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Measurement of Additive Effects of Cyclodextrins on Fibrin Gelation by Using Quartz-Crystal Microbalance

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The processes of fibrin polymerization and gelation were examined by using QCM method through the change in frequency (ΔF) and impedance (ΔR). Two different fibrinogen concentrations have been used; lower one without the gelation (0.1 mg/ml) and higher one with the gelation (2.0 mg/ml). Fibrinogen-immobilized 9MHz quartz-crystal was immersed in the fibrinogen solutions, and then 0.01 unit/ml of thrombin was added to initiate the reaction. At the low concentration, ΔR was proportional to ΔF in the progress of fibrin polymerization and/or the side-by-side association of protofibrils. At the high concentration, ΔR steeply increased with increase in ΔF due to the fibrin gelation. Furthermore, additive effects of α-, β- and γ-cyclodextrin (CD) on the fibrin gelation were investigated. CDs are torus-like molecules with a hydrophobic internal cavity and hydrophilic external surface and are widely used as solubilizer or stabilizer in many products such as foods, pharmaceuticals and cosmetics. It is revealed that the addition of CDs, especially β-CD, not only retarded the fibrin gelation but also changed the viscoelastic properties of the formed gel.
Key words: fibrinogen, fibrin, gelation, cyclodextrin, quartz-crystal microbalance

1. INTRODUCTION

The final stage of blood coagulation reaction is the fibrinogen-fibrin conversion induced by the action of thrombin. This reaction is initiated by the release of fibrinopeptides A and B (FPA and FPB) and finally produces three-dimensional gel network (fibrin gel). The formation of fibrin gel proceeds in a stepwise manner: activation of fibrinogen to originate the fibrin monomer and half staggered axial polymerization of fibrin monomers (protofibril formation) in the first step, and side-by-side association of protofibrils (fiber growth) resulting the gel formation in the succeeding step [1,2].

Recently, it has been found that cyclodextrins (CDs) retarded the fibrin gel formation [3,4]. CDs are cyclic oligosaccharide consisting of 6, 7 or 8 glucopyranose units joined by α-(1,4)- glycosidic linkages (respectively named α-, β-, and γ-CD). They are torus-like molecules with a hydrophobic internal cavity and hydrophilic external surface. The hydrophobic cavity is capable of forming inclusion complexes with various guest molecules and the hydrophilic external surface generates good water solubility. With its characteristic property, CDs are widely used as solubilizer or stabilizer in many products such as foods, pharmaceuticals and cosmetics.

Quart-crystal microbalance (QCM) has been used as a very sensitive mass sensor because its frequency decreases with mass adsorption on the crystal in the order of nanogram [5-8]. In another variation, QCM can be used to examine the viscoelastic properties at/near the crystal surface by the measurement of impedance at resonance [9] or dissipation factor [10,11]. In this study, the processes of fibrin polymerization and gelation were measured by using QCM method. The additive effects of α-, β-, and γ-CD on these processes were analyzed through the changes both in the adsorbed mass (as ΔF) and the viscosity (as ΔR) of fibrin layers on the crystal surface.

2. EXPERIMENTAL

Bovine fibrinogen was purchased from Sigma-Aldrich Co. and was dissolved into a phosphate buffered saline solution PBS (155 mM NaCl, 3.9 mM K_2HPO_4, 0.7 mM KH_2PO_4, pH 7.4) without further purification. After dialysis against PBS overnight, final concentrations of the solution were adjusted to 0.1 and 2.0 mg/ml, which was determined from an absorbance at 280nm using an extinction coefficient of 1.5 ml/(mg·cm). α-, β- and γ-CD (Sigma-Aldrich) dissolved into the PBS were added to the fibrinogen solution at a final concentration of 8 mM. Thrombin (Wako Pure Chemical) PBS solution was added to the fibrinogen solution at a final concentration of 0.01 NIH units/ml.

The QCM measurements were made by the commercially available QCM-system (Seiko EG&G, QCA922) equipped a handmaid temperature controls.

Gold coated AT-cut quartz crystal (9 MHz) was used as the electrode. Fibrinogen molecules were immobilized on the electrode surface covered with a self-assembled monolayer of dithiobis(succinimidyl hexanoate) (Dojindo). The fibrinogen immobilized electrode was immersed in 250 μl of the fibrinogen solution. Immediately after the addition of 2.5 μl of 1 NIH units/ml thrombin solution, the frequency (F) and impedance (R) at resonance were measured at 25 °C. The relation between ΔF and mass change (Δm) of the adsorbed rigid materials onto the electrode is expressed by the following equation [10]:

$$\Delta F = \left(-2F_0^2 / A\sqrt{\rho\mu}\right) \cdot \Delta m \qquad (1)$$

where F_0 is the fundamental frequency of the QCM (9 MHz), A is the area of electrode (1.96×10^{-1} cm^2), ρ and μ are the density (2.65 g/cm^3) and shear modulus (2.95×10^{11} dyn/cm^2) of quartz, respectively. Thus, a frequency decrease of 1Hz corresponds to a mass increase of *ca.*1 ng on the QCM. When the electrode was immersed into a liquid, the quartz crystal was affected by the viscosity (η_L) and density (ρ_L) of liquid, yielding the change in R. The relation among R, η_L and ρ_L is expressed by the following equation [11]:

$$R = \left(A/k^2\right) \cdot \sqrt{2\pi\rho_L\eta_L} \qquad (2)$$

where k is the quartz electromechanical coupling coefficient.

3. RESULT AND DISCUSSION

Figure 1 shows time courses of ΔF and ΔR of the fibrinogen immobilized QCM in the 0.1 and 2.0 mg/ml fibrinogen solution. Immediately after the addition of thrombin denoted as elapsed time t=0, the decrease in ΔF and the increase in ΔR were observed in progress of fibrin polymerization. The initial changes in ΔF and ΔR were almost same for both concentrations, but the saturated vales at the high concentration were larger than those at the low concentration. After the measurements, fibrin gelation was observed only for the high concentration. The relation between ΔF and ΔR at the same t is shown in Fig. 2. At the low concentration, the plot was linear, and ΔR was proportional to ΔF. At the high concentration, the plot curved upward around ΔF=400 Hz. The turning point was correspond to t=50 min in Fig. 1 and was consistent with clouding point of the fibrinogen solution (data not shown). This result means that the formation of fibrin gel increases the viscosity near the electrode surface, resulting in increase in ΔR.

Figure 3 shows the time courses of ΔF and ΔR for the 0.1 mg/ml fibrinogen solution in the presence α-, β-, and γ-CD. While the change in both ΔF and ΔR were retarded by the addition of CDs in the early stage t<40 min, its extent was larger than that of the control sample in the order of γ- < α- < β-CD. The initial changes in ΔF and ΔR represent the polymerization of fibrin monomers (protofibril formation) and/or the side-by-side association of protofibrils (fiber growth). CDs showed the retardation effects on these reactions, and the effects were consistent with those obtained by dynamic light scattering measurements [3]. The relation between ΔF and ΔR at the same t is shown in Fig. 4. In the range of ΔF < 100 Hz, all plots were linear and almost overlapped. With increasing ΔF, the plots curved slightly upward in the presence of CDs, especially β-CD. This result indicates that a viscous layer was formed on the electrode surface. The amount of fibrinogen immobilized on the electrode surface (1.96×10^{-1} cm^2) was estimated from a difference in frequency (*ca.* 100 Hz) between the immobilized and the bare electrode. On the assumption that fibrinogen molecules (10 nm in diameter) were immobilized on the

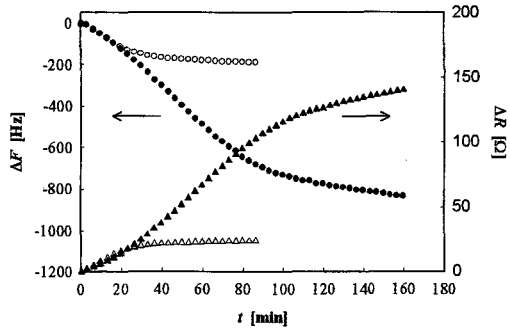

Fig. 1 Time courses of ΔF (circle) and ΔR (triangle)of the fibrinogen immobilized QCM in the 0.1 (open) and 2.0 mg/ml (closed) fibrinogen solution.

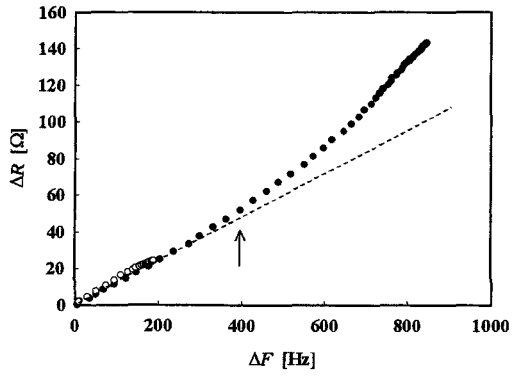

Fig. 2 Relation between ΔF and ΔR at the same t for the 0.1 (○) and 2.0 mg/ml (●) fibrinogen solution. The plot was curved upward at the arrow.

surface like a tail, the surface would be filled up by the molecules. Since there were not sufficient spaces to develop the lateral aggregation of protofibrils, the thickening of fibrin fiber would be inhibited. The growth of fibrin fibers of the control sample was hindered and suspended with deplete of fibrin monomers, which were consumed to form fibrin fibers in bulk solution, showing small changes of ΔF and ΔR. On the contrary, CDs produced very thin and branching fibrin fibers [13]. Therefore, the growth of fibrin fiber continued for a long time, showing large changes in ΔF and ΔR.

Figure 5 shows the time courses of ΔF and ΔR for the 2.0 mg/ml fibrinogen solution in the presence α-, β-

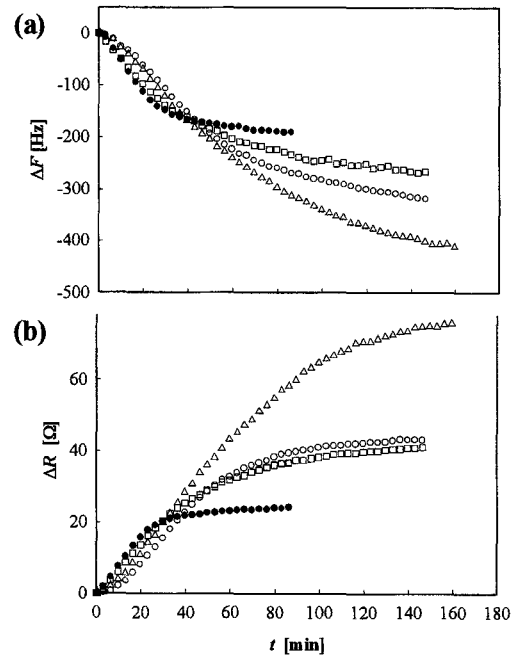

Fig. 3 Time courses of ΔF and ΔR for the 0.1 mg/ml fibrinogen solution in the presence α- (○), β- (△) and γ-CD (□). The CDs were added to the fibrinogen solution at a concentration of 8 mM. Closed circles (●) represent the control sample without CD.

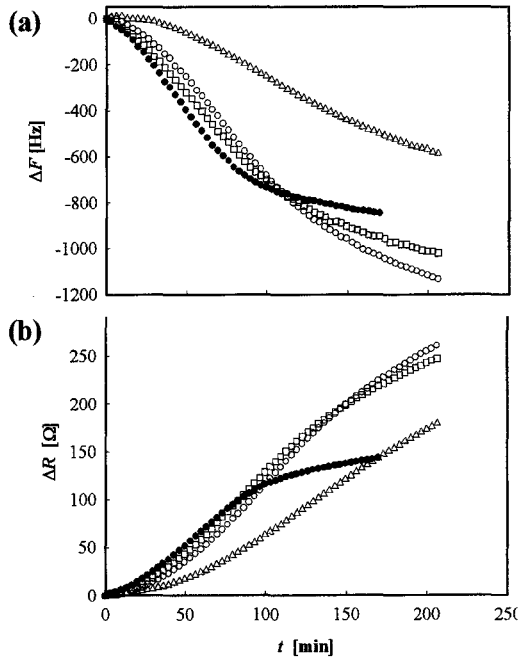

Fig. 5 Time courses of ΔF and ΔR for the 2.0 mg/ml fibrinogen solution in the presence α- (○), β- (△) and γ-CD (□). The CDs were added to the fibrinogen solution at a concentration of 8 mM. Closed circles (●) represent the control sample without CD.

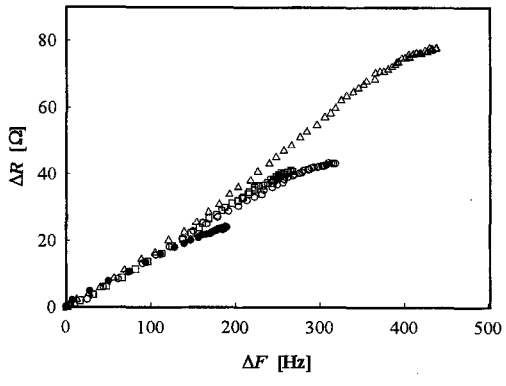

Fig. 4 Relation between ΔF and ΔR at the same t for the 0.1 mg/ml fibrinogen solution with CDs. Meanings of the symbols are the same as those in Fig. 3.

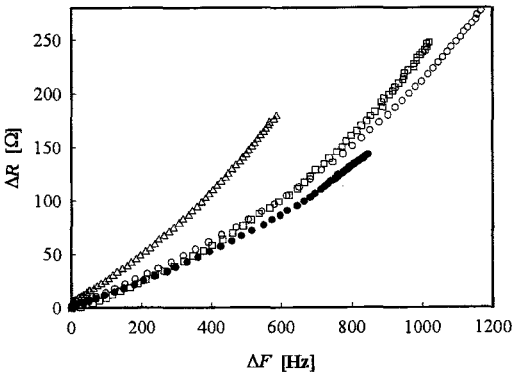

Fig. 6 Relation between ΔF and ΔR at the same t for the 2.0 mg/ml fibrinogen solution with CDs. Meanings of the symbols are the same as those in Fig. 5.

and γ-CD. Except for β-CD, the time courses were similar to those obtained at the low concentration (in Fig. 3). The changes of ΔF and ΔR significantly decreased in the presence of β-CD. The relation between ΔF and ΔR at the same t is shown in Fig. 6. The plots curved upward due to the fibrin gelation and the degree of curvature was increased in the presence of CDs, especially β-CD. This result indicates that CDs affect not only the fibrin polymerization but also the viscoelastic properties of the formed gel. It has been found that β-CD hinders the lateral aggregation of fibrin fibers and a very week and imperfect network is formed [3]. The very week gel with β-CD would induce the marked increase in ΔR against ΔF.

In conclusion, the processes of fibrin polymerization and gelation were examined by using QCM method through ΔF and ΔR. Two different fibrinogen concentrations have been used; lower one without gelation (0.1 mg/ml) and higher one with gelation (2.0 mg/ml). The value of ΔR was proportional to ΔF in the progress of fibrin polymerization and steeply increased with fibrin gelation. The additive effects of α-, β-, and γ-CD on these processes were investigated. The addition of CDs, especially β-CD, not only retarded the fibrin gelation but also changed the viscoelastic properties. These results were consistent with the recent our results obtained by dynamic light scattering. It is promised that QCM is a useful method to analyze a gelation process.

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