Frequency dependence of seed-dependent amyloid formation of β2-microglobulin under ultrasonic field

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1. Introduction

In today's aging society, neurodegenerative diseases such as Alzheimer's disease (AD) have become serious social issues. AD and other neurodegenerative diseases are caused by formation of aggregates of specific proteins *in vivo*. Proteins play their biological role *in vivo* by folding into a thermodynamically stable native conformation (folding reaction). However, as aging progresses, it becomes more difficult to maintain proteostasis, leading to misfolding reaction instead of the proper folding reaction. Misfolded proteins can form insoluble aggregates called amyloid fibrils, which cause a disease called amyloidosis[1]. Amyloidosis is a general term for diseases derived from deposition of amyloid fibrils on tissues and their toxicities, causing motor and cognitive dysfunction.

Recently, detection of amyloid fibrils, which exist in extremely small amounts *in vivo*, has been attracting attention as a significant contribution to the early diagnosis of amyloidosis. Previous studies have suggested that detection of amyloid fibrils by ultrasonic irradiation with an optimized irradiation condition is an effective method for enhancing the detection sensitivity and shortening the detection time. In the acceleration mechanism of amyloid fibril formation by ultrasonic irradiation, the radial motion of cavitation bubbles generated in the solution by ultrasound is important. Its behavior strongly depends on the frequency of the ultrasonic field. Indeed, it has been reported that primary nucleation of amyloid fibrils under ultrasonic field depends on the ultrasonic frequency[2], suggesting that the detection sensitivity and the time for detection of the amyloid seeds under the ultrasonic field may also depend on the ultrasonic frequency. Although optimization of the ultrasonic frequency can result in sensitive detection of the amyloid seeds, the frequency dependence of the seed-dependent amyloid-formation, which is called seeding reaction, has not yet been studied. In this study, we investigated the frequency dependence of the seeding reaction of β2-microglobulin (β2m)[3], the causative protein of dialysis-related amyloidosis, using a sonoreactor, originally developed in this study.

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2. Experimental Methods

We developed the sonoreactor for the amyloid fibril assay, as schematically shown in Fig. 1. We molded many identical plastic sample plates as shown in Fig. 2, where eighteen 135-μL wells 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 container filled with water. The frequency of ultrasonic field was changed by changing the oscillator. The amyloid fibril formation was measured by the thioflavin-T (ThT) fluorescence assay[4], 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 by a revolver so as to apply identical ultrasonic energy to each well.

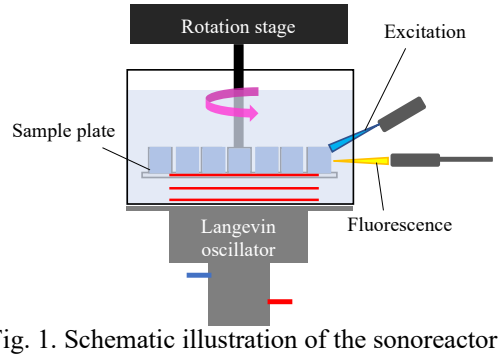

Fig. 1. Schematic illustration of the sonoreactor for the amyloid fibril assay.

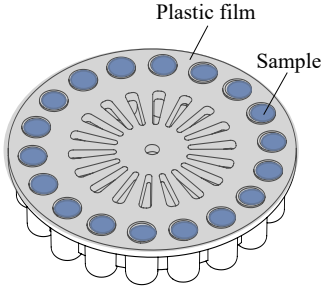

Fig. 2. Schematic illustration of the sample plate used for experiments.

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 2.5 μM β2m monomer, 20 mM HCl, 150 mM NaCl, and 5 μM ThT. The preformed β2m seeds were added to the sample solution with a concentration of 1 nM.

3. Result and Discussion

We measured the time course of ThT fluorescence intensity of β2m samples with and without seeds under ultrasonication with frequencies of 63 and 241 kHz, as shown in Fig. 3. Clearly, amyloid seeds (red lines) accelerated amyloid fibril formation compared with the monomer solution without seeds (black lines) due to the seeding effect. The lag time, which is defined as the time when the value of the normalized ThT fluorescence intensity reaches 0.5, under different solution conditions and ultrasonic frequencies is shown in the Fig. 4. The average lag time of the monomer sample among six independent solutions under ultrasonic frequencies of 63 and 241 kHz were nearly the same (11.4 and 11.0 h, respectively). Since the detection sensitivity of amyloid seeds can be discussed based on the ratio of the lag times of samples with and without seeds, the frequency dependence of the seeding reaction can be evaluated by equally adjusting the lag time for the monomer sample at different frequencies. In the presence of the seeds (red bars), the lag time is significantly longer at the frequency of 241 kHz than at 63 kHz, suggesting that the seed-detection sensitivity is higher at 63 kHz than at 241 kHz.

Our results indicate that lower ultrasonic frequencies are more suitable for detecting amyloid seeds with higher sensitivity. This is due to the difference in the cavitation characteristics in ultrasonic fields. The shear force generated near the point of bubble collapse under lower frequencies is greater than under higher frequencies, because the maximum radius of cavitation bubble is larger under lower frequencies. The shear force contributes to the fragmentation of amyloid fibrils, leading to the sensitive seed detection by amplifying the seeds effectively. In contrast, under high-frequency ultrasonic field, less fragmentation occurs, and the seed amplification by ultrasonic fragmentation is insufficient to improve the detection sensitivity. To further discuss the frequency dependence of the detection sensitivity, it is important to investigate the efficiency of fragmentation under different frequencies through microscopic observations of formed fibrils.

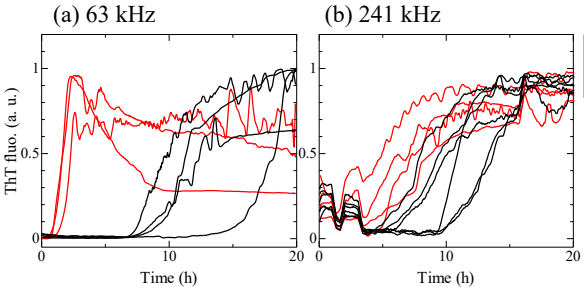

Fig. 3. ThT time-course curves of β2m solutions including seeds with concentrations of 1 nM (red lines), and monomer solutions (black lines) under ultrasonication with frequencies of (a) 63 kHz, and (b) 241 kHz, respectively.

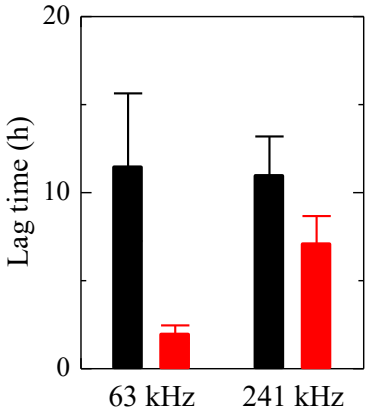

Fig. 4. Comparison of the lag time of β2m solutions including seeds with concentrations of 1 nM (red bars), and monomer solutions (black bars) under ultrasonication with frequencies of 63 kHz, and 241 kHz. The error bars denote the standard deviation for six independent measurements.

4. Conclusion

We investigated the frequency dependence for the detection sensitivity of amyloid seeds using β2m, cause protein of dialysis-related amyloidosis. The experiments were performed using the originally developed multichannel sonoreactor. The experimental results indicate that lower ultrasonic frequencies are more suitable for detecting amyloid seeds than higher frequencies with greater sensitivity.

References

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