



**INDIAN INSTITUTE OF TECHNOLOGY (BHU)**  
**VARANASI**  
Varanasi – 221005

---

**CERTIFICATE**

It is certified that the work contained in the thesis titled *Studies on protein folding, stability and amyloid formation/dissociation: Effect of selected Biomolecules* by **Debanjan Kundu** has been carried out under my supervision and that this work has not been submitted elsewhere for a degree. It is further certified that the student has fulfilled all the requirements of Comprehensive Examination, Candidacy and SOTA for the award of PhD. Degree.

**Signature:**

(Prof. Vikash Kumar Dubey)  
Supervisor  
Indian Institute of Technology (BHU) Varanasi



# INDIAN INSTITUTE OF TECHNOLOGY (BHU) VARANASI Varanasi – 221005

---

## DECLARATION BY THE CANDIDATE

I, **Debanjan Kundu**, certify that the work embodied in this thesis is my own bonafide work and carried out by me under the supervision of **Prof. Vikash Kumar Dubey** from **July 2018 to July 2022**, at the School of Biochemical Engineering, Indian Institute of Technology (BHU) Varanasi. The matter embodied in this thesis has not been submitted for the award of any other degree/diploma. I declare that I have faithfully acknowledged and given credits to the research workers wherever their works have been cited in my work in this thesis. I further declare that I have not will fully copied any other's work, paragraphs, text, data, results, etc., reported in journals, books, magazines, reports dissertations, theses, etc., or available at websites and have not included them in this thesis and have not cited as my own work.

**Date:**

**Signature of the Student**

**Place:** Varanasi

(Debanjan Kundu)

## CERTIFICATE BY THE SUPERVISOR

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

Supervisor  
(Indian Institute of Technology (BHU))

## COPYRIGHT TRANSFER CERTIFICATE

**Title of the Thesis:** Studies on protein folding, stability, amyloid formation/dissociation: Effect of selected biomolecules

**Name of the Student:** Debanjan Kundu

### Copyright Transfer

The undersigned hereby assigns to the Indian Institute of Technology (Banaras Hindu University) Varanasi all rights under copyright that may exist in and for the above thesis submitted for the award of the *Doctor of Philosophy*

**Date:**

Signature of Student

**Place:** Varanasi

Debanjan Kundu

**Note:** However, the author may reproduce or authorize others to reproduce material extracted verbatim from the thesis or derivative of the thesis for the author's personal use, provided that the source and the Institute's copyright notice are indicated.

*Dedicated to my parents, extended family, and  
all my mentors in Biology.....*

## Acknowledgement

As I draw near the end of this enthralling journey of obtaining my PhD degree, I want to express my gratitude and heartfelt thanks to all the people who have helped me accomplish this journey. I would like to thank the administration, the Central Instrumentation Facility (CIF) and the Param Shivay Supercomputing Facility available at the Indian Institute of Technology (BHU) Varanasi.

I would like to express my sincere gratitude to my supervisor, **Prof. Vikash Kumar Dubey**, for his constant guidance, encouragement, and motivation. He has always been a source of motivation and inspiration with his wise words and strong sense of belief. He never failed to instill a strong sense of work ethics in me throughout my journey during various phases of my research and helped me put a conclusion to my work as well. Further, I would like to thank other members of RPEC, **Dr. Sanjay Kumar** and **Dr. Gyan Prakash Modi**, for their insights into my work and their efforts to increase the overall quality of my work outputs.

I am also thankful to **Dr. Avneesh Singh Parmar**, Associate Professor, Department of Physics, IIT (BHU), and his group for Dynamic Light Scattering (DLS) experiments. Further, I also thank **Mr. Nirmal Mallick** for helping me with my Atomic Force Microscopy (AFM) experiments and all other staff of the CIF, IIT (BHU). Further, I also would like to thank **Dr. Manoj Kumar Bharty**, Associate Professor, Institute of Science, Department of Chemistry, BHU and his group for kindly allowing me to use the Fluorescence spectrophotometer for my initial experiments.

I have been surrounded by some outstanding seniors, batchmates, and juniors throughout my PhD journey. I would like to especially thank **Dr. Sunita Yadav**, who played an instrumental role during the preliminary days of my PhD journey. Further, I would like to thank my batchmate and labmate, **Ms. Kumari Prerna**. She has played an essential role in my growth as a budding researcher. Among my other labmates, I would like to thank **Ms. Preeti Ranjan**, **Mr. Manash Sarma**, **Ms. Naveena Menpadi**, and **Mr. Kushal Bora & Mr. Umesh Kushwaha** for their constant support and vigour during our stay together in the Cellular Biochemistry Laboratory. The acknowledgement would be

incomplete without mentioning other juniors who completed their IDD and M.Tech projects from our lab. *Umesh Bhati, Daniel Pegu, Digvijay Pratap, Mihir Anand, Shruti Pandey, Neeraj Moitra, Shalini Mishra, Bhavya Rajan, Ria Sonigra, Kshitij Sinha, Tanuj Handa and Abhishek Sahu*, all of whom gave me enough opportunities to sharpen my mentoring skills with all the help and assistance they asked for from time to time. During one of the critical phases of our research journey, the COVID-19 pandemic, *Dr. Subhomoi Borkotoky* emerged as one of our most considerable help, whose substantial contribution to my computational biology studies would always stay with me. During the last few months of our PhD journey, especially after the resumption of work post-pandemic, *Dr. Jay Prakash* played an essential role in shaping the first draft of my thesis and other scientific discussions. I would also like to extend my appreciation and gratitude to *Dr. Monu Pande* for her support and guidance through different phases of my study.

Lastly, I would like to thank my ever supportive and loving parents, *Mr. Susanta Kundu & Mrs. Sangita Kundu*, without whose blessings I would not have been able to complete my journey, and all my relatives and cousins whose constant love and support were there throughout. All my friends from all parts of the world special mention to *Niharika, Aritrajit, Attrayee* and *Mahamaya*. My other batchmates and lab alumni, *Dr. Kamalesh Verma & Dr. Gundappa Saha*, for their guidance and scientific discussion on various matters. Successive Coordinators of my School, *Prof. Subir Kundu* and *Prof. Pradeep Srivastava*, and all the support staff and other faculties who were always there to support me and provide me with any help whenever required.

Debanjan Kundu  
July 2022

## Table of Contents

<b>CONTENTS</b>	<b>PAGE NO</b>
<b>CHAPTER 1</b>	<b>1</b>
<b>INTRODUCTION AND REVIEW OF LITERATURE ON PROTEIN FOLDING, AMYLOIDOSIS AND NEURODEGENERATION*</b>	<b>1</b>
Abstract	1
1.1 Introduction to Protein Folding	2
1.2- History and clinical relevance of amyloidosis	3
1.3- Intermediate Pathways in Protein aggregation	4
1.4- Cellular mechanisms against protein misfolding	7
1.5- Amyloids and associated diseased conditions	8
1.6 Amyloid formation mechanism	12
(A) From Amyloid Precursor Protein to Amyloid $\beta$ - sheet formation:	12
(B) Formation of amyloids <i>in vitro</i> :	13
1.7- Structural characteristics of amyloids	16
1.8- Factors modulating the amyloid formation	19
(A) Hydrophobicity and secondary structure:	19
(B) Sequence of Amino Acids:	20
(C) Unfolded areas in the protein	21
1.9- Nucleotide imbalance and neurodegeneration	21
(A) Purinergic Signaling	25
(B) Nucleosides, Nucleotides and Neurodegenerative diseases	25
Adenosine	26
Adenosine Triphosphate (ATP)	26
Guanine-based purines (GBPs)	27
(C) Pyrimidines and Neurodegeneration:	28
(D) Drug development against amyloid diseases: Current and future strategies	29
1.10- Model Proteins used in this study	34
Hen Egg White Lysozyme (HEWL)	34
1.11- Scope and Objectives of the current study	35
<b>CHAPTER 2</b>	<b>37</b>
<b>INTERACTION OF SELECTED BIOMOLECULES AND METABOLITES WITH AMYLOIDOGENIC PROTEINS*</b>	<b>37</b>

Abstract	37
2.1: Introduction	38
2.2 Methodology	39
2.2.1 Preparation of model proteins:	39
2.2.2 Preparation of Ligands	40
2.2.3 ADME analysis of ligands	40
2.2.4 Molecular Docking using Autodock Tools	41
2.2.5 Visualisation and analyses	41
<b>3.0 Results</b>	<b>42</b>
3.1 Docking analysis with hen egg-white lysozyme	42
3.2 Docking analysis with Amyloid $\beta$ peptides	49
3.3 SWISS ADME analysis of molecules	53
<b>4.0 Discussion and Conclusion</b>	<b>54</b>
<b>CHAPTER 3</b>	<b>55</b>
<b>Nucleosides and Nitrogenous Bases Inhibit Protein Amyloidosis: A Biophysical and Computational Analysis Using Hen Egg White Lysozyme as Model System*</b>	<b>55</b>
Abstract	55
3.1. Introduction	56
3.2. Materials and Methods	57
3.2.1 Materials	57
3.2.2 Methods	57
3.2.2.1 Buffer, Protein and metabolites solutions	57
3.2.2.2 Hen Egg White Lysozyme Amyloid formation	58
3.2.2.3 Thioflavin T assay	58
3.2.2.3 ANS Assay	59
3.2.2.4 Aggregation Index measurements	59
3.2.2.5 Dynamic Light Scattering	59
3.2.2.6 Atomic Force Microscopy (AFM)	59
3.2.2.8 Molecular Dynamics and Simulation	60
3.2.2.9 Statistical analyses using OriginPro (Learning Edition)	61
3.3 Results	61
3.3.1 HEWL aggregates have a higher growth rate in near physiological pH than pH 12.2	61
3.3.2 ThT and ANS assay at near physiological pH	63
3.3.3 Aggregation index at near physiological pH	64
3.3.4 ThT and ANS assay at pH 12.2	64
3.3.5 Aggregation index at pH 12.2	66
3.3.6 Dynamic Light Scattering Study (DLS)	66



3.3.7 AFM imaging of HEWL oligomers /amyloids	70
3.3.8 Aggregation prone regions and pH-dependent disorder	70
3.3.9 Molecular Dynamics and Simulation	72
3.3.9.1 RMSD, RMSF and Radius of Gyration analyses	72
3.3.9.2 SASA, DSSP and hydrogen bond analyses	73
3.3.9.3 MM/PBSA energy analyses	75
3.4. Discussion	78
3.5 Concluding remarks	82
<b>Chapter 4</b>	<b>84</b>
<b>Investigating Potential Mechanisms of Selected Intracellular Metabolites on Preformed Lysozyme Oligomers at Physiological pH*</b>	<b>84</b>
Abstract	84
4.1 Introduction	85
4.2 Materials and Methods	86
4.2.1 Materials	86
4.2.2 Preparation of stock solutions	87
4.2.3 Generation of HEWL oligomers	87
4.2.4 Tryptophan fluorescence and fluorescence quenching	87
4.2.5 Endpoint Thioflavin T assay and Thioflavin T kinetics	88
4.2.6 Light scattering by protein aggregates	89
4.2.7 Atomic Force Microscopy (AFM)	89
4.2.8 Statistical analyses using OriginPro (Learning Edition)	90
4.3 Results:	90
4.3.1 Selected Intracellular metabolites enhance the amyloid formation of HEWL	90
4.3.2 Atomic Force Microscopy imaging	91
4.3.3 Light Scattering via UV and Turbidity assay	91
4.3.4 Selected Intracellular metabolites quench tryptophan fluorescence of HEWL	93
4.3.5 HEWL shows a saturating elongation and fragmentation-dominated aggregation pathway at physiological pH	94
4.3.6 ThT Kinetic Analysis	97
4.4 Discussion	99
4.5 Conclusion	102
<b>CHAPTER 5</b>	<b>104</b>
<b>POTENTIAL ALTERNATIVES TO CURRENT CHOLINESTERASE INHIBITORS: AN <i>IN-SILICO</i> DRUG REPURPOSING APPROACH*</b>	<b>104</b>
Abstract	104

5.1 Introduction	105
5.2 Methodology	108
<b>5.2.1 Protein Preparation:</b>	108
5.2.2 Target Site Identification for Molecular Docking	109
5.2.3 Selection and Preparation of Ligands	110
5.2.4 Compound Screening and Molecular Docking	111
5.2.5 Molecular Dynamic Simulation Studies	111
<b>5.2.6-MM/PBSA Free Energy Analysis</b>	112
5.2.7-Visual analysis	112
<b>5.2.8-ADME and Toxicity analyses</b>	113
5.3-Results	113
5.3.1-Docking results with Acetylcholinesterase of Homo sapiens	113
5.3.2 Docking results with Butyrylcholinesterase of Homo sapiens	116
5.3.3 Molecular Simulation Results	118
5.3.3.1 Simulation of Acetylcholinesterase	119
5.3.2.2 Simulation of Butyrylcholinesterase	120
5.3.4 ADME analysis results	122
5.4 Discussion	123
5.5 Conclusion	125
<b>CHAPTER 6</b>	<b>126</b>
<b>CONCLUSION &amp; FUTURE SCOPE</b>	126-132
<b>REFERENCES</b>	133-143
<b>APPENDIX A</b>	<b>144</b>
LIST OF PUBLICATIONS FROM THESIS WORK	144
<b>APPENDIX B</b>	<b>145</b>
CONFERENCES & WORKSHOPS	145

## List of Abbreviations

AchE	Acetylcholinesterase
AD	Alzheimer's Disease
ADME	Absorption Distribution Metabolism Excretion
AFM	Atomic Force Microscopy
ANS	8-Anilino-naphthalene-1-sulfonic acid
ATP	Adenosine triphosphate
A1R	Adenosine 1 Receptor
A2R	Adenosine 2 Receptor
BuChE	Butyrylcholinesterase
CT	Computed Tomography
CTF	C-terminal Fragment
CTP	Cytidine Triphosphate
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic Acid
dNTP	deoxynucleotide triphosphates
DTT	Dithiothreitol
DMT	Disease Modifying Therapies
ER	Endoplasmic Reticulum
EM	Electron Microscopy
GPB	Guanine Based Purines

HD	Huntington's Disease
HEWL	Hen Egg White Lysozyme
LGA	Lamarckian Genetic Algorithm
MDS	Molecular Dynamics and Simulation
NMR	Nuclear Magnetic Resonance
NPT	Number Pressure Temperature
NVT	Number Volume Temperature
PD	Parkinson's Disease
PDB	Protein Data Bank
PET	Positron Emission Tomography
PLIP	Protein Ligand Interaction Profiler
PME	Particle Mesh Ewald
R <sub>g</sub>	Radius of Gyration
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
RNA	Ribonucleic Acid
SASA	Solvent Accessible Surface Area
ThT	Thioflavin T
TTP	Thymidine Triphosphate
UV	Ultraviolet
UTP	Uridine Triphosphate
VDW	Van Der Waals
XRD	X-Ray Diffraction

## List of Tables

- Table 1.1-** Amyloid diseases affecting the nervous system of the patients and causing neurodegenerative conditions
- Table 1.2-** Non-neuropathic systematic amyloidosis conditions occurring in humans, their associated protein structural details
- Table 1.3-** Defective mutations associated with various genes in pyrimidine metabolism and their relative conditions
- Table 1.4-** Purine and Pyrimidine based drug development and their results in some neurological conditions
- Table 2.1-** Aggregation prone regions in hen egg-white lysozyme, their amino acid composition and grid coordinates for docking
- Table 2.2-** Details of various interactions between the amino acids and ligand when grid box no. 1 (25-32: LGNWVCAA) of HEWL. Detailed interaction of top 5 ligands based on binding energy is also given. N/A applies Not Applicable.
- Table 2.3-** Details of various interactions between the amino acids and ligand when grid box no. 2 (53-61: YGILQINSR) of HEWL. Detailed interaction of top 5 ligands based on binding energy is also given. N/A applies Not Applicable.
- Table 2.4-** Details of various interactions between the amino acids and ligand when grid box no. 3 (80-92: CSALLSSDITASV) of HEWL was fixed for the docking. Detailed interaction of top 5 ligands based on binding energy is also given. N/A applies Not Applicable.
- Table 2.5-** Details of various interactions between the amino acids and ligand when grid box no. 4 (105-110: MNAWVA) of HEWL was fixed for the docking. Detailed interaction of top 5 ligands based on binding energy is also given. N/A applies Not Applicable.
- Table 2.6-** Details of various interactions between the amino acids and ligand when grid box no.5 (120-124: VQAWI) of HEWL was fixed for the docking. Detailed interaction of top 5 ligands based on binding energy is also given. N/A applies Not Applicable.
- Table 2.7-** Details of various interactions between the amino acids and ligand when grid box no. 1 (15-20: QKLVFFA) of Amyloid  $\beta$  peptide was fixed for the docking. Detailed interaction of top 5 ligands based on binding energy is also given. N/A applies Not Applicable.
- Table 2.8-** Details of various interactions between the amino acids and ligand when grid box no. 2 (30-42: IIGLMVGGVVIA) of Amyloid  $\beta$  peptide was fixed for the docking. Detailed interaction of top 5 ligands based on binding energy is also given. N/A applies Not Applicable.
- Table 2.9-** Lipinski's parameters for drug likeliness and ADMET properties of chosen ligands and the approved standard drugs. Log P value represents the lipophilicity of the molecule, Log S value represents the water solubility, TPSA is Total Polar Solvent Accessibility, and BA is the oral bioavailability of a drug
- Table 3.1-** ThT Kinetics profiling of HEWL at 20 and 100 $\mu$ M concentration at pH 7.4 and pH 12.2

- Table 3.2-** Dynamic Light Scattering analysis of physiological and alkaline pH HEWL aggregates with and without ligands
- Table 3.3-** Computed solvent-accessible surface areas of the HEWL and the HEWL-metabolites complexes
- Table 3.4-** Details of Secondary structure of apoprotein and holoprotein complexes computed using DSSP tool
- Table 3.5-** MM/PBSA binding free energy analyses of the various ligands with hen egg-white lysozyme
- Table 4.1-** The approximate number of binding sites and Stern-Volmer Coefficient ( $K_{SV}$ ) of the various ligands in the current study
- Table 4.2-** The combined  $k_n k_+$  rates for various systems under consideration and the potential mode of action of various ligands in the study,  $n_c$  is 2.0 used as a global constant for nucleus size,  $t_{1/2}$  is the time in which half of the maximum intensity is reached
- Table 4.3-** The approximate lag time in hours and potential mechanism of ligands in seeded reaction
- Table 5.1-** Small molecule drugs used in the current study, their usage and year of approval by the FDA
- Table 5.2-** Details of the proteins used for docking, the sites of docking and their respective coordinates and references
- Table 5.3-** Details of various ligand interactions, respective binding and intermolecular energy and inhibitor constant in Acetylcholinesterase from *Homo sapiens* (*HsAChE*). Donepezil is the positive control which is already an approved acetylcholinesterase inhibitor by FDA.
- Table 5.4-** Details of various ligand interactions, respective binding and intermolecular energy and inhibitor constant in Butyrylcholinesterase from *Homo sapiens* (*HsBuChE*). Donepezil is the positive control which is already an approved acetylcholinesterase inhibitor by FDA. VDW is Van Der Waal's interactions.
- Table 5.5-** MM/PBSA free energy analysis of Donepezil, Brexipiprazole and Pimavanserin in complex with butyrylcholinesterase, SASA is Surface Accessible Surface Area.
- Table 5.6-** MM/PBSA free energy analysis of Donepezil, Brexipiprazole and Siponimod in complex with acetylcholinesterase, SASA is Surface Accessible Surface Area
- Table 5.7-** ADMET analysis of top 5 drugs across both the proteins taken in this study. We analysed the drugs for their drug likeliness and the following Lipinski's rule and its various parameters

## List of Figures

- Fig 1.1:** Representations of different models to explain the protein folding process
- Fig 1.2-** Alois Alzheimer and his patient, Auguste Deter, the first person reported to have died from Alzheimer's disease (AD)
- Fig 1.3-** The various stages in the protein folding pathway
- Fig 1.4-** Diagrammatic representation of the protein folding process, the polypeptide's various intermediate states can form and take up during the folding process.
- Fig 1.5-** Classical sigmoidal kinetics, which is typical of the protein aggregation process.
- Fig 1.6-** Diagram showing two proteolytic pathways of the amyloidogenic precursor protein
- Fig 1.7-** Illustration of the de novo pathway highlights the incorporation of nucleoside triphosphates (NTPs) and dNTPs in RNA and DNA.
- Fig 1.8-** A Graphical representation of the action of pyrimidines and their role in improving cognitive functions and memory in the case of neurodegeneration.
- Fig 1.9-** A general illustration of the central hypothesis of the present study.
- Fig 1.10:** Model proteins visualised in Pymol used in this study
- Fig 2.1:** Ligplot images showing both hydrogen and hydrophobic interactions by (A) Guanine and (B) GMP with aggregation-prone sites of hen egg-white lysozyme.
- Fig 2.2:** Ligplot images showing both hydrogen and hydrophobic interactions by (A) Thymine and (B) GMP with second aggregation-prone sites of hen egg-white lysozyme.
- Fig 2.3:** Ligplot images showing both hydrogen and hydrophobic interactions by (A) Coumarin and (B) Guanine with third aggregation-prone sites of hen egg-white lysozyme.
- Fig 2.4:** Ligplot images showing both hydrogen and hydrophobic interactions by (A) GMP and (B) Guanine with aggregation-prone sites of hen egg-white lysozyme.
- Fig 2.5:** Ligplot images showing both hydrogen and hydrophobic interactions by (A) Guanine and (B) UMP with first aggregation-prone sites of amyloid  $\beta$  peptide.
- Fig 2.6:** Ligplot images showing both hydrogen and hydrophobic interactions by (A) Guanine and (B) UMP with aggregation-prone sites of amyloid  $\beta$  peptide.
- Fig 3.1-** Hen egg-white lysozyme aggregation trends at different time points
- Fig 3.2-** Thioflavin T and ANS intensity of 50  $\mu$ M HEWL samples incubated with selected nitrogenous bases and nucleosides at pH 7.4.
- Fig 3.3-** Thioflavin T and ANS intensity of 50  $\mu$ M HEWL samples incubated with selected nitrogenous bases and nucleosides at pH 12.2
- Fig 3.4-** Thioflavin T of HEWL samples incubated with various ligands at 1:10 concentration after 120 hours
- Fig 3.5-** Aggregation Index of HEWL incubated with bases and nucleosides at pH 7.4 and 12.2

- Fig 3.6-** Dynamic Light Scattering of Hen Egg White Lysozyme aggregates with and without ligands after 120 hours of incubation at **(A)** pH 7.4 and **(B)** pH 12.2.
- Fig 3.7-** AFM images of HEWL amyloid aggregates/fibrils in the absence and presence of ligands after 72 hours of incubation at pH 12.2
- Fig 3.8-** Histogram analysis of AFM images showing Heights Vs Counts of various systems in **(A)** pH 12.2 and **(B)** 7.4
- Fig 3.9-** Data from DispHscan Web server
- Fig 3.10-** Molecular Dynamics and simulation analyses of HEWL and HEWL-metabolites complexes for 100 ns run
- Fig 4.1-** Thioflavin T intensity after adding ligands to preformed HEWL oligomeric solutions. Thioflavin T intensity after **(A)** 0hr **(B)** 24hrs and **(C)** 72 hrs
- Fig 4.2-** Atomic Force Microscopy imaging of control sample and various other HEWL solutions in the presence of various ligands and **(B)** Histogram profiling showing the average heights and counts of the various samples
- Fig 4.3-** Turbidity of various performed HEWL early oligomeric solutions (120 hrs) after addition of ligands at different time points
- Fig 4.4-** Thioflavin T kinetics of HEWL at various concentrations
- Fig 4.5-** Tryptophan fluorescence quenching by various ligands
- Fig 4.6-** Thioflavin T Kinetics Analysis in **(A)** Unseeded HEWL oligomeric solutions and **(B)** Seeded HEWL oligomeric solutions with and without various ligands at desired concentrations.
- Fig 5.1-** Interaction of *Homo sapiens* Acetylcholinesterase (HuAChE) with **(A)** Deutetrabenazine and **(B)** Donepezil as seen in Discovery Studio Visualizer.
- Fig 5.2-** Interaction of *Homo sapiens* Butyrylcholinesterase (HuBuche) with **(A)** Brexipirazole and **(B)** Pimavansarin as seen in Discovery Studio Visualizer.
- Fig 5.3-** Molecular Dynamics and Simulation of AChE and BuChE **(A)** RMSD and **(B)** Radius of Gyration of AChE **(C)** RMSD and **(D)** Radius of gyration of BuChE **(E)** RMSF of Acetylcholinesterase and **(F)** RMSF of BuChE
- Fig 6.1-** The principle behind the potential binding of small molecules within disordered regions within the protein and their action of preventing further notorious interactions of the protein-based on which we performed our computational study
- Fig 6.2-** Protein aggregation phases
- Fig 6.3-** Possible mechanism of metabolites at various stages of HEWL aggregation.
- Fig 6.4-** 3D diagrams of interaction between FDA-approved drugs and critical enzymes in Alzheimer's Disease. The drugs could be potential cholinesterase inhibitors
- Fig 6.5-** Significant findings of the current study and future scope of research in bridging the connection between the role of intracellular metabolites and proteinopathies



## Preface

Protein misfolding and aggregation is not only a great intellectual challenge but also of excellent therapeutics importance. Protein misfolding is associated with various pathological conditions in humans, including neurodegenerative diseases like Alzheimer's (AD), Parkinson's (PD), and prion-related protein misfolding diseases. Drug discovery processes for protein misfolding diseases are still a tremendous scientific challenge as the protein folding process is still an enigma, and the underlying processes are still poorly understood. Protein amyloids usually form stable  $\beta$ -sheet-rich structures, as reported in most cases. Amyloid fibrils represent a very stable class of structures in terms of thermodynamics. Among various schools of the hypothesis, the toxicity of oligomeric intermediates is well established and accepted compared to matured fibrils. Currently, therapeutic development for protein misfolding diseases focuses on these oligomeric intermediates.

The thesis reports some critical findings related to protein amyloid formation/dissociation in the presence of a few selected metabolites using integrated computational and biophysical methods. The work provides fundamental insights into the effect of these selected metabolites on protein amyloid formation and critical insight into pathological conditions arising from disturbed homeostasis with neurodegenerative symptoms.

We have utilized extensive computational methods like molecular docking and simulation to analyse the interactions of various nucleosides, nitrogenous bases and nucleotides with aggregation-prone zones of model amyloid protein, *i.e.* Hen egg White Lysozyme (HEWL). We further analyzed the binding interactions and molecular docking statistics to identify the best binding molecules. The hypothesis for this work mainly revolved around the fact that interaction of various intracellular metabolites with aggregation-prone zones within the protein would prevent intracellular interactions within these regions, which could drive the protein away from aggregation-mediated pathways under stress conditions. We have identified a few small intracellular metabolites like nucleosides and nitrogenous bases with the best docking parameters within the aggregation-prone regions of HEWL. The selected metabolites show a good binding affinity and potential anti-amyloid properties. The encouraging results with few compounds prompted us to test the hypothesis experimentally.

Subsequently, we examined the effect of the best computationally identified compounds for anti-amyloid properties using HEWL amyloid as a model system. HEWL amyloid is well characterized at pH 12.2 and widely reported in the literature. We have also characterized HEWL amyloid at near physiological pH, *i.e.* 7.4. Our results showed that amyloid fibrillation is more pronounced in near physiological pH than at pH 12.2. Further, we also report a lower formation of oligomers and, subsequently, matured fibrils of HEWL in the presence of both nitrogenous bases (Cytosine, Guanine, Thymine and Uracil), nucleosides (Adenosine and Guanosine) in a time-dependent manner. We also validated the results using supporting data like aggregation index, dynamic light scattering (DLS) studies and atomic force microscopy (AFM) imaging. We also performed and interpreted the RMSD, RMSF, SASA, and secondary structure analyses through molecular dynamics and simulation of the selected holoprotein complexes after binding these compounds with amyloid-prone regions. We concluded that the selected nucleosides and nitrogenous bases potentially suppress (slow down) primary nucleation processes under these experimental conditions.

Furthermore, we have also explored the impact of these metabolites on the preformed early oligomeric state of HEWL. We attempted to unravel the HEWL amyloid kinetics in near physiological pH and determine the critical microscopic stages of HEWL aggregation. The HEWL aggregation followed a Saturation Elongation and Fragmentation model. We report that upon adding ligands on preformed HEWL amyloids, there is a sharp increase in the Thioflavin T intensity of the samples indicating the rapid formation of matured fibrils upon adding the chosen ligands. The observation was repeated at three different time points. Our AFM analyses also supplemented this. On the contrary, our turbidity and UV scattering analyses showed a decreasing trend, which could be explained by the overall reduction of scattering intensity due to large aggregates. We further elucidated using the Thioflavin T kinetics assay that in the presence of the metabolites, the HEWL aggregation half-time is shortened compared to HEWL aggregation without metabolites. Further, there is an increase in combined nucleation and elongation rate. Considering these two factors, we concluded that the selected metabolites enhance and promote fibrillation processes at this stage. Although in seeded reactions where we had anticipated quickening up of overall reaction and shortening of lag phase, the addition of ligands along with preformed seeds to HEWL monomers showed varied results. Adenosine and Cytosine did not decrease lag time compared to control samples. On the contrary, Guanine, Guanosine and Thymine increased overall lag time. This important observation indicates that the metabolites modify the lag phase period in aggregation kinetics, depending much upon the stage of protein aggregation they encounter in solution.

Since it is now established that protein aggregation remains the critical process behind the occurrence of notorious forms of protein aggregation diseases, some additional *in vivo* factors also direct the overall pathogenicity of the diseased state. In the last chapter of the thesis, we have used *in silico* methods to decipher potential novel inhibitors of Acetylcholinesterase (AChE) and Butyrylcholinesterase (BuChE) by drug repurposing strategy, which are vital enzymes identified as factors aggravating the pathophysiology of Alzheimer's Disease (AD). We performed molecular docking in critical domains of these two enzymes with some of the recently approved drugs by FDA for various other neurological and psychiatric diseases. Subsequently, we employed molecular dynamics (MD) simulations for further insights. We report Brexpiprazole and Deutetrabenazine as potential BuChE and AChE inhibitors by their high binding affinity and capacity to interact with critical residues of the oxyanion hole and active catalytic site (CAS), indicating a possible dual mechanism of enzyme inhibition. The results are encouraging for other studies *in vivo* as potential enzyme inhibitors for AD treatment.

The results from this thesis open up some critical areas for a better understanding of protein aggregation, especially in correlation with loss in the homeostasis of intracellular metabolites. The work depicted here also gives us insights into potential mechanisms of small ligands, how they could act as protein aggregation modifiers, and areas where more useful therapeutics could be developed.