Acta Scientific MICROBIOLOGY (ISSN: 2581-3226)

Volume 5 Issue 4 April 2022

Research Article

Expression and Characterization of *in vitro* Aggregates and Inclusion Bodies of α-synuclein in *E. coli*

Sudeepa Srichandan[1], Kripa N Nand[2*], Amulya K Panda[1*] and Pratima Ray[3]

[1]*Product Development Cell, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi, India*

[2]*Department of Biological Sciences, RPI, New York, USA*

[3]*Faculty of Science, Jamia Hamdard University, New Delhi, India*

***Corresponding Author:** Amulya K Panda and Kripa N Nand, Product Development Cell, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi, India and Department of Biological Sciences, RPI, New York, USA.

DOI: 10.31080/ASMI.2022.05.1044

Received: March 03, 2022

Published: March 24, 2022

Abstract

α-synuclein (SNCA gene) aggregation in neuronal cells causes Parkinson's disease (PD), one of the most common neurodegenerative diseases. Mutations in the SNCA gene locus and accumulation of metal ions are the major hallmarks in PD patients. Inclusion bodies of α-synuclein were expressed in *E. coli* by incorporating mutations in α-synuclein and supplementing metal ions in the culture media. The inclusion body aggregates were purified and analyzed for the presence of amyloid type structures using thioflavin binding. The structure and functional characteristics of inclusion body aggregates formed during protein expression in *E. coli* makes them a suitable model to understand the mechanism of amyloid formation. Soluble α-synuclein was aggregated to form amyloid type aggregates *in vitro*. Inclusion bodies of α-synuclein showed similar structure and similar kinetics of formation as that of *in vitro* aggregates of α-synuclein. Formation of α-synuclein as inclusion bodies in *E. coli* and its amyloidogenic characteristics can be used to understand the process of protein aggregation. This information will be useful in discovery of next generation inhibitors for Parkinson's disease.

Keywords: α-synuclein; Aggregation; Inclusion Body; Amyloids; Parkinson's Disease

Introduction

Advancement in medical research has lead to recognition of a specific class of diseases known as the 'conformational diseases' [1]. Conformational diseases includes diverse disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, Prion diseases or transmissible spongiform encephalopathies, systemic amyloidosis and Type 2 diabetes, where conformational rearrangement of a specific protein leads to the aggregation and deposition of the protein [2]. Understanding protein aggregation in conformational diseases not only help in understanding the disease progression but also in the development of suitable treatment modality.

Proteinaceous intracellular inclusions called Lewy bodies, are the hallmark of α-synucleinopathies. Lewy bodies contain a large number of molecules, the most predominant being α-synuclein [3]. α-synuclein is a small acidic protein of 14 kDa molecular weight consisting of only 140 amino acid residues that are highly conserved in vertebrates. It has three distinct regions: membrane binding N-terminal region where all the clinical mutations responsible for its aggregation are reported, Central NAC (non-

amyloid -component) hydrophobic region responsible for the oligomerization and fibrillation and disordered acidic C-terminal region. α-synuclein attains different conformations and oligomeric states, maintained in a dynamic equilibrium, affected by factors that either accelerate or prevent fibrillation [4,5]. The SNCA gene encodes α-synuclein whose genomic duplication or triplication is responsible for the autosomal-dominant PD along with some point mutations in the N-terminal of the gene locus A30P, E46K, H50Q, G51D, A53T prevelent in PD cases [6-8].

α-synuclein displays conformational flexibility i.e. it exists in different conformations and oligomeric states in a dynamic equilibrium, regulated by factors that either accelerates or inhibits protein aggregation [9,10]. α-synuclein adopts different aggregation states depending upon different conditions such as change in pH, temperature, oxidative stress, post translational modification, genetic modification, presence of phospholipids, fattyacids and metal ions. It has been hypothesized that the toxic species might be the amyloid like insoluble fibrils or soluble protofibrillar intermediates [11,12].

In most cases of PD, both genetic and environmental factors have a combinatorial effect in its epidemiology. There are evidences that sustained usage of toxic chemicals, heavy metals, use of herbicides and pesticides are thought to be major risk factors in PD [13]. Metals have systematically involved in the pathology of PD mainly by changing metal-protein interaction and metal distribution. Prolonged exposure to metal ions like zinc (Zn), copper (Cu), manganese (Mn) and iron (Fe) induce neurodegeneration [14,15]. Cellular homeostasis of iron (Fe), copper (Cu) and manganese (Mn) has a key role in the normal functioning the brain specifically the central nervous system. However they remains as a major source of catalyst that generates reactive oxygen species (ROS). ROS production induces oxidation, misfolding and aggregation of essential proteins. Cellular mechnaisms to control these damages caused by ROS are affected upon ageing, which is prevalent in Alzheimer's disease, Parkinson's disease and Amyotrophic lateral scerosis [16,17]. α-synuclein in association with Cu changes its redox properties resulting of ROS production. α-synuclein contains an Fe binding site at it's 5' region, which probably binds to Fe and increases it's expression with increase in Fe concentration [18,19]. Lewy bodies found in parkinsonian substantia nigra have shown to have elevated levels of Al and Fe, specifically Fe(III) and Fe(III) binding protein ferritin [20,21] This information indicates that metal ion can be used to induce aggregation during α-synuclein expression.

Over expression of proteins in *Escherichia coli* often results in their aggregation leading to the formation of insoluble refractile particles known as Inclusion bodies (IBs) [22]. Fourier transform infrared (FTIR) spectroscopic and X-ray diffraction analysis of inclusion body aggregates have shown to have the characteristic cross- β structures as found in amyloid fibrils [23]. Like amyloid aggregation, inclusion bodies are formed in a nucleation based phenomenon and consists of variable amounts of natively folded, partially folded and totally unfolded form of the heterologous protein. Inclusion body aggregates are highly dynamic in nature and are characterized by the continuous addition and removal of polypeptide chains [24]. Protein molecules participating in inclusion body formation can reversibly disaggregate and fold into its native form [25,26]. Apart from being used as a source of therapeutic protein [27], inclusion body aggregates produced in *E. coli* has been used as a model to conduct structural and functional analysis of amyloids [28].

The objective of the present investigation was to express α-synuclein as inclusion bodies in *E. coli* and use it as model to understand amyloid aggregation. Mutations in α-synuclein gene and presence of metal ions during protein expression were used to express α-synuclein as inclusion body aggregates in *E. coli*. These inclusion body aggregates were analyzed for amyloid characteristics. *In vitro* aggregates of soluble α-synuclein were compared to that of inclusion body aggregates to find structural similarity between them. The results indicates that inclusion body aggregates of α-synuclein clearly resemble to amyloid aggregates and can be used as a model in understanding the pathology associated with α-synucleinopathy.

Materials and Methods

Expression of α-synuclein under normal condition

The synthetic gene of WT-Syn and M-Syn was commercially synthesized by Genscript corporation, Piscataway, NJ, USA in pET 28a vector. Using standard protocol, the plasmid was directly transformed into competent *E. coli* BL21 (DE3) pLysS cells. Transformed cells were grown at 37 ºC until the OD at 600 nm reached 0.8 the expression of recombinant protein was induced

using 1 mM of asopropyl *β*- D thiogalactopyranoside (IPTG, Sigma-Aldrich, St.Louis, USA) and allowed to grow for 4 hours post induction. Protein expression was checked using 12% SDS-PAGE and confirmed by Western blot using rabbit polyclonal antibody specific for α-synuclein purchased from Krishgen biosystem (Signalway Antibody, Baltimore, USA).

Expression of α-synuclein under ionic stress condition

The chloride salts of the metal ions Al (III), Mn (III), Fe (III), Ca (III), Mg (III) and sulphate salt of Cu (II), Zn (II) (Sigma-Aldrich, St. Louis, USA) were prepared in 1 M stock in MQ. Different concentrations of each metal ion were added to the culture media at the time of induction to find out their role in IB formation during α-synuclein expression. Cells were grown at 37 ºC and induced at OD of 0.8 at 600 nm, in presence of metal ions for expression of α-synuclein.

Inclusion body purification and analysis

Inclusion bodies (IBs) were isolated from induced *E. coli* cells by resuspending cells in ice-cold lysis buffer (50 mM Tris-HCl, pH-8.5, 1 mM EDTA and 1 mM PMSF) and homogenized. The homogenous suspension was sonicated on ice (Q 700 sonicator, QSONICA, USA) using sonicator pulse for 10 min (1 sec on, 1 sec off) at 50% output. The cell lysate was centrifuged (Eppendorf Centrifuge 5810 R) at 10,000 rpm for 30 min to sediment IBs. The resulting supernatant and pellet were resuspended in 2X SDS-sample buffer (0.15 M Tris-HCl, pH-6.8, 5% sodium dodecyl sulfate (SDS), 25% glycerol, 12.5% β-mercaptoethanol, 0.025% bromophenol blue) and incubated at 96ºC for 10 min. Samples were resolved by 0.1% SDS-12% PAGE for Coomassie staining as well as Western blot analysis.

Thioflavin-S staining

E. coli cells expressing WT-Syn and M-Syn, both in normal conditions and in presence of Fe (III) were tested for Thioflavin-S dye binding followed by fluorescence microscopy and flow cytometry. Both induced and un-induced cells were resuspended in 200 µl of 125 µM of Th-S in PBS, pH 7.4 and incubated at room temperature for 15 min. The cells were harvested by centrifugation at 6000 rpm for 10 min. The cells were washed with 200 µl of PBS, and finally resespended in PBS for analysis.

Fluorescence microscopy

E. coli cells expressing WT-Syn and M-Syn, both in normal conditions and in presence of Fe (III) after staining with Thioflavin-S dye were imaged using Nikon eclipse Ti-S. 10 µl of resuspended cells were deposited on a glass slide and covered with a cover slip. Images were obtained under UV light using excitation wavelength from 350-400 nm and emission wavelength from 400-500 nm.

Flow cytometry

Flow cytometry of the *E. coli* cells expressing WT-Syn and M-Syn, both in normal conditions and in presence of Fe (III) after staining with Thioflavin-S dye was done to check the formation of inclusion bodies as amyloid specific dye binding results in characteristic spectral alteration. Cells were gated (P1) using forward scatter (FSC) and side scatter (SCC) signals. Cells in P1 were analyzed for green-yellow fluorescence emission measured on an FL1 detector with 530/30 nm band pass filter. Data was analyzed using the Facs Diva software (BD Biosciences, USA).

Purification of M-Syn

M-Syn was purified from the sonication supernatant using affinity chromatography using Ni-NTA agarose column as the recombinant proteins have histidine tag incorporated into them. Pre-packed Ni-NTA agarose column (GE Healthcare, USA) 5 ml was used for the purification of the recombinant proteins. Column was washed Mili Q (MQ) water and equilibrated with 50 mM Tris-HCl, pH 8.5. Sonication supernatant was centrifuged and loaded onto the column. The column was washed with equilibration buffer and samples were eluted using the imidazole step gradient. The presence of protein was further confirmed by SDS-PAGE. Elutes containing protein were pooled and concentrated with using concentrator (Amicon ultra centrifugal filter unit, Merk Millipore, USA) with 3 KDa cut off. Concentrated samples were loaded onto size exclusion chromatographic column (SEC), Superdex 75 PG (GE Healthcare, USA). The column was equilibrated with 20 mM Tris-HCl, pH 8.5. 2 ml of concentrated sample was loaded and eluted with 120 ml of 20 mM Tris-HCl, pH 8.5. The presence of protein was further confirmed by SDS-PAGE. Inclusion bodies of M-Syn (100 l) were solubilized in 900 l of 8 M urea at RT for 2 hours. The solubilized inclusion bodies were centrifuged at 15,000 rpm (rotor SA-300, Sorvall RC 6 Plus, Thermo Scientific) for 30 mins to remove the insolubilized inclusion bodies. Refolding was carried out in pulsatile manner using 50 mM Tris-HCl, pH 8.5. Samples were analyzed by SDS-PAGE, concentrated and run on Superdex 75 PG size exclusion column.

In vitro aggregation

In vitro aggregates were formed by incubation of M-Syn at 37 ºC with continuous stirring. Purified M-Syn at an initial concentration of 100 µM in PBS, pH 7.4 was incubated at 37 ºC under constant stirring at 200 rpm to initiate aggregation. Samples were collected at 1 h, 6 h, 12 h and 24 h intervals for Th-T binding assay.

Thoiflavin-T fluorescence

Thioflavin-T amyloid dye binding was checked by measuring the fluorescence measurements using Cary Eclipse Spectrophotometer, Varian, Australia. 10 µl of the protein mixture and 10 µl of IBs of M-Syn induced in presence of Fe (III) were added to cuvette containing 1 ml of 20 µM of Th-T in 20 mM PBS, pH 7.4 and 150 mM NaCl. The solutions were excited at 440 nm and emission spectra were scanned between 460 nm to 600 nm using a 5 nm slit width.

Dynamic light scattering

DLS measurements were performed for M-Syn *in vitro* aggregates using Zetasizer APS (Malvern instuments, USA). Size analysis of IBs of M-Syn induced in presence of Fe (III) was done using Malvern Mastersizer hydro 2000S (Malvern instuments, USA). IBs samples were injected into the detection chamber with 5% obscurity and scanned 3 times. Size distribution of IBs was analyzed by plotting a graph between percentage population and their size.

Results

Expression of recombinant WT-Syn and M-Syn in *E. coli*

The SNCA gene locus encodes for α-synuclein and many point mutations in this are associated with PD. The WT-Syn codes for α-synuclein protein and M-Syn codes for α-synuclein protein with five point mutations (A30P, E46K, H50Q, G51D, A53T). The genes were commercially synthesized by Genscript corporation, Piscataway, NJ, USA in pET 28a vector. The recombinant α-synuclein protein (WT-Syn and M-Syn) was expressed in *E. coli* BL21 (DE3) pLysS cells. Transformed cells were grown and induced at 37ºC at OD of 0.8 at 600 nm with 1 mM IPTG. Induced cells were harvested by centrifugation and IBs were extracted from both induced cells by sonication in lysis buffer followed by centrifugation. SDS-PAGE and Western blot analysis showed a promiant band at ~ 17 kDa size in the induced samples (Figure 1).

α-synuclein is a highly negative charged protein as a result of which its abnormal binding to SDS leads to unusual mobility on SDS-PAGE. SDS-PAGE and Western blot analysis showed that 90% of the protein was expressed in soluble fraction. In case of M-Syn some fraction of the protein was also expressed as inclusion bodies. This proves that presence of point mutations in the SNCA gene locus induces α-synuclein aggregation, both as inclusion bodies in *E. coli* and human brain.

Figure 1: SDS-PAGE and Western Blot analysis of sonication supernatant and pellet of *E. coli* cells expressing WT-syn and M-syn. Lane M represents molecular weight markers. Panel (a) and (b) represents SDS-PAGE and Western blot analysis of expression of WT-Syn. Panel (c) and (d) represents SDS-PAGE and Western blot analysis of expression of M-Syn. Lanes are represented as Lanes 1 and 3: sonication supernatant of induced *E. coli* expressing WT-Syn and M-Syn respectively, Lanes 2 and 4: pellet after sonication of induced *E. coli* expressing WT-Syn and M-Syn respectively. Lane M represents molecular weight markers in kDa.

Effect of ionic stress and metal ions on α-synuclein aggregation during expression

Lewy bodies that are hallmark of PD, have high content of Fe and Al indicating the increased accumulation of these metal ions during the diseased condition as compared to normal tissues [3]. Presence of certain metal ions accelerates the rate of α-synuclein fibrillation *in vitro* such as Al(III), Cu(II), Cd(II) and Fe(III) [29]. α-synuclein acts as a cellular ferrireductase, catalyze the reduction of Fe (III) to Fe (II) inside the dopaminergic neurons. The loss of this activity impairs the functioning neurons and increases levels of Fe (III) inside the cells (30,31). So it can be hypothesized that aggregation of α-synuclein and abnormal increase in metal ion concentration inside the cells are interconnected. To check whether presence of metal ions induces the aggregation of α-synuclein, the media was supplemented with certain metal ions just before induction. The chloride salts of the metal ions Al (III), Mn (II), Fe (III), Ca (II), Mg (II) and sulphate salt of Cu (II), Zn (II) with a final concentration of 2 mM was used to check their effect on protein aggregation. The

cells were induced at 37 ºC at OD of 0.8 at 600 nm with 1 mM IPTG. SDS-PAGE and Western blotting analysis (Figure 2a and 2b) of the harvested cells showed IB formation in both WT-Syn and M-Syn cells in presence of Fe (III) ions where no inclusion bodies were formed in presence of other metal ions.

Figure 2a: SDS-PAGE analysis of *E. coli* cells expressing WT-Syn in presence of different metal ions. Panels (a) and (c) represents SDS-PAGE of cells expressing WT-Syn induced in presence of different metal ions respectively. Panels (b) and (d) represents Western blot of cells expressing WT-Syn induced in presence of different metal ions respectively. Lanes are represented as Lane 1,3,5,7: sonication supernatant of induced cells expressing WT-Syn, Lanes 2,4,6,8: pellet after sonication of induced cells expressing WT-Syn, Lane M represents molecular weight markers in kDa.

Figure 2a: SDS-PAGE analysis of *E. coli* cells expressing WT-Syn in presence of different metal ions. Panels (a) and (c) represents SDS-PAGE of cells expressing WT-Syn induced in presence of different metal ions respectively. Panels (b) and (d) represents Western blot of cells expressing WT-Syn induced in presence of different metal ions respectively. Lanes are represented as Lane 1,3,5,7: sonication supernatant of induced cells expressing WT-Syn, Lanes 2,4,6,8: pellet after sonication of induced cells expressing WT-Syn, Lane M represents molecular weight markers in kDa.

This can be attributed to property of amyloidogenic proteins as chaperons of redox metals. The increase in local concentration of the protein favours IBs formation and point mutations in the SNCA gene locus increases the aggregation propensity of the protein. So after induction of protein expression, oligomerization of α-synuclein starts because of molecular crowding and prescence of Fe (III) accelerates the formation of much larger oligomers resulting in amyloid fibrils. This results in higher IB formation in M-Syn in comparison to that of WT-Syn.

Expression of α-synuclein as inclusion body aggregates

During recombinant protein expression in *E. coli* the intracellular protein concentration increases resulting in insoluble protein deposits as IBs. They have highly ordered, amyloid like conformations [1]. Many amyloids specific dyes are used for the detection of amyloid aggregates however most commonly used are Congo red (CR) and Thiflavins (Th-T and Th-S) [32]. Staining with Th-S has an advantage over other dyes that it binds to amyloid fibrils only and not the soluble monomers or oligomers. Th-S also has the ability to cross the cell membrane and bind to the amyloid fibrils without interfering with the amyloid pathway. So, Th-S staining followed by flow cytometry can be used as potential method to detect amyloid aggregation inside living bacterial cells [33]. Amyloid dyes binding with amyloid fibrils increases the fluorescence intensity without change in the emission or excitation spectra. This fluorescence emission caused by the dye binding to aggregate can be visualized using fluorescence microscopy. As can be seen in figure 3 both WT-Syn and M-Syn cells grown in presence of Fe (III) when stained with Th-S shows bright green-yellow fluorescence against dark background when illuminated with UV-light. This shows that presence of Fe (III) initiates fibrillation of α-synuclein in WT-Syn, which becomes prominent in M-Syn, because of mutations at A30P, E46K, H50Q, G51D, A53T.

Figure 3: Fluorescence microscopy detection of *E. coli* cells expressing WT-Syn and M-Syn stained with Th-S. Panel (a) represents only *E. coli* cells stained with Th-S. Panel (b) represents un-induced *E. coli* cells expressing M-Syn stained with Th-S. Panels (c) and (e) represents induced WT-Syn and M-Syn in normal conditions and stained with Th-S. Panels (d) and (f) represents induced WT-Syn and M-Syn in presence of Fe (III) and stained with Th-S.

As fluorescence microscopy based Th-S fluorescence detection cannot be used for large-scale analysis, flow cytometry was used for the analysis of complete cell population and detection of Th-S binding to IBs inside the cell. The fluorescence properties of bacterial cells expressing IBs were analyzed in presence and absence of Th-S. Fluorescence measurements was done using excitation wavelength of 355 nm and emission collected at 530 nm.

Figure 4 shows that there is no significance difference in fluorescence between the bacterial cells with and without Th-S, indicating the absence of any background fluorescence. This allowed the threshold (P2) for gating the fluorescent cell population in the presence of Th-S. For WT-Syn, bacterial cells induced in presence of Fe (III), 67.6% of the cells were found in P2. For M-Syn, bacterial cells induced without Fe (III), 63.3% of the cells were found in P2, which increased to 82.6% when cells were induced in presence of Fe (III). The results correlate with the fluorescence data indicating the fibrillation or oligomerization of α-synuclein in presence of point mutations and Fe (III) ions. Flow cytometry data also suggests that cells staining with Th-S provide a sensitive and selective approach for the identification of intracellular amyloid like aggregates or inclusion bodies.

Figure 4: Flow cytometry detection of *E. coli* cells expressing WT-Syn and M-Syn stained with Th-S. Panel (A) corresponds to forward scatter (FSC) vs. side scatter (SSC) dot-plots showing the P1 gate. Cells in P1 were analyzed by fluorescence emission at 530 nm upon excitation at 355 nm. Panel (B) corresponds to cell frequency histograms of *E. coli* BL21 cells in the absence of Th-S. Panel (C) corresponds to cell frequency histograms of *E. coli* BL21 cells stained with Th-S. Panels (D) and (G) corresponds to cell frequency histograms of *E. coli* BL21 cells expressing WT-Syn and M-Syn induced in normal conditions in the absence of Th-S respectively. Panel (E) and (H) corresponds to cell frequency histograms of *E. coli* BL21 cells expressing WT-Syn and M-Syn induced in normal conditions stained with Th-S respectively. Panel (F) and (I) corresponds to cell frequency histograms of *E. coli* BL21 cells expressing WT-Syn and M-Syn induced in presence of Fe (III) stained with Th-S respectively.

Amyloid aggregation takes place in a nucleation dependent manner in which aggregates of the protein acts as seeds for further fibrillation. As can be concluded from the above results point mutations enhance the binding of Fe with α-synuclein. There are certain reports, which show the binding of Fe-α-synuclein *in vitro* in single point mutation variants [34-37]. To check if the five point mutations together increases the Fe dependent aggregation or any structural changes in the protein which increase it's Fe binding, the M-Syn was purified from both the soluble fraction and IBs.

Purification of recombinant α-synuclein (M-Syn)

M-Syn was purified from both the soluble fraction and IBs. M-syn was purified from the sonication supernatant using affinity chromatography using Ni-NTA agarose column as the recombinant proteins have histidine tag incorporated into them. Sonication supernatant was centrifuged before loading onto pre-packed His Trap FF 5 ml column. After washing column with buffer, bound proteins were eluted by step gradient using imidazole. Figure 5a shows the SDS-PAGE analysis of elutes of M-Syn. As can be seen, the recombinant protein eluted with 250 and 500 mM imidazole concentration. The fractions were pooled and concentrated by using Amicon concentrator (3 kDa cut-off). Concentrated protein was run on Superdex 75 PG size exclusion column (Figure 5b). Purified and concentrated protein was analyzed by SDS-PAGE (Figure 5c). Purified M-Syn when run on Superdex 75 PG size exclusion column was eluted just after the void volumn. The corresponding elute when run on gel shows a band at a lower molecular weight. This shows that M-Syn protein is not monomeric in nature and is forming soluble aggregates in solution. The formation of soluble aggregates can be attributed to the presence of mutations, which increased the aggregation propensity of the protein.

Figure 5a: SDS-PAGE analysis of elutes of affinity chromatography of M-Syn. Purification of M-Syn from sonication supernatant using affinity chromatography. Lanes are represented as Lanes 1 and 2: represents the load and flow through respectively. Lanes 3 to 7: represents eluted fractions(wash, 50 mM, 100 mM, 250 mM, 500 mM imidazole respectively). Lane M represents molecular weight markers in kDa.

Figure 5b: Elution profile of M-Syn using size exclusion chromatography column. Purified M-Syn was further purified using size exclusion chromatography. Y-axis represents the absorbance at 280 nm (mAU) representing the protein content in various elution fractions collected. X-axis represents the volume (ml) of total run.

Figure 5c: SDS-PAGE analysis purified M-Syn. Lanes are represented as Lanes 1 represents elute from the first peak. Lane 2 represents purified M-Syn protein from the second peak. Lane M represents molecular weight markers in kDa.

M-Syn was also purified from inclusion bodies from cells induced in presence of Fe (III), by solubilizing in 8 M urea. The solubilized proteins were refolded by pulsatile renaturation method and analyzed using SDS-PAGE (Figure 6a).

M-Syn was purified with 95% purity from inclusion bodies. The refolded and concentrated M-Syn protein was run on Superdex 75 PG size exclusion column. Figure 6b shows the chromatogram of the run. The protein eluted just after the void volumn similar to that of soluble expressed M-Syn showing the presence of soluble aggreagtes. Mutations in the α-synuclein protein results in forming oligomeric forms of the protein. So it can be concluded that these soluble aggregates increases propensity for Fe and act as a seed for the initiation of aggregation of the protein into inclusion bodies.

Figure 6a: SDS-PAGE analysis of purified M-Syn from inclusion bodies. Solubilized M-Syn was refolded and concentrated. Lanes are represented as Lane 1- pellet after centrifugation after refolding, Lane 2- concentrated refolded M-Syn. Lane M represents molecular weight markers in kDa.

Figure 6b: Elution profile of solubilized and refolded M-Syn from IBs using size exclusion chromatography column. Solubilized M-Syn was refolded and concentrated and run on size exclusion chromatography. Y-axis represents the absorbance at 280 nm (mAU) representing the protein content in various elution fractions collected. X-axis represents the volume (ml) of total run.

Characterisation of IBs and *in vitro* aggregates of α-synuclein

Protein aggregation is a common phenomenon, which results when intracellular misfolded protein interacts with each other. This is one of the reasons leading to protein aggregation during expression, causing formation of IBs. IBs are formed in a nucleation-based phenomenon similar to that of amyloid aggregates and the protein aggregates act as seeds for aggregation of similar proteins. The similarities between IBs and amyloid aggregates provide a prospect of the molecular pathway involved in their formation [1]. *In vitro* aggregates were prepared by continuous agitation of monomeric protein samples at 37 ºC. Samples were collected at 1 h, 6 h, 12 h and 24 h intervals to check the formation of aggregates. Similarly M-Syn expressing bacterial cells induced in presence of Fe (III) was harvested at 1 h, 2 h, 3 h and 4 h post induction to study the kinetics of the IB formation. Size distribution of the different samples was analyzed to identify the nucleation and growth phase of IB formation.

As can be seen in figure 7 the size distribution pattern remained constant for 4 h duration after induction. There were no distinct nucleation and growth phases present during the IB formation. It can be interpreted that the aggregates are formed because of a continuous seeding of aggregated molecules inside the cells during expression. Multiple nucleation seeds are formed in a single cell of *E. coli* during protein expression and growth of these seeds occurred for a short duration. The size of the seed got saturated and new seed were for formed during expression period. The maximum size of the IBs after 4h of induction is in the range of 0.4 to 1 Similarly size distribution of the *in vitro* aggregates after 1h of incubation shows nucleation of aggregation. This is the lag phase where soluble aggregates are formed followed by the elongation phase where insoluble aggregates are seen. The elongation phase starts after 6h of incubation. The size of the aggregates increases till 12 and achieves a steady state till 24h where all protein molecules precipitates out. The maximum size of the aggregates after 12h of incubation is in the range of 0.3 to 1 As can be seen in the figure 7 IBs and *in vitro* aggregates have similar size after different incubation time. This could be due to the formation of unfolded intermediates during *in vivo* misfolding and *in vitro* denaturation.

Figure 7: Size distribution pattern of IBs and *in vitro* aggregates of M-Syn. Panel (a) represents the size distribution pattern of M-Syn IBs induced in presence of Fe (III) isolated from induced cells harvested at 2h, 3h and 4h after induction. Panel (b) represents the size distribution pattern of *in vitro* aggregates of M-Syn harvested at 6h, 12h and 24h after induction.

The kinetics of IB formation and *in vitro* aggregates were monitored by Th-T binding assay as fluorescence of Th-T increases upon binding to -sheet secondary structure. Samples from different time points were added to 20 of Th-T and the solutions were excited at 440 nm and emission spectra were scanned between 460 nm to 600 nm. Binding of Th-T with amyloid fibrils alters the emission maxima of the dye to 482 nm.

As can be seen in the figure 8 the fluorescence intensity increased for *in vitro* aggregates in comparison to free dye. It also showed that with increase in incubation time larger aggregates were formed as evident from increased fluorescence intensity with time. How ever, IBs isolated at different time points after induction showed similar intensity conforming that multiple nucleation seed were formed during the inclusion body formation. So, it can be concluded that as α-synuclein forms amyloid fibrils when incubated *in vitro* in the similar way α-synuclein forms IBs when expressed in *E. coli* with mutation in the SNCA gene and induced in the presence of Fe (III).

Figure 8: Fluorescence emission spectra of Th-T binding IBs and *in vitro* aggregates. Panel (a) represents M-Syn IBs induced in presence of Fe (III) isolated from induced cells harvested at 2h, 3h and 4h after induction. Panel (b) represents *in vitro* aggregates of M-Syn harvested at 6h, 12h and 24h after induction.

Discussion

In recent years, α-synuclein has shown to be the main regulator of PD pathology. In both familial and sporadic forms of PD oligomeric or aggregated forms of α-synuclein is the major contributing factor in the formation of Lewy bodies [3]. Along with the protein component evidences shows the presence of certain metal ions specifically Fe(III) and Fe(III) binding protein ferritin in the lewy bodies [20]. Both the genetic and environmental factors often have a combinatorial effect in causing the diseased condition in PD. Although the homeostasis of redox active metals iron (Fe) and copper (Cu) plays important role in the normal functioning of the brain they catalyze the formation of reactive oxygen species (ROS), which are pathogenic to the cells. These ROS further increases the toxic effect of the protein aggregates inside the tissue or cellular compartments.

The intrinsically unfolded protein α-synuclein has a high affinity to bind metal ions. Expression of α-synuclein is regulated by iron in the presence of iron response element (IRE) at the 5' untranslated region (UTR) of the α-synuclein transcript [18]. Iron regulatory proteins (IRPs) help in maintaining the level of iron inside the cells. These are cytoplasmic RNA-binding proteins, which controls the translation of proteins involved in iron uptake, storage and release by binding to the IRE. This IRP-IRE binding regulates the translational activation of ferritin, ferroportin 1, and transferrin receptor depending on the absence and presence of Fe.

Human α-synuclein contains a similar IRE at its 5' UTR as found in human ferritin and ferroportin [19,38]. This provides evidence that Fe regulates translation of the protein by the IRP-IRE signaling pathway. Expression of WT-Syn in *E. coli* did not form inclusion bodies but expression in the presence of Fe formed inclusion bodies. This was confirmed from fluorescence microscopy data supported by flow cytometry data. This proves that Fe regulates the expression of α-synuclein. The inclusion body formation further increased when mutations were induced in the SNCA gene locus. Amylogenic proteins are thought to act as chaperons of redox metals i.e. they have more affinity for the metal ions. Point mutations in the SNCA gene make the protein aggregation prone or amylogenic increasing its affinity for Fe (III). This is supported by certain reports which shows increase in Fe induced aggregation of α-synuclein because of mutations [34,35]. This Fe- α-synuclein complex acts as a nucleation seed for the formation of oligomers and amyloid fibrils. Earlier reports have shown formation of larger oligomers of α-synuclein upon addition of Fe during *in vitro* aggregation of the protein [29,36]. Presence of iron is known to cause posttranslational modification of the α-synuclein protein such as nitration and phosphorylation, increasing its tendency to aggregate. Iron also induces toxicity by ROS accumulation mediated by oxidative stress. Kinetics of α-synuclein aggregation during *in vitro* aggregate formation and IB formation indicated that the smaller nucleates leads to amyloid formation. So the pathology of PD can be attributed to the interaction of α-synuclein to Fe synergistically damaging the cells by deleterious mechanisms. The toxic effect of the aggregates is enhanced by many other factors such as mitochondrial dysfunction, disruption of electron transport chain, presence of ROS and failure of the intracellular proteolytic systems. Inclusion body aggregate formation in *E. coli* thus can be used as a model to simulate *in vivo* aggregation. This provides a viable alternative to evaluate the action of various drugs like substances, which can ultimately be used to control aggregation.

Conclusion

α-synuclein forms IBs when expressed in *E. coli* with mutation in the SNCA gene and in the presence of Fe (III). Mutations in the SNCA gene locus, which encodes for the α-synuclein protein, induce oligomerization of the protein when local concentration of the mutated protein increases. Mutant α-synuclein forms soluble aggregates, which acts as a seed for the initiation of amyloid aggregation of α-synuclein. Presence of Fe (III) ions in the medium enhances the aggregation. This can be attributed to the property of the amylogenic proteins as chaperons of redox metals. This Fe- α-synuclein complex formed acts as a nucleation seed for the formation of oligomers and amyloid fibrils, leading to the formation of Lewy bodies.

Conflict of Interest

All the Authors declare that there is no conflict of interest.

Acknowledgement

This research funded by Department of Science and Technology, Government of India. I would like to thank National institute of Immunology and Jamia Hamdard University of their cooperation in completing this research work.

Bibliography

1. A Ramón., *et al*. "Inclusion bodies: not that bad". *Frontiers in Microbiology* 5.56 (2014).

2. A Surguchev., *et al*. "Conformational diseases: Looking into the eyes". *Brain Research Bulletin* 81 (2010): 12-24.

3. TF Outeiro., *et al*. "Dementia with Lewy bodies: an update and outlook". *Molecular Neurodegeneration* 14.5 (2019).

4. MG Spillantini., *et al*. "α-Synuclein in Lewy bodies". *Nature* 388 (1997): 839-840.

5. B Dehay., *et al*. "Targeting α-synuclein for treating Parkinson's disease: mechanistic and therapeutic considerations". *Lancet Neurology* 14 (2015): 855-866.

6. MC Chartier-Harlin, *et al*. "α-synuclein locus duplication as a cause of familial Parkinson's disease". *Lancet* 364 (2004): 1167-1169.

7. Polymeropoulos MH., *et al*. "Mutation in the alpha-synuclein gene identified in families with Parkinson's disease". *Science* 276.80 (1997): 2045-2047.

8. CM Lill., *et al*. "Genetics of Parkinson's disease". *Molecular and Cellular Probes* 30 (2016): 386-396.

9. B Fauvet., *et al*. "α-Synuclein in central nervous system and from erythrocytes, mammalian cells, and *Escherichia coli* exists predominantly as disordered monomer". *Journal of Biological Chemistry* 287 (2012): 15345-15364.

10. L Pieri., *et al*. "Fibrillar α-synuclein and huntingtin exon 1 assemblies are toxic to the cells". *Biophysics Journal* 102 (2012): 2894-2905.

11. L Pieri., *et al*. "Structural and functional properties of prefibrillar α-synuclein oligomers". *Scientific Reports* 6 (2016): 24526.

12. M Ingelsson., *et al*. "Alpha-synuclein oligomers—Neurotoxic molecules in Parkinson's disease and other lewy body disorders". *Frontiers in Neuroscience* 10 (2016): 408.

13. ER Dorsey., *et al*. "The Parkinson pandemic—A call to action". *JAMA Neurology* 75 (2018): 9-10.

14. PL Eleonora Carboni., *et al*. "Insights on the interaction of alpha-synuclein and metals in the pathophysiology of Parkinson's disease". *Metallomics* 7 (2015): 395-404.

15. L Mezzaroba., *et al*. "The role of zinc, copper, manganese and iron in neurodegenerative diseases". *Neurotoxicology* 74 (2019): 230-241.

16. K Acevedo., *et al*. "Redox active metals in neurodegenerative diseases". *Journal of Biological Inorganic Chemistry* 24 (2019): 1141-1157.

17. Scudamore., *et al*. "Increased oxidative stress exacerbates α-synuclein aggregation *in vivo"*. *Journal of Neuropathology and Experimental Neurology* 77 (2018): 443-453.

18. AL Friedlich., *et al*. "The 5′-untranslated region of Parkinson's disease α-synuclein messenger RNA contains a predicted iron responsive element". *Molceular Psychiatry* 12 (2007): 222-223.

19. B Chen., *et al*. "Interactions between iron and α-synuclein pathology in Parkinson's disease". *Free Radical Biology and Medicine* 141 (2019): 253-260.

20. F Molina-Holgado., *et al*. "Metals ions and neurodegeneration". *BioMetal*s 20 (2007): 639-654.

21. P Lingor., *et al*. "Alpha-synuclein and iron: two keys unlocking Parkinson's disease". *Journal of Neural Transmission* 124 (2017): 973-981.

22. FA Marston., *et al*. "The purification of eukaryotic polypeptides synthesized in *Escherichia coli*". *Biochemical Journal* 240 (1986): 1-12.

23. NS de Groot., *et al*. "Amyloids in bacterial inclusion bodies". *Trends in Biochemical Sciences* 34 (2009): 408-416.

24. Cano-Garrido., *et al*. "Supramolecular organization of protein-releasing functional amyloids solved in bacterial inclusion bodies". *Acta Biomaterial* 9 (2013): 6134-6142.

25. MM Carrió., *et al.* "Construction and deconstruction of bacterial inclusion bodies". *Journal of Biotechnology* 96 (2013): 3-12.

26. E Gifre-Renom., *et al*. "The biological potential hidden in inclusion bodies". *Pharm* 12 (2020): 157.

27. P Singhvi., *et al*. "Bacterial Inclusion Bodies: A Treasure Trove of Bioactive Proteins". *Trends in Biotechnology* 38 (2020): 474-486.

28. A de Marco., *et al*. "Bacterial inclusion bodies are industrially exploitable amyloids". *FEMS Microbiology Review* 43 (2019): 53-72.

29. VN Uversky., *et al*. "Metal-triggered structural transformations, aggregation, and fibrillation of human α-synuclein: a possible molecular link between parkinson′s disease and heavy metal exposure". *Journal of Biological Chemistry* 276 (2001): 44284-44296.

30. P Davies., *et al*. "Alpha-synuclein is a cellular ferrireductase". *PLoS One* 6 (2011): e15814-e15814.

31. Q He., *et al*. "Alpha-synuclein aggregation is involved in the toxicity induced by ferric iron to SK-N-SH neuroblastoma cells". *Journal of Neural Transmission* 118 (2011): 397-406.

32. MP Jackson., *et al*. "Why are functional amyloids non-toxic in humans?". *Biomolecules* 7 (2017): 71.

33. S Ventura., *et al.* "Screening protein aggregation in cells using fluorescent labels coupled to flow cytometry". Protein Misfolding Dis. Methods Protoc., Springer, New York, (2019): 195-212.

34. Zhu ZJ., *et al*. "Differential interaction between iron and mutant alpha-synuclein causes distinctive Parkinsonian phenotypes in Drosophila". *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 1862.4 (2016): 518-525.

35. Ostrerova-Golts., *et al*. "The A53T α-Synuclein mutation increases iron-dependent aggregation and toxicity". *The Journal of Neuroscience* 20.16 (2000): 6048-6054.

36. Peng Y., *et al*. "Binding of alpha-synuclein with Fe (III) and with Fe (II) and biological implications of the resultant complexes". *Journal of Inorganic Biochemistry* 104.4 (2010): 365-370.

37. Joppe K., *et al*. "The contribution of iron to protein aggregation disorders in the central nervous system". *Frontiers in Neuroscience* (2019).

38. E Tan., *et al*. "Iron regulatory protein (IRP)-iron responsive element (IRE) signaling pathway in human neurodegenerative diseases". *Molecular Neurodegeneration* 12.75 (2017).