# Control of Morphology and Subsequent Toxicity of Aβ Amyloid Fibrils through the Dequalinium-induced Seed Modification

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Amyloid fibril formation of amyloid β/A4 protein (Aβ) is critical to understand the pathological mechanism of Alzheimer's disease and develop controlling strategy toward the neurodegenerative disease. For this purpose, dequalinium (DQ) has been employed as a specific modifier for Aβ aggregation and its subsequent cytotoxicity. In the presence of DQ, the final thioflavin-T binding fluorescence of Aβ aggregates decreased significantly. It was the altered morphology of Aβ aggregates in a form of the bundles of the fibrils, distinctive from normal single-stranded amyloid fibrils, and the resulting reduced β-sheet content that were responsible for the decreased fluorescence. The morphological transition of Aβ aggregates assessed with atomic force microscope indicated that the bundle structure observed with DQ appeared to be resulted from the initial multimeric seed structure rather than lateral association of preformed single-stranded fibrils. Investigation of the seeding effect of the DQ-induced Aβ aggregates clearly demonstrated that the seed structure has determined the final morphology of Aβ aggregates as well as the aggregative kinetics by shortening the lag phase. In addition, the cytotoxicity was also varied depending on the final morphology of the aggregates. Taken together, DQ has been considered to be a useful chemical probe to control the cytotoxicity of the amyloid fibrils by influencing the seed structures which turned out to be central to develop therapeutic strategy by inducing the amyloid fibrils in different shapes with varied toxicities.

**Key Words :** Aβ, Dequalinium, Seeding effect, Amyloid fibrils, Alzheimer's disease

## Introduction

Alzheimer's disease (AD) is a representative neurodegenerative disorder predominantly affecting cholinergic neurons with clinical manifestations of cognitive deficits involving memory, orientation, judgment, and reasoning.[1-3] The brain of AD patients contains a large number of amyloid deposits known as senile plaques in which amyloid β/A4 proteins (Aβ) with 40 amino acid residues (Aβ40) has constituted the major component along with another extended form of Aβ with 42 amino acid residues (Aβ42).[4-6] As suggested by the amyloid cascade hypothesis,[7] the Aβ aggregates in a fibrillar state have been recognized as a critical phenomenon to understand the molecular etiology of AD. However, the amyloidosis mechanism has not been fully understood; therefore no effective treatment strategy for AD has been suggested. Many attempts have been made to understand the mechanism and find chemical compounds that influence the amyloid fibril formation reaction of Aβ.[8-12] Those compounds have been also expected to be used as probes to assess basic assembly mechanism of the peptide leading to the amyloid fibril formation.

Dequalinium (DQ), 1,1'-(1,10-decanediyl)-bis-[4-amino-2-methylquinolium] (Fig. 1A), was demonstrated to cause the protofibril formation of α-synuclein, a pathological component of Parkinson's disease, by inducing the selective protein self-oligomerization.[13] The resulting protofibrils were shown to affect cell viability by influencing membrane stability. The compound has been also shown to exert anti-tumor effect by selectively accumulating in mitochondria.[14] In fact, DQ affects crucial enzymes for cellular biogenesis such as mitochondrial $F_1$-ATPase, protein kinase C, calmodulin-dependent phoshodiesterase, and calcium activated $K^+$-channel.[15-18]

In this paper, we have described an effect of dequalinium as a modifier of Aβ aggregation by promoting and stabilizing the multimeric seed structures of Aβ. By serving as a nucleation center, the DQ-induced granular species gave rise to the Aβ aggregates which were different from the normal amyloid fibrils obtained in the absence of DQ in terms of their morphologies and cytotoxicities.

## Materials and Methods

**Materials.** Human Aβ40 was purchased from Sigma-Aldrich Co. (St. Louis, USA) or Peptide Institute (Osaka, Japan). Dequalinium was obtained from Sigma. The carbon-coated copper grid (300 mesh) and uranyl acetate were from Samchang Inc. and Electron Microscopy Sciences, respectively. Microcon-10 filter was purchased from Millipore Co. Human dopaminergic neuroblastoma cell line, SH-SY5Y, was a gift from Dr. Chung (Yonsei Univ., Korea). DMEM, trypsin-EDTA, and penicillin-streptomycin were provided by Gibco and fatal bovine serum was obtained from Hyclone. MTT, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium, for cell viability assay was purchased from Sigma. Materials used in this study were supplied from Sigma in the highest grade, unless otherwise mentioned.

**Preparation of Aβ aggregates.** Aβ was dissolved in 0.02% NH₃, and stored in aliquots at −70 °C. The dissolved peptide was diluted to 20 $\mu$M in 20 mM Mes, pH 6.5, and incubated in the absence or presence of DQ without shaking at either 37 °C or room temperature. Dequalinium stock was prepared in 50% ethanol at the highest concentration of 10 mM and diluted with 50% ethanol for subsequent use. The aggregative kinetics was monitored with thioflavin-T binding fluorescence at 485 nm with an excitation at 450 nm. The thioflavin-T binding fluorescence of the Aβ aggregates was obtained by combining the samples collected during the aggregative process and 2.5 $\mu$M thioflavin-T in 50 mM glycine at pH 8.5.

**Isolation of DQ-induced granular species of Aβ.** Granular forms of Aβ induced by dequalinium were isolated with a membrane filter of molecular weight cut-off limit at 10,000 Da. The peptide was dissolved in the NH₃ and diluted with 20 mM Mes, pH 6.5, to a final concentration of 20 $\mu$M, which was incubated for 5 hours at 37 °C in the presence of 100 $\mu$M DQ. The filter was pre-washed with water and 20 mM Mes buffer prior to the filtration. The peptide solutions were filtered through the pre-washed filter via a centrifugation at 12,000 × g for 20 min.

**Seed-dependent amyloid fibril formation of Aβ in the presence of the DQ-induced granular aggregates.** The DQ-induced self-oligomerized granules were employed as the seed for the Aβ fibrillation. The granular species of Aβ obtained with 100 $\mu$M DQ were added to Aβ monomer solution (20 $\mu$M) at 3%, and the mixtures were incubated at 37 °C. The aggregative kinetics was monitored with the thioflavin-T binding fluorescence.

**Cell culture and cell viability test.** Human dopaminergic SH-SY5Y cells were cultured in DMEM including 10% fetal bovine serum in the presence of penicillin-streptomycin at 37 °C in 5% CO₂. The cells were subcultured whenever they reached 80% of confluency. Following trypsinization with 1 mL of trypsin-EDTA at 37 °C, the cells were plated in 96-well plate at 5 × 10⁴ cells/well and incubated for 48 hours. The cells were further incubated in the presence of the Aβ amyloid fibrils at 37 °C for overnight. Cell survival was evaluated with tetrazolium salt-extraction method.[19] MTT, 3-[4,5-dimethyldiazol-2-yl]-2,5-diphenyltetrazolium bromide, was added to the culture medium at a final concentration of 1 mg/mL and incubated for 3 hours at 37 °C. The active mitochondria in living cells should cleave the tetrazolium ring into a visible dark-blue formazan product. To measure absorbance of the product at 570 nm, MTT extraction buffer containing 20% SDS and 50% N,N-dimethylformamide, pH 4.7, was added to each sample (100 $\mu$L into 100 $\mu$L medium in 96-well plate including 20 $\mu$L of 5 mg/mL MTT) to dissolve the formazan grains. All the data related to the cytotoxicity were obtained from three separate experiments.

**Miscellaneous.** The Aβ aggregates were visualized with either a transmission electron microscope (TEM; H7100, Hitachi) or atomic force microscope (AFM; XE-150, PSIA). An aliquot (5 $\mu$L) containing the aggregates was adsorbed onto a carbon-coated copper grid (300 mesh) and air-dried for 1 min. Following a negative staining with 2% uranyl acetate for 1 min, the aggregates were examined under TEM. For AFM analysis, the aggregate-containing aliquot was placed on a freshly cleaved mica, and left for 1 to 2 min for the aggregates to be adsorbed. After displacement of the droplet with water and the removal of excessive water, the aggregates were analyzed with AFM.

Secondary structures of the Aβ aggregates were monitored with circular dichroism (CD) spectroscopy (Jasco J-715) in 20 mM potassium phosphate (pH 7.5) at 20 °C. The spectra were obtained within a 0.1-mm pathlength quartz cell between 195 nm and 250 nm with a step resolution of 1.0 nm, band width of 1.0 nm, and scan speed of 20 nm/min. All of the spectra were obtained from an average of five scans.

## Results and Discussion

Dequalinium effect on the amyloid fibril formation of Aβ has been examined. When incubated alone, Aβ showed a time-dependent increase in the thioflavin-T binding fluorescence with a lag period of around 20 hours at 37 °C (Fig. 1). As DQ concentration increased, however, the final thioflavin-T binding fluorescence intensities became significantly suppressed in a dose-dependent manner. The decrease in the final fluorescence would indicate that the final Aβ aggregates have been produced to less amount. Alternatively, the amyloid fibrils may have different distinctive structures from the cross β-sheet conformation which has been
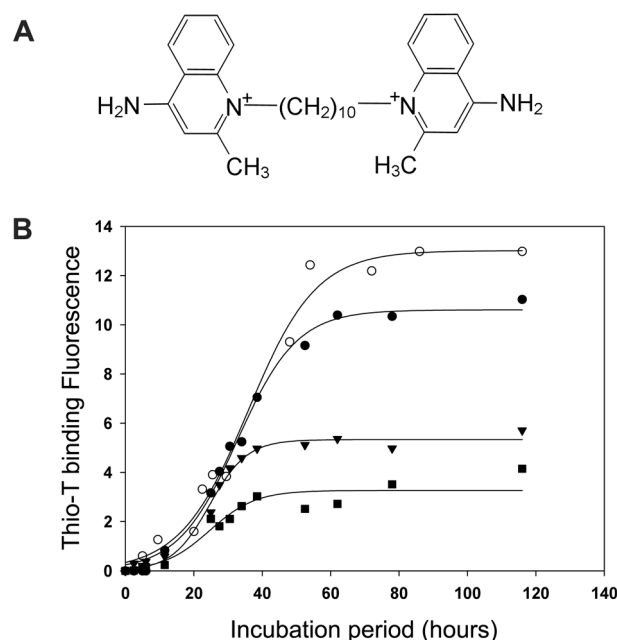


**Figure 1.** Aβ aggregation kinetics in the presence and absence of DQ. (A) Structure of dequalinium; (B) Aβ prepared in 0.02% NH₃ was quiescently incubated at 20 $\mu$M in 20 mM Mes, pH 6.5, at 37 °C in the absence (○) and presence of various concentrations of DQ at 20 $\mu$M (●), 100 $\mu$M (▼), and 200 $\mu$M (■). The aggregation kinetics was monitored with the thioflavin-T binding fluorescence at 485 nm with an excitation at 450 nm.
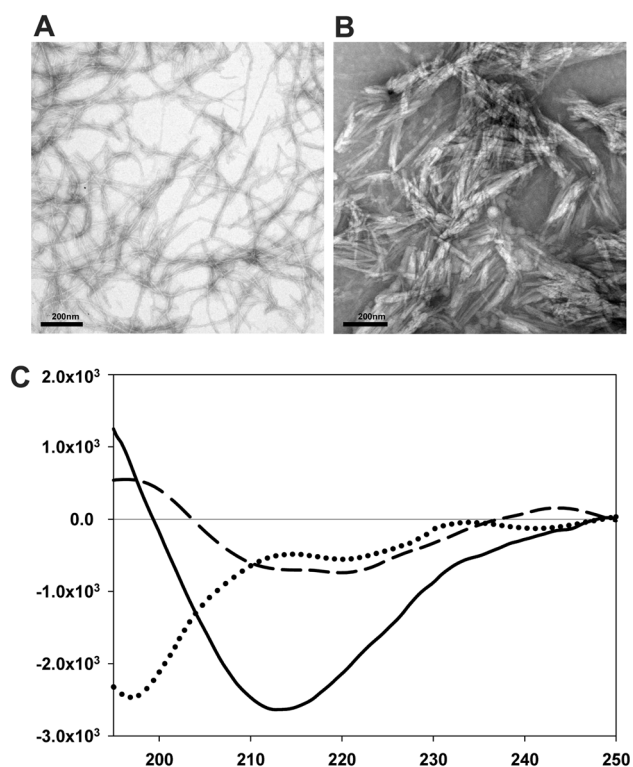
**Figure 2**. Morphologies of Aβ aggregates prepared in the presence and absence of DQ and their secondary structure analyses. The final Aβ aggregates obtained in the absence (A) and presence (B) of DQ were examined with a transmission electron microscope following a negative staining with 2% uranyl acetate on a carbon-coated copper grid (300 mesh). Secondary structures of the aggregates were monitored with circular dichroism spectroscopy in 20 mM potassium phosphate (pH 7.5) at 20 °C (C). Dotted, solid, and broken lines represent the spectra of the Aβ monomer, the Aβ amyloid fibrils obtained without DQ, and the Aβ aggregates prepared with DQ, respectively.

suggested to accommodate thioflavin-T to exert the binding fluorescence.

To confirm the final states, transmission electron microscope and circular dichroism (CD) spectroscopy were employed to analyze the final fibrillar structures and the underlying secondary structures of the Aβ aggregates, respectively. The Aβ aggregates obtained in the absence of DQ showed a typical morphology of amyloid fibrils which appears thin single-stranded fibrils with clean surface under transmission electron microscope (Fig. 2A). In the presence of DQ, however, the Aβ aggregates appeared to be chopped-wood like structure with thick bundles of the fibrils (Fig. 2B). Secondary structures of the protein aggregates were also analyzed with CD spectroscopy. The Aβ monomer exhibited mostly the random structure with a minimum ellipticity at 197 nm (Fig. 2C). As the Aβ fibrillation proceeded in the absence of DQ, the obvious β-sheet conformation was induced from the previous random structure as the minimum ellipticity has shifted to 219 nm (Fig. 2C), which could be readily recognized by thioflavin-T binding fluorescence (Fig. 1). In the presence of DQ, however, the β-sheet content decreased as the minimum ellipticity at 219 nm monitored with the Aβ aggregates increased (Fig. 2C). It was, therefore, the final morphology with bundles of the fibrils and the altered underlying secondary structure of the DQ-induced Aβ aggregates that should be responsible for the decreased thioflavin-T binding fluorescence as observed in Figure 1.

In order to examine molecular causes responsible for the dramatic alteration in the final structures of the Aβ aggregates obtained in the presence or absence of DQ, the morphological transition of Aβ aggregates has been followed with AFM (Fig. 3). In the absence of DQ, the single-stranded Aβ fibrils (Fig. 3b and 3c) have been gradually
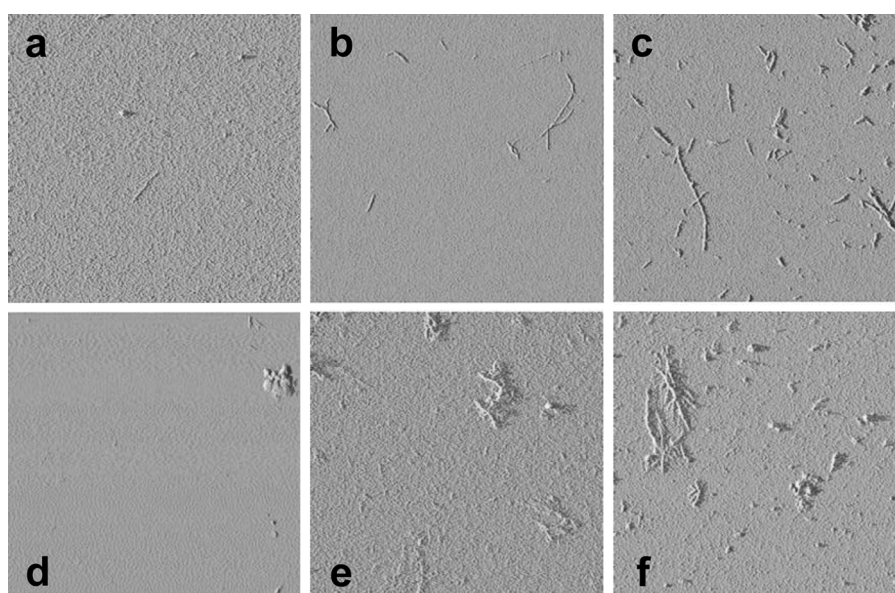


**Figure 3**. Structural transitions of Aβ aggregates obtained in the presence and absence of DQ. The Aβ aggregates collected at various time points during the aggregation kinetics in the absence (a, b, c) or presence (d, e, f) of 100 μM DQ were examined with AFM. After the aggregates were adsorbed on freshly cleaved mica with extra materials washed away, the aggregates were examined with AFM on tapping mode.

developed from the isolated round-shape granules as observed in Figure 3a. In the presence of DQ, however, the Aβ aggregates with multiple fibrils bundled together (Fig. 3e and 3f) appeared to be induced from the initial peptide aggregates in which the round-shape granules were associated to form the multimeric structure (Fig. 3d). In other words, the bundled structure of Aβ aggregates obtained with DQ-treatment has not been produced by lateral association of the preformed single-stranded fibrils, but instead they were resulted from the initial multimeric seed structures to which free Aβ has been accreted to multiple sites in parallel (Fig. 3e). Therefore, the initial seed structures of Aβ aggregates should determine their final morphologies by providing appropriate nucleation centers. In other words, the seed effect could influence not only the aggregation kinetics by shortening the lag phase, but also the final morphology of the protein/peptide aggregates.

The seeding effect of the DQ-induced initial Aβ aggregates has been investigated to assess whether they affect the final Aβ fibrillar structures as well as the aggregation kinetics. The seeds were prepared by incubating monomeric Aβ with 100 μM DQ for 5 hours at 37 °C (Fig. 4A inset).

The seed was added into the Aβ monomer solution at 20 μM to the amount of 3%, and incubated at 37 °C for more than 100 hours. Compared with a control of the Aβ aggregation obtained in the absence of the seed, the lag period was significantly reduced, indicating that the DQ-induced Aβ aggregates have exerted the seeding effect for the development of Aβ aggregation. As expected from the DQ-induced Aβ aggregation, the final thioflavin-T binding fluorescence was also significantly suppressed as the seeds were included in the aggregation mixture (Fig. 4A). The final morphology of the seed-induced Aβ aggregates was distinctive from the normal amyloid fibrils of Aβ obtained in the absence of the seeds (Fig. 4B and C). Those seeded-aggregates appeared more like the DQ-induced Aβ aggregates with the bundled fibrillar structures (Fig. 4C). The data clearly shows that the altered morphology of the DQ-induced Aβ aggregates could be due to the initial Aβ aggregates which act as the seeds during the Aβ aggregation. In other words, the final morphology of Aβ amyloid fibrils could be manipulated by modifying the seed structures.

The cytotoxicities of the Aβ aggregates prepared in the absence and presence of DQ were monitored with SH-SY5Y cells employing MTT assay. While the normal Aβ amyloid fibrils exerted the cytotoxicity by 28%, the seeded-Aβ aggregates showed the toxicity by only 12% (Fig. 5). The reduced cytotoxicity seemed to be due to the altered morphology of the seeded-aggregates since polymorphism of the amyloid fibrils has been suggested to be responsible for the varied cellular toxicities.[20-22] This data indicates that the cytotoxicity of amyloids could be controlled by manipulating the seed structures which would lead to multiplicity
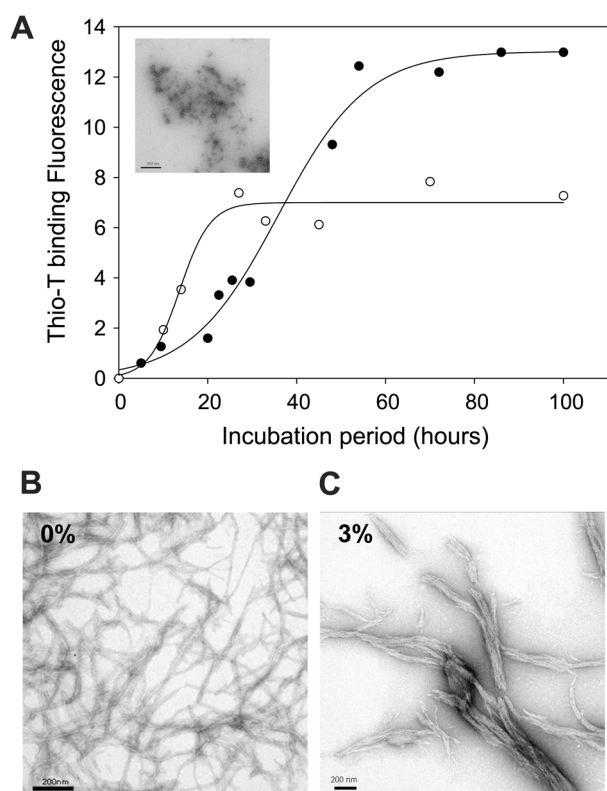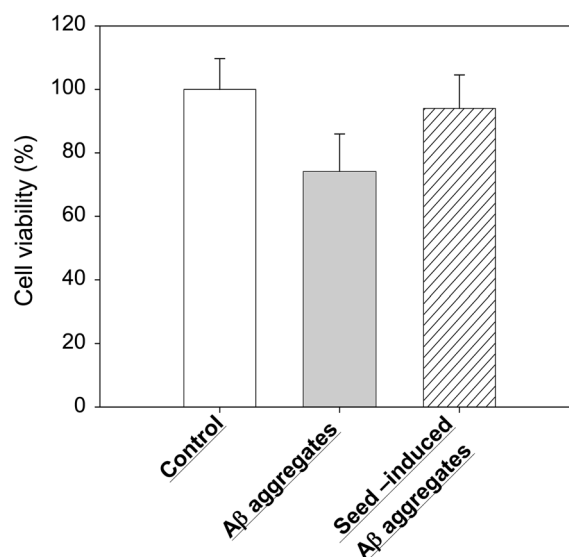


**Figure 4**. Seeding effect of the DQ-induced granular species for Aβ aggregate formation. The DQ-induced granular species of Aβ were obtained by incubating the monomeric peptide (20 μM) with 100 μM DQ in 20 mM Mes, pH 6.5, for 5 hours. The Aβ aggregation was carried out in the presence (○) or absence (●) of the granules to the amount of 3%. The thioflavin-T binding fluorescence was monitored with the aliquots obtained during the aggregations at 485 nm with an excitation at 450 nm (A). The final Aβ aggregates collected without (B) and with (C) the seeds were revealed with TEM.



**Figure 5**. Cytotoxicities of the Aβ aggregates generated with and without the DQ-induced seeds. The cell death of human dopaminergic SH-SY5Y cells was monitored with MTT assay in the presence of the Aβ aggregates obtained without (gray bar) and with (oblique bar) the DQ-induced seeds at 3% (Fig. 4B and C). The control cell survival has been shown in white bar. The cell viability for each condition was estimated with three separate experiments.

in the amyloid fibrils in shapes.

In fact, a number of different therapeutic strategies toward AD have been proposed in relation to Aβ,[23,24] which include a suppressed Aβ production, elimination of the existing amyloids from the affected brain, and blocking of the initial conformational change of Aβ to its pathological β-sheet structure and subsequent transition to oligomers, proto-fibrils, or fibrils. Several small molecules have been shown to be capable of inhibiting Aβ aggregation, including enoxaparin, nicotine, and curcumin.[20-22] In this respect, DQ examined in this study is unique since the compound has changed eventual fibrillar structures exerting varied cyto-toxicities by altering the seed structures *via* facilitated association of the initial granular aggregates. In other words, DQ would exhibit its regulatory effect on the cytotoxicity of Aβ aggregates by changing the final morphology while other compounds have been suggested to play therapeutic roles simply by inhibiting the aggregation process. There-fore, DQ has been proposed to be a useful chemical probe to control the cytotoxicity of the amyloid fibrils.

In conclusion, this investigation provides information that the seeds have acted as templates for the Aβ aggragation which could determine the final morphology of the aggre-gates and influence the aggregation kinetics by shortening of the lag period as well. In addition, the cytotoxicity of the Aβ aggregates has been also varied depending on their morphologies. It is, therefore, the seed structures that could be central to develop therapeutic strategy by inducing the amyloid fibrils in different shapes with varied cytotoxi-cities.

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