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# Relationship between ultrasonically induced aggregation phenomenon of amyloid β peptides and pressure of ultrasonic harmonics

アミロイドβペプチドの超音波誘起異常凝集現象と高調波音圧との関係

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## 1. Introduction

Alzheimer's disease (AD) remains central issue on the aging society. AD is one of neurodegenerative diseases developed with aging and is known as a major cause of dementia. The number of AD patients is predicted to grow significantly. However, the pathogenic mechanism of AD is still unclear and hence, efficient drugs or treatment approaches to AD have not been achieved.

AD is characterized by formation of neurofibrillary tangle, neuronal loss, and extracellular aggregation and deposition of amyloid β peptides (Aβ). Thus, it is widely recognized that the pathogenesis of AD is deeply related with the aggregation behavior of Aβ peptides. Aβ is released from amyloid precursor protein by two proteases, known as β- and γ-secretases. Aβ consists of ~40 amino acid residues and their molecular weights are about 4.5 kDa. Aβs form fibrillar aggregates, called amyloid fibrils, through self-association. These aggregates have highly regular cross β-sheet structures [1].

In general, the onset age of AD is higher than 60's. This long-term aggregation characteristics of Aβ peptides prevented us from clarifying the pathogenic mechanism of AD. Therefore, it is significantly important to develop a methodology to accelerate aggregation of the peptides.

Recently, Goto and his co-workers have displayed that ultrasonic irradiation induce the formation of amyloid fibrils for $\beta_2$-microglobulin [2, 3]. This phenomenon has been also reported for other amyloidosis proteins, including α-synuclein [4] and prion [5]. Therefore, we here focus our attention on acceleration of aggregation of Aβ peptides by ultrasonication and investigation of ultrasonically induced aggregation behavior for Aβ. The accelerated aggregation reaction will be caused by abnormally enhanced nucleation by ultrasonic irradiation. However, this mechanism remains unclear. For identifying the cause for dominant nucleation mechanism, it is efficient to investigate acoustic-pressure dependence of the phenomenon quantitatively. Here, we study relationship between ultrasonically induced aggregation phenomenon and acoustic-pressure of fundamental waves and harmonics. We used two-type ultrasonic transducers with fundamental frequencies of 26 and 200 kHz. The thioflavin-T (ThT) assay is adopted for evaluation of growth of amyloid fibrils.

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## 2. Experiment Procedure

The ultrasonic transducer was located in a water tank, where temperature is kept at ~15 °C. Several microtubes (1.5 ml vol.) were located above the transducer. The acoustic pressures of fundamental and harmonics modes were measured in individual microtubes using a 1-mm diameter handmade PZT probe. Obtained acoustic-pressure value was calibrated using a needle-type hydrophone.

$A\beta_{1\text{-}40}$ was used throughout this study. The lyophilized Aβ was dissolved in dimethyl sulfoxide with stirring at 200 rpm for 5 min and then, diluted by acetate buffer saline (pH 4.6) including 0.2 M NaCl to obtain final concentration of 100 μg/ml. A 500-μl Aβ solution was poured in the microtubes and then performed ultrasonication for them. A single ultrasonication sequence consisted of 5-min ultrasonication and 1-min incubation. This 6-min sequence was repeated for 5 h.

Effect of ultrasonic irradiation on aggregation of Aβ was evaluated using the ThT fluorescence assay. ThT was dissolved into 50 mM glycine-NaOH buffer (pH 8.5) to obtain the final concentration of 5 μM. The solution was wrapped in aluminum foil and stocked at 4 ºC before use. From the microtubes subjected to the ultrasonication, an aliquot of 5 μl was taken and mixed with 50 μl of ThT solution in a quartz cell. The fluorescence intensity of ThT was then measured as the fluorescence wavelength at 485 nm with excitation wavelength at 450 nm.

The morphologies of the Aβ aggregates caused by ultrasonication were observed using an atomic-force microscopy (AFM): A 10 μl solution was dropped onto a freshly cleaved mica plate, dried for 5 min, rinsed by ultrapure water, and dried to make the substances in the solution attached on the mica plate. The tapping-mode measurement was adopted with a silicon cantilever with the stiffness of 40 N/m, showing the resonance frequency near 300 kHz. The scan frequency was 0.5 kHz.

## 3. Result and Discussion

**Figure 1** shows effect of ultrasonic irradiation on the aggregation of the Aβ peptide as well as those of the 1200-rpm stirring and incubation procedures. The ThT fluorescence intensity remained low values with incubation treatment, and it increases a little at 3 h with the stirring treatment. However, it jumps up with the ultrasonic irradiation after ~2 h lag time. In addition, amyloid fibrils and spherical aggregates were observed using AFM after ultrasonication for 5 h (**Fig. 2**), which were not found in solutions by the incubation and stirring treatments. This indicates that ultrasonic irradiation can induce aggregation of Aβ.

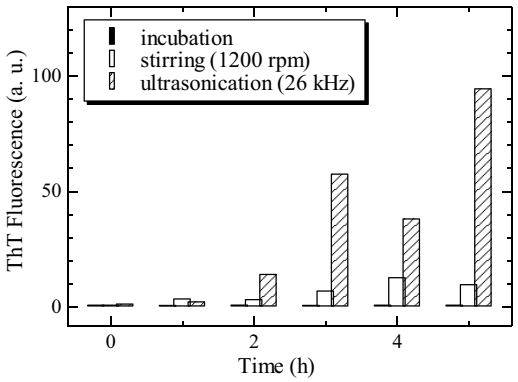


Fig.1 Change in the ThT fluorescence intensity in Aβ solution with ultrasonication (stripe bar), stirring (open bar) and incubation (solid bar) treatments.

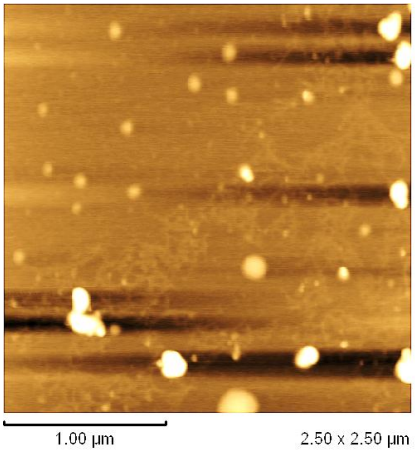


Fig. 2 The AFM image of Aβ aggregates caused by the ultrasonication sequence for 5 h.

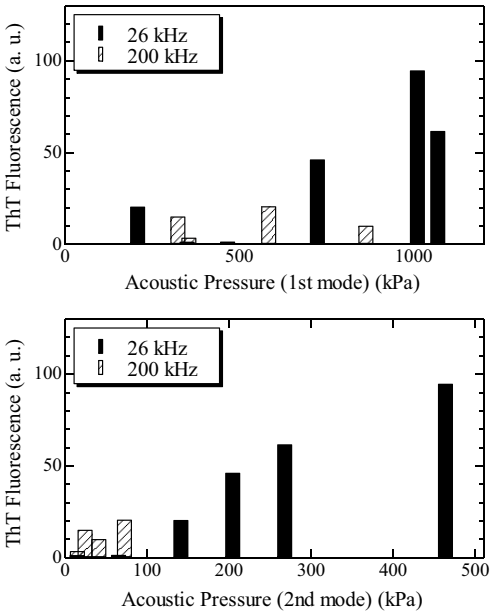


Fig. 3 Relationship between ultrasonically induced aggregation phenomenon of Aβ and acoustic pressures of 1st and 2nd modes.

Relationship between ultrasonically induced aggregation phenomenon and acoustic-pressure of 1st and 2nd modes was investigated using 26 and 200 kHz transducers (**Fig. 3**). It should be noted that the acoustic pressure of 1st mode fails to show a good correlation with the ThT level, whereas that of the 2nd mode showed a positive correlation with it. These results suggest that this phenomenon should be deeply related with cavitation phenomenon, because cavitations enhance intensity of harmonics [6-8]. Thus, high energy barrier for the nucleation of Aβ may be lowered by energy emission accompanied by collapse of cavitation bubbles.

## 4. Conclusion

We revealed that second-harmonic acoustic-pressure dominates the aggregation behavior of $A\beta_{1\text{-}40}$ peptides. This indicates significant contribution of cavitation to the nucleation of the peptide. This result will contribute to the initial diagnosis of AD.

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