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Molecular Mechanisms Underlying the Flavonoid-Induced Inhibition of α-Synuclein Fibrillation[†]

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ABSTRACT: The molecular mechanism underlying the flavonoid-induced inhibition of α-synuclein fibrillation was thoroughly examined by various biochemical and biophysical approaches. The noncovalent binding of the inhibitory flavonoids to α-synuclein and the covalent modification by the flavonoid quinone led to the restriction of the conformational changes in this natively unfolded protein and to the stabilization of soluble flavonoid-modified species of α-synuclein (monomers and oligomers). All of these factors rather than a single one contribute to the inhibition of WT α-synuclein fibrillation induced by the flavonoid. The structural requirements that appear necessary to provide a flavonoid the ability to inhibit α-synuclein fibrillation were determined to be vicinal dihydroxyphenyl moieties, irrespective of the ring position where they are located. Flavonoids with three vicinal hydroxyl groups exhibited enhanced inhibitory effects on α-synuclein fibrillation. The antioxidant activities of flavonoids were generally correlated with their in vitro inhibitory effects on α-synuclein fibrillation. The flavonoids inhibiting α-synuclein fibrillation and stabilizing the protein monomeric conformation can serve as a model for the development of therapeutic drugs in combating Parkinson's disease.

α-Synuclein is widely expressed in the brain, and predominantly localized at presynaptic terminals (*1*, *2*). Human α-synuclein is a small acidic protein composed of 140 a.a. residues, lacking both cysteine and tryptophan residues. The central part of the protein (residues 61−95) is a highly hydrophobic and amyloidogenic NAC region, which was first identified as the non-A*β* component of Alzheimer's disease (AD) (*3*). The C-terminal region (residues 96−140) is rich in acidic residues (Glu and Asp) and in prolines, and contains three conserved tyrosine residues. Deletions of this region predispose α-synuclein toward fibrillogenesis (*4*−*6*).

α-Synuclein is found in both soluble and membrane-associated fractions of the brain with transient dynamic interactions with vesicles (*7*, *8*). In aqueous solution, α-synuclein has no stable tertiary or secondary structure; it is a typical intrinsically disordered protein, as demonstrated by a variety of techniques, such as circular dichroism spectroscopy (CD),[1] small-angle X-ray scattering (SAXS), Fourier transform infrared spectroscopy (FTIR), and NMR (*9*, *10*). The association with lipid molecules or phospholipid membranes promotes changes in the secondary structure of the protein from unstructured (random-coil-like) to the α-helical conformation (*11*−*14*). The normal physiological functions of α-synuclein are still unknown. However, as a member of the class of natively unfolded proteins (*15*−*17*), α-synuclein is potentially involved in various biological activities and cell regulation and can interact with multiple targets due to its exceptional conformational plasticity (*18*−*22*).

Amyloid fibrils made of α-synuclein are the main proteinaceous constituents of Lewy bodies accumulated inside of dopaminergic neurons in the brains under pathological conditions such as Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy (*23*, *24*). α-Synuclein fibrils have similarity to all amyloid fibrils core made of a cross-beta structure (*10*, *25*, *26*). The mechanism of α-synuclein fibrillogenesis has been thoroughly studied in vitro (*27*−*31*). It has been demonstrated that the first step of fibrillogenesis is the formation of a partially folded intermediate that promotes the self-association of the protein and the formation of various oligomeric species (*9*). With time, some transient oligomers form fibrils whereas others lead to insoluble amorphous aggregates and soluble stable oligomers. The outcome of the branched α-synuclein aggregation pathway can be modulated by various factors. Among factors that accelerate α-synuclein fibrillation are certain pesticides (*32*−*34*), metals (*35*−*37*), polycations (*38*, *39*), glycosaminoglycans (*40*, *41*), and macromolecular crowding (*42*−*44*). Acceleration typically arises from conditions that increase the concentration of the critical amyloidogenic intermediate. Furthermore, a number of factors that lead to inhibition of α-synuclein

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[1]Abbreviations: ThT, thioflavin T; DMSO, dimethyl sulfate; ATR-FTIR, attenuated total reflectance Fourier transform infrared spectroscopy; UV, ultraviolet; CD, circular dichroism; SAXS, small-angle X-ray scattering; IEF, isoelectric focusing; ESI-MS, electrospray ionization mass spectrometry; EM, electron microscopy; AFM, atomic force microscopy; TEAC, Trolox-equivalent antioxidant capacity; SDS−PAGE, sodium dodecylsulfate−polyacrylamide gel electrophoresis; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide.

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fibrillation have been identified, including either chemical modifications of the protein through methionine oxidation (*45*, *46*), tyrosine nitration (*47*, *48*), and by catecholamines (*49*−*51*), or the presence of β- and γ-synucleins (*52*, *53*), high concentrations of alcohols (*54*) and osmolytes (*55*). The presence of membranes and vesicles has been reported to both accelerate and inhibit fibrillation, and this probably reflects the varying conditions used in the in vitro experiments, such as lipid compositions and lipid to protein ratios (*56*).

We showed previously that a variety of flavonoids inhibited α-synuclein fibrillation, and most of the strong inhibitory flavonoids disaggregate preformed fibrils (*57*). In this paper, we analyzed the molecular mechanisms underlying the inhibition of α-synuclein fibrillation by flavonoids.

MATERIALS AND METHODS

Materials. All flavonoids were obtained from INDOFINE chemical Co., Inc. Thioflavin T (ThT) was obtained from Sigma-Aldrich. All other chemicals were analytical grade and obtained from Fisher chemicals or VWR scientific.

Expression and Purification of Wild Type α-*Synuclein and* α-*Synuclein Mutants*. Wild type (WT) human recombinant α-synuclein containing plasmid *pRK172* was previously transfected into *Escherichia coli* BL21 (DE3) cells (obtained from R. Jakes and M. Goedert) and purified as described previously (*38*).

Mutagenesis was carried out on the pRK172 plasmid containing the WT human α-synuclein insert. The two truncated mutants (Syn30−140 and Syn1−103) as well as the triple mutant (Y125F/Y133F/Y136F) were created using the QuikChange site-directed mutagenesis protocol (Stratagene). Purification was carried out as described above for WT α-synuclein. The only modification made on the procedure of purification for Syn1−103 is that cation ion-exchanging chromatography (Hitrap SP FF 5 mL, Pharmacia) was used instead of anion-exchange. The resultant α-synuclein protein and the mutants were judged to be >95% pure following SDS−polyacrylamide electrophoresis, size exclusion chromatography, and MS analysis. Protein concentration was determined spectrophotometrically for the two truncated mutants. The triple mutant (Y125F/Y133F/Y136F) has no significant UV absorbability, and the concentration was determined by Bradford assay and gel electrophoresis using Image J software.

Aggregation/Fibrillation Studies and ThT Assays. Lyophylized α-synuclein was prepared for use by dissolution at high pH (approximately 10.5) in dilute NaOH for 5−10 min and adjusted to pH 7.4 prior to centrifugation at 95 000 rpm with a Beckman Airfuge ultracentrifuge to remove any aggregated material. Flavonoids were dissolved in DMSO to make a stock solution at a concentration of 5, 10, or 20 mM and to make a final solution of protein and flavonoid in 1% DMSO unless indicated otherwise. All of the protein final solutions consisted of PBS with 0.02% NaN_3 added to avoid bacteria growth.

For plate-reader assays, the protein solution was mixed with flavonoids to give final concentrations of protein 35 μM and flavonoids 50 μM. Three replicates of each sample were prepared in a 96-well plate with a volume of 120 μL per well and a 3 mm Teflon bead to provide agitation (120 rpm, 20 mm diameter). The assays were performed in a fluorescent plate reader (Labsystems Fluoroskan Ascent CF). Measurements were taken at 30-min intervals with excitation at 444 nm and emission monitored at 485 nm.

For manual assays, the protein solution was mixed with flavonoids to give final concentrations of protein 70 μM and flavonoids 100 μM. Protein samples were stirred at 37 °C with a mini-Teflon stir bar. Aliquots of 5 or 10 μL were removed from the incubated solution and added to 1 mL of 10 μM ThT solutions in 20 mM phosphate buffer (pH 7.4) as a function of time to monitor the fibrillation kinetics. ThT fluorescence was recorded at 482 nm with excitation at 450 nm and slits of 5 nm for both excitation and emission using a FluoroMax-3 spectrofluorometer (Jobin Yvon Horiba).

The in vitro kinetics of α-synuclein fibril formation show the classic sigmoidal behavior attributed to a nucleation-dependent polymerization. For comparing the fibrillation kinetics, the intensity of ThT fluorescence was plotted as a function of time and fitted by a sigmoidal curve described by the following equation using SigmaPlot:

$$Y = y_i + m_i x + \frac{y_f + m_f x}{1 + \mathbf{e}^{-[(x - x_0)/\tau]}}$$

where Y is the fluorescence intensity, x is time, and x_o is the time to 50% of maximal fluorescence. Thus, the apparent first-order rate constant, k_{app}, for the growth of fibrils is given by $1/\tau$, and the lag time is given by $x_o - 2\tau$.

Electron Microscopy Measurements. Transmission electron microscopy (TEM) was used to estimate the size and structural morphology of α-synuclein. Aliquots of 5 μL sample were deposited on Formvar-coated 300 mesh copper grids (Ted Pella) and incubated for 5−10 min. Salts were washed out with distilled water, and samples were dried, negatively stained with 1% (w/v) uranyl acetate, and visualized on a JEOL JEM-100B transmission electron microscopy operated at 80 kV. Typical magnifications ranged from ×75 000 to 300 000. The grids were thoroughly examined to obtain an overall evaluation of the samples. Images were produced with Gattan Digital Micrograph software.

Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) was carried out on an Amersham Phast system separation and control unit with PhastGel gradient 8−25 polyacrylamide gels. SDS buffer (0.20 M tricine, 0.20 M Tris, 0.55% SDS, pH 8.1) strips (Amersham) were used. Gel electrophoresis samples were prepared by the addition of 3 parts of protein solution to 1 part SDS buffer (0.25 M Tris, 8% SDS, 60% glycerol, 0.08% bromophenol blue, pH 6.8) and boiled for 3−5 min. The 10−225 kDa protein markers were purchased from USB Corporation (Cleveland, Ohio, USA).

Isoelectric Focusing. Isoelectric focusing (IEF) was carried out on an Amersham Phast system separation and control unit with IEF polyacrylamide gels (pI 4−6.5). pI standards contain proteins as follows: human carbonic anhydrase B, 6.55; bovine carbonic anhydrase B, 5.85; β-lactoglobulin A, 5.20; soybean trypsin inhibitor, 4.55; glucose oxidase mannitol, 4.15; methyl red (dye), 3.75; amyloglucosidase, 3.50; pepsinogen, 2.80.

Chromatography. Size exclusion (SEC) HPLC measurements were done as follows. An aliquot of each sample was removed from the α-synuclein incubation at various times, and the insoluble material was removed by centrifugation for 20 min at 14 000 rpm. Sample volumes of 40 μL of supernatant were eluted from a TSK-GEL G4000SWXL size exclusion column (7.8-mm inner diameter × 30 cm) in 20 mM phosphate buffer, pH 7.0, and 100 mM Na_2SO_4 using a Waters 2695 separations module with a Waters 996 photodiode array detector. The HPLC system was controlled, and data were collected and analyzed by

Millennium software. The column was eluted at a flow rate of 0.5 mL/min, and the absorbance of the mobile phase was monitored over the wavelength range from 220 to 450 nm with a bandwidth of 1.2 nm. The retention times were calibrated with the following protein molecular mass standards: ribonuclease A (13.6 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), albumin (bovine serum) (67 kDa), aldolase (158 kDa). The void volume was determined with blue dextran 2000 (~2000 kDa).

RP-HPLC was performed using an analytical Vydac C18 reverse-phase HPLC column (Separations Group, Hesperia, CA) with a flow rate of 700 μL/min. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) unless indicated otherwise. A linear gradient was employed with a rate of change at 2%B/min.

Determination of Equilibrium Dissociation Constants of α-Synuclein- Flavonoid Complexes. The apparent equilibrium dissociation constants (K_d) for ligand-α-synuclein complexes were determined from measurements of UV absorbance changes in flavonoids and by Tyr fluorescence quenching in α-synuclein. For estimation of dissociation constants, 10 samples with different α-synuclein concentrations ranging from 0 to 8 times K_d were prepared in PBS. Absorption spectra over the range from 220 to 500 nm were measured using a UV-2401 PC UV−visible recording spectrophotometer (Shimadzu, Japan) immediately following the addition of 30 μM flavonoid to the samples. The binding constant, K_d, was calculated with the Benesi-Hilderbrand equation.

Tyr quenching experiments were done on FluoroMax-3 spectrofluorometer (Jobin Yvon Horiba) using quartz cuvettes (Hellma) with a 1-cm excitation light path. The light source was a 150-W xenon lamp. Emission spectra were recorded from 305 to 550 nm with excitation at 270 nm, in increments of 1 nm, with integration time 0.2 s, and 1 nm slits for both excitation and emission. Emission spectra were measured immediately following the addition of various concentrations of flavonoids to α-synuclein.

Circular Dichroism Spectroscopy. Far-UV CD spectra were collected on an Aviv 60DS spectrophotometer using a 0.02-cm path length cell. CD spectra were recorded with a step size of 1 nm, a bandwidth of 1.5 nm, and an averaging time of 5 s. α-Synuclein concentration of 1 mg/mL was used for all CD spectral analyses, and an average of five scans after subtracting the appropriate buffer was obtained.

Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) Spectroscopy. ATR-FTIR data were collected on a ThermoNicolet 670 spectrometer equipped with an MCT detector. The samples were spread out and dried as a thin film on a germanium IRE, with a constant flow of nitrogen gas. Between 256 and 1024 interferograms were collected for each sample. Samples were 5−10 μL of 70 μM protein in PBS. Data analysis was performed with the GRAMS32 software from Galactic Industries.

Electrospray Ionization Mass Spectrometry (ESI-MS) and Trypsin Digestion. Samples for mass spectrometry analysis were desalted with C8 reverse phase column and eluted out with 80% acetonitrile adjusted with TFA to a final pH at 2.0. Injection was carried out via a Harvard Apparatus (Holliston, MA) syringe pump at a flow rate of 20 μL/min. Mass spectra were obtained using Micromass ZMD electrospray mass spectrometer operating in positive ionization mode. The source temperature was set to 80 °C, and the capillary voltage was 3.25 kV. Protein molecular weight was determined from m/z by Masslynx software.

Trypsin digestion of samples containing 2 mg/mL of α-synuclein in fibrillation assay buffer (PBS, 0.02% NaN_3, 1% DMSO) in the absence and in the presence of 200 μM of baicalein was done overnight at room temperature.

Detection of H_2O_2 using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit. The kit was purchased from Molecular Probes, Inc. Reactions containing 50 μM Amplex Red reagent, 0.1 U/mL HRP and the indicated amount of H_2O_2 in 50 mM sodium phosphate buffer, pH 7.4, were incubated for 30 min at room temperature. In the presence of peroxidase, the Amplex Red reagent reacts with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin. Resorufin has an absorption maximum of approximately 571 nm, with an extinction coefficient of 54 000 cm^{-1} M^{-1}.

RESULTS

Fibrillation of α-Synuclein in the Presence of Various Flavonoids. Previously, 48 flavonoids belonging to several classes with structures differing in ring substituents and in the nature/extent of alkylation have been tested for their ability to inhibit the fibrillation of α-synuclein in vitro (*57*). These flavonoids included: 18 flavones, 12 flavonols, 4 flavanones, 6 isoflavones, 2 dihydroflavonols, 3 catechins, and 3 anthraquinones. We showed that different flavonoids affected the α-synuclein fibrillation to a different extent. On the basis of the results of this study, some flavonoids, such as 22−357, were classified as weak inhibitors, as they slightly inhibited the fibrillation of α-synuclein, increasing the lag time 2−3-fold in comparison with that of the control. Other flavonoids, such as quercetin, were classified as good inhibitors, noticeably inhibiting α-synuclein fibrillation, with a lag time 4−5-fold longer than that of the control. Finally, several flavonoids (such as baicalein, eriodictoyl, 6-HP, 22-324, myricetin, EGCG, T-415, 22-340/tricetin, and 22-341) were classified as strong inhibitors since in their presence α-synuclein fibrillation was completely inhibited (*57*). The molecular mechanisms underlying the inhibition of α-synuclein fibrillation by several flavonoids were analyzed below.

Auto-Oxidation of Flavonoids. Flavonoids, such as baicalein, undergo auto-oxidation in the presence of oxygen. Since the rate of oxidation (half-life, $t_{1/2}$) depends on the flavonoid concentration, we used the same flavonoid concentrations as those utilized earlier under the incubation conditions inducing α-synuclein fibrillation (*57*). Auto-oxidation kinetics and changes in absorbance spectra of several flavonoid are shown in Figure 1. A rough correlation between the half-lives and the reported half-wave potentials of the first oxidation wave was found by Yang et al. (*58*). However, the reported redox-potential values of the flavonoids were found to vary in reports of different groups, likely due to the differences in methods of assessment and other experimental details. For instance, myricetin was reported to have a redox-potential of 0.06 V by Van Ackers et al. (*59*), and 0.03 V by Yang's group (*58*).

From the monoexponential decrease in the absorbance at λ_{max}, most inhibitory flavonoids had a half-life in the range of a few hours under the usual incubation conditions, depending on the individual structure. For example, quercetin had a half-life of only about 1 h, and kaempferol with one less hydroxyl group on ring B had a little longer half-life. Luteolin, with a structure similar to quercetin but without the 3-OH, had a half-life of more than 10 h. Wogonin, a flavonoid that does not have a catechol structure on ring B, was found to be almost completely resistant

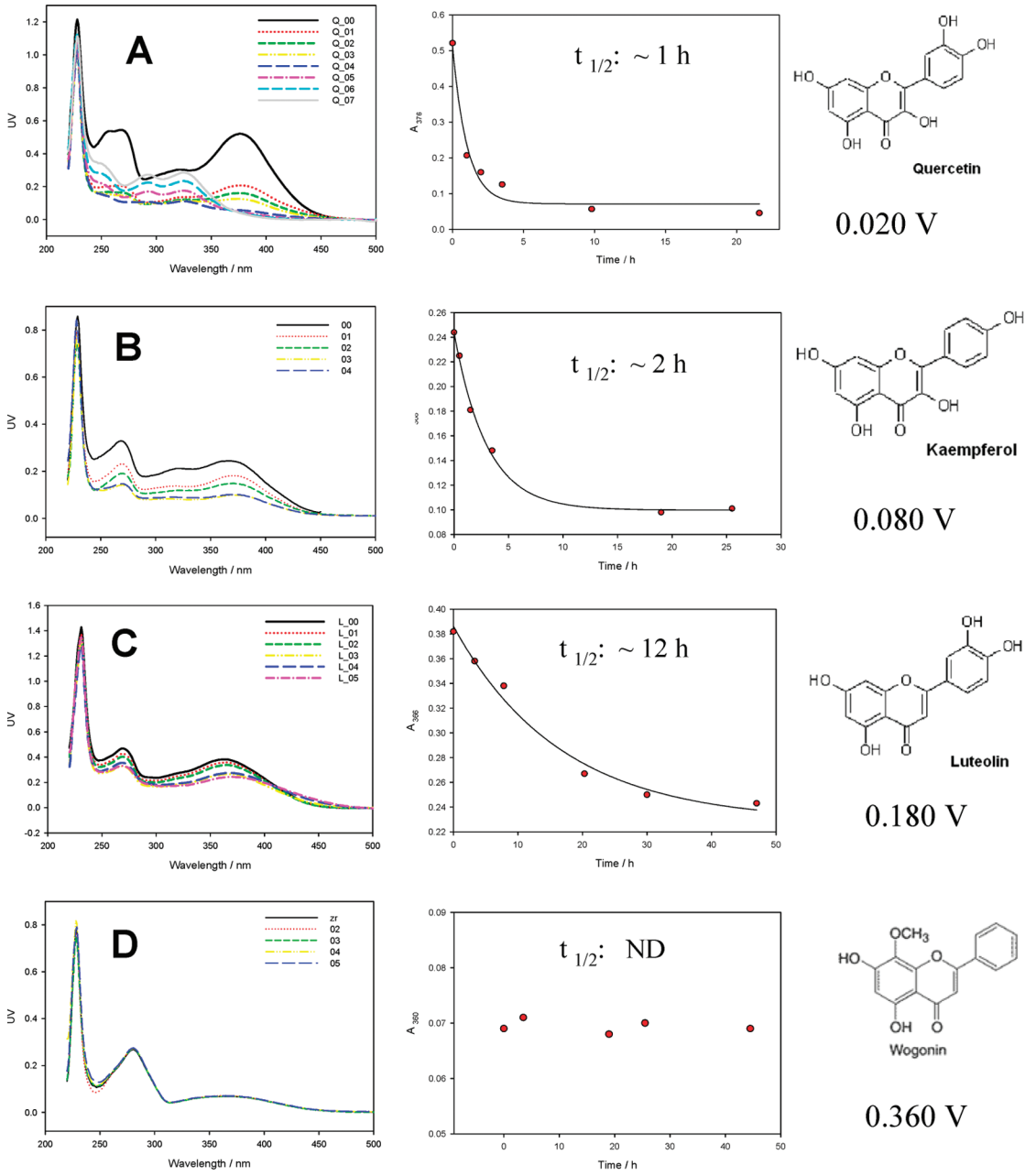

FIGURE 1: Auto-oxidation of several flavonoids from the screening pool monitored by changes in UV−vis absorbance as a function of incubation time. (A) Quercetin; (B) kaempferol; (C) luteolin; (D) wogonin. The reported half-wave potentials of the first oxidation wave are also listed on the right side.

to oxidation under the incubation conditions used. The wogonin oxidation was hard to detect even after 3 days of incubation. In general, the tendency of a flavonoid to auto-oxidize depended on the availability of phenolic hydrogens and on the possibility of stabilization of the resulting phenoxyl radicals via hydrogen bonding or by expanded electron delocalization.

Since most inhibitory flavonoids have half-lives in the range of a few hours under the usual incubation conditions, a significant amount of the flavonoid would be oxidized during the nucleation and early fibrillation stages of α-synuclein aggregation (~20 h). The major oxidation product is the corresponding quinone(s), since other species such as the semiquinone are much shorter lived. Such quinones are expected to be susceptible to nucleophilic attack by the amine groups on the side chains of proteins, leading to covalent modification (for more discussion see below). Other derivatives include radical-induced polymerized and degraded products, which were detected through RP-HPLC (Min Zhu, unpublished data).

Effects of Aged Flavonoids on α-Synuclein Fibrillation. To investigate the effect of aged flavonoids on the inhibition of α-synuclein fibrillation, various concentrations of aged baicalein (that is, 10, 20, 30 μM) were added to the 35 μM α-synuclein solution, and the differences in the lag time of the fibrillation kinetics were estimated. The compound was preincubated for various times to obtain solutions of the differently aged compound containing one or two predominant species. Figure 2A shows the changes in the ThT fluorescence intensity during the α-synuclein fibrillation in the presence of various concentrations of fresh and differently aged baicalein. The kinetics of the α-synuclein fibrillation alone in 0.5% DMSO is also shown for comparison. The lag time of the fibrillation for each condition was plotted against the concentration of the compound, as shown

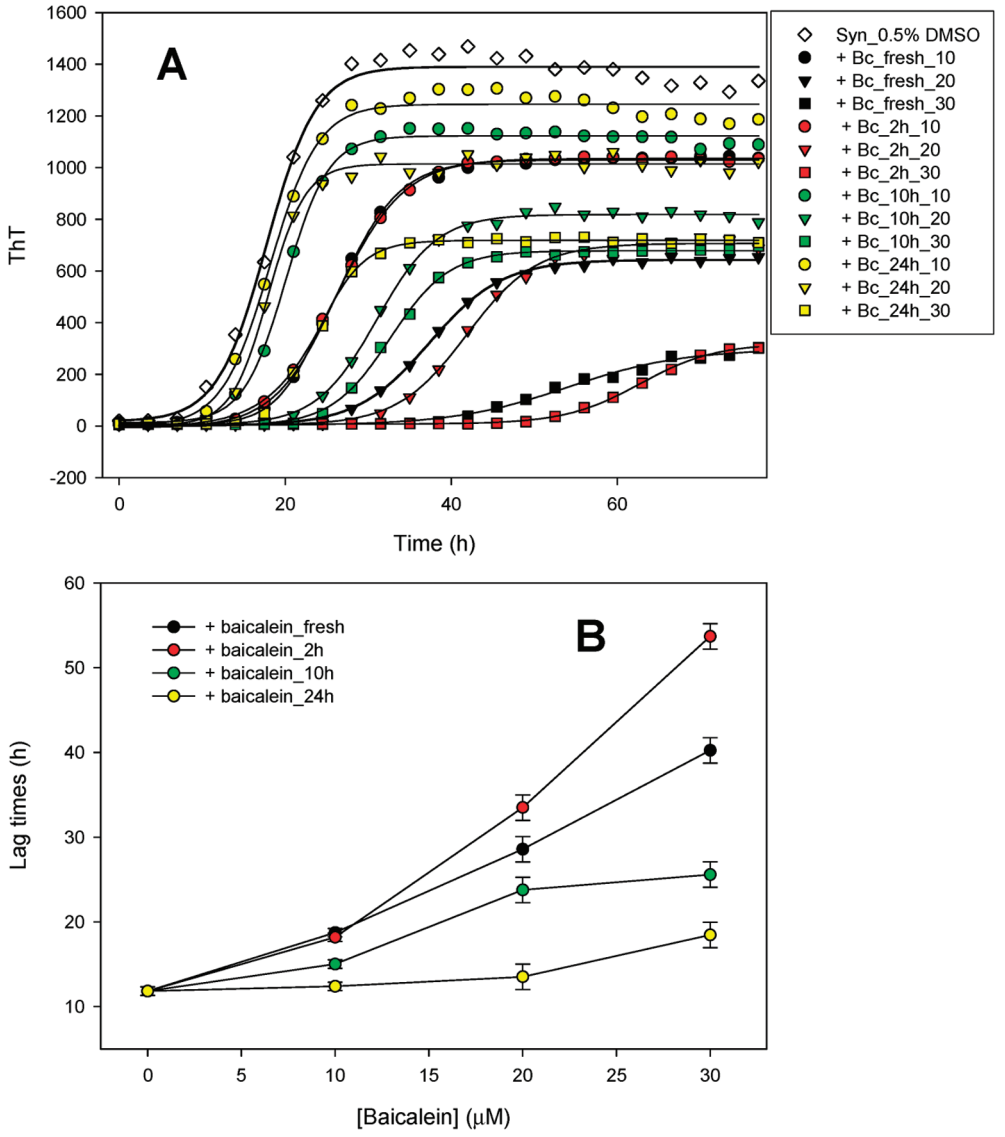

FIGURE 2: The effect of aged species of baicalein on the kinetics of α-synuclein fibrillation. (A) ThT fluoresence of α-synuclein fibrillation in the presence of baicalein with and without preincubation at various concentrations, with the kinetics of the fibrillation of α-synuclein alone in 0.5% DMSO shown for comparison (open diamonds). (B) The lag time of the fibrillation kinetics under each condition is plotted against the concentration of baicalein.

in Figure 2B. The analysis revealed that the inhibitory efficiency of this flavonoid was proportional to its concentration. Longer aged baicalein (i.e., 10 h, 24 h, 76 h) exhibited a weaker inhibitory effect on the α-synuclein fibrillation, whereas baicalein preincubated for 2 h exhibited a stronger inhibitory effect than the fresh flavonoid. This was manifested by the prolonged lag time in the presence of 20 and 30 μM of the aged baicalein. This phenomenon correlated well with the changes in the UV absorbance associated with the auto-oxidation of baicalein (Figure S1, Supporting Information). At around 2−4 h of incubation, some transient oxidation product(s), most likely quinone(s), were formed. The weaker inhibition induced by the longer-preincubated compound can be explained by the fact that longer-preincubated compounds likely corresponded to the radical-induced polymerized or degraded products that are not as effective inhibitors as quinones. The fact that baicalein preincubated for 2 h inhibited fibrillation stronger than the untreated compound suggested that the flavonoid quinone is the major species in the inhibition of α-synuclein fibrillation.

To address the question whether the auto-oxidation of flavonoids was required for inhibiting α-synuclein fibrillation, the same experiment was carried out under the anaerobic conditions, at which the auto-oxidation is assumed to be mostly prevented (*60*). This analysis revealed that in the absence of oxygen the baicalein-induced inhibition of α-synuclein aggregation was much weaker, indirectly demonstrating that the oxidized product(s) generated during auto-oxidation of baicalein played an important role in the inhibition of α-synuclein fibrillation (data not shown). Collectively, it can be concluded that auto-oxidation of the flavonoid is required for the effective inhibition of α-synuclein fibrillation.

α-*Synuclein Oxidation by Hydrogen Peroxide Produced in the Presence of Inhibitory Flavonoids*. To investigate whether flavonoid-mediated inhibition of α-synuclein fibrillation was because of the chemical modification of α-synuclein, electrospray ionization mass spectrometry (ESI-MS) was applied to the α-synuclein samples incubated in the presence of the inhibitory flavonoids. It was observed that α-synuclein was significantly oxidized after incubation with inhibitory flavonoids, such as

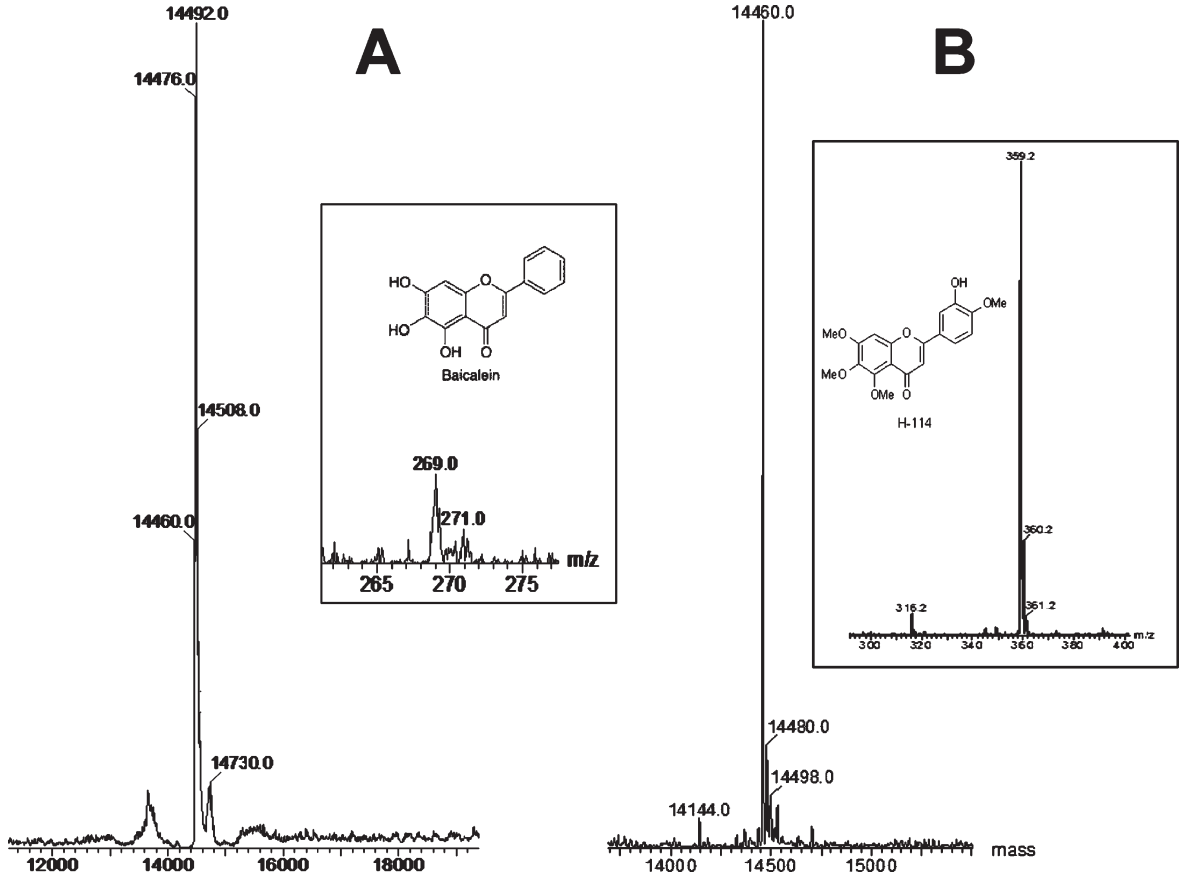

FIGURE 3: Electrospray ionization mass spectra (ESI-MS) of α-synuclein incubated in the presence of inhibitory flavonoid, baicalein (A) and noninhibitory flavonoid, H-114 (B). Insets show the MS of oxidized baicalein and intact H-114, respectively.

baicalein. In fact, several species with variously increased molecular masses were observed and the increase in mass was proportional to the addition of 16 × N (N = 1, 2, and 3, Figure 3A). On the other hand, in the presence of noninhibitory flavonoids, such as H-114, α-synuclein was not chemically modified, as shown by the unchanged MW peak at 14460 ± 2 in Figure 3B.

All amino acids are susceptible to oxidation, although their reactivities vary greatly (*61*). Methionines (Met) and cysteins (Cys) are among the most readily oxidized amino acids. Human α-synuclein has no Cys but has four methionines, Met1, Met5, Met116, and Met127, all located outside the repeat-containing region. Oxidation of α-synuclein by incubation in the presence of 4% H_2O_2 for 20 min resulted in the oxidation of all four methionines to the sulfoxides, and no other oxidative modifications, as confirmed by mass spectrometry (*45*). In fact, much lower concentrations of H_2O_2 (up to 0.1%, 27 mM) were found to be sufficient to oxidize all the four methionines in the presence of 5 mM NaN_3, about the same concentration that was applied to prevent bacterial growth in protein fibrillation assays.

It is well-known that most flavonoids are potent antioxidants. Then, why does the presence of the flavonoid mediate the oxidation of the protein under the incubation conditions? It was reported that hydrogen peroxide (H_2O_2) is formed during incubation of tea catechins such as epigallocatechin gallate (EGCG) (*62*). EGCG is one of the inhibitory flavonoids found in our screening study (*57*). Therefore, the Amplex Red hydrogen peroxide/peroxidase assay kit was used to monitor the hydrogen peroxide formation during the incubation of inhibitory flavonoids in the absence and the presence of α-synuclein.

As shown in Figure 4A, 100 μM baicalein alone generated H_2O_2 rapidly during the first few hours of incubation. The amount of produced H_2O_2 was stable during the remaining incubation time. On the other hand, for 70 μM α-synuclein alone, a negligible amount of H_2O_2 was generated. For the sample of 100 μM baicalein in the presence of 70 μM α-synuclein, the kinetics of H_2O_2 formation was similar to the flavonoid alone during the first few hours; however, more H_2O_2 was generated and a maximum H_2O_2 concentration was achieved at around 10 h of incubation, corresponding to the middle of the nucleation phase of α-synuclein fibrillation (Figure 4B, open circles). The detected amount of H_2O_2 was decreased gradually during the rest of incubation process, which was most readily explained by the H_2O_2 consumption through the α-synuclein oxidation. When the concentration of baicalein was changed to lower or higher than 100 μM (that is, 30 and 200 μM, respectively), and the concentration of α-synuclein was maintained unchanged, the kinetics of α-synuclein fibril formation were similar to that with 100 μM baicalein; that is, α-synuclein did not form fibrils within 3 days of incubation. The kinetics of H_2O_2 formation was similar to that detected with 100 μM baicalein (Figure 4A); that is, the level of H_2O_2 generated exceeded that from the flavonoid alone, and maximum was reached at about 10 h of incubation, suggesting that α-synuclein could act as a catalyst for H_2O_2 generation.

H_2O_2 formation was also measured for α-synuclein with other flavonoids at the end of the incubation period (Figure 4C). The concentrations of H_2O_2 generated from the protein alone, baicalein alone, and α-synuclein-baicalein, are shown for comparison. In the presence of other inhibitory flavonoids, such as 6-HP, significant amounts of H_2O_2 were generated. On the other

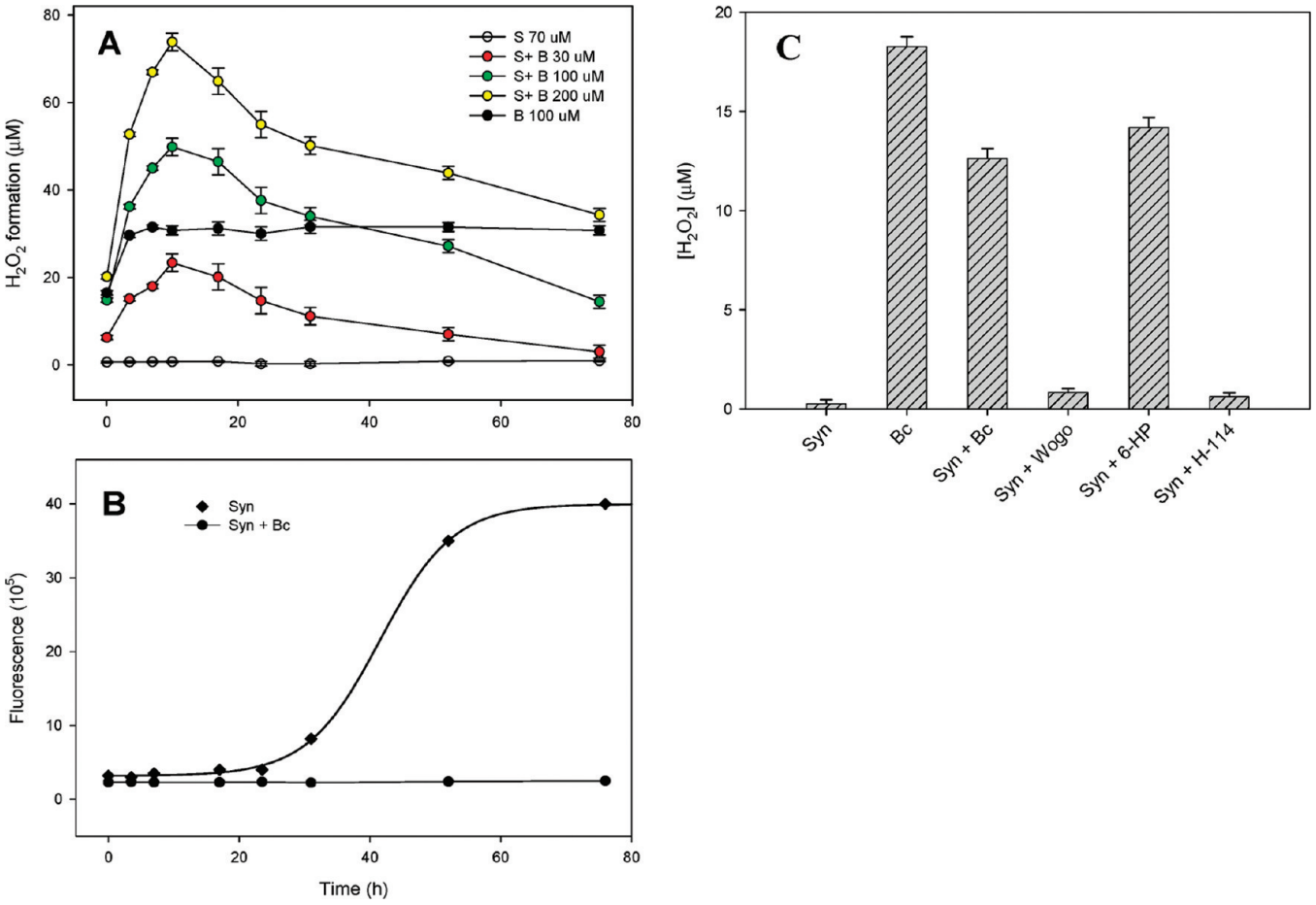

FIGURE 4: Hydrogen peroxide formation during flavonoid oxidation. (A) The kinetics of H_2O_2 formation during the auto-oxidation of baicalein in the absence and in the presence α-synuclein. (B) The kinetics of α-synuclein (70 μM) fibril formation in the absence and in the presence of baicalein at concentrations higher than 30 μM, by ThT fluorescence assays were shown for comparison. (C) H_2O_2 formation during the auto-oxidation of various flavonoids in the presence of α-synuclein at the end of reaction.

hand, in the presence of noninhibitory flavonoids, such as wogonin and H-114, the H_2O_2 formation was negligible. Noninhibitory flavonoids had a low auto-oxidation tendency, as observed by the MS analysis of the aged flavonoid solution, which revealed the predominant peak with the molecular mass of 359 corresponding to the intact compound (Figure 3B, inset). Thus, the amount of H_2O_2 generated was correlated with the oxidation of flavonoids. Not surprisingly, the amount of H_2O_2 generated was also consistent with the degree of Met oxidation in α-synuclein as exemplified with the intact α-synuclein MS peak in Figure 3.

The Effect of Catalase on the Flavonoid-Induced Inhibition of α-Synuclein Fibrillation. Earlier, we established that the fibrillation of α-synuclein at neutral pH was inhibited by methionine oxidation (*45*), although the inhibition mechanism is still not clear. To investigate whether flavonoid-induced inhibition of α-synuclein fibrillation is due to the α-synuclein oxidation, catalase (a well-known H_2O_2 scavenger) was added during the incubation of α-synuclein with baicalein. The presence of 1 μM catalase did not significantly affect either the kinetics of α-synuclein fibrillation or the baicalein-induced inhibition of α-synuclein fibrillation (Figure 5A). However, this amount of catalase was sufficient for both the successful prevention of the H_2O_2 formation during the incubation of baicalein (Figure 5B) and for the prevention of the Met oxidation of α-synuclein (confirmed by MS, data not shown). Since the presence of catalase did not affect baicalein-induced inhibition of α-synuclein fibrillation, Met oxidation in α-synuclein was not a prerequisite for the flavonoid-induced inhibition of the protein fibrillation.

The MS in the lower MW range also showed that the presence of catalase could not prevent the oxidation of baicalein (similar to that shown earlier in the inset of Figure 3A), demonstrating that auto-oxidation of the flavonoid occurs prior to H_2O_2 formation and that oxidized form(s) of the flavonoid is essential for the inhibition of the fibrillation process. In addition, the presence of catalase did not prevent the formation of the flavonoid-stabilized soluble oligomeric forms of α-synuclein, as revealed by SDS–PAGE. In fact, this analysis showed similar levels of monomers, dimers, and oligomers formed in both α-synuclein-baicalein samples, with and without catalase (Figure 5). These results suggested that the stabilized soluble species were a result of the oxidized baicalein (probably the quinone(s)) binding to α-synuclein, leading to the intermolecular cross-linking of modified protein.

The Effects of Radicals and DMPO on the Flavonoid-Induced Inhibition of α-Synuclein Fibrillation. Flavonoids can serve as antioxidants since they are able to reduce highly oxidizing free radicals by the hydrogen atom donation. The aroxyl radical (Fl-O•) may react with a second radical, acquiring a stable quinone structure. However, the aroxyl radicals could also interact with oxygen, generating quinones and superoxide anion, rather than terminating chain reactions (*63*). Thus, H_2O_2 can be generated during the auto-oxidation of the flavonoid (*64*). The presence of H_2O_2 and a reduced metal, such as Fe (II)

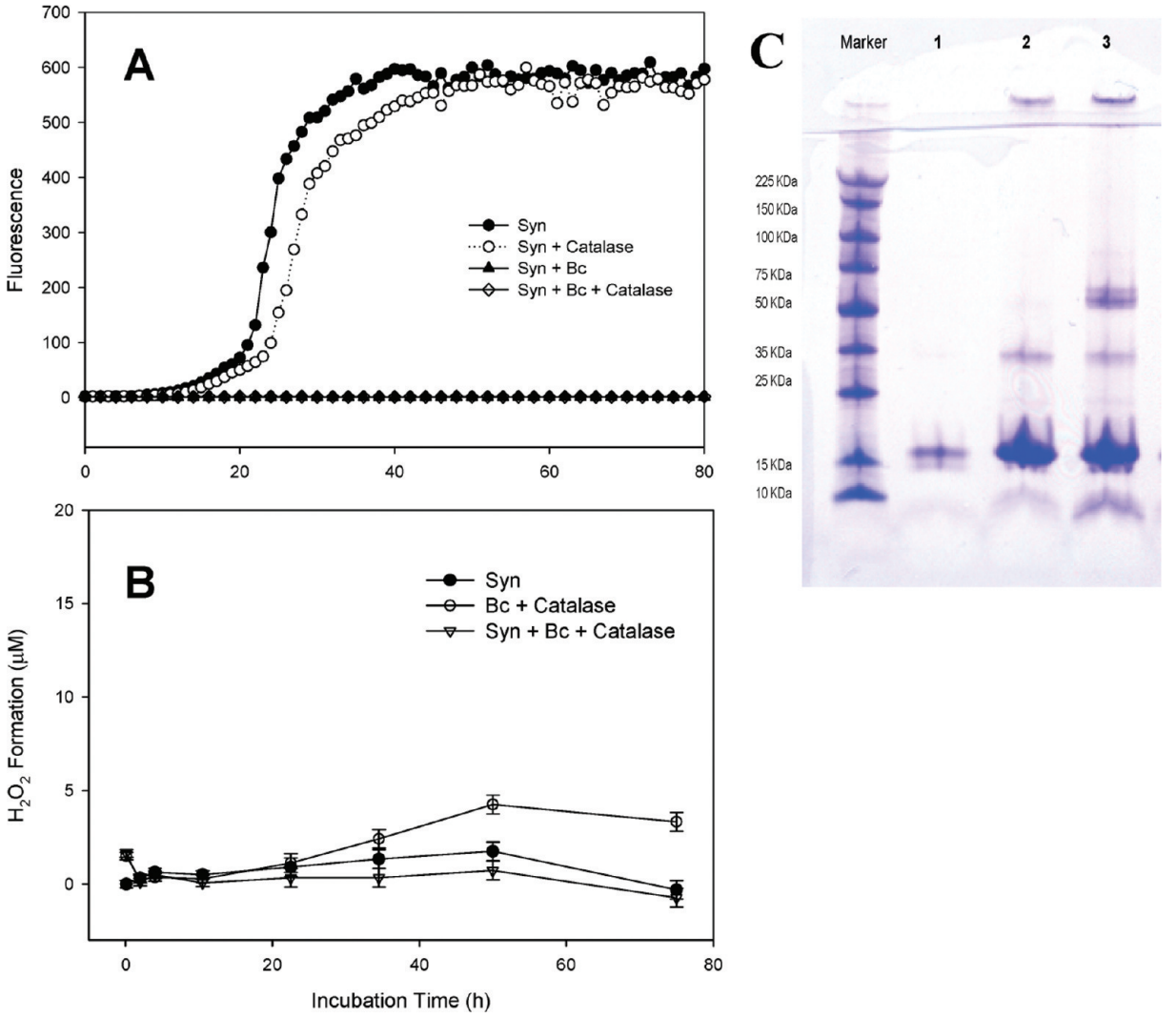

FIGURE 5: Effect of catalase on α-synuclein fibrillation. (A) The effect of catalase on the kinetics of α-synuclein fibrillation and on the baicalein-induced inhibition of α-synuclein fibrillation. Data for the α-synuclein fibrillation in the presence of baicalein and catalase coincide with the data of α-synuclein fibrillation in the presence of baicalein. (B) The corresponding kinetics of H_2O_2 formation (see details in Materials and Methods) during incubation (C) SDS−PAGE of α-synuclein-baicalein incubated in the presence of 1 μM catalase (lane 3). Incubated α-synuclein (lane 1) and α-synuclein-baicalein (lane 2) were shown for comparison. The protein band around 60 kDa for the α-synuclein-baicalein-catalase sample came from catalase.

complex, can further lead to the hydroxyl radical (HO•) production through the Fenton reaction. Trace catalytic metals are ubiquitous in salt and buffer solutions. Typical concentrations of iron are ~1 μM, while that of copper is ~0.1 μM (*65*). These levels of metals can make significant contributions to metal-mediated oxidative pathways.

In order to examine the presence of free radicals that would be generated during the flavonoid auto-oxidation, the effects of the nitrone spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) on the baicalein-induced inhibition of α-synuclein fibrillation was studied (see Figure 6, triangles). The effect of DMPO on the kinetics of α-synuclein fibrillation in the absence of flavonoids is also shown in Figure 6 (circles). The concentration of DMPO, as high as 400 mM, did not have a pronounced effect on α-synuclein fibrillation. However, DMPO weakened the baicalein-induced inhibition of α-synuclein fibrillation. In fact, in the presence of both DMPO and baicalein, α-synuclein was able to successfully fibrillate with a lag time around 20 h (2-fold longer than that of the control). The effective fibril formation in the α-synuclein-baicalein sample incubated in the presence of DMPO was further

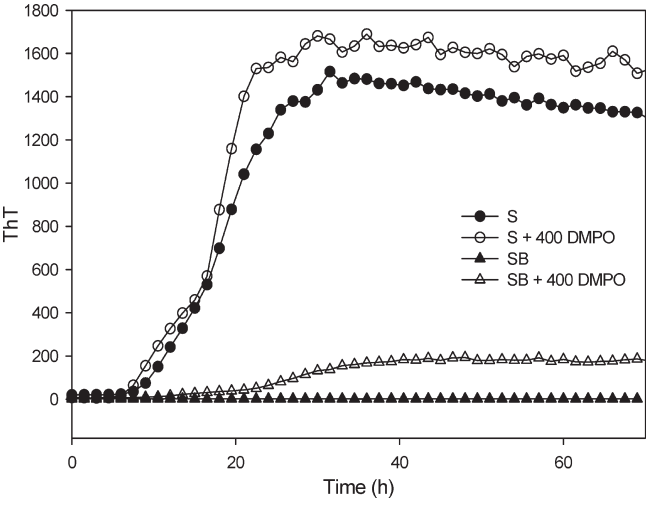

FIGURE 6: The effect of DMPO on the baicalein-induced inhibition of α-synuclein fibrillation (triangles); the effect of DMPO on the kinetics of α-synuclein fibrillation (circles) is shown for comparison.

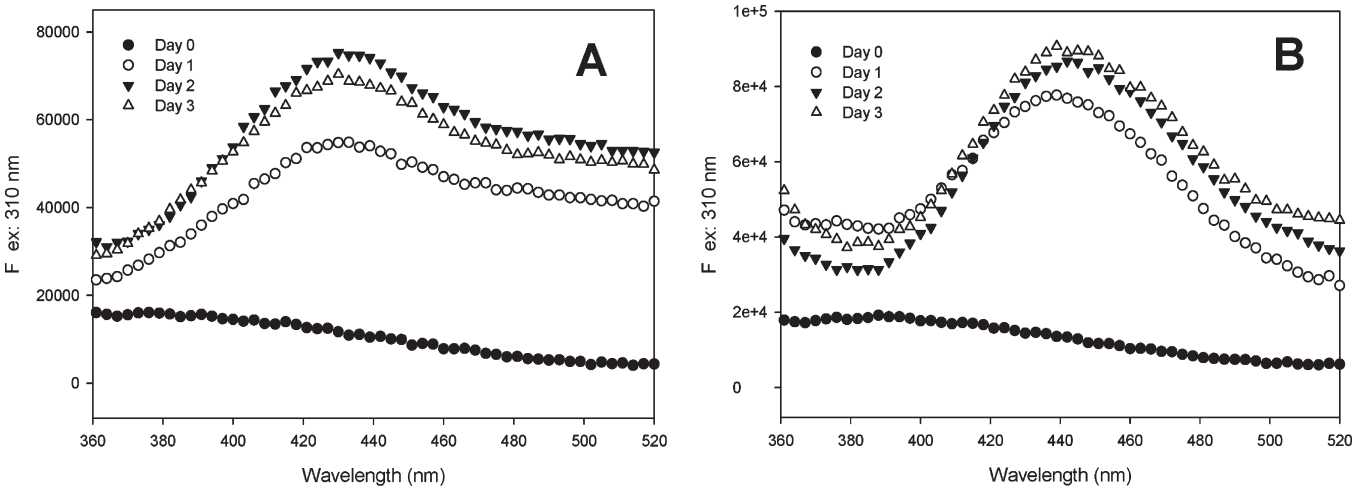

FIGURE 7: Fluorescence emission spectra of α-synuclein and flavonoid complexes formed during 3 days of incubation. (A) α-Synuclein with baicalein. (B) α-Synuclein with 22−324.

confirmed by EM analysis. The fact that the inhibition was not completely abolished by DMPO is most likely due to the short lifetime of the DMPO-radical adduct(s). Furthermore, the presence of DMPO decreased the formation of the flavonoid-stabilized α-synuclein soluble species. In particular, no oligomeric protein bands were detected in SDS−PAGE (data not shown), indicating that formation of free radicals, such as $O_2^{\bullet}$, $HO^{\bullet}$, etc., can be involved in the protein oligomerization. Such radicals are likely to result in the formation of dityrosine or protein-baicalein adduct(s). Interestingly, both L-dopa and dopamine, two drugs used in the treatment of PD, are known to produce free radicals during normal metabolism (*66*, *67*).

Dityrosine Detection During α-Synuclein Incubation with Flavonoids. Dityrosine arises from the protein oxidation and is produced by covalent bond formation between two Tyr side chains involving tyrosyl radicals (*68*, *69*). The reduction potential of the TyrO•/TyrOH couple is 0.88 V, indicating that the coupled reaction needs to be highly oxidative to be thermodynamically favorable. In this regard, superoxide radical if it exists could be one of the species that fulfill this requirement. In order to check for the dityrosine formation, fluorescence emission was measured with excitation at 310 nm during the incubation of α-synuclein and various flavonoids. A typical dityrosine emission peak is around 410 nm with excitation at 310 nm (*70*). In the case of α-synuclein and baicalein, there was no detectable fluorescence at zero time of incubation. However, fluorescence with the maximum at >430 nm was detected after the prolonged incubation. Intensity of this fluorescence increased with time (Figure 7A). Baicalein itself, fresh and aged, did not exhibit any fluorescence at this spectral region (data not shown). The emission peak observed for the incubated α-synuclein-baicalein sample was not consistent with the characteristics typical for the dityrosine fluorescence, indicating the formation of α-synuclein-baicalein adduct(s).

Similarly, in the case of α-synuclein and 22−324 (another strong inhibitor), no obvious fluorescence was observed at zero time of incubation. A noticeable fluorescence with the maximum >440 nm was detected after the prolonged incubation that increased with time (Figure 7B). Interestingly, in the absence of protein, 22−324 itself exhibited strong emission at around 440 nm during the auto-oxidation (data not shown), indicating that α-synuclein was able to partially quench fluorescence of the 22−324 oxidized form(s). The emission peak at 440 nm could correspond to the complex(s) or adduct(s) of α-synuclein and 22−324. Fluorescence emission of α-synuclein incubated with various noninhibitory flavonoids, such as wogonin and 22−323, was also studied in the same way. It was noticed that the emission maximum varied depending on flavonoid and on the binding interaction between the flavonoid and the protein. The results of these studies demonstrated that there was no significant dityrosine formation in the process of the flavonoid-induced inhibition of α-synuclein fibrillation, whereas tyrosine-flavonoid adduct formation may be possible (see Discussion).

Examination of α-Synuclein-Flavonoid Adducts by Mass Spectroscopy and IEF. To analyze the covalent modification of the protein by flavonoids, the α-synuclein-baicalein sample incubated for 3-days was analyzed by ESI-MS. The MS spectrum (Figure 3) revealed a trace amount (less than 5%) of covalently modified protein, with the peak at 14730 ± 2. The low abundance of α-synuclein-baicalein quinone adduct(s) in the sample precluded accurate mass analysis. In order to get a better signal of the α-synuclein-baicalein adduct, ESI-MS was applied for RP-HPLC purified fractions of the incubated sample. The MS spectrum (Figure 9A) reflected an elevated level (~30%) of the covalently modified protein. The adduct peak of 14714 ± 2, about 16 less than the sum of the molecular masses of α-synuclein and baicalein, was attributed to the covalent modification through Schiff base between the amine group(s) of α-synuclein and the baicalein quinone(s). The peak of 14732 ± 2 could be attributed either to imine formation between Met-oxidized α-synuclein and baicalein quinone(s) or to Michael addition between the protein and the quinone. However, the precise determination of the mechanism of adduct formation was complicated by the fact that the mass difference arising from the two addition mechanisms and that from Met oxidation (s) is very close. One of the explanations for the relatively low levels of covalent modifications is that the modifications (e.g., Schiff base) are reversed and readily lost under the acidic conditions of the mass spectrometry. This was verified by running MS on $NaBH_4$ treated α-synuclein baicalein after incubation. Figure 9B shows a robust increase in the adduct level (~70%) with the peak around a mass of 14730 ± 2, suggesting Schiff base formation.

Since the MS results revealed that covalent modification occurred via imine (Schiff base) formation and/or Michael addition, IEF gels were run to further determine if the possible binding site(s) of the inhibitory flavonoid on α-synuclein were

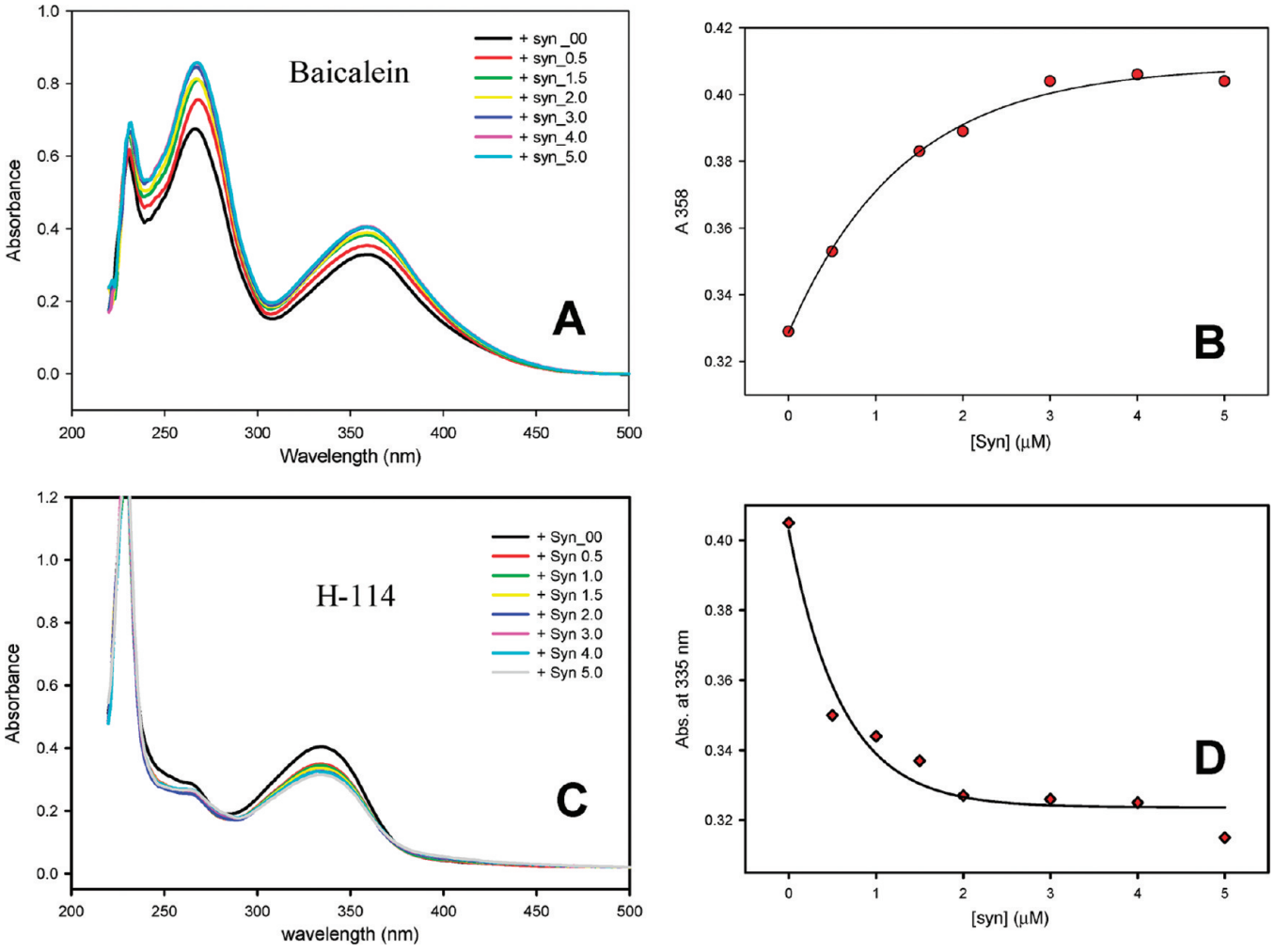

FIGURE 8: UV−vis absorbance of the flavonoid (A, baicalein; C, H-114) titrated with α-synuclein. Changes in λ_{max} of the flavonoid as a function of α-synuclein concentrations (B, baicalein; D, H-114).

amine group(s) in the protein or not. The IEF gel (Figure 9C) showed a clear dark band for the monomer protein control sample at 4.67, which corresponds to the α-synuclein pI value. For the α-synuclein samples incubated in the presence of baicalein, there were two more bands around 4.5 and 4.4, suggesting that the baicalein quinone(s) form Schiff base with at least two amine groups in the protein (most likely those of the Lys residues). Similar results were also seen for the α-synuclein samples incubated with several other inhibitory flavonoids. Note, for α-synuclein monomer, the minor bands, the top band on IEF gel (lane 4) at pI ~ 5.8 and the lower bands between 4.55 and 4.6, resulted from products of proteolysis occurring during the storage, corresponding to the minor bottom band at ~10 kDa on SDS−PAGE (lane 1). The fragments with the increased pIs can correspond to the C-terminally truncated fragments of α-Syn, which show the increase in pI because C-terminal is rich in acidic residues. Alternatively, some of these bands could correspond to the reaction adducts with the pI changed.

Identification of α-Synuclein Sites of Flavonoid Attachments. ESI-MS was run after trypsin digestion on the incubated sample of α-synuclein in the presence and in the absence of baicalein (Figure 10). Major peaks of the unmodified α-synuclein were labeled as the corresponding peptide fragments (see the labeling rules in the figure legend). In this way, the intensity of m/z peak at 560.2 ± 1.0 (P9) appeared to be the one with least change when the superimposed spectra of the two digested samples were compared, indicating no modification on the fragment P9. On the other hand, several peaks of the spectrum of α-synuclein-baicalein sample exhibited a dramatic decrease in the intensity (red) compared with that of unmodified protein (black). The most significantly affected peptide fragments include Ak.P3, P3_P4, P5+TK, P6+TK, P10, which are listed in the right side of Table 3. Several new peaks appeared in the spectrum of digested α-synuclein-baicalein sample (red). In the lower m/z field of Figure 10, the peak at 267.2 ± 1.0 corresponds to the oxidized baicalein quinone, and two other new peaks at 341.2 ± 1.0, 359.1 ± 1.0 are most likely from baicalein derivatives. Figure 10 also shows the decrease in the intensity of m/z peak at 1430.1 ± 1.0, accompanied by the appearance of two new peaks at 1435.4 ± 1.0 and 1440.6 ± 1.0 that can be due to the oxidation of the two Met residues at the C-terminus (P10) of the protein.

Among all the new peaks listed in Table 3, the most pronounced one at m/z 684.7 ± 1.0 was attributed to the fragment P3.TK modified with baicalein quinone (Figure 10). The appearance of this peak correlated with a more than 50% decrease in the intensity of peaks at 536.7 ± 1.0 and 1072.7 ± 1.0, both of which correspond to the fragment AK.P3. Two other new peaks, one at 976.7 ± 1.0, the other at 1090.1 ± 1.0, were also estimated to be P3.TK related fragments with modification by baicalein quinone. Therefore, Lys 21 was estimated to be the target-binding sites of the highest probability on α-synuclein by baicalein quinone(s).

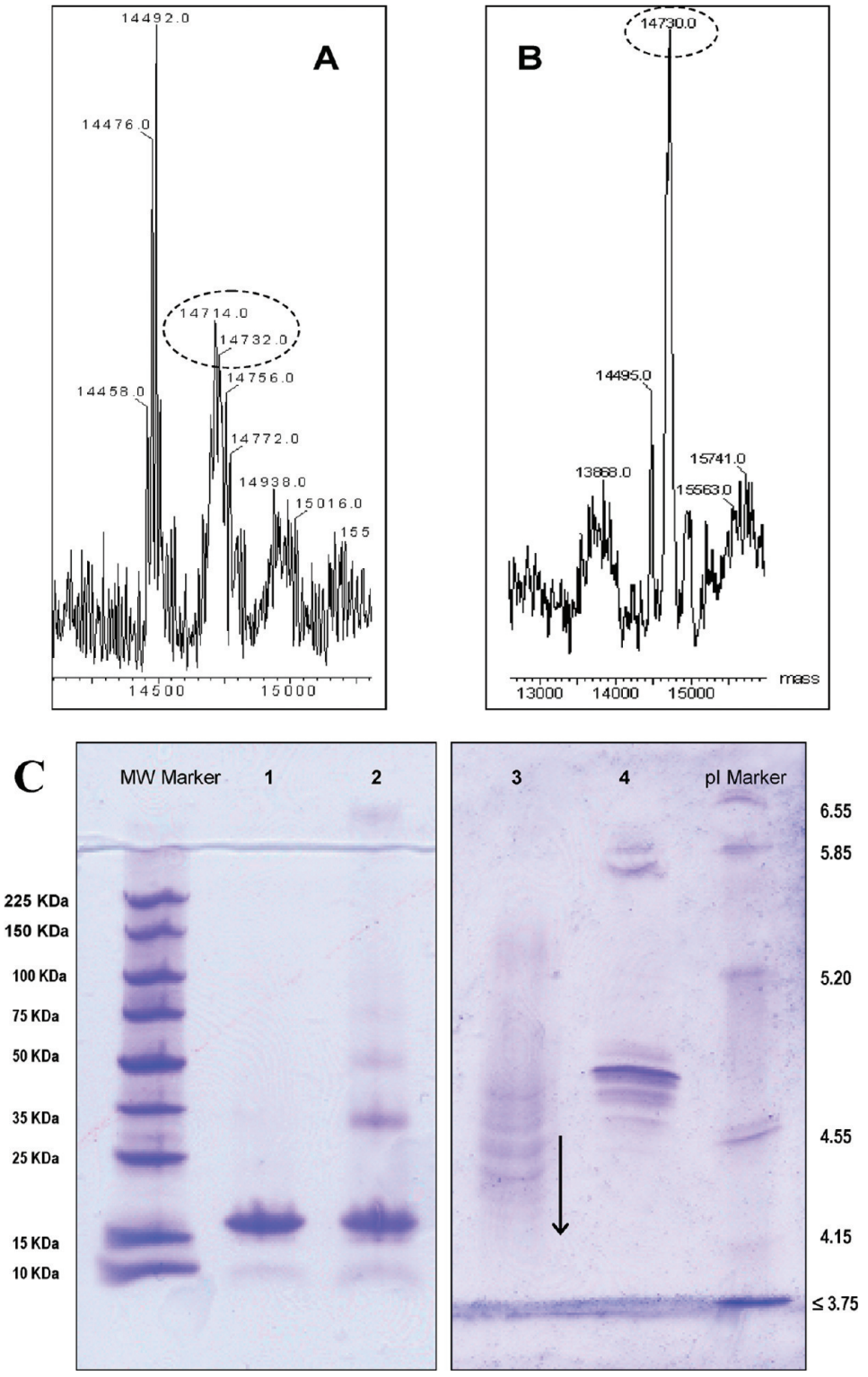

FIGURE 9: The MS spectra of the incubated α-synuclein-baicalein after RP-HPLC purification (A) and after $NaBH_4$ treatment (B), respectively. (C) IEF gel (right) on the incubated α-synuclein-baicalein (lane 3), α-synuclein monomer (lane 4); SDS−PAGE on the same sample is presented for comparison (left), α-synuclein monomer (lane 1) and the incubated α-synuclein-baicalein (lane 2).

Similarly, all other new peaks and the peaks with significantly decreased intensity were listed in Table 3 with font in bold to emphasize either the well-correlated changes or the key peptide(s) that were involved in the modification. The possible binding site(s) and mechanism of addition(s) on the protein were also listed through calculations of the estimated m/z values. In this way, several other Lys residues were also proposed to be involved in the modification, including Lys 23, 43, or 34 in an order of decreasing probability. Besides the ε-NH_2 group on the side chain of lysine residues, several new peaks (1098.7 ± 1.0, 1616.1 ± 1.0, 1441.7 ± 1.0) are attributed to the adduct formation between baicalein quinone(s) and α-NH_2 group at the N-terminus of the protein. Considering α-NH_2 has a pK_a value of 9.28, lower than that of ε-NH_2 (10.54), the involvement of α-NH_2 in the modification is not surprising. Interestingly, two of the new peaks with m/z at 1098.7 ± 1.0 and 1616.1 ± 1.0, could be attributed to

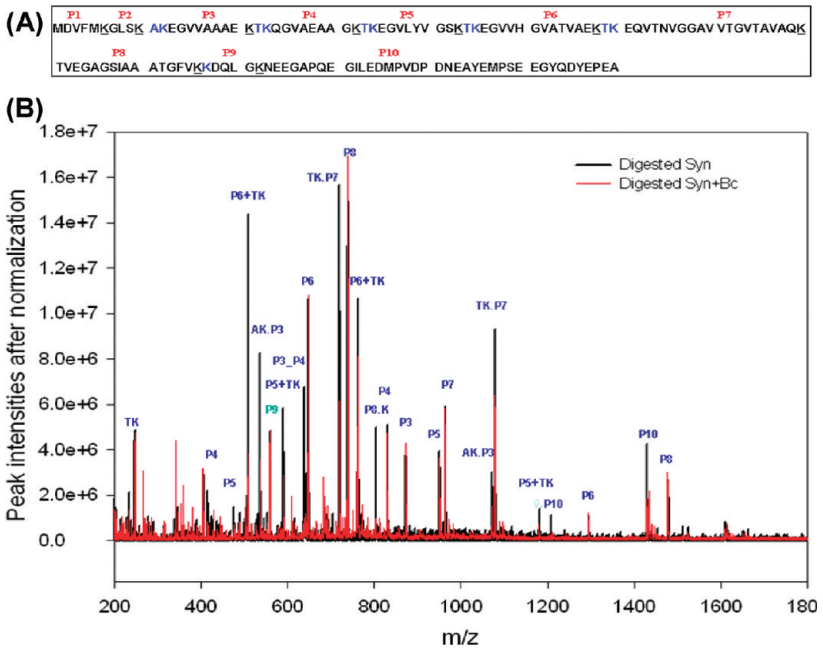

FIGURE 10: The MS spectra of α-synuclein-baicalein digested with trypsin. (A) α-Synuclein sequence with peptide fragments of more than 3 a.a. residues numerically labeled. Peak labeling of the fragments with missed cleavage site(s) follows the rules: Pn.TK represents that the position of TK (the 2 a.a. residues) are behind the peptide Pn in the protein; Pn+TK, the position of TK can be either before or after Pn; Pn_Pm represents the fragment containing Pn up to *Pm*. (B) Comparison of the MS spectra of incubated α-synuclein-baicalein (red) and α-synuclein (black) alone after trypsin digestion. Peak intensities are normalized according to the concentration of α-synuclein initially used in each of the sample. Major peaks of the spectrum of unmodified α-synuclein were labeled as the corresponding peptide fragments.

the cross-linking of two N-terminal peptide fragments (P1 or P1.P2) with one molecule of baicalein quinone. Furthermore, the peak at 1098 ± 1.0 was proposed to arise from intermolecular cross-linking between P1 and P1.P2, via baicalein quinone through formation of two Schiff bases. Since protein−protein cross-links could be formed also by the interaction of amine groups from two different protein chains with the carbonyl group (C═O) of the quinone(s), modification in this way probably contributed to the baicalein stabilized dimer and oligomer of α-synuclein.

Besides the amine groups in α-synuclein, the MS results also showed that Tyr 39 is involved in the covalent modification, as observed with more than 50% decrease in the intensity of peaks related to fragments P5, P5+TK, and the appearance of a new peak at 610.1 ± 1.0, corresponding to the baicalein quinone modified P5 fragment via Michael addition. Given the comparable pK_a value of the side chain of Lys (10.54) and Tyr (10.46), it is possible that Tyr acts as a good nucleophile as Lys. Taken together, several target binding sites of baicalein quinone(s) on α-synuclein can be proposed, including ε-NH_2 on Lys 21 (23, 43, or 34 with less probability), α-NH_2 at the N-terminus, and Tyr 39.

Determination of Equilibrium Dissociation Constants (K_d) of α-Synuclein−Flavonoid Complexes. UV−vis spectrophotometric titration experiments were carried out to measure the dissociation constants (K_d) for the α-synuclein-flavonoids complexes. The binding of baicalein to α-synuclein resulted in characteristic changes in the absorbance spectrum of the compound. In particular, an increase in the intensity at 358 nm was clearly observed. Thus, by spectral titration, the binding of baicalein to α-synuclein had a K_d of 0.80 ± 0.05 μM (Figure 8A, B). The K_d value was confirmed by Tyr fluorescence quenching assay (K_d = 0.78 ± 0.03 μM) (Figure S2, Supporting Information). Interestingly, for some flavonoids the formation of flavonoid−α-synuclein binding complex resulted in the increase of the flavonoid extinction coefficient, whereas for others results in the decrease the flavonoid extinction coefficient. This finding reflects the difference in $\pi-\pi^*$ electronic transitions depending on flavonoid structure and protein binding site environment. For example, for the noninhibitory flavonoid H-114, a decrease in the intensity of UV absorbance at 335 nm was observed after the addition of protein. The interaction of α-synuclein with this flavonoid was shown to be characterized by K_d of 0.42 ± 0.05 μM (Figure 8C,D).

Table 1: Dissociation Constants (K_d) of α-Synuclein and Flavonoid Complexes

inhibitory activities	names of flavonoid	appr K_d (SD ± 0.05 μM)
strong	baicalein	0.80
	6-HP	0.48
	22-324	0.38
good	quercetin	0.65
	D-406	0.25
	D-116	0.60
weak	22-344	0.62
	D-258	0.30
	22−336	0.35
noninhibitors	kaempferol	0.60
	D-407	0.50
	H-114	0.42

Table 1 lists the values of dissociation constants (K_d) for complexes of α-synuclein with flavonoids with different inhibitory activities. The spectral titration of flavonoids with α-synuclein indicated that α-synuclein binds the flavonoids with the dissociation constants in the submicromolar range. The similarity in binding is expected because all flavonoids have a very similar structure (Figure S3, Supporting Information) and the differences in binding are attributed to the modifications of the flavone rings.

Table 2: Effect of Inhibitory Flavonoids on the Kinetics of the Two Truncated and Triple Mutant Y125F/Y133F/Y136F of α-Synuclein Compared with that of WT α-Synuclein[a]

	WT		Syn (30−140)		Syn (1−103)		Y125F/Y133F/Y136F	
	control	+ FL	control	+ FL	control	+ FL	control	+ FL
lag time (hours)	10−15	NA	∼20	NA	∼5	10−60	∼15	20−30
ThT intensity	100%	neg	∼10%	neg	∼5%	∼1%	∼100%	10−30%

[a] Note: for WT and NT α-synucleins, NA means no lag time was detected in 3 days of incubation and the intensity of ThT fluorescence was negligible.

Table 3: Comparison of the Peak Changes in the MS Spectra of Digested α-Synuclein in the Presence and Absence of Baicalein

detected new mlz (±1.0)	estimated *m/z* (±1.0)	involved peptides	target binding site(s)	mechanism of addition	decreased mlz (±1.0)	involved peptides
684.7	(1102.6 + 269)/2 = 685.8	**P3.TK**	**K21**	**M**	**536.7, 1072.7**	**AK.P3**
976.7	(1687 + 259)/2 = 978	P2.AK,**P3.TK**	**K21** or K10 or K12	**M**		
1090.1	(1914.0 + 269)/2 = 1091.5	**P3_P4**	**K21** or K23	**M**	**639.1** 415.7, 830.4	**P3_P4** P4
759.4	(1059.5 + 269 + 951.5)/3 = 760	P4 + TK and PS	K23 or K32 with Y39	cross-linking		
610.1	(951.5 + 269)/2 = 610.3	**P5**	**Y39**	**M**	**476.1**, 951.6	**P5**
1448.0	1180.6 + 269 = 1449.6	**P5** + **TK**	K34 or **K43**	**M**	**590.8**, 1180.6	**P5** + **TK**
1435.4	1180.6 + 269 − 16 = 1433.6 and/or	P5 + TK and /or	K34 or **K43Y39**	**S**	**590.8**, 1180.6 and /or	**P5** + **TK** and/or
	(4286.7 + 16)/3 = 1434.2	**P10**_1 M-ox	-----	-----	**1430.1**; 1208.6	**P10**; P9.P10
984.2	(2686.5 + 269)/3 = 985	P5.TK, **P6.TK**	**K43** or K45 or K58	M	1180.6 **509.0**, 763.0	P5 + TK **P6** + **TK**
1441.7	1171.6 + 269 = 1440.6 or 1187.6 + 269 − 16 = 1440.6	**P1**.P2_1M-ox or **P1.**P2_2M-ox	**α-NH2** or K6	**M** or S	578.3	P1.P2
1440.6	(4286.7 + 32)/3 = 1439.6	**P10**_2 M-ox	-----	-----	**1430.1**; 1208.6	**P10**; *P9*.P10
1098.7	(786.4 + 1171.6 + 269 − 32)/2 = 1097.5	**P1**_1M-ox and **P1.P**2_i M-ox	**α-NH_2** with **α-NH_2** K6	**S & S** intermolecular cross-linking	578.3	P1.P2
1616.1	(1386.7 + 1606.9 + 269 − 32)/2 = 16515.3	**P1**.P2.AK_2 M-ox and **P8.K**	**α-NH_2** with K96	**S & S** intermolecular cross-linking	578.3 804.0	P1.P2 **P8.K**

Examination of α-Synuclein-Flavonoid Interactions Using α-Synuclein Mutants. α-Synuclein mutants were used to clarify which regions of the protein are important for the flavonoid-induced inhibition of α-synuclein fibrillation. To this end, truncated mutants, the N-terminal truncated Syn (30−140), two C-terminal truncated Syn (1−103; 1−122) and triple Y125F/Y133F/Y136F mutant were analyzed. Table 2 represents the effect of inhibitory flavonoids on the fibrillation kinetics of α-synuclein mutants compared with that of the WT protein.

In comparison with the WT α-synuclein, Syn (30−140) formed fibrils with a similar lag time but with much lower ThT fluorescence intensity (∼10%). On the other hand, Syn (1−103) formed fibrils with a much shorter lag time (∼5 h) than WT α-synuclein, and ThT fluorescence intensity was even lower, (< 5%) as compared with the WT protein. The EM image of the incubated samples showed a plenty of fibrils, suggesting that the lower ThT fluorescence signals result from an altered binding between ThT and fibrils made of the N- or C-terminal truncated synuclein, leading to a lower quantum yield of the fluorophore rather than less amount of fibril formation.

In the presence of baicalein and other strong inhibitors of the WT α-synuclein fibrillation, the fibrillation of the Syn (30−140) was also inhibited. This clearly showed that the lack of the N-terminus did not affect the flavonoid-induced inhibition of the protein fibrillation, suggesting that N-terminus was not crucial for the inhibitory interaction between the protein and the flavonoid. However, the inhibitory effects of the same flavonoids on the fibrillation of Syn (1−103) were significantly weakened. This was shown by the increase in ThT fluorescence and the decrease in lag-times, as well as by the detection of the well-grown fibrils by EM. For example, in the presence of eriodictyol and T-415, the lag time was only ∼10 h; in other words, the inhibition was almost completely abolished. In the presence of baicalein, the lag time was ∼60 h. Similar results were obtained on another C-terminally truncated mutant, Syn (1−122), indicating that the C-terminus of α-synuclein plays an important role in the inhibitory interaction between the protein and the flavonoids. The fibrillation kinetics of the N- and C-terminally truncated mutants in the presence or in the absence of flavonoids are shown in Figure S4 (Supporting Information).

Given the fact that both of the C-terminally truncated α-synuclein mutants (1−103 and 1−122) lack three tyrosine residues (125, 133, 136), and that the flavonoid-induced inhibition of fibrillation of these mutants was much weaker than that of the WT protein, the three tyrosine residues were proposed to be involved in the inhibition of the WT α-synuclein fibrillation induced by the flavonoids. To examine this hypothesis, the fibrillation of a triple mutant of α-synuclein, Y125F/Y133F/Y136F, was analyzed (Table 2). In the absence of flavonoids, this mutant formed fibrils with kinetics similar to that of the WT α-synuclein. Importantly, the inhibitory effects of baicalein and other strong inhibitors of the WT α-synuclein fibrillation on the fibril formation of the triple mutant were almost completely abolished (Table 2). The presence of the mature fibrils in the Y125F/Y133F/Y136F mutant samples incubated with flavonoids was also confirmed by EM analysis. These findings strongly indicated that the C-terminal tyrosine residues contributed to the flavonoid-induced inhibition of WT α-synuclein fibrillation. To verify what role of Tyr residues is in the inhibition of fibrillation by flavonoids, we determined K_d of α-synuclein

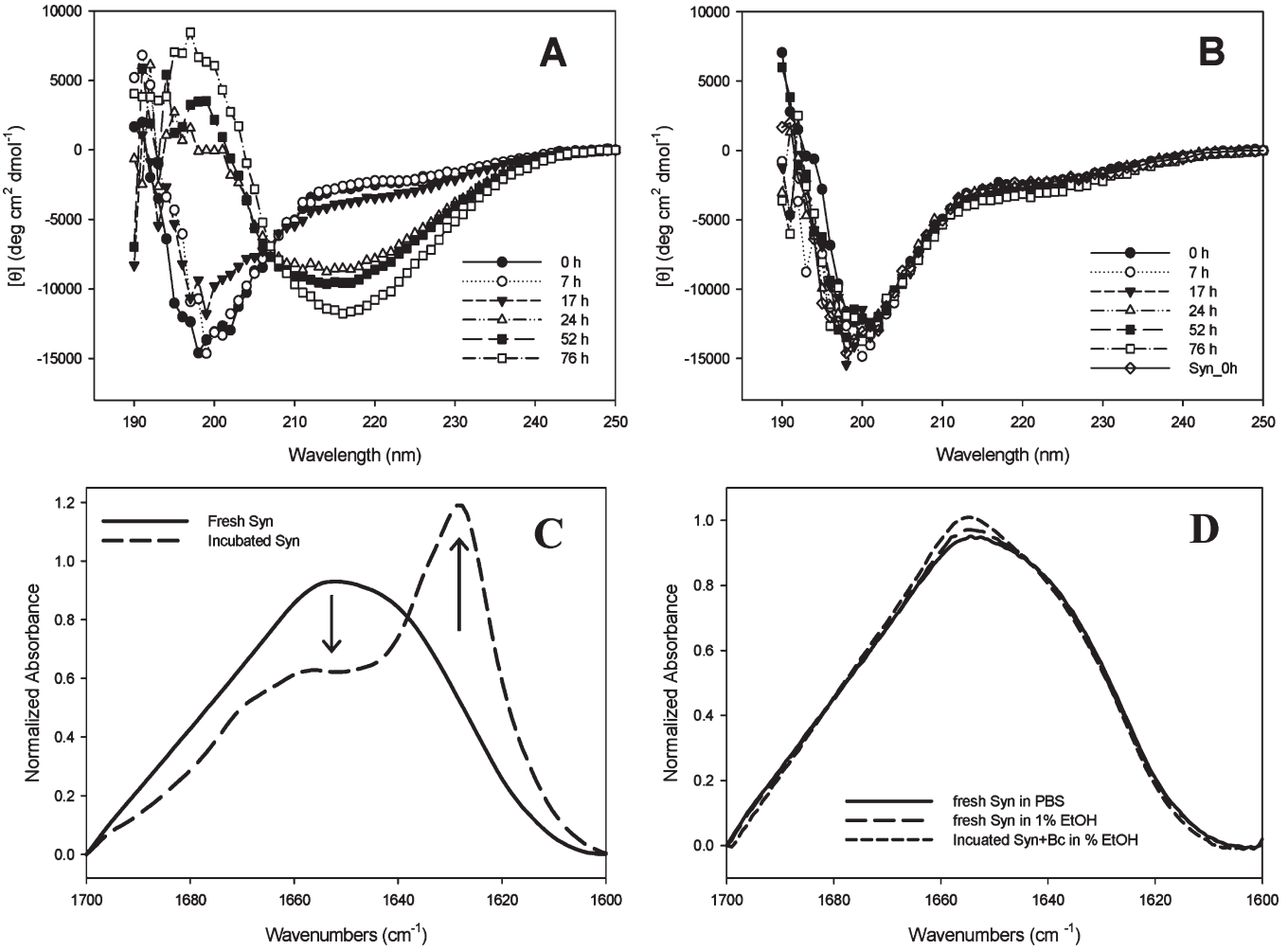

FIGURE 11: Secondary structure analyses of α-synuclein in the presence of baicalein. (A, B) The far-UV CD spectra of 70 μM α-synuclein in the absence (A) and in the presence (B) of 100 μM baicalein (in EtOH) during 3 days of incubation. (C, D) The ATR-FTIR spectra of α-synuclein in the absence (A) and in the presence (B) of baicalein (1% EtOH) before and after incubation.

Y125F/Y133F/Y136F−baicalein complex, which was twice lower than the K_d of WT−baicalein complex (Figure S5, Supporting Information).

Structural Characterization of the Baicalein-Stabilized Species. Since α-synuclein is an intrinsically disordered protein, circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopies were used to determine the structural change in the protein associated with the flavonoid binding. As DMSO is the standard solvent for preparing stock solutions of compounds for drug discovery, all the samples for structural analysis were originally prepared in PBS solution containing 1% DMSO. However, the presence of DMSO alone had a very strong contribution to both far-UV CD and FTIR spectra. Therefore, in our structural studies we utilized low percentage of ethanol (1−2%) instead of DMSO. These low ethanol concentrations did not interfere with the spectral measurements. More importantly, the addition of 1−2% ethanol did not affect the kinetics of α-synuclein fibrillation in the absence or in the presence of baicalein (data not shown).

Samples of α-synuclein incubated without or with baicalein were taken at 0, 7, 17, 24, 52, and 76 h of incubation. The far-UV CD spectrum of freshly prepared α-synuclein in PBS containing 2% EtOH exhibited characteristic features typical of an essentially unfolded polypeptide chain; that is, an intense minimum in the vicinity of 196 nm, with the absence of characteristic bands in the 210−230 nm region (Figure 11A, black circles). The shape of the far-UV CD spectrum changed during incubation (0−76 h), especially at 197 and 218 nm, reflecting a gradual transition from the natively unfolded structure via a set of partially folded structures to a structure with significant β-sheet (Figure 11A, open squares). On the other hand, no significant changes were observed in the far-UV CD spectra of α-synuclein incubated with 100 μM baicalein (2% ethanol), suggesting that baicalein stabilizes α-synuclein in its natively unfolded conformation (Figure 11B).

FTIR represents a powerful method for the investigation of protein secondary structure. The main advantage of this approach in comparison with CD is that FTIR is much more sensitive to β-structure. Attenuated total reflectance FTIR and hydrated thin films can be applied for samples with low protein concentrations (*71*). The FTIR spectra for α-synuclein incubated alone showed a characteristic transition from the natively unfolded structure to a structure with significant β-sheet (Figure 11C). Consistent with the results from the CD measurements, no significant structural changes were observed for α-synuclein incubated in the presence of baicalein (Figure 11D), further supporting the hypothesis that baicalein stabilized natively unfolded conformation of α-synuclein. When higher concentration of baicalein was used, some structural changes were observed (Figure S6, Supporting Information) corresponding to

the decrease of unfolded and α-helical conformation and the increase of loops and turns. The peak at 1625 cm^{-1} can be attributed to Schiff base formation rather than β-sheet formation at least partially because it disappears in low pH.

DISCUSSION

Flavonoid Inhibition of α-Synuclein Fibrillation via Quinone Adduct Formation. The molecular mechanism underlying the inhibition of α-synuclein fibrillation induced by the flavonoid was thoroughly examined using various biochemical and biophysical probes. Polyphenols, such as baicalein and other flavonoids, are reductive substances that are readily oxidized to quinones by oxygen, while the quinones are very reactive that can react with the side-chain amino groups of proteins. Most flavonoids which effectively inhibited the α-synuclein fibrillation had a half-life of oxidation in the range of a few hours under the usual incubation conditions, depending on the individual structure. The major oxidation products were the corresponding quinones. Other derivatives included radical-induced polymerized and degraded products, which were detected through RP-HPLC (Min Zhu, unpublished data).

A free radical spin trap DMPO weakened baicalein-induced inhibition of α-synuclein fibrillation and decreased the formation of soluble flavonoid-modified protein species. In particular, no SDS-resistant oligomers were detected in the SDS−PAGE experiments, indicating that free radicals (such as O_2•, HO•, flavonoid radicals, etc.) generated during the flavonoid auto-oxidation were involved in the protein oligomerization. Such radicals were likely to result in the formation of dityrosine or protein-baicalein adduct(s). A typical dityrosine emission peak has a maximum at around 410 nm with excitation at 310 nm (*68*, *69*). However, the incubated α-synuclein-inhibitory flavonoid samples exhibited an emission peak at a wavelength >430 nm, suggesting formation of α-synuclein-flavonoid adduct(s).

The fact that 2 h preincubated baicalein exhibited the strongest inhibitory effect, as well as the fact that under anaerobic conditions baicalein-induced inhibition was much weaker, demonstrated that the oxidized product(s), that is, the flavonoid quinone(s), played an important role in the inhibition of α-synuclein fibrillation. Thus, auto-oxidation of the flavonoid was required for the inhibition of α-synuclein fibrillation. The weaker inhibition induced by more aged flavonoid (i.e., aged for 10, 24, or 76 h) was most likely due to the presence of other flavonoid derivatives generated during the prolonged incubation and due to the disappearance of quinones.

The H_2O_2 formation was detected during the auto-oxidation of the inhibitory flavonoids, which would be expected to result in α-synuclein oxidation. A considerable amount of Met oxidation in α-synuclein was indeed detected by MS during the incubation with the flavonoids. Catalase (a H_2O_2 scavenger) prevented the oxidation of α-synuclein but did not affect the inhibitory effect of the flavonoids, demonstrating that flavonoid-induced inhibition of α-synuclein fibrillation is independent of Met oxidation in α-synuclein. The fact that catalase did not prevent the auto-oxidation of the flavonoid or the formation of the flavonoid-stabilized soluble α-synuclein species suggested that the stabilization was likely due to the binding of the flavonoid quinone to α-synuclein leading to the intermolecular cross-linking of the modified protein. This was confirmed by MS and IEF results, revealing the adduct formation between baicalein quinone and NH_2 groups of the protein.

The covalent modification of the protein by the flavonoid quinone mostly corresponds to the formation of Schiff base, which is not stable and disappear in acidic pH as confirmed by MS and FTIR, whereas MS spectra on the $NaBH_4$-pretreated sample revealed an enhanced amount of the α-synuclein-quinone adducts, additionally proving Schiff base formation. However, some adduct peaks could also result from Michael addition. In some cases, the precise determination of the mechanism of adduct formation was complicated by the fact that the mass differences arising from the two addition mechanisms and that from the methionine oxidation were identical. The IEF gel of the incubated sample suggested that 2−3 amine groups in the protein were involved in the covalent modification. MS of trypsin digested baicalein-treated α-synuclein samples revealed several modification sites corresponding to Lys 21, the N-terminal or α-amino of Met 1, Tyr 39, and Lys 23, 43, and 34. The fact that no covalent modifications in α-synuclein were observed for incubated samples with noninhibitory flavonoids further confirmed that covalent modification of the protein played an important role in the inhibition. Our results parallel the finding of other researchers that polyphenols and dopamine reacts with synuclein or other proteins through quinone adduct formations (*50*, *51*, *60*).

Interactions of Flavonoids with α-Synuclein Mutants. To clarify which region of the protein might be involved in the flavonoid binding and the inhibition of protein fibrillation, fibrillation of several α-synuclein mutants was analyzed. ThT fluorescence analysis showed that in the presence or in the absence of flavonoids the N-terminally truncated Syn (30−140) exhibited fibrillation kinetics similar to that of the WT protein. This suggested that the N-terminus did not affect either the amyloidogenic property of the protein or the flavonoid-induced inhibition of the protein fibrillation. On the other hand, the flavonoid-induced inhibition was almost completely abolished in the fibrillation of the C-terminally truncated Syn (1−103) and Syn (1−122), suggesting that the C-terminus of the protein played a role in the interaction with the compounds (Table 2). Because the C-terminally truncated α-synuclein mutants formed fibrils fast, it can be argued that inhibition by flavonoid may not be detected. Therefore, we examined the effects of flavonoids on the full-length triple Y125F/Y133F/Y136F mutant that in the absence of flavonoids had very similar fibrillation kinetics to WT Syn. The choice of this mutant was also dictated by the possible role of Tyr in flavonoid binding either through stacking or H-bonding. The binding of baicalein to the mutant was weakened ($K_d \sim 1.2$ μM versus 0.7−08 μM for WT). The small changes in K_d were likely due to the preservation of stacking interactions between the flavonoid and Phe residues. In contrast to the small K_d changes, the inhibition of fibrillation was completely abolished in this triple Tyr mutant. The lack of inhibitory effects of flavonoids on the fibrillation of triple Y125F/Y133F/Y136F mutant in comparison with the fibrillation of the WT protein proves that the C-terminal Tyr residues of the protein contributed to the flavonoid-induced inhibition of α-synuclein fibrillation and H-bonding was important for inhibition. Because H-bonding was not essential in the initial binding of the flavonoid, it may be required for other steps in the inhibition such as later binding events, catalysis (covalent modification by flavonoids), or folding.

Mechanism of Flavonoid Inhibition of α-Synuclein Fibrillation. It has to be emphasized that inhibition by flavonoids should be distinguished from a simple binding as well as a nonspecific modification by quinones or radicals. On the basis

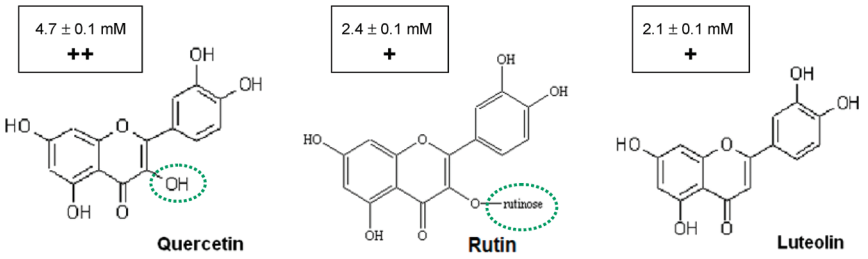

FIGURE 12: The influence of the antioxidant activity on the flavonoid induced inhibition of α-synuclein fibrillation. The comparison is made among the three flavonoids, with the inhibitory activity and the reported VEAC value listed beside the structure.

of the results presented in the paper, we argue that the inhibition by a flavonoid is a complex process and involves several successive steps. The first step is a noncovalent binding of a flavonoid at a specific binding site. Since monomeric α-synuclein has a mostly unfolded structure it can readjust conformation of the binding site depending on the precise structure of a flavonoid. We showed that all flavonoids examined (Table 1 and Figure S3, Supporting Information) have K_d values in the range of 200−800 nM. This is a very strong binding considering the fact that the protein is mostly unfolded. Differences in K_d values can be explained by variation in the position of the flavonoid hydroxyl groups: when 3′-OH is located in phenyl ring it results in higher affinity, whereas 3-OH in benzopyrone decreases binding. Unfortunately, the binding is not detectable by FTIR or CD which are not sensitive to local structural changes and/or these structural changes are minimal. Considering the ring-like shape of α-synuclein conformations detected by NMR (*72*), the binding of flavonoids may occur in the C-terminal region that interacts with the N-terminal part, and thus minimal structural changes would be required. Because there is no correlation between K_d value and inhibition, other steps are required for inhibition to occur. The second and the third steps of inhibition are the restricted oxidation of flavonoids (quinone formation) and the reversible (Schiff base) covalent modification of Lys 21 (and to a lesser degree Lys 23, 34, 43) or irreversible Michael addition to Tyr 39. Two vicinal OH groups are required for quinone formation and attachment to α-synuclein. The increase of production of hydrogen peroxide in the presence of α-synuclein indicates that protein (or protein-residual metal complex) catalyzes the reaction. The oxidation reaction by hydrogen peroxide also involves radical production and may result in other mechanism of flavonoid attachments to the protein. However it can be considered as a minor site reaction because the addition of catalase does not affect the flavonoid-induced inhibition of α-synuclein. At this point, we cannot distinguish how precisely inhibition occurs: the modification of Tyr 39 or Lys 21(23, 34, 43) may stabilize the unfolded conformation, or preclude fibril assembly. Most of bulky group modification of the N-terminal region and especially the central part of the protein abolish fibril formation and leads to the accumulation of oligomers (L. Munishkina, unpublished results). Bulky groups may disfavor the fibril core beta-sheet formation through steric hindrance. Our results on identification of regions involved in either the attachment of flavonoids (MS of trypsin digested protein-baicalein adducts) or flavonoid inhibition of α-synuclein fibrillation (mutant studies) strongly demonstrated the existence of communication between the N- and C-terminal regions. The C-terminal tyrosines may be involved in the flavonoid binding as well as in the catalysis of Schiff base formation or Michael addition of flavonoids to Lys 21 (23, 34, 43) or Tyr 39. Stacking and hydrogen bonding of the C-terminal tyrosines to flavonoids are most likely requisites for such binding and/or catalysis.

Taken together our data suggested that the oxidation and binding of the inhibitory flavonoids to α-synuclein lead to their attachment by Schiff base or Michael addition that restricts the conformational changes in the protein or preclude fibril assembly as revealed by mostly unchanged far-UV CD and FTIR spectra and detection of α-synuclein soluble species (monomers and oligomers).

Analysis of the Relationship between Antioxidant and Inhibitory Activities of the Flavonoids. Since it has been determined that auto-oxidation of the flavonoid is required for the inhibition of α-synuclein fibrillation, it was quite possible that the antioxidant activities of flavonoids were correlated with the in vitro inhibitory activities. Several research groups supported the presumption that the fibrillation inhibitors act by virtue of the antioxidant and/or metal-chelating activities (*73*, *74*). Furthermore, resveratrol and other polyphenols were reported to act as antioxidants in a variety of cellular types to protect against the cytoxicity induced by the amyloid ensembles (*75*, *76*).

Our study revealed that the antioxidant activities of flavonoids were related to their inhibitory effects on the in vitro α-synuclein fibrillation. In fact, flavonoids with three vicinal hydroxyl groups exhibited the enhanced inhibitory effects on α-synuclein fibrillation compared to those with two vicinal hydroxyl groups. Consistently, flavonoids with three vicinal hydroxyl substitutions usually were reported to have higher antioxidant capacity.

The comparison of the quercetin (good inhibitor) structure with structures of weak inhibitors luteolin and rutin (Figure 12) showed that quercetin has five hydroxyl groups (including the 3′,4′ dihydroxy structure in the B ring) as well as the 2,3-double bond in the C ring and the 4-oxo function. This creates a structural advantage, which confers the compound a high antioxidant activity, characterized by a relatively high Trolox-equivalent antioxidant capacity (TEAC) value of 4.7 ± 0.1 mM (*77*).

The glycosylation of flavonoids reduced their antioxidant activity when compared to the corresponding aglycones. As in rutin (quercetin 3-rutinoside), blocking the 3-hydroxyl group in the C ring of quercetin with a sugar substituent decreased the flavonoid antioxidant activity to a TEAC value of 2.4 ± 0.1 mM, most likely due to the steric hindrance, and the coplanarity loss of the B ring, as well as the loss in the full delocalization potential (*77*). Removing of the 3-OH group in the C ring of quercetin (as in luteolin) decreased the antioxidant activity to a TEAC value of 2.1 ± 0.1 mM (*78*). Consistent with the relative weak antioxidant activities, both rutin and lueteolin were found in our study to be weak inhibitors of α-synuclein fibrillation.

However, there are also examples suggesting that the correlation between the inhibitory activities and the antioxidant

activities of flavonoids is not perfect. For instance, the flavanone eriodictyol, bearing a saturated heterocyclic C ring and a consequent lack of conjugation between the A and B rings, in contrast to the flavones and flavonols, had a relatively low antioxidant activity (*77*, *79*), but high efficiency for the α-synuclein amyloid inhibition.

One important factor leading to the confounding of the correlation between the antioxidant activity and inhibitory activities is the discrepancy in the reported antioxidant activities originating from different methods of assessment, varying substrate systems, as well as different concentrations of active antioxidants. For example, it has been reported by Yang et al. that baicalein had a much lower oxidation potential compared to luteolin. This conclusion was made based on the measurement of the half-wave potential ($E_{1/2}$) of the first oxidation wave of flavonoids by flow-through column electrolysis. Accordingly, baicalein has stronger antioxidant activity than luteolin. However, Kim and Lee reported that baicalein had a much lower vitamin C equivalent antioxidant capacity (VCEAC) value than luteolin in the assay using free blue/green 2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals (*79*).

Taken together, antioxidant activities of flavonoids are roughly correlated with their in vitro inhibitory effects on α-synuclein fibrillation despite a few exceptions, which could be due to the variation in the reported antioxidant activities of flavonoids from different groups using different assays.

Flavonoids as a Model for the Development of Drugs against Parkinson's Disease. Most of tested flavonoids bind to α-synuclein with K_d less than 1 μM showing that they can be useful for the design of chemical compounds stabilizing monomeric conformation of α-synuclein. At present, it is not known what conformations of α-synuclein are cytotoxic and can cause the onset of neurodegeneration in Parkinson's disease. However, fibrillar and some oligomeric forms are the major suspects as they can increase membrane permeability in vivo and in vitro experiments (*80*). It has been suggested that increased membrane permeability may result in several events such as the release of cytochrome *c* and triggering apoptosis, the decrease in the mitochondrial membrane potential, the increase in the Ca^{2+} influx, and the release of ROS that affect the normal neuron function and lead to cell death. Small chemical compounds like flavonoids can change the equilibrium existing between various α-synuclein conformations and stabilize monomeric conformation of α-synuclein that is less pathogenic than fibrillar and oligomeric forms. Most of the inhibitory flavonoids stabilize both monomeric and oligomeric forms (with ~ 1:1 or 1:2 ratio) (*57*), and the mechanism of their strong inhibition is mostly due to the formation of Schiff base. The challenge is to design new compounds that can more efficiently stabilize monomeric conformations (without Schiff base formation). The flavonoid structures can serve as a starting model for the intelligent design of drugs specifically binding to α-synuclein and preventing Parkinson's disease.

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SUPPORTING INFORMATION AVAILABLE

Figures illustrating the UV−vis absorbance spectra of baicalein as a function of incubation time; tyrosine fluorescence quenching by baicalein; structures of flavonoids arranged by their inhibitory effects on α-synuclein fibrillation; binding effects of inhibitory flavonoids on the fibrillation kinetics of α-synuclein (30−140) and α-synuclein (1−103); effect of tyrosines baicalein binding to on α-synuclein; and FTIR analysis of Schiff base formation in α-synuclein-baicalein comlex. This material is available free of charge via the Internet at http://pubs.acs.org.

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