CHAPTER 7

STUDY OF INHIBITION OF α-SYNUCLEIN AGGREGATION USING CROWDING AGENTS

Study of inhibition of α-Synuclein aggregation using crowding agents

7.1. Abstract:

Macromolecular crowding is one of the essential cellular environment elements that can influence the aggregation mechanism of α-Synuclein (αS). The experimental investigation of the effect of various inert crowding agents/crowders on the behaviour of αS protein has attracted increased attention in recent research. However, attention has not been given in the molecular level *in-silico* analysis of αS in a crowded environment. Therefore, in this computational work, Polyethylene glycol (PEG) is used to create a crowded environment (one with 5 PEG molecules; other with 10 PEG molecules) surrounding αS protein, and the resulting complexes are subjected to Molecular Dynamics Simulation (MDS). The trajectories resulting from the MDS are used to analyze the effects induced by PEG in the conformational and structural dynamics of αS during the simulation time. The conformational snapshots from the two crowded environments compared with the control show that the amount of α-helices in the secondary structure of αS decreases as simulation time progresses. The RMSD of the three systems shows that the stability of the αS protein is more in the 10PEG-αS system than in the 5PEG-αS system when compared with the control. It is also observed that the amount of anti-parallel β-sheets is highest in the 10PEG-αS system than in the 5PEG-αS system and the control. This study has thus helped us to understand that αS protein is sensitive towards an inert crowding environment and hence this crowding environment affects structural and conformational properties of the protein.

7.2. Introduction:

An animal cell's internal environment is very crowded since it contains a large number of macromolecules, such as proteins, nucleic acids, ribosomes, and carbohydrates. This implies that other macromolecular species are not able to occupy a sizable portion of the intracellular space. The cytoplasm of an organism contains between 80 and 400 mg/ml of macromolecules, according to estimates [632]. Between 10% and 40% of the volume of physiological fluids is taken up by all macromolecules. Increased bulk-viscosity, the presence of excluded volume effects, and the possibility of

both particular and non-specific intermolecular interactions are all effects of the crowded environment. The term "Macromolecular Crowding" was first used by Minton in 1981 to describe the effects of excluded volume from macromolecules, and throughout the past three decades, he has produced several important foundational investigations. Macromolecular crowding is a term used to describe the total excluded volume of all macromolecules inside the cells. The behavior of proteins in cellular contexts can be significantly impacted by macromolecular crowding and confinement [633, 634]. Macromolecule concentrations inside of cells can exceed 400 g/L. In the cellular environment, macromolecules take up a lot of space and exert non-specific forces on nearby molecules. It is generally known that these forces can have a big impact on how proteins behave. Molecular crowding has been shown to have an impact on protein structure and function in experiments. For instance, cytochrome c takes on an unfolded shape at low pH. The protein changes into a nearly natural molten globule state when the crowding agent dextran is added to the sample. Additionally, it has been demonstrated *in vitro* that crowding increases the activity of phosphoglycerate kinase (PGK). The crowded conditions inside of cells also affect diffusional behaviour of proteins, which affects how quickly they fold, associate with the other molecules, and move inside of cells. Numerous in vitro tests conducted by us and others have demonstrated that the presence of macromolecular crowders increases protein stability [635, 636].

However, less research has been conducted on how macromolecular crowding affects the structure and the conformational dynamics of IDPs. These proteins can be exceedingly flexible in non-denaturing circumstances and they lack stable tertiary structures. The occurrence of neurodegenerative diseases is caused by these IDPs. The structural and conformational analysis of the monomeric αS protein to comprehend the conformational changes that lead to the aggregated state is significantly vital because it has been demonstrated that the monomeric form of αS protein gradually aggregates into fibrils and plays a role in PD pathogenesis [637, 638]. It has been investigated how different environmental and physical factors affect the folding of αS. The behaviour of macromolecular crowding on the structure of αS has been studied using a variety of crowding agents, including Polyethylene Glycol (PEG), Ficoll, Dextran, and alcohol, among others. To imitate intracellular crowding density, PEGs, which are water-soluble, chemically inert straight-chain polymer with straightforward repeating subunits, and biocompatible polymers, are frequently used as macromolecular crowding agents [639,

640]. The PEG and other crowders can speed up enzyme reactions, change reaction byproducts, prevent macromolecules from thermal denaturation, hasten protein folding, and aid in nucleic acid renaturation. Additionally, they can protect macromolecules against thermal denaturation [641-643]. PEG is employed in the purification of proteins and produces the precipitation of proteins at high concentrations (30%). In order to highlight the function of PEG in the fibrillation and aggregation kinetics of αS, numerous experimental experiments have been conducted. However, no analysis at the computational or molecular levels has been carried out in this regard. The PEG molecules were used as crowders in this computational study's explicit simulation of the αS protein system using the AMBER force field and Molecular Dynamics Simulation. In order to arrive at conclusions, the structural and dynamic changes of the αS molecule are investigated in the presence of the PEG molecules over a range of simulation time periods.

7.3. Materials and Methods

7.3.1 System Preparation:

7.3.1.1. Preparation of receptor:

The αS monomeric 3-D structure (PDB ID: 1XQ8) [275] retrieved from RCSB Protein Data Bank [502, 503] was used to prepare receptor molecule.

7.3.1.2. Preparation of crowding agent:

The structure of PEG molecules have been constructed with the help of xleap module of AMBER [478]. In one of the complexes, 5 molecules of PEG, each containing 10 monomer units are used so that the polymer will be flexible enough to coil upon itself but short enough so that we use multiple PEGs that could independently probe different binding locations on the surface of αS monomer. In another complex, 10 molecules of PEG, each containing 10 monomer units are used.

7.3.1.3. Preparation of the complexes:

A system of αS and PEG was constructed for this work comprising of these components: (a**) α-Synuclein (control)**; (b) αS monomer in crowding environment of 5 PEG molecules named as **5PEG-α-Synuclein**; (c) αS monomer in crowding environment of

10 PEG molecules named as **10PEG-α-Synuclein**. Using the antechamber protocol, the selected solution structure was further curated in xleap. This includes bcc charge addition, frcmod file generation, and complicated system in explicit and implicit solvation. The topology and the coordinate files for both systems were then created separately. We used explicit solvation to perform MD simulations on the complex systems. The relevant topology and parameter input files were also produced for the binding free energy study.

7.3.2. Setup for MD simulations:

The AMBER ff99SB force field [74] in the AMBER 14 Leap module [478] was used to simulate the complex system. Using the explicit TIP3P (transferable intermolecular potential with 3 points) water model [480-482], the αS monomer was solvated in a cubic periodic box. A salt concentration of 150 mM kCl, the approximate k^+ concentration in the mammalian cell, was added to the water box via xleap module of AMBER. The 10-mer PEG10 chains were derived from the LIMP-2–PEG2 simulations [641]. The PEG2 in the crystal structure was preserved and is read as PEG molecule for this study. Five molecules of PEG were randomly placed around the αS monomer/dimer to build the crowding environment. Thus the final system consists of αS, surrounded by PEG molecules, water and kCl ions. The whole system was neutralised by injecting the necessary amount of counter ions and the strong van der Waals were eliminated by energy minimization. The later part of the MD experiment followed a standard methodology, as discussed in *Section 4.3.2.*

7.4. Results and Discussion:

7.4.1. Analysis of the conformational changes observed during different intervals of simulation period:

α-Synuclein (Control): The conformational snapshots (depicted in **Figure 7.1(A)**) ranging from 0 ns to 100 ns of the αS (control) structure shows that: the N-terminal domain (red-colored portion) of the protein gets twisted further as the simulation progresses, the starting end of the structure in the N-terminal domain gets far apart from the C-terminal domain and the amount of α-helices decreases as the simulation progresses.

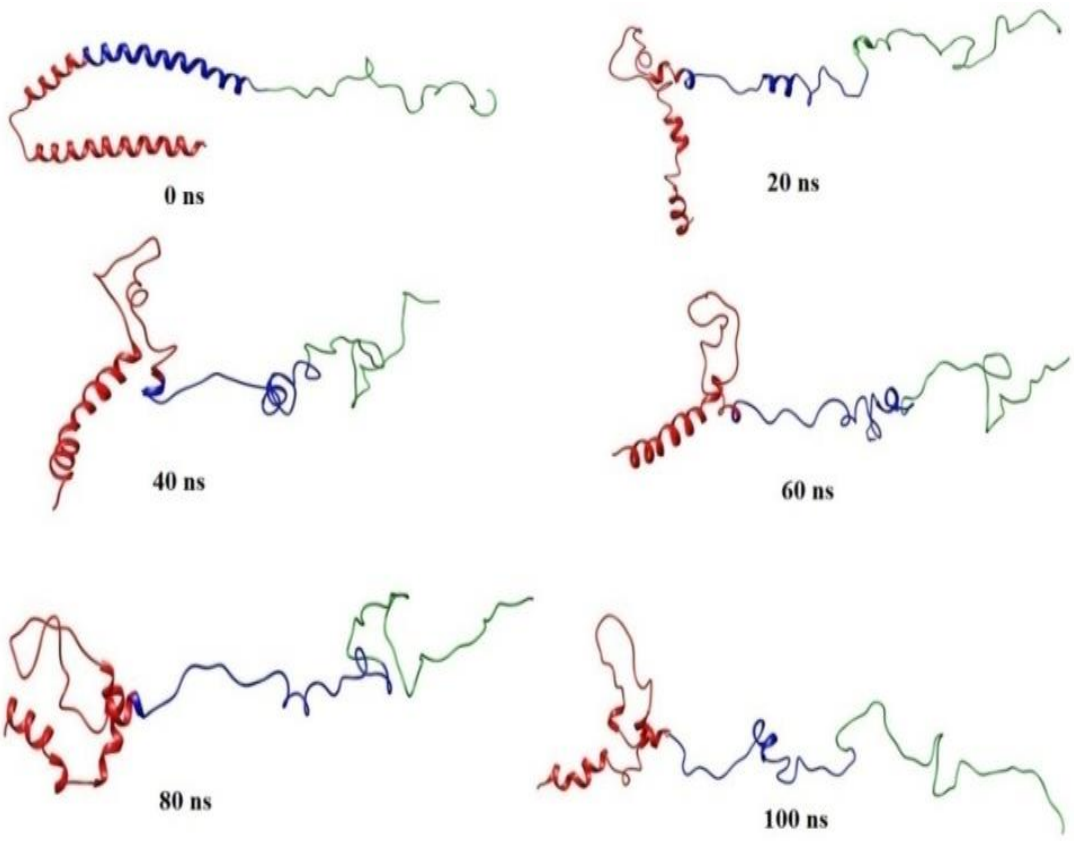

Figure 7.1(A). *Conformational snapshots of α-Synuclein Monomer (control) structure from 0 ns to 100 ns of simulation time period.*

5PEG-α-Synuclein: The conformational snapshots (depicted in **Figure 7.1(B)**) ranging from 0 ns to 100 ns of the 5PEG-α-Synuclein structure shows that: the C-terminal domain (green-colored portion) of the protein gets more twisted in shape as the simulation progresses compared to the initial structure and the control structure, the N-terminal is less coiled or twisted as compared to the N-terminal of the control structure, the amount of α-helices present in the N-terminal and NAC domain (blue-colored portion) decreases as the simulation progresses.

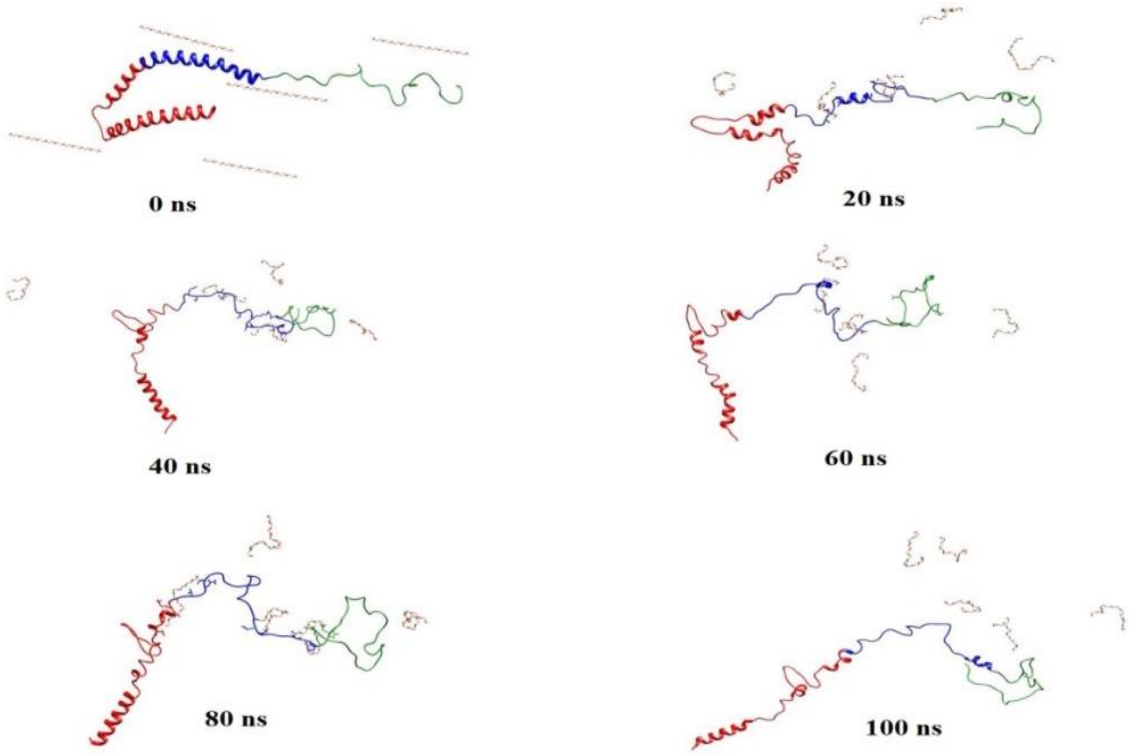

Figure 7.1(B). *Conformational snapshots of 5PEG-α-Synuclein Monomer structure from 0 ns to 100 ns of simulation time period*

10PEG-α-Synuclein: The conformational snapshots (from **Figure 7.1(C)**) ranging from 0 ns to 100 ns of the 10PEG-α-Synuclein structure shows that: the N-terminal domain of the protein gets more coiled as the simulation progresses compared to the initial structure, the C-terminal domain is less coiled as compared to the N-terminal of the control structure, the amount of α-helices present in the N-terminal and NAC domain decreases as the simulation progresses as compared to the initial structure. It is also observed that the overall amount of α-helices in the αS structure is comparatively lesser in this crowded environment of 10 PEG molecules as compared to the control structure and the crowded environment of 5 PEG molecules.

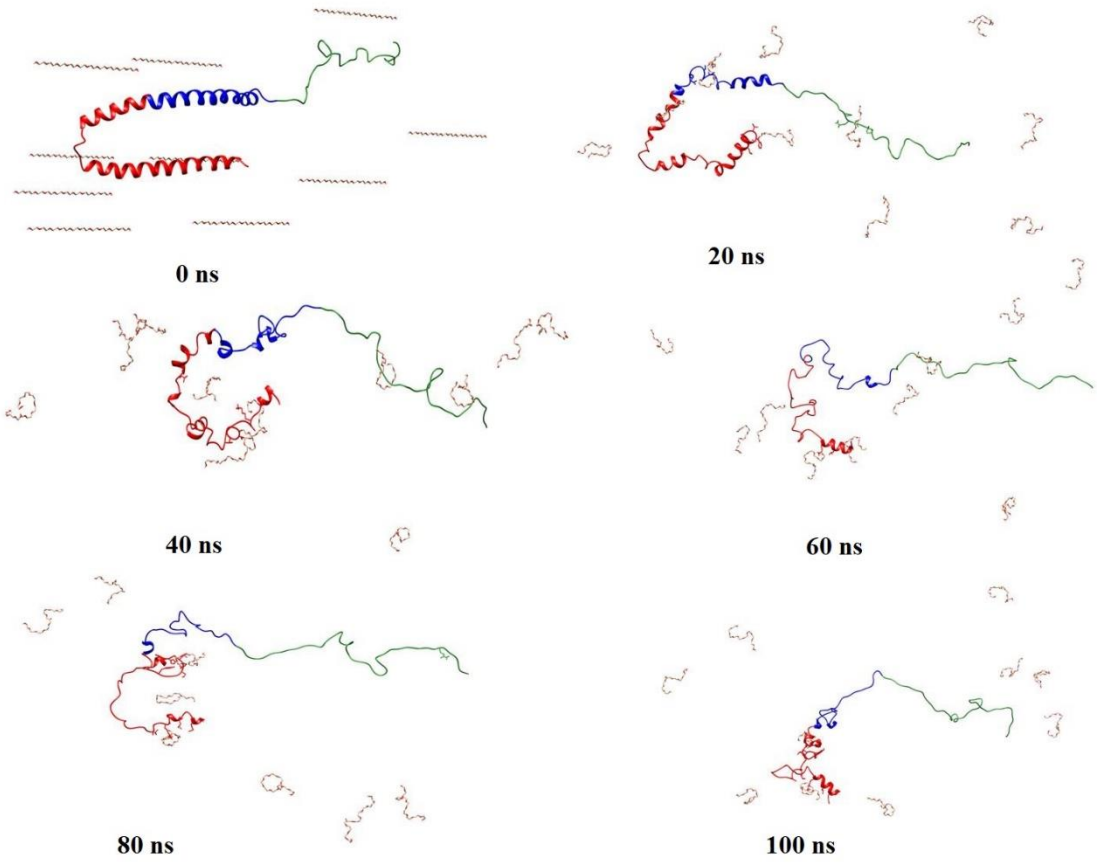

Figure 7.1(C). *Conformational snapshots of 10PEG-α-Synuclein Monomer structure from 0 ns to 100 ns of simulation time period.*

The conformational snapshots obtained for the α-Synuclein (control), the 5PEG-α-Synuclein and 10PEG-α-Synuclein at various time periods of MD Simulation (**Figures 7.1(A), 7.1(B), 7.1(C)**) shows that the α-helical content in the secondary structure of the αS protein decreases gradually as the simulation time progresses. It is also noted that the α-helices were found to greatly reduce in the more crowded environment (with 10 PEG molecules) compared to the control and the less crowded environment (with 5 PEG molecules) and this was also ascertained from the secondary structure analysis.

7.4.2. Structural properties of the complexes from the MD simulation

7.4.2.1. Root Mean Square Deviation (RMSD) Analysis:

The RMSD values of all the C_α atoms of the αS components were calculated for the three monomer complexes referenced to their starting structures to assess the stability. **Figures**

7.2(A), 7.2(B) and 7.2(C) depicts the RMSD calculated with respect to time period for the α-Synuclein (control), 5PEG-α-Synuclein and 10PEG-α-Synuclein respectively. The RMSD analysis helped us to understand the stability of the three systems. The αS protein was observed to be unstable in the control (**Figure 7.2**) as there was no presence of crowding agent. In the case of the less crowded environment (with 5 PEG molecules) the αS protein was stable as compared to the control. However, in the presence of more crowders in the third system (with 10 PEG molecules), the αS protein was even more stable compared to the control. Thus, it is inferred that when PEG molecules were added to the environment surrounding the αS protein, it was in a more compact environment with restricted randomness of the molecules. In this manner, the stability of the αS was affected.

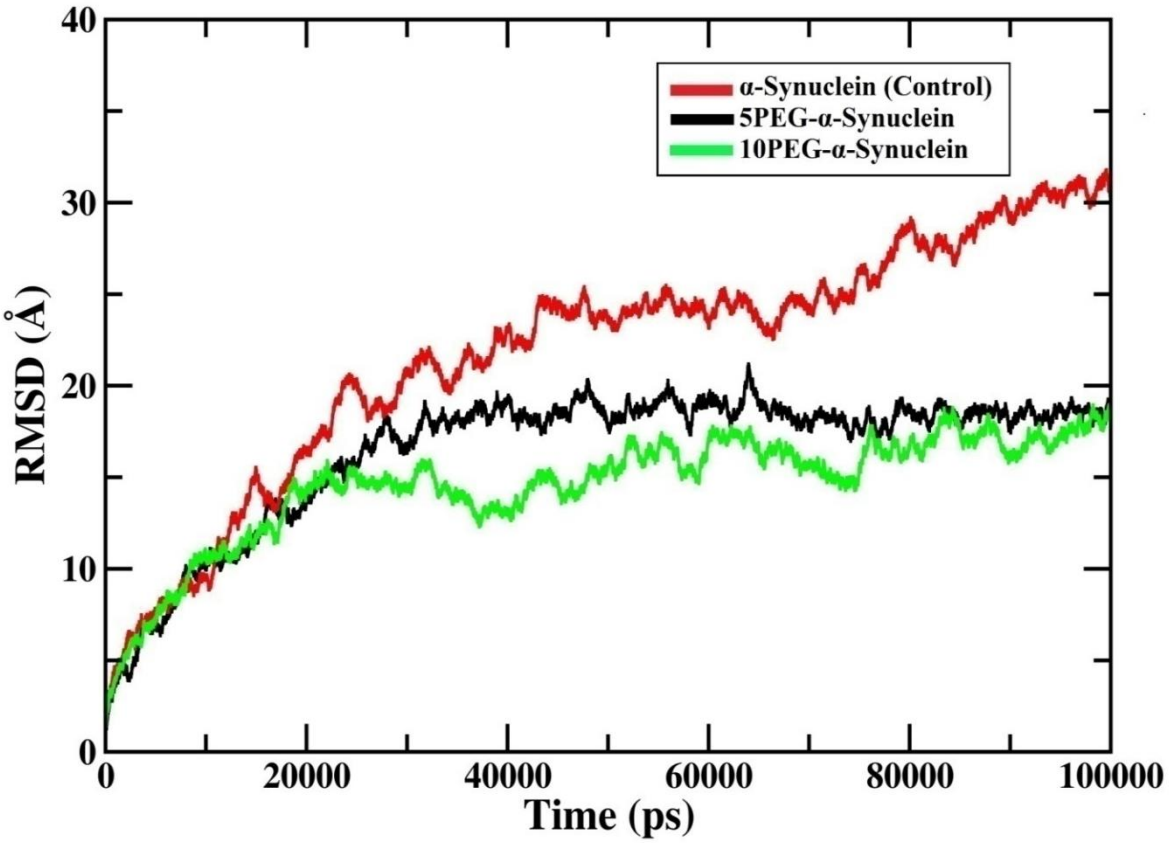

Figure 7.2. *Root Mean Square Deviation (RMSD) analysis with respect to simulation time period for α-Synuclein (control), 5PEG-α-Synuclein and 10PEG-α-Synuclein structure.*

7.4.2.2. Root Mean Square Fluctuation (RMSF) Analysis:

The RMSF values of all the C_α-atoms referenced to their starting structures of the three monomer systems were determined to assess individual residue flexibility during the simulation time. RMSF per residue is typically plotted versus residue number, and can indicate structurally which amino acids in a protein/peptide contribute the most to a molecular motion. **Figure 7.3** depicts the RMSF calculated with respect to time period for the α-Synuclein (control), 5PEG-α-Synuclein monomer and 10PEG-α-Synuclein monomer. The RMSF analysis helped us to understand the fluctuation of the residues of the three systems. From **Figure 7.3**, it was observed that the residues of the α-Synuclein (control) had overall less fluctuation. However, the in the presence of less crowders in the second system (with 5 PEG molecules), the αS protein had more room for randomness and hence higher fluctuation values at different intervals of the simulation period. But the overall fluctuation of the residues was less in the third system (with 10 PEG molecules), as the αS protein had more less for randomness. Thus the RMSF values were found to be in conformation with the RMSD values.

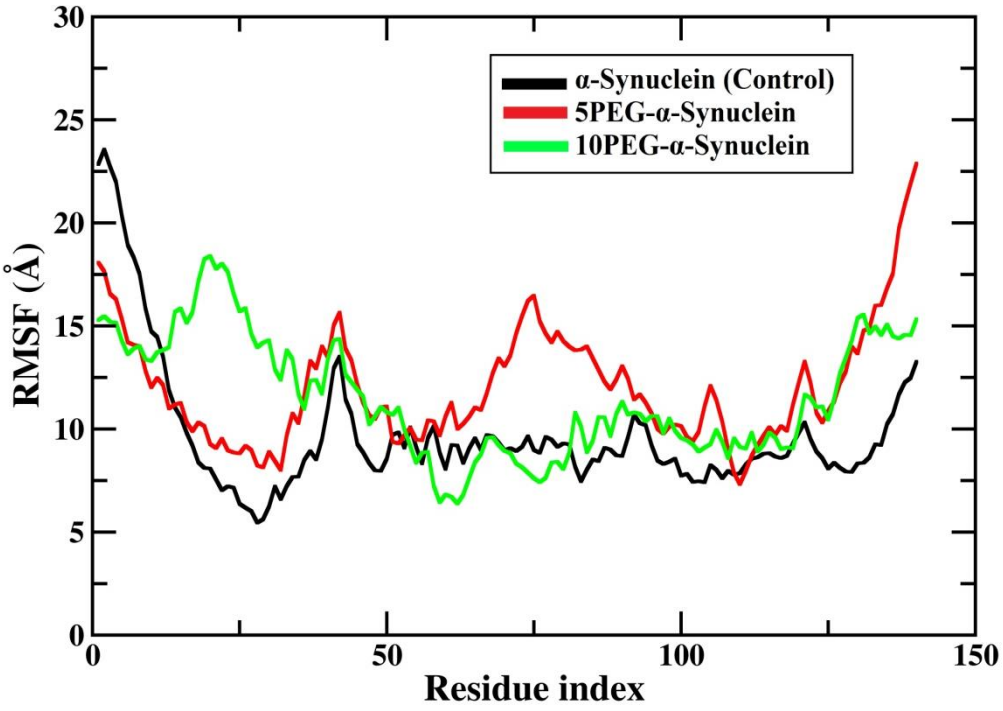

Figure 7.3. *Root Mean Square Fluctuation (RMSF) analysis with respect to simulation time period for α-Synuclein (control), 5PEG-α-Synuclein and 10PEG-α-Synuclein structure.*

7.4.2.3. Radius of gyration (R_g) Analysis:

R_g is frequently used to calculate the total distance between each atom in a given biomolecule and its common axis or centre of gravity. R_g serves as a measure of protein structural compactness. The R_g values for the control, 5PEG-α-Synuclein and 10PEG-α-Synuclein structure systems are shown in **Figure 7.4**. According to the R_g study, the αS protein is more compact in control than in the presence of crowded environments. In both the crowded systems, the αS protein adopts a different type of folding at different intervals of the simulation time period. Besides, the changes we have seen in the R_g values are the reflections endured by the various conformations of αS structure and their molecular interactions during the course of the simulation.

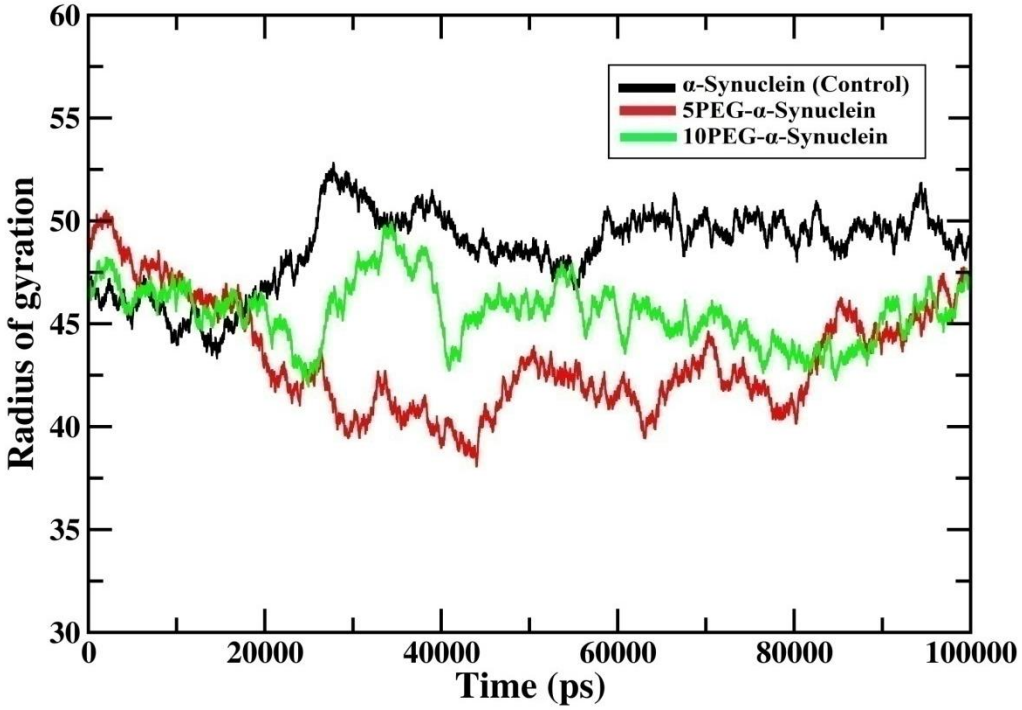

Figure 7.4. *Radius of Gyration (R_g) analysis with respect to simulation time period for α-Synuclein (control), 5PEG-α-Synuclein and 10PEG-α-Synuclein structure.*

7.4.3. Secondary structure analysis:

The secondary structure analysis for all three monomer complexes of αS was carried out using the Kabsch and Sander algorithm incorporated in their DSSP (Dictionary of Secondary Structure for Protein) program. The secondary structure variation of each residue as a function of frame numbers for the three monomer

complexes have been depicted in **Figure 7.5** and **Figure 7.6 (A), 7.6 (B) and 7.6 (C).** The analysis of secondary structure of the three systems helps us to infer that the amount of α-helices decreases and the amount of anti-parallel β-sheets increases the concentration of PEG (crowders) increase as compared to the αS (control) Monomer. This analysis of secondary structure of αS Monomer can be compared with the study performed by Menon and Mondal, 2022 where they have calculated percentage of secondary structure components (α-helices and β-sheets) residue-wise in the conformational ensembles of αS [644]. The addition of crowding agents (5PEG and 10PEG) have resulted in the appearance of β-sheets structures in the C-terminal of the αS protein. The appearance of β-sheets in the secondary structure of IDPs are a marker of aggregation and many studies have also revealed that the presence of crowders many induce the aggregation process of αS [645-649]. However, the effect of different crowders on different IDPs is different and it cannot be ascertained from the secondary structure analysis that addition of PEG can accelerate the aggregation of αS protein. But it can definitely be said that the crowded environment of PEG has potential in effecting the aggregation mechanism of αS protein.

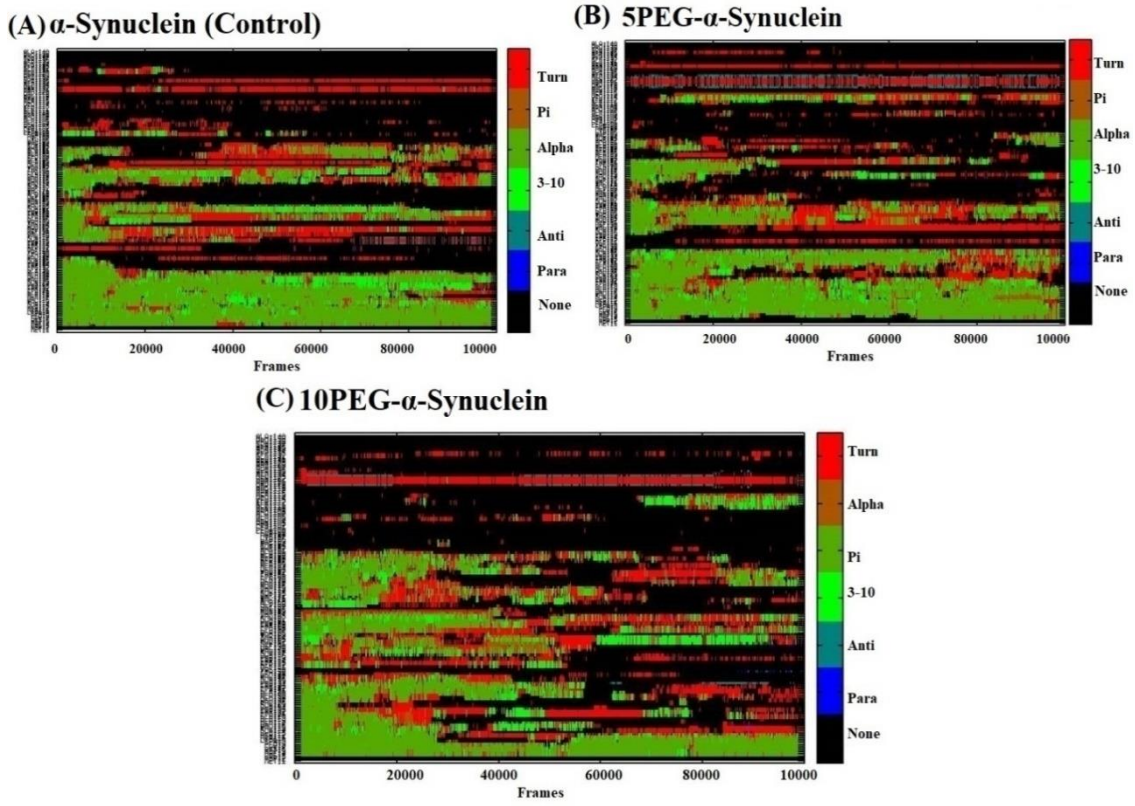

Figure 7.5. *Analysis of secondary structure for (a) α-Synuclein (control) Monomer, (b) 5PEG-α-Synuclein and (c) 10PEG-α-Synuclein structure throughout the simulation period.*

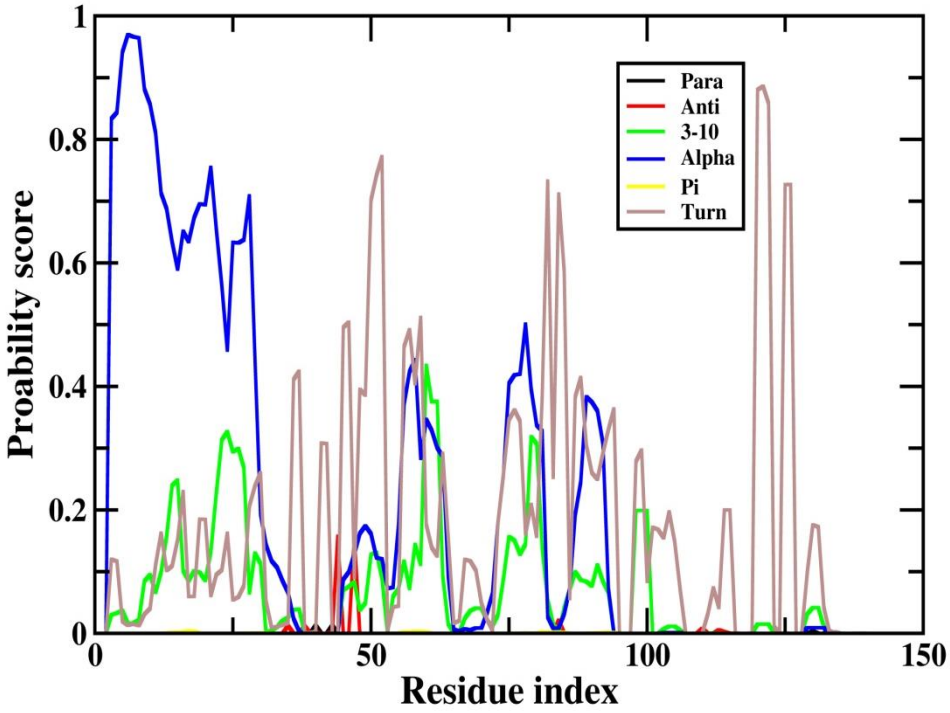

Figure 7.6(A). *Probability score of Secondary structure analysis with respect to residue indices for α-Synuclein (control) Monomer throughout the simulation period.*

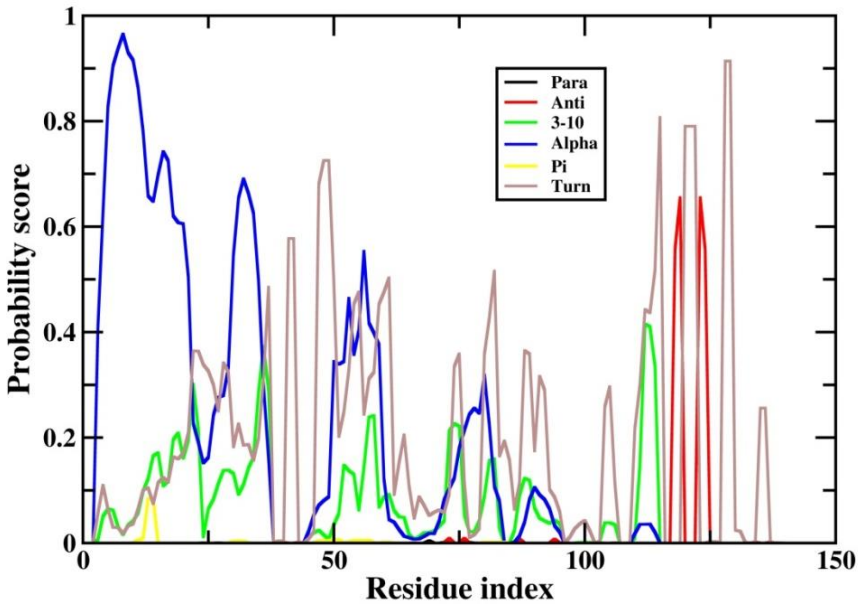

Figure 7.6(B). *Probability score of Secondary structure analysis with respect to residue indices for 5PEG-α-Synuclein throughout the simulation period.*

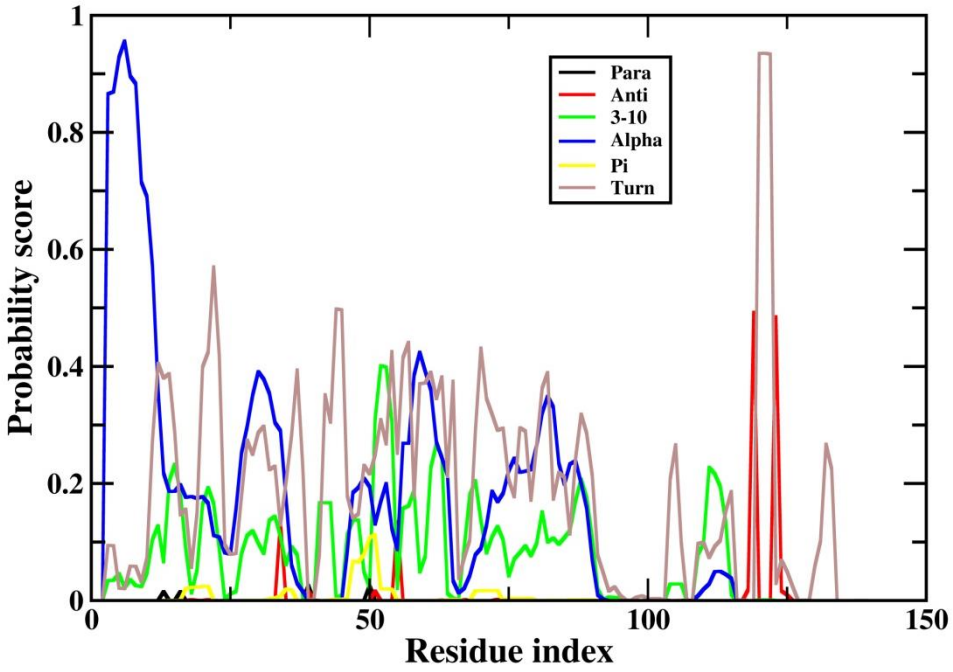

Figure 7.6(C). *Probability score of Secondary structure analysis with respect to residue indices for 10PEG-α-Synuclein throughout the simulation period.*

7.4.4. Analysis of Diffusion Coefficient:

To calculate the self-diffusion coefficient of αS monomer in different environments of the solution, the Einstein relation have been used as shown in the equation 7.1.

$$D = 1\ 6 \lim t \rightarrow \infty\ d/dt \langle MSD \rangle \quad \textbf{(7.1)}$$

In the above equation, <MSD> represents averaged mean square displacement. But the linear relationship between the <MSD> and time *t* allows for graphical methods to determine the *D* using the relation MSD=2nDt in which n represents the dimension. The Diffusion coefficient values calculated for the αS monomer in different environments were summarized in **Table 7.1**. The Diffusion coefficient value for αS in Control was found to be lower as compared the value observed in 5PEG- α-Synuclein complex. But the diffusion coefficient value for 10PEG-α-Synuclein complex solution tend to decrease from the value for the 5PEG-α-Synuclein complex which is probably due to structural changes developed in the molecule. Similar diffusion trend have been noticed in an experiment performed by M. Kakati et. al. [650], where they observed that in the beginning, the structure of αS is more compact and its diffusion is affected by the

presence of water molecules. However, they have observed that gradually with increase in concentration of ethanol molecules, the intermolecular interaction between water and αS decreases.

Table 7.1. *Diffusion coefficient comparison for α-Synuclein at non-crowded and crowded environments.*

System	Slope	Diffusion Coefficient(D) (m^2/s)
α-Synuclein (control)	0.0313	0.0521
5PEG-α-Synuclein	0.0664	0.1106
10PEG-α-Synuclein	0.0538	0.0896

7.5. Conclusion:

This study was performed to understand the structural and conformational changes undergone by the αS protein in the presence of crowders. The RMSD analysis have shown that the stability of the αS protein is more in the more crowded environment (10 PEG molecules) and in the less crowded environment (5 PEG molecules). From the RMSF analysis, it was observed that the residues of the αS (control) had overall more fluctuation than the two crowded systems. According to the R_g analysis, the αS protein is found to be more compact in the control than in the presence of crowded environments. The conformational snapshots have provided insights on the effect of addition of crowder PEG in the structural and conformational dynamics of αS protein at different percentage of crowding environments. The secondary structure analysis have helped to infer that the presence of crowding agent PEG has led to the gradual and overall decrease in the amount of α-helices in the N-terminal and NAC domain of the αS protein. The secondary structure analysis also showed that there is an increase in the presence of anti-parallel β-sheet structure of the αS protein in the presence of crowders as compared to the αS (control) protein. This parameter indicates that crowders play a role in affecting the aggregation of αS protein. Thus this study has helped us to understand the role of crowding agent PEG in the structural and conformational properties of αS protein. Thus, it can also be remarked that the αS protein is sensitive to the presence of inert crowding environment.