Phthalocyanine Tetrasulfonates Affect the Amyloid Formation and Cytotoxicity of α-Synuclein†

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ABSTRACT: α-Synuclein is a pathological component of Parkinson's disease by constituting the filamentous component of Lewy bodies. Phthalocyanine (Pc) effects on the amyloidosis of α-synuclein have been examined. The copper complex of phthalocyanine tetrasulfonate (PcTS−Cu^{2+}) caused the self-oligomerization of α-synuclein while Pc−Cu^{2+} did not affect the protein, indicating that introduction of the sulfonate groups was critical for the selective protein interaction. The PcTS−Cu^{2+} interaction with α-synuclein has occurred predominantly at the N-terminal region of the protein with a K_d of 0.83 μM apart from the hydrophobic NAC (non-Aβ component of Alzheimer's disease amyloid) segment. Phthalocyanine tetrasulfonate (PcTS) lacking the intercalated copper ion also showed a considerable affinity toward α-synuclein with a K_d of 3.12 μM, and its binding site, on the other hand, was located at the acidic C-terminus. These mutually exclusive interactions between PcTS and PcTS−Cu^{2+} toward α-synuclein resulted in distinctive features on the kinetics of protein aggregation, morphologies of the final aggregates, and their in vitro cytotoxicities. The PcTS actually suppressed the fibrous amyloid formation of α-synuclein, but it produced the chopped-wood-looking protein aggregates. The aggregates showed rather low toxicity (9.5%) on human neuroblastoma cells (SH-SY5Y). In fact, the PcTS was shown to effectively rescue the cell death of α-synuclein overexpressing cells caused by the lactacystin treatment as a proteasome inhibitor. The anti-aggregative and anti-amyloidogenic properties of PcTS were also demonstrated with alcohol dehydrogenase, glutathione *S*-transferase, and amyloid β/A4 protein under their aggregative conditions. The PcTS−Cu^{2+}, on the other hand, promoted the protein aggregation of α-synuclein, which gave rise to the fibrillar protein aggregates whose cytotoxicity became significant to 35.8%. Taken together, the data provided in this study indicate that PcTS/PcTS−Cu^{2+} could be considered as possible candidates for the development of therapeutic or prophylactic strategies against the α-synuclein-related neurodegenerative disorders.

Parkinson's disease (PD)[1] exhibiting resting tremor, bradykinesia (slowness of initial movement), rigidity, and postural instability has been pathologically characterized by selective loss of dopaminergic neurons in the substantia nigra pars compacta and the presence of cytoplasmic inclusions called Lewy bodies (*1*, *2*). α-Synuclein is the major constituent of the Lewy bodies along with ubiquitin and neurofilaments (*3*, *4*). α-Synuclein is genetically linked to PD. Two independent missense mutations of its gene were isolated from a few pedigrees of familial PD, which resulted in substitutions of alanine at either residue 30 or residue 53 with proline and threonine (Ala30Pro and Ala53Thr), respectively (*5*, *6*). Overexpression of the protein in mouse or fly led to the behavioral deficits reminiscent of human PD with selective degeneration of dopaminergic neurons, although the fibrous protein aggregates were observed only in *Drosophila melanogaster* (*7*, *8*). These facts clearly indicate that α-synuclein is a pathological component of the disease.

Screening for α-synuclein interactive small molecules, therefore, should provide us a means to control the neurodegenerative disorder by influencing the Lewy body formation, even though it has been debated whether the amyloid deposit is responsible for the actual cell death (*9*, *10*). The amyloid deposit has been suggested to be a simple detoxification end product of toxic oligomeric intermediates which would form amyloid pores on membranes (*11*, *12*). Various α-synuclein interactive small chemicals have been reported, which affected the amyloid formation in vitro. Coomassie

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[1] Abbreviations: Aβ, amyloid β/A4 protein; AD, Alzheimer's disease; ADH, alcohol dehydrogenase; BCA, bicinchoninic acid; CBB, Coomassie brilliant blue; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; GST, glutathione *S*-transferase; HRP, horseradish peroxidase; MTT, 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, non-Aβ component of AD amyloid; PAGE, polyacrylamide gel electrophoresis; Pc, phthalocyanine; PcTS, phthalocyanine tetrasulfonate; PD, Parkinson's disease; PrP-res, protease-resistant prion protein; PrP-sen, protease-sensitive prion protein; SDS, sodium dodecyl sulfate.

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brilliant blue- (CBB-) G and R facilitated the protein aggregation of α-synuclein, which resulted in aggregates with different morphologies of wormlike and filamentous structures, respectively (*13*). α-Synuclein could be divided into three regions in its primary structure (*14*, *15*): the N-terminal region (residues 1−60) which has been shown to form amphipathic α-helices upon membrane interaction (*16*) is followed by a hydrophobic stretch (residues 61−95) also known as NAC [non-Aβ component of Alzheimer's disease (AD) amyloid] because the segment was found in the senile plaque of AD as the second major constituent (*17*). The protein ends with the acidic C-terminus (residues 96−140) which is most variable among synuclein isoforms including β- and γ-synucleins (*18*). The CBB-G and -R binding to α-synuclein was independent of the acidic C-terminus. Eosin was another dye which exhibited specific interaction with α-synuclein among various dyes, including Congo red, thioflavin-S and -T, Rhodamine 6G, and Phenol red (*19*). The eosin was shown to interact with the hydrophobic NAC region as well as the acidic C-terminus, which resulted in protein self-oligomerization and eventual fibrous protein aggregation (*19*). Pesticides such as rotenone, paraquat, and dieldrin were demonstrated to accelerate the fibrillization of α-synuclein although the rotenone was suggested to cause the PD-like status by affecting mitochondrial complex I (*20*−*22*). In addition, other chemicals such as trimethylamine *N*-oxide and diethyl dithiocarbamate were also reported to influence the self-interaction of α-synuclein (*24*, *25*).

In this report, phthalocyanine (Pc)/phthalocyanine tetrasulfonate (PcTS) interactions of α-synuclein have been investigated in terms of their effects on the protein self-oligomerization and aggregation. In addition, cytotoxicities of the chemical-induced protein aggregates and the chemical effects on transiently overexpressed α-synuclein within human neuroblastoma cells (SH-SY5Y) were also examined. The PcTS, a cyclic tetrapyrrole compound, has been shown to exhibit antiscrapie activity by presumably suppressing the formation of abnormal protease-resistant prion protein (PrP-res) from the normal protease-sensitive form (PrP-sen) (*25*). Direct interaction between PcTS and PrP-res was suggested to be responsible for its regulatory role in the transmissible spongiform encephalopathy (*26*). In fact, tetrapyrroles have been suggested to contain prophylactic or therapeutic potential for noninfectious diseases of protein folding and aggregation, such as PD and AD. They are highly selective in protein interaction and available in various chemical structures (*27*−*32*). They are sufficiently lipophilic, which is a prerequisite for crossing the blood−brain barrier, and exhibit low toxicities for medical applications (*25*, *33*−*35*). In this study, therefore, molecular details on the interaction between α-synuclein and Pc/PcTS have been carefully examined to develop eventual preventive or therapeutic strategies for α-synuclein-related neurodegenerative disorders, including PD, AD, dementia with Lewy bodies (DLB), and multiple system atrophy.

MATERIALS AND METHODS

Materials. Recombinant α- and β-synucleins were prepared according to the previously described procedures (*36*, *37*). Clones of the two mutant forms of α-synuclein (Ala53Thr and Ala30Pro) were generous gifts from Dr. J. Kim (Yonsei University, Korea). N-Terminally truncated α-syn61−140 was prepared from a clone of the glutathione *S*-transferase fusion construct (kindly provided by Dr. H. Lim at Catholic University, Korea) as described elsewhere (*38*). The C-terminal truncation of α-synuclein to obtain α-syn97 was carried out with an endoproteinase Asp-N treatment, and the modified protein was purified according to the procedure previously described (*39*). The protein assay kit employing bicinchoninic acid (BCA) was obtained from Pierce. PcTS was from ICN Biomedical Inc. Pc, Pc−Cu^{2+}, Pc−Fe^{2+}, Pc−Zn^{2+}, Pc−Mn^{2+}, PcTS−Cu^{2+}, and PcTS−Ni^{2+} were from Fluka. PcTS−Al^{3+}, PcTS−Fe^{3+}, and PcTS−Zn^{2+} were provided by Frontier Scientific. Coupling reagent of *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ), eosin, lactacystin, alcohol dehydrogenase, and glutathione *S*-transferase were from Sigma. S-Sepharose and glutathione−sepharose were purchased from Pharmacia. Precast gels for 10−20% Tricine−SDS−PAGE were provided by Novex. 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 (DMEM), penicillin−streptomycin, and trypsin−EDTA were obtained from Gibco. Fetal bovine serum and Lipofectamine Plus reagent were provided by Hyclone and Invitrogen, respectively. Antibodies such as monoclonal anti-α-synuclein antibody and horseradish peroxidase (HRP) conjugated anti-mouse IgG secondary antibody were from Transduction Laboratories. ECL western blotting detection reagent was from Amersham Biosciences. Materials for protein purifications and other reagents used in this study were supplied from Sigma, unless otherwise mentioned.

Analysis of Self-Oligomerization of α-Synuclein in the Presence of Various Pc and PcTS. α-Synuclein (5 μM) was preincubated with various concentrations of the Pc/PcTS in 20 mM Mes, pH 6.5, for 30 min at 37 °C. While the metal complexes of PcTS were prepared in the Mes buffer, the Pc complexes were stored in dimethyl sulfoxide (DMSO). Following addition of 0.3 mM EEDQ originally stored in DMSO, the chemical cross-linking reactions proceeded for another 1 h at 37 °C while concentration of the organic solvent was kept at less than 10% in the final reaction mixtures (*40*). 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-HCl, pH 8.45, by mixing at an 1:1 (v/v) ratio. After being boiled for 5 min, the samples were analyzed with the precast gel for 10−20% Tricine−SDS−PAGE, and the ladder formation was visualized with silver staining by Morrissey (*41*). For the experiments of the competition study, self-oligomerization of α-synuclein was carried out with eosin (0.1 mM), copper (0.5 mM), or PcTS−Cu^{2+} (50 μM). The PcTS was added to the reaction mixtures at various concentrations to observe prevention of the self-oligomerization of α-synuclein. The mixtures were analyzed with 10−20% Tricine−SDS−PAGE as described.

Dissociation Constants between PcTS/PcTS−Cu^{2+} and Synucleins. Various synucleins including α-synuclein, β-synuclein, α-syn61−140, and α-syn97 were separately incubated at 5 μM with various concentrations of PcTS/PcTS−Cu^{2+} (0.05−2.5 mM) in 20 mM Mes, pH 6.5, for 40 min at 37 °C in a final reaction volume of 200 μL. Protein-bound tetrapyrroles were separated from the unbound forms by

using a centrifuge column procedure, also called Penefsky column, packed with Sephadex G-25 (coarse) (*42*). Preswollen gel packed in a 3 mL syringe was compressed by centrifugation at 600*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 additional 1.5 min at the same speed. The synucleins in the recovered sample were quantified with MicroBCA assay, while the amounts of PcTS and PcTS−Cu^{2+} were estimated by measuring absorbances at 610 and 620 nm, respectively. Saturation curves were drawn between total amounts treated and the bound tetrapyrroles. Dissociation constants between the synucleins and PcTS/PcTS−Cu^{2+} were obtained from double-reciprocal plots of the saturation curves.

Analysis of Protein Aggregations in the Presence of PcTS/PcTS−Cu^{2+}. Protein aggregation of α-synuclein was monitored with either turbidity or thioflavin-T binding fluorescence (*43*, *44*). α-Synuclein was incubated with PcTS/PcTS−Cu^{2+} (50 μM) at two different concentrations of 0.2 and 1 mg/mL in 20 mM Mes, pH 6.5, at room temperature under continuous shaking at 150 rpm with an orbit shaker (Red Rotor, Hoefer Scientific Inc.). Turbidity was estimated by measuring the absorbance of the incubation mixture at 405 nm. Amyloid formation of α-synuclein (1 mg/mL) in the presence and absence of 50 μM PcTS was evaluated with thioflavin-T binding fluorescence at 485 nm with an excitation at 440 nm. During the incubation, aliquots (20 μL) were combined with 5 μM thioflavin-T in 50 mM glycine, pH 8.5, to a final volume of 100 μL. The fluorescence was measured with an FL500 microplate fluorescence reader (Bio-Tek Instruments). Morphologies of the final protein aggregates were examined with a transmission electron microscope (H7100, Hitachi). Aliquots (5 μL) 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 (*45*). Cytotoxicities of the protein aggregates of α-synuclein which were obtained following 160 h of incubation in the presence and absence of 50 μM PcTS/PcTS−Cu^{2+} and sufficient dialysis against 20 mM Mes, pH 6.5, were examined with human neuroblastoma cells (SH-SY5Y). The cells were treated with the aggregates corresponding to approximately 5 μM α-synuclein for 24 h, and the mitochondrial activities of live cells were evaluated with the tetrazolium salt extraction method (see below).

In addition, PcTS effects on protein aggregations of alcohol dehydrogenase (ADH), glutathione *S*-transferase (GST), and amyloid β/A4 protein (Aβ) were also examined with turbidity measurement. Protein solutions of ADH (4 μM) and GST (6.8 μM) were prepared in phosphate-buffered saline (PBS) consisting of 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 at pH 7.5. They were heated to 65 °C, and light scattering of the solutions were measured in the presence and absence of 50 μM PcTS by observing absorbances at 360 nm within a thermostatic cell holder of a Beckman spectrophotometer (DU650). Absorbance of the tetrapyrrole itself was subtracted to obtain the turbidities solely due to aggregations of the proteins. Peptide solutions of Aβ (157 μM) were prepared in PBS in the presence and absence of 50 μM PcTS and subjected to shaking incubation with the orbit shaker (150 rpm) at room temperature. The turbidities were monitored at 405 nm during 10 h of incubation.

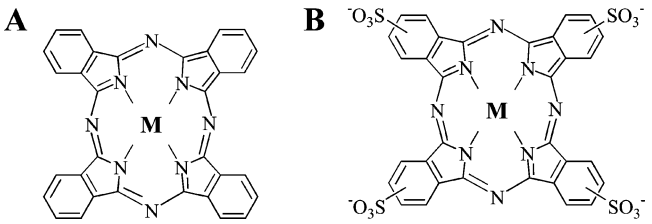

FIGURE 1: Structures of phthalocyanine (A) and phthalocyanine tetrasulfonate (B). Letters M in the middle of the structures represent intercalated metal ions.

Overexpression of α-Synuclein in the SH-SY5Y Cell. Human dopaminergic neuroblastoma cells (SH-SY5Y) were grown in DMEM containing 50 units/mL penicillin and 50 μg/mL streptomycin supplemented with 10% fetal bovine serum in 5% CO_2 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 μg) 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% CO_2 and 95% air. After change to the medium containing 50 units/mL penicillin, 50 μg/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/well).

Overexpression of α-synuclein inside the cells was examined with Western blotting. The cell lysates were prepared with sonication (10 s each for three times) in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10 μM leupeptin, 1 μg/mL aprotinin, and 10 μM pepstatin A. Soluble and particulate fractions were obtained via centrifugation of the lysate at 15000*g* for 15 min at 4 °C. The extract of each fraction was subjected to 15% SDS−PAGE, and the gels were transferred to PVDF membrane and incubated with the monoclonal antibody to α-synuclein (1:2000). The membrane was then incubated with HRP-conjugated anti-mouse IgG secondary antibody (1:2000), and bands were visualized on X-ray film by using the ECL system (*37*).

Cytotoxicity Assay. The cells transfected with vectors containing either α-synuclein or no additive were grown to 80% confluence on a 24-well plate. Then, PcTS−Cu^{2+} was treated at two different concentrations of 5 and 10 μM and incubated for an additional 24 h at 37 °C. Cell survival was estimated with the tetrazolium salt extraction method (*46*). Since living cells with active mitochondria cleave the tetrazolium ring into a visible dark blue formazan reaction product, 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the culture medium at a final concentration of 1 mg/mL, and the resultant medium was incubated for 3 h at 37 °C. To measure absorbance at 570 nm, MTT extraction buffer containing 20% SDS and 50% *N*,*N*-dimethylformamide, pH 4.7, was added to each sample (400 μL into 400 μL of medium in a 24-well plate including 80 μL of 5 mg/mL MTT) to dissolve the formazan grains. The cells were further incubated at 37 °C overnight, and absorbance was measured at 570 nm with the extraction

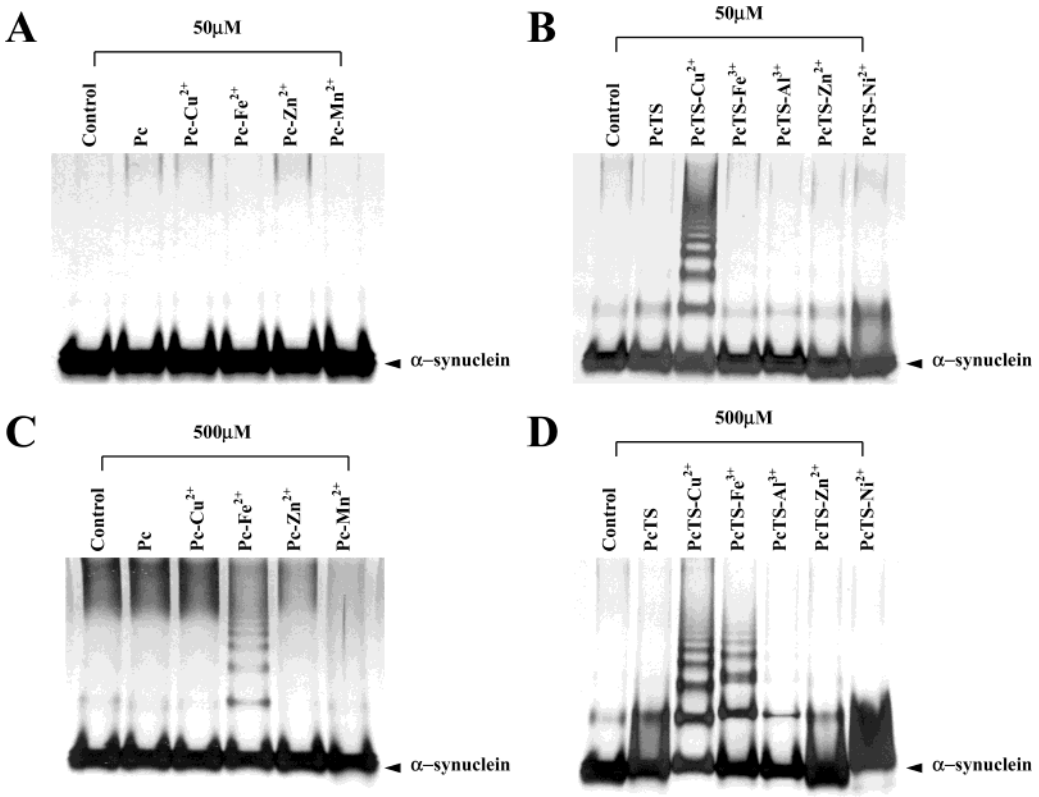

FIGURE 2: Self-oligomerization of α-synuclein in the presence of various metal complexes of Pc and PcTS. α-Synuclein (5 μM) was preincubated with various metal complexes of Pc (A and C) and PcTS (B and D) at two different concentrations of 50 μM (A and B) and 0.5 mM (C and D). The self-oligomers were cross-linked with 0.3 mM EEDQ for 1 h at 37 °C. After termination of the reactions with the sample buffer of Tricine−SDS−PAGE, the ladder formation was visualized with silver staining. The metal complexes examined are denoted on the top of each panel.

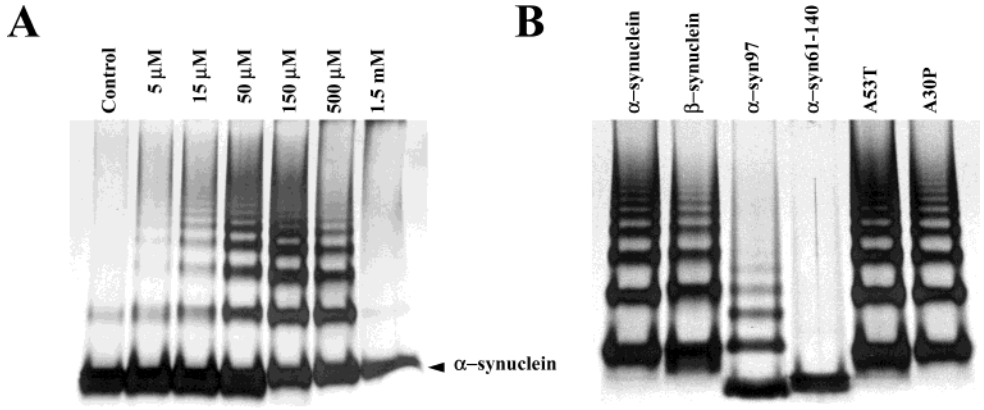

FIGURE 3: Self-oligomerization of α-synuclein with various concentrations of PcTS−Cu^{2+} (A) and the oligomerization of various synucleins in the presence of 50 μM PcTS−Cu^{2+} (B). The experiment of protein self-oligomerization of α-synuclein (see Materials and Methods) in the presence of EEDQ was carried out with various concentrations of PcTS−Cu^{2+} as indicated on the top of panel A. The lane marked as control shows the self-oligomerization of α-synuclein in the absence of the compound. The monomeric band of α-synuclein is indicated with an arrowhead on the right side (A). Protein self-oligomerizations of various isoforms of α-synuclein were examined with 50 μM PcTS−Cu^{2+} (B).

buffer containing the medium as a blank. All of the data related to the cytotoxicity were obtained from three separate experiments.

The PcTS effect on the lactacystin-induced cell death of SH-SY5Y cells was examined in the presence and absence of overexpressed α-synuclein. Human cDNA of α-synuclein within pcDNA3.0 and mock plasmid were separately transfected into the cells at 4 μg each. After the trypsinization, the cells were plated in a 24-well plate at 1.5×10^5 cells/well and incubated for 36 h. A proteasome inhibitor of lactacystin was then treated at either 5 or 10 μM, and the cells were further incubated for an additional 12 h in the presence and absence of 5 or 10 μM PcTS. Cell viability was assessed with the MTT assay.

Statistical Analysis. All of the statistical analyses were performed with ANOVA and Duncan's multiple range test by using the SAS package. A limit of the statistical significance was selected at a p-value of <0.05.

RESULTS

Self-Oligomerization of α-Synuclein in the Presence of Various Metal Complexes of Pc and PcTS. Molecular

interactions between Pc/PcTS (Figure 1) and α-synuclein were evaluated on the basis of whether the compounds induced the protein self-oligomerization in the presence of a coupling reagent, EEDQ. The oligomers were revealed as a ladder formation on a silver-stained gel of 10−20% Tricine−SDS−PAGE. Among various metal complexes of Pc and PcTS tested at a final concentration of 50 μM (Figure 2A,B), a discrete ladder formation was observed only with PcTS−Cu^{2+} (Figure 2B). To exhibit this selective interaction, therefore, not only the negative charges provided by the sulfonate groups but also the copper chelation which might alter the tetrapyrrole into a unique molecular structure were demonstrated to be essential factors. At the higher final concentration of 0.5 mM, both Pc−Fe^{2+} and PcTS−Fe^{3+} also induced the protein self-oligomerization (Figure 2C,D) whereas Pc−Cu^{2+} was yet to cause the oligomerization (Figure 2C). These data indicate that the sulfonate group substitution on the iron complexes is not as critical as that on the copper complex of PcTS to exert the α-synuclein interaction.

The PcTS−Cu^{2+} interaction of α-synuclein was further examined because not only the self-oligomerization was induced at the lower concentration but also the sulfonate group requirement was distinctive among other compounds. When the concentrations were varied, the ladder formation due to the protein self-oligomerization started to be observed from 5 μM PcTS−Cu^{2+}, which corresponded to an 1:1 molar ratio with α-synuclein at the fixed concentration of 5 μM (Figure 3A). The ladder formation was most prominent at 150 μM of the tetrapyrrole and decreased thereafter. Since the decreased ladder formation did not raise the intensity of the monomeric band of α-synuclein, the oligomers appeared to form large complexes at higher concentrations, which would not be resolved within the gradient gel. Binding site(s) of PcTS−Cu^{2+} on α-synuclein responsible for the self-oligomerization was (were) assessed with various isoforms of the protein, including β-synuclein, α-syn97, α-syn61−140, and the two mutant forms of Ala53Thr and Ala30Pro. β-Synuclein which differs from α-synuclein especially at the acidic C-terminus was also self-oligomerized in the presence of 50 μM PcTS−Cu^{2+} (Figure 3B, lane 2), indicating that the PcTS−Cu^{2+} interaction was independent of the C-terminal region. When a C-terminally truncated α-synuclein, α-syn97, lacking all of the amino acids from residue 98 to the end was tested, self-oligomerization of the modified protein was still observed as another ladder formation (Figure 3B, lane 3). On the other hand, N-terminal truncation of α-synuclein to the α-syn61−140 completely abolished the tetrapyrrole-induced protein self-oligomerization (Figure 3B, lane 4). In addition, the PcTS−Cu^{2+}-induced self-oligomerization of α-synuclein was not affected by the presence of the NAC peptide fragment (data not shown). Taken together, the tetrapyrrole interaction of α-synuclein has been directed to the N-terminal region apart from the hydrophobic NAC segment. The two mutations, however, did not influence the PcTS−Cu^{2+}-induced protein self-oligomerization (Figure 3B, lanes 5 and 6). These data clearly indicate that the PcTS−Cu^{2+} interaction has been directed toward the N-terminal region of α-synuclein and independent of the acidic C-terminus.

Dissociation Constants between PcTS−Cu^{2+}/PcTS and α-Synuclein. To evaluate molecular affinity, dissociation

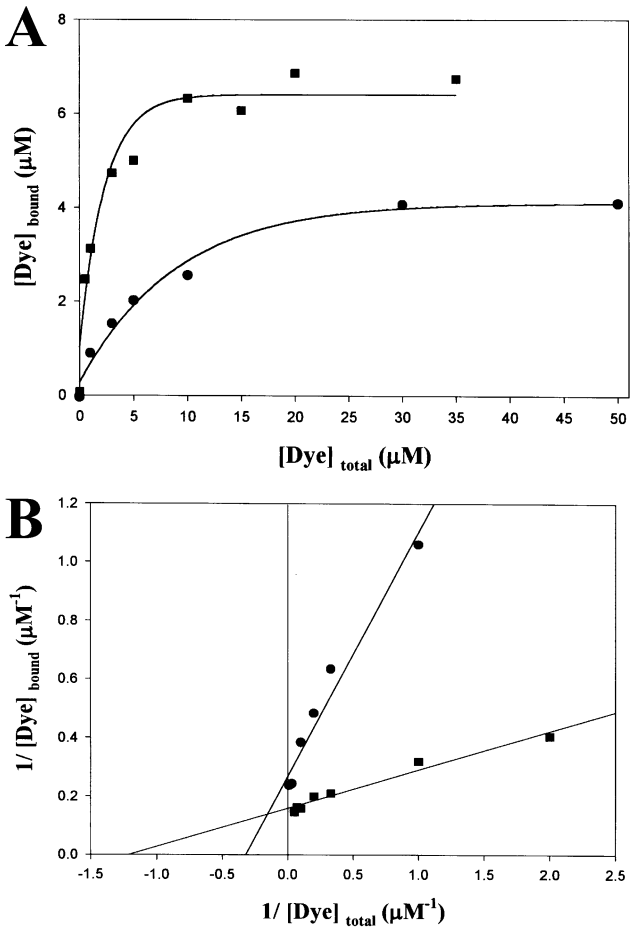

FIGURE 4: Saturation curves (A) of α-synuclein with PcTS/PcTS−Cu^{2+} and their corresponding double reciprocal plots (B). α-Synuclein (5 μM) was incubated with various concentrations of either PcTS (●) or PcTS−Cu^{2+} (■) in 20 mM Mes, pH 6.5. Protein-bound tetrapyrroles and the unbound forms were separated with the centrifuge column packed with Sephadex G-25 (coarse) as described in Materials and Methods. After quantifying the bound tetrapyrroles by measuring absorbances between 610 and 620 nm, saturation curves were drawn between the total amounts of the compounds and the amounts of the protein-bound forms (A). Double reciprocal plots of the saturation curves were redrawn to obtain dissociation constants of K_d (B).

constants between α-synuclein and PcTS in the presence and absence of the chelated Cu^{2+} ion were obtained via saturation curves (Figure 4A) and corresponding double-reciprocal plots (Figure 4B). Following incubation between the protein and the tetrapyrroles, the bound PcTS/PcTS−Cu^{2+} was collected with the centrifuge column packed with Sephadex G-25. The PcTS−Cu^{2+} interaction exhibited a high affinity with a K_d of 0.83 μM. Unexpectedly, however, the PcTS without the metal chelation, which did not induce the protein self-oligomerization (Figure 2B,D), was shown to interact with α-synuclein with a considerable K_d of 3.12 μM. By performing a competition study, the PcTS interaction of α-synuclein was examined and compared with the PcTS−Cu^{2+} interaction. The eosin-induced protein self-oligomerization which was previously shown to depend on both NAC and the C-terminal acidic region (*19*) was suppressed in the presence of PcTS from the molar ratio of 1:1.5 between eosin (0.1 mM) and the compound (Figure 5A), indicating that the PcTS binding appeared to be independent of the PcTS−Cu^{2+} interacting N-terminal region. The copper-induced self-

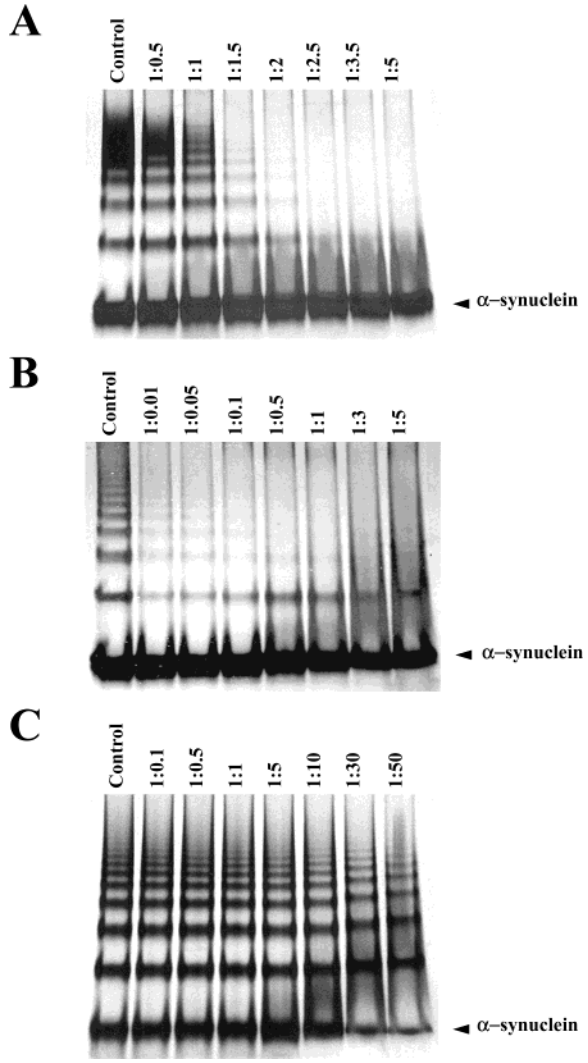

FIGURE 5: Competitive effects of PcTS on the protein self-oligomerizations of α-synuclein in the presence of eosin (A), copper (B), and PcTS−Cu^{2+} (C). The experiment of protein self-oligomerization of α-synuclein (5 μM) was conducted in the presence of either 0.1 mM eosin (A), 0.5 mM copper (B), or 50 μM PcTS−Cu^{2+} (C). During the reaction, the PcTS was added at various molar ratios to the fixed amount of each oligomerizing agent as indicated on the top of each panel. The self-oligomers were visualized as ladders on the silver-stained gradient gels. Lanes labeled with control show the self-oligomerizations of α-synuclein by the oligomerizing agents in the absence of PcTS. Protein bands corresponding to monomeric α-synuclein are indicated with arrowheads on the right side of each panel.

oligomerization which was demonstrated to be a C-terminal-dependent phenomenon (*39*) was instantaneously inhibited by the PcTS treatment from an 1:0.01 (copper:PcTS) molar ratio (Figure 5B). This fact implied that the PcTS binding site would be located at the acidic C-terminus. Finally, the PcTS effect on the PcTS−Cu^{2+}-induced self-oligomerization was examined. As shown in Figure 5C, the self-oligomerization was barely affected by PcTS even at the highest concentration of 2.5 mM (1:50 = PcTS−Cu^{2+}:PcTS), indicating that the PcTS and PcTS−Cu^{2+} interactions with α-synuclein were mutually independent. To provide additional evidence for the selective interaction of PcTS, direct binding studies between the tetrapyrrole and various synucleins, including β-synuclein, N-terminally truncated α-syn61−140, and C-terminally truncated α-syn97, were performed by obtaining the dissociation constants (Table 1). The K_d value of 3.12 μM between PcTS and α-synuclein was not affected by the N-terminal truncation since the α-syn61−140 also showed an almost identical K_d of 3.22 μM toward the compound. When β-synuclein was tested, however, the K_d was increased to 26.9 μM, which was somewhat expected because the C-terminal acidic region was most variable between α- and β-synucleins. The C-terminal specific interaction was confirmed by the observation that PcTS did not even bind to the C-terminally truncated α-syn97 although the compound was varied between 1 and 200 μM, which was the same range as employed in the other binding studies. Taken together, therefore, it is pertinent to consider that the PcTS and the PcTS−Cu^{2+} interactions with α-synuclein are mutually exclusive by preferentially recognizing the C-terminal and the N-terminal regions, respectively.

Table 1: Dissociation Constants between PcTS and Synucleins

	α-synuclein	N-terminally truncated α-syn61−140	C-terminally truncated α-syn97	β-synuclein
K_d (μM)	3.12	3.22	ND[a]	26.9

[a] ND, not determined.

Protein Aggregation of α-Synuclein in the Presence of PcTS and PcTS−Cu^{2+}. PcTS/PcTS−Cu^{2+} effects on the protein aggregation of α-synuclein were studied with aggregation kinetics and morphologies of the final protein aggregates. The time course of protein aggregation was followed at two different protein concentrations (0.2 and 1 mg/mL) by observing turbidity at 405 nm (Figure 6A,B). The PcTS−Cu^{2+} increased the final turbidity of α-synuclein at 1 mg/mL in 20 mM Mes, pH 6.5, by less than 30% from its absence without affecting the lag phase. The PcTS, on the other hand, significantly alter the aggregation kinetics of α-synuclein by prolonging the lag phase for more than three times and reducing the final turbidity by 45% (Figure 6A). The aggregation-promoting activity of PcTS−Cu^{2+} as expected from its effect on the self-oligomerization (Figures 2 and 3) became obvious when the protein aggregation was carried out at the lower concentration of α-synuclein (0.2 mg/mL). The PcTS−Cu^{2+} shortened the lag phase by half and increased the final turbidity by three times, while the PcTS completely suppressed the aggregation (Figure 6B). In fact, the PcTS was also demonstrated to exhibit its suppressive effect against the amyloid formation as well since it inhibited the thioflavin-T binding fluorescence of the final aggregates of α-synuclein at 1 mg/mL by more than 50% (Figure 6C). The thioflavin-T binding fluorescence known to be quantitative to estimate amyloid formation was not applicable to the PcTS−Cu^{2+}-induced protein aggregation because the copper-chelated compound interfered with the fluorescence by directly interacting with thioflavin-T even in the absence of amyloid.

To evaluate the specificity of anti-aggregative effect of PcTS, protein aggregations of nonamyloidogenic proteins, such as ADH and GST, and amyloidogenic Aβ were examined in the presence of the tetrapyrrole at 50 μM by observing turbidity at either 360 or 405 nm (Figure 7). The PcTS significantly inhibited the heat-induced protein aggregations of ADH and GST by 72% and 32% at 65 °C, respectively (Figure 7A,B). In addition, the final turbidity

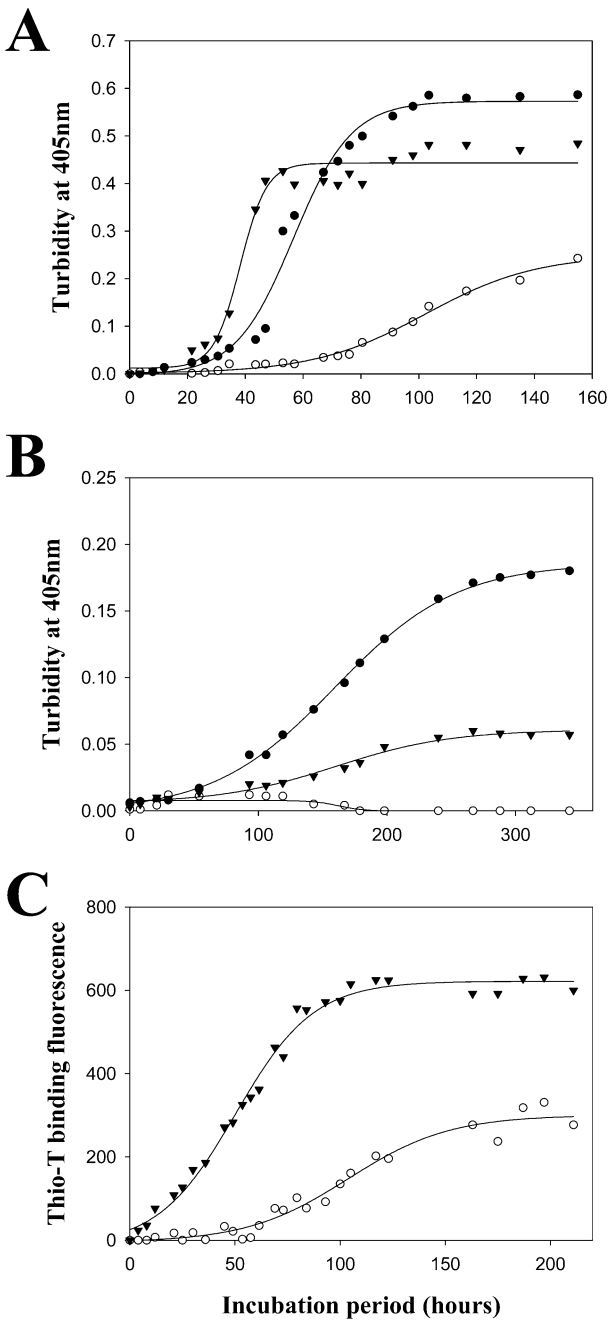

FIGURE 6: Aggregation kinetics of α-synuclein in the presence of PcTS and PcTS−Cu^{2+}. α-Synuclein was incubated in the absence (▼) and presence of either 50 μM PcTS (○) or 50 μM PcTS−Cu^{2+} (●) at two different protein concentrations of 1 mg/mL (A and C) and 0.2 mg/mL (B) in 20 mM Mes, pH 6.5, at room temperature with continuous shaking. The protein aggregation and the amyloid formation were evaluated with the turbidity by measuring the absorbance at 405 nm (A and B) and the thioflavin-T binding fluorescence at 485 nm with excitation at 440 nm (C), respectively.

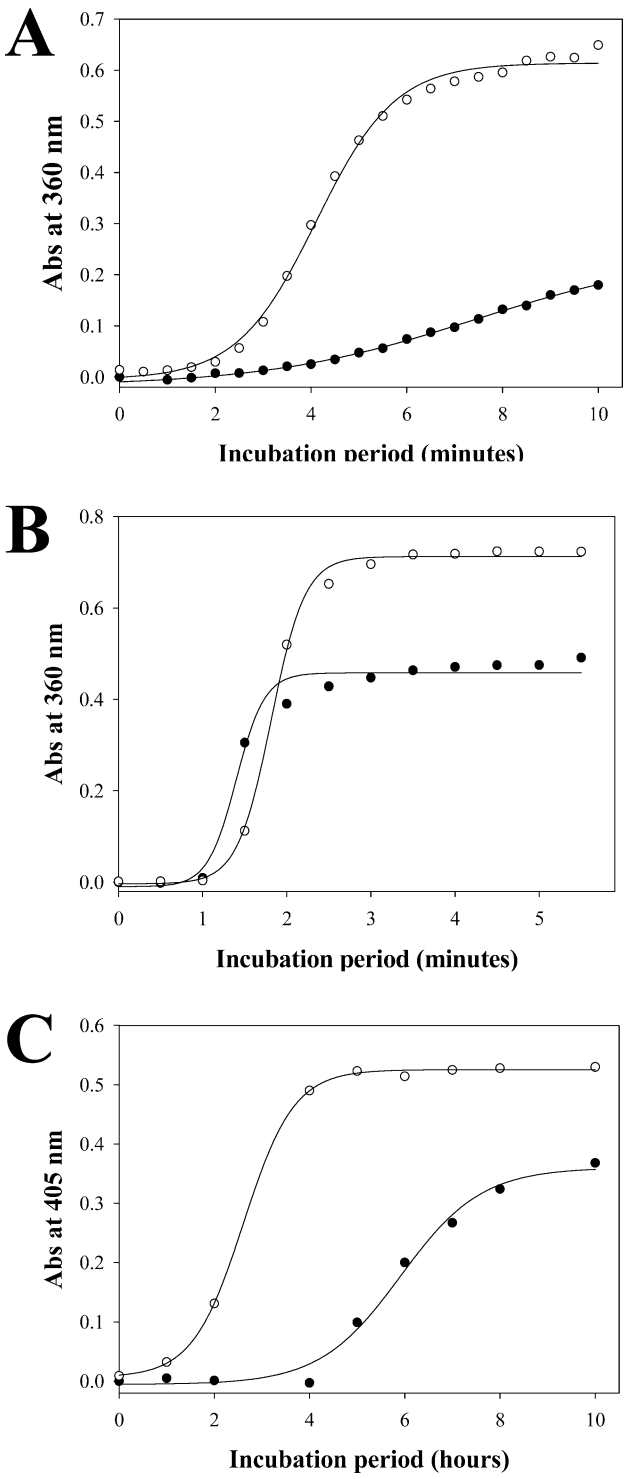

FIGURE 7: Chemical chaperone effect of PcTS on protein aggregations of ADH (A), GST (B), and Aβ (C). Protein solutions of ADH (4 μM) and GST (6.8 μM) in PBS, pH 7.5, were subjected to heat treatment at 65 °C, and light scattering of the solutions was monitored at 360 nm in the presence (●) and absence (○) of 50 μM PcTS (A and B). Peptide solution of Aβ (157 μM) in PBS was incubated with continuous shaking at room temperature in the presence (●) and absence (○) of 50 μM PcTS. The turbidity was followed at 405 nm during the incubation (C).

due to the aggregation of the amyloidogenic Aβ peptide was also effectively suppressed by 31% along with a prominent extension of the lag phase by more than 2-fold (Figure 7C). This result clearly indicates that PcTS could be considered as a chemical chaperone which might control protein aggregations in general.

In Vitro Cytotoxicity of the Tetrapyrrole-Induced Protein Aggregates and the Tetrapyrrole Effect on the Overexpressed α*-Synuclein inside SH-SY5Y Cells.* Although the PcTS interaction to α-synuclein in particular had appeared inhibitory against the protein aggregation, the presumption needed to be adjusted after analyzing morphologies of the final aggregates. The final protein aggregates of α-synuclein in the absence of the tetrapyrroles formed fibrils with a rather

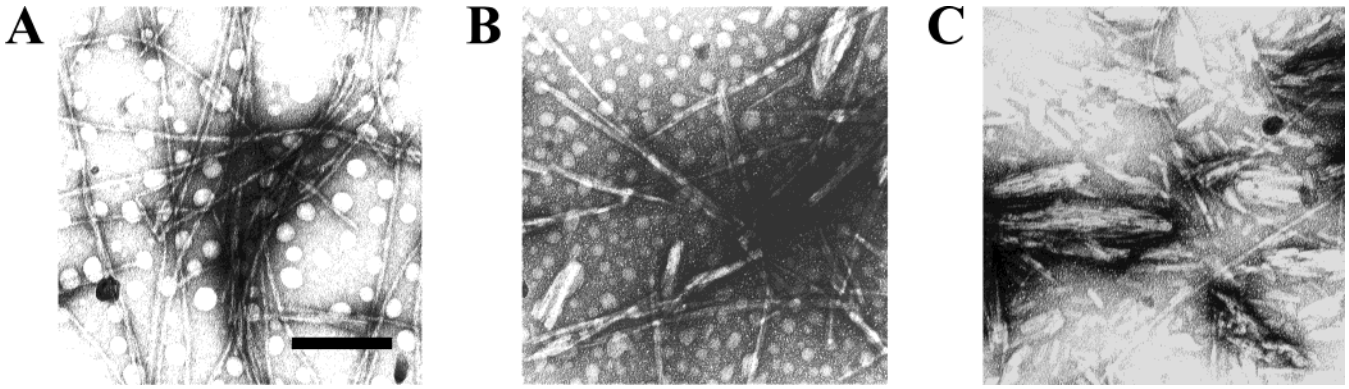

FIGURE 8: Morphologies of the final protein aggregates of α-synuclein prepared in the absence (A) and presence of either PcTS−Cu^{2+} (B) or PcTS (C). Following 160 h of incubation, the final protein aggregates of α-synuclein (1 mg/mL) obtained in the absence and presence of PcTS/PcTS−Cu^{2+} were observed with a transmission electron microscope (H7100, Hitachi) after negative staining with 2% uranyl acetate. The scale bar represents 0.312 μm.

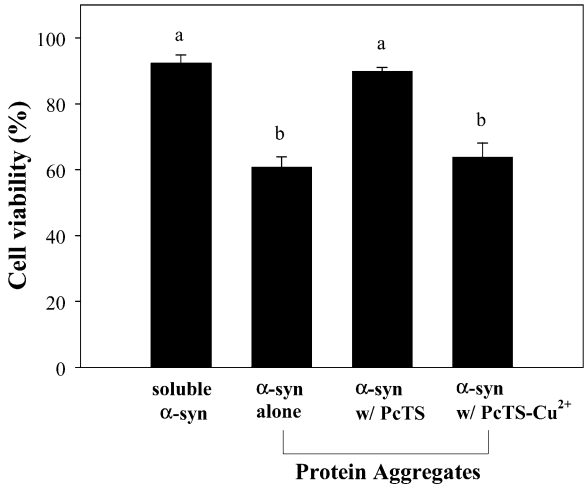

FIGURE 9: Cytotoxicities of the tetrapyrrole-induced protein aggregates. The SH-SY5Y cells were treated for 24 h with the protein aggregates corresponding to 5 μM α-synuclein, which were prepared for the morphological analysis shown in Figure 8. Cell viability was assessed with the MTT assay of three separate experiments. Soluble α-synuclein and the protein aggregates obtained in the absence and presence of PcTS/PcTS−Cu^{2+} were analyzed as indicated in the panel. Values of percent cell viability are the mean ± SE, and means with the same superscripts are not significantly different at $p < 0.05$ by Duncan's multiple range test.

smooth surface (Figure 8A). The aggregates prepared with PcTS−Cu^{2+} were also fibrous with a somewhat sharp surface appearance (Figure 8B). The aggregates obtained in the presence of PcTS, on the other hand, exhibited structures resembling chopped-wood fragments (Figure 8C). The lower values in both turbidity and thioflavin-T binding fluorescence obtained in the presence of PcTS, therefore, might be due to the distinctive morphology in addition to actual amounts of the protein aggregates. These observations indicate that the discrete α-synuclein interactions of PcTS and PcTS−Cu^{2+} would be responsible for the differences in shapes of the final aggregates as well as the aggregation kinetics.

Cytotoxicities of the tetrapyrrole-induced protein aggregates obtained following 160 h of incubation of α-synuclein in the presence and absence of PcTS/PcTS−Cu^{2+} were assessed with SH-SY5Y cells and the MTT assay (Figure 9). While soluble α-synuclein barely affected the cell (6.6% cytotoxicity), the fibrous aggregates of α-synuclein corresponding to an approximate protein concentration of 5 μM prepared in the absence of the tetrapyrroles augmented the toxicity by 34.4%. Intriguingly, the aggregates obtained with the PcTS did not show any significant toxic effect (only by 9.5%), whereas the fibrous aggregates by the PcTS−Cu^{2+} exhibited 35.8% of cytotoxicity. These data indicate that morphologies of the protein aggregates could affect the cells as far as in vitro cytotoxicity was concerned, although the amount of protein aggregates should be involved in the toxicity as well.

Then, we turned to examine tetrapyrrole effects on transiently overexpressed α-synuclein inside SH-SY5Y cells, which could put therapeutic or preventive values on the compounds. To demonstrate a possible suppressive effect of PcTS against cytotoxicity, a proteasome inhibitor of lactacystin was employed as an aggregation-promoting factor for the overexpressed α-synuclein to cause the cell death. It was reported that lactacystin was shown to cause nigral degeneration with inclusion body formation of α-synuclein in rats (*47*). Lactacystin affected the mock-transfected SH-SY5Y cells in a dose-dependent manner by giving rise to cell viabilities of 89.7%, 73.9%, and 57.3% at 0, 5, and 10 μM, respectively (Figure 10A). When the α-synuclein overexpressing cells were treated with the inhibitor, the toxic effect of lactacystin was significantly enhanced by showing cell viabilities of 89.0%, 59.0%, and 44.5% at the same concentrations of 0, 5, and 10 μM, respectively (Figure 10B). Surprisingly, the augmented toxicities became almost completely protected to their control levels in the presence of PcTS in a dose-dependent manner (Figure 10). The enhancement of toxicity due to the α-synuclein overexpression was 1.57- and 1.3-fold at 5 and 10 μM lactacystin, respectively (Table 2). At 5 μM lactacystin, the 1.57-fold enhancement was alleviated to 1.08- and 0.98-fold at 5 and 10 μM PcTS, respectively (Table 2). The augmented toxicity by 29.7% (1.3-fold) at 10 μM lactacystin was completely suppressed even at 5 μM PcTS with the 0.91-fold enhancement. These results clearly suggest that PcTS could control the toxicity due to the α-synuclein overexpression and proteasomal inhibition by possibly influencing the protein aggregation process. On the other hand, however, the PcTS−Cu^{2+} effect on the overexpressed α-synuclein within the cells was not adequately evaluated because the compound itself caused a substantial amount of cell death (data not shown).

The tetrapyrrole effects on protein self-association of α-synuclein overexpressed inside the cells were examined

Table 2: Lactacystin-Induced Cytotoxicities in the Presence and Absence of α-Synuclein Overexpression in the SH-SY5Y Cells

	5 μM lactacystin + PcTS (μM)			10 μM lactacystin + PcTS (μM)		
	0	5	10	0	5	10
% cytotoxicity without α-synuclein overexpression	26.1	28.6	28.9	42.8	42.5	38.0
% cytotoxicity with α-synuclein overexpression	41.0	31.0	28.2	55.5	38.6	36.0
enhancement of cytotoxicity upon α-synuclein overexpression (fold)	1.57	1.08	0.98	1.30	0.91	0.95

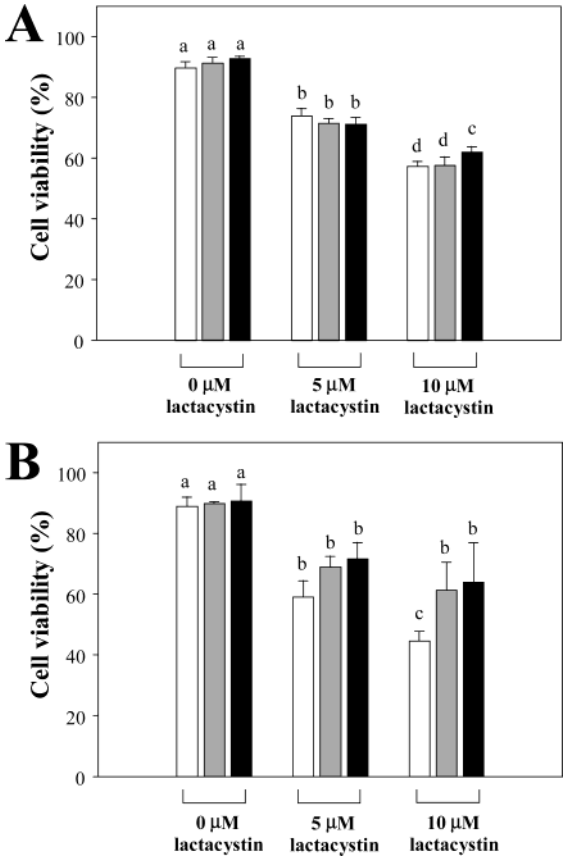

FIGURE 10: Protective effect of PcTS against the lactacystin-induced cell death of SH-SY5Y cells in the absence (A) and presence (B) of overexpressed α-synuclein. The transfected SH-SY5Y cells with either the human cDNA of α-synuclein or mock plasmid were incubated for 36 h before lactacystin was treated at 0, 5, and 10 μM as indicated on the panels. The cells were further incubated for an additional 12 h in the presence of 0 μM (open bar), 5 μM (shaded bar), and 10 μM (filled bar) PcTS as shown in each panel. The cell viabilities were estimated with three separate experiments of the MTT assay. Values are the mean ± SE, and means with the same superscripts are not significantly different at $p < 0.05$ by Duncan's multiple range test.

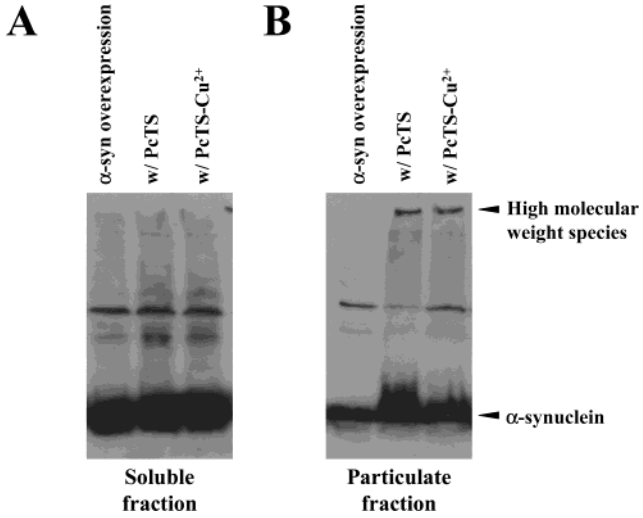

FIGURE 11: Western blot analysis of overexpressed α-synuclein within the SH-SY5Y cells treated with PcTS/PcTS−Cu^{2+}. The cells treated with or without the tetrapyrroles were collected with trypsin−EDTA and sonicated three times for 10 s each. Soluble and particulate fractions were separated via centrifugation at 15000*g* for 15 min at 4 °C. They were subjected to 15% SDS−PAGE, and the gels were transferred to the PVDF membrane. The membrane was successively incubated with monoclonal antibody to α-synuclein and HRP-conjugated anti-mouse IgG secondary antibody. The protein bands were visualized on X-ray film by using the ECL system. Bands corresponding to the high molecular weight species and the monomeric α-synuclein are indicated with the arrowheads.

with Western blotting of the PcTS/PcTS−Cu^{2+}-treated cell extracts by using anti-α-synuclein antibody. The immunoreactive protein bands in the soluble cell extracts prepared in the presence and absence of the tetrapyrroles showed identical distributions (Figure 11A). The particulate fractions obtained with the PcTS and PcTS−Cu^{2+} treatments, however, revealed the SDS-resistant high molecular weight species along with protein smears around the monomeric band of α-synuclein. The particulate fraction prepared in the absence of the chemical treatment, on the other hand, did not yield the high molecular weight species on the gel (Figure 11B). This fact implied that the tetrapyrroles permeated into the cells, and α-synuclein became one of the targets to be self-associated. As a matter of fact, the permeation of the compounds was actually observed as blue dots scattered inside the cells under the microscope (Olympus AX70TRF) at 200× magnification (data not shown).

In this study, PcTS and PcTS−Cu^{2+} were demonstrated to exhibit specific interactions with α-synuclein and influence the protein aggregation, which resulted in altered aggregation kinetics and distinct shapes of the final aggregates whose cytotoxicities also varied accordingly. The tetrapyrrole effects on the protein were confirmed at the cellular level as well, where the PcTS was shown to protect the α-synuclein overexpressing cells from the cell death induced by the proteasome inhibitor treatment. Taken together, the PcTS/PcTS−Cu^{2+} could be considered as possible candidates for drug development against the α-synucleinopathies, including PD and AD.

DISCUSSION

Phthalocyanine compounds have been applied to various areas from industrial purposes to biomedical applications, including dyes for paints, textiles, and plastics, inks, catalysts, materials for photoconducting devices, electrochromic displays, fuel cells, and even cancer phototherapy (*48*). Since these compounds were suggested to act as antiscrapie drugs and possibly influence amyloidogenic proteins during their aggregation processes (*25*, *26*), phthalocyanine (Pc) and its related structures have been examined in this study with

respect to their pro- to anti-aggregative effects on the PD-related protein of α-synuclein. Introduction of four sulfonate groups to Pc with the copper ion chelated in the middle of the structure exhibited highly selective interaction with α-synuclein leading to the protein self-oligomerization, while $Pc-Cu^{2+}$ without the substituted sulfonate groups did not affect the protein to be self-interactive even at the relatively high concentration of 0.5 mM. The sulfonate groups, therefore, have been considered to be critical for the protein interaction by converting lipophilic Pc into a hydrophilic and multianionic form of PcTS. The hydrophobicity, however, was not solely responsible for the protein to be self-interactive because both $Pc-Fe^{2+}$ and $PcTS-Fe^{3+}$ also induced the self-oligomerization of α-synuclein at the higher concentration (0.5 mM) regardless of the sulfonate substitution. It is pertinent to consider that the sulfonation could provide not only negative charges which affect the hydrophobicity but also alteration in structures of the tetrapyrroles. These altered chemical and physical properties are attributable to the specific interaction with α-synuclein and subsequent protein aggregation in the presence and/or absence of appropriate metal chelation. Alternatively, molecular structures of the tetrapyrroles could direct the substituted sulfonates to exhibit the α-synuclein interaction.

Copper intercalation to PcTS also provided the tetrapyrrole with special properties for the protein aggregation of α-synuclein and eventual cytotoxicity. The intercalation has shifted the chemical's regional preference for α-synuclein interaction from the C-terminal acidic region to the N-terminal region. The $PcTS-Cu^{2+}$ interaction, therefore, might alter the natively unfolded structure of α-synuclein to be self-oligomerized (*49*, *50*). In addition, the PcTS and $PcTS-Cu^{2+}$ interactions with α-synuclein produced different aggregation kinetics and distinct morphologies of the final protein aggregates. Taken together, the copper chelation has definitely changed the molecular properties of PcTS to be more amyloidogenic toward α-synuclein. As far as morphology of the protein aggregates was concerned, the fibrous aggregates of α-synuclein obtained in the presence and absence of $PcTS-Cu^{2+}$ were more devastating to the cells than the chopped-wood structures formed with PcTS. In other words, transition from the filamentous aggregates to the fragmented forms in the absence and presence of PcTS, respectively, actually prevented the cytotoxicity. In addition to the amount of amyloids, therefore, shapes of the protein aggregates could be considered to play a critical role on the toxicity.

As others have already suggested (*51*−*53*), transient overexpression of α-synuclein inside the cells did not exhibit the toxic effect as much as expected. Sometimes the overexpression was reported to be proliferative instead (*54*). This controversy will be clarified only after physiological function(s) of α-synuclein has (have) been unequivocally established. When the α-synuclein overexpressing cells were treated with PcTS in the presence of lactacystin, the cell death induced by the proteasomal inhibitor was significantly protected. It was shown that the PcTS permeated into the cells and caused high molecular weight protein aggregates of the overexpressed α-synuclein, which appeared to contradict the suppressive effect of PcTS against the protein aggregation of α-synuclein observed in vitro (Figure 6). As demonstrated in Figure 8, however, the compound exhibited a sort of special interaction with α-synuclein in particular producing the protein aggregates with distinctive morphology which might not be appropriate to be evaluated with the conventional in vitro assays (*45*). By taking the distinctive PcTS-induced protein aggregates into consideration (Figure 8), therefore, the tetrapyrrole compound could be considered to exert its protective effect against the cytotoxicity of the α-synuclein overexpression by producing nontoxic and nonfibrillar protein aggregates rather than actual suppression of the protein aggregation. Additionally, this study has also informed us of a close relationship between proteasomal inhibition and the cytotoxicity of the overexpressed α-synuclein by possibly causing abnormal protein accumulation and subsequent aggregation.

In the case of $PcTS-Cu^{2+}$, we have observed that the compound augmented a small toxicity induced by the overexpressed α-synuclein. Unfortunately, however, the data were not presented because the $PcTS-Cu^{2+}$ showed considerable toxicity by itself, which made its enhancement statistically insignificant. Concerning the cellular effects of PcTS/$PcTS-Cu^{2+}$, direct and specific interaction between the tetrapyrroles and α-synuclein within the cells needs to be further examined. It is still possible that the tetrapyrroles could not reach α-synuclein inside the cells as effectively as expected because of other tetrapyrrole binding proteins or reduced effective concentration of the compounds due to their self-association (*25*, *26*, *32*, *55*). Alternatively, α-synuclein would be just one of several intracellular proteins influenced by the tetrapyrroles.

On the basis of the data provided in this report, the tetrapyrroles have been demonstrated to be useful for future drug development. Reflecting general interests toward the compounds among researchers, a large variety of phthalocyanines and their methods of preparation are available (*56*). Since their protein interactions have also been introduced in the literature (*25*−*32*), optimization of the compounds could be achieved to make them applicable for controlling α-synuclein and its toxic consequence. The PcTS in particular could be useful as an anti-amyloidogenic compound not only because it suppresses protein aggregations in general as observed with the nonamyloidogenic and amyloidogenic proteins but also because it has a tendency to convert α-synuclein which is prone to form the toxic fibrous aggregates into the nontoxic forms. In addition, the tetrapyrrole shows little toxicity to the cells by itself and exhibits fairly high specificity toward the protein. In the case of $PcTS-Cu^{2+}$, its selective and high-affinity interaction with α-synuclein would make the compound or its derivatives effective materials controlling the amyloidosis despite its own chemical toxicity. These compounds, therefore, await extensive examination to develop into eventual therapeutic and preventive strategies against the α-synuclein-related neurodegenerative disorders.

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