Ultrasonic analysis on early stage of amyloid fibril formation of hen egg white lysozyme

Kichitaro Nakajima[1†], Eriko Asanuma[1], Tomoki Ota[1], and Hirotsugu Ogi[1]
([1]Grad. Sch. Eng., Osaka Univ.)

1. Introduction

Amyloid fibrils are aggregates of denatured proteins with ordered structures and needle-like morphologies, typically 10 nm in diameter and 1 μm in length, respectively. They deposit on the biological tissue in vivo and cause severe diseases, such as Alzheimer's disease, Parkinson's disease, and dialysis-related amyloidosis[1]. From the physicochemical perspective, amyloid fibrils show a crystal-like nature. The formation process of amyloid fibrils in a supersaturated monomer solution exhibits nucleation-dependent phase transition[2], composed of a relatively long lag time and subsequent rapid fibril growth. In other words, primary nucleation from the monomers is a rate-limiting step of amyloid formation. Thus, inhibiting the nucleation of amyloid fibrils is an ideal strategy to eradicate severe diseases caused by amyloid fibrils. The primary nucleation process is, however, not fully understood.

To investigate the kinetics of amyloid formation, the fluorescence molecules, which specifically detect the ordered structure of amyloid fibrils, are widely used[3]. However, this method quantitates an amount of formed fibrils. Thus, it is challenging to investigate the change in protein molecules during a lag time for nucleation.

In this study, we focus on ultrasound spectroscopy as an analytical tool to analyze the nucleation of amyloid fibrils. Ultrasound spectroscopy has been applied to research the properties of polymer solutions, including its phase transition[4,5], showing its efficacy in detecting the change in solution properties. This method has, however, never been applied to study amyloid nucleation.

Then, we construct an ultrasound measurement system in conjugation with a fluorometer and evaluate the applicability of this system for the study of primary nucleation of amyloid fibrils. The experimental system allows us to simultaneously measure ultrasound and fluorescence spectra of a protein solution during amyloid formation. Using this system, we study the amyloid formation of hen egg white lysozyme, one of the representatives of amyloidogenic proteins.

E-mail: †k.nakajima@prec.eng.osaka-u.ac.jp

2. Materials and Methods

We constructed the experimental setup for ultrasonic measurements of protein solutions, as shown in **Fig. 1**. The sample solution is in a quartz cell with an optical and acoustic path of 10 mm. A thin polyvinylidene fluoride (PVDF) film is attached to the side of the quartz cell using epoxy glue and is used as an ultrasound transducer and receiver. The resonant frequency of the PVDF film is approximately 20 MHz. The voltage pulse was applied to the PVDF transducer to generate an ultrasound pulse, which propagates in a sample solution. The ultrasound pulse reflected another side of the cell is detected using the ultrasonic receiver and recorded using the oscilloscope with a sampling rate of 4 GHz. The temperature of the sample solution was controlled by a Peltier element to be constant at 37 °C.

The lyophilized powder of hen egg white lysozyme was dissolved in a HCl solution with a concentration of 10 mM. The guanidium chloride was added to the solution to induce denaturation of protein molecules. The concentration of guanidium

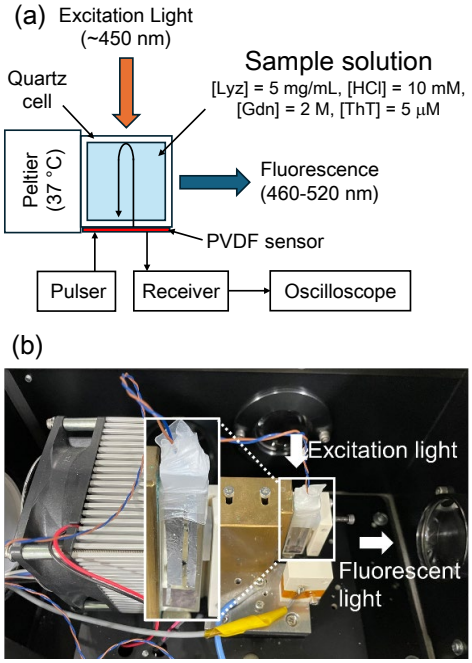

Fig. 1 (a) Schematic illustration and (b) picture of the experimental setup for simultaneous measurement of ultrasonic and fluorescence spectra.

chloride was adjusted to 2 M. The final concentration of hen egg white lysozyme was 5 mg/mL. The thioflavin-T (ThT) dye, which specifically binds to structures of amyloid fibrils and emits intense fluorescence, was also added to the sample solution with a concentration of 5 μM.

For the measurement, the prepared protein solution was added to the quartz cell with the PVDF film. The quartz cell was placed on the optical path of a fluorospectrometer (Hitachi, F-7100). The wavelength of excitation light is ~450 nm, and the fluorescence intensity is scanned from 430-550 nm. Additionally, the intensity of the light scattered from the sample solution is monitored. The ultrasonic measurements are repeated every 5 minutes.

3. Results and Discussion

Figure 2(a) and (b) show the time-course curves of group velocity of ultrasound and intensities of ThT fluorescence and scattered light of the sample solution, respectively. The group velocity suddenly increases immediately after the beginning of measurement and gradually increases over the experiment, as shown in **Fig. 2(a)**. The intensity of the scattered light starts increasing from 0 hours and hits the plateau at ~5 hours, as shown in **Fig. 2(b)**, indicating the formation of some aggregates in the solution. The increase in the ThT fluorescence intensity means that the aggregates formed are amyloid fibrils, as the ThT fluorescence intensity indicates the amount of protein aggregates with an ordered structure.

It should be noted that the ultrasound velocity and ThT fluorescence intensity showed different time evolutions, indicating the possibility that ultrasound spectroscopy detected the change in the protein molecules before amyloid formation during the nucleation phase. Furthermore, a gradual increase in the sound velocity after 10 hours, during which the fluorescence intensity remained constant, presumably suggests the formation of higher-order structures among amyloid fibrils, such as gel-like structures. These preliminary data should be further analyzed, for example, by decomposing the pulse waveforms into different frequency components to scrutinize the phase velocities and attenuations at various frequencies. Furthermore, microscopic observations of the formed products will help interpret the meaning of a change in the ultrasonic parameters.

Acknowledgment

The authors are grateful to Professor Yuji Goto and Associate Professor Keiichi Yamaguchi in Osaka University, and Daicel Corporation.

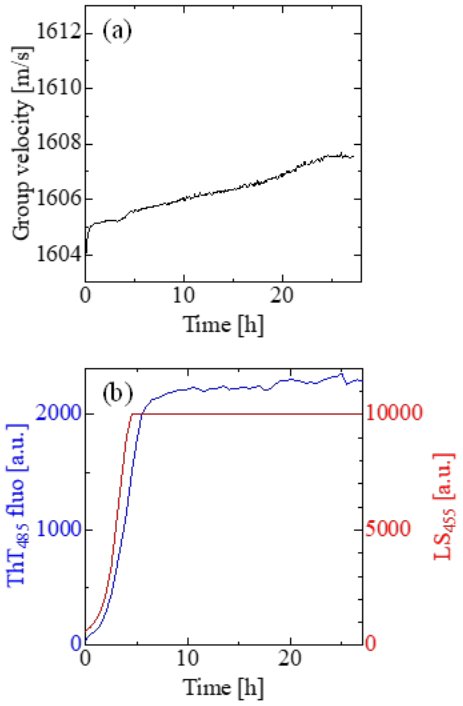

Fig. 2 Time-course curves of (a) group velocity of ultrasound propagating the protein solution and (b) ThT fluorescence (blue) and light scattering (red) intensities during amyloid formation of hen egg white lysozyme.

References

1) T. P. J. Knowles, M. Vendruscolo, and C. M. Dobson, *Nat. Rev. Mol. Cell Biol.* **15**, 384-396 (2014).
2) Y. Yoshimura et al., *Proc. Natl. Acad. Sci. USA.* **109**, 14446-14451 (2012).
3) H. Naiki, K. Higuchi, M. Hosokawa, and T. Takeda, *Anal. Biochem.* **177**, 244-249 (1989).
4) T. Klacic, A. Jugl, M. Pekar, and D. Kovacevic, *Macromolecules* **56,** 1434−1445 (2023).
5) S. Okura, K. Tsuji, and T. Norisue, *Ultrasonics* **126**, 106807 (2022).