To Investigate the Effect of Salt Concentration on Ovalbumin Aggregation

A dissertation submitted

in partial fulfillment of the requirements

for the degree of

M. Sc. (Chemistry)

Submitted by

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July 2017

CERTIFICATE

This is to certify that dissertation entitled, "**To Investigate the Effect of Salt Concentration on Ovalbumin Aggregation**" being submitted by **Ms. Damanpreet Kaur** in partial fulfilment of requirements for the award of the degree of **Masters of Science** in **Chemistry** and being submitted to School of Chemistry and Biochemistry, Thapar University, Patiala is a bonafide work carried out by her under our supervision. The work has reached the standard necessary for submission, The content of this dissertation have not been submitted to any other university or institute for the award of any degree or diploma.

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CANDIDATES DECLARATION

I hereby declare that the work being presented in the dissertation entitled **"To Investigate the Effect of Salt Concentration on Ovalbumin Aggregation"** in partial fulfilment of the requirements for the award of the degree of **Masters of Science** in **Chemistry** and being submitted to School of Chemistry and Biochemistry, Thapar University, Patiala is my own work during the period of January to July 2017 under the supervision of **Dr. Mily Bhattacharya**. I have not submitted the contents embodied in this dissertation for the award of any degree.

Damanpreet

Damanpreet Kaur

Date: 21/7/16

It is certified that the above statement made by the student is correct to the best of my knowledge and belief.

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Date: 21/7/16

Abstract

In this work, we investigated the fibrillation of a 44.5 KDa egg white glycoprotein, Ovalbumin, as a function of salt (NaCl) concentration using intrinsic (Tryptophan) and extrinsic (ANS and ThT) fluorophores. The conformational changes were monitored by using Fluorescence and CD spectroscopic techniques. Both the studies indicate that there is an increase in β-sheet and decrease in α-helix content of the protein as the concentration of salt increases, indicating unfolding of protein. This finding demonstrates that aggregation of Ovalbumin is induced by the salt.

Keywords: ovalbumin, aggregation, amyloid, fibrillation, protein.

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Fig.4.6 ANS fluorescence anisotropy monitored at (a) 0 mM NaCl, (b) 50 mM NaCl (c) 150 mM NaCl

Fig. 4.7 Far-UV CD spectra of Ovalbumin (a) with 150 mM NaCl (b) with 0 mM NaCl (c) Kinetics of the loss of helicity at 222 nm during aggregation of ovalbumin

Chapter-1 Introduction

Proteins are diverse and most abundant biological macromolecules, playing important roles in all aspects of cell structure and function, for example, Hemoglobin which is an important iron containing protein complex in red blood cells of vertebrates and it carries oxygen from lungs to body tissues. Amino acids are the building blocks of proteins; therefore their physical and chemical properties are determined by constituent amino acids. There are 20 naturally occurring amino acids that are found in all the proteins. The properties of each amino acid are determined by its specific side chain.

Proteins have different structural levels as its biological function is due to its conformation. The primary structure is the sequence of amino acids that form a polypeptide chain. The secondary structure of protein consists of regular and repeated folding of polypeptide backbone with sequence of amino acids linked by hydrogen-bonding. The two types of secondary structure of proteins are α-helix and β-sheet. In α-helix, the polypeptide backbone coils around an imaginary helix axis while in β-sheet the polypeptide chain is fully extended with interchain hydrogen bonding forming pleated sheet.

1.1 Protein Folding

Various models have been proposed to describe the folding process of protein from unfolded state to its native structure. Protein Folding Funnel model is one of the very well known model that sketches the folding process of protein. It assumes that the native structure of protein corresponds to minimum free energy.

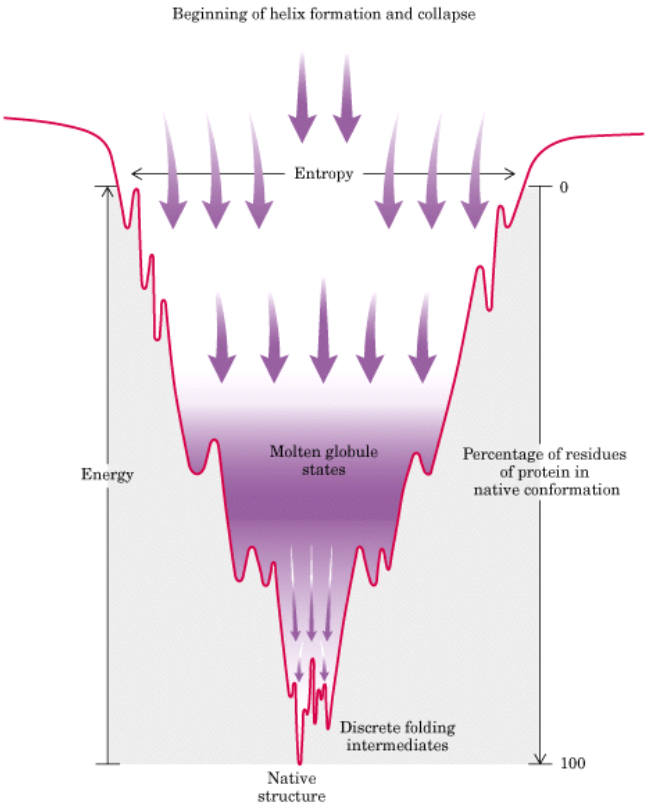


Fig. 1.1 The free energy protein folding funnel [1]

The protein folding funnel model (Fig. 1.1) describes that protein folding can progress via multiple routes rather than using a single pathway. The unfolded protein has high entropy along high free energy where high entropy corresponds to there being a large number of possible conformational states. At the bottom of the funnel, the free energy is at a minimum and there is only one conformational state available to the protein molecule.

1.2 Protein Aggregation

It is the abnormal polymerization of misfolded or unfolded protein into aggregates. The aggregations of misfolded protein to form insoluble fibers that are resistant to degradation are termed as **Amyloid**. These Amyloid fibrils are highly ordered and stable protein aggregates. Amyloid formation involves the structural rearrangement of the native state into a cross β-sheet rich fibrillar conformation (Fig. 1.2).

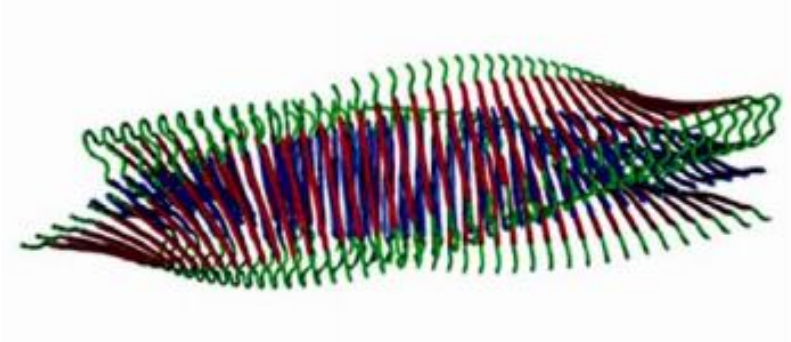


Fig. 1.2 Structural Model of Amyloid Fibril [2]

This uncontrolled self-assembly of proteins is disruptive as it hinders the folding and normal functioning of proteins. In amyloid associated diseases, Amyloid fibrils are deposited in tissues, forming insoluble structures and sometimes replacing cells. It is one of the most challenging fields as aggregation of certain proteins causes many neurodegenerative diseases, including Alzheimer disease, Parkinson disease, Huntington disease etc. [3]. The association of protein aggregation with neurodegeneration motivates to elucidate the detailed molecular aspects of protein aggregation, including mechanisms and pathways of aggregation of the relevant peptides and proteins.

1.2.1 Mechanism of Protein Aggregation

Aggregation of proteins can arise from a number of complex mechanisms. A general pathway for the formation of these aggregates is shown below (Fig. 1.3).

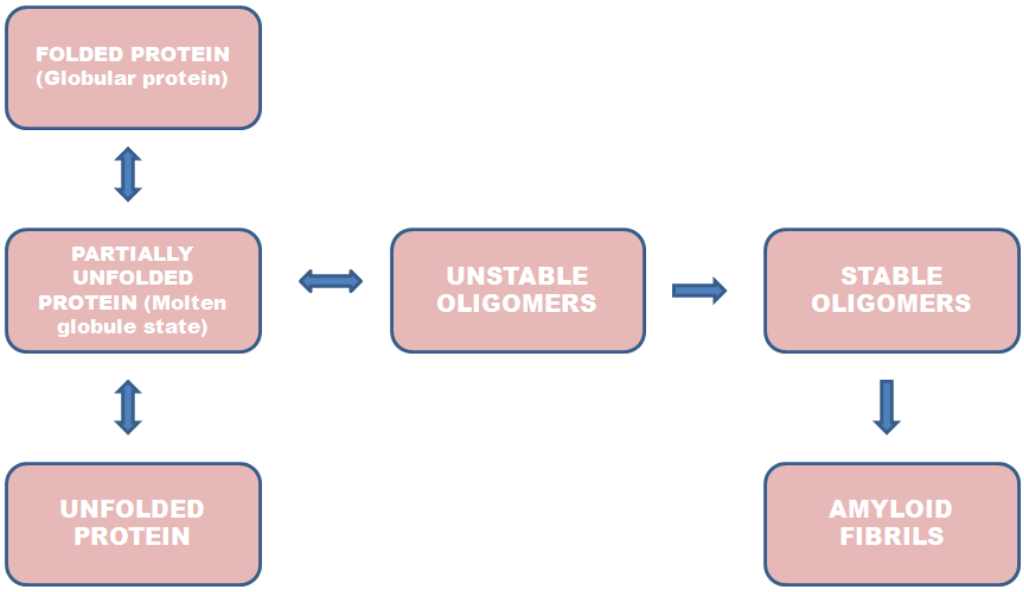


Fig. 1.3 Schematic representation of formation of protein aggregation

The key step in Amyloid formation is destabilization of native protein structure. [4]. Once the native structure of protein is destabilized, there is formation of partially unfolded protein intermediate which is known as the molten-globule state. The molten-globule state of the protein or the partially unfolded state retains some degree of secondary structure but is completely devoid of compact tertiary structure. Such a partially unfolded state with exposed hydrophobic regions has turned out to be an important intermediate in both protein folding and protein aggregation processes. The partially-unfolded protein molecules associate to form oligomeric aggregates that lead to formation of Amyloid fibrils.

In the protein folding funnel model (section 1.2) there is some ruggedness along the energy landscape that can trap the partially folded protein, i.e., protein in metastable state that is transiently populated. Accumulation of these partially folded proteins is the key step towards protein aggregation

The molten-globule state of the protein or the partially unfolded state retains some degree of secondary structure but is completely devoid of compact tertiary structure.[5] Such a partially

unfolded state with exposed hydrophobic regions, has turned out to be an important intermediate in both protein folding and protein aggregation processes.

1.3 Methods to destabilize protein structure

It is important to explore the destabilization of protein as the partially unfolded state of protein might serve as a precursor for protein aggregate and Amyloids. These factors enhance the accumulation of a significant population of partially folded intermediates which otherwise are inaccessible under normal conditions.

pH- Small changes in pH of protein can add or remove H^+ ions from side chain thereby inducing many structural changes and unfolding the protein by disrupting H-bonding and salt bridge ,i.e. , ionic interaction between Amino acid side chain.

Temperature- High temperature can be used to disrupt hydrogen bonds and non-polar hydrophobic interactions. This occurs because high temperature increases the kinetic energy and causes the molecules to vibrate so rapidly and violently that the bonds are disrupted.

Ionic strength- The increase in ionic strength tends to screen the electrostatic interaction among charged amino acids. For the globular proteins at pH 7.0, small elongated aggregates are formed at low ionic strength, while at high ionic strength larger aggregates appear to be formed by random association of the small aggregates. [6]

However, there might be some intrinsic factor that may cause the aggregation of protein such as mutation of amino acid. Mutation of specific residues in proteins may increase the aggregation rate as the protein will become unstable and may lose its function. [7]

Many model proteins have been studied to understand the mechanism of Amyloid formation such as Lysozyme [8] and Bovine Serum Albumin [9] as a function of surfactant concentration. In this research project, we have used Ovalbumin (Chicken Egg White Albumin) as the model protein to study the effect of salt concentration on its aggregation.

1.4 Ovalbumin (Chicken Egg White Albumin)

Ovalbumin is a glycoprotein found in the egg white of the chicken egg and comprises 54% of the total proteins of egg white. It has a molecular weight of 44.5 kDa and isoelectric point (pI) of

4.5. It consists of 385 amino acid residues. [10]. It is composed of three β-sheets and nine α-helices (Fig. 1.4). It has multifunctional properties such as it is widely used in food industry as a gelling agent and as an emulsifier. [11]

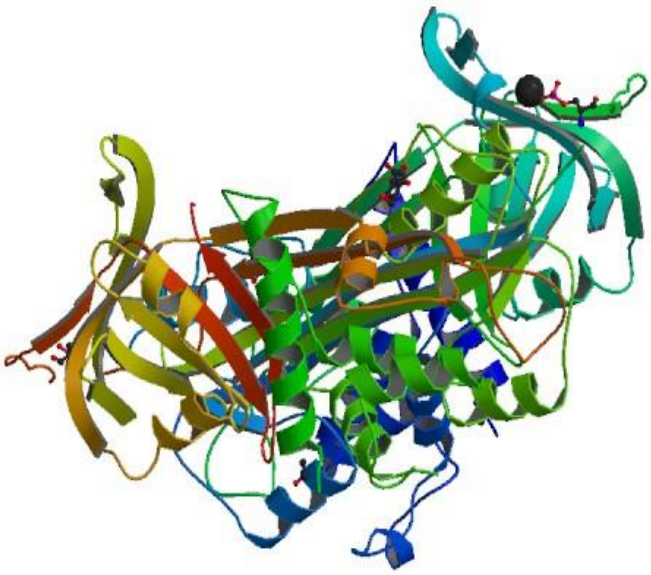


Fig1.4 Crystal structure of Ovalbumin at 1.95 angstrom resolution from Protein Data Bank (PDB ID: 1OVA)

Table 1.1 Amino acid present in Ovalbumin calculated from the sequence obtained from Protein Data Bank (PDB ID: 1OVA)

Symbol	Amino Acid	Number Found	Symbol	Amino Acid	Number Found
A	Alanine	35	K	Lysine	20
R	Arginine	15	M	Methionine	16
N	Asparagine	17	F	Phenylalanine	20
D	Aspartate	14	P	Proline	14
Q	Glutamine	15	S	Serine	38
E	Glutamate	33	T	Threonine	15
G	Glycine	19	Y	Tyrosine	10
H	Histidine	7	V	Valine	31
I	Isoleucine	25	W	Tryptophan	3
L	Leucine	32	C	Cysteine	6

Ovalbumin contains three intrinsically fluorescent residues, i.e., it contains three tryptophan residues and one disulphide linkage involving cysteine residues. [12]. Recently it has been show that Ovalbumin tends to undergo molten globule state under acidic pH condition. [13]. In this work, we used Ovalbumin as the model protein to study the detailed conformational and size changes as a function of salt concentration by using Circular Dischroism (CD) and Fluorescence spectroscopic techniques.

Protein aggregation is one of the most challenging fields since a large number of human disease, including Alzheimer's disease, Parkinson's disease, Huntington's disease as well as the prion diseases, are related to self-assembly of protein into Amyloid fibril that are characterized by the presence of deposits of fibrillar aggregates. The process of fibrillation depends on several factors such as pH, which affects the protein net charge and ionic strength of the solution influencing the electrostatic interactions in proteins. [14]

Pal *et al.*, reported the fibrillation of Ovalbumin induced by the biomineralization of two alkali halides KCl, NaCl in the Langmuir_Blodgett (LB) film of ovalbumin. FTIR spectroscopic study of the amide band in LB films as well as CD spectroscopy qualitatively indicates the increase in β- sheet content in the presence of salt, suggesting unfolding of the protein. They suggested that the ion attachment to the peptide chain leads to unfolding .Their finding demonstrated that the fibrillation of ovalbumin is induced by the biomineralization of alkali halides. [15]

Bhattacharya *et al.*, 2011 demonstrated the fibrillation proneness of different conformational isomers of bovine serum albumin (BSA), an all α-helical protein. Different pH conditions and ionic strengths were used to study the process using fluorescence and circular dichroism (CD) spectroscopy. At low pH and higher protein concentration, the partially folded structures associated to form oligomers that converted into ordered amyloid-like fibrils when incubated at high temperature. They elucidated the mechanism of fibril formation by monitoring the kinetics of structural changes during the aggregation process.(Fig. 2.1)

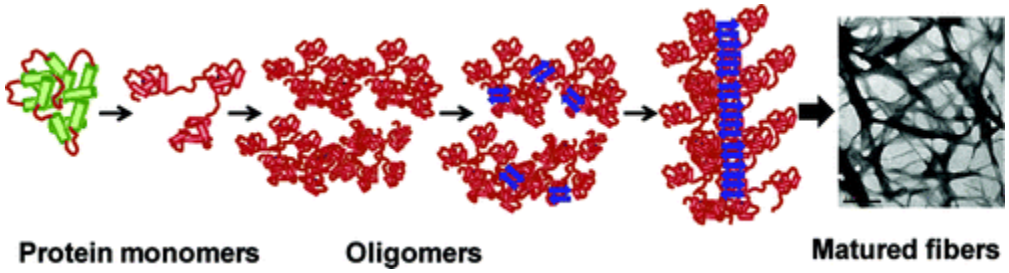


Fig. 2.1 Aggregation Pathway and Fibril Formation in BSA

Both conformational and size changes were observed by measuring the time dependence of fluorescence intensity and anisotropy of intrinsic fluorophore, i.e., tryptophans and several extrinsic fluorophores during the aggregation. CD spectroscopy was employed to monitor the changes in secondary structure of protein during fibrillation. Findings suggest that the conformational conversion that occurs in the oligomers serve as amyloidogenic precursors and precedes the overall fibrillar growth.

Bhattacharya *et al*., 2011 described the structural properties of both native and partially unfolded molten-globule state of Ovalbumin using multiple spectroscopic tools. Time resolved fluorescence measurements provided important structural and dynamic revelation about the native and molten-globule forms. Fluorescence anisotropy decay analysis showed that there is a conformational swelling from the native to the molten-globule state of Ovalbumin. Additionally, stopped-flow fluorescence experiments revealed that the conformational transition from the native to the molten-globule state proceeds in a stepwise manner. [16]

Lara *et al*., 2012 demonstrated the fibrillation pathway of Ovalbumin protein and reported the simultaneous formation of several types of fibrils, with structural and physical differences. They compared the kinetics of fibrils formation in two conditions: with and without addition of 50 mM NaCl and followed the kinetics of fibrils growth by atomic force microscopy (AFM), static and dynamic light scattering (SLS, DLS), and small-angle X-ray scattering (SAXS). [17]

Sarell *et al*., 2013 discussed consequences of co-aggregation of different proteins upon the structure, stability, and toxicity of the resulting Amyloid aggregates, including the role of co-aggregation in expanding the repertoire of oligomeric and fibrillar structures and how this can affect their biological and biophysical properties. [18]

The motivation for the present work is to combine the process of aggregation of protein with the effect of ionic strength. Thus, our work aims to unravel the detailed information on kinetics of conformational changes and size variation during aggregation under the variation of salt

concentration using intrinsic (tryptophan) and extrinsic fluorophores by using Ovalbumin as the model protein.

Chapter -3 Materials and Methodology

This chapter deals with the materials and methodologies, which includes chemicals, glass wares and instruments, that were used to carry out the research work presented in this report.

3.1 Materials

3.1.1 Reagents and Chemicals Used

Ovalbumin (Chicken Egg White Albumin), 8-Anilino-1-naphthalenesulfonic acid (ANS), Thioflavin T (ThT), Sodium chloride, Buffer reagents such as, Glycine, Disodium phosphate dihydrate were obtained from Sigma-Aldrich and used as received.All the aqueous solutions of protein, buffers, fluorophores, salt and the aggregation samples were prepared using Milli-Q water (Millipore).

3.1.2 Apparatus

Micro-pipettes, tips, falcon tubes (15 mL and 50 mL), beaker (50 mL), spatula, kimwipes, microcentrifuge tubes (2 mL, 1.5 mL & 0.5 mL). Cuvettes (UV-Vis Absorption cuvette 10 x 10 mm, Fluorescence cuvette 10 x 2 mm & Circular Dichroism cuvette 2 mm; Fig.3.1).

UV-Vis Absorption cuvette 10 x 10 mm

Circular Dichroism cuvette 10 × 2 mm

Fluorescence cuvette 10 x 2 mm

Fig. 3.1 Different types of cuvettes

3.2 Instrument Used

3.2.1 Analytical balance

To weigh accurate quantities of compounds, analytical balance was used (Shimadzu AY 220; Fig. 3.2). It consists of a large pan which is highly sensitive with multiple weighing units such as mg, g etc.

Fig.3.2 Analytical Balance

3.2.2 pH Meter

The pH meter (ELICO LI 120; Fig. 3.3) is a laboratory device to determine or adjust the pH of a solution/buffer by adding an acid or base to achieve desired value of pH. It includes a highly sensitive pH electrode that measures the concentration of H^+ ions in the solution. Solution containing more H^+ ion will be acidic while the solution containing more OH^- ions will be basic. In this experiment, it has been used to adjust the pH of buffers used for protein solution. measures the pH of solution.

Fig. 3.3 pH Meter

3.2.3 Heating Block

Heating block (Rivotek; Fig. 3.4) consists of carved (an array of partially hollow tube-like structures) aluminium blocks that are used to hold microcentrifuge tubes containing samples. The device causes a uniform heating in the sample. The observed temperature is less than the set temperature. In this report, the sample temperature was 65 ± 1 °C for which the temperature was set at 75 °C. For uniform and reproducible results, the instrument was allowed to heat up for 2 hours so that it gets equilibrated. In order to check the temperature, a thermometer was put inside a water-filled microcentrifuge tube in such a way that a convex bulb was formed at the brim of the tube without allowing the water to fall. The temperature was recorded till it became constant at ~65 °C.

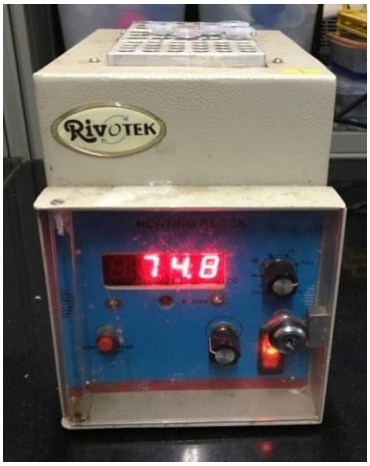


Fig. 3.4 Heating Block

3.2.4 Fluorimeter

Horiba Scientific Fluoromax-4 spectrofluorometer (JobinYvon; Fig.3.5) was used to measure the steady-statefluorescence intensities and anisotropy of protein samples to monitor the aggregation kinetics.

Fig. 3.5 Flourimeter

It in an emission spectroscopic technique and is based on the principle of fluorescence. When a molecule absorbs light, an electron is promoted to a higher excited singlet state, the electron in the excited orbital is paired (by opposite spin) to the second electron in the ground-state orbital. The molecule can partially dissipate its energy by undergoing conformational changes and relaxed to the lowest vibrational level of the excited state in a process called vibrational relaxation. If the molecule is rigid and cannot vibrationally relax to the ground state, it then returns to the ground state by emitting light, the process is known as fluorescence. The lifetime of fluorescence is nearly 10 ns.

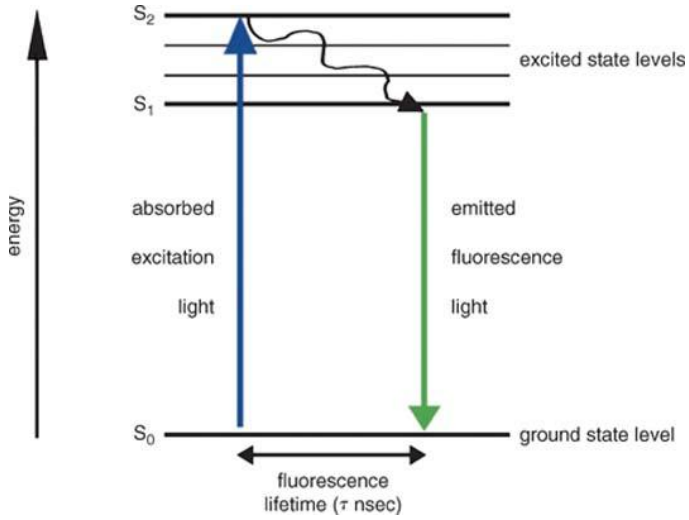
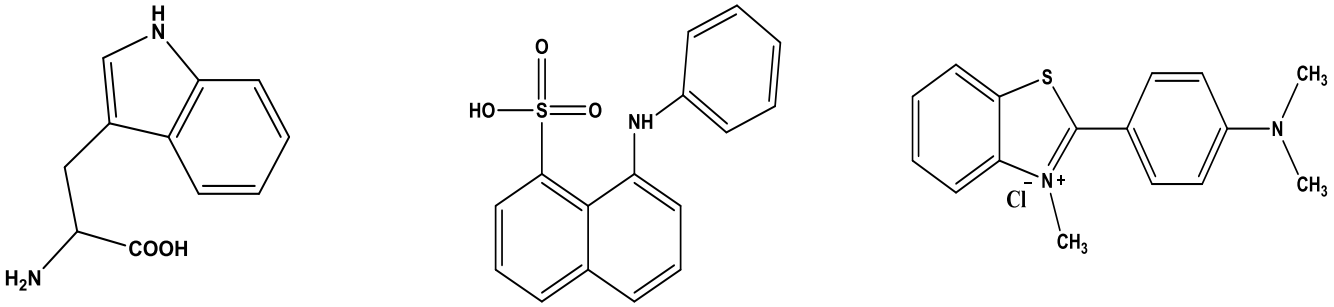


Fig. 3.6 Jablonski diagram illustrating phenomenon of fluorescence

In proteins, the dominant intrinsic fluorophore is tryptophan (Fig. 3.7 (a)) which contains an indole ring. It absorbs near 280 nm and emits ~340 nm. The emission of indole may be blue-shifted if the group is buried within a native protein, and its emission may shift to longer wavelengths (red shift) when the protein is unfolded. Extrinsic fluorophores such as 8-Anilinonaphthalene-1-sulphonic acid (ANS) (Fig. 3.7 (b)) and Thioflavin T (ThT) (Fig. 3.7 (c)) are also used to study structural changes in protein. For instance, ANS is highly fluorescent when it binds to the hydrophobic pocket of a protein and ThT is widely used to detect the presence of cross β-sheet rich Amyloid aggregates formed due to self-assembly of misfolded protein.



(a) **(b)** **(c)**

Fig. 3.7 (a) Structure of Tryptophan, (b) Structure of ANS, (c) Structure of ThT

Fluorescence anisotropy is also an important aspect of fluorescence spectroscopy. Steady-state fluorescence anisotropy measurements provide information about the overall size and shape of proteins. It is based on the principle of photoselective excitation of fluorophores by polarized incident light. Upon excitation with the polarized light, one selectively excites those fluorophore molecules whose absorption transition dipole is parallel to the electric vector of the excitation. The fluorescence anisotropy (r) is defined as

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

Where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities of the vertically (||) and horizontally (⊥) polarized emission, when the sample is excited with vertically polarized light.

3.2.5 Circular Dichroism Spectrophotometer

CD spectrophotometer Chirascan (Applied Photophysics; Fig.3.6) was used to measure the protein absorbance and to investigate the structural changes in protein samples in the far-UV region. The instrument is continuously flushed with nitrogen to avoid absorption by oxygen in the far-UV region.

Fig. 3.8 Circular Dichroism Spectrophotometer

Circular dichroism spectroscopy is a differential absorption spectroscopy in which a circularly polarized light is allowed to pass through an optically active compound. Circularly polarized light can be represented as sum of two circularly polarized components of equal magnitude, one rotating counter clockwise (left circularly polarized light) and other clockwise (right circularly polarized light). When this circularly polarized light is passed through an optically active compound, the absorbance of LCP (A_L) is different from absorbance of RCP (A_R). After passing through the sample, each component is still circularly polarized, however, the radii of electric field vector for one of the components is different, as a result, combination of these two components would now result in an elliptically polarized light. Ellipticity is defined as the arc tangent of the ratio of the minor axis to the major axis of the ellipse, i.e., $\Theta = \tan^{-1}(b/a)$.

$$Circular\ Dichroism = \Delta A\ (\lambda) = A\ (\lambda)\ LCP - A\ (\lambda)\ RCP$$

In proteins the chromophores of interest includes the peptide linkage, aromatic amino acid side chains and disulphide linkages. The secondary structure of protein is observed in 180 – 240 nm, i.e., in the far-UV region, is primarily due to peptide bond which can differentiate between diferrent secondary structure such as α – helix, β – sheet and random coil.

3.3 Methodologies

3.3.1 Preparation of Buffers

3.3.1.1 Phosphate buffer

A stock solution (500 mM) of disodium phosphate dibasic was prepared in milli-Q water. The pH of the buffer was adjusted to 7.01 at 25 °C. From this stock solution, 50 mM phosphate buffer was prepared by 10-fold dilution of stock solution. The pH of this buffer was also adjusted to 7.01 at 25 °C. Another buffer solution was prepared of 5 mM for the preparation of protein stock solution by the 10-fold dilution of 50 mM buffer. The pH of 5 mM was adjusted to 7.01 ± 0.1 at 25 °C.

3.3.1.2 Glycine – HCl buffer

A stock solution of 500 mM was prepared of glycine – HCl buffer. The pH of the buffer was adjusted to 2.2 at 25 °C. From this stock solution, 50 mM of glycine- HCl buffer of pH 2.2 was prepared by 10-fold dilution of stock solution for three different salt concentrations.

3.3.2 Preparation of Protein Stock solution

The stock solution of Ovalbumin (chicken egg white albumin) was dissolved in freshly prepared phosphate buffer of 5 mM of pH 7 to give a stock solution of 1 mM and was stored at 4 °C. The actual concentration of stock solution was determined by measuring the absorbance of tryptophan at 280 nm using CD spectrophotometer. The molar extinction coefficient of Ovalbumin is 30957 M^{-1} cm^{-1} .[19]

3.3.3 Preparation of Salt Stock solution

A stock solution of 5 M of NaCl was prepared. From this stock solution, 1 M of NaCl was prepared by 5-fold dilution of NaCl stock solution. Variable salt concentrations used to study the aggregation of Ovalbumin were 0 mM, 50 mM and 150 mM. The dilution of 1 M NaCl for preparation of different concentrations of salt was done by using Glycine – HCl buffer of pH 2.2. 50 mM of NaCl was prepared by 20 fold dilution of 1 M NaCl. 150 mM of NaCl was prepared by 6.66 fold dilution of 1 M NaCl. Similarily, 200 mM of NaCl was prepared by 5 fold dilution of 1 M NaCl.

3.3.4 Preparation of Protein Aggregation samples

For the aggregation experiment the protein stock solution of 1 mM was diluted to 100 uM using 50 mM Glycine-HCl buffer of pH 2.2 in a 2 mL microcentrifuge tube. The protein samples were incubated with three different concentrations of NaCl, i.e., 0 mM, 50 mM and 150 mM. These samples were then heated to 65 ± 1° C in a heating block preset at the required temperature without agitation.

3.3.4 Preparation of Aggregation Dilution buffers

3.3.4.1 Tryptophan Dilution Buffer

It was prepared for three different concentrations of NaCl, i.e., 50 mM, 150 mM, and 200 mM. For 50 mM of NaCl, dilution buffer was prepared by 20-fold dilution of 1 M NaCl using of glycine-HCl buffer of pH 2.2 of 50 mM prepared from 500 mM glycine-HCl buffer stock. Similarily for 50 mM of NaCl, dilution buffer was prepared by 20-fold dilution of 1 M NaCl and 6.66-fold dilution for 150 mM NaCl diluton buffer

3.3.4.1 Thioflavin T Dilution Buffer

Similar to tryptophan dilution buffer, ThT dilution buffer was prepared for three different concentrations of NaCl. A stock solution of 10 mM of ThT was prepared. In order to attain the

concentration of ThT as 10 µM in aliquots of 600 µL of aggregation reaction mixture, the required concentration of ThT in dilution buffer was 11.11 µM.

3.3.4.1 ANS Dilution Buffer

Similar to ThT dilution buffer, ANS dilution buffer was prepared for 50 mM, 150 mM and 200 mM of NaCl. A stock solution of 10 mM of ANS was prepared. In order to attain the concentration of ANS as 10 µM in aliquots of 600 µL of aggregation reaction mixture, the required concentration of ANS in dilution buffer was 11.11 µM.

3.3.5 Fluorescence Experiments

All the steady state fluorescence measurements were performed on Horiba Scientific Fluoromax-4 spectrofluorometer (JobinYvon) at room temperature (~25°C),. The cuvette used for fluorescence experiments was fluorescence cuvette of 10 × 2 mm. the sample volume for all the fluorescence reading was 600 µL with final concentration of protein kept constant at 10 µM. For Tryptophan and ANS fluorescence experiment fluorescence intensity and anisotropy were collected. For ThT experiment, fluorescence intensity was collected.

3.3.5.1 Tryptophan fluorescence

The tryptophan intensity and anisotropy were measured to determine the conformational and size changes in the monomeric protein conformers. The following parameters were adjusted for monitoring tryptophan fluorescence intensity: λex = 295 nm, λem = 320-450 nm, excitation band pass = 1 nm, emission band pass = 1.5 nm, integration time = 0.5 s, No. of Accumulations = 2. For monitoring tryptophan fluorescence anisotropy: λex = 295 nm, λem = 340 nm, excitation band pass = 2 nm, emission band pass = 4 nm. For tryptophan fluorescence measurements, aliquots of protein aggregation sample were taken out at regular intervals and were diluted by 10 fold to 10uM using tryptophan dilution buffer of pH 2.2 such that the final volume is 600 uL. The experiment was repeated for all the three concentrations of NaCl.

3.3.5.2 ANS fluorescence

The following parameters were adjusted for monitoring ANS fluorescence intensity: λex = 350 nm, λem = 400 - 600 nm, excitation band pass = 0.5 nm, emission band pass = 2 nm, integration time = 0.5 s, No. of Accumulations = 2. For monitoring ANS fluorescence anisotropy: λex = 350 nm, λem = 475 nm, excitation band pass = 1.5 nm, emission band pass = 4 nm. For ANS fluorescence measurement, aliquots of protein aggregation sample were taken out at regular intervals and were diluted by 10 fold to 10uM using ANS dilution buffer of pH 2.2 such that the final volume is 600 uL. The experiment was repeated for all the three concentrations of NaCl.

3.3.5.3 ThT fluorescence

The following parameters were adjusted for monitoring ANS fluorescence intensity: λex = 450 nm, λem = 460 - 550 nm, excitation band pass = 1 nm, emission band pass = 4 nm, integration time = 0.5 s, No. of Accumulations = 2. For ThT fluorescence intensity measurement, aliquots of protein aggregation sample were taken out at regular intervals and were diluted by 10 fold to 10uM using ThT dilution buffer of pH 2.2 such that the final volume is 600 uL. The experiment was repeated for all the three concentrations of NaCl.

3.3.6 Circular Dichroism spectroscopy

The far-UV CD spectra were recorded for protein samples at room temperature. The protein aggregation sample with a concentration of 100uM was diluted by 40 fold at pH 2.2 leading to a final concentration of 2.5uM, which was taken in a quartz cuvette with a path length of 2mm. The following parameters were adjusted for monitoring the secondary structural changes: λ (scan range) = 198-250 nm, step = 1 nm, time per point = 0.5 s, bandwidth = 1 nm, No. of Accumulations =5. The spectra were corrected with buffer baseline subtraction.

The conformational changes in Ovalbumin as a consequence of salt concentration were characterized using steady-state fluorescence and CD spectroscopy. To investigate the changes in Size and conformation of ovalbumin as a function of salt concentration, the fluorescence properties of intrinsic (tryptophan) and extrinsic (ANS and ThT) fluorophores were measured. Since all the fluorophores are environment-sensitive, any conformational changes in the protein due to a change in concentration of salt can be monitored.

The Ovalbumin stock solution was prepared freshly every time before setting up the protein aggregation reaction. In order to find out the actual concentration of the stock solution, it was suitably diluted and absorbance was recorded (Fig. 4.1). The absorbance at 280 nm was used to calculate the stock solution concentration using the Lambert-Beer law and the dilution factor was also included (See Materials and Methods).

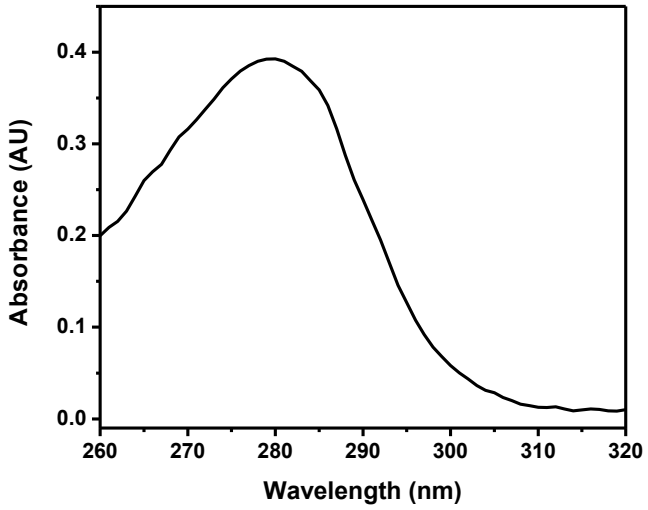


Fig. 4.1 UV-Vis absorption spectrum of Ovalbumin in phosphate buffer (pH 7, 50 mM) at room temperature

4.1 Steady-state fluorescence study

The fluorophores are highly sensitive to its environment; therefore changes observed in the fluorescence intensity can be correlated to the conformational changes in protein. [20] The fluorescence anisotropy measurements reveal information about the general size and the rigidity of the probe bound to the biomolecules.

4.1.1 Aggregation monitored by ThT fluorescence

The aggregation of ovalbumin as a function of NaCl concentration was investigated by changes in the ThT fluorescence intensity. ThT fluorophore is an amyloid detector which when bound to amyloid aggregate, shows an enhancement in the fluorescence intensity with an emission peak observed at 480 nm. [21]. The measurement of ThT fluorescence kinetics gives the rate of formation of cross β-sheet-rich amyloids.

The protein samples were incubated with 0, 50 and 150 mM of NaCl at pH 2.2 and were heated to 65 °C. The ThT fluorescence intensity of the samples was recorded at regular intervals. Figure 4.2 shows a typical progression of ThT fluorescence spectra as a function of salt concentration, suggesting the formation of amyloid-like aggregates. For the plots with NaCl concentration with 50 mM and 150 mM, the ThT intensity increased very sharply and reached the maximum, in comparison to plot with 0 mM NaCl concentration. Such enhancement in the ThT fluorescence intensity observed at the end of process compared to the intensity observed before the sample was incubated at 65 °C, indicated an increase in ThT binding sites, therefore formation of cross β-sheet-rich Amyloid aggregates.

The ThT fluorescence intensity at 480 nm at each aggregation time-point was fitted using mono-exponential function. The kinetics of Amyloid aggregation in the absence of salt, $k = \sim 253 \times 10^{-3}$ min^{-1}, at 50 mM concentration of NaCl, $k = \sim 144 \times 10^{-3}$ min^{-1} and at 150 mM concentration of NaCl, $k = \sim 187 \times 10^{-3}$ min^{-1}. The observed rate constants indicate that the salt facilitates the formation of Amyloid aggregates.

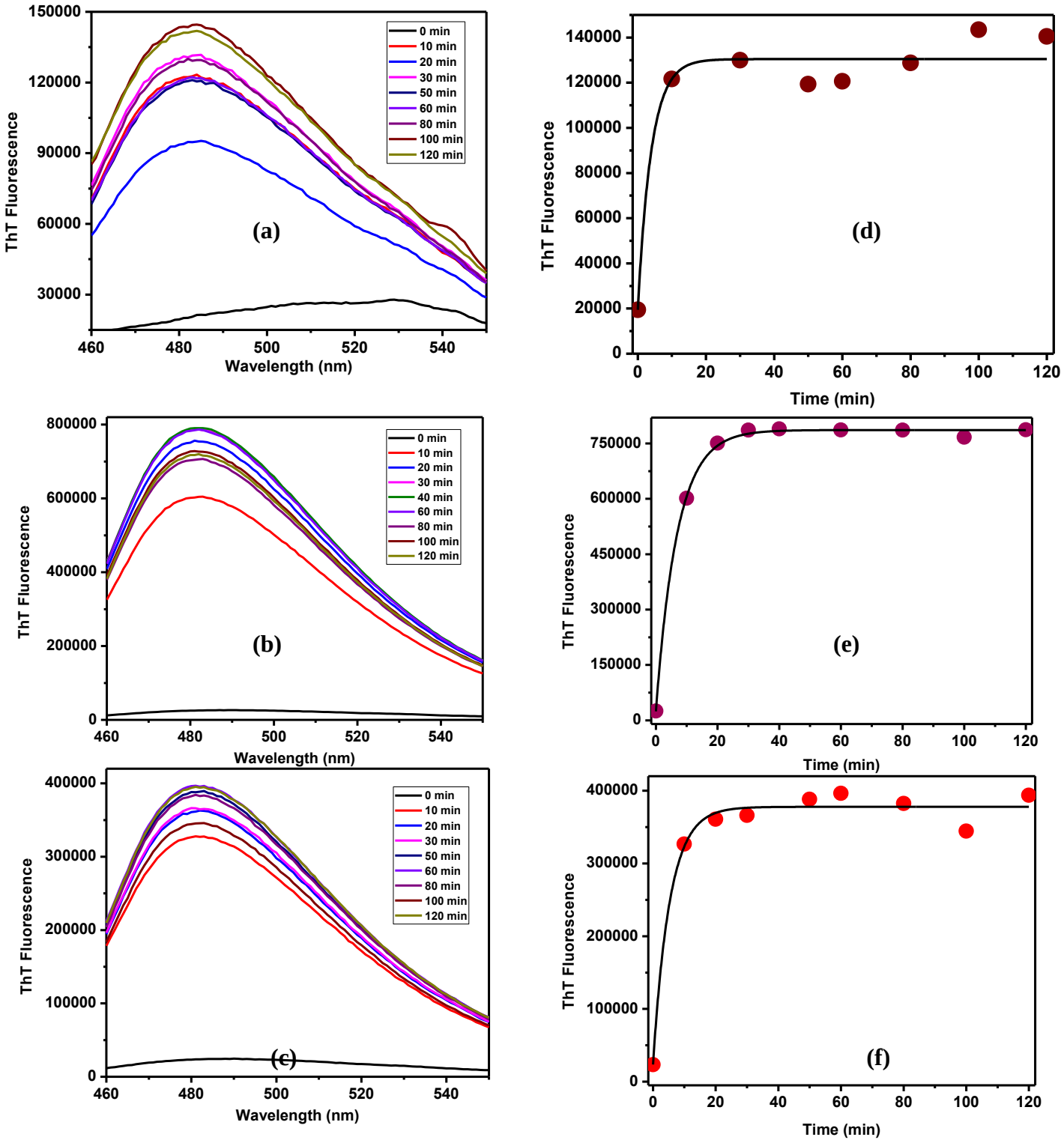


Fig. 4.2 Amyloid aggregation of ovalbumin at pH 2.2 (a) 0 mM NaCl, (b) 50 mM NaCl (c) 150 mM NaCl ,monitored by change in ThT fluorescence. ThT fluorescence kinetics at (d) 0 mM NaCl, (e) 50 mM NaCl (f) 150 mM NaCl -ThT fluorescence intensity at 480 nm at each aggregation time-point was fitted using mono-exponential function. The black line represents mono-exponential fit. The goodness of the fit lies between 0.95 - 0.98.

4.1.2 Aggregation monitored by Tryptophan fluorescence

The changes in the fluorescence intensity and anisotropy of the three intrinsic tryptophan residues were monitored during fibrillation process. The Trp fluorescence intensity was recorded at 340 nm. Unlike ThT and ANS fluorescence intensities, Trp fluorescence intensity did not exhibit any significant changes for the 0 min (before heating) and aggregated samples (after heating). Hence, apparent rate constant was not estimated.

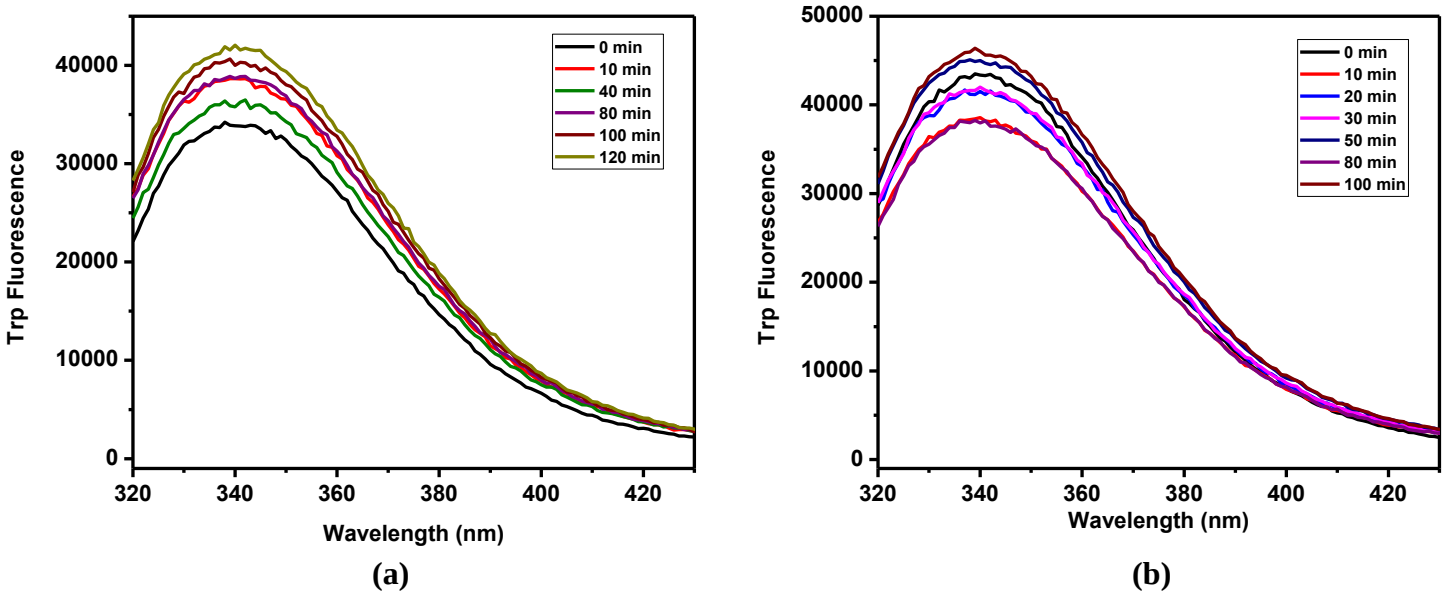


Fig. 4.3 Amyloid aggregation of ovalbumin at pH 2.2 (a) 0 mM NaCl, (b) 150 mM NaCl ,monitored by change in tryptophan fluorescence

A significant enhancement in the tryptophan fluorescence anisotropy was observed that reached saturation as fibrillation progressed (Fig.4.6), indicating the formation of large-sized aggregates. The apparent rate constant obtained for changes in tryptophan anisotropy for 0 mM of NaCl, k = 254×10^{-3} min^{-1}, for 150 mM of NaCl, k = 128×10^{-3} min^{-1}.

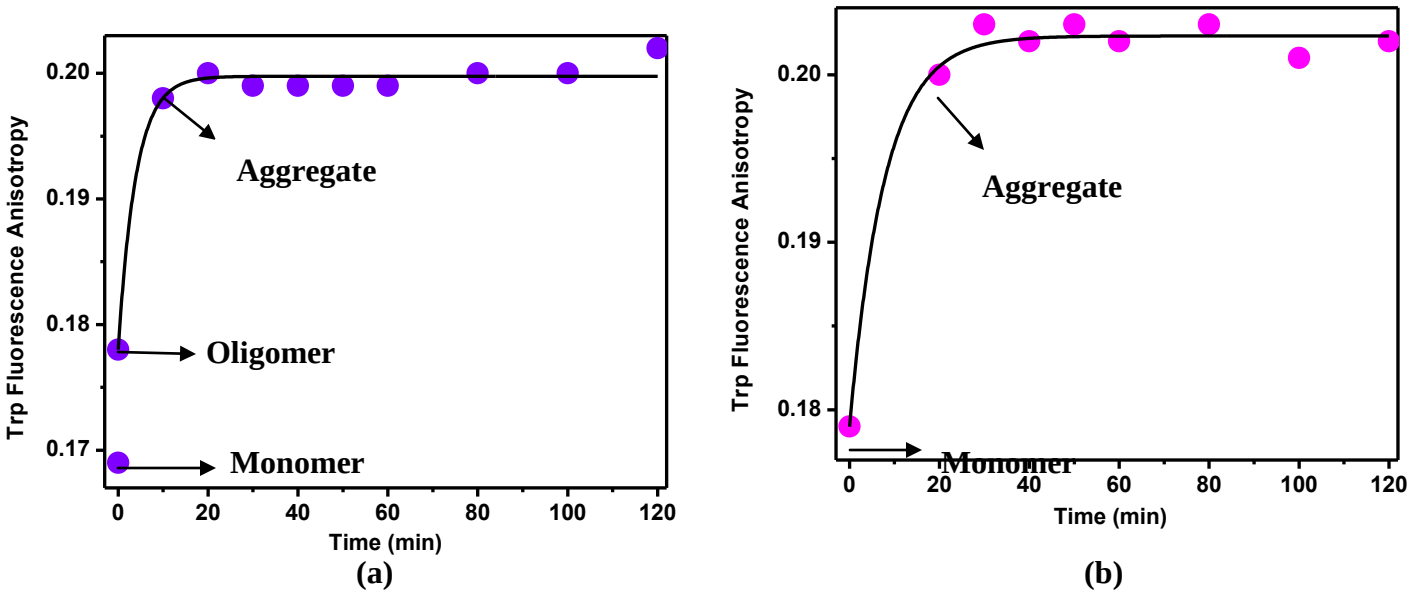
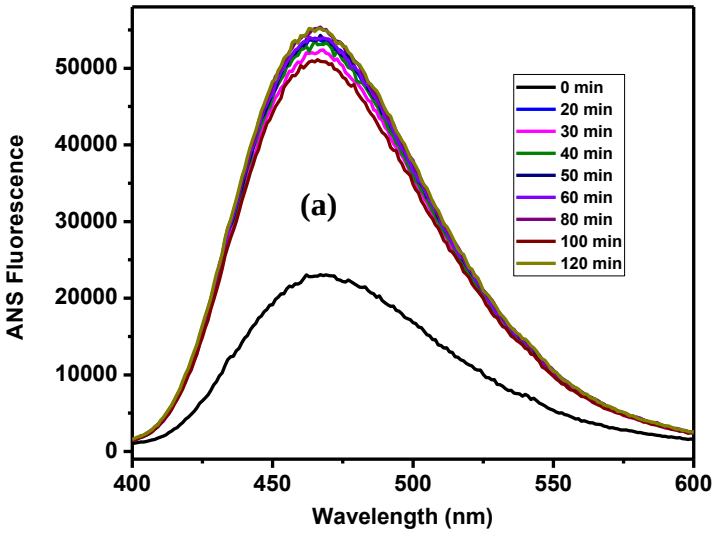


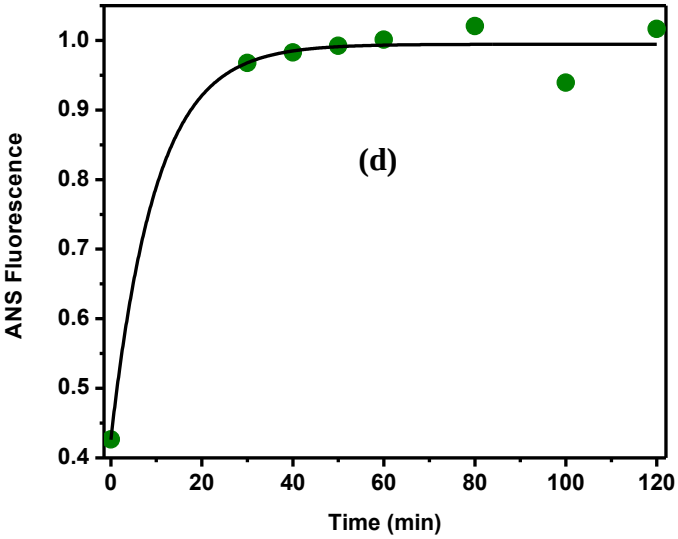
Fig. 4.4 Tryptophan fluorescence anisotropy monitored at (a) 0 mM NaCl, (b) 150 mM NaCl

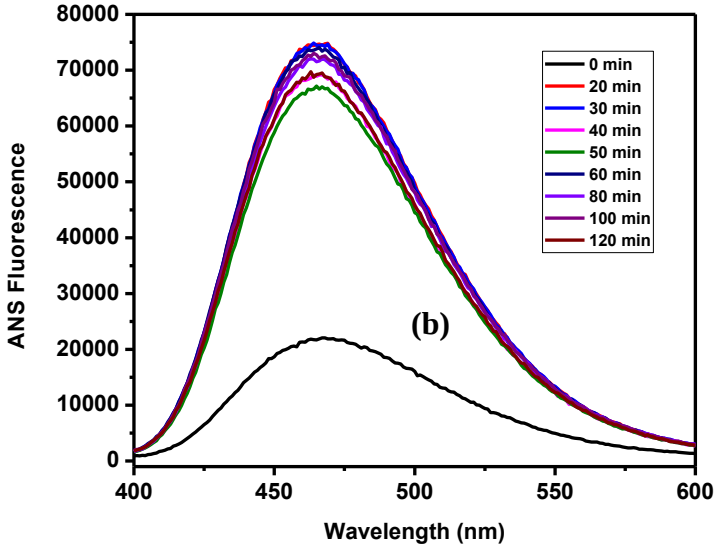
4.1.3 Aggregation monitored by ANS fluorescence

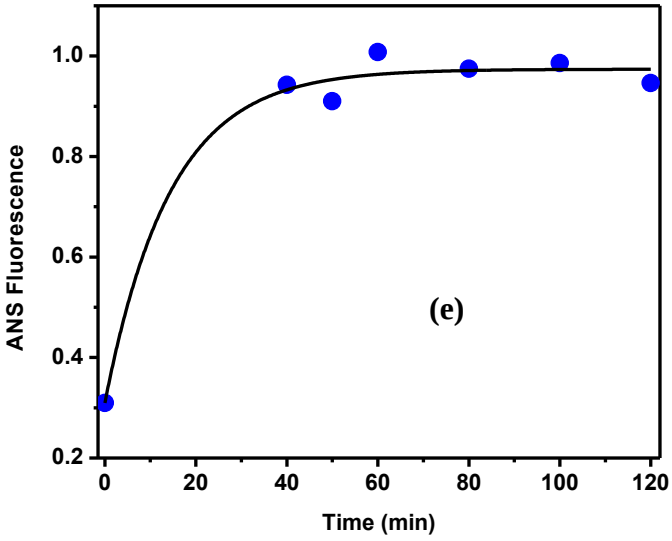
ANS intensity kinetics gives the measure of the rate of formation of hydrophobic pockets as a function of aggregation whereas ANS anisotropy kinetics gives a measure of the rate of size growth. ANS is an extrinsic fluorophore which is weakly fluorescent in aqueous environment but becomes strongly fluorescent when located in a hydrophobic environment. [22]. In this work, the protein samples were incubated with 0, 50 and 150 mM of NaCl at pH 2.2 and were heated to 65 °C. The ANS fluorescence intensity and anisotropy of the samples were recorded at regular intervals. A sharp enhancement in the ANS emission at 475 nm (Fig. 4.3). Oligomerization was also observed for the 50 mM and 150 mM NaCl samples even before the samples were heated, i.e., 0 minute..

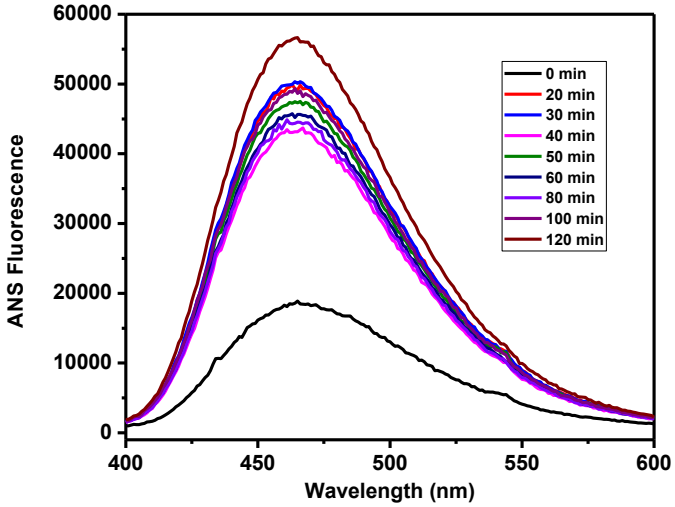
The ANS fluorescence intensity at 475 nm at each aggregation time-point was fitted using mono-exponential function. The kinetics of Amyloid aggregation obtained for 0 mM of NaCl, k = ~102 × 10^{-3} min^{-1}, at 50 mM concentration of NaCl, k = ~69.5 ×10^{-3} min^{-1} and at 150 mM concentration of NaCl, k = ~58.2 × 10^{-3} min^{-1}. The observed rate constants indicate that with increase in salt concentration, there is a decrease in the hydrophobic pockets. It suggests that the salt concentration is hindering the generation of hydrophobic pockets.


ANS Fluorescence
Wavelength (nm)
(a)
0 min
20 min
30 min
40 min
50 min
60 min
80 min
100 min
120 min

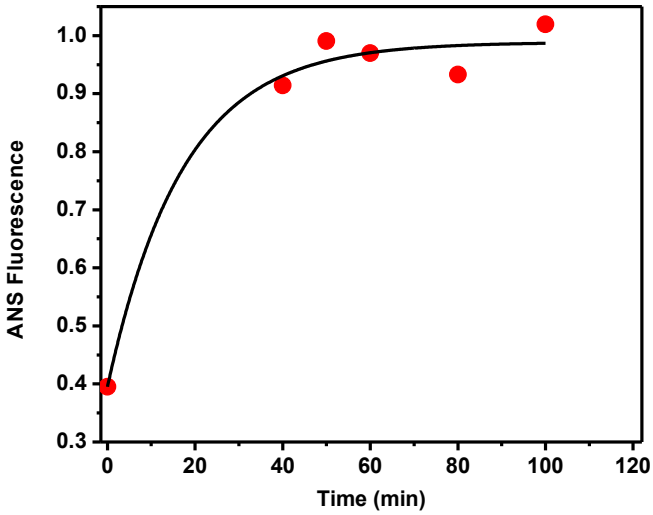

ANS Fluorescence
Time (min)
(d)


ANS Fluorescence
Wavelength (nm)
(b)
0 min
20 min
30 min
40 min
50 min
60 min
80 min
100 min
120 min


ANS Fluorescence
Time (min)
(e)



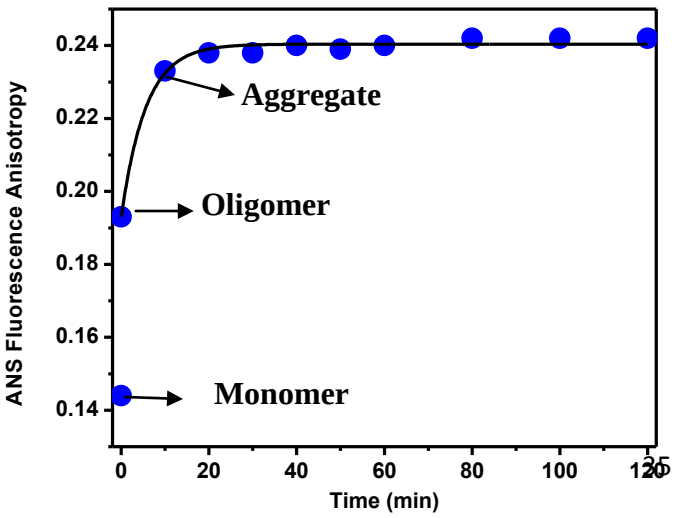
(c)



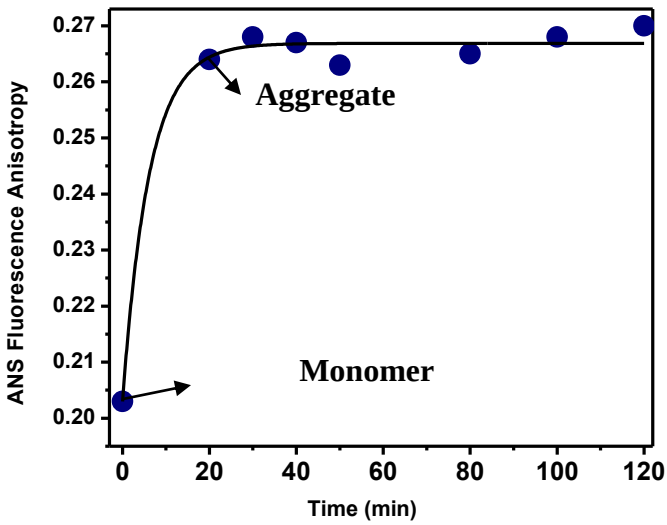
(f)

Fig. 4.5 Amyloid aggregation of ovalbumin at pH 2.2 (a) 0 mM NaCl, (b) 50 mM NaCl (c) 150 mM NaCl ,monitored by change in ANS fluorescence. ANS fluorescence kinetics at (d) 0 mM NaCl, (e) 50 mM NaCl (f) 150 mM NaCl -ANS fluorescence intensity at 475 nm at each aggregation time-point was fitted using mono-exponential function. The black line represents mono-exponential fit. The goodness of the fit lies between 0.95 - 0.98.

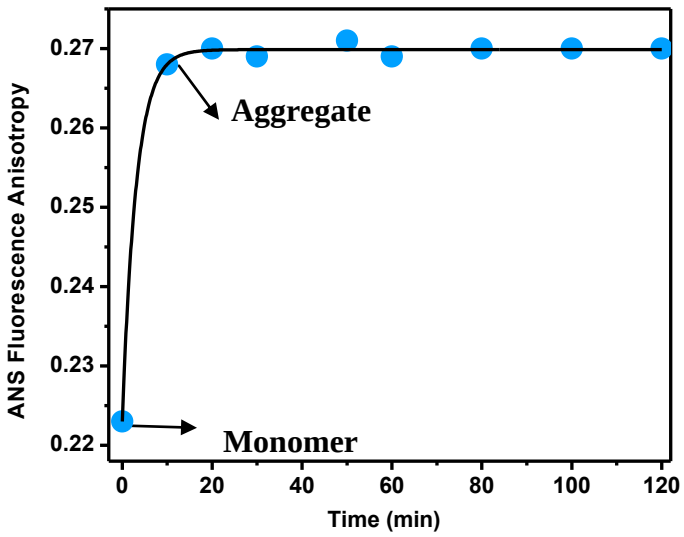
A significant enhancement in the ANS fluorescence anisotropy was observed (Fig. 4.4) that reached saturation as fibrillation progressed, indicating the formation of large-sized aggregates. The apparent rate constant obtained for changes in ANS anisotropy for 0 mM of NaCl, k = 179× 10^{-3} min^{-1}, for 50 mM of NaCl, k = 163 × 10^{-3} min^{-1}, for 150 mM of NaCl, k = 323× 10^{-3} min^{-1}.Comparing the rate constants for all the three concentrations, the size growth is fastest in protein solution containing 150 mM of NaCl. The rate constant for fluorescence intensity and anisotropy suggests that increase in overall size is much faster than the conformational changes of the aggregates.



(a)



(b)



(c)

Fig. 4.6 ANS fluorescence anisotropy monitored at (a) 0 mM NaCl, (b) 50 mM NaCl (c) 150 mM NaCl

4.2 CD spectroscopic study: Changes in secondary structure

We performed the far-UV CD experiments to investigate and analyze the secondary structural content of ovalbumin at different salt concentrations. As expected, a moderate reduction in the helicity was observed in the native form of ovalbumin in the presence of NaCl (Fig.4.7). At pH 2.2 and 150 mM NaCl, after the sample was incubated at 65 °C, a successive loss in the helicity is revealed by the far-UV CD spectra (Fig. 4.5(a)) which may suggest that the formation of both β-sheet and unordered conformation occur with the loss of α-helical conformation. This also suggests that the partially unfolded intermediates having variable secondary structural content exist in solution after heating which afterwards lead to the formation of β-sheet-rich amyloid aggregates as evidenced by an increase in ThT fluorescence. On the other hand, in absence of NaCl, there is an abrupt loss in α-helix structure suggesting the absence of existence of partially unfolded conformers in the solution. The average rate constant for loss of helicity at 222 nm (Fig. 4.5 (c)) was determined to be ~ 323 × 10^{-3} min^{-1}.

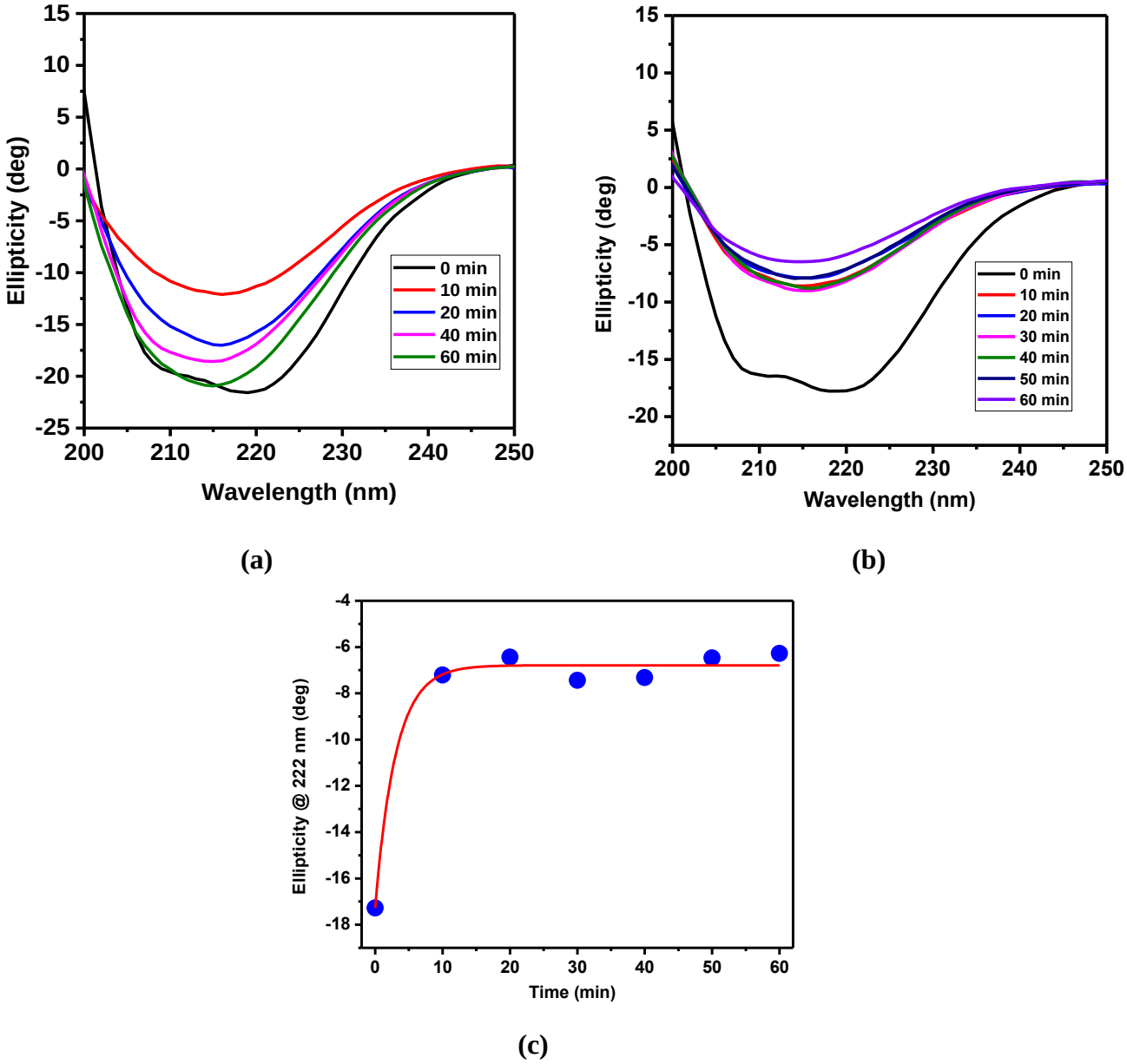


Fig. 4.7 Far-UV CD spectra of Ovalbumin (a) with 150 mM NaCl (b) with 0 mM NaCl (c) Kinetics of the loss of helicity at 222 nm during aggregation of ovalbumin (single exponential fit)

From all the experimental data and kinetics plots, it appears that oligomer formation is the first key step in ovalbumin aggregation under these solution conditions. Also, the size growth of the aggregates appears to be faster than the conformational changes that leads to the formation of cross b-sheet-rich amyloid aggregates.

Conclusion

The fibrillation of Ovalbumin induced by NaCl was investigated using steady-state fluorescence and CD spectroscopy to describe the early steps that are involved in the fibrillation event. The salt-induced unfolding of protein is confirmed by a pronounced increase in β-sheet to α-helix ratio in the presence of salt by studying CD spectra. The fluorescence study suggested that oligomer formation is the first key step in ovalbumin aggregation under these solution conditions. Also, the size growth of the aggregates appears to be faster than the conformational change that leads to the formation of cross b-sheet-rich amyloid aggregates that might have important inference in the study of unfolding and aggregation of other important physiological proteins.

Chapter-6 References

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