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Research Report

Role of Advanced Glycation on Aggregation and DNA Binding Properties of α-Synuclein

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Abstract. Parkinson's disease (PD) is a neurodegenerative disease with multiple etiologies. Advanced glycation end products (AGEs) accumulate in the aging brain and could be one of the reasons for age-related diseases like PD. Oxidative stress also leads to the formation of AGEs and may be involved in neurodegeneration by altering the properties of proteins. α-Synuclein is involved in pathogenesis of PD and there are limited studies on the role of AGE-α-synuclein in neurodegeneration. We studied the aggregation and DNA binding ability of AGE-α-synuclein *in vitro*. α-Synuclein is glycated using methylglyoxal and formation of AGE-α-synuclein is characterized using fluorescence studies, intrinsic tyrosine fluorescence, and fructosamine estimation. The results indicated that AGE-α-synuclein aggregates into smaller globular-like aggregates compared to fibrils formed with native α-synuclein. Further, it is found that AGE-α-synuclein induced conformational changes in scDNA from B-form to B-C-A mixed conformation. Additionally, AGE-α-synuclein altered DNA integrity as evidenced by the melting temperature, ethidium bromide, and DNAse I sensitivity studies. AGE-α-synuclein converted biphasic T_m to higher monophasic T_m. The T_m of AGE-α-synuclein-scDNA complex is more than that of native α-synuclein-scDNA complex, indicating that AGE-α-synuclein stabilized the uncoiled scDNA. AGE-α-synuclein could stabilize the uncoiled scDNA, as shown by the decrease in the number of ethidium bromide binding molecules per base pair of DNA. DNAse I sensitive studies indicated that both AGE-α-synuclein-scDNA and α-synuclein-scDNA are resistant to DNAse I digestion. The relevance of these findings to neuronal cell death is discussed.

Keywords: AGEs, α-synuclein, DNA conformation, glycation, Parkinson's disease, protein aggregation

INTRODUCTION

Parkinson's disease (PD) is an age-related neurodegenerative disease characterized by progressive neuronal loss in the substantia nigra region of the brain. The etiology of PD is still not clear [1]. α-Synuclein is an important protein expressed in neurons and pathologically involved in PD [2–4] and is localized in both the synapse and nucleus [5]. The normal function of α-synuclein is not understood yet, but studies showed that α-synuclein may be involved in synaptic development and vesicular binding [6–9]. α-Synuclein localization in the nucleus indicates a possible role for the protein in the nucleus [10–13]. Kontopoulos and colleagues [14] showed the localization of α-synuclein in nucleus in flies. Several studies have shown that α-synuclein binds to DNA [15–19]. The mechanism of α-synuclein in neuropathology is debatable [20]. Studies have also shown that aggregation of α-synuclein is the pathological event in the development of PD [21, 22]. α-Synuclein (140 aa) is natively in random coil conformation. The protein conformation is

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altered in disease condition and is in the aggregated form leading to neuronal cell death. Several factors like metals, oxidative stress, failure of proper protein degradation, and mutation in α-synuclein are linked to altered function of α-synuclein. Protein modifications like phosphorylation and glycation are linked to the α-synuclein aggregation process.

Advanced glycation end products (AGEs) are formed when reducing sugars like glucose, react with amino groups of proteins/lipids/nucleic acids through non-enzymatic glycation [23, 24]. In the aging process, by the continuous and constant exposure of α-synuclein to normal levels of glucose in circulation may favor the formation of AGE-α-synuclein and may aggravate further due to various insults and exposures including oxidative stress [25]. AGEs and α-synuclein are found in the brains of PD patients and are co-localized [26]. Recent studies have indicated that AGEs induce cross-linking in α-synuclein and thus accelerate aggregation of α-synuclein [26, 27].

Oxidative stress is known to damage biomolecules like DNA and proteins leading to neuronal cell death [28]. AGEs are formed during hyperglycemia and oxidative stress conditions [26]. AGEs are a heterogeneous group of molecules formed via Maillard reactions and Schiff's base formation from the non-enzymatic glycation of reducing sugars with free amino groups of proteins, lipids, and nucleic acids. The high concentration of reducing sugars like glucose, fructose, and reactive dicarbonyls, such as methylglyoxal (MGO), glyoxal, 3-deoxy glucosone, are responsible for the glycation of proteins in brain [23, 29]. The role of MGO in inducing oxidative stress in aging and neurodegenerative disorders is widely debated [30, 31]. Also the role of free radicals and oxidative stress is widely implicated in the pathogenesis of PD [32]. Several factors like oxidative stress, depletion of antioxidant levels, and trace metal homeostasis increase the reactive molecules responsible for advanced glycation and this reaction may be involved in the pathogenesis of neurodegeneration, but the mechanisms are unclear [33–36].

Hence, in the present study, we investigated the effect of glycation and AGE formation on the aggregation and DNA binding properties of α-synuclein.

MATERIALS AND METHODS

α-Synuclein was purchased from r-peptide (USA). Supercoiled pUC18 DNA (cesium chloride purified, 90% supercoiled structure, scDNA) was purchased from Banglore Genei, India. Agarose, HEPES, Tris, and EDTA were purchased from SISCO Research Laboratories. MGO, ethidium bromide, copper grids, aurinetricarboxylic acid (ATA), and $MgCl_2$ were purchased from Sigma (USA). Uranyl acetate was purchased from BDH Chemicals.

Preparation of AGE-α-synuclein and its characterization

Preparation of AGE-α-synuclein

AGE-α-synuclein was prepared by incubating 60 μM of α-synuclein with 60 mM MGO in 10 mM PBS buffer (pH 7.4) at 37°C for 144 h under sterile conditions. An aliquot (10 μl) from the incubation mixture was taken and made to 500 μl in PBS buffer pH 7.4 for the fluorescence analysis. The formation of AGE-α-synuclein was analyzed for every 24 h days monitoring its fluorescence at Ex 340 nm and Em: 360–500 nm using HITACHI spectrofluorometer.

Circular dichroism of AGE-α-synuclein

The secondary conformation of α-synuclein and AGE-α-synuclein was recorded on JASCO J700 spectropolarimeter at 25°C with 2 mm cell length in the wavelength range between 200–320 nm in Tris-Cl buffer (5 mM, pH 7.4). The sample that was incubated with MGO for 144 h, was subjected to CD studies. The secondary conformation for each spectrum was the average of four scans.

Intrinsic Tyrosine fluorescence

Tyrosine intrinsic fluorescence spectra were recorded on a HITACHI 2000 spectrofluorimeter in quartz with a 1 cm excitation light path. For tyrosine intrinsic fluorescence α-synuclein containing solution was excited at 275 nm and emission monitored in the range from 295 nm to 350 nm. The maximum emission was observed at 304 nm. The concentration of α-synuclein for the intrinsic tyrosine fluorescence was kept at 2 μM. In the present investigation, tyrosine intrinsic fluorescence was used to understand glycation induced folding in α-synuclein. The sample that was incubated with MGO for 144 h was subjected to intrinsic tyrosine fluorescence.

Analysis of fructosamine in AGE-α-synuclein

The amount of glycated ketoamine in AGE-α-synuclein was estimated by fructosamine assay. The 144 h incubated sample (50 μl) was mixed with 1 ml of carbonate buffer (pH 10.8) containing 0.25 mM

NBT (Nitroblue tetrazolium) and incubated for 10 min. Absorbance of the above reaction mixture was measured at 530 nm at 5th and 10th minute. Fructosamine formation was compared with the standard DMF (1-deoxy-1-morpholine-D-Fructose).

Trypsin digestion

Proteolytic cleavage of proteins has been used as a probe of protein conformation and stability [37, 38]. We analyzed the proteolytic cleavage of AGE-α-synuclein with trypsin in comparison with native α-synuclein. Trypsin digestion of α-synuclein and AGE-α-synuclein were carried out according to the method described by Hegde et al. [16]. α-Synuclein and AGE-α-synuclein were incubated with Trypsin (2 μg) in Tris-HCl buffer (pH 7.4) for 1 h and 2 h. After proteolytic cleavage, the samples were immediately frozen at −20°C. The trypsin digestion pattern was analyzed on 12% Tricine SDS-PAGE and stained with silver staining. Electrophoresis was carried out according to the method described by Laemmli et al. [39].

Aggregation of AGE-α-synuclein

Preparation of α-synuclein and AGE-α-synuclein for aggregation studies

α-Synuclein (50 μM) was dissolved in 20 mM Tris-HCl pH (7.4) and continuously stirred using magnetic bead (Teflon coated) in glass vials. We studied the aggregation as a function of time (0–96 h) and aliquots were taken at regular intervals of 8 h starting for 0 h to 96 h for thioflavin-T and TEM study. AGE-α-synuclein (50 μM) was also subjected to aggregation similar to α-synuclein and the aliquots were collected at regular intervals of 8 h starting from 0 h to 96 h.

Thioflavin-T assay

Thioflavin-T assay for the aliquots of the aggregation samples were carried out to monitor the α-synuclein aggregation. Thioflavin-T specifically binds to protein aggregates. The aliquots of α-synuclein aggregates were diluted to 10 μM in 50 mM glycine-NaOH buffer (pH 9.0). Assay solutions contained 10 μM Thio-T and α-synuclein at a concentration of 5 μM in 50 mM glycine-NaOH buffer, pH 9.0. Thioflavin-T fluorescence was measured at an Ex: 446 nm and Em: 482 nm using F4500 Hitachi fluorescence spectrometer. The background thioflavin-T fluorescence intensity was subtracted from the experimental values.

Transmission electron microscopy study

Transmission electron microscopy (TEM) studies were conducted to detect the presence of aggregates. 5 μL of incubated sample is placed on carbon coated copper grid (200 mesh size) and allowed for one min and excess sample was blotted with filter paper. This process was repeated 2–4 times after which the grids were negative stained by adding a drop of 1% uranyl acetate (pH 5.1) on the grid and blotted with a filter paper after 10 s. The grids were completely dried so as to avoid moisture for TEM examination [40]. Four individual experiments were carried out for each sample. The samples were examined with a JEOL 1010 transmission electron microscope and the images were photographed.

DNA binding properties of AGE-α-synuclein

Circular dichroism (CD) studies

The secondary conformation of scDNA in the presence of α-synuclein and AGE-α-synuclein (at DNA/α-synuclein mass ratios 1 : 4) was recorded on JASCO J700 spectropolarimeter at 25°C, with 2 mm cell length in the wavelength range between 200–320 nm in Tris-Cl buffer (5 mM, pH 7.4). The secondary conformation for the each spectrum was the average of four scans.

Thermal denaturation studies

The melting temperature (T_m) profiles of the α-synuclein and AGE α-synuclein binding to scDNA were recorded in HEPES buffer (10 mM, pH 7.4) using spectrophotometer equipped with a thermostat programmer and data processor (Amarsham Biosciences, HongKong). The melting profiles were recorded with increase of 1°C/min in the temperature range of 25–95°C.

Ethidium bromide (EtBr) binding studies

The changes in the integrity of scDNA upon α-synuclein and AGE α-synuclein binding were studied by EtBr study. EtBr bound in moles per base pair of DNA was measured in Tris-Cl (10 mM, pH 7.4) using HITACHI F-2000 Fluorescence Spectrofluorimeter. The fluorescence was measured using a constant amount of scDNA (0.5 μg) with increasing amounts of EtBr. The fluorescence measurements were monitored with an Ex: 535 nm and Em: 600 nm using 10 mm path length. The maximum amount of EtBr bound per base pair of DNA was calculated using Scatchard plots of 'r' vs 'r/Cf', in the DNA–EtBr reaction mixture at various titration intervals when increasing amount of EtBr was titrated to constant amount of DNA [41, 42].

The concentration of bound EtBr in 1.0 mL dye-DNA mixture (Cb′) were calculated using the equation:

$$Cb' = Co'[(F - Fo)/(V \times Fo)]$$

where
Co = EtBr (pmoles) present in the dye-DNA mixture,
F = observed fluorescence at any point of dye-DNA mixture,
Fo = observed fluorescence of EtBr with no DNA,
V = experimentally derived value, ratio of bound EtBr/free EtBr at saturation point.

The concentration of free dye (Cf′) was then calculated by the relation

$$Cf' = Co' - Cb',$$

where Cf′, Co′, and Cb′ were expressed in pmoles. The amount of bound EtBr/base pair 'r' was calculated by

$$r = Cb'(\text{pmoles})/\text{DNA concentration (pmoles of base pair)}.$$

A plot with r vs r/cf is plotted, point where the straight line intersects the X-axis is denoted as 'n'. 'n' is the maximum amount of dye bound per base pair (n), where $Cf = Cf' \times 10^{15}$ M.

DNAse I sensitivity assay

DNAse I digests the DNA and the sensitivity of DNAse I digestion is a marker of DNA integrity. scDNA incubated with α-synuclein and AGE-α-synuclein in the mass ratio of 1 :2 for 12 h and treated with DNAse I. The digestion of scDNA was monitored using spectrofluorimeter using ethidium bromide.

Statistical analysis

Data are expressed as mean ± SEM of triplicates. Statistical analysis of data was performed using one-way analysis of variance (ANOVA) with a Tukey's multiple comparison post-test and significance at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

RESULTS

Preparation and characterization of AGE-α-synuclein

We characterized the AGE-α-synuclein by fluorescence detection assay to confirm the glycation of α-synuclein. The native α-synuclein did not show any fluorescence (Fig. 1). However, AGE-α-synuclein showed an increase in fluorescence, confirming AGE formation in α-synuclein (Fig. 1).

Next we studied the secondary conformation of AGE-α-synuclein by Circular dichroism spectroscopy. α-Synuclein normally exists in random coil conformation (Fig. 2). The CD spectra inferred that AGE-α-synuclein also exists in random coil conformation (Fig. 2). The data indicated that AGE formation in α-synuclein did not alter the secondary confirmation.

Further we studied the folding pattern of AGE-α-synuclein by intrinsic tyrosine fluorescence assay. α-Synuclein contains four tyrosine residues and there was no tryptophan. Hence, the tyrosine fluorescence was used to monitor folding of α-synuclein [43]. The intrinsic tyrosine fluorescence was decreased for AGE-α-synuclein, while intrinsic tyrosine fluorescence was increased for native α-synuclein (Fig. 3). The data indicated that tyrosine molecules in AGE-α-synuclein might have buried inside due to folding changes in AGE-α-synuclein compared to native α-synuclein.

We next performed the analysis of fructosamine formation in AGE-α-synuclein. Glycation involves formation of fructosamine in the reaction between MGO and amino groups of protein. To characterize the glycation of α-synuclein, fructosamine content was estimated. In native α-synuclein, fructosamine level was negligible, but in AGE-α-synuclein, fructosamine concentration was 38.6 mmoles/50 μg of protein (Fig. 4).

We also performed the trypsin digestion pattern of AGE-α-synuclein. Trypsin cleaves proteins into peptides at carboxyl ends of lysine and arginine. Fig. 5 indicated trypsin digestion pattern of α-synuclein and AGE-α-synuclein. Trypsin completely cleaved native α-synuclein (Fig. 5). But AGE-α-synuclein partly cleaved by trypsin as evidenced by an intense tryptic peptide bands seen between 3.5 and 14.3 kD region (Fig. 5, lane 5, 6).

Aggregation of AGE-α-synuclein

We performed these series of experiments to understand the kinetics of aggregation of AGE-α-synuclein compared to native α-synuclein. We first conducted Thioflavin-T binding assay to map the formation of aggregates in the aggregation assay. Fig. 6 shows three phases of aggregation kinetics of α-synuclein as a function of time (0–96 h). The thioflavin-T data indicated a lag period of 24 h, with static fluorescence intensity indicating the presence of monomers. After 24 h, there was an intermediate phase (96 h) where oligomers and related intermediate forms might have formed. After 96 h, there was a saturated phase, where fully matured

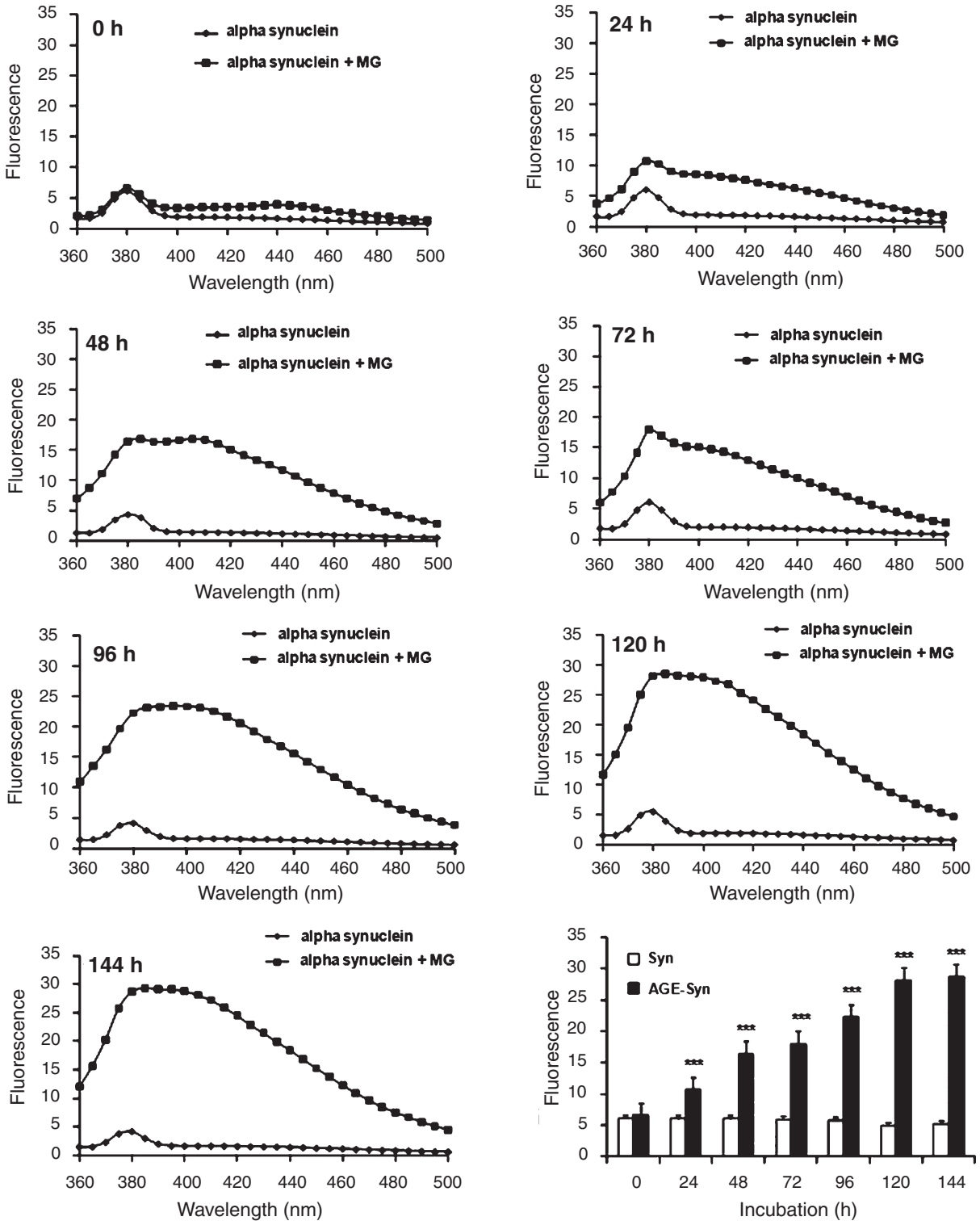

Fig. 1. Kinetics of α-synuclein glycation in the presence of MGO as a function time. Values are expressed as mean ± SEM of triplicates and significant at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ in comparison to '0' h synuclein control.

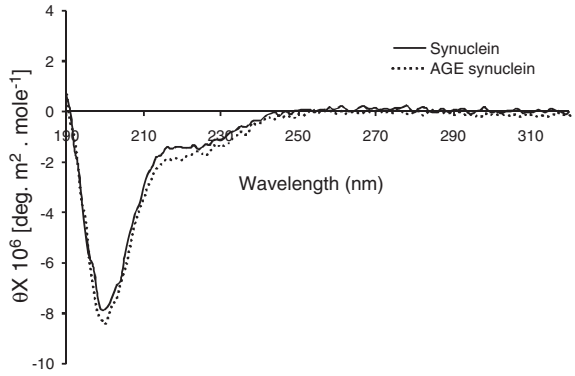

Fig. 2. CD spectra of native α-synuclein and AGE-α-synuclein. Both native α-synuclein and AGE-α-synuclein shows random coil conformation.

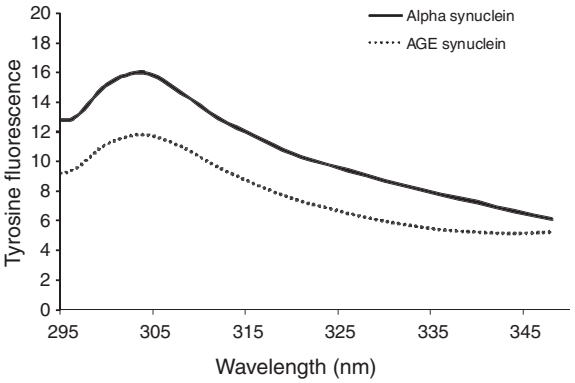

Fig. 3. Intrinsic tyrosine fluorescence of native α-synuclein and AGE-α-synuclein. Glycation of α-synuclein decreased the intrinsic tyrosine fluorescence indicating folding of AGE-α-synuclein compared to native α-synuclein.

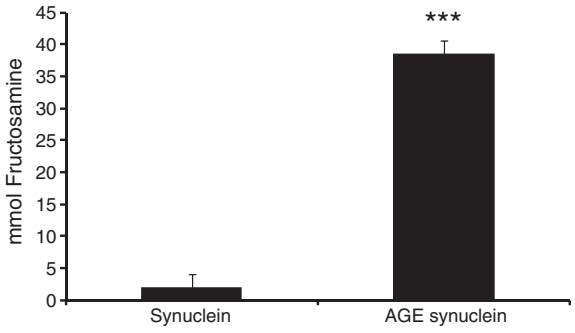

Fig. 4. Quantification of fructosamine in native α-synuclein and AGE-α-synuclein. Values are expressed as mean ± SEM of triplicates and are significant at $^{***}p < 0.001$.

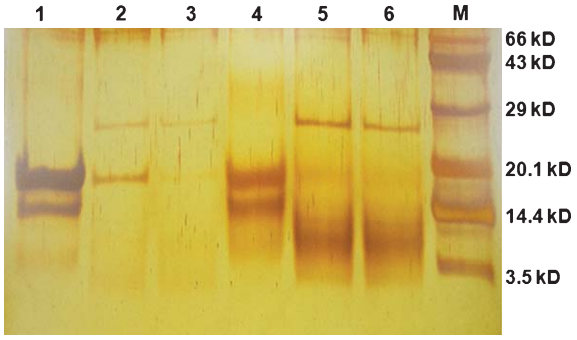

Fig. 5. Native α-synuclein and AGE α-synuclein digestion with trypsin. 1) α-synuclein alone; 2) α-synuclein digested with trypsin for 1 h; 3) α-synuclein digested with trypsin for 2 h; 4) AGE α-synuclein; 5) AGE α-synuclein digested with trypsin for 1 h; and 6) AGE α-synuclein digested with trypsin for 2 h. Proteolytic cleavage of α-synuclein and AGE-α-synuclein with trypsin showed different digestion pattern indicating that glycation altered folding of α-synuclein.

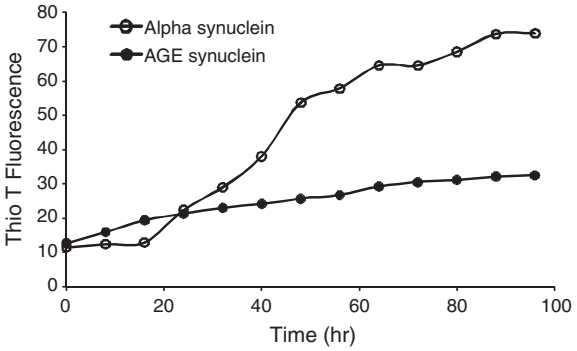

Fig. 6. Thioflavin T binding assay: Native α-synuclein (50 μM) and AGE α-synuclein (50 μM) dissolved in 20 mM Tris-HCl pH (7.4) and the aggregation is carried out at 37°C. The fluorescence of thioflavin-T is recorded with Ex:446 nm and Em 482 nm respectively. AGE-α-synuclein aggregation kinetics also showed a sigmodial curve, but different from that of native α-synuclein. The fluorescence intensity of AGE-α-synuclein was less than that of control alpha synuclein.

fibrils were formed. AGE-α-synuclein did not show three phases. The data indicated that AGE-α-synuclein was resistant for aggregation into fibrils (Fig. 6).

We next performed analysis to understand the morphological difference of aggregates formed between α-synuclein and AGE-α-synuclein. The TEM data in Fig. 7 clearly shows that there were no aggregates at 0 h in both α-synuclein and AGE-α-synuclein. At 24 h, oligomers were formed. At 48 and 96 h, the matured aggregates were formed (Fig. 7). The TEM data indicated that AGE-α-synuclein showed less number of aggregates.

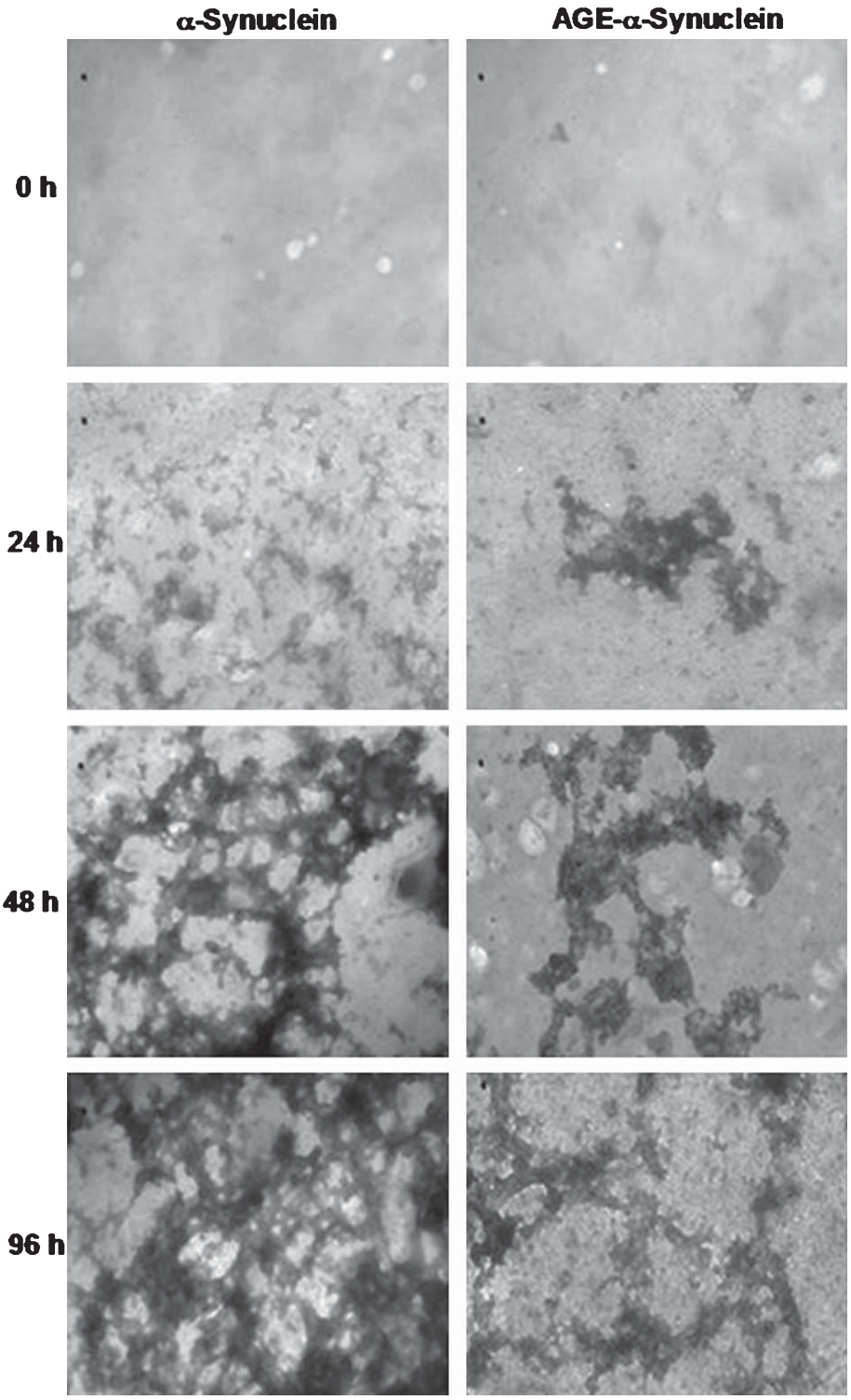

Fig. 7. Transmission electron microscopic study: Native α-synuclein and AGE α-synuclein (50 μM) aggregated at 37°C in Tris-Cl buffer (pH 7.4). The aliquots are taken at 0, 24, 48 and 96 h and analyzed for presence or absence of aggregates. Compared to native α-synuclein, AGE-α-synuclein showed less number of aggregates with globular like structures than that of fibrils.

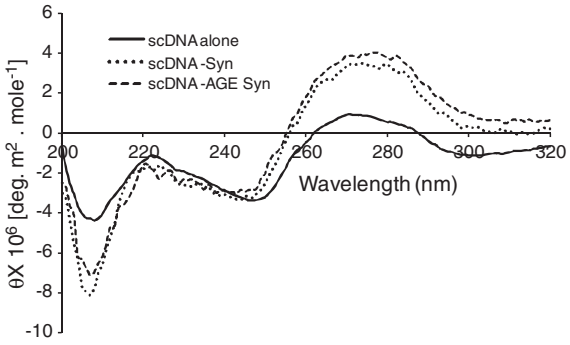

Fig. 8. CD spectra of native α-synuclein and AGE-α-synuclein interaction with scDNA. scDNA is in B-form, native α-synuclein and AGE-α-synuclein induced B-C-A mixed conformation in scDNA.

DNA binding properties of AGE-α-synuclein

In order to determine if the DNA binding properties of AGE-α-synuclein were altered, we first performed Circular dichroism (CD) studies. In CD spectrum, scDNA showed characteristic B-DNA conformation, having a positive peak at 272 nm and a negative peak at 245 nm (Fig. 8). In the presence of α-synuclein, scDNA CD spectrum was altered in the near UV region with an increased 210 nm and 220 nm negative peaks (Fig. 8). The spectra of scDNA-α-synuclein complex are subtracted from the α-synuclein spectra alone of same concentrations. Taken together, these changes indicate that α-synuclein induces B-C-A mixed transition in scDNA [44]. AGE-α-synuclein also induced B-C-A mixed conformation in scDNA (Fig. 8).

We next performed thermal denaturation studies to understand the integrity of scDNA due to AGE synuclein binding. The melting temperature (T_m) profile of scDNA showed characteristic biphasic pattern. The first transition (T_{m1}) was due to relaxation of supercoils in the scDNA and the second transition (T_{m2}) was due to opening of double strands into single strands (Fig. 9). The T_m values of scDNA were $T_{m1} = 54°C$ and $T_{m2} = 86°C$ (Fig. 9). α-Synuclein and AGE α-synuclein interaction with scDNA changed the biphasic pattern to monophasic T_m. The monophasic T_m values of scDNA-α-synuclein and scDNA-AGE α-synuclein were 77°C and 79°C respectively. This data indicated that both native α-synuclein and AGE α-synuclein altered DNA integrity.

We next determined the integrity of scDNA by measuring the number of EtBr molecules bound per base pair of DNA using Scatchard plot. The number of EtBr molecules bound per base pair of scDNA alone was 0.28 (Fig. 10). The number of EtBr molecules

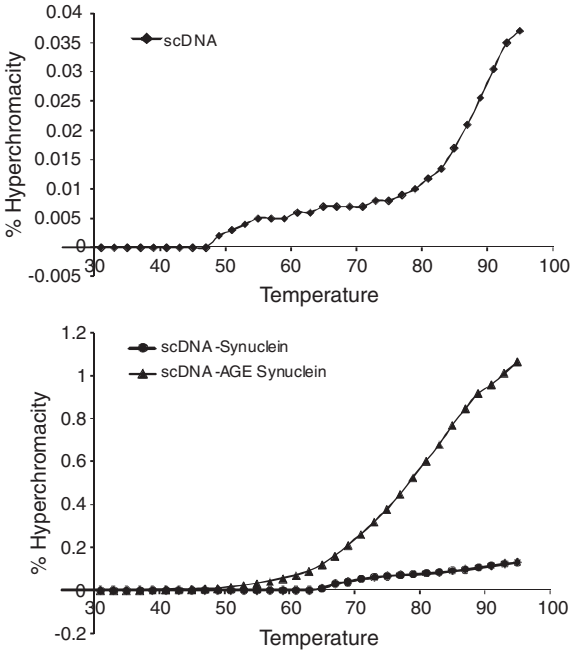

Fig. 9. Melting temperature (T_m) profile of native α-synuclein and AGE α-synuclein interaction with scDNA. T_m values of scDNA were $T_{m1} = 54°C$ and $T_{m2} = 86°C$. Both native α-Synuclein and AGE α-synuclein converted biphasic Tm to monophasic Tm with 77°C and 79°C respectively.

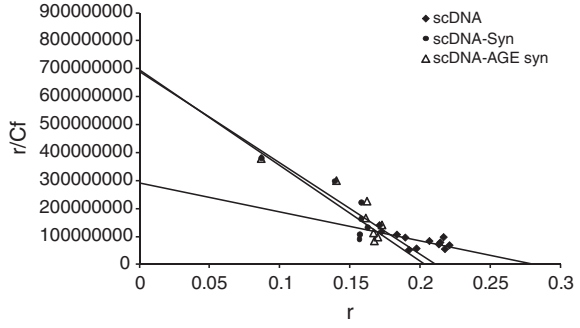

Fig. 10. scDNA was incubated with α-synuclein and AGE α-synuclein in the mass ratios of 1:4 in 10 mM Tris-Hcl (pH 7.4) at 37°C for 12 h. Ethidium bromide binding pattern to incubated samples along with the control scDNA is investigated by titrating with increasing concentration of EtBr. Using scatchard plot EtBr molecules bound/base pair are calculated. The number of EtBr molecules bound per base pair of scDNA, scDNA-α-synuclein and scDNA-AGE α-synuclein were 0.28, 0.21, and 0.2, respectively.

bound per base pair of DNA for scDNA-α-synuclein and scDNA-AGE α-synuclein were 0.21 and 0.20 respectively (Fig. 10). The decrease in number of EtBr molecules bound per base pair indicated the binding of α-synuclein and AGE α-synuclein to scDNA.

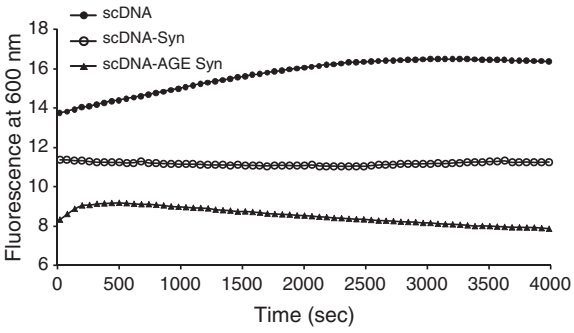

Fig. 11. DNAse I sensitivity to scDNA, α-synuclein-scDNA complex and AGE α-synuclein-scDNA complex. A saturated reaction mixture of scDNA and EtBr in the ratio of 1 :1 ratio (W/W, 0.5 μg scDNA + 0.5 μg EtBr) is treated with DNAse I (0.5 μg/mL). Similarly α-synuclein-scDNA complex and AGE α-synuclein-scDNA complex is treated with DNAse I. Both scDNA-α-synuclein and scDNA-AGE-α-synuclein showed resistance to DNAse I.

We further studied the changes in the integrity of scDNA due to binding of AGE-α-synuclein by DNase I sensitivity assay. scDNA alone treated with DNase I showed an increase in the fluorescence indicating sensitivity of scDNA to DNAse I digestion. scDNA-α-synuclein and scDNA-AGE-α-synuclein complexes treated with DNAse I showed static fluorescence intensity as a function of time (Fig. 11). The data in sighted that scDNA-α-synuclein and scDNA-AGE-α-synuclein showed resistance to DNAse I.

DISCUSSION

AGE formation is observed during PD. The role of metals, free radicals, and oxidative stress are widely implicated in the pathogenesis of PD [32, 45]. AGEs trigger toxic pathways in neuronal cells leading to the neuronal cell death [34]. Castellani and colleagues [46] reported immunoreactivity to AGEs in autopsy samples of brain regions in with Lewy bodies. Munch et al. [26] also reported that AGEs and α-synuclein are co-localized in PD brain samples. They showed that AGEs are involved in the cross-linking of α-synuclein and accelerating aggregation of α-synuclein [26]. In a recent report by Choi and Lim [47], AGEs are shown to form in an MPTP mouse model of PD. In this mouse model, α-synuclein co-localizes with AGEs of N^{ε}-(carboxymethyl)lysine (CML) and N^{ε}-(carboxyethyl)lysine (CEL) in brain. All the above studies indicated that α-synuclein may also become glycated in PD.

α-Synuclein is involved in the pathology of PD. α-Synuclein protein aggregates and forms Lewy bodies in the brains of PD [48]. The role of nuclear localization of α-synuclein has recently been the focus of attention. α-Synuclein is shown to interact with DNA and histone proteins in the nucleus altering the normal functions of DNA. In the context of the above findings, we analyzed the role of glycation in the aggregation and DNA binding properties of α-synuclein. α-Synuclein is glycated using MGO as a glycating agent. MGO is formed as an intermediate of glucose during Maillard and Schiff's base formation in all tissues including brain and more so during hyperglycemic conditions and oxidative stress [49]. Our results show that AGE-α-synuclein had similar random coil conformation as that of native α-synuclein (Fig. 2). Aggregation kinetics showed that AGE-α-synuclein forms more globular-like aggregates than the fibrillar form aggregates. The above results are in accordance with that of Lee et al. [50] where they showed that glycated α-synuclein aggregates into small oligomeric forms. Chen and coworkers [51] also showed that glycation of α-synuclein with ribose resulted in the formation of molten globule-like structure in *in vitro* aggregation. Our results, along with other studies, indicate that glycation of α-synuclein results in the formation of oligomeric or globular structures. Recently, it has been debated that oligomeric forms of α-synuclein are more toxic than that of fibrillar structures. As glycation may lead to the formation of oligomeric forms of α-synuclein and since oligomeric forms are more toxic, it becomes important to focus on the role of AGE-α-synuclein in the pathogenesis of PD.

The other major issue is the biology of nuclear localization of α-synuclein. Hegde and collaborators [16] reported first time that α-synuclein bind to DNA. Additionally, the authors showed that α-synuclein alter the DNA conformation from B-form to altered B form and also uncoils the supercoiled DNA to open a circular form [16]. It was previously shown that DNA binding alters the aggregation properties of α-synuclein [6, 18]. But there are no reports showing the effect of glycation on α-synuclein DNA binding properties. In our present study, plasmid supercoiled DNA is selected as a model system for DNA binding studies. Plasmid supercoiled DNA is analogous to eukaryotic small supercoiled DNA pockets and is known to be involved in gene expression. AGE-α-synuclein induced conformational change in scDNA from B-form to B-C-A mixed conformation. Also AGE-α-synuclein altered DNA integrity as evidenced by the melting temperature, ethidium bromide, and DNAse I sensitivity studies. AGE-α-synuclein converted biphasic T_m to higher monophasic T_m similar to that of native α-synuclein.

The T_m of AGE-α-synuclein-scDNA complex is more than that of native α-synuclein-scDNA complex, indicating that AGE-α-synuclein stabilized the uncoiled scDNA. AGE-α-synuclein and α-synuclein stabilization of the uncoiled scDNA is indicated by the decrease in the number of ethidium bromide binding molecules per base pair of DNA. DNAse I sensitive studies showed that both AGE-α-synuclein-scDNA and α-synuclein-scDNA are resistant to DNAse I digestion.

In conclusion, our study indicates that AGE-α-synuclein aggregates into smaller globular-like aggregates compared to fibrils formed with native α-synuclein. The binding association of AGE-α-synuclein with DNA and alteration of the secondary conformation suggests that AGE-α-synuclein may alter the DNA structure in the nucleus and thus may influence gene expression. Further *in vivo* studies in animal models are required to establish the pathological role of AGE-α-synuclein.

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REFERENCES

[1] Gallagher DA, Schapira AH (2009) Etiopathogenesis and treatment of Parkinson's disease. *Curr Top Med Chem* **9**, 860-868.

[2] Bisaglia M, Mammi S, Bubacco L (2009) Structural insights on physiological functions and pathological effects of alpha-synuclein. *FASEB J* **23**, 329-340.

[3] Fasano M, Lopiano L (2008) Alpha-synuclein and Parkinson's disease: a proteomic view. *Expert Rev Proteomics* **5**, 239-248.

[4] Jellinger KA (2009) A critical evaluation of current staging of alpha-synuclein pathology in Lewy body disorders. *Biochim Biophys Acta* **1792**, 730-740.

[5] Maroteaux L, Campanelli JT, Scheller RH (1988) Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal. *J Neurosci* **8**, 2804-2815.

[6] Hegde ML, Vasudevaraju P, Rao KJ (2010) DNA induced folding/fibrillation of alpha-synuclein: new insights in Parkinson's disease. *Front Biosci* **15**, 418-436.

[7] Bayer TA, Jäkälä P, Hartmann T, Egensperger R, Buslei R, Falkai P, Beyreuther K (1999) Neural expression profile of alpha-synuclein in developing human cortex. *Neuroreport* **10**, 2799-2803.

[8] Kaplan B, Ratner V, Haas E (2003) Alpha-synuclein: its biological function and role in neurodegenerative diseases. *J Mol Neurosci* **20**, 83-92.

[9] Di Rosa G, Puzzo D, Sant'Angelo A, Trinchese F, Arancio O (2003) Alpha-synuclein: between synaptic function and dysfunction. *Histol Histopathol* **18**, 1257-1266.

[10] Schneider BL, Seehus CR, Capowski EE, Aebischer P, Zhang SC, Svendsen CN (2007) Over-expression of alpha-synuclein in human neural progenitors leads to specific changes in fate and differentiation. *Hum Mol Genet* **16**, 651-666.

[11] Mori F, Tanji K, Yoshimoto M, Takahashi H, Wakabayashi K (2002) Immunohistochemical comparison of alpha- and beta-synuclein in adult rat central nervous system. *Brain Res* **941**, 118-126.

[12] Specht CG, Tigaret CM, Rast GF, Thalhammer A, Rudhard Y, Schoepfer R (2005) Subcellular localisation of recombinant alpha- and gamma-synuclein. *Mol Cell Neurosci* **28**, 326-334.

[13] Yu S, Zuo X, Li Y, Zhang C, Zhou M, Zhang YA, Uéda K, Chan P (2004) Inhibition of tyrosine hydroxylase expression in alpha-synuclein-transfected dopaminergic neuronal cells. *Neurosci Lett* **367**, 34-39.

[14] Kontopoulos E, Parvin JD, Feany MB (2006) Alpha-synuclein acts in the nucleus to inhibit histone acetylation and promote neurotoxicity. *Hum Mol Genet* **15**, 3012-3023.

[15] Hegde ML, Gupta VB, Anitha M, Harikrishna T, Shankar SK, Muthane U, Subba Rao K, Jagannatha Rao KS (2006) Studies on genomic DNA topology and stability in brain regions of Parkinson's disease. *Arch Biochem Biophys* **449**, 143-156.

[16] Hegde ML, Jagannatha Rao KS (2003) Challenges and complexities of alpha-synuclein toxicity: new postulates in unfolding the mystery associated with Parkinson's disease. *Arch Biochem Biophys* **418**, 169-178.

[17] Hegde ML, Rao KS (2007) DNA induces folding in alpha-synuclein: understanding the mechanism using chaperone property of osmolytes. *Arch Biochem Biophys* **464**, 57-69.

[18] Cherny D, Hoyer W, Subramaniam V, Jovin TM (2004) Double-stranded DNA stimulates the fibrillation of alpha-synuclein *in vitro* and is associated with the mature fibrils: an electron microscopy study. *J Mol Biol* **344**, 929-938.

[19] Goers J, Manning-Bog AB, McCormack AL, Millett IS, Doniach S, Di Monte DA, Uversky VN, Fink AL (2003) Nuclear localization of alpha-synuclein and its interaction with histones. *Biochemistry* **42**, 8465-8471.

[20] Waxman EA, Giasson BI (2009) Molecular mechanisms of alpha-synuclein neurodegeneration. *Biochim Biophys Acta* **1792**, 616-624.

[21] Uversky VN, Eliezer D (2009) Biophysics of Parkinson's disease: structure and aggregation of alpha-synuclein. *Curr Protein Pept Sci* **10**, 483-499.

[22] Beyer K, Ariza A (2007) Protein aggregation mechanisms in synucleinopathies: commonalities and differences. *J Neuropathol Exp Neurol* **66**, 965-974.

[23] Negre-Salvayre A, Salvayre R, Augé N, Pamplona R, Portero-Otín M (2009) Hyperglycemia and glycation in diabetic complications. *Antioxid Redox Signal* **11**, 3071-3109.

[24] Sensi M, Pricci F, Andreani D, Di Mario U (1991) Advanced nonenzymatic glycation endproducts (AGE): their relevance to aging and the pathogenesis of late diabetic complications. *Diabetes Res* **16**, 1-9.

[25] Sandyk R (1993) The relationship between diabetes mellitus and Parkinson's disease. *Int J Neurosci* **69**, 125-130.

[26] Münch G, Lüth HJ, Wong A, Arendt T, Hirsch E, Ravid R, Riederer P (2000) Crosslinking of alpha-synuclein by advanced glycation endproducts–an early pathophysiological step in Lewy body formation? *J Chem Neuroanat* **20**, 253-257.

[27] Shaikh S, Nicholson LF (2008) Advanced glycation end products induce *in vitro* cross-linking of alpha-synuclein and

accelerate the process of intracellular inclusion body formation. *J Neurosci Res* **86**, 2071-2082.

[28] Jenner P (2003) Oxidative stress in Parkinson's disease. *Ann Neurol* **53** Suppl 3, S26-S36; discussion S36-S38.

[29] Brownlee M, Vlassara H, Cerami A (1984) Nonenzymatic glycosylation and the pathogenesis of diabetic complications. *Ann Intern Med* **101**, 527-537.

[30] Desai KM, Chang T, Wang H, Banigesh A, Dhar A, Liu J, Untereiner A, Wu L (2010) Oxidative stress and aging: is methylglyoxal the hidden enemy? *Can J Physiol Pharmacol* **88**, 273-284.

[31] Kuhla B, Lüth HJ, Haferburg D, Boeck K, Arendt T, Münch G (2005) Methylglyoxal, glyoxal, and their detoxification in Alzheimer's disease. *Ann N Y Acad Sci* **1043**, 211-216.

[32] Koutsilieri E, Scheller C, Grünblatt E, Nara K, Li J, Riederer P (2002) Free radicals in Parkinson's disease. *J Neurol* **249** Suppl 2, II1-5.

[33] Jomova K, Vondrakova D, Lawson M, Valko M (2010) Metals, oxidative stress and neurodegenerative disorders. *Mol Cell Biochem* **345**, 91-104.

[34] Vicente Miranda H, Outeiro TF (2010) The sour side of neurodegenerative disorders: the effects of protein glycation. *J Pathol* **221**, 13-25.

[35] Ko SY, Lin YP, Lin YS, Chang SS (2010) Advanced glycation end products enhance amyloid precursor protein expression by inducing reactive oxygen species. *Free Radic Biol Med* **49**, 474-480.

[36] Fang F, Lue LF, Yan S, Xu H, Luddy JS, Chen D, Walker DG, Stern DM, Yan S, Schmidt AM, Chen JX, Yan SS (2010) RAGE-dependent signaling in microglia contributes to neuroinflammation, Abeta accumulation, and impaired learning/memory in a mouse model of Alzheimer's disease. *FASEB J* **24**, 1043-1055.

[37] Nadig G, Ratnaparkhi GS, Varadarajan R, Vishveshwara S (1996) Dynamics of ribonuclease A and ribonuclease S: computational and experimental studies. *Protein Sci* **5**, 2104-2114.

[38] Fontana A, Zambonin M, Polverino de Laureto P, De Filippis V, Clementi A, Scaramella E (1997) Probing the conformational state of apomyoglobin by limited proteolysis. *J Mol Biol* **266**, 223-230.

[39] Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

[40] Thomas TJ, Bloomfield VA (1985) Quasielastic laser light scattering and electron microscopy studies of the conformational transitions and condensation of poly(dA-dT).poly(dA-dT). *Biopolymers* **24**, 2185-2194.

[41] Scatchard G (1949) The attraction of proteins for small molecules and ions. *Ann N Y Acad Sci* **51**, 660-672.

[42] Chatterjee B, Rao GRK (1994) Superhelical density of goat mitochondrial DNA: Fluorescent studies. *Indian J Biochem Biophys* **31**, 77-79.

[43] Uversky VN, Li J, Fink AL (2001) Metal-triggered structural transformations, aggregation, and fibrillation of human alpha-synuclein. A possible molecular NK between Parkinson's disease and heavy metal exposure. *J Biol Chem* **276**, 44284-44296.

[44] Gray DM, Taylor TN, Lang D (1978) Dehydrated circular DNA: circular dichroism of molecules in ethanolic solutions. *Biopolymers* **17**, 145-157.

[45] Duracková Z (2010) Some current insights into oxidative stress. *Physiol Res* **59**, 459-469.

[46] Castellani R, Smith MA, Richey PL, Perry G (1996) Glycoxidation and oxidative stress in Parkinson disease and diffuse Lewy body disease. *Brain Res* **737**, 195-200.

[47] Choi YG, Lim S (2010) N()-(carboxymethyl)lysine linkage to α-synuclein and involvement of advanced glycation end products in α-synuclein deposits in an MPTP-intoxicated mouse model. *Biochimie* **92**, 1379-1386.

[48] Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M (1997) Alpha-synuclein in Lewy bodies. *Nature* **388**, 839-840.

[49] Sian J, Dexter DT, Lees AJ, Daniel S, Agid Y, Javoy-Agid F, Jenner P, Marsden CD (1994) Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. *Ann Neurol* **36**, 348-355.

[50] Lee D, Park CW, Paik SR, Choi KY (2009) The modification of alpha-synuclein by dicarbonyl compounds inhibits its fibril-forming process. *Biochim Biophys Acta* **1794**, 421-430.

[51] Chen L, Wei Y, Wang X, He R (2010) Ribosylation rapidly induces alpha-synuclein to form highly cytotoxic molten globules of advanced glycation end products. *PLoS One* **5**, e9052.