Acceleration of amyloid fibril formation by multichannel sonochemical reactor

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Typeset with jjap2.cls in Alzheimer’s disease and the type of constituent protein: The misfolded proteins form observed in the formation of amyloid fibrils regardless of monomeric proteins are adsorbed to the ends of the nuclei, followed by an elongation reaction in which the nucleus, and the fibril formation reaction takes a long time. In order to promote amyloid fibril formation, considerably higher concentrations of protein compared to biological concentrations and agitation such as stirring and rotation have been used. The fibril formation behavior can be monitored by a fluorometric analysis using thioflavin T (ThT), which specifically binds to the β-sheet structure in amyloid fibrils and emits high-intensity fluorescence. An increase in the ThT fluorescence intensity therefore indicates the increase in formed fibrils.

Ultrasound irradiation is a beneficial method to significantly promote the nucleation of amyloid fibrils. It repeatedly creates positive and negative pressures in the solution. The mechanism of accelerated nucleation reaction is explained by the interaction between cavitation bubbles generated by ultrasound wave and dissolved proteins. The cavitation bubbles grow under negative pressure, and a large amount of protein molecules are trapped on the surfaces of the bubbles by their hydrophobic sites. Then, when the acoustic pressure switches to positive pressure, the bubbles contract and collapse in a short time, so that the adsorbed protein molecules are collected at contraction points of the bubbles and condensed locally, and at the same time, a transient temperature increase due to adiabatic compression of the gas in the bubble occurs, which accelerates the nucleation reaction.

We have developed a high-throughput fluorescence spectroscopy system that can monitor the formation process of amyloid fibrils while performing ultrasound stimulation and named it "HANABI" (Handai amyloid burst inducer). In this system, a 96-well microplate containing protein solutions is placed near the water surface in a water bath, and three ultrasound generators simultaneously irradiate the microplate from below in the water bath. We applied the HANABI system to various proteins and found that it was effective for studying their fibril formation behavior. However, there are several problems with the HANABI system: The powerful ultrasonic waves emitted from the three ultrasonic generators fixed at the bottom of the water bath generate a large number of cavitation bubbles in the bath, which scatter the ultrasonic waves, resulting in uneven ultrasonic energy input to each well of the microplate. In addition, the temperature in the bath fluctuates due to ultrasound, resulting in poor reproducibility.

To solve these problems, we developed a multichannel ultrasonic sonochemical reactor with fluorescence measurement system, in which the water bath was removed and one transducer was placed in each well of a 96-well microplate (Fig. 1). As a result, we successfully detected the amyloid fibril formation of β2-microglobulin (β2m) and the low concentration seed of β2m. We name this improved HANABI system "HANABI-2000". In the previous study, we have shown that the developed multichannel ultrasound reactor is

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capable of inducing fibrillation reactions of Aβ1–40 and α-synuclein solutions. Here, the applicability of the developed system to amyloid assay measurements was investigated in more detail, not only for Aβ1–40 and α-synuclein, but also for β2m and lysozyme from egg white. We confirmed that the multichannel sonochemical reactor can significantly promote the formation of amyloid fibrils of these proteins, and that the reaction rate of each well can be controlled by adjusting the ultrasound irradiation conditions individually.

2. Experimental methods and instruments

2.1 Materials and protein preparation

Lyophilized-powder Aβ1–40 peptides were purchased from Peptide Institute (No. 4307-v). Recombinant human β2m protein with an additional methionine residue at the N terminus and recombinant human α-synuclein were expressed in E. coli and purified as described elsewhere.8,39,40 Lysozyme, dimethyl sulfoxide (DMSO), phosphate-buffer saline (PBS), NaCl, and Thioflavin-T (ThT) were purchased from Wako Pure Chemical Industries Ltd.

The powder of Aβ1–40 was dissolved in DMSO, and the solution was diluted with 100 mM PBS mixed with NaCl and ThT to obtain a final concentration of 5 µM of Aβ1–40, 100 mM of NaCl, and 5 µM of ThT at pH 7.4. (The volume ratio of DMSO to PBS is 1:4.)

Lyophilized powdered α-synuclein was dissolved in 0.8 M phosphate buffer (pH 6.9) containing ThT to obtain a solution with a final concentration of 7.0 µM of α-synuclein with 5 µM ThT.

Lyophilized powder β2m was dissolved in 10 mM HCl (pH 2.0) containing NaCl and ThT to obtain a final concentration of 8.5 µM of β2m with 100 mM NaCl and 5 µM ThT.

Powder lysozyme from egg white (No. 20841-54, Nacalai Tesque Inc.) was dissolved by 3 M guanidinium chloride containing HCl and ThT to obtain a final concentration of 35 µM of lysozyme with 10 mM NaCl and 5 µM ThT.

2.2 Multichannel sonochemical reactor

We have developed a spectroscopic multichannel sonochemical reactor, as shown in Fig. 1.36 A single piezoelectric lead zirconate titanate (PZT) transducer is placed in each well of a 96-well microplate and it irradiates the sample solution in the well through a thin (0.1 mm) plastic sheet with ultrasound, and the fluorescence measurement is performed from the back surface. The PZT transducers are driven in turn by a 0.3-ms-long burst signal via a fast switching device. (b) Schematic of a single PZT transducer and one well of a 96-well plate.

2.3 AFM measurement

The morphology of the aggregates formed in the solution was observed by atomic force microscopy (AFM) using an instrument produced by Shimadzu Corporation (SPM A9600). The tapping mode was used with a silicon cantilever (stiffness: 40 N/m, resonant frequency: 300 kHz). The ultrasonically irradiated solution was dropped onto the mica with the new surface and incubated for 15 min. Then, it was washed with 100 µl of ultrapure water and dried before AFM observation.

3. Results and Discussion

Figure 2(a) shows the time course of ThT fluorescence intensity of Aβ1–40 solution measured by the HANABI-2000 system. These are the results of simultaneous measurements of 12 wells in one microplate. Although there seems to be some variation in the change in fluorescence intensity, the variation is small enough when we focus on the initial rise time of the fluorescence intensity (Fig. 2(b)). The rapid increase in the fluorescence intensity corresponds to the onset of amyloid fibril formation. For comparison, changes in the ThT fluorescence intensity were measured under shaking and quiescent conditions (Figs. 2(c) and (d), respectively). When
the threshold of the lag time to amyloid formation is set to 0.1 of the maximum ThT intensity, the average values of the lag
time obtained in the multichannel sonochemical reactor, shaking,
and quiescent are 1.7, 5.1 and 23 h, respectively. Therefore,
our developed multichannel sonochemical reactor can
significantly accelerate the amyloid fibril formation of Aβ₁₋₄₀
peptide.

Figures 3 (a), (c), and (e) show the results of the mea-
surements for α-synuclein, β₂m, and lysozyme, respectively.
All protein solutions showed a rapid increase in the ThT flu-
orescence intensity within 15 h when using our multichannel
sonochemical reactor. We used the coefficient of vari-
ation (CV) value as a measure of reproducibility, which is
the standard deviation divided by the average value. Table I
shows the average lag time, standard deviation, and the CV
value. It can be seen that the CV values are very low for the
measurements for all samples, indicating high reproducibil-
ity among different wells. For example, in our previous study
with the high-throughput fluorescence spectroscopy system
(previous HANABI system), the CV value for β₂m solution
was about 20% at the optimal conditions under plate move-
ment, which was reduced to 14% in the HANABI-2000 in-
strument. It is also important to note that AFM observation
confirmed the formation of amyloid fibrils in all sample sol-
tions as shown in Fig. 4. Although it is reported that ultra-
sound irradiation causes fragmentation of amyloid fibrils, sufficiently long amyloid fibrils up to 5 µm were observed in
the present study. Therefore, the ultrasound irradiation by
the multichannel sonochemical reactor developed in this study
contributes to the elongation of amyloid fibrils rather than
their fragmentation.

On the other hand, under shaking condition, the ThT flu-
orescence intensity did not increase in some wells even after
280 h (Figs. 3(b), (d), and (f)), and the lag time variation
was significantly large (Table I). These results indicate
that the HANABI-2000 system developed in this study not only
promotes the formation of amyloid fibrils of various proteins,
but also achieves high reproducibility.

Finally, we investigated the effect of the voltage applied to
the PZT transducer on the fibril formation reaction. Figure 5
shows the relationship between the inverse of the lag time and
the applied voltage for amyloid fibril formation using Aβ₁₋₄₀
peptide. The result indicates that as the applied voltage in-
creases, the lag time becomes shorter, and the fibril forma-
tion reaction can be controlled by adjusting the applied volt-
Therefore, it is considered that there is an optimal ultrasound pressure for the diagnosis of each disease, and the multichannel sonochemical system developed here, which can irradiate the solution in each well at different pressure, will highly contribute to the early stage diagnosis of neurodegenerative diseases.

4. Conclusion

We throughout examined the usefulness of our developed multichannel sonochemical reactor, HANABI-2000. It is capable of highly accelerating amyloid fibril formation of various proteins with high reproducibility among many wells of a microplate. The amyloid fibril formation sometimes takes more than 300 h under the traditional shaking condition, but our multichannel sonochemical reactor has succeeded in shortening the assay time to less than 20 h. In this system, ultrasonic-irradiation conditions (amplitude, frequency, duration, and so on) on each PZT transducer can be easily adjusted, allowing simultaneous measurement of different conditions for various proteins. Therefore, the multichannel sonochemical reactor will be a powerful tool for studying amyloid fibril formation and diagnosis of neurodegenerative diseases.

![AFM images](a) Aβ_{1–40}, (b) α-synuclein, (c) β2m, and (d) lysozyme, respectively, after ultrasonic irradiation by the HANABI-2000 system. The scale bars indicate 1 μm.

Fig. 4. AFM images of (a) Aβ_{1–40}, (b) α-synuclein, (c) β2m, and (d) lysozyme, respectively, after ultrasonic irradiation by the HANABI-2000 system. The scale bars indicate 1 μm.

![Graph](Fig. 5. Relationship between voltage applied to piezoelectric transducer and inverse of lag time in fibril formation reaction of Aβ_{1–40} peptide.)

Fig. 5. Relationship between voltage applied to piezoelectric transducer and inverse of lag time in fibril formation reaction of Aβ_{1–40} peptide.

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