The effects of ultrasonic cavitation and environmental factors on amyloid formation

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1. Introduction

Amyloid fibrils are insoluble aggregates of denatured protein with crystal-like morphology, causing various amyloidosis such as Alzheimer's disease, Parkinson's disease, and dialysis-related amyloidosis (DRA). Although proteins *in vivo* usually exist as soluble monomers with native state, the monomers become denatured state and form amyloid fibrils at an early stage of amyloidosis. Amyloid formation causes *in vivo* the death of nerve cells and eventually leads to significant biological malfunction. Therefore, it is crucial to prevent amyloid formation before the onset of clinical symptoms. To predict the onset of amyloidosis, it is necessary to analyze the amyloid formation *in vitro*.

Amyloid formation occurs from supersaturated solution of denatured monomer whose concentration exceeds the solubility, following a nucleation-growth two-step mechanism[1)]. Due to the high energy barrier of the nucleation step, amyloid formation takes a long time *in vitro*. Recently, ultrasonication has emerged as a promising method for accelerating amyloid formation[2)]. This is attributed to the catalytic effects related to ultrasonic cavitation bubble[3)]. Effectively utilizing ultrasonication to control amyloid formation is expected to contribute to early diagnosis of diseases and to the elucidation of disease onset mechanisms. However, while amyloid formation experiments are sometimes conducted at high temperature above 50 °C[4)], the effect of cavitation bubble at high temperatures on amyloid formation has not been fully investigated.

In this study, we aim to investigate the effects of ultrasonic cavitation and environmental factors, such as temperature, on amyloid formation using a originally developed sonoreactor. Our focus is on β_2-microglobulin (β2m), the protein responsible for DRA. Although β2m amyloid fibrils in patients are observed at neutral pH, β2m amyloid formation *in vitro* has been challenged because β2m exhibits the amyloid-resistant native state. Therefore, we investigate the β2m amyloid formation at neutral pH under ultrasonication at elevated temperatures to induce denaturation.

2. Experimental Method

In this study, β2m monomers expressed in *Escherichia coli* were purified by liquid-phase chromatography. For the experiment, the β2m monomers were diluted by sodium phosphate buffer with pH of 7.0 to be the monomer concentration of 0.5 mg/mL. The solution also includes 1000 mM sodium chloride, and 5 μM thioflavin-T (ThT) dye. ThT was used for evaluating amyloid fibril formation because it specifically binds to β-sheet structure of amyloid fibril and emits high intensity fluorescence.

For the ultrasonic irradiation, we used the sonoreactor equipment, as shown in **Fig. 1**. We designed a Langevin oscillator with the resonant frequency of ~23 kHz. The sample solution was injected into the plastic plate which has 18 wells placed in an axial symmetry manner. The plate was sealed with a plastic film. A chirp burst voltage signal was applied to the Langevin oscillator. During the ultrasound experiment, the sample solution was repeatedly irradiated with ultrasound for 1.0 s and stopped for 1.5 s. The ThT-fluorescence intensity was measured every ~50 s from the side of the each well.

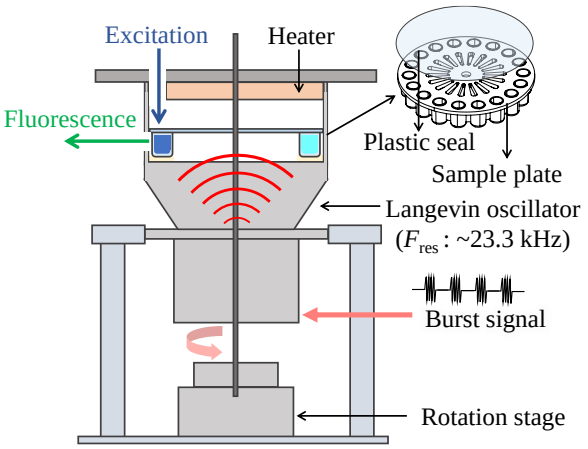

Fig. 1 Schematic diagram of the developed sonoreactor for amyloid formation assays.

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3. Results and Discussion

We measured the time course of ThT fluorescence intensity of the β2m sample between 50°C and 80°C under ultrasonic irradiation, as shown in **Fig. 2**. **Fig. 2(a)** shows that ThT fluorescence intensity increased in samples between 60°C and 80°C, indicating amyloid formation. On the other hand, no amyloid formation was observed at 50°C and 55°C within 20 h.

The lag time for amyloid formation exhibits an intriguing temperature dependency, as shown **Fig. 2(b)**. In this study, the lag time is defined as the time when the normalized ThT fluorescence intensity reaches 20% of its the maximum value within 20 h. Amyloid formation occurred within 1 h at temperatures between 60°C and 80°C. The lag times decreased with increasing temperature, indicating that amyloid formation was accelerated at higher temperatures. However, at 55°C, which is 5°C lower than 60°C, no amyloid formation was observed within 20 h.

The physical properties of water change with temperature, thereby altering the dynamics of cavitation bubbles in the ultrasonic field and potentially affecting the amyloid formation. Subsequently, we conducted numerical simulations to investigated the bubble dynamics based on the Keller-Misis equation[5], which describes the radial motion of a spherical bubble in the acoustic field, under the conditions at 50°C and 80°C, as shown **Fig. 3**. It is found that the increase of temperature allows bubble to attain a larger maximum size(**Fig. 3(a)**), resulting in higher energy during its collapse such as a local temperature increase in the gas inside bubble, and the power of the shock wave emitted from the collapse center(**Fig. 3(b)**).

In the β2m amyloid formation experiment under acoustic filed, the temperature affects both the state of β2m monomer and the dynamics of bubble. Although β2m is typically in an amyloid-resistant native state at neutral pH, the fraction of denatured monomer increases at higher temperatures, and solubility decreases[4]. Additionally, the hydrophobic interactions of protein are enhanced with increasing temperature, facilitating amyloid formation.

On the other hand, the bubble dynamics simulation explains that a change in temperature allows the bubble to grow more extensively and potentially elevates the energy associated with the bubble collapse event. The nucleation step, the rate-limiting step in amyloid formation, is facilitated at the surface of ultrasonic cavitation. Thus, the more extensive growth of the bubble enhances amyloid formation. Moreover, the increase in energy associated with the bubble collapse event may induce more substantial mechanical stress on the b2m protein in both denatured and native states.

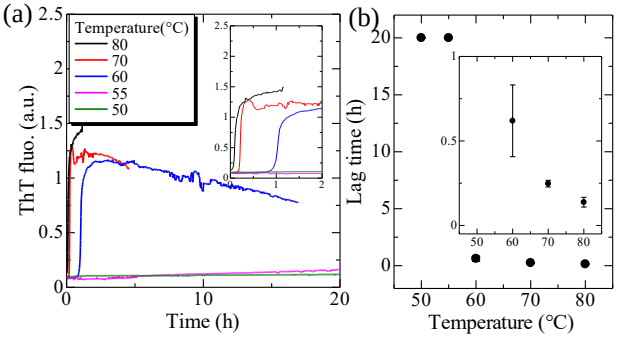

Fig. 2 (a) Time-course curves of ThT fluorescence between 50°C and 80°C. (b) The lag time with different temperature.

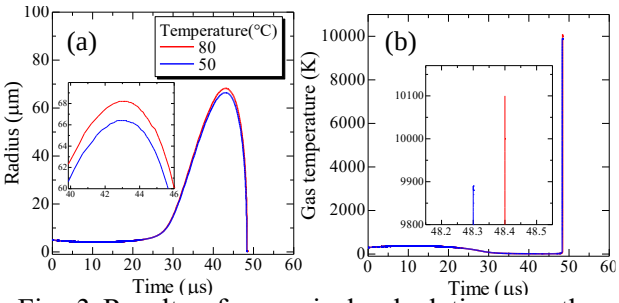

Fig. 3 Results of numerical calculations on the dynamics of (a) bubble radius and (b) temperature of the gas inside of the bubble at 50°C and 80°C.

Additionally, a decrease in surface tension and an increase in saturated vapor pressure of water due to a temperature increase lowers the threshold of the pressure for bubble generation[6], resulting in an increase in the number of cavitation bubbles in the solution, which could further enhance amyloid formation. Thus, the temperature dependence of β2m amyloid formation under ultrasonic field can be attributed to the combined effects of the direct influence of temperature on the β2m protein and the changes in the dynamics of cavitation.

4. Conclusion

We investigated the effects of cavitation bubble and environmental factors on amyloid formation using β2m at neutral pH. The results indicate that the β2m amyloid formation can be enhanced by a crossover between ultrasonic cavitation and environmental factors.

Acknowledgments

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