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Drastic acceleration of fibrillation of insulin by transient cavitation bubble

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ABSTRACT

Amyloid-fibril formation of proteins can be accelerated by ultrasonic irradiation to the peptide solutions. Although this phenomenon contributes to understanding pathogenic behavior of amyloidosis, its physical mechanism has not been clarified, because several factors (cavitation, temperature increase, stirring effect, and so on) related to ultrasonic irradiation can participate in the fibrillation reaction. Here, we independently study contributions of the possible factors, using insulin, which is extremely stable and then suitable for the mechanism clarification. We find that the optimized ultrasonic irradiation can drastically accelerate the fibrillation reaction; the time for completing the reaction is shortened compared with the high-speed (1200 rpm) stirring agitation by a factor of 430. The fibrillation reaction proceeds only when the subharmonic-mode intensity exceeds a threshold, indicating generation of the transient cavitation bubbles. Our results reveal that not the temperature increase but the transient cavitation bubbles work as the dominant accelerator of the fibrillation reaction.

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1. Introduction

Amyloid fibrils of proteins are deeply associated with serious diseases called amyloidosis [1], including Prion disease [2,3], Alzheimer's disease [4,5], Parkinson disease [6,7], and so on. Many studies revealed that amyloid fibrils show common characteristics, being independent of element monomers: They show needle-like morphologies with diameter of ∼10 nm [8–10]. They possess secondary structure composed of the cross-β sheet [11], which can be recognized by the amyloid-binding dye, thioflavin-T (ThT) [12]. Furthermore, monomers generally transform into the fibril through nucleation-growth reaction [13,14]. The nucleation reaction is the rate-limiting step with much higher energy barrier than that of the growth reaction [15]. After the nucleation, fibrils explosively grow from the nucleus. Because it is required a number of experiments and statistical data analysis for clarifying the biological characteristics of the amyloid fibril, an acceleration methodology for nucleation is the key for the progress in the protein-aggregation field of study. It also allows us to evaluate the effect of an inhibitor for aggregation quickly.

Recently, it was reported that the amyloid-fibril formation can be promoted by ultrasonic irradiation to the monomer solution [16–18]. This reaction enables us to form the fibrils in a short time.

In our previous study [19], we found out an optimum ultrasonic frequency near 30 kHz for inducing amyloid-β (Aβ) fibrils and succeeded in marked acceleration of the nucleation reaction with the optimized ultrasonic irradiation (OUI). While this study will contribute to effective fabrication of neurotoxic aggregates related to Alzheimer's disease, Aβ is unsuitable for clarifying the acceleration mechanism; the possible factors which affect the fibrillation reaction during ultrasonic irradiation are (i) collapse of transient cavitation bubble, (ii) temperature increase in bulk solution, and (iii) stirring agitation of the ultrasound. Because Aβ peptide is sensitive to all the factors, especially to the solution temperature, it is never straightforward to separately investigate their contributions. (For example, Aβ causes spontaneous nucleation at slightly elevated temperatures (∼40 °C) even without agitation.).

We then focus on insulin as a target protein in this study. It exhibits a native-folded structure and remains extremely stable under neutral pH near room temperature; it is difficult to be transformed into fibrils with standard agitations. Furthermore, it has been widely used as a model protein for studying amyloid fibrils [20–22] because of its low cost, so that we can compare our results with previous reports. Throughout this study, we used the ThT assay for monitoring the fibrillation reaction in the sample solution, which is a standard methodology for studying the amyloid fibril formation. (When proteins form fibril-like aggregates

composed of the cross-β-sheet structure, the ThT molecules bind them and cause the fluorescence.).

First, fibrillation reactions with a high-speed (1200 rpm) stirring agitation were carried out at various temperatures under neutral pH to determine activation energies for nucleation and growth at this condition. In previous studies on amyloid aggregation phenomena, stirring agitation was usually used with stirring speeds of ~500 rpm. For example, Cremades and coworkers [23] used the stirring agitation for aggregation of α-synuclein with 200-rpm stirring speed, and Yoshimura and coworkers [24] used 600-rpm stirring agitation to induce the fibrillation of β_2-microgulobulin. Therefore, the stirring speed of 1200-rpm used in this study is significantly higher than used in the previous papers.

Second, OUI with frequency of 26 kHz was applied to the sample solution, and the reaction speed was compared with other agitation methods, including the high-speed stirring agitation and the high-temperature (70° C) incubation. Third, the ultrasonic irradiation experiments were performed with various acoustic-intensity OUIs to study relationships among the solution-temperature increase, the acoustic field, and the fibrillation-reaction rate. The results show that the fibrillation reaction occurs only when the subharmonic-wave intensity exceeds a threshold, but it was unaffected by the temperature increase of the bulk solution. Because subharmonic-wave intensity comes larger when the bubble behavior shows motion of transient cavitation bubble [25], we conclude that the dominant fibrillation accelerator should be the transient cavitation bubble.

2. Materials and methods

2.1. Preparations of sample and ThT solutions

Insulin can fibrillate efficiently under a low pH, and most studies used an acidic buffer, including hydrochloric acid with pH lower than 2 [26]. However, because we need ultrastable insulin against fibrillation, we used neutral pH (pH 7.4), so that spontaneous formation of amyloid fibril hardly occurs.

Lyophilized-powder insulin from bovine pancreas (Sigma Aldrich, I1882) was dissolved into dimethyl sulfoxide (DMSO), and the solution was stirred for 15 min at 200 rpm with a magnetic stirrer. It was then diluted by 100-mM phosphate buffer solution (PBS) containing 100 mM NaCl and again stirred for 5 min to dissolve insulin with the buffer. The final concentration of insulin was adjusted to 100 μM. The volume ratio of DMSO and PBS in the sample solution was 1:19. DMSO, PBS, and NaCl were purchased from Wako Pure Chemical Industries, Ltd.

ThT powder was first dissolved into 50-mM Glycine-NaOH buffer (pH = 8.5) to obtain 1-mM ThT solution. It was stored as stock solution at 4 °C by wrapping with aluminum foil for avoiding photobleach. Just before using the solution, it was further diluted by the Glycine-NaOH buffer to 5 μM.

2.2. Ultrasonic irradiation experiment

Our laboratory-built experimental system for the ultrasonic irradiation experiments is shown in Fig. 1(a). The ultrasonic transducer with the fundamental frequency of 26 kHz (KAIJO, 43103) was placed in the water bath filled with degassed water treated by a degassing instrument (KAIJO, 40006A) to avoid the scattering loss of acoustic wave caused by cavitation bubbles there. The microtubes containing the sample solution were set above the transducer and irradiated with ultrasonic wave through the degassed water. The degassed-water temperature in the water bath was controlled by a temperature control system (THOMAS KAGAKU Co., Ltd. (TRL-107NH)). Fig. 1(b) shows change in the nor-

malized subharmonic intensity during increasing the output power level of the ultrasonic generator instrument up to its maximum.

The sample solutions were irradiated with the ultrasonic wave for 1 min and incubated for 4 min. This 5 min sequence was repeated for ~7 h. Every ~0.5 h, a 5-μl sample solution was picked up and mixed with the 5-μM ThT solution, and its fluorescence intensity was measured with fluorospectrometer manufactured by JASCO (FP-6200) at ambient temperature. Wavelength of the excitation light was 450 nm, and the spectrum of the emitted light was aquired between 440 and 500 nm and the maximum value (near 485 nm) was recorded as the ThT level. Note that ThT was added to the sample solution just before the fluorescence measurement, so that deactivation of ThT molecules caused by ultrasonic irradiation does not occur in our experiments.

2.3. Measurement of temperature and acoustic field in sample tube

The temperature change in the microtube was measured by a non-contact radiation thermometer (KEYENCE, FT-H10) every 0.1 s during the ultrasonic irradiations. The acoustic pressure was measured with the hand-made PZT needle-type probe (~2-mm diameter) by inserting it into the microtube containing only the buffer solution without insulin before the fibrillation experiment. The acquired waveforms were imported to PC through the digitizer (National Instruments, USB-5133), and then, fast Fourier transformation (FFT) was applied to them to obtain their amplitude spectra.

2.4. TEM observation

The TEM observation was performed with H-7650 system manufactured by HITACHI Corp. at the acceleration voltage of 80 kV. A sample solution was put on a carbon-coated grid and incubated for 1 min, and then, it was stained with 2% ammonium molybdate for negative-stain observation. Acquisition magnitude was between 8000 and 20,000.

3. Results

3.1. Activation energies of fibrillation reaction under high-speed stirring agitation

Fibrillation reactions under the 1200-rpm stirring agitation were monitored by the ThT fluorescence assay under various temperatures as shown in Fig. 2. They show the typical two-step aggregation reaction; the ThT level steeply increases after a lag time and reaches the saturation level. The morphology of aggregates at the saturated stage observed by TEM shows fibril structures as shown in Fig. 2. The fluorescence intensity should be proportional to the fibril concentration $[F]$, and its time course should obey the nucleation-growth two-step model [13]

$$[F] = [M]_0 \left[1 - \frac{k_n + k_g[M]_0}{k_n \exp(k_n + k_g[M]_0)t + k_g[M]_0} \right]. \tag{1}$$

We fitted this theory to the experiments to extract the rate constants for nucleation k_n and growth k_g. Here, $[M]_0$ denotes the initial monomer concentration (=100 μM). At each temperature, three independent measurements were performed for confirming reproducibility. The Arrhenius plots for nucleation and growth are shown in the inset of Fig. 2, from which we obtained the activation energies for nucleation and growth to be 734 and 49.1 kJ/mol, respectively.
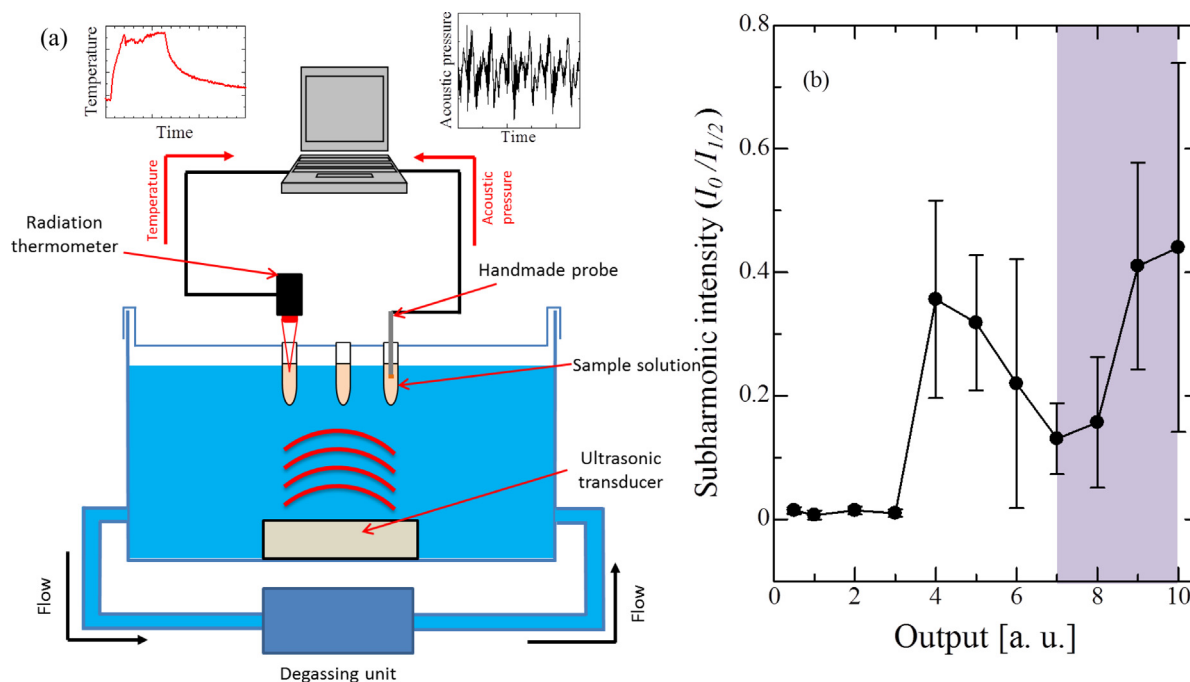
Fig. 1. (a) Schematic illustration of our laboratory-build experimental system for the ultrasonic irradiation experiments. Microtubes containing insulin sample solution are set above the ultrasonic transducer with fundamental frequency of 26 kHz, which is the optimized to induce the aggregation reaction [19]. Ultrasonic wave are irradiated through the degassed water to avoid loss of acoustic power in the water bath. Temperature and acoustic fields in the microtubes are measured by a non-contacting radiation thermometer and handmade PZT needle probe, respectively. The top of the microtube is open only when the temperature profile is measured. (b) Change in the normalized subharmonic intensity as the output power level of the ultrasonic generator increases. The shaded region indicates the output level used in the aggregation reaction.
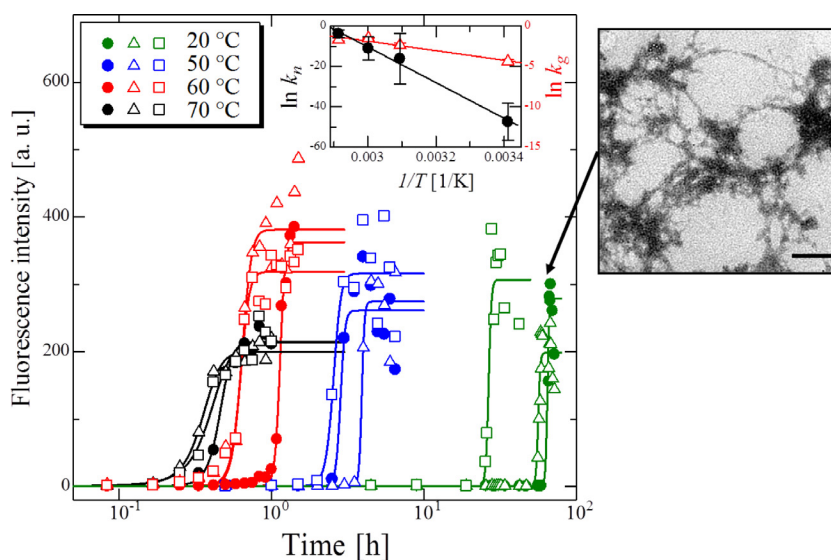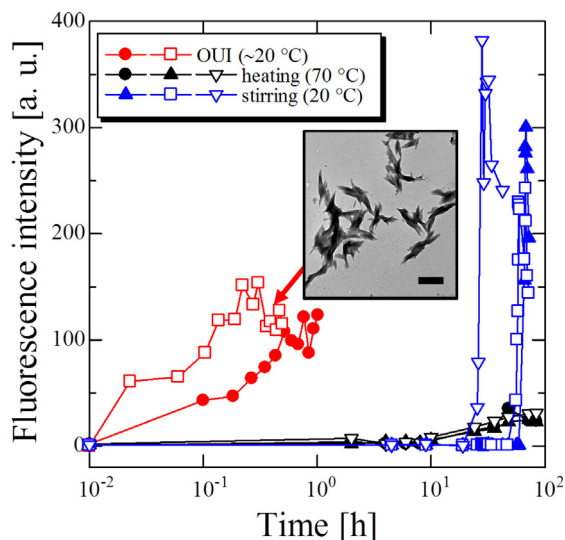

Fig. 2. Evolutions of the ThT fluorescence intensity with the 1200-rpm stirring agitation under four various temperatures. Inset shows the Arrhenius plot for the nucleation and growth reactions, where the reaction velocity constants for nucleation k_n (h^{-1}) and for growth k_g (h^{-1} μM^{-1}) are extracted by fitting the theoretical curve to each experiment. The TEM image shows the morphology of the aggregates formed under 20 °C. The scale bar length is 200 nm.

3.2. OUI dramatically accelerates the insulin fibrillation.

The aggregation experiments were performed under the OUI condition keeping the water-bath temperature at 10 °C. Stirring and heating agitations were also applied to the samples for comparisons. Typical results of the ThT evolution are shown in Fig. 3 (a). The results for the 1200-rpm stirring agitation, where the fibrillation takes near 50 h, are again shown in Fig. 3(a). In the case of the heating agitation at 70 °C, the fluorescence remained unchanged for 10 h and then slightly increased up to 100 h, but

no fibril formation was observed. Surprisingly, the fluorescence intensity saturates within ~30 min under the OUI agitation. The TEM image in inset of Fig. 3 (b) shows fibril morphology made by OUI. The fibril length appears to be shorter than that made under the high-speed stirring agitation because of the breakup effect of ultrasonic wave [27,28]. This effect will originate from the shock wave emitted at the bubble collapse.

The time at which the fluorescence intensity becomes the half maximum value is about 7 min under OUI, whereas it is about 50 h under the high-speed stirring agitation, despite that the

Fig. 3. Evolutions of the ThT fluorescence intensity caused by OUI, stirring, and heating agitations. The solution temperatures during applying agitations are shown in the labels. (The temperature for the OUI is the average during the 5-min sequence composed of 1-min ultrasonic irradiation and 4-min incubation, being identical to the temperature under the stirring agitation.) Inset shows a TEM image of fibrils formed under the OUI agitation. The scale bar length is 200 nm.

average temperature of the sample solution under OUI ($\sim$20 °C) was identical to the sample temperature under the high-speed stirring agitation. Therefore, the reaction time for fibrillation is shortened by the OUI agitation by a factor of $\sim$430.

3.3. Relationship between fibrillation and subharmonic component

The subharmonic intensity significantly becomes larger when the output level of the ultrasonic generator exceeds level 4 (Fig. 1(b)), temporally decreases between levels 4 and 7, and again increases over level 7. This two-threshold-based behavior of the subharmonic intensity was previously reported by Neppiras [29] and was interpreted as follows: The first increase of the subharmonic intensity is caused by the non-linear resonant oscillation of the stable bubble. The second increase beyond level 7 indicates appearance of the transient bubbles; bubbles cause oscillations at the subharmonic component until the transient movement. This was also confirmed by Eller and Flynn [30], theoretically. In our aggregation experiments, insulin cannot be fibrillated at the first subharmonic peak, and we then used the output levels between 7 and 10 (shaded region in Fig. 1(b)), so that we expect that many transient bubbles participate into the aggregation reaction. However, it is important to note that the acoustic field inside the sample tube is highly affected by the setting of the tube, not the driving power from the instrument but the subharmonic intensity inside the tube should reflect the actual ultrasonic intensity there.

OUIs with various amplitudes in this power region were applied to the sample solutions under 10 °C bath-water temperature to investigate contribution of acoustic field to the fibrillation reaction. In Fig. 4(a), four examples of the ThT time course are shown, where two showed positive for ThT within 30 min and two remained negative for 6 h. (We here use "positive" when the ThT level is significantly higher than the baseline ($> \sim$50).) Fig. 4(b) shows the corresponding temperature change in the sample solutions. Importantly, there is no obvious difference in the temperature profile; the sample-solution temperature reaches the maximum value of $\sim$35 °C during ultrasonic irradiation, and then cooled down to the base temperature for the subsequent incubation period. Thus,

this result highly indicates that the solution temperature can not be the dominant factor for the fibrillation acceleration.

The relationship between the fibrillation reaction and acoustic field is investigated by acquiring waveforms in the microtubes. Fig. 4(c) shows the FFT spectra for the corresponding measurements to Fig. 4(a) and (b). The vertical axis is the normalized by the fundamental peak height (I_1). We find obvious difference in the FFT spectrum between ThT-positive and -negative experiments; the subharmonic-mode intensities are always larger in ThT-positive cases. Here, we focus on the peak intensity of the subharmonic mode ($I_{1/2}$), and investigated the relationship between the subharmonic intensity and ThT level at 1 h as shown in Fig. 4 (d). Clearly, the fibrillation reaction shows the threshold-based behavior about the subharmonic intensity ($I_{1/2}/I_1 > \sim 0.1$), and because the threshold is close to that of the generation of the transient bubbles (Fig. 1(b)), the dominant factor of the fibrillation acceleration should be related to the transient cavitation bubble.

4. Discussion

First, we discuss the high stability of insulin. The activation energy for nucleation (734 kJ/mol) under the high-speed stirring agitation is higher than that of Aβ_{1-40} peptide determined under quiescent condition (61.9 kJ/mol) [19] by a factor of 12. This is a remarkable difference, because this value is the activation energy under the high-speed stirring condition at 1200 rpm. Such an extreme stirring condition will decrease the apparent activation energy, and we expect that the activation energy for nucleation of insulin under a quiescent condition will be much larger. Moreover, the ratio of the activation energy for nucleation to that for growth is considerably larger for insulin (=15) than for Aβ_{1-40} peptide (=7.9), confirming the view that the nucleation reaction of insulin hardly proceeds. Thus, insulin possesses ultrastable structure near room temperature under neutral pH and is an ideal protein for investigating the fibrillation-acceleration mechanism.

It is surprising that the reaction time for fibrillation is shortened by a factor of 430 when we apply the OUI agitation compared with the high-speed stirring agitation, because the high-speed stirring agitation must increase the number of the association event of the monomers and proceed the fibrillation reaction much faster than in quiescent condition. Ultrasonic irradiation also produces such a stirring effect for enhancing monomer association event (factor (iii) in Introduction). However, OUI drastically accelerates the reaction, indicating that the factor (iii) cannot be a principal accelerator.

Next, we discuss the temperature effect. As shown in Fig. 4(b), the maximum temperature inside the sample tube is below 40 °C, but the fibrillation reaction is completed within 30 min for some cases. On the other hand, the incubation of the solution at 70 °C cannot cause the fibrillation reaction over 100 h (Fig. 3(a)). Therefore, the temperature of the bulk solution cannot be the dominant factor.

Because the fibrillation reaction proceeds only when the subharmonic-mode intensity exceeds the threshold as shown in Fig. 4(d), the transient bubbles should contribute to the fibrillation reaction. In sufficiently high amplitude acoustic field, the bubbles are generated during the negative-pressure phase, and then they repeat expansion and collapse events with the driving acoustic-pressure fluctuation [31]. Sonochemical reactions are generally promoted by the collapse event because it creates the hot spot ($\sim$10,000 K [32]), depending on the acoustic pressure, and produces hydroxyl radicals and shock waves [33,34], causing the degradation reactions of organic compounds. Acceleration of the fibrillation reaction, however, means promotion of polymerization of protein monomers, and it is an opposite reaction to the general
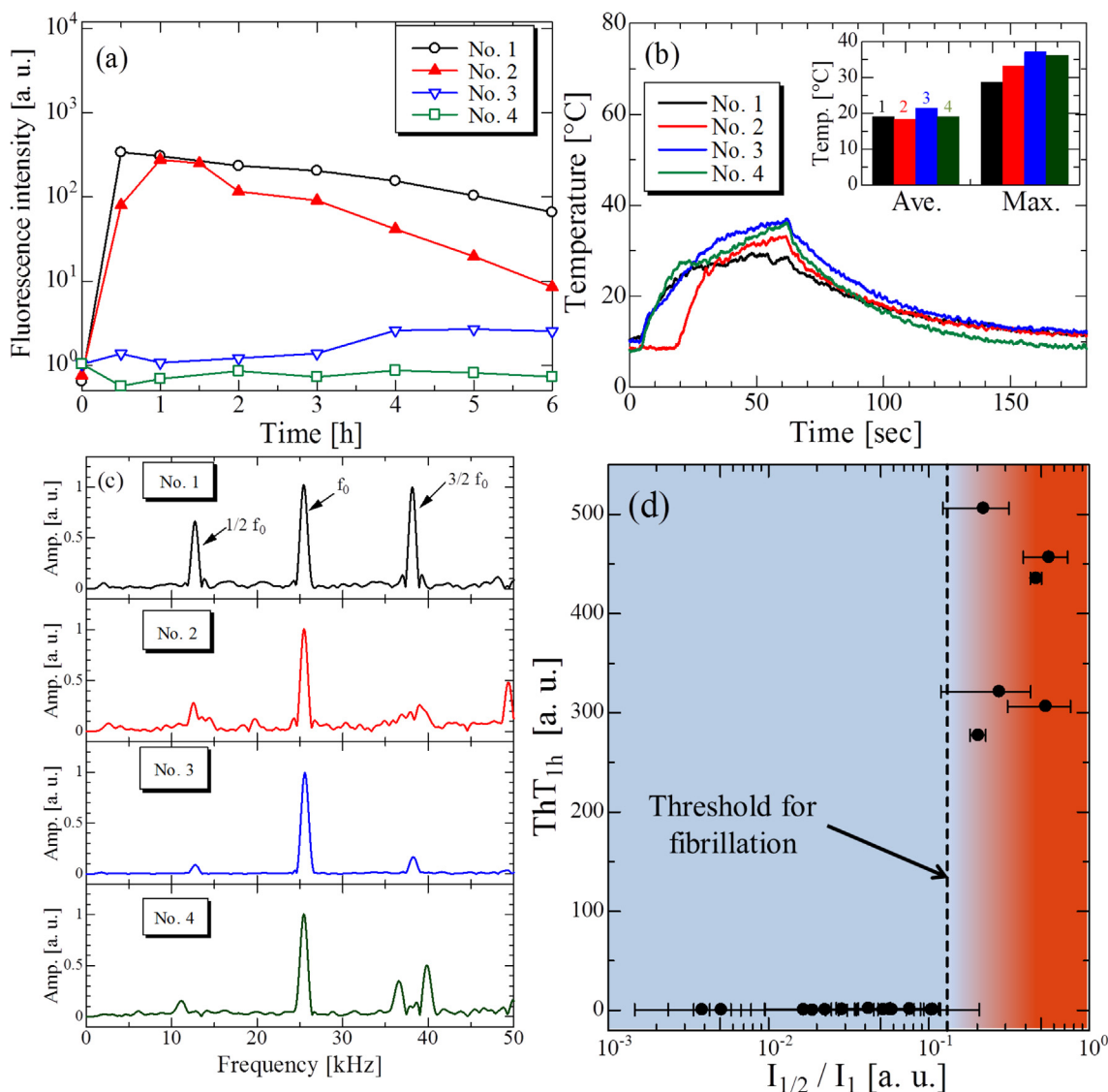
Fig. 4. (a) Evolutions of the ThT fluorescence of four samples exposed to OUI. The same power (the maximum of the instrument) was applied to the ultrasonic transducer for measurements No. 1 and No. 2, and 75% power of the maximum was used for measurements No. 3 and No. 4. (b) Temperature profiles of each sample in (a) measured from the beginning of ultrasonic irradiation. Inset shows average and maximum temperatures of the four measurements (the same color is used for individual curve.) (c) FFT spectra of wave forms in sample solutions of the four measurements. The vertical axis is normalized by the fundamental peak height. (d) Relationship between normalized subharmonic intensity ($I_{1/2}/I_0$) and the ThT fluorescence intensity. Each plot denotes the ThT level at 1 h after start of the experiment. The error bar denote the standard deviation among three normalized subharmonic intensities which are calculated from the waveforms measured at different times.

sonochemical reaction. We previously suggested the acceleration mechanism focused on the transient-bubble movement [19]: In the bubble-growth phase, the monomers are attached on the bubble surface because their hydrophobic amino acid residues interact with the gas–water interface [35]. And then, they are gathered to the center of the bubble in the subsequent bubble-contraction phase, and they are transiently exposed to the high-temperature and high-pressure field at the bubble-collapse event. This event achieves the fibril nucleation. We consider that this mechanism applies to the fibrillation-accelerated phenomenon of insulin.

Finally, we propose insulin as the acoustic-field probe in solutions. In high-power ultrasound experiments, measurement of the acoustic-energy in solution is a key to quantitatively study the ultrasonic effects. It is, however, difficult to evaluate the acoustic energy under such a high-power excitation because inserting a sensor probe disturbs the acoustic field and, more seriously, the sensor probe is easily deteriorated in a high-power acoustic field. The present study finds that insulin shows the threshold-based

structure-transition behavior with the generation of transient bubbles, and the existence of the transient bubbles can be evaluated within ∼30 min by monitoring the ThT level of the solution. In addition to the simple measurement, it is advantageous that insulin is an inexpensive and safety protein. Furthermore, no need for inserting a sensor object in solution, which varies the acoustic field there. Thus, a new methodology for evaluating the acoustic field is made possible using insulin as a probe of the transient bubble.

5. Conclusions

In this study, we used insulin as a model protein for investigating the mechanism of ultrasonically accelerated aggregation reaction of protein, because it shows ultra-stable native structure near room temperature under neutral pH. Actually, the high-speed-stirring experiment revealed extremely high energy barrier for fibril nucleation to be 734 kJ/mol, which is larger than that of amyloid

β peptide by a factor of 12. The optimum ultrasonic irradiation condition accelerates the fibrillation reaction 430-folded faster than in the high-speed stirring agitation. We found that the fibrillation reaction proceeded only when the subharmonic intensity inside the sample solution exceeded a threshold value $(I_{1/2}/I_1 > \sim 0.1)$, indicating that there is a critical bubble size for the aggregation reaction. Once the acoustic pressure exceeds the threshold, the fibrillation reaction was completed within 30 min, being independent of the acoustic power. These results highly confirm that the dominant accelerator is the cavitation bubbles. Furthermore, insulin can be a new probe for evaluating the presence of the transient cavitation bubble in a high-power acoustic experiment.

Acknowledgements

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