Highly sensitive detection of β2-microglobulin seeds by ultrasonic irradiation

超音波照射による凝集加速反応を利用した、
β2M タンパク質の高感度なシード検出

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1. Introduction

Amyloidosis is a general term for diseases caused by amyloidogenic proteins such as Alzheimer's disease and Parkinson's disease. We study the impact of ultrasound on amyloidogenic protein by seeding experiments. It is important to diagnose amyloidosis before toxic fibrils form. This is because oligomers form prior to fibrillation, and detection of oligomers can help to preempt the onset of the disease. In this study, we evaluate the seed detectability of our ultrasonication diagnostic system using artificially created seeds.

β_2-microglobulin (β2M : 11.8 kDa) was used as the target protein. It is the causative agent of dialysis-related amyloidosis, a disease that occurs when it accumulates in the bloodstream[1]. Patients with long-term kidney disease must remove their waste products by dialysis. However, β2M molecules are too small to be removed by dialysis, resulting in increase of its blood concentration and accumulation in the tissue. If the seeds can be detected before it aggregates, the β2M adsorption column (KANEKA MEDICAL PRODUCTS : Lixelle)[2] can be used to interfere with its progress.

Normally, amyloidogenic proteins need a long time to form fibrils. Hence, we apply ultrasonic wave to further accelerate the aggregation reaction. Previously, our group discovered that an optimum ultrasound with the frequency near 30 kHz can promote aggregation dramatically[3][4]. This phenomenon is mainly caused by the transient cavitation bubbles made by ultrasonic irradiation[5], on which hydrophobic peptides are attached, and they are highly condensed and heated at the subsequent bubble collapse. We consider that this mechanism also applies to small β2M aggregates, which are expected to exhibit hydrophobic regions. The formation of neurotoxic amyloid fibrils was evaluated by thioflavin T (ThT), which specifically binds the cross-β structure of amyloid fibrils[6] and emits strong fluorescence.

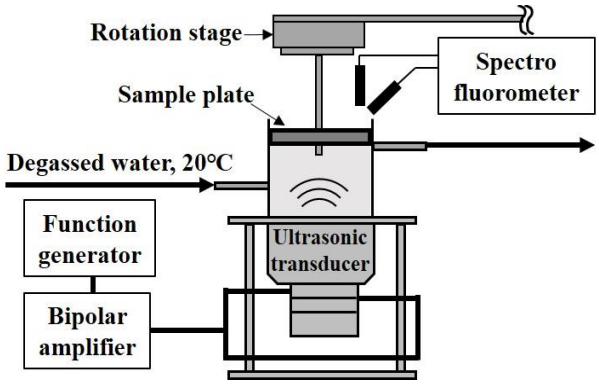

Fig. 1 Schematic illustration of experimental system.

2. Experimental procedure

For preparing the ThT solution, the ThT powder was dissolved with 50 mM glycine-NaOH buffer (pH 8.5) solution containing 100 mM NaOH to obtain the final 1 mM ThT solution.

First, the powder β2M was dissolved by 0.1 M Glycine-HCl buffer solution (pH2.2) by stirring at 200 rpm for 10 min. Second, the ThT solution was added to the β2M solution and stirred at 200 rpm for 5 min to be final β2M and ThT concentrations of 10 μM and 5 μM, respectively. This solution was prepared as the β2M monomer solution.

For preparing the seed solution as the model target, post-experimental fibrils formed from the monomer solution were used: The fibrils were crushed by ultrasonic irradiation for 1 min. We confirmed the formation of the cross-β aggregates by the fluorescence at 483 nm (ThT emits 483 fluorescence with 450 nm excitation when it binds to the cross-β structure.). This solution was frozen at -

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40 ℃ just before the experiments. Before use, this solution was dissolved with β2M monomer solution to an intended seeds concentration.

We developed the experimental system for ultrasonic irradiation as shown in **Fig.1**. The Langevin type ultrasonic transducer with the fundamental frequency of 30 kHz was tightly fixed on the bottom face of the stainless-steel cylinder with a screw. The reaction cylinder was filled with degassed water for avoiding loss of the acoustic energy caused by cavitation bubbles there. Degassed-water temperature was kept at 20 ℃. The sinusoidal voltage generated by the function generator was amplified and input to the ultrasonic transducer. The sequence of 30-s ultrasonic irradiation and 60-s incubation was repeated.

The microplate cut to the appropriate size was used for the multichannel experiment. This sample plate has 8 wells. The monomer solutions and the solutions containing the seeds were poured into 5 channels and 3 channels, respectively, and then the microplate was sealed with a plastic film. This sample plate was rotated by a rotation stage to make the ultrasonic irradiation uniformly. The rotation speed was 2 Hz, and we measured the fluorescence intensity every 10 min using the bundle fiber type spectrofluorometer

3. Results and Discussion

Changes in the ThT fluorescence intensity in the wells without the seed (monomer only solution) and those in the wells of the monomer solution containing different amount of seeds are shown in **Fig. 2**. We normalized the fluorescence value corresponding to the maximum values within 30 h of each well (those intensities of monomers are normalized based on the largest value of them). We used the time, $T_{0.1}$, at which the normalized ThT fluorescence level becomes 0.1, for evaluating the seeds concentration. **Fig. 2** plots the averages of 5 or 4 data. The $T_{0.1}$ values of 10 nM, 1 nM and 100 pM seeds solutions are 3.61, 11.5 and 18.9 h, respectively. **Fig. 3** shows averages and standard deviations of $T_{0.1}$. From this result, it is clear that different seeds solutions can be distinguished within ~20 h. The β2M concentration in serum ranges 1.0 ~ 7.8 μM [7]. Because the seed concentration of 10 nM in the present study is evaluated from the original monomer concentration, the actual seed concentration is expected to be much lower. For example, if a single seed is composed of 100 monomers, the seed concentration will be 0.1 nM. Thus, our result suggests importance in the practical diagnosis.

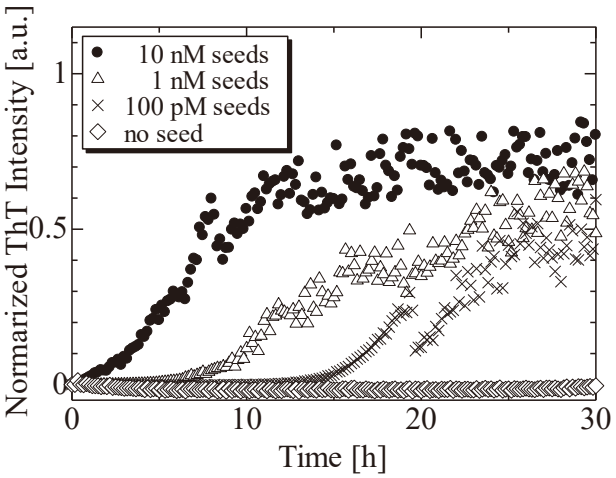

Fig. 2 Differences of the ThT time course between different seeds' concentrations. 10 nM seeds solution means 5-μM β2M monomer solution including β2M 10 nM seed and others is same.

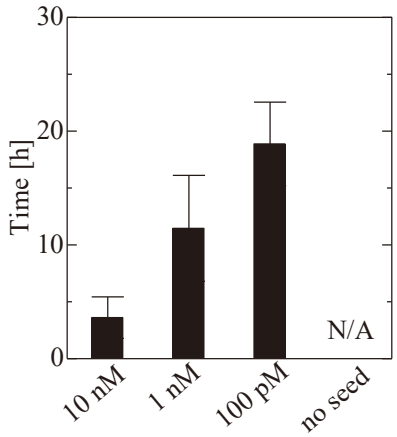

Fig. 3 Seed concentration dependency of $T_{0.1}$.

4. Conclusion

We developed the seeding-reaction-acceleration system with ultrasonic irradiation, and demonstrated that ultrasonication can significantly accelerate the seeding reaction. This method will be useful for the amyloidosis diagnosis.

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