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Seed-dependent Accelerated Fibrillation of α-Synuclein Induced by Periodic Ultrasonication Treatment

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Abstract α-Synuclein is the major component of Lewy bodies and responsible for the amyloid deposits observed in Parkinson's disease. Ordered filamentous aggregate formation of the natively unfolded α-synuclein was investigated *in vitro* with the periodic ultrasonication. The ultrasonication induced the fibrillation of α-synuclein, as the random structure gradually converted into a β-sheet structure. The resulting fibrils obtained at the stationary phase appeared heterogeneous in their size distribution, with the average length and height of 0.28 μm±0.21 μm and 5.6 nm±1.9 nm, respectively. After additional extensive ultrasonication in the absence of monomeric α-synuclein, the equilibrium between the fibril formation and its breakdown shifted to the disintegration of the preexisting fibrils. The resulting fragments served as nucleation centers for the subsequent seed-dependent accelerated fibrillation under a quiescent incubation condition. This self-seeding amplification process depended on the seed formation and subsequent alterations in their properties by the ultrasonication to a state that accretes the monomeric soluble protein more effectively than their reassociation of the seeds back to the original fibrils. Since many neurodegenerative disorders have been considered to be propagated *via* the seed-dependent amyloidosis, this study would provide a novel aspect of the significance of the seed structure and its properties leading to the accelerated amyloid formation.

Keywords: α-Synuclein, natively unfolded protein, amyloidosis, fibrillation, ultrasonication, neurodegenerative disorder

α-Synuclein is the major filamentous constituent of Lewy bodies, a pathological hallmark of Parkinson's disease (PD) [10, 11]. When incubated *in vitro*, α-synuclein has undergone structural alteration from its natively unfolded state to cross β-sheet conformation, which results in the filamentous aggregate formation. Understanding the aggregation process is important because it could provide means to control the amyloidosis-related toxicity observed in neuronal cell death. Despite its importance, however, the molecular mechanism of the α-synuclein aggregation process remains unclear. The filamentous aggregate formation of α-synuclein has been considered to be a nucleation-dependent process [16]. It could be seeded by a previously self-assembled species of α-synuclein acting as a nucleus. Its growth kinetics, therefore, is dependent upon the seed concentration, whereas a critical concentration of the α-synuclein monomer at the stationary phase is independent of the seed [16]. During fibril formation, natively unfolded α-synuclein is believed to adopt a partially unfolded intermediate structure and transform into highly ordered fibrils. Under optimized conditions of pH and temperature for this partially unfolded intermediate, the α-synuclein fibril formation could be accelerated [13]. Macromolecular crowding, metal ions, and small chemicals also facilitated the fibril formation [5, 14, 15]. Some chemicals, however, induced α-synuclein to protofibrils instead of the eventual fibril formation [4].

Ultrasound is a mechanical wave causing periodic vibration of particles in solution [9]. In liquid, its velocity is about 1,000–1,600 m/sec and its wavelength ranges from micrometers to centimeters. Ultrasound, therefore, cannot directly affect the energy levels of molecules. Instead, ultrasonication could be used for preparing the seeds from preformed fibrils. This effect has been successfully applied to the replication of infectious prion proteins [2]. Recently, it has been demonstrated that ultrasound may influence protein structure and induce filamentous aggregate formation [6, 12]. Stathopulos *et al.* [12] demonstrated that ultrasonication led various proteins to aggregates that resembled amyloids. The proteins were likely to be destabilized and unfolded by the ultrasonication, and changed into the partially unfolded intermediate structures. Ohhashi *et al.* [6] investigated the ultrasonication effect on β_2-microglobulin, a protein

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responsible for dialysis-related amyloidosis. The ultrasonication triggered β_2-microglobulin to the amyloid fibrils even at neutral pH, which gave rise to thicker fibrils than those generally obtained at pH 2.5 [6]. The β_2-microglobulin fibril formation was suggested to be facilitated by the agitation caused by ultrasonication [6]. In this report, the ultrasonication effect on α-synuclein has been investigated in terms of the accelerated and seed-dependent fibrillation of the natively unfolded protein.

MATERIALS AND METHODS

Expression and Purification of α-Synuclein

Recombinant α-synuclein was prepared according to the procedure previously described [3, 7, 18]. Briefly, α-synuclein overexpressed in *E. coli* BL21(DE3) was completely purified *via* heat treatment, DEAE-Sephacel anion-exchange, Sephacryl S-200 size-exclusion, and S-Sepharose cation-exchange chromatography steps. Purified α-synuclein was dialyzed against 20 mM Mes at pH 6.5 and stored at −20°C in aliquots.

Ultrasonication-induced α-Synuclein Fibrillation

α-Synuclein was subjected to ultrasonication by using the ultrasonic transmitter employed in the previous study [6]. In brief, a water bath-type ultrasonic transmitter (ELESTEIN SP070-PG-M, Elekon, Tokyo) contained approximately 12 l of water under a thermostatic condition at 37°C. The frequency of the ultrasonic transmitter was 17–20 kHz and the power output was 350 watts. All α-synuclein (1 mg/ml) fibrilization reactions were monitored in 20 mM Mes (pH 6.5) at 37°C. The samples were subjected to the periodic ultrasonication with a 10 min cycle (1 min for ultrasonication followed by 9 min of quiescent incubation in the absence of sonication) during the entire incubation periods. For the experiment of elucidating the effect of quiescent incubation versus number of sonications on the fibril formation, the quiescent incubation period was varied from 0.5 min to 9 min. Fibril formation was monitored with thioflavin-T binding fluorescence (Hitachi F-4500 fluorescence spectrophotometer). During the incubation, aliquots (5 μl) were combined with 1 ml of 5 μM thioflavin-T prepared in 50 mM glycine at pH 8.5. The sample was kept for 5 min at room temperature with light protection before the light emission was measured at 483 nm with an excitation at 450 nm.

Seed-dependent Fibril Formation of α-Synuclein

For the seeding experiment, the fragments of α-synuclein fibrils obtained as the final products of extensive ultrasonication were combined with α-synuclein monomer at a 1:9 (v/v) ratio to give a 1 mg/ml final protein concentration. The mixture was incubated at 37°C under a quiescent condition without sonication in the absence of agitation. For the experiment with agitation, monomeric α-synuclein (1 mg/ml) was subjected to shaking incubation at 37°C with an orbit shaker rotating at 200 rpm.

Atomic Force Microscopy (AFM) Measurement

Samples containing the fibrils were dropped in aliquots (1–2 μl) on a freshly cleaved mica and allowed to be adsorbed for 5 min. The mica surface was washed with distilled water (40 μl) and then dried completely [8]. The sample was observed through Veeco Metrology Multimode Atomic Force Microscopy.

Circular Dichroism (CD) Spectroscopy Measurement

Far-UV CD spectra of α-synuclein were measured by a Jasco spectropolarimeter J-600 [17]. During the ultrasonication experiment, aliquots were collected and diluted with 10 mM potassium phosphate to reach 10 μM α-synuclein at pH 7.0 for the CD measurement.

RESULTS AND DISCUSSION

Ultrasonication-induced Fibril Formation of α-Synuclein

Monomeric α-synuclein was prepared in 20 mM Mes, pH 6.5. Prior to each protein aggregation experiment, the α-synuclein preparation had been filtered through 0.22 μm to eliminate any impurities that might serve for nonspecific seeds during the fibril formation. α-Synuclein (1 mg/ml) prepared in an Eppendorf tube was placed in the ultrasonication bath, and subjected to the periodic ultrasonication with a 10 min cycle (1 min sonication and 9 min under a quiescent incubation) [6]. The aggregation kinetics was monitored with the thioflavin-T binding fluorescence. In fact, the ultrasonication induced the fibrillation of α-synuclein, whereas the quiescent incubation did not give rise to fibril formation at all (Fig. 1A). The aggregation kinetics by ultrasonication exerted a typical curve of amyloid fibril formation, which consists of lag, elongation (growth), and stationary phases. Transition from the lag to the elongation phase required around 20 h of the periodic ultrasonication. In the elongation phase, the filamentous aggregate formation drastically increased and reached to the maximum at the stationary phase within less than 10 h. Natively unfolded state of α-synuclein was found to show random structure in the CD spectrum. This random structure gradually converted into a β-sheet structure as the ultrasonication proceeded (Fig. 1B). The minimum ellipticity at 195 nm, characteristic for the random structure, was shifted upward whereas the minimal point at 217 nm, representing β-sheet structure, became evident in the spectra.

The ultrasonication-induced filamentous aggregate formation appears to be dependent upon the quiescent incubation period within the periodic ultrasonication cycle rather than the actual number of applied sonications. The lag periods

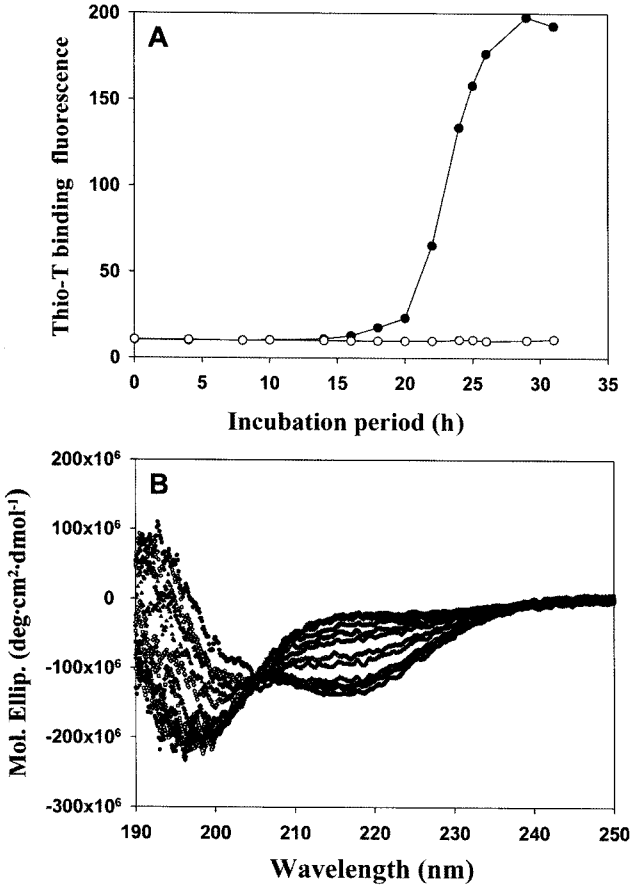

Fig. 1. Ultrasonication-induced α-synuclein fibrillation kinetics (**A**) and circular dichroism spectroscopic analysis (**B**).
α-Synuclein (1 mg/ml) was incubated in a 2 ml final volume with (●) and without (○) ultrasonication. Filamentous aggregate formation was evaluated with 5 μM thioflavin-T binding fluorescence (**A**). During ultrasonication-induced fibrillation, the secondary structure of α-synuclein was analyzed with CD spectroscopy (**B**).

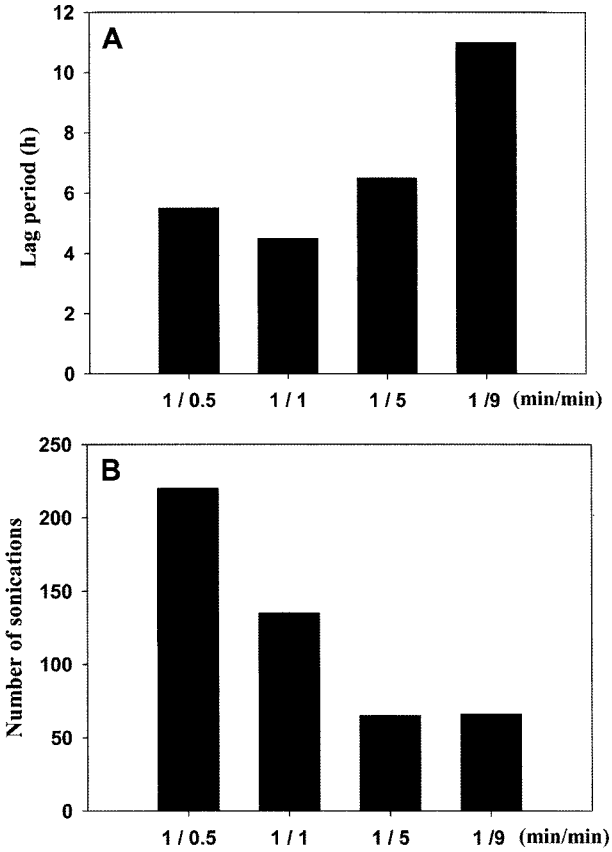

Fig. 2. Lag periods of fibril formation with variations in the periodic ultrasonications.
α-Synuclein (1 mg/ml) was subjected to the periodic ultrasonications in a final volume of 0.5 ml with variation in the quiescent incubation periods (30 sec, 1 min, 5 min, and 9 min) following the sonication at a fixed duration of 1 min. The individual lag period was evaluated (**A**). The number of sonications during the lag periods were calculated and compared with each other (**B**).

of the filamentous aggregate formation were monitored for a set of different reaction mixtures with various quiescent incubation periods, from 30 sec, 1 min, 5 min to 9 min, following a fixed sonication period of 1 min. Although the lag period became increased from 5 to 11 h as the duration of ultrasonication cycle was increased between 1.5 min (1 min/0.5 min) and 10 min (1 min/9 min) (Fig. 2A), the actual number of sonications required for the transition between the lag and the elongation phase were dramatically reduced from 220 to 66 cycles, respectively (Fig. 2B). This fact indicates that less numbers of sonications are required for the filamentous aggregate formation as the quiescent incubation period increases. The structural transition of α-synuclein responsible for exposing an interactive patch for subsequent protein-protein interaction might occur especially during the quiescent period following sonication. In other words, the ultrasonication-dependent fibril formation may depend on the quiescent incubation following sonication, instead of the sonication itself.

Comparison Between Fibrillations of α-Synuclein with and without the Ultrasonication

The ultrasonication-induced α-synuclein fibrillation was compared with a fibrillation induced by agitation in the absence of the ultrasonication treatment (Fig. 3). Soluble monomeric α-synuclein was subjected to shaking incubation at 37°C with an orbit shaker at 200 rpm. When the lag periods were compared, the ultrasonication-induced fibril formation exerted the longer lag phase of around 20 h, whereas the agitation-induced fibril formation showed only 5 h of lag phase. The longer lag phase indicates that the actual nucleus formation was somewhat less favored for the ultrasonication-derived fibrillation, although the condition would increase collision frequency among the proteins. This might be partly due to the destabilization effect of ultrasonication toward the nucleus formation responsible for the shortening of the lag period.

For the growth phase, the ultrasonication and the agitation required 10 and 16 h to reach their corresponding stationary phases with two different final thioflavin-T

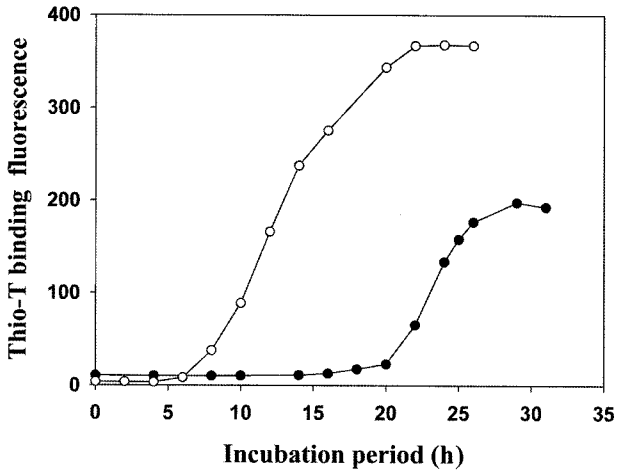

Fig. 3. Aggregation kinetics of α-synuclein obtained with either agitation (○) or ultrasonication (●).
α-Synuclein (1 mg/ml) was incubated under a shaking condition (200 rpm, 37°C) in the absence of ultrasonication, and monitored with 5 mM thioflavin-T binding fluorescence. The ultrasonication-induced amyloid kinetics obtained in Fig. 1 was compared.

binding fluorescence intensities. The ultrasonication led to the final aggregates with the binding fluorescence that was one half of the fluorescence intensity obtained with the agitation-induced fibrillation of α-synuclein (Fig. 3). It would suggest that the ultrasonication causes possible disintegration or fragmentation of preexisting fibrils along with actual fibril formation. In other words, the elongation of fibrils and their breakdown might exist in equilibrium, at least for the beginning of the stationary phase, although an extensive ultrasonication favored the fragmentation in the absence of monomeric α-synuclein (see below).

AFM Images of the Ultrasonication-induced Protein Aggregates of α-Synuclein

The ultrasonication-induced protein aggregates obtained during the elongation and the stationary phases of the aggregation kinetics were examined with AFM. From the aggregates collected at the beginning of the elongation phase (Fig. 1A), rather short α-synuclein fibrils were observed (Fig. 4A). Average height of the fibrils was 3.6 nm±1.3 nm. These short fibrils were responsible for the increase of β-sheet CD spectra. As the fibrils elongated, the intensity of β-sheet spectra at 217 nm became stronger. Right after the α-synuclein aggregation kinetics reached the stationary phase, we clearly identified the ultrasonication-induced fibrils under AFM (Fig. 4B). The average length of ultrasonication-induced fibrils was 0.28 μm±0.21 μm and the maximum length of the fibril reached about 1 μm. The presence of the maximum length fibril being three times longer than the average length and the heterogeneous size distribution of the fibrils ought to be related to the fact that those longer fibrils have been broken down to shorter ones by the ultrasonication, which could serve as new templates for subsequent seed-dependent fibril growth. This self-seeding amplification process of the fibrillation along with the breakdown of the preexisting fibrils would contribute to the rapid increase in the kinetic curve and the heterogeneous distribution of resulting fibrils. At the stationary phase, the average height of the fibrils was 5.6 nm±1.9 nm, which was increased by 55% from the initial short fibrils. In fact, complete fragmentation of those fibrils was accomplished with an extended period of the periodic ultrasonication. Following an additional 30 h of ultrasonication toward the fibrils initially found at the stationary phase, the thick and short fibrillar fragments were obtained with an average length of less than 0.16± 0.06 μm (Fig. 4C). Average height of the fragments was 7.5 nm±1.6 nm, which was increased by 30% from that of the previous fibrils obtained at the beginning of the stationary phase (Fig. 4B). The increase in the average height indicates that α-synuclein fibrils had been further developed into mature forms until the monomeric protein was exhausted before the fibrils were actually broken into the fragments (Fig. 4C). Ultrasonication, therefore, not

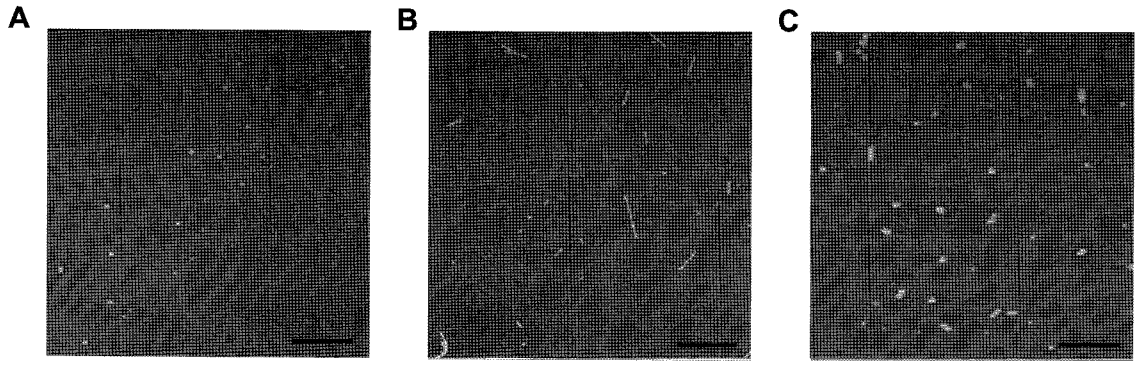

Fig. 4. Atomic force microscope images of ultrasonication-induced α-synuclein aggregates.
The aggregates obtained at the initial period of the elongation phase (**A**) and the stationary phase of the kinetics (**B**). The fibrillar fragments obtained with additional 30 h of the periodic ultrasonication after the aggregation kinetics reached the stationary phase were visualized (**C**). Scale bars represent 1 μm.

only induced the fibril formation by presumably affecting the conformation of α-synuclein and/or increasing the collision frequency among molecules, but also caused the fragmentation of the preexisting fibrils.

Seed-dependent Amyloid Fibril Formation of α-Synuclein in the Presence of the Ultrasonication-derived Fragments

The fibrillar fragments derived *via* the extensive ultrasonication, however, appeared to lose their potential to be reassociated into the mature fibrils, since longer fibrils were not found among the homogeneous distribution of the fragments (Fig. 4C) and the thioflavin-T binding fluorescence actually decreased significantly from the maximum value at the beginning of the stationary phase (data not shown). This might be due to ultrasonication-related effects on the fragments, such as local heating effect on the protein aggregates. Instead, the altered property of the fibrillar fragments has been examined for whether it could serve as an effective nucleation center to recruit monomeric soluble α-synuclein for the formation of eventual protein fibrils.

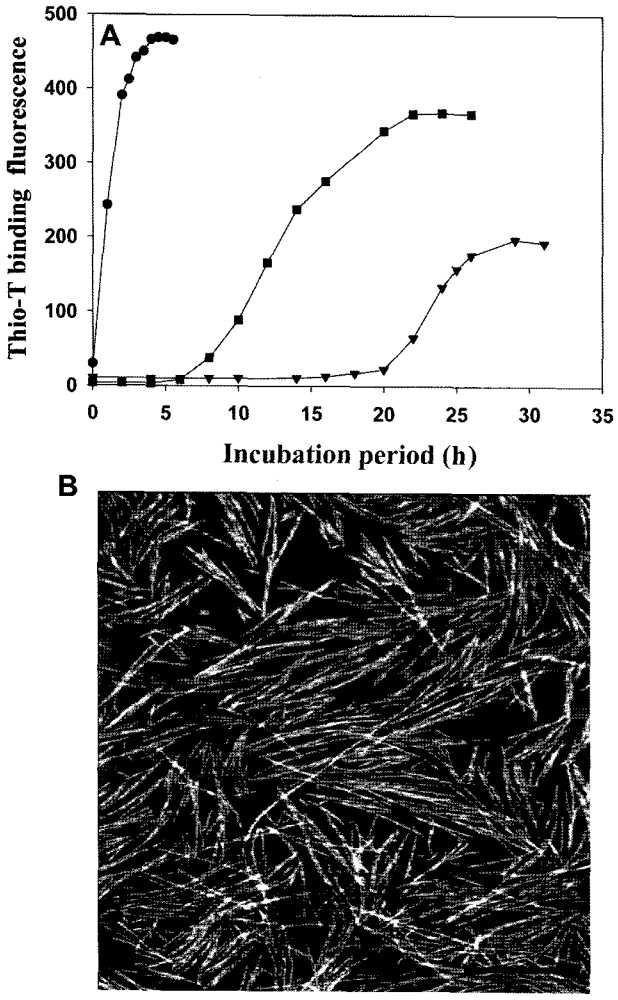

Fig. 5. Seed-dependent aggregation kinetics of α-synuclein (**A**) and the final fibrillar aggregates (**B**).

Seed-induced aggregation kinetics of α-synuclein was obtained in the presence of the ultrasonication-induced fibrillar fragments (**A**). Monomeric α-synuclein (1 mg/ml) was co-incubated with the ultrasonication-induced fragments of fibrils as seeds in 10% (w/w) (●). The aggregation kinetics obtained with the agitation (■) (Fig. 3) and the ultrasonication (▼) (Fig. 1A) are also shown for comparison. Fibril formation was evaluated with 5 μM thioflavin-T binding fluorescence. Morphology of the final seed-induced protein aggregates was observed with AFM (**B**). Scale bar represents 1 μm.

Seeding effect of the ultrasonication-derived fragments on the filamentous aggregate formation of α-synuclein was investigated under a quiescent incubation condition (Fig. 5A). The reaction was monitored in 20 mM Mes, pH 6.5, at 37°C without sonication. As soon as the seeds were introduced to 10% of total protein concentration (1 mg/ml), the thioflavin-T binding fluorescence was instantaneously increased without any lag period and the aggregation kinetics was finished within 4 h, while the agitation-induced aggregation required more than 20 h to reach the stationary phase with an apparent lag phase of 5 h (Fig. 5A). This dramatic facilitation of the fibril formation appeared to be highly dependent upon the seeds derived from the ultrasonication treatment of the mature fibrils. The final intensity of thioflavin-T binding fluorescence at the stationary phase went up twice as high as the fluorescence intensity obtained with the ultrasonication-induced α-synuclein fibrillation (Fig. 5A). Since thioflavin-T binding fluorescence was emitted only when the dye bound to fibrils, the level of mature fibrils generated by the seed-dependent fibrillation procedure was expected to be higher than the amount of fibrils obtained with the ultrasonication treatment [1]. When the final protein aggregates derived from the seeds were examined with AFM, the extensive α-synuclein filamentous aggregates were definitely visualized (Fig. 5B). These facts suggest that ultrasonication could produce the seeds from the preformed fibrils, which provide new templates for the subsequent facilitated filamentous aggregate development.

Taken together, our study clearly demonstrates that the protein fibril formation of α-synuclein has been facilitated by the seeds obtained from the ultrasonication treatment of the preexisting fibrils. This self-seeding amplification process depends on the fragmentation and subsequent alteration in the property of resulting fragments to a state that accretes the monomeric soluble protein more effectively than their reassociation back to the original fibrils. Since many neurodegenerative disorders have been considered to be propagated *via* the seed-dependent process of amyloidosis, this study would provide a novel aspect of the importance of the seed structure and their properties to participate in the amplified amyloid formation. In addition, the ultrasonication method could be applicable to enrich the amyloid fibrils for eventual production of functionalized protein nanofibrils.

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