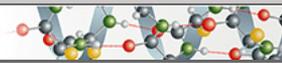


**Protein Structure and Folding:
Dequalinium-induced Protofibril
Formation of α -Synuclein**

PROTEIN STRUCTURE
AND FOLDING



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Dequalinium-induced Protofibril Formation of α -Synuclein*

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α -Synuclein is the major constituent of Lewy bodies, a pathological signature of Parkinson disease, found in the degenerating dopaminergic neurons of the substantia nigra pars compacta. Amyloidosis generating the insoluble fibrillar protein deposition has been considered to be responsible for the cell death observed in the neurodegenerative disorder. In order to develop a controlling strategy toward the amyloid formation, 1,1'-(1,10-decanediyl)-bis-[4-amino-2-methylquinolinium] (dequalinium), was selected and examined in terms of its specific molecular interaction with α -synuclein. The protein was self-oligomerized by dequalinium, which gave rise to the ladder formation on *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine/SDS-PAGE in the presence of a coupling reagent of *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline. The double-headed structure of dequalinium with the two cationic 4-aminoquinolium rings was demonstrated to be critical for the protein self-oligomerization. The dequalinium-binding site was located on the acidic C-terminal region of the protein with an approximate dissociation constant of 5.5 μ M. The protein self-oligomerization induced by the compound has resulted in the protofibril formation of α -synuclein before it has developed into amyloids. The protofibrils were demonstrated to affect the membrane intactness of liposomes, and they have also been shown to influence cell viability of human neuroblastoma cells. In addition, dequalinium treatment of the α -synuclein-overexpressing cells exerted a significant cell death. Therefore, it is pertinent to consider that dequalinium could be used as a molecular probe to assess toxic mechanisms related to the amyloid formation of α -synuclein. Ultimately, the compound could be employed to develop therapeutic and preventive strategies toward α -synucleinopathies including Parkinson disease.

α -Synuclein is the major constituent of Lewy bodies, a pathological hallmark of Parkinson disease (PD),² found within the dopaminergic cells in the substantia nigra pars compacta (1–4). Physiological function

of the protein is virtually undefined, although its involvement in synaptic plasticity has been suggested (5). α -Synuclein is the first protein shown to be genetically linked to PD. Three independent missense mutations of the gene were isolated from a few pedigrees of familial PD (6–8). Two of those resulted in substitutions of alanine at either residue 30 or residue 53 with proline and threonine (A30P and A53T), respectively (6, 7). The third mutation showed a drastic substitution from acidic glutamate at residue 46 to basic lysine residue (E46K) (8). In addition, triplication of the α -synuclein gene was observed in an American family of mixed northern European origin with autosomal dominant young onset PD (9). Experimentally, overexpression of α -synuclein in mice and flies caused behavioral deficits reminiscent of human PD with selective degeneration of dopaminergic neurons (10, 11). These facts clearly indicate that α -synuclein is a pathological component of PD. The protein is also related to other neurodegenerative disorders, collectively known α -synucleinopathies including Alzheimer disease (AD), dementia with Lewy bodies, and multiple system atrophy (12, 13).

α -Synuclein has been known to be a “natively unfolded” protein (14, 15). When incubated *in vitro*, however, the protein tends to aggregate and form the fibrils known as amyloids in which the unstructured protein has aligned to form cross- β -sheet conformation (16–18). The underlying mechanism for the amyloid formation remains to be clarified. Amyloid formation of the mutant form of A53T was facilitated as compared with the wild-type α -synuclein, whereas the other A30P was not as effective (19–21). It has been debated whether the amyloids are responsible for the cell death observed in the degenerative diseases. It was suggested that oligomeric intermediates obtained prior to the amyloid formation would be a culprit for the toxicity by possibly forming the amyloid pores on membranes (22–24). On the other hand, various forms of protein aggregates from granular to filamentous structures were hypothesized to exert different potentials for the cell death, depending on their morphologies (25–27).

α -Synuclein could be divided into three regions in its primary structure (28, 29). The N-terminal region (residues 1–60) has been demonstrated to form amphipathic α -helices upon membrane interaction (30), suggesting that the protein could be involved in synaptic plasticity by participating in membrane dynamics (31). Since all of the amino acid substitutions observed in the mutant forms are exclusively localized on this region, deficits in its membrane interaction could have pathological implications, if any (32). The hydrophobic middle segment (residues 61–95) is also known as the non-A β component of AD amyloid, since the peptide fragment has been found in the senile plaques of AD as the second major constituent next to the primary amyloid β /A4 protein

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² The abbreviations used are: PD, Parkinson disease; A β , amyloid β /A4 protein; AD, Alzheimer disease; AFM, atomic force microscope; C4-DQ, tetramethylene bis-(4-aminoquinolium iodide); C6-DQ, hexamethylene bis-(4-aminoquinolium iodide); C8-DQ, octamethylene bis-(4-aminoquinolium iodide); C10-DQ or dequalinium, 1,1'-(1,10-decanediyl)-bis-[4-amino-2-methylquinolinium]; C14-DQ, dequalinium

analogue with C14-linker; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; MITT, 3-(4,5-dimethylidiazol-2-yl)-2,5-diphenyltetrazolium bromide; TEM, transmission electron microscopy; Mes, 4-morpholineethanesulfonic acid.

Dequalinium Effects on α -Synuclein

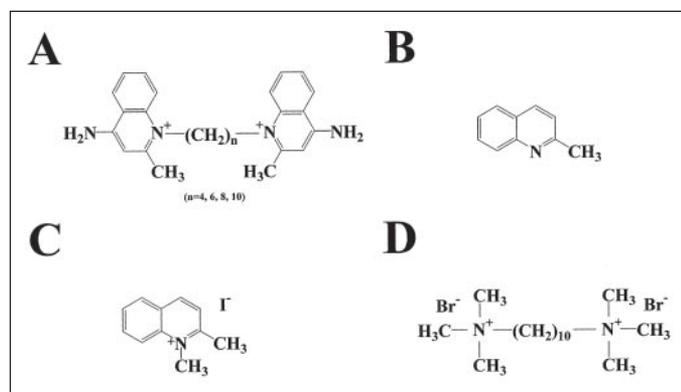


FIGURE 1. Chemical structures of dequalinium (A), quinaldine (B), quinaldinium iodide (C), and decamethonium bromide (D).

(A β) (33). The protein ends with the acidic C terminus (residues 96–140), which is most variable among synuclein isoforms including β - and γ -synucleins (5). This protein has been demonstrated to experience multiple ligand interactions. This property could be due to its “natively unfolded” structure, which is prone to be stabilized upon various ligand interactions (34). In particular, we have been interested in screening for α -synuclein interactive small chemicals that could be used to control the amyloidosis eventually. Recently, phthalocyanine tetrasulfonate, previously proposed as an antiscrapie agent (35), was shown to interact with α -synuclein via selective binding to the acidic C terminus, and it prevented the cytotoxicity on SH-SY5Y cells caused by the overexpression of α -synuclein in the presence of a proteasomal inhibitor, lactacystin (36). The copper complex of phthalocyanine tetrasulfonate-Cu²⁺, on the other hand, influenced the protein to be self-oligomerized by interacting with the N-terminal region and stimulated the amyloid formation (36). Coomassie Brilliant Blue-G and -R augmented the protein aggregation of α -synuclein and gave rise to two different shapes of the aggregates, such as worm-like and filamentous structures, respectively (26). Their interactions were independent of the acidic C terminus. Eosin was another specific dye for the α -synuclein interaction, which led to the facilitated protein self-oligomerization and fibrillization (37). Pesticides such as rotenone, paraquat, and dieldrin also enhanced the fibrillization of α -synuclein (38–40). Other chemicals including trimethylamine *N*-oxide and diethyl dithiocarbamate also induced the protein to be self-interactive (41).

1,1'-(1,10-Decanediy)bis-[4-amino-2-methylquinolinium] (dequalinium (C10-DQ)) is an amphipathic cation that contains two cationic aminoquinaldinium rings separated by a 10-carbon methylene bridge (Fig. 1) (42). Dequalinium previously used as a topical antimicrobial agent exhibited anti-tumor activity by selectively accumulating in mitochondria (43). The compound has been demonstrated to interact with several enzymes such as mitochondrial F₁-ATPase, protein kinase C, calmodulin-dependent phosphodiesterase, and calcium-activated K⁺ channel (44–48). In this report, dequalinium interaction of α -synuclein has been investigated in terms of its effects on induction of protein self-oligomerization and the stabilization of the protofibrils. Molecular details of the interaction, therefore, could provide a means to assess toxic mechanisms related to the amyloid formation of α -synuclein.

EXPERIMENTAL PROCEDURES

Materials—Dequalinium (C10-DQ), dequalinium analogue with C14-linker (C14-DQ), quinaldine, quinaldinium iodide, and decamethonium bromide were purchased from Sigma. Other dequalinium analogues with varying length of the hydrocarbon chain, such as tetrameth-

ylene bis-(4-aminoquinaldinium iodide) (C4-DQ), hexamethylene bis-(4-aminoquinaldinium iodide) (C6-DQ), and octamethylene bis-(4-aminoquinaldinium iodide) (C8-DQ), were synthesized. Commercially available diols were converted to diiodides such as 1,4-diiodobutane, 1,6-diiodohexane, and 1,8-diiodooctane. They were alkylated with a 2–3-fold excess of 4-aminoquinaldine in 4-methyl-2-pentanol. After refluxing for 156 h at 170 °C, the precipitates were washed three times with acetone. The precipitates were then dissolved in methanol and treated with charcoal. The products were then recrystallized in ethanol and subjected to ¹H NMR.

C4-DQ: ¹H NMR (Me₂SO-*d*₆, 300 MHz) δ 8.46 (d, *J* = 8.4 Hz, 1H), δ 8.27 (d, *J* = 8.7 Hz, 1H), δ 8.05 (t, *J* = 7.7 Hz, 1H), δ 7.75 (t, *J* = 7.7 Hz, 1H), δ 6.75 (s, 1H), δ 4.53 (brt, 2H), δ 2.79 (s, 3H), δ 1.98 (m, 2H).

C6-DQ: ¹H NMR (Me₂SO-*d*₆, 300 MHz) δ 8.44 (d, *J* = 7.5 Hz, 1H), δ 8.17 (d, *J* = 8.7 Hz, 1H), δ 8.01 (t, *J* = 7.4 Hz, 1H), δ 7.73 (t, *J* = 7.4 Hz, 1H), δ 6.73 (s, 1H), δ 4.48 (brt, 2H), δ 2.74 (s, 3H), δ 1.76 (m, 2H), δ 1.55 (m, 2H).

C8-DQ: ¹H NMR (CDCl₃, 300 MHz) δ 8.44 (d, *J* = 7.2 Hz, 1H), δ 8.15 (d, *J* = 9.3 Hz, 1H), δ 8.02 (t, *J* = 8.0 Hz, 1H), δ 7.73 (t, *J* = 7.5 Hz, 1H), δ 6.73 (s, 1H), δ 4.45 (t, *J* = 7.7 Hz, 2H), δ 2.50 (s, 3H), δ 1.74 (m, 2H), δ 1.46 (m, 2H), δ 1.36 (m, 2H).

Recombinant α - and β -synucleins were prepared according to the procedures previously described (49, 50). Clones of the two mutant forms of α -synuclein (A53T and A30P) were generous gifts from Dr. J. Kim (Yonsei University, Korea). The C-terminally truncated α -syn97 was obtained via an endoprotease Asp-N treatment of α -synuclein based on the previously described procedure (51). The N-terminally truncated α -syn61–140 was prepared from a clone of glutathione *S*-transferase fusion construct as described elsewhere (52). A protein assay kit employing bicinchoninic acid (BCA) was obtained from Pierce. The coupling reagent *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) and the proteasome inhibitor lactacystin were from Sigma. *S*-Sepharose and glutathione-Sepharose were purchased from Amersham Biosciences. Precast gels for 10–20% Tricine/SDS-PAGE were provided from Novex. The carbon-coated copper grid (300 mesh) and uranyl acetate were from Ted Pella Inc. and Electron Microscopy Sciences, respectively. Dulbecco's modified Eagle's medium, penicillin/streptomycin, and trypsin-EDTA were obtained from Invitrogen. Fetal bovine serum and Lipofectamine PlusTM reagent were provided by Hyclone and Invitrogen, respectively. Antibodies, such as monoclonal anti- α -synuclein antibody and horseradish peroxidase-conjugated anti-mouse IgG secondary antibody, were from Transduction Laboratories. ECL Western blotting detection reagent was from Amersham Biosciences. Materials for protein purification and reagents used for other purposes in this study were supplied from Sigma, unless otherwise mentioned.

Analysis of Self-oligomerization of α -Synuclein in the Presence of Dequalinium and Its Analogues— α -Synuclein (5 μ M) was preincubated with various concentrations of C10-DQ and C14-DQ in 20 mM Mes, pH 6.5, for 30 min at 37 °C. The protein was also incubated with dequalinium analogues such as C4-DQ, C6-DQ, and C8-DQ along with quinaldine, quinaldinium iodide, and decamethonium bromide at a fixed concentration of 1.5 mM. Dequalinium and its analogues were prepared in 50% ethanol. The dequalinium-induced protein self-oligomerization was also carried out with various α -synuclein related proteins such as β -synuclein, α -syn97, α -syn61–140, and the two mutant forms (A53T and A30P) at a concentration of 5 μ M. Following the addition of 0.3 mM EEDQ originally stocked in Me₂SO, the chemical cross-linking reactions proceeded for another 1 h at 37 °C while keeping concentration of the organic solvent less than 10% in the final mixtures (53).

The reactions were terminated with a sample buffer of Tricine/SDS-PAGE consisting of 8% SDS, 24% glycerol, 0.015% Coomassie Blue G, and 0.005% phenol red in 0.9 M Tris-Cl, pH 8.45, by mixing at an 1:1 (v/v) ratio. After boiling for 5 min, the samples were analyzed with a precast gel for 10–20% Tricine/SDS-PAGE, and the ladder formation was visualized with the silver staining procedure by Morrissey (54).

Analysis of Protein Aggregation—Protein aggregation of α -synuclein was monitored with either turbidity and/or thioflavin-T binding fluorescence (55, 56). α -Synuclein (1 mg/ml) was incubated with either various concentrations of dequalinium or its analogues (C4, C6, and C10) at a fixed concentration of 50 μ M in 20 mM Mes, pH 6.5, at room temperature under continuous shaking at 200 rpm with an orbit shaker (Red Rotor; Hoefer Scientific Inc.). Turbidity was estimated by measuring absorbance of the incubation mixture at 405 nm. Amyloid formation of α -synuclein was evaluated with thioflavin-T binding fluorescence at 485 nm with an excitation at 440 nm. During the incubation, aliquots (20 μ l) were combined with 5 μ M thioflavin-T in 50 mM glycine, pH 8.5, to a final volume of 100 μ l. The fluorescence was measured with FL500 Microplate Fluorescence Reader (Bio-Tek Instruments). The protein aggregates were visualized with a transmission electron microscope (H7100; Hitachi). Aliquots (5 μ l) of the aggregates were adsorbed onto a carbon-coated copper grid (300-mesh) and air-dried for 1 min. After negative staining with 2% uranyl acetate for another 1 min, the aggregates were observed with the electron microscope (27). For the analysis with atomic force microscope (AFM; XE-150, PSIA), an aliquot (5 μ l) was placed on freshly cleaved mica (thickness 0.3 mm). Following adsorption of the protein aggregates (1–2 min), the droplet was displaced with 100 μ l of Millipore-filtered water. After removing excess water with a filter paper, the aggregates were examined with AFM.

Cytotoxicity—Human dopaminergic neuroblastoma cells (SH-SY5Y) were grown in Dulbecco's modified Eagle's medium containing 50 units/ml penicillin and 50 μ g/ml streptomycin supplemented with 10% fetal bovine serum in 5% CO₂ at 37 °C. Cells were cultured to 80% confluence on a 60-mm culture dish, and subjected to transient transfection with a mammalian expression vector (pcDNA 3.0) containing human cDNA of α -synuclein. The vector (2 μ g) was mixed with Lipofectamine Plus™ reagent according to the manufacturer's procedure and added onto the culture dish in the presence of serum-free medium. The transfection was carried out for 3 h at 37 °C under humidified 5% CO₂ and 95% air. After a change to the medium containing 50 units/ml penicillin, 50 μ g/ml streptomycin, and 10% fetal bovine serum, the cells were further incubated for 24 h. Following trypsinization with 1 ml of trypsin-EDTA for 1 min at 37 °C, the cells were plated onto a 24-well plate (2×10^5 cells/well). Overexpression of α -synuclein inside the cells was examined with Western blotting. The cell lysates were prepared with 1% Triton X-100 in 50 mM Tris-Cl, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μ M leupeptin, 1 μ g/ml aprotinin, and 10 μ M pepstatin A. The extracts were subjected to 15% SDS-PAGE, and the gels were transferred to polyvinylidene difluoride membrane and incubated with the monoclonal antibody to α -synuclein (1:2,000). Following horseradish peroxidase-conjugated anti-mouse IgG secondary antibody treatment, the bands were visualized on x-ray film by using the ECL system (50).

The effect of dequalinium on the α -synuclein-overexpressing SH-SY5Y cells was examined. The cells were plated in a 24-well plate at 1.5×10^5 cells/well. After cell growth reached 80–90% confluence, human cDNA of α -synuclein within pcDNA3.0 and a mock plasmid were separately transfected into the cells at 0.5 μ g each in the presence

and absence of 0.5 μ M dequalinium, and incubated for 24 h at 37 °C. Cell survival was estimated with the tetrazolium salt extraction method (57) and trypan blue exclusion assay. Since living cells with active mitochondria cleave the tetrazolium ring into a visible dark blue formazan reaction product, 3-[4,5-dimethyliazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added to the culture medium at a final concentration of 1 mg/ml and incubated for 3 h at 37 °C. To measure absorbance at 570 nm, the MTT extraction buffer containing 20% SDS and 50% *N,N*-dimethylformamide, pH 4.7, was added to each sample (400 μ l into 400 μ l of medium in a 24-well plate including 80 μ l of 5 mg/ml MTT) to dissolve the formazan grains. The cell survival was monitored with absorbance at 570 nm by taking the extraction buffer containing the medium as a blank. In addition, trypan blue exclusion of the viable cell was estimated with 0.4% trypan blue by mixing the cell at a 1:1 (v/v) ratio. Stained cells were counted in hemocytometer under an inverted microscope. All of the data related to the cytotoxicity were obtained from two sets of three separate experiments.

Miscellaneous—Dissociation constant between α -synuclein and dequalinium was obtained by incubating α -synuclein at 8.7 μ M with various concentrations of dequalinium (0–25 μ M) in 20 mM Mes, pH 6.5, for 30 min at room temperature in a final reaction volume of 200 μ l. Protein-bound dequalinium was separated from the unbound form by using a centrifuge column procedure (Penefsky column) packed with Sephadex G-25 (coarse) (58). Preswollen gel packed in a 3-ml syringe was compressed via centrifugation at $600 \times g$ (HA1000-3 by Hanil Industrial Co., Incheon, Korea) for 1 min. The reaction mixture was loaded on the top of the dehydrated gel and centrifuged for an additional 1.5 min at the same speed. The amounts of protein in the collected samples were quantified with a micro-BCA assay, which gave rise to the protein recovery of 52% on average. The amount of dequalinium was estimated by measuring absorbance at 327 nm with an extinction coefficient of $\epsilon = 2.80 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. A saturation curve was drawn between the amounts of total dequalinium and its protein-bound form. A dissociation constant between α -synuclein and dequalinium was obtained from a double-reciprocal plot of the saturation curve.

Structural alterations of α -synuclein in the presence of various concentrations of dequalinium were monitored with CD spectroscopy (JASCO J-715) after 30 min of preincubation between α -synuclein (2 μ M) and dequalinium in 20 mM potassium phosphate, pH 7.5, at 20 °C. Structural transition of the protein upon liposome treatment was also observed with the CD spectroscopy in the presence and absence of 10 μ M dequalinium. The contents of secondary structures were evaluated with the program provided by the manufacturer.

Liposome was prepared with a lipid mixture of phosphatidic acid and phosphatidylcholine at a mass ratio of 1:1. A total of 5 mg of the mixture was dissolved in 1 ml of chloroform and dried under nitrogen gas with vortex to increase surface area of the resulting lipid film. After treating the film at 60 °C for 30 s, 20 mM sodium phosphate (pH 7.2) was added to 1 ml, and vortexed vigorously in the presence of glass beads. Following sonication of the sample in the absence of the beads, the resulting cloudy solution was subjected to repeating extrusion (15–17 times) through a 0.1- μ m membrane equipped in the miniextruder (Avanti Polar Lipids Inc.) at 50–60 °C. The resulting large unilamellar liposome was separated from free lipids with Sephacryl S-200 size exclusion chromatography. Morphological alterations of the liposome were examined with TEM or AFM under various experimental conditions.

All of the statistical analyses were performed with analysis of variance and Duncan's multiple range test by using the XLSTAT-Pro. A limit of the statistical significance was selected at *p* value of <0.05.

Dequalinium Effects on α -Synuclein

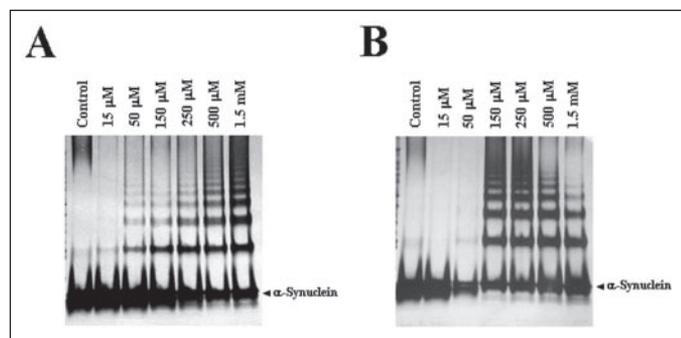


FIGURE 2. Self-oligomerization of α -synuclein in the presence of various amounts of dequalinium (C10-DQ) and C14-DQ. α -Synuclein (5 μ M) was preincubated for 30 min at 37 $^{\circ}$ C in 20 mM Mes, pH 6.5, in the presence of C10-DQ (A) and C14-DQ (B) at various concentrations as denoted at the top. Chemical cross-linking was carried out with 0.3 mM EEDQ by incubating for another 1 h. The reactions were terminated with the SDS-PAGE sample buffer and subjected to 10–20% Tricine/SDS-PAGE. Proteins on the gel were visualized via the silver staining. The monomeric α -synuclein band is also indicated with an arrowhead.

RESULTS

Self-oligomerization of α -Synuclein in the Presence of Dequalinium—Dequalinium interaction of α -synuclein was evaluated in terms of whether it induced the protein self-oligomerization in the presence of a coupling reagent of 0.3 mM EEDQ (53). As shown in Fig. 2A, the protein was self-oligomerized by dequalinium from 50 μ M at a molar ratio of 1:10 (α -synuclein/C10-DQ), which appeared in ladders on 10–20% Tricine/SDS-PAGE. The ladder formation became stronger as the dequalinium concentration was raised to 1.5 mM at a ratio of 1:300. On the other hand, the self-oligomerization induced by a dequalinium analogue with a longer carbon chain of C14-linker (C14-DQ) responded abruptly to the chemical concentration and exerted a biphasic response. The ladder formation appeared suddenly at 150 μ M C14-DQ and started to disappear from 500 μ M (Fig. 2B). This abnormal behavior could be due to possible cooperative interaction of the compound to the protein and subsequent nonspecific protein aggregation, which might suppress the ladder formation on the gel at the higher concentrations of C14-DQ. The phenomenon of ligand-induced self-oligomerization of α -synuclein has been demonstrated to be a highly selective process between the protein and various small chemicals, such as eosin, erythrosine B, Coomassie Brilliant Blue (G and R), and the copper-containing phthalocyanine tetrasulfonate in addition to A β 25–35 and the divalent copper ion (26, 36, 37, 45, 51, 53). The dequalinium interaction of α -synuclein, therefore, could be also considered as another highly selective process of molecular recognition.

In order to demonstrate the molecular selectivity, the chain length between the two quinaldinium rings in dequalinium (C10-DQ) was varied from 4 carbon units (C4-DQ) to 6 (C6-DQ) and 8 (C8-DQ) carbon units. These dequalinium analogues with different chain length were synthesized and employed to induce the protein self-oligomerization of α -synuclein. At a fixed chemical concentration of 1.5 mM, the ladder formation was revealed from C8-DQ as the chain extended to C10-DQ (Fig. 3A). Since quinaldine, quinaldinium iodide, and decamethonium bromide (Fig. 1) did not cause the self-oligomerization (Fig. 3B), the double-headed structure with the two cationic quinaldinium rings optimally separated by a hydrocarbon chain with appropriate chain length turned out to be a prerequisite for the selective α -synuclein interaction leading to the protein self-oligomerization.

Binding between Dequalinium and α -Synuclein—The specific interaction site of dequalinium on α -synuclein responsible for the protein self-oligomerization was pursued by employing various isoforms of α -synuclein, including β -synuclein, C-terminally truncated α -syn97,

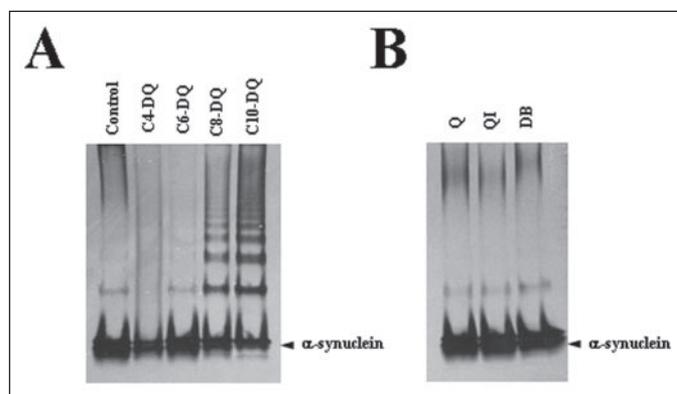


FIGURE 3. Self-oligomerization of α -synuclein in the presence of dequalinium and its analogues. The self-oligomerization of α -synuclein (5 μ M) was examined with the dequalinium analogues, such as C4-DQ, C6-DQ, C8-DQ, and C10-DQ, at a fixed concentration of 1.5 mM (A). The oligomerization was also observed with quinaldine (Q), quinaldinium iodide (QI), and decamethonium bromide (DB) at the same concentration of 1.5 mM (B). Following the chemical cross-linking with 0.3 mM EEDQ, the protein products were analyzed with 10–20% Tricine/SDS-PAGE and silver staining.

N-terminally truncated α -syn61–140, and the two mutant forms of A53T and A30P. The ladder formation was observed with all of the isoforms except the C-terminally truncated α -syn97 (Fig. 4), indicating that the dequalinium binding site should be localized on the acidic C-terminal region. Ionic interaction between the negatively charged acidic region and the cationic quinaldinium rings might be one of the critical factors for the selective interaction. Since β -synuclein, which contains the similar acidic C terminus as α -synuclein with a distinctive amino acid composition, still gave rise to the same self-oligomerization in the presence of dequalinium, a pivotal role of the charged interaction was also appreciated (Fig. 4).

In order to evaluate molecular affinity, α -synuclein was incubated with various concentrations of dequalinium, and the protein-bound compound was separated from its free form by employing the centrifuge column packed with Sephadex G-25. By plotting a saturation curve and its corresponding double-reciprocal plot (Fig. 5), an approximate dissociation constant between α -synuclein and dequalinium was obtained as 5.5 μ M, indicating that the molecular interaction has exerted a considerable affinity. This binding of dequalinium caused a prominent effect on the structure of α -synuclein as shown in Fig. 6. Structural alteration of α -synuclein upon the dequalinium treatment was examined with CD spectroscopy. The minimum ellipticity at 197 nm increased as dequalinium concentration was raised to 20 μ M in the presence of 2 μ M α -synuclein (Fig. 6). Dequalinium itself, however, was not optically active. These data indicated that the protein appeared to experience a significant structural transition by reducing the content of random coil with a tendency to increase β -sheet structure.

Protein Aggregation of α -Synuclein in the Presence of Dequalinium—Protein aggregation of α -synuclein was monitored with turbidity by observing absorbance at 405 nm as well as thioflavin-T binding fluorescence at 485 nm with excitation at 440 nm. As dequalinium concentrations increased (1.4, 14, and 140 μ M), the final turbidities were also increased from a control with prominent shortening of the lag phases on the aggregation kinetics of α -synuclein (Fig. 7A). Intriguingly, however, the final amyloid formation evaluated with the thioflavin-T binding fluorescence was decreased instead as the dequalinium concentrations were increased, although the lag phases were consistently reduced in the presence of the compound (Fig. 7B). The increased fluorescence, especially during the initial period of the aggregation in the presence of dequalinium, was found to be due to the granular protein aggregates of α -synuclein that have been

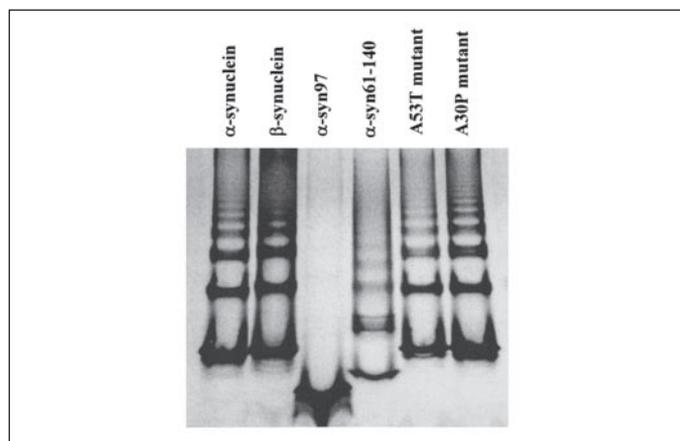


FIGURE 4. **Dequalinium-induced protein self-oligomerization of various α -synuclein-related proteins.** α -Synuclein-related proteins ($5 \mu\text{M}$ each) such as β -synuclein, α -syn97, α -syn61–140, and the two mutant forms (A53T and A30P) were subjected to chemical cross-linking with 0.3 mM EEDQ in the presence of 1.5 mM dequalinium. The self-oligomers were analyzed with 10–20% Tricine/SDS-PAGE and visualized with silver staining.

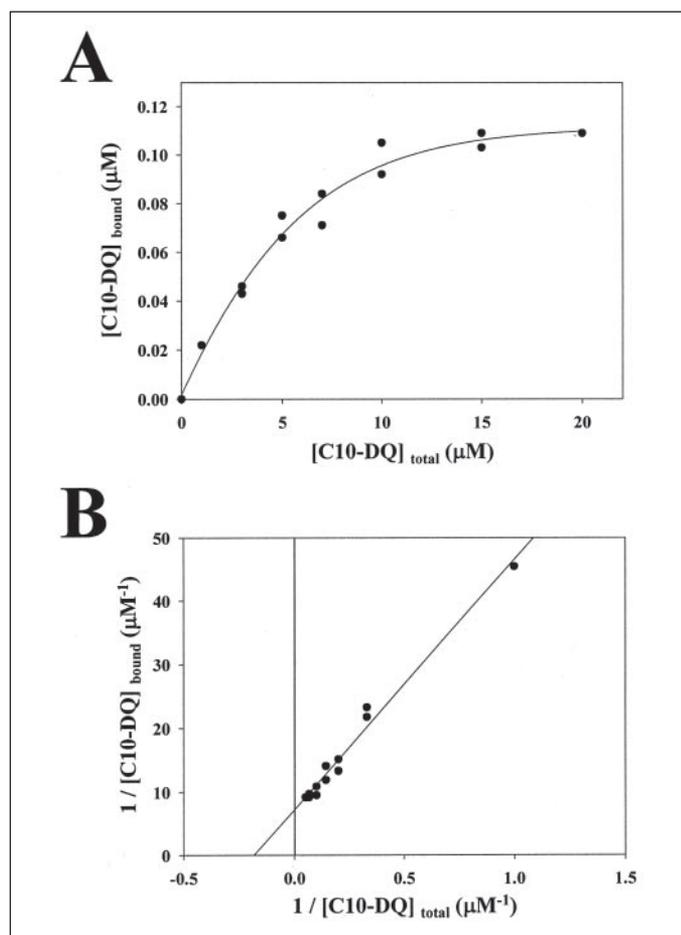


FIGURE 5. **Saturation curve of α -synuclein by dequalinium (A) and its corresponding double-reciprocal plot (B).** α -Synuclein ($8.7 \mu\text{M}$) was incubated with various concentrations of dequalinium (0 – $25 \mu\text{M}$) in 20 mM Mes, pH 6.5, for 30 min at room temperature. Protein-bound dequalinium was separated from the unbound form by using the centrifuge column packed with Sephadex G-25 (coarse). The dehydration of the gel and the collection of the dequalinium-bound protein were achieved with a low speed centrifugation ($600 \times g$) for 1 and 1.5 min, respectively. The protein-bound dequalinium was quantified by observing absorbance at 327 nm with $\epsilon = 2.80 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. A saturation curve was drawn between total amounts of dequalinium initially treated and amounts of the protein-bound forms (A). The double-reciprocal plot was redrawn from the saturation curve (B).

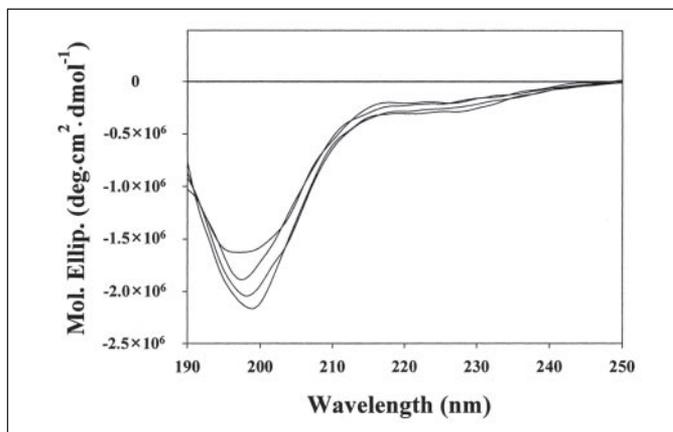


FIGURE 6. **CD spectra of α -synuclein in the presence of dequalinium.** α -Synuclein ($2 \mu\text{M}$) was examined in the absence and presence of dequalinium at various molar ratios, such as 1:1, 1:5, and 1:10 in 20 mM potassium phosphate, pH 7.5, with CD spectroscopy.

observed often within the protofibril fraction (data not shown). Although thioflavin-T binding fluorescence has been employed to monitor amyloid formation in general, the amyloids are not the exclusive materials for the dye to be bound. In fact, the thioflavin-T binding fluorescence was routinely observed even with only the granular forms of α -synuclein aggregates (27). These observations indicated that dequalinium could facilitate the protein aggregation by inducing the granular aggregates, whereas the final amyloid formation was somewhat suppressed. The results showing enhanced protein aggregation with reduced amyloid formation were consistently observed when other dequalinium analogues were employed to induce the protein aggregation. As the length of hydrocarbon chain was varied among C4, C6, and C10 at a fixed concentration of $50 \mu\text{M}$, the final turbidity was increased with apparent reduction in the lag period (Fig. 7C), whereas the final thioflavin-T binding fluorescence obtained in the presence of the analogues was lower than that of the aggregates prepared with α -synuclein alone (Fig. 7D). These data suggest that dequalinium could induce the oligomeric intermediates of α -synuclein via the facilitated protein self-oligomerization, whereas their subsequent progress into the final amyloids has been somewhat prevented.

Induction of the Protofibrils of α -Synuclein by Dequalinium—The oligomer-inducing property of dequalinium was investigated by examining the protein aggregates of α -synuclein collected at a stationary phase of the aggregation kinetics following a sufficient period of incubation. In the absence of dequalinium, only the fibrillar protein aggregates were observed (Fig. 8A). The granular forms that appeared as “white circles” with an average diameter of about 40 nm , however, were present in the aggregates as dequalinium was included in the incubation mixtures at three different concentrations of 1.4 , 14 , and $140 \mu\text{M}$ (Fig. 8, B–D). These “white circles” have been frequently observed during various protein aggregation studies with α -synuclein in the presence of various chemical ligands (27, 36). The protein aggregates obtained in the absence and presence of the compound were also observed with AFM. Whereas α -synuclein itself produced the fibrillar aggregates as an exclusive product (Fig. 8E), dequalinium ($50 \mu\text{M}$) also gave rise to the small granules ($\sim 40 \text{ nm}$ diameter) in addition to the incomplete fibrillar structures (Fig. 8F). By measuring various granular structures revealed under either TEM or AFM, their average diameters were estimated as 42 ± 6 and $39 \pm 9 \text{ nm}$, respectively. Protofibrils, on the other hand, were defined as the node-containing chainlike structures with an average width of $43 \pm 4 \text{ nm}$ and a length shorter than 500 nm . The protofibrils

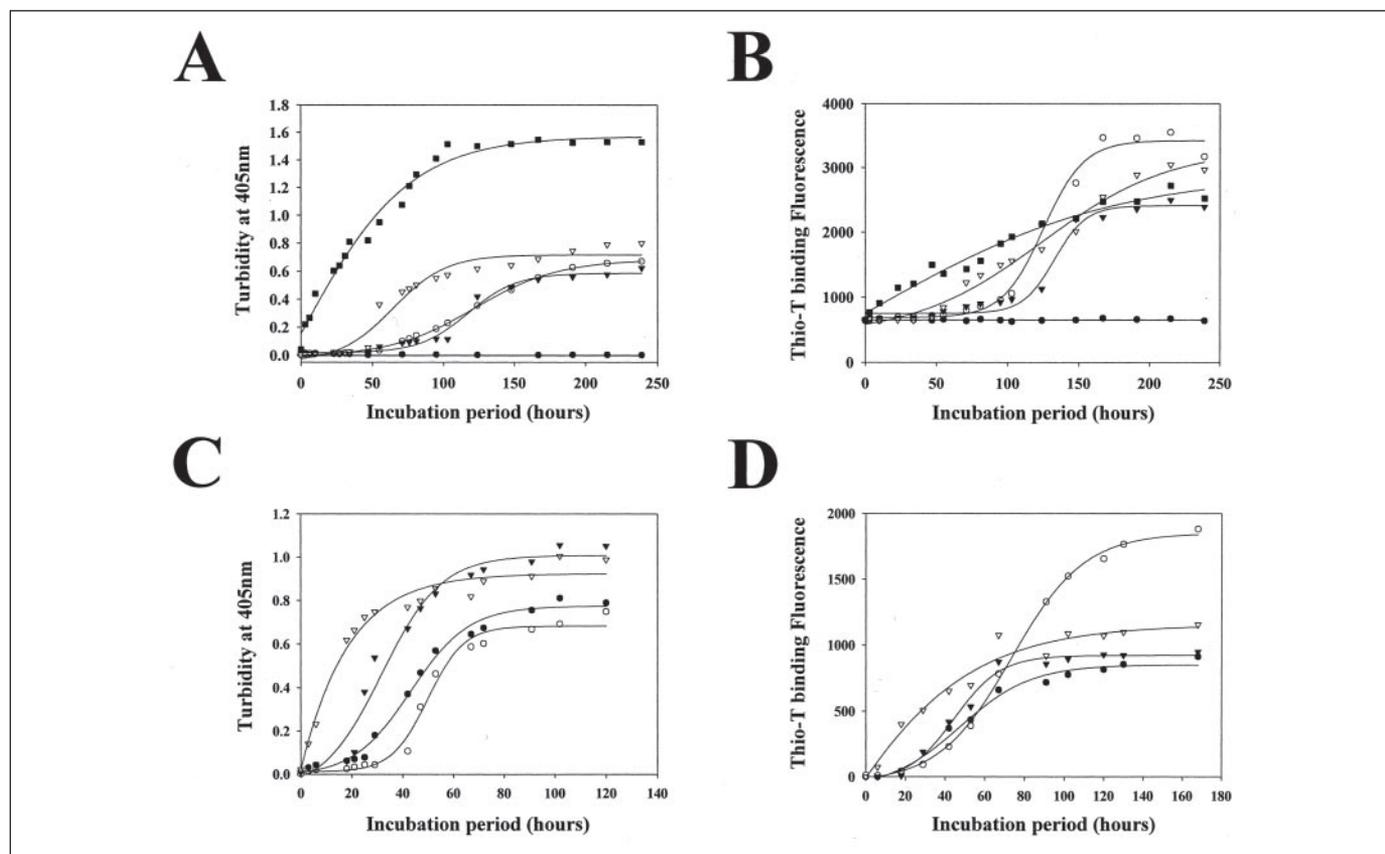


FIGURE 7. **Aggregation kinetics of α -synuclein with various concentrations of dequalinium (A and B) and various dequalinium analogues (C and D).** α -Synuclein (1 mg/ml) was incubated in the absence (○) and presence of dequalinium at various concentrations, such as 1.4 μ M (●), 14 μ M (▼), and 140 μ M (■) in 20 mM Mes, pH 6.5. Protein aggregation was monitored with turbidity by observing absorbance at 405 nm during the incubation (A). Amyloid formation was also evaluated with thioflavin-T binding fluorescence (see "Experimental Procedures") (B). ●, compound incubated at 140 μ M in the absence of α -synuclein. The protein (1 mg/ml) was also incubated in the absence (○) and presence of the dequalinium analogues such as C4-DQ (●), C6-DQ (▼), and C10-DQ (▽) at a fixed concentration of 50 μ M in 20 mM Mes, pH 6.5. The aggregation kinetics was followed by measuring turbidity (C) and thioflavin-T binding fluorescence (D).

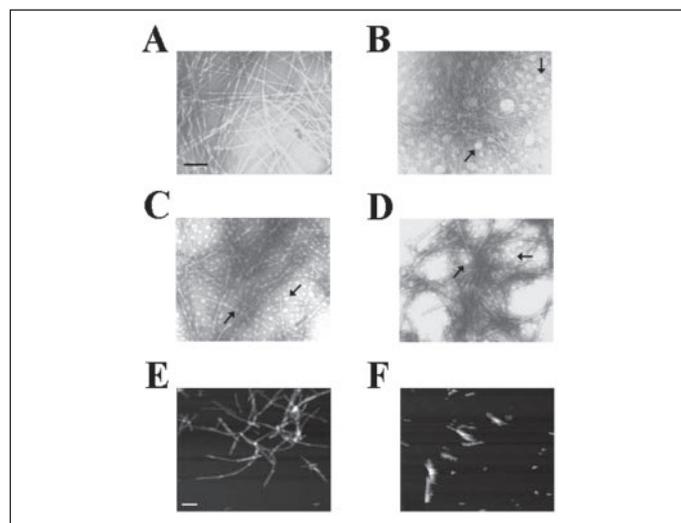


FIGURE 8. **Morphologies of the final protein aggregates of α -synuclein prepared with dequalinium.** Following sufficient incubation for more than 240 h, the protein aggregates of α -synuclein obtained in the absence (A) and presence of dequalinium at 1.4 μ M (B), 14 μ M (C), and 140 μ M (D) were examined with a transmission electron microscope after negative staining with 2% uranyl acetate on a carbon-coated copper grid (200-mesh). The scale bar (A) represents 200 nm for all of the TEM images. The granular protein aggregates are indicated with arrows (B–D). Another set of protein aggregates of α -synuclein was prepared in the absence (E) and presence (F) of 50 μ M dequalinium for the analysis of atomic force microscope. Aliquots (5 μ l) were placed on freshly cleaved mica (mica plate, 0.3 mm). Following adsorption, the remaining droplet was displaced with distilled water. AFM images (scale bar of 200 nm) were obtained with XE-150, PSIA.

appeared to result from lateral associations of the granular structures. Intriguingly, an average width of amyloid fibrils was reduced to 35 ± 11 nm from that of the protofibrils. In addition, the node structures in the protofibrils were smoothed in the amyloids. Unfortunately, the morphological relationship between the protofibrils and the amyloids could not be evaluated until currently unknown association mechanisms from the protofibrils to the amyloids have been identified. Since the granular aggregates were observed along with the protofibrils in the presence of dequalinium (Fig. 8F), they could be considered as oligomeric intermediates that might develop into amyloids via the protofibril formation (23, 24). Dequalinium, therefore, could be considered as an inducer and/or stabilizer of the oligomeric intermediates by partly preventing subsequent fibrillization of α -synuclein.

The dequalinium-mediated oligomeric intermediate formation of α -synuclein was examined with size exclusion chromatography of Sephacryl S-200 (Fig. 9). The protein aggregation of α -synuclein (2 mg/ml) was performed in the absence and presence (5 and 50 μ M) of dequalinium (Fig. 9, inset). Prior to the incubation, the protein solutions were filtered through a 0.22- μ m syringe filter to remove any high molecular weight species. It was confirmed that these protein samples did not contain any materials eluted at the void volume of the size exclusion chromatography. Following 24 h of further incubation after the kinetics reached their stationary phases, as indicated with arrows in the inset (Fig. 9), the aggregates were separately subjected to the gel filtration chromatography. The monomeric α -synuclein was hardly

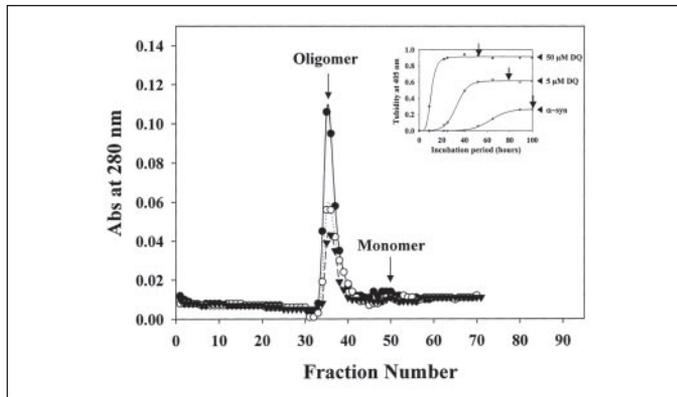


FIGURE 9. Oligomer formation of α -synuclein in the presence of dequalinium. The protein aggregates of α -synuclein (2 mg/ml) prepared in the absence (\blacktriangledown) and presence of dequalinium at 5 μ M (\circ) and 50 μ M (\bullet) were collected during the stationary phases of the aggregation kinetics at the points indicated with arrows (inset). The mixtures were subjected to gel filtration chromatography with Sephacryl S-200 in 20 mM Mes, pH 6.5. Protein elution was monitored with absorbance at 280 nm. The positions where oligomers and monomers have been eluted are also indicated.

observed from the three samples. The aggregates prepared with α -synuclein alone exhibited the shortest peak at the void volume where the oligomeric forms were expected to be eluted. Intriguingly, the oligomer-containing peak was increased as dequalinium level was raised. In the presence of 50 μ M dequalinium, the highest peak due to the oligomeric protein aggregates was obtained at the void volume, although the condition caused most rapid protein aggregation with the highest final turbidity (Fig. 9). This finding data clearly indicated that dequalinium favored the oligomer formation and also prevented further development into the amyloids.

The oligomers of α -synuclein obtained with dequalinium were collected from the size exclusion chromatography and examined under AFM. The image contained the aggregates of not only granular structures but also protofibrils in which small round-shaped granules were serially aligned to form a chainlike structure (Fig. 10, A and B). Since the protofibril fraction was suggested to contain the amyloid pores, which could affect membranes, liposomes were prepared and treated by the protofibrils in order to check their influence on membrane intactness. Generally, the liposomes treated with the protofibrils experienced drastic shrinkage in size when compared with a control (Fig. 10, C and D). On average, diameters of liposomes were reduced by 36% from 81.1 to 51.6 nm in the absence and presence of the protofibrils, respectively. In addition, some of the boundaries of protofibril-treated liposomes were disrupted to result in diffused structures, as indicated with arrows (Fig. 10D). The membrane-disrupting effect of the protofibrils was evaluated with live cells (SH-SY5Y) as well by observing the penetration of trypan blue into the cells. The protofibril-treated cells were stained by the dye to the higher proportion when compared with the control cells treated with monomeric α -synuclein (Fig. 10, E and F). The data indicated that those protofibrils could affect cell viability by possibly influencing the membrane intactness.

Dequalinium-induced Cytotoxicity of the α -Synuclein-overexpressing Cells—Cell death of the SH-SY5Y cells overexpressing α -synuclein was examined in the presence of dequalinium to show that the membrane-disrupting toxic activity of the oligomeric intermediates could be reflected within the cell. The cells were transfected with pcDNA containing the human α -synuclein gene in the presence and absence of dequalinium and incubated for 24 h at 37 $^{\circ}$ C within a humidified CO₂ (5%) incubator. The expression of the protein was confirmed with Western blot. Cell survival was monitored with both MTT and trypan blue exclusion assays. Compared with the control in which a mock

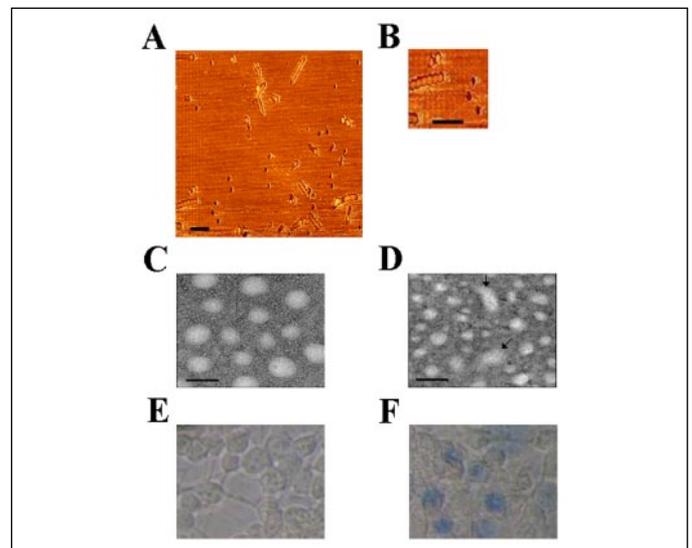


FIGURE 10. Protofibrils of α -synuclein induced by dequalinium and their effects on liposomes and neuroblastoma cells. The dequalinium-induced oligomeric forms of α -synuclein were collected from the void volume of the gel filtration chromatography packed with Sephacryl S-200 (Fig. 9). The oligomeric protein aggregates of α -synuclein were observed with AFM (A). Protofibrils in the oligomeric protein aggregates were magnified (B). Scale bar (A and B), 200 nm. Liposomes were treated without (C) and with (D) the protofibrils, subjected to a negative staining with 2% uranyl acetate, and examined under TEM. Scale bar, 200 nm. The trypan blue staining of neuroblastoma cells (SH-SY5Y) previously treated without (E) and with (F) the protofibrils was examined under an inverted light microscope. Trypan blue (0.4%) prepared in the phosphate-buffered saline was used to stain the permeabilized cells.

plasmid was transfected, the α -synuclein overexpression slightly affected the cell viability by 11% based on the results of the MTT assay (Fig. 11). In the presence of 0.5 μ M dequalinium, however, the α -synuclein-overexpressing cells were demonstrated to experience a significant cell death, exerting 54% of the cell viability from 77% of the control. The cell death assessed with the trypan blue exclusion also showed that dequalinium caused another significant cell death of the α -synuclein-overexpressing cells by 26% (Fig. 11), which showed a good correlation with the death evaluated by the MTT assay. Taken together, the data suggested that the overexpressed α -synuclein could exhibit its toxic effect in the presence of dequalinium by presumably forming the protofibrils and affecting membrane stability, although the possibilities that the reagent could affect intracellular ATP generation and phosphorylation level by directly inhibiting F₀F₁-ATPase or protein kinase C, respectively, should not be completely excluded (45, 46, 60).

Since both dequalinium and α -synuclein have been considered to influence membranes via a common amphipathic nature, their individual and mutual effects on the membranes were investigated with AFM and CD spectroscopy. During 12 h of incubation with dequalinium at 10 μ M, the liposomes appeared significantly distorted on their morphologies examined under AFM (Fig. 12, A and B). Coexistence with α -synuclein (1 μ M), however, restored the liposomes to their original shapes (Fig. 12, A and C). This observation indicated that dequalinium interaction with the lipid membranes could be prevented by α -synuclein via selective interaction between the molecules. In addition, membrane interaction of α -synuclein was also demonstrated to be modulated by the compound. The random structured protein was confirmed to show a significant structural transition to increase α -helical content upon the liposome interaction (Fig. 12D). Intriguingly, the altered structure of α -synuclein in the presence of the membranes was shown to experience a dramatic structural transition upon the dequalinium treatment to the original spectrum, with an increased minimum ellipticity around 197 nm (Fig. 12D), indicating that the membrane-

Dequalinium Effects on α -Synuclein

bound α -synuclein could be removed and subsequently influenced by the compound. These morphological and structural analyses indicated that the individual potentials of α -synuclein and dequalinium to interact with lipid membranes were abolished via their specific intermolecular interactions, which could lead to the eventual protofibril formation. Since the protofibrils could be formed inside the cells overexpressing α -synuclein in the presence of dequalinium and they have been shown to exert cytotoxicity *in vitro*, the compound could be used as a molecular probe to assess toxic mechanism of α -synuclein during the amyloidosis.

DISCUSSION

α -Synuclein has experienced the protein self-oligomerization in the presence of dequalinium. The double-headed structure of the compound with two cationic 4-aminoquinolium rings separated by a hydrophobic hydrocarbon chain of 10 carbon units appeared to be critical for the self-oligomerization. Its interaction to α -synuclein was local-

ized on the acidic C-terminal region. It is not clear, however, whether the two cationic quinolium rings could occupy two separate sites on one protein or bridge between two proteins. Its mode of interaction could depend on the chain length as well. The chain, however, cannot be extended too long, because additional hydrophobicity would cause rather abnormal protein self-interaction as observed with C14-DQ. Alternatively, a certain solution structure of dequalinium prior to its protein engagement could influence the molecular interaction with α -synuclein (42).

α -Synuclein has been suspected to have certain local structures to accommodate the structural characteristics of dequalinium, although the protein has been known to exist in a "natively unfolded" state (14, 15). This suggestion has been supported by various studies employing small chemicals responsible for specific α -synuclein interactions (26, 36, 37, 51, 53, 62). Although structural plasticity of this unstructured protein would play a key role to interact with various small ligands, the protein might not exist in a fully extended completely denatured state. The CD spectrum of α -synuclein showing predominant random coil structure does not necessarily mean that the protein would not form any local structures distinctive from either α -helix or β -pleated sheet. Recently, it was demonstrated that α -synuclein could exist in a stable compact structure in addition to the elongated state, depending on pH (63, 64). When the protein recognizes its specific ligand, however, the protein would exhibit structural plasticity to accommodate the ligand. It is this altered structure of α -synuclein that could develop into the final protein aggregates in the presence of the specific ligand. As a result, the final protein aggregates of the single so-called "natively unstructured" protein could end up with various forms of amyloids depending on ligands treated (26, 27, 36). Our data indicated that α -synuclein has experienced structural transition upon the dequalinium treatment, which is necessary for the intermolecular interaction leading to the protein aggregation.

It has been controversial whether intracellular overexpression of α -synuclein actually causes the cell death under a cultured condition (65–68). Although it might depend on the cell types studied, our transient α -synuclein overexpression did not affect the SH-SY5Y cells. It has required an additional executor, such as lactacystin as a proteasome inhibitor, in order to exert cell death (36). In this study, dequalinium has

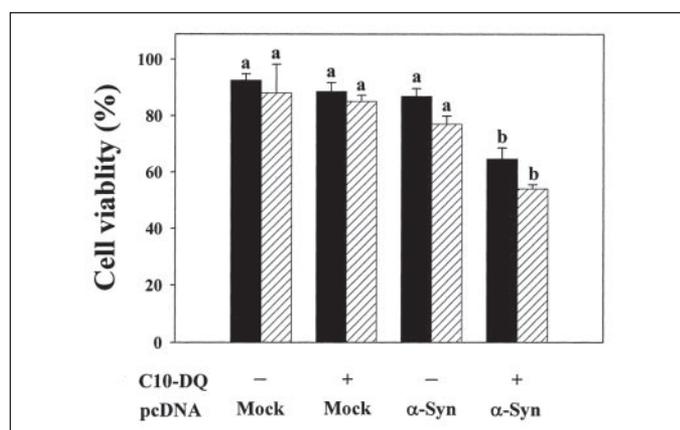
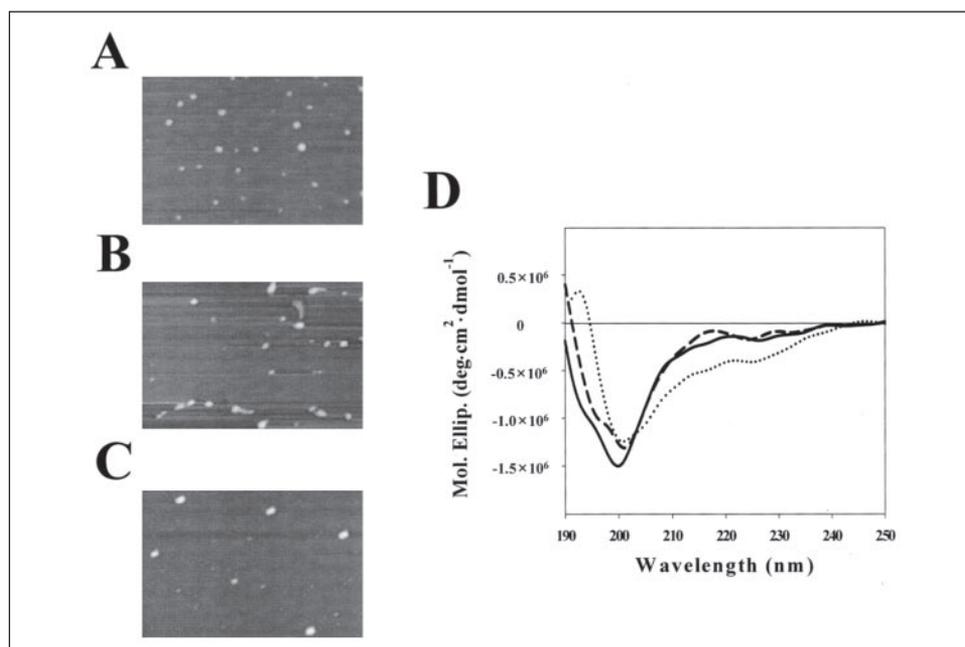


FIGURE 11. Dequalinium-induced cytotoxicity of the α -synuclein overexpressing SH-SY5Y cells. The cells were transfected with pcDNA containing either nothing or the human α -synuclein gene in the presence and absence of $0.5 \mu\text{M}$ dequalinium and incubated for 24 h. The cytotoxicities of the compound to the cells were assessed with both MTT (black bar) and trypan blue exclusion assays (oblique bar) (see "Experimental Procedures"). The cell viabilities of each condition were estimated with two sets of three separate experiments. Values are the mean \pm S.E., and the means labeled with the same superscripts are not significantly different at $p < 0.05$ by Duncan's multiple range test.

FIGURE 12. Liposome interactions of dequalinium and α -synuclein assessed with AFM (A–C) and CD spectroscopy (D). Liposomes were incubated in the absence (A) and presence (B and C) of $10 \mu\text{M}$ dequalinium without (B) and with (C) α -synuclein ($1 \mu\text{M}$) in 20 mM sodium phosphate, pH 7.4, during 12 h of continuous shaking at room temperature. Then the liposomes were examined under AFM. For the structural analyses of α -synuclein (D), the protein ($10 \mu\text{M}$) was subjected to CD spectroscopy in the absence (solid line) and presence (dashed and dotted lines) of liposomes with (dashed line) and without (dotted line) $100 \mu\text{M}$ dequalinium in 20 mM sodium phosphate, pH 7.4.



been shown to cause the cell death of the α -synuclein-overexpressing SH-SY5Y cells via presumably direct interaction with the protein in the absence of the additional executor. However, since the compound itself could also affect the cell survival by influencing various critical enzymes, such as F_0F_1 -ATPase and protein kinase C or direct accumulation inside mitochondria responsible for the elevated level of reactive oxygen species, the dequalinium effect on the cytotoxicity could not be overestimated solely on the basis of the protofibril formation of α -synuclein inside the cell. It awaits more direct evidence for the dequalinium interaction of α -synuclein inside the cell, leading to the intracellular protein aggregation.

Based on this study, dequalinium has been proposed to act as an inducer and/or stabilizer of the protofibrils of α -synuclein. Induction of the stable α -synuclein oligomers has been also reported in other studies. Methionine oxidation and nitration of α -synuclein were shown to inhibit the fibrillization of α -synuclein by forming soluble oligomers (69, 70). Concerning the pathological significance of the soluble oligomers as an intermediate during amyloidosis, recently the soluble A β oligomers have acquired more attention than the A β deposits as a pathological component responsible for the neuronal cell death and subsequent memory loss observed in early AD (71). However, a possibility that dequalinium could disaggregate preformed fibrillar protein aggregates into protofibrils cannot be completely excluded, as demonstrated with rifampicin and baicalein (72, 73). Nonetheless, the protofibril-based toxic mechanisms could be evaluated with the dequalinium-induced protein aggregation. Membrane instability of the plasma membrane and other intracellular organelles could be a part of the toxic causes observed with the protofibrils, as shown in this study. Alternatively, membrane itself could influence amyloidogenic proteins to be more pathogenic (74). The protofibrils could be formed inside the cells overexpressing α -synuclein in the presence of dequalinium via their mutual intermolecular interaction, which could suppress their individual potentials to interact with membranes. Taken together, it is pertinent to consider that dequalinium could be useful as a chemical probe to understand molecular mechanisms of the cell death caused by the protofibrils and/or amyloids of α -synuclein. In addition, the data provided in this study would put emphasis on the protofibrils, not the final amyloids, of amyloidogenic proteins as a real culprit for the neuronal cell death observed in various neurodegenerative disorders (59, 61).

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