Pressure and frequency dependences of mechanical and chemical effects by ultrasonic cavitation on amyloid fibril formation

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1. Introduction

In today's aging society, neurodegenerative diseases such as Parkinson's disease have become a serious social problem. These diseases, called amyloidosis[1)], are supposed to be caused by the formation of amyloid fibrils, which are aberrant aggregates of specific proteins existing in the body. Currently, nuclear magnetic resonance imaging and positron emission tomography[2)] have been employed as diagnostic methods for these diseases. However, these diagnostic methods evaluate the progression of clinical symptoms by observing amyloid fibrils that have already formed, and it is difficult to cure the disease after exposure to clinical symptoms due to the nature of the disease[2)]. Therefore, it is necessary to develop an early diagnosis method before the formation of amyloid fibrils.

Recently, detection of amyloid-fibril seeds in biological fluids has attracted attention as an early diagnosis method for amyloidosis. Previous research succeeded in distinguishing a type of amyloidosis based on the detection of amyloid seeds in cerebrospinal fluids using the protein-misfolding-cyclic-amplification method, which amplifies the seeds by shaking sample solutions[3)]. Meanwhile, our research group has demonstrated that ultrasonic irradiation is a promising method for amyloid-fibril assays[4)]. Ultrasonic irradiation to solutions of amyloidogenic proteins highly accelerates amyloid fibril formation and sensitively detects the seeds compared to the shaking method, owing to the effects of ultrasonic cavitation. It is expected that amyloid fibril formation under ultrasonic irradiation strongly depends on the pressure and frequency of ultrasound. Indeed, we reported that the spontaneous formation of amyloid fibrils, where amyloid fibrils are formed through primary nucleation from monomers, depends on ultrasonic irradiation conditions[5)]. However, seed-dependent amyloid formation, where amyloid fibrils are mainly formed by elongation of pre-existing seeds, has not been studied systematically. In this study, we investigate ultrasonic pressure and frequency dependences of seed-dependent fibril formation of β_2-microglobulin (b2m), the causative protein of dialysis-related amyloidosis, using an ultrasonic reactor originally developed in this study.

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2. Experimental material

We developed the sonoreactor for amyloid fibril assays. In addition, we developed a special circular plate to fit this device, including its mold, where eighteen wells with the volume of 150 mL are located for sample solution. After pouring the sample solution into each well, all wells were sealed by a plastic film with a thickness of 0.1 mm. A Langevin oscillator was fixed to the bottom plate of the cylindrical water bath. The frequency of ultrasonic field was changed by replacing the oscillator. The amyloid fibril formation was measured by the thioflavin-T (ThT) fluorescence assay[6)], an amyloid-specific dye. During the ultrasonic experiment, sample solutions were repeatedly irradiated with the ultrasound for 20 ms with an interval of 800 ms. The ThT fluorescence intensity of each sample was measured every 2 min. The plastic plate was rotated to homogenously apply ultrasonic energy to each well.

In this study, β2m monomers expressed in *Escherichia Coli* BL21 were purified and used for the experiments. For the amyloid fibril formation experiment, we prepared sample solutions including 8.5 μM β2m monomer, 20 mM HCl, 80 mM NaCl, and 5 μM ThT. The preformed β2m seeds were added to the sample solution with a concentration of 1 pM.

3. Results and Discussion

We measured the time course of the ThT fluorescence intensity of β2m monomer and seeded sample under ultrasonication with various applied voltages of 10, 30, 70, and 120 V with the frequency of 48 kHz as shown in Fig. 1. The lag time, which is defined as the time when the value of the ThT fluorescence intensity reaches 20% of the maximum value, was measured under different solution conditions and ultrasonic conditions. The average lag time of the monomer sample among six independent solutions under the ultrasonic frequency of 48 kHz was 28.3, 7.3, 3.8, and 1.5 h, at applied voltages of 10, 30, 70, and 120 V, respectively. The lag-time ratios of monomer sample to seeded sample, which represent the seed-detection sensitivity under certain conditions, were 2.3, 3.5, 1.0, and 1.0, respectively. Thus, the seed-detection sensitivity was the highest at a moderate applied voltage and decreased with higher voltages. Similar results were

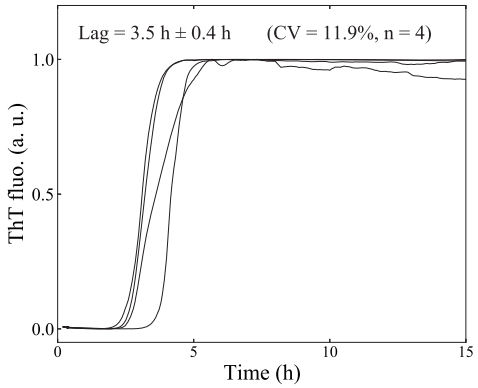

Fig. 1. Example of ThT time-course curves of β2m solutions.

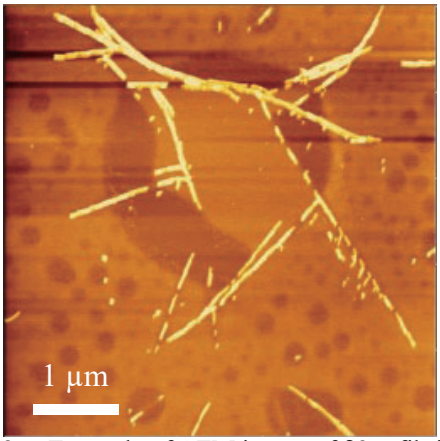

Fig. 2. Example of AFM image of β2m fibrils.

obtained at other frequencies of 26, 109, 241, and 450 kHz. Investigation of the seed-detection sensitivity at the optimum voltage for each frequency reveals that lower frequencies exhibit higher seed-detection sensitivity than higher frequencies.

The seed-detection sensitivity is determined by the trade-off between the primary nucleation and the fragmentation of formed fibrils; the former is a reaction in which monomers form nuclei of amyloid fibrils, and the latter is a reaction in which longer fibrils are broken down into shorter fragments. Ultrasonic irradiation to protein solutions enhances both reactions owing to the effects ultrasonic cavitation[5]. Within them, enhancement of the primary nucleation deteriorates the seed-detection sensitivity because it directly produces fibrils from monomers without the seeds. On the other hand, enhancement of fragmentation improves the sensitivity due to the rapid amplification of preformed fibrils by increasing the number of active termini, where fibril growth takes place.

We independently investigated the balance between the enhancement of primary nucleation and the fragmentation at various frequencies and acoustic pressures. To investigate the effect of ultrasonication on the fragmentation, we prepared solutions including long fibrils that were grown under quiescence. After irradiating the sample solutions with ultrasound under different acoustic pressures at frequencies of 48 kHz and 450 kHz for 10 minutes, the distribution of fibril length in each solution was measured by atomic force microscopy (AFM), as shown in Fig. 2. As the applied voltage was increased at each frequency, the fragmentation suddenly appeared above a certain voltage. These results indicate that the fragmentation occurs with a threshold which depends on the frequency. The fragmentation is attributed to the shear force during cavitation collapse, which is suggested to have a threshold value. In contrast, the ThT curves show that the lag time of the sample solutions without seeds becomes shorter as the acoustic pressure increases. The gas-liquid interface is considered to be a factor to enhance nucleation[7], and a larger bubble radius at a higher acoustic pressure is thought to accelerate nucleation by increasing the surface area accessible to the monomer. Results show that fragmentation is more dominant at lower frequencies than at higher frequencies with respect to the balance between fragmentation and nucleation effects, resulting in higher seed-detection sensitivity.

3. Conclusion

We investigated the frequency and acoustic pressure dependence of the detection sensitivity of amyloid seed using β2m, the protein causing for dialysis-related amyloidosis. Experiments were performed using an originally developed multichannel sonoreactor. The results show that the balance between enhancements of the primary nucleation and the fragmentation is critical for the detection of amyloid seeds and that lower ultrasonic frequencies are more suitable.

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