t)$ varies from the stretched exponential form to the power-law one with β decreasing to zero and τ_s diverging.

2.3. CLSM Experiment.

The real space images of the network structure were obtained by a confocal scanning laser microscope (LSM510, Carl Zeiss Co., Ltd., Germany). A 100 µl of sample solution was poured into a dish, which had a glass bottom of 0.12 mm or less thickness. The dish was capped and incubated at 37.0 °C until complete the gel formation. Then, the fibrin gel was stained by pouring 1ml of 1.6 mM fluorescein isothiocyanate (FITC) in a 0.15 M PBS buffer (pH 8.0) to the dish for about 3 hours and washed extensively by a fresh buffer without FITC. The obtained image was analyzed by a box counting method to determine the fractal dimension of the network structure. A self-similar structure was expressed using the fractal dimension D_f defined as

$$M(R) \sim R^{Df}. \quad (6)$$

Here, R is the specific length and M the mass in the range R. The details of the experiments and analyses of the CLMS were described elsewhere [3].

3. RESULTS AND DISCUSSION

3.1. Gelation Dynamics Observed by DLS.

The gelation time was deduced from the correlation function $g^{(2)}(t)$-1 as judged by the crossover behavior from the stretched decay function (Eq. 5) to the power law one (Eq. 4). Typical examples of the double logarithmic plot of $g^{(2)}(t)$-1 for 0.025 NIHunits/ml thrombin sample were shown in Fig. 1. The upper and bottom figures correspond to the samples containing 0.2 M and 1.0 M urea, respectively. The numbers inside the figures are elapsed time t_e (min) and the lines are fitted functions to Eq. 4 or Eq. 5. For 0.2 M urea sample the $g^{(2)}(t)$-1 showed the stretched exponential behavior for t_e < 99 min which corresponds to that the sample is in a sol state because of existing of the characteristic decay time, whereas it showed the power law one for $t_e \geq$ 99 min with diminishing the initial amplitude. The power law behavior of $g^{(2)}(t) - 1$ means the network obtains a self-similar structure on a large scale compared with molecular size, i.e. the formation of fibrin gel is completed at this time. The fitting parameters τ_s and β of the stretched exponential formula for 0.2 M and 1.0 M urea were shown in Fig. 2. The results of 0.2 M urea showed a

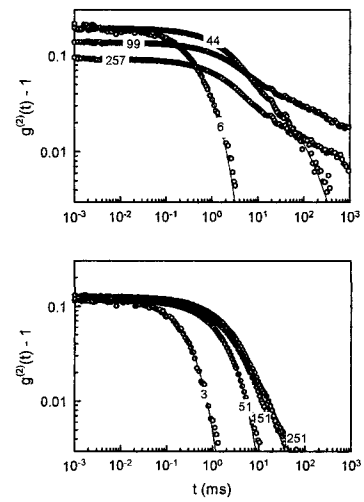

Fig. 1. Double logarithmic plots of the time correlation function $g^{(2)}(t)$ −1 for fibrinogen solutions containing 0.2 M urea (upper) and 1.0 M urea (bottom). The numbers inside the figures indicate the elapsed time, t_e (min). The concentration of thrombin is 0.025 NIHunits/ml.

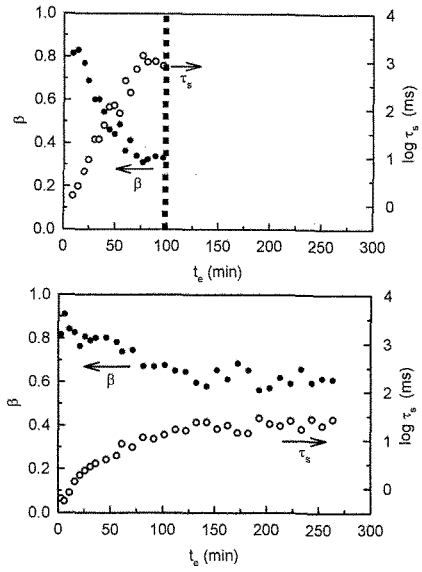

Fig. 2. Decay time τ_s and exponent β obtained with Eq. 5 for 0.2 M urea (upper) and 1.0 M urea (bottom). The dotted line indicates the gelation time.

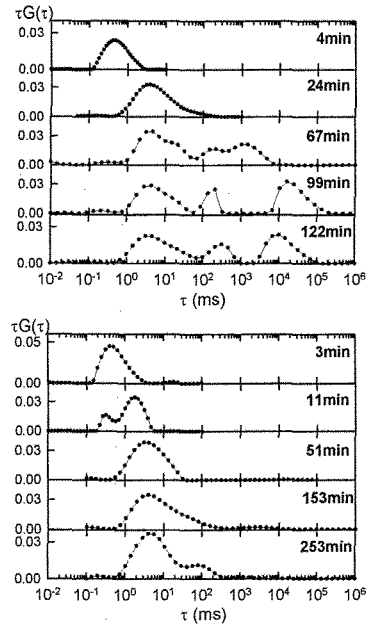

Fig. 3. Distribution function of decay time τG(τ) for 0.2 M (upper) and 1.0 M (bottom) urea samples.

rapid increase of τ_s and decrease of β as time elapsed. The dotted line indicates the gelation time determined from the crossover behavior of $g^{(2)}(t)$-1. For 1.0 M urea sample the τ_s and β showed gradual changes and showed saturation behaviors. The existence of a finite relaxation time τ_s means the solution is in a sol state, i.e. it is confirmed that the fibrin gel formation was inhibited by the addition of 1.0 M urea for this fibrinogen-thrombin sample. This can be also seen in Fig. 1 that the decay time does not change after 150 min.

Figure 3 represents the time evolution of the decay time distribution τG(τ) which clearly shows the suppression of fibrin gel formation for 1.0 M urea sample. The mono modal peak at t_e = 4 min for 0.2 M urea sample and at t_e = 3 min for 1.0 M urea sample represent the fibrin monomers or the fibrin oligomers. For 0.2 M sample at the elapsed time from 4 to 24 min, the protofibril formation occurred as can be seen in the peak shift toward the slightly slow decay times ($\tau = 10^0$ - 10^1 ms). At t_e = 99 min, the slowest mode, which is result of a gel cluster formation, was observed and this time corresponds to the gelation time. The intermediate peaks at around $\tau = 10^1$ - 10^3 ms indicate the relaxation process of fibrin fibers (gel mode). This stepwise gelation process of 0.2 M urea sample was identical with 0 M urea sample (data not shown) except for the elapsed time. For 1.0 M sample the slight peak shift was observed, however, the slower peaks ($\tau > 10^2$ ms) did not appear in the whole experimental time. This observation implies that the protofibril formation took place, while the fiber formation (lateral aggregation of protofibrils) was inhibited by the addition of 1.0 M urea.

3.2. Gelation Time and Fractal Dimension

The urea concentration dependence of the gelation time determined by DLS is summarized in Fig. 4 as indicated by circles. The filled and open circles correspond to the samples of 0.033 NIHunits/ml and 0.025 NIHunits/ml thrombin, respectively. The lines are drawn to guide eye. In both thrombin concentrations the gelation time was delayed with increasing the urea concentration. Gelation times showed a divergence tendency as a function of urea concentration that the inhibition of gel formation occurred at around 0.2 M urea for 0.025 NIHunits/ml thrombin sample (dotted line) and at around 0.8 M urea for 0.033 NIHunits/ml thrombin sample (plain line). The suppression of gel formation depends on both the thrombin concentration and the urea concentration.

Figure 4 also shows fractal dimension D_f as indicated by triangles which were obtained by the correlation function using the Muthukumar's theory, described as $1-\phi = d(d+2-2D_f)/2(d+2-D_f)$ [8,9]. Here, d corresponds to the dimension and ϕ is the power low exponent in Eq. 4. As the urea concentration increases the fractal dimension increased with a leveling off tendency. The magnitude of the fractal

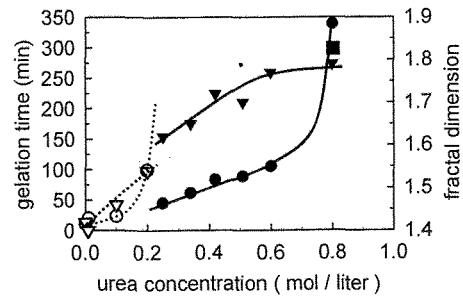

Fig.4. Urea concentration dependence of the gelation time (○,●) and fractal dimension (△,▲) determined by DLS. The thrombin concentrations are 0.025 NIHunits/ml (open symbols) and 0.033 NIHunits/ml (filled symbols). The square (■) corresponds to the fractal dimension determined by 3-d images of CLSM (Fig. 5).

dimension becomes large as increase of urea concentration. Usually, smaller amount of thrombin gives a slower gelation process with more homogeneous network structure corresponding to a large fractal dimension. These results indicate that the network structure becomes more homogeneous with slow gelation time by increasing of the urea. This point is discussed by the real space images in the next section.

3.3. Real Space Observation by CLSM

In the previous paper [3], we demonstrated that the fractal dimension of the 3-d network structure could be determined on 3-d images obtained by CLSM, and the fractal dimension of fibrin gel was determined using a 3-d box-counting method. The resultant values were coincident well with the results of DLS and Fourier transform analyses. Figure 5 shows the constructed 3-d image for 0.8 M urea with 0.033 NIHunits/ml thrombin sample. In comparison with the results without urea, which are shown in previous publication (Fig. 2 of Ref. [3]), one can easily identify the thinner fiber diameter with a sparse network structure for 0.8 M urea sample. The crosslinking distances of the network for 0.8 M urea is longer than the samples without urea. Unfortunately, further quantitative analyses, such as the fiber diameter distribution and the Fourier transformation for evaluation of power spectrum as carried out in Ref. [3], were not employed in this image because of the resolution limit of thin fibers. Even for the low resolution image, the fractal dimension was tentatively determined as 1.8 by the 3-d box-counting method and plotted in Fig. 4 as indicated by filled square. The homogeneous network structure composed of the fine fibers is result of the addition of urea. It indicates that the aggregation of protofibrils to form fibrin fibers is inhibited by urea. This fact is in agreement with DLS results, where the fiber formation is suppressed as can be seen in Fig. 3. Therfore the larger fractal dimensions obtained for higher urea concentrations could be result of the better homogeneity of the network structure.

3.4. Effect of Urea for Lateral Aggregation

The addition of urea leads to a slow gelation process, and the resultant network structure is sparse one composed of fine fibers, although the stepwise manner of fibrin gel formation is identical in those low urea concentrations as shown in Fig. 3. For a sufficient amount of urea the gel formation was suppressed. This is because, as clearly shown in Fig, 3, the lateral aggregation of protofibrils to from fibrin fibers was inhibited by the sufficient amount of urea.

The spontaneous gel formation is known as the cleavage of fibrinopeptides A and B from fibrinogen, the formation of protofibrils from fibrins, the lateral aggregation of the fiber formation from protofibrils, and the succeeding network formation [10-13]. Among these steps it is expected that the local structure of water should have an important role. If one assumes that urea molecules destroy the water structure, the gelation dynamics could be modified in a great extent. Indeed, the significant effect of urea

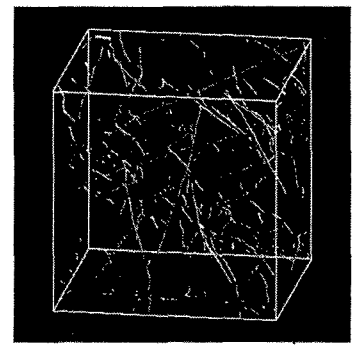

Fig. 5. Three-dimensional image of hydrated fibrin gel observed by CLSM for 0.8 M urea with 0.033 NIHunits/ml thrombin. The image size is 25.6 x 25.6 x 25.6 μm.

for the fibrin gel formation and the gel structure were illuminated clearly in this study. Further studies with adjusted thrombin activity for fibrin gelation will give precise roles of hydrogen bondings on fibrin gelation.

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