Development of miniaturized sonoreactor for amyloid fibril assays

Tina Kondo[1‡], Kichitaro Nakajima[2*], Keiichi Yamaguchi[2], Yuji Goto[2] and Hirotsugu Ogi[2] ([1]Dept. Eng. , Osaka Univ; [2]Grad. Sch. Eng. , Osaka Univ)

1. Introduction

In today's aging society, amyloidosis, such as Alzheimer's disease, is a serious problem. The early diagnosis and prediction of the onset of these diseases are attracting attentions. The cause of these diseases is aberrant aggregates of proteins, called amyloid fibril. Normally, proteins play their biological functions in vivo by folding into stable native structures[1)]. However, proteins may misfold and form amyloid fibrils instead to proper folding[2)]. These amyloid fibrils deposit on organs and tissues, leading to amyloidosis. Because amyloid formation is placed at the top of the onset cascade of amyloidosis, the early diagnosis of these diseases is essential to eradicate amyloidosis.

Recent studies show that the detection of amyloid fibrils included in biological fluids, such as blood or cerebrospinal fluid, and the prediction of the onset of amyloidosis can contribute to the early diagnosis of amyloidosis. It has been demonstrated that ultrasonic irradiation to protein solutions is a promising method for the detection of amyloid fibrils and the prediction of the onset of amyloidosis[3, 4)]. Regarding the detection of amyloid fibrils, it is suggested that ultrasonic irradiation can improve the detection sensitivity of amyloid seeds and shorten a time for the detection[3)]. For the prediction of the onset of these diseases, it is necessary to analyze the effects of biological substances affecting formation of amyloid fibrils. Although the effects of biological substances on the primary nucleation, which possesses a high energy barrier, should be investigated, it fails to proceed spontaneously under physiological conditions. To overcome this challenge, ultrasonic enhancement of amyloid fibril formation has been leveraged as an effective method for identifying factors[4)], which control amyloid fibril formation in vivo.

For the study on amyloid fibrils, we have developed several sonoreactors to effectively induce amyloid formation by ultrasonic irradiation and have demonstrated their availability not only for fundamental research on biophysics of amyloid fibrils, but also for the clinical study on amyloidosis. In this study, we developed a miniaturized sonoreactor optimized for the amyloid fibril assays, which allows us to irradiate protein solutions with ultrasound without a water bath and monitor an evolution of amyloid formation through the fluorescence measurement. The performance of the developed sonoreactor was evaluated using β_2-microglobulin (β2m), the protein responsible for dialysis-related amyloidosis, by means of the thioflavin-T (ThT) fluorescence assay and atomic force microscopy (AFM).

2. Developed device and experimental method

We developed a miniaturized sonoreactor for amyloid fibril assays, as shown in **Fig. 1**. We designed and made a Langevin oscillator whose resonant frequency is approximately 23 kHz. The upper part of this oscillator is hollow, where a plastic plate including protein solutions can be placed, as shown in **Fig. 2**.

The plastic plate possesses eighteen wells placed in an axial symmetry manner. After injecting the sample solutions into each well, all wells were sealed with a plastic film. The sample solutions in the plate were irradiated with ultrasound with a waveform of the chirp burst signal whose frequency ranges from 23.0 kHz to 23.5 kHz. The duty cycle was composed of 0.5-s irradiation and 1.0-s rest. We added the acoustic coupling agent between the oscillator and the bottom of the plate. The plate was rotated by a rotating stage. The sample solutions in the plate were homogenously irradiated with ultrasound from the bottom, making the resultant kinetics of amyloid formation uniform. To monitor

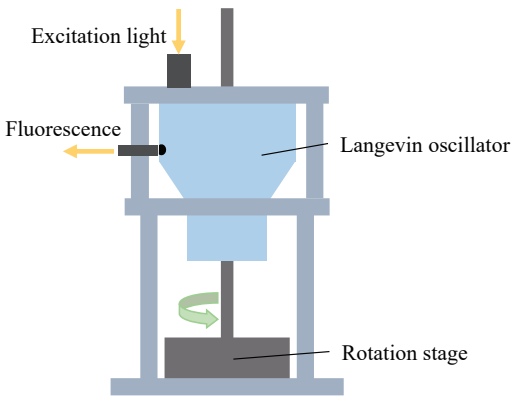

Fig. 1 Schematic diagram of the miniaturized sonoreactor for amyloid fibril assays.

E-Mail: ‡kondo@qm.prec.eng.osaka-u.ac.jp,
*k.nakajima@prec.eng.osaka-u.ac.jp

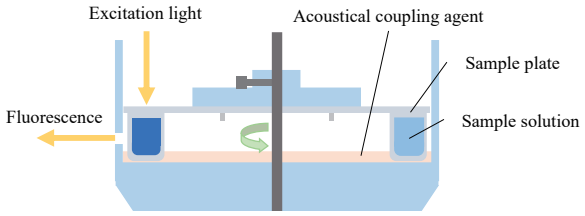

Fig. 2 Schematic diagram of the inside of the Langevin oscillator.

amyloid fibril formation, the ThT fluorescence intensity of the sample solution was measured. A light emitting diode (LED) for excitation light irradiation was placed above the plate, and an optical fiber for the fluorescence detection of the sample solution was placed on the side of the oscillator. The light emitted from the LED passes through short pass filters with the cut-off wavelength of 450 nm, which was used as the excitation light. The fluorescence signal emitted from the sample solution passes through long pass filters with the cut-off wavelength of 475 nm because the ThT fluorescence possesses a peak fluorescence wavelength of ~482 nm[5]. The fluorescence intensity was measured by a photomultiplier. The ThT fluorescence intensity of each sample solution was measured approximately every 23 seconds until the end of the experiment.

In this study, β2m monomer expressed by *E. coli* was used as the amyloidogenic protein. β2m was dissolved into 10 mM HCl. We prepared the sample solutions including β2m monomer, NaCl, HCl, and ThT.

3. Result and discussion

The ThT fluorescence intensity of the β2m solution was measured to evaluate performance of the developed sonoreactor. The solution concentrations were [β2m] = 0.3 mg/mL, [NaCl] = 150 mM, [HCl] = 20 mM, and [ThT] = 5 μM. An applied voltage to the ultrasonic oscillator was 250 $V_{p\text{-}p}$. About 2 mL of the acoustic coupling agent was added. To confirm whether fibrils were formed, β2m solution after ultrasonic irradiation and β2m monomer solution without ultrasonic irradiation were observed by AFM.

Figure 3 shows the results of the ThT fluorescence measurement of β2m solution. The lag time, defined as the time when the ThT fluorescence intensity reaches 20% of the maximum intensity in this paper, was calculated, as shown in Fig. 3(b). The ThT fluorescence intensity increased in all wells, confirming that the developed miniaturized sonoreactor can induce amyloid fibril formation. In addition, comparison of AFM images between the β2m samples before and after ultrasonic irradiation shown in **Fig. 4** indicates that the aggregates seen in Fig. 4(a) were formed by ultrasonic irradiation. It has been found that it takes more than 10 hours to form amyloid fibril without ultrasonic irradiation[3]. Therefore, this experimental result shows that the amyloid fibril formation is accelerated by ultrasonic irradiation using the developed instruments. The coefficient of variant (CV), which represents the variation in reaction time, was about 15%, a good value.

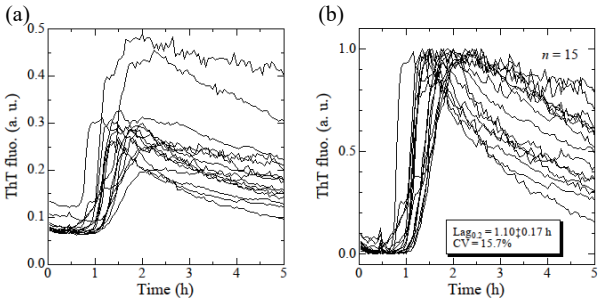

Fig. 3 Experimental results of β2m fibril formation using the developed ultrasonic instrument. (a) ThT time-course curves of β2m monomer (raw data), (b) ThT time-course curves of β2m monomer (after normalization)

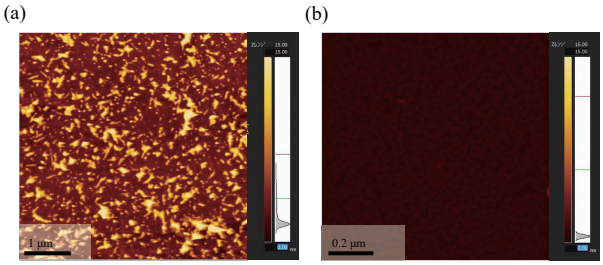

Fig. 4 AFM images of the (a) β2m aggregates formed under ultrasonication and (b) β2m monomer solution.

4. Conclusion

We worked on developing miniaturized instrument to form fibril by ultrasonic irradiation with observing the process of fibril formation. We also evaluated the performance of the instrument by the experiment using β2m.

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