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High-throughput sonochemical reactor for accelerative amplification of ultratrace amyloid-fibril seeds

極微量アミロイド線維シードの加速的増幅のための
ハイスループット超音波化学反応装置

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1. Introduction

Amyloid fibrils, which are protein aggregates with ordered β-sheet structure and needle-like morphology, deeply relate to onset of intractable diseases such as Alzheimer's disease and Parkinson's disease called amyloidosis. Though the causative protein is different among the amyloidoses, onset process of the diseases has a common mechanism in a view point of protein aggregation: soluble protein monomers which exert biological function in healthy body form the insoluble amyloid fibrils, deposit on the physiological tissue, and finally cause malfunction of the tissue by their neurotoxicity. The tissue malfunction is irreversible damage in most cases. Thus, it is important to early detect a risk of the amyloid-fibril formation for mitigating the amyloidosis.

Formation processes of the amyloid fibrils are essentially divided into an initial nucleation and a subsequent fibril growth. The initial nucleation is a long-term reaction, being a rate-limiting step of the fibril formation. Conversely, the fibril growth rapidly completes after the nucleation. This character is evidently explained by which addition of the preformed fibril, so-called 'seed', immediately induces the fibril formation without a lag time for the nucleation. This fact implies that formation of the seed in vivo triggers the onset and progression of amyloidosis. Therefore, detection of the seeds in biological fluids such as blood and cerebral spinal fluid is promising method for the early diagnosis of the amyloidosis. It is, however, difficult to detect the seeds for the reason of that the seed amount in the biological fluids is ultratrace before the onset of diseases.

Previously, we demonstrated that the ultrasonic irradiation to protein solution drastically accelerates the nucleation through sonochemical effect of the transient cavitation bubble[1,2]. As other aspects of the ultrasonic effect, ultrasonication to the mature fibrils induces fragmentation of long fibrils into shorter ones[3]. In this study, we ultrasonically amplify the amyloid seeds to rapidly detect a tiny amount of seeds by the high-throughput sonochemical reactor which we developed. β_2-microglobulin (β2m), which is a causative protein of dialysis-related amyloidosis[4] with the molecular weight of ~11.8 kDa, is adopted as the amyloidogenic protein. We investigated reaction times to the fibril formation of β2m monomer in the developed sonochemical reactor with a different amount of seed. Both of the fibril formation of β2m with and without seed were accelerated by the effect of ultrasonication. The β2m monomer solution with a 0.4-fmol (0.4×10^{-16} mol) seed completed the fibril formation in a shorter time than that in solution without seed. These results showed that the sonochemical reactor enable us to detect ultratrace seeds at an accelerated rate.

2. Experimental Methods

We developed the sonochemical reactor which can simultaneously react 36 samples, as schematically shown in Fig. 1. The piezoelectric lead-zirconate-titanate (PZT) transducers with a quartz part at the tip is located above the 96-well plate including sample solution in each well. The PZT transducer is excited its resonance with the frequency of ~30 kHz in an axial-oscillation mode

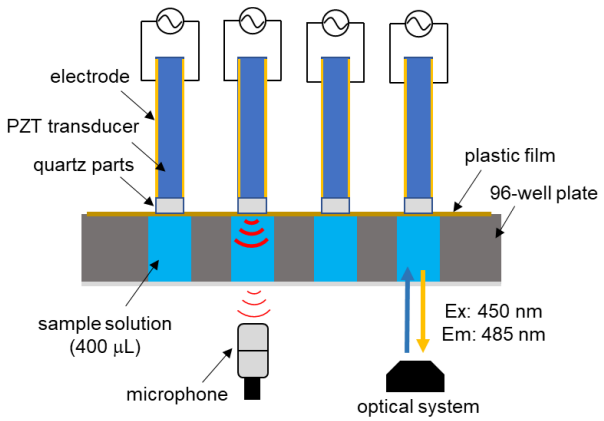

Fig.1. Schematic side view of the developed sonochemical reactor.

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by transverse piezoelectric effect, irradiating the sample solution with the ultrasound. Amplitude and frequency of the applied voltage to the transducer is determined by the acoustic calibration using a microphone. The ultrasonic irradiation for 0.3 s is iteratively performed with an interval of ~30 s for each sample. The fibril formation of the sample is monitored by fluorescence measurement using the fibril-specific dye, thioflavin-T (ThT), which emits strong fluorescence by selectively binding with the fibril[5]. The fluorescence intensity is measured every 10 min by the excitation and emission wavelength of 450 and 485 nm, respectively.

β2m is expressed in *Escherichia Coli* BL21 and purified as previously described[6]. The lyophilized β2m monomers are dissolved into ultrapure water. The solution is filtrated by the membrane filter with the pore diameter of 220 nm to eliminate preformed aggregates, if any. The β2m monomer concentration is decided by its absorption coefficient at 280 nm of 19300 M^{-1} cm^{-1}. The final concentration of the monomer is 8.5 μM (0.1 mg/mL). The sample solution also includes 20 mM HCl, 150 mM NaCl, 5 μM ThT, and different amount of the seed, being its pH of 2.0. The seed is prepared by breaking down the preformed fibrils into shorter ones with the ultrasonication 5 times for 1 s. The sample solution is dispensed to the 96-well plate with the volume of 400 μl for each well.

3. Results and Discussion

We investigated the fibril formation reaction of β2m monomer with 40-fmol, 0.4-fmol seeds (n = 6) in the monomer equivalent amounts, and without seed (n = 12) by the developed reactor. The representative ThT time-course curves are shown in Fig. 2(a). In case without seeds, increase in the ThT fluorescence intensity was observed only in 6 samples among 12 samples within 12 h. Meanwhile, the all samples with seeds increased their ThT intensity within 12 h in a sigmoidal manner, which is a typical time-course curve of the fibril formation[5]. The time when ThT intensity reaches a half of the maximum, t_{half}, decreases with an increase in the seed amount as shown in Fig. 2(b). The result also indicates the success to discern between the sample with 0.4-fmol seed and that without seeds. We further compared the t_{half} in cases with and without ultrasonication, as shown in Fig. 2(a). It clearly shows the acceleration effect of the ultrasonic irradiation for both the samples with and without seeds, indicating that the ultrasonic irradiation accelerates not only spontaneous nucleation of the monomer molecules, but also fibril elongation originated from the added seeds. The nucleation reaction in the monomer solution is optimally accelerated by the effect of ultrasonic cavitation with

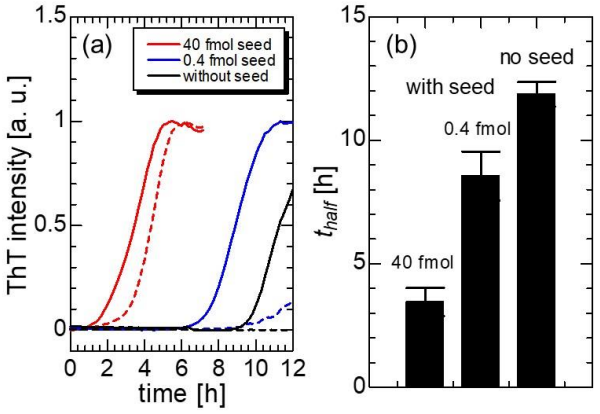

Fig.2. (a) Time-course curve of ThT fluorescence intensity with different amount of seeds. Solid and broken curves correspond to the condition with and without ultrasonic irradiation, respectively. (b) Relationship between the seed amount and t_{half} . The error bars denote the standard deviation (n = 6).

the frequency of ~30 kHz as previously reported [1]. For the fibril growth, the ultrasonic fragmentation of longer fibrils into shorter ones effectively amplifies the amount of active seed number because the fibril elongates by attaching the monomers to the seed terminals[7,8]. Our experimental results demonstrated that the sonochemical reactor succeeded in detecting 0.4-fmol seed with the effective amplification through the sonochemical effects.

4. Conclusion

We investigated the amyloid-fibril formation of β2m under ultrasonic irradiation with and without seed using the developed sonochemical reactor which is able to ultrasonically test 36 samples simultaneously. The reactor effectively accelerated the fibril formation in the sample solution, resulting in the accelerative detection of 0.4 fmol amyloid-fibril seeds. Our results indicate the applicability of the sonochemical reactor toward the early-stage diagnosis of amyloidosis such not only the dialysis-related amyloidosis, but also Alzheimer's and Parkinson diseases.

References

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