Various metal ions promote formation of α -Synuclein fibrils with different shapes and cytotoxicities.

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Research Article

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Abstract

α -Synuclein (α -Syn) aggregates are key components of intracellular inclusion bodies characteristic of Parkinson's disease (PD) and other synucleinopathies. Metal ions have been considered as the important etiological factors in PD since their interactions with α -Syn alter the kinetics of fibrillation. In the present study, we have systematically explored the effects of Zn^{2+} , Cu^{2+} , Ca^{2+} , and Mg^{2+} cations on α -Syn fibril formation. Specifically, we determined fibrillation kinetics, size, morphology, and secondary structure of the fibrils and their cytotoxic activity. While all cations accelerate fibrillation we observed distinct effects of the different ions. For example, Zn^{2+} induced fibrillation by lower t_{lag} and higher k_{app} and formation of shorter fibrils, while Ca^{2+} ions lead to formation of longer fibrils, evidences by dynamic light scattering and atomic force microscopy studies. Additionally, the morphology of formed fibrils was different. Circular dichroism studies revealed higher content of antiparallel β -sheets in fibrils formed in the presence of Zn^{2+} . Interestingly, cell viability studies indicated nontoxicity of α -Syn fibrils formed in the presence of Zn^{2+} ions, while the fibrils formed in the presence of Cu^{2+} , Ca^{2+} , and Mg^{2+} were cytotoxic. Our results revealed that α -Syn fibrils formed in the presence of different divalent cations have distinct structural and cytotoxic features and indicate a protective role of Zn^{2+} in the formation of neurotoxic α -Syn assemblies.

Introduction

α -Synuclein (α -Syn) is a highly conserved 140-residue intrinsically disordered protein, which is abundantly present in presynaptic terminals in the brain¹. Misfolding and aggregation of α -Syn in neurons and glial cells, as intracellular inclusions called Lewy bodies, is a pathological characteristic of a group of neurodegenerative diseases called synucleinopathies, mainly among them is Parkinson's disease (PD)^{2,3}. Others include Lewy body dementia (LBD)⁴ and multiple system atrophy (MSA)⁵. α -Syn is composed of three separate regions: 1) the amphipathic N-terminus (residues 1–60), which is positively charged and adopts an α -helical structure when interacting with biological membranes; 2) the central highly hydrophobic self-aggregating sequence called NAC (non-amyloid β component; residues 61–95), which is the part involved in the formation of β -sheet during fibrillation; and 3) the acidic C-terminus (residues 96–140), which contains a number of negatively charged residues and is the main binding site for metal ions⁶. α -Syn is natively unfolded and lacks a unique structure and adopts multitude of conformations with no persistent secondary structure in its native monomeric state^{7,8}. Although still controversial, it is believed that α -Syn plays a role in regulation of synaptic vesicle recycling via interacting with negatively charged lipids, where metal ions can interfere⁹. It has been shown that Ca^{2+} binding by α -Syn can change the charge distribution of the negatively charged C-terminus and consequently modulate the α -Syn-membrane interactions⁹.

On the other hand, interactions between α -Syn and metal ions have been indicated to favor α -Syn folding, oligomerization, aggregation, and fibrillation^{10,11}. High concentrations of Zn^{2+} , Cu^{2+} , and Ca^{2+} were detected in α -Syn fibrillar deposits in parkinsonian *substantia nigra*^{12,13}. Therefore, binding of various

metal ions to α -Syn has been largely explored in order to elucidate the molecular mechanism underlying α -Syn fibrillation acceleration and pathogenesis of induced diseases^{10,12,14,15}. Mapping the α -Syn-Zn²⁺ interactions using NMR spectroscopy located the ions binding site around Asp121, Asn122 and Glu123 with Asp121 as the main anchoring site and another lower affinity binding site on His50 in the unstructured N terminal domain¹⁶. Studies of the binding sites of Cu²⁺ ions on α -Syn revealed that the N terminus is the primary binding site involving His50 as the main anchor, while C terminus with negatively charged residues acts as a secondary binding site with a much lower binding affinity⁶. Ca²⁺ and Mg²⁺ were found to bind the acidic C terminal domain¹⁷⁻¹⁹.

In spite of the efforts of identifying the binding sites of metal ions on α -Syn and their accelerating effect on α -Syn fibrillation, a comparison of the cytotoxicities of the fibrils induced by metal ions has been remained largely unexplored. Therefore, in the current comparative study we scrutinized more precisely the morphology, structures and cytotoxicity of α -Syn fibrils formed in the presence of Zn²⁺, Cu²⁺, Ca²⁺, or Mg²⁺ ions. We tried to answer this question that whether polymorphically different α -Syn fibrils formed in presence of various metal ion inducers could have different toxicities. The findings of this research can pave the way for the elucidation of the role of the diverse inducers in the α -Syn fibrillation and PD pathology.

Results

Metal ions accelerated α -synuclein fibril formation

Effect of metal ions on kinetic of α -Syn amyloid formation was evaluated using the standard ThT fluorescence assay (Figure 1a). ThT data were normalized and then fitted using Eq. 1 (Table 1). According to the obtained results, presence of Zn²⁺, Cu²⁺, Ca²⁺, and Mg²⁺ accelerated rate of α -Syn fibril formation. A considerable decrease in t_{lag} was observed for the α -Syn fibrillation kinetics in presence of Zn²⁺ ions, which was accompanied by a higher k_{app} . Fibril formation in the presence of Cu²⁺, Ca²⁺, and Mg²⁺ was nearly parallel and followed a similar trend with the Mg²⁺ sample having a slower rate compared to the other two ions. Control sample in the absence of the ions has a lag phase of 18 h. Interestingly, Zn²⁺ seemed to enhance the kinetic of α -Syn fibril formation by nearly 5 times compared to the control sample.

Then the Congo red (CR) binding assay was performed to further confirm α -Syn fibril formation and to quantitatively evaluate the formation of the β -sheets during fibrillation. As shown in Figure 1b, presence of amyloid fibrils in the control sample caused a higher CR absorbance accompanied by a red shift. Interestingly, we observed a shoulder at around 540 nm for Zn²⁺, Cu²⁺, Ca²⁺, and Mg²⁺ samples, what can be assigned to the higher presence of amyloid fibrils. Molar concentration of the bound CR, as a quantitative criterion for the amount of amyloid fibrils, was calculated using Eq. 2 and presented in Figure 1c. According to the obtained results, presence of Zn²⁺, Cu²⁺, Ca²⁺, and Mg²⁺ led to a marked increase of the amount of amyloid fibrils compared with control sample. The presence of metal ions led to higher

yield of α -Syn fibril formation. However, no significant difference was detected between fibrils formed in presence of various metal ions.

Fibrils of α -Syn formed in presence or absence of Zn^{2+} , Cu^{2+} , Ca^{2+} , and Mg^{2+} were also analyzed using ANS binding assay²⁰. Based on the results displayed in Figure 1d, fibrils in the control sample resulted in a minor increase in ANS fluorescence intensity along with a blue shift indicative of more hydrophobic clusters formed upon fibrillation. Presence of Mg^{2+} led to similar changes. However, Zn^{2+} , Cu^{2+} , and Ca^{2+} resulted in greater changes in hydrophobic patches of α -Syn fibrils.

Table 1					
Kinetic parameters of α -Syn fibril formation in presence and absence of each metal ion. The parameters were calculated based on kinetic studies displayed in Fig. 1 using Eq. 1.					
	Control	Zn^{2+}	Cu^{2+}	Ca^{2+}	Mg^{2+}
t_{lag} (h)	18 ± 0.26	2 ± 0.56	9 ± 0.20	6 ± 0.26	13.5 ± 0.50
k_{app} (h^{-1})	0.07 ± 0.01	0.33 ± 0.05	0.12 ± 0.06	0.25 ± 0.05	0.17 ± 0.03

Various metal ions induced formation of different α -synuclein fibril polymorphs

We monitored the size evolution of the fibrils using DLS in the comparable conditions with kinetic studies. Figure 2 displays the mean size (nm) of α -Syn fibrils formed through a time scale. α -Syn aggregates formed in presence of Zn^{2+} ions rapidly started growing after 4 h and their sizes remained nearly constant after 7 h. However, Cu^{2+} , Ca^{2+} , and Mg^{2+} presented delayed increase in size while longer fibrils were formed compared to Zn^{2+} , with Cu^{2+} and Ca^{2+} resulted in formation of the longest fibrils.

Since a remarkable difference in the lengths of α -Syn fibrils formed in presence and absence of Zn^{2+} , Cu^{2+} , Ca^{2+} , and Mg^{2+} was detected, further analysis on the size and morphology of the fibrils was performed using atomic force microscopy. AFM images were obtained from final stage fibril samples (Figure 3). Fibril lengths were quantified using Nova software and displayed in Table 2. Interestingly, it was observed that except for the Zn^{2+} , the other samples presented well-defined mature fibrils with various distribution of lengths and arrangements. Presence of Zn^{2+} resulted in a striking difference in length of the fibrils. It seemed Zn^{2+} caused formation of shorter fibrils. In contrast, Ca^{2+} induced formation of longer fibrils.

Table 2

Average length of α -Syn fibrils formed in presence and absence of metal ions. The lengths were determined using AFM images.

	Control	Zn ²⁺	Cu ²⁺	Ca ²⁺	Mg ²⁺
Average length (μm)	0.48 $\pm$ 0.09	0.20 $\pm$ 0.04	0.72 $\pm$ 0.31	0.94 $\pm$ 0.21	0.49 $\pm$ 0.22

Zn²⁺ ions induced formation of more antiparallel β -sheets

The differences observed in polymorphisms of α -Syn fibrils formed in presence or absence of various metal ions promoted us to evaluate the secondary structures of the fibrils using CD spectroscopy (Figure 4). A negative band at 217 nm in the CD spectra of the fibrils clearly indicate formation of β -sheets. In contrast, the α -Syn monomers are dominated by random coil structures, evidenced by a negative band at 197 nm²¹.

Contents of secondary structure elements were computed using DICHROWEB CONTILL algorithm and summarized in Table 3. The total amount of β -sheets increases in the fibril samples compared to α -Syn monomer. However, the increase in the contents of β -sheets is associated with the decrease in the amounts of unordered moieties. Nevertheless, the analysis of the amounts of two types of strands (strand 1 and strand 2) clearly shows that there is a considerable increase in the amount of strand 1 in fibrils formed in presence of Zn²⁺ compared with the other cations (65.4% for Zn²⁺ sample versus 29.2% for Cu²⁺, what is the highest amount after the Zn²⁺ sample). CD data were further analyzed using a recently developed online web server (<http://bestsel.elte.hu>), which provides more accurate analysis of CD spectra. Based on the results displayed in Table 3, there is a remarkable increase in the amount of antiparallel β sheets in fibrils formed in the presence of Zn²⁺ compared with the control samples and the fibrils formed in presence of other cations. The fibrils formed in presence of Zn²⁺ ions presented greater tendency to form antiparallel β -sheets compared to Cu²⁺, Ca²⁺, and Mg²⁺. A more appropriate criterion would be the ratio of parallel/antiparallel β -sheet presented in the last row of the Table 3, which shows the great difference between the arrangement of β -sheet contents in the fibrils formed in presence and absence of Zn²⁺, Cu²⁺, Ca²⁺, and Mg²⁺.

Table 3

The secondary structure contents of α -Syn monomer or fibrils formed in presence and absence of each studied cation. The analyses were performed using DICHROWEB CONTILL algorithm and SMP180 reference data set and BeStSel online web server.

DICHROWEB CONTILL algorithm						
	α -Syn Monomer	Control	Zn ²⁺	Cu ²⁺	Ca ²⁺	Mg ²⁺
α -Helix	11.2	9.2	0.0	5.5	14.4	11.5
Total β -sheet	27.5	34.7	79	42.7	28.7	30.8
Strand 1	15.5	22.8	65.4	29.2	18.0	18.6
Strand 2	12.0	11.9	13.6	13.5	10.7	12.2
Turns	13.9	13.2	6.7	11.6	13.7	13.0
Unordered	47.4	42.9	14.4	40.1	43.2	44.8
BeStSel						
α -Helix	2.0	6.3	0.0	15.9	6.2	9.0
Antiparallel β sheet	28.9	22.6	76.0	20.5	19	17.9
Parallel β sheet	4.0	8.6	3.6	2.9	8.4	8.7
Turn	16.8	15.0	9.1	16.2	13.6	15.7
Others	48.3	47.5	11.3	44.5	52.8	48.8
Parallel/Antiparallel ratio	0.1	0.4	0.1	0.1	0.4	0.5

Zn²⁺ ions induced formation of nontoxic α -synuclein fibrils

Significant differences between the fibrils formed in presence and absence of Zn²⁺, Cu²⁺, Ca²⁺, and Mg²⁺ stimulated us to check the cytotoxicity of fibrils using MTT assay (Figure 5). It was interesting to note that 10 or 20 μ M of fibrils formed in presence of Zn²⁺ did not change cell viability. However, fibrils of the control sample and fibrils formed in presence of Cu²⁺, Ca²⁺, and Mg²⁺ led to significant decrease in cell viability at both tested concentrations, what remains in agreement with earlier published reports²²⁻²⁴. We also checked the cytotoxicity of the studied cations at the concentration the cells receive when treated with α -Syn fibrils and detected no toxicity (data not shown).

Discussion

There has been a considerable attention on the effect of metal ions on α -Syn fibril formation^{10,15,25,26}. In the present study, we uncovered the relation between α -Syn fibril structure and their cytotoxicity. It was observed that all Zn^{2+} , Cu^{2+} , Ca^{2+} , and Mg^{2+} cations accelerated α -Syn fibril formation but with different rates inducing the formation of various polymorphs of α -Syn fibrils. We found that fibrils with higher amount of antiparallel β -sheets were more cytotoxic compared to fibrils containing its smaller amounts.

Studies of fibrillation kinetics demonstrated that the cations induced acceleration of fibril formation with shorter lag phase along with higher k_{app} , what suggests shorter nucleation times and higher rates of elongation. The Zn^{2+} was the most effective in accelerating fibril formation compared with other studied cations. The results of this study are consistent with the pioneering work published by Uversky et al., which indicated stimulation of α -Syn fibril formation and also induction of conformational changes in α -Syn by cations¹⁰. During years of intense research on the interactions of various metal ions with α -Syn, it has been observed that the acidic C terminal domain of α -Syn (residues 96–140) containing numerous negatively charged residues is an important binding site for metal ions²⁷. Furthermore, His50 in the N terminal unstructured region has been determined as a binding site for Zn^{2+} and Cu^{2+} ions^{6,16}. The higher aggregation propensity of α -Syn upon metal ions binding is due to the reduction of electrostatic charges, what leads to different degrees of neutralization of the charged N- and C-terminal regions and consequently the more energetically favorable fibril formation^{15,28,29}. We also observed formation of more hydrophobic clusters and β -sheets through ANS and CR binding studies, respectively, in fibrils produced in presence of the tested cations. Therefore, the Zn^{2+} , Cu^{2+} , Ca^{2+} , and Mg^{2+} ions drove α -Syn fibril formation at various rates, what could also cause the formation of different types of fibrils.

Interesting observation was that fibrils formed in presence of Zn^{2+} were considerably shorter than the other fibrils. This was supported by AFM studies revealing great variations in the fibrils morphology particularly of their length, particularly sizes of the fibrils formed in presence of Zn^{2+} being the shortest and fibrils formed in presence of Ca^{2+} being the longest (more than 4 times longer than the former). The shorter fibrils formed in the presence of Zn^{2+} and the corresponding shorter lag phase can imply formation of a higher number of nuclei in presence of Zn^{2+} compared to Cu^{2+} , Ca^{2+} , and Mg^{2+} . Furthermore, considering the longer fibrils formed in presence of Ca^{2+} ions, one can suggest that interaction of Ca^{2+} with α -Syn can result in formation of lower number of nuclei compared to Zn^{2+} .

Evaluation of contents of secondary structure in the fibrils formed in presence and absence of Zn^{2+} , Cu^{2+} , Ca^{2+} , and Mg^{2+} disclosed the interesting observation that Zn^{2+} ions induced formation of higher amounts of antiparallel β -sheets compared with the other three ions. Shorter α -Syn fibrils containing higher amounts of antiparallel β -sheets in presence of Zn^{2+} ions are evidenced by studies suggesting that antiparallel β -strands can contain lower number of β -strands in comparison to parallel β -sheets³⁰, which can imply that they are shorter. The observed differences in the secondary structure contents of fibrils formed

in presence of Zn^{2+} , Cu^{2+} , Ca^{2+} , and Mg^{2+} can be attributed to the variations in the binding sites and unique nature of each cations interaction with α -Syn monomers. It has been determined that Zn^{2+} in lower concentrations (up to 1:1 α -Syn: Zn^{2+} ratio) binds mainly to Asp121, Asn122 and Glu123 in the C-terminal region. However, at the concentration we tested (500 μM i.e. 1:5 α -Syn: Zn^{2+} ratio), the His50 is also involved as a lower affinity binding site for Zn^{2+} with its imidazole ring as the anchoring group for Zn^{2+} binding¹⁶. It has been suggested that Zn^{2+} can bridge adjacent strands of α -Syn to fulfill its coordination sphere, which was not confirmed by the results of studies of Valiente-Gabioud. The binding of Zn^{2+} to α -Syn through His50 has not been fully elucidated and questions remained unanswered regarding Zn^{2+} coordination. Although it has been revealed that the hydroxyl of Tyr can also provide stable Zn^{2+} complexes in the case of murine amylin³¹. Therefore, Tyr39 located at the vicinity of His50 probably can fulfill Zn^{2+} coordination sphere.

The interactions between α -Syn and Cu^{2+} follow different mechanics. Although Cu^{2+} binds to N-terminal region of α -Syn and His50, the binding site is different. Exploring the interaction between α -Syn and Cu^{2+} has revealed 1MDVFMKGLS9 and 48VAHGV52 as the main binding sites of Cu^{2+} in the N-terminal domain of α -Syn^{6,15}. Studies of the interactions between Ca^{2+} and Mg^{2+} with α -Syn indicated the acidic C-terminal region as the binding site¹⁷. Therefore, the differences in the binding mode and the engaged amino acids can explain the differences observed in fibril conformation and morphology. We thought that morphologically different fibrils could display different levels of cytotoxicity.

α -Syn fibrils released from dead neurons into extracellular space can be cytotoxic to neighboring neurons³². It has been shown that α -Syn fibrils can bind plasma membranes of neuronal cells and induce cell death³³. We observed cytotoxicity of α -Syn fibrils formed in absence of metal ions, which was comparable to the published reports^{22,23}. Enhanced cytotoxicity of α -Syn fibrils formed in presence of Cu^{2+} has also been reported²⁴. Furthermore, we also observed higher cytotoxicity of α -Syn fibrils formed in presence of Ca^{2+} and Mg^{2+} . However, nontoxicity of the fibrils induced by Zn^{2+} was interesting. Since based on previous studies which indicated that the length of amyloid fibril fragments plays a key role for their cytotoxicity³⁴ and regarding the shorter fibrils formed in presence of Zn^{2+} ions compared to control fibrils (AFM and DLS studies), we expected to detect greater cytotoxicity of these fibrils. Facing this, we turned to CD studies and performed an accurate analysis of the fibrils conformations and noticed a considerable difference in the contents of antiparallel beta sheets in fibrils formed in presence of Zn^{2+} ions in comparison with other fibrils. Considering the different binding sites of Zn^{2+} , Cu^{2+} , Ca^{2+} , and Mg^{2+} on α -Syn, one can suggest that various types of oligomers may form giving rise to different morphologies of α -Syn fibrils with differing cytotoxicity. Additionally, a recently published paper reported the release of toxic oligomeric species from α -Syn fibrillar ends, which can cause cytotoxicity³⁵. This along with the fact that there are a number of receptors at the cell membrane, which bind to β -sheet rich conformers such as cellular prion protein³⁶, led us to the hypothesis that maybe there are specific receptors at the cell surface for particular types of α -Syn oligomers, whose activation upon binding of the oligomers can transduce

various deadly or survival intracellular cell signals. Alternatively, polymorphically different fibrils could have different seeding activities once entered the cells³⁷. Therefore, the authors suggest that the type of β -sheet formed in fibrils can affect the interactions of fibrils with the potential receptors at the cell surface and consequently their cytotoxicity. Furthermore, the amino acid stretches exposed on the sides of α -Syn fibrils can define their interactomes at the neuronal cell membrane³⁸, and regarding the different binding sites of various metal ions, i.e. not the same exposed amino acid stretches at α -Syn fibril sides, it can be suggested that involvement of different interaction partners can be the basis of the observed differences between different fibrillar structures and cytotoxicities of α -Syn fibrils.

Figure 6 displays a schematic representation of the findings in the current paper. To conclude, fibrils produced in presence of Zn^{2+} , Cu^{2+} , Ca^{2+} , and Mg^{2+} ions presented different kinetic parameters, morphology, secondary structure, and cytotoxicity. These can be tracked down to the different mode and binding sites of each cation's interaction with α -Syn monomers. Particularly, in the case of Zn^{2+} ions we observed a considerably higher amount of antiparallel β -sheets compared to parallel ones and interestingly no cytotoxicity of the corresponding fibrils. The authors suggest a possible protective role for Zn^{2+} ions in neurons, which can neutralize pathogenic high concentrations of α -Syn and prevent formation of cytotoxic fibrils. The characterization of toxicity or of protective mechanism has not been addressed in the current study. However, the authors also suggest that there may be some receptors that favor specific polymorphs of amyloid fibrils and disfavor others. The receptors and the corresponding downstream signaling activation can determine the neuron's fate, either survival or death.

Materials And Methods

Materials

Thioflavin T (ThT), tris(hydroxymethyl)aminomethane, phenylmethylsulfonyl fluoride (PMSF), Congo red, 8-Anilino-1-naphthalenesulfonic acid (ANS), and 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) were purchased from Sigma (St.Louis, MO, USA). KCl, NaCl, ZnCl_2 , CuCl_2 , CaCl_2 , and MgCl_2 , ethylenediaminetetraacetic acid (EDTA), ammonium sulfate, NaH_2PO_4 , and Na_2HPO_4 , and KH_2PO_4 were obtained from Merck. pT7-7 asyn WT plasmid containing α -Syn gene was obtained from addgene. Isopropyl β -D-1-thiogalactopyranoside (IPTG) was purchased from CinnaGen. The HiTrap Q FF anion exchange chromatography column was from GE Healthcare. Dulbecco's Modified Eagles Medium (DMEM), fetal bovine serum (FBS), and trypsin were all from Gibco. Penicillin-streptomycin solution was purchased from BIO-IDEA. The cell culture flasks and plates were obtained from SPL.

Expression and purification of α -synuclein

α -Syn was expressed in *Escherichia coli* BL21(DE3) transfected with pT7-7 asyn WT plasmid according to Hoyer *et al.* with some modifications^{39,40}. Briefly, pT7-7 asyn WT plasmid containing *E. coli* cells were grown in Luria broth (LB) overnight in the presence of 100 $\mu\text{g}/\text{ml}$ ampicillin and used as a pre-culture next

day. Upon reaching the OD₆₀₀ of 0.6, the cells were induced to express α-Syn using 1 mM IPTG for 4 h, while the culture flasks were incubated in 37 °C and shaken at 180 rpm. Then, the cells were harvested by centrifugation at 6,000 rpm for 5 min at 4 °C to obtain the cell pellet. Following re-suspension of the cell pellet in lysis buffer (20 mM Tris base pH 8.0, 1mM EDTA, and 1 mM PMSF), sonication at 50 W (10 s on and 10 s off) was performed. Then, the cells suspended in the lysis buffer were placed in boiling water for 20 min followed by centrifugation at 18,000 ×g for 30 min at 4 °C. The supernatant was collected and ammonium sulfate was slowly added until reaching 0.36 g/ml. Following stirring for 30 min at 4 °C, the suspension was centrifuged at 18,000 ×g for 20 min at 4 °C. The pellet was resuspended in 20 mM Tris buffer (pH 8.0) and loaded onto a HiTrap Q FF anion exchange chromatography column. α-Syn was eluted at 300 mM NaCl and its purity was confirmed by SDS-PAGE. The purified α-Syn was dialyzed against 20 mM Tris base buffer (pH 7.5) and its concentration was determined by measuring its absorbance at 275 nm ($\epsilon_{275} = 5600 \text{ M}^{-1} \text{ cm}^{-1}$)⁷. It is worth mentioning that the purified α-Syn used in our studies does not contain any additional motifs, *i.e.* His tag, that could interact with the metal ions used in our experiments.

Fibril formation

To study the effect of different cations (Zn²⁺, Cu²⁺, Ca²⁺, and Mg²⁺) on α-Syn fibril formation, 100 μM of the protein in 20 mM Tris buffer (pH 7.5) was incubated in presence and absence of 500 μM of each cation at 37 °C under 1,000 rpm stirring. As a control α-Syn was incubated in Tris buffer alone. Kinetic studies were performed followed by characterization of the fibrils upon completion of the fibril formation.

Thioflavin T fluorescence assay

Kinetic of α-Syn fibril formation was assessed in presence of 0 and 500 μM of each metal ion using ThT fluorescence assay^{41,42}. ThT fluorescence assays were carried on a Cary Eclipse VARIAN fluorescence spectrophotometer (Mulgrave, Australia). The measurements were performed by adding 10 μl of 100 μM α-Syn to 490 μl of 25 μM ThT solution followed by 5 min incubation in room temperature. ThT sample was prepared as a 10 mM stock solution and diluted to 25 μM in 25 mM sodium phosphate buffer (pH 6.5) and filtered through a 0.2 μm filter. ThT was excited at 450 nm and the emission was monitored at 465-550 nm. The excitation and emission slits were set at 5 and 10 nm, respectively. The obtained data from ThT fluorescence were fitted using the following equation⁴³:

$$F = F_{\min} + \left(\frac{F_{\max}}{1 + e^{-\left[\frac{(t-t_0)}{\tau}\right]}} \right) \quad \text{Eq. 1}$$

where $F_{\min}$ and $F_{\max}$ define the fluorescence intensities at time 0 and saturation phase of incubation, respectively. t is the incubation time and t_0 is the time that 50% of maximal fluorescence is obtained. The value of τ was determined by nonlinear regression. Apparent growth rate constant (k_{app}) and lag phase time (t_{lag}) were calculated as $1/\tau$ and $t_0 - 2\tau$, respectively⁴⁴. The kinetic studies were performed in triplicate.

ANS binding assay

For each measurement, 490 μl ANS solution was mixed with 10 μl of end stage α -Syn fibrils. ANS was excited at 350 nm and emission spectra were recorded between 400 and 600 nm. Excitation and emission slits were set at 5 and 10 nm, respectively.

Congo Red adsorption

Congo red solution was prepared in Congo red buffer containing 5 mM potassium phosphate and 150 mM NaCl (pH 7.5). Upon completion of α -Syn fibril formation, 25 μl of each sample was added to 475 μl of 20 μM Congo red solution. Following 30 min incubation in room temperature, absorbance spectra in the range 400-600 nm were collected. The amount of Congo red bound to fibrils, as an index of amount of fibrils, was determined using the following equation⁴⁵:

$$\text{CRB (M)} = (\text{A540}/25295)/(\text{A488}/46306) \quad \text{Eq. 2}$$

where CRB defined the molar concentration of bound Congo red, and 25,295 and 46,306 present the molar extinction coefficients of bound and unbound Congo red, respectively.

Circular dichroism spectroscopy

Secondary structural changes of α -Syn at various time points during fibril formation in presence of 0 and 500 μM of each metal ion (Zn^{2+} , Cu^{2+} , Ca^{2+} , and Mg^{2+}) was studied using far UV (190-260 nm) circular dichroism spectroscopy on an AVIV 215 spectropolarimeter. The protein was diluted to a final concentration of 20 μM and placed in 0.1 cm path length cuvettes. The CD data were finally analyzed using CDSD software. We used DICHROWEB server CONTILL algorithm and the SMP180 reference data set to further analyze the contents of secondary structure elements⁴⁶⁻⁴⁹. Furthermore, we used the BeStSel web server to accurately analyze the CD spectra^{50,51}.

Dynamic light scattering

To analyze the size distribution of α -Syn assemblies at various time points during fibril formation we performed dynamic light scattering (DLS) measurements using a Zeta plus (Brookhaven Instruments). The solutions were filtered through a 0.2 μ m syringe filter. Final concentration of each sample was 8 μ M (starting concentration equivalent) in the cuvette. The measurements were performed by a laser of 657 nm and at fixed detector angle of 90 ° in room temperature (25 °C). DLS experiments were performed in triplicate.

Atomic force microscopy

α -Syn fibrils formed in presence and absence of 500 μ M of each studied cation were also analyzed by atomic force microscopy. Briefly, 10 μ l of 100 fold diluted α -Syn fibril samples were placed on a freshly cleaved mica and dried at room temperature. Then, images were obtained in semicontact mode using an Atomic Force Microscopy (NTEGRA, NT-MDT, Russia) followed by processing the images by Nova software (version 1.26.0.1443).

Cell studies

Human neuroblastoma SH-SY5Y cells were a kind gift from Dr. Saeed Karima (Department of Clinical Biochemistry, Shahid Beheshti University of Medical Sciences, Tehran, Iran). The cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. SH-SY5Y cells were cultured in T25 flasks (SPL, South Korea) at a density of 20,000 cell/cm² and subcultured using 0.25% (w/v) trypsin and 0.03% (w/v) EDTA in Dulbecco's Phosphate Buffered Saline (DPBS) to detach cells. The cells were incubated at 37 °C in a humidified atmosphere (95%) along with 5% CO₂.

Effect of α -Syn fibrils formed in presence or absence of 500 μ M Zn²⁺, Cu²⁺, Ca²⁺, and Mg²⁺ on SH-SY5Y neuroblastoma cells were evaluated using MTT assay. The cells were harvested and seeded at 5,000 cells/well in a 96-well cell culture plate (SPL, South Korea) and incubated for 24 h at 37 °C to allow cells to attach. Then, the cells were treated with 0, 10, and 20 μ M of fibrils formed in presence or absence of 500 μ M Zn²⁺, Cu²⁺, Ca²⁺, and Mg²⁺ and incubated for 24 h. Then, the culture media were aspirated and the cells were treated with fresh media containing 0.25 mg/ml MTT. Following incubation for 3 h at 37 °C, the culture media were removed and 100 μ l DMSO was added to each well to dissolve the formed formazan crystals. Absorbance of the formed colorful solution was measured at 570 nm using an Elisa reader spectrophotometer (BioTek, USA). Cell viability was calculated using the following equation⁵²:

$$\text{Viable cells (\%)} = (\text{Abs of sample} \times 100) / (\text{Abs of control}) \quad \text{Eq. 3}$$

Statistical analysis

All the results were average of at least three independent experiments and represented as mean $\pm$ standard error of mean (SEM). The statistical analysis was carried out using Microsoft Office Excel Version 2016. One-way analysis of variance (ANOVA) and Tukey's post-hoc analysis were used to compare means for continuous variables. A p -value of 0.05 was considered as the level of statistical significance.

Declarations

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Author Contribution

D.A. conceived of and designed the study, performed statistical analysis, dynamic light scattering and protein purification, and collaborated in ThT fluorescence assay, circular dichroism spectroscopy and atomic force microscopy. F.M. carried out ThT fluorescence assay, ANS binding assay, Congo Red Absorption, atomic forced microscopy analysis and cell studies, and collaborated in study design, circular dichroism spectroscopy and protein purification. A.G carried out circular dichroism spectroscopy. F. M. M. visualized the results of this study. M.P. and H.D. provided support for protein purification. G.R., V.M., T.H., J.T., and A.A.S. provided useful reagents and helpful discussions. G.R. and A.A.S. supervised and coordinated the study. D.A. and F.M. organized the experiments and wrote the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing Interest Statement

The authors report no conflict of interest.

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Figures

Figure 1

α -Syn fibril formation in presence and absence of each metal ion. **a)** For kinetic studies, 100 μ M α -Syn was incubated under constant shaking (1000 rpm) in 20 mM Tris buffer (pH 7.5) supplemented with 500 μ M of the mentioned metal ions at 37 °C. As a control (Ctrl) α -Syn was incubated in Tris buffer alone. Fibril formation was monitored by 25 μ M ThT fluorescence excited at 450 nm. **b)** Congo Red binding absorbance spectra of α -Syn fibrils formed in presence and absence of the metal ions. **c)** The amount of fibrils formed compared to control calculated based on bound CR using Eq. 2. **d)** Effect of each cation on hydrophobicity of α -Syn fibrils. α -Syn (100 μ M) was incubated in the presence and absence of 500 μ M of chloride salts of the metal ions in 20 mM Tris buffer (pH 7.5) at 37 °C and 1,000 rpm. ANS fluorescence measurements were performed on end stage fibrils of each sample. CR: Congo red solution. ANS: ANS solution.

Figure 2

Effect of each metal ion on size evolution of α -Syn fibrils. The fibrils were analyzed by DLS at the denoted times. The error bars represent the standard deviations of three independent experiments.

Figure 3

AFM images of α -Syn fibrils formed in presence and absence of each studied cation. The end stage fibrils formed in Tris buffer or in presence of Zn^{2+} , Cu^{2+} , Ca^{2+} , and Mg^{2+} . The scale bars represent 500 nm.

Figure 4

Far-UV CD spectra of α -Syn monomer and fibrils formed in presence and absence of each cation. The fibrils formed by 100 μ M α -Syn in 20 mM Tris buffer (pH 7.5) or in presence of 500 μ M of the metal cations were analyzed. The measurements were carried out in room temperature (25 $^{\circ}$ C). See materials and methods for details.

Figure 5

Cytotoxicity of fibrils formed in presence and absence of each metal ion. SH-SY5Y cells were treated by α -Syn fibrils formed in Tris or in presence of the metal ions. The end stage fibrils were separated by centrifugation and after correction for concentration were used in cytotoxicity studies. The cells were treated by **(a)** 10 μ M and **(b)** 20 μ M of fibrils (corresponding to the starting monomer concentration). Cell viability was determined based on ability of cells to metabolize MTT. Fb: α -Syn fibrils. * $p < 0.05$ significant difference compared with the control group. # $p < 0.05$ significant difference compared to the Fb group.

Figure 6

Schematic representation displaying the effect of various cations on α -Synuclein fibril formation. Various metal ions induced accelerated rate of α -Syn fibrillation and formation of polymorphically different fibrils. Analysis of secondary structure revealed a relation between the type of β -sheets and morphology of the fibrils. Moreover, fibrils with higher amounts of antiparallel β -sheets were found to be less cytotoxic compared to fibrils harboring higher amounts of parallel β -sheets.