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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

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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.

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

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Debanjan Kundu
July 2022

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List of Abbreviations

AchE	Acetylcholinesterase
AD	Alzheimer's Disease
ADME	Absorption Distribution Metabolism Excretion
AFM	Atomic Force Microscopy
ANS	8-Anilinonaphthalene-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
Rg	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

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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 β-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 While 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.