Molecular basis of amyloid- β chaperone activity of lipocalin-type prostaglandin D synthase (L-PGDS)

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**MOLECULAR BASIS OF AMYLOID- β CHAPERONE ACTIVITY OF
LIPOCALIN-TYPE PROSTAGLANDIN D SYNTHASE (L-PGDS)**

BHUVANESWARI KANNAIAN (G1503075A)

SCHOOL OF BIOLOGICAL SCIENCES

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LIPOCALIN-TYPE PROSTAGLANDIN D SYNTHASE (L-PGDS)**

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SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological University

in partial fulfillment of the requirement for the degree of

Doctor of Philosophy

2019

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AUTHORSHIP ATTRIBUTION STATEMENT

This thesis contains materials from 2 papers published in the following peer-reviewed journals in which I am listed as an author.

Chapter 3 and 4 are published as **Kannaian, B.**, Sharma, B., Phillips, M., Chowdhury, A., Manimekalai, M. S. S., Adav, S. S., Ng, J. T. Y., Kumar, A., Lim, S., Mu, Y., Sze, S. K., Gruber, G., Pervushin, K. Abundant neuroprotective chaperone Lipocalin-type prostaglandin D synthase (L-PGDS) disassembles the Amyloid-beta fibrils. Scientific reports, 9 (1), 12579 (2019). DOI: 10.1038/s41598-019-48819-5.

The contributions of the co-authors are as follows:

Bhuvaneswari Kannaian prepared samples for all the experiments and performed all biophysical and NMR experiments and wrote the manuscript. Bhargy Sharma prepared conjugates and Margaret Phillips captured TEM images for conjugates. Anup chowdury helped in preliminary biophysical studies. Malathy S.S. Manimekalai performed SAXS experiments with Bhuvaneswari Kannaian and analyzed the data. Sunil S.Adav and Bhuvaneswari Kannaian prepared samples for proteomics and analyzed the data. Justin T.Y. Ng performed the molecular dynamic simulations. Ambrish Kumar and Sierin Lim provided ferritin samples. Konstantin Pervushin devised the experimental plan and supervised the study. Bhuvaneswari Kannaian, Bhargy Sharma, Margaret Phillips, Malathy S.S. Manimekalai, Gerhard Gruber, Sunil S. Adav, Siu K. Sze, Justin T.Y. Ng, Yuguang Mu and Konstantin Pervushin wrote and edited the manuscript. All authors reviewed, revised and approved the manuscript.

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The contributions of the co-authors are as follows:

Margaret Phillips performed and analyzed data from UV/vis, NMR and Peroxidase assay and wrote the manuscript. Bhuvaneswari Kannaian prepared L-PGDS protein sample and performed and analyzed the ThT assay. Justin Ng Tze Yang and Mu Yuguang performed the heme docking and MD simulation. Ralf Kather and Jeffrey R. Harmer performed and analyzed the EPR data. Konstantin Pervushin supervised the project. All authors contributed towards editing the manuscript.

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ABBREVIATIONS

AD	Alzheimer's disease
Aβ40	Amyloid beta 1-40
Aβ (25-35)	Amyloid beta (25-35)
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
ACG	Automatic gain control
AL	Light chain amyloidosis
ALS	Amyotrophic lateral sclerosis
AICD	APP intracellular domain
BBB	Blood brain barrier
BMRB	Biological Magnetic Resonance Data Bank
BR	Bilirubin
CTFα	C-terminal fragments α
CTFβ	C-terminal fragments β
CSP	Chemical shift perturbation
CpSRP43	Chloroplast signal recognition particle
DNA	Deoxy ribonucleic acid
DTT	1,4-Dithiothreitol
DLS	Dynamic light scattering
DSS	4, 4-dimethyl-4-silapentane-1-sulfonic acid
EOAD	Early-onset Alzheimer's disease

<i>E.coli</i>	<i>Escherichia coli</i>
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
EDC	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide
FA	Formic acid
FAD	Familial Alzheimer's disease
FAS-1	Fascin like domain-1
FPLC	Fast protein liquid chromatography
FTLD	Frontotemporal lobar degeneration
FUS	Fused in sarcoma
GSH	Glutathione
L-PGDS	Lipocalin- type prostaglandin D synthase
HD	Huntingtin's disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HTT	Huntingtin gene
HFIP	1,1,1,3,3,3-Hexafluoro-2-propanol
HPLC	High-performance liquid chromatography
H-PGDS	Hematopoietic - prostaglandin D synthase
His-tag	Histidine tag
Hsp	Heat shock proteins
HSQC	Heteronuclear single quantum coherence spectroscopy
Hsc	Heat shock cognate
HCD	Higher-energy collisional dissociation

IDE	Insulin degrading enzyme
IPTG	Isopropyl β -D-1-thiogalactopyranoside
IMAC	Immobilized metal affinity chromatography
ITC	Isothermal titration calorimetry
IC50	The half maximal inhibitory concentration
KDa	Kilodalton
LB	Lewy bodies
LOAD	Late-onset Alzheimer's disease
LC-MS	Liquid chromatography–mass spectrometry
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization-Time of flight
MD	Molecular dynamics
MOX HCl	Methoxamine hydrochloride
Ni-NTA	Nickel–Nitriloacetic acid
NMR	Nuclear Magnetic resonance spectroscopy
NFT	Neurofibrillary tangles
NSAIDs	Non-steroidal anti-inflammatory drugs
PSEN1	Presenilin 1
PSEN2	Presenilin 2
PD	Parkinson's disease
PD	Proteome discoverer
PDB	Protein data bank
PGH2	Prostaglandin H2
PGD2	Prostaglandin D2

PQC	Protein quality control
RNA	Ribonucleic acid
RBP	RNA binding protein
ROS	Reactive oxygen species
R_g	Radius of gyration
SAXS	Small angle X-ray scattering
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SP	Senile plaques
SPR	Surface plasmon resonance
SOD	Superoxide dismutase
Sulfo-NHS	N-hydroxysulfosuccinimide
TB	Terrific broth
TCEP	Tris (2-carboxyethyl) phosphine hydrochloride
TFA	Trifluoroacetic acid
TEM	Transmission electron microscopy
TDP-43	TAR DNA binding protein-43
TRiC	TCP-1 ring complex
ThT	Thioflavin T
TGFBβIp	Transforming growth factor β induced protein
UPS	Ubiquitin proteasome system
UV-vis	Ultraviolet-visible
WT	Wild type

ABSTRACT

Misfolding and aggregation of specific proteins are involved in the neurodegenerative proteinopathies like Alzheimer's disease (AD), Parkinson's disease (PD) and Amyotrophic lateral sclerosis (ALS), etc. AD is an irreversible, progressive neurodegenerative disease which causes memory loss and detrimental effects in speaking, mood, and cognitive skills. The amyloid β peptides undergo a conformational change from their soluble monomeric forms to insoluble aggregates, which is believed to be the crucial pathogenesis associated with Alzheimer's disease. Molecular chaperones are the nanomachinery which primarily aids in protein folding and refolding of misfolded or damaged proteins. Lipocalin-type prostaglandin D synthase (L-PGDS) is found to have chaperone activity in addition to the well-established enzymatic and lipophilic ligand carrier activities.

Here, we establish the role of L-PGDS as a chaperone by inhibiting the A β aggregation and by disaggregating the preformed fibrils. The protective role of L-PGDS as a chaperone was determined for A β 40 and A β (25-35) using Thioflavin T assay and fluorescence microscopy. L-PGDS inhibits the A β 40 aggregation by targeting the primary and secondary nucleation. C65A mutant also exhibited inhibitory activity against A β 40 and A β (25-35) aggregation indicating that C65 is not the only critical residue for the chaperone activity of L-PGDS. Interaction between L-PGDS and the monomeric A β 40 was established using Nuclear magnetic resonance (NMR) spectroscopy and Small-angle X-ray scattering (SAXS) techniques. HSQC titration of L-PGDS and monomeric A β 40 identified the possible binding interface. The results revealed that the C-terminus of A β 40 is mainly involved in the interaction with L-PGDS. SAXS results confirm that L-PGDS is rigid and

remains as a monomer in solution and does not aggregate even at a concentration of 4mg/ml. The L-PGDS-A β 40 complex showed a $\sim 1\text{\AA}$ increase in the radius of gyration (R_g) compared to the Apo form, and the shape shows an additional domain occupied by the N-terminus of A β 40. A representative model for L-PGDS-A β 40 complex generated by classical molecular dynamics simulations showed that the N-terminal residues (1-16) of A β 40 are not in contact with L-PGDS and are extended which is in agreement with the SAXS model. Moreover, the contact map generated from the MD model showed several residues that were identified by the NMR titration to be in the interaction site. Thus, our MD model is in agreement with the experimental NMR data and SAXS model.

In addition to the protective role of molecular chaperones, they are also found to be involved in the disaggregation of preformed fibrils. In this context, L-PGDS has also performed well for dismantling the preformed fibrils of A β 40 and A β (25-35) as monitored by Thioflavin T assay and Transmission electron microscopy (TEM). Since L-PGDS showed chaperone activity for dismantling the fibrils, L-PGDS was used to dissolve the protein aggregates collected from the brain tissue of Alzheimer's disease patients. The insoluble protein aggregates were treated with L-PGDS, Formic acid, and Hexafluoroisopropanol. Proteomic analysis of L-PGDS treated sample identified 187 proteins, which include several crucial proteins that are commonly found in the AD brain samples. L-PGDS is a highly abundant, ubiquitously expressed, small chaperone protein which inhibits the A β aggregation and disintegrates the preformed fibrils of A β without ATP consumption. On the whole, both the protective role and the disaggregase role of L-PGDS as a chaperone is discussed in this study thereby elucidating the possible mechanism of action.

ORGANIZATION OF THIS THESIS

This thesis consists of five chapters in total. The first chapter introduces the concept of protein misfolding and aggregation in both neurological and non-neurological amyloid diseases. The mechanism and importance of molecular chaperones in such conditions have been discussed. The overview of AD includes the critical amyloid cascade hypothesis and APP processing. A brief introduction on the chaperone protein, L-PGDS is also presented in this chapter.

The second chapter of this thesis describes all the experimental methods and materials used in this study. Thioflavin T assay (ThT), microscopy techniques, Small-angle X-ray scattering (SAXS), Nuclear magnetic resonance spectroscopy (NMR), High-performance liquid chromatography (HPLC), Dynamic light scattering (DLS), and proteomics have been used to investigate the inhibitory and disaggregase activity of L-PGDS.

The third chapter displays the results regarding the protective role of L-PGDS as a chaperone for amyloid aggregation. This chapter includes the optimization of protein expression and purification. NMR, SAXS, and molecular dynamics simulation confirms the interaction between L-PGDS and monomeric A β 40 and reveals a model for L-PGDS-A β 40 complex. ThT assay and fluorescence images exhibit the inhibitory role of L-PGDS for A β aggregation.

The fourth chapter explains the results obtained for the disaggregase activity of L-PGDS for amyloid peptides. ThT assay, Transmission electron microscopy (TEM) images, HPLC, and DLS results exhibit that L-PGDS efficiently dismantles the preformed fibrils of A β . Proteomic analysis of the L-PGDS treated insoluble protein aggregates of human AD

brain discussed in this chapter displays the ability of L-PGDS to solubilize several crucial proteins aggregated in AD brain.

The final chapter provides the overall conclusions made from the study explained in this thesis. Possible future directions for this project are also described in this chapter.

CHAPTER 1

INTRODUCTION

1.1. Protein folding and misfolding

After protein translation, the majority of the nascent polypeptide chains must fold into a specific three-dimensional structure, undertaking the intricate tertiary and quaternary folds to perform their biological functions. However, approximately 20-30% of the eukaryotic proteins are intrinsically unstructured and acquire a fold upon binding to its interacting partners [1]. Anfinsen's theory on protein folding states that the amino acids in the protein carry all the information needed for protein folding. He also claimed that the physicochemical properties of the amino acids direct the protein to fold into its lowest energy conformation, which is thermodynamically stable.

Protein folding happens rapidly because the amino acids interact locally by weak, non-covalent bonds, thereby restricting the conformational space of a protein and thus pushing it to enter into an energy funnel. Hydrophobic interactions play a critical role in protein folding by burying the hydrophobic amino acids to the core of the protein, whereas the hydrophilic residues are exposed to the solvent. Though most of the proteins fold into their correct native conformation, some proteins have bifurcated pathways, with different energy minimized conformations, instead of a single pathway [1-3]. As a result of the ruggedness in the free energy surface navigated during folding, few kinetically trapped and misfolded structures accumulate transiently before reaching the favorable downhill path. Here, chaperones come into play in preventing the intermolecular contacts, which results in

the formation of oligomers, amorphous aggregates, β -sheet fibrils, and thus promote proper native folding (Fig. 1.1).

Figure 1.1: Energy landscape for protein folding

The unfolded protein entering the energy landscape either attains the native conformation through intramolecular interactions or misfolded structures (amorphous aggregates, oligomers, and amyloid fibrils) by intermolecular interactions. Chaperones assist in proper folding to the native structure and also prevent aggregation. Figure adapted from [1].

Protein folding occurs either immediately while the new polypeptide is still attached to the ribosome or in the endoplasmic reticulum (ER) once the newly formed polypeptide chain is transported from the ribosome. If required, molecular chaperones and other folding catalysts aid in the proper folding of the nascent polypeptide chain. Adequately folded

proteins are transported to the Golgi apparatus, whereas the misfolded proteins are exported to the cytosol and then degraded by the Ubiquitin-proteasome system (UPS) (Fig. 1.2) [3].

Figure 1.2: Schematic representation of protein folding and misfolding.

The newly translated protein transported into the ER lumen undergoes folding and is then transported to Golgi through vesicle formation. Misfolded proteins are transported out for Ubiquitin proteasome system degradation. Figure adapted from [3].

Although most of the proteins undergo folding spontaneously, some proteins are less capable of folding and are prone to misfolding and aggregation. Especially, larger proteins with multi-domains, expose the hydrophobic residues to solvent while folding, bringing them to off-pathway interactions, which might result in aggregation [2]. The highly crowded cell environment with the total concentration of cytosolic proteins reaching 300-400 g/L makes the protein folding even more challenging *in vivo* [1]. In some cases, mutations might also result in the formation of misfolded proteins that accumulate [4]. It is

critically important to maintain proteome integrity and protein homeostasis (Proteostasis). To maintain proteostasis, the aggregates need to be degraded by Protein quality control (PQC) where molecular chaperones play an important role. The unique cell structure of the neurons make PQC complicated in it compared to other cells [5]. Disturbance in proteostasis can lead to several diseases such as peripheral amyloidosis, cystic fibrosis, cancer, type 2 diabetes, lysosomal storage disease and neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Amyotrophic lateral sclerosis (ALS), Frontotemporal lobar degeneration (FTLD) [2].

1.2. Amyloidosis

Amyloidosis is a collective term for the group of diseases in which the proteins aggregate to form extracellular, β -pleated amyloid fibrils in tissues ultimately leading to the tissue damage or even failure of the organ [6]. Different proteins are involved in each kind of amyloid disease, and 27 different proteins have been identified till date as the cause of amyloid diseases. Although the primary structure of these proteins is different, they all can form β -pleated, insoluble amyloid fibrils in common [7]. Amyloidosis is classified into two distinct categories, such as systemic and localized amyloidosis based on the location of amyloid deposition. In Systemic amyloidosis, the amyloid protein deposits are found in several tissues and organs of the body. Localized amyloidosis is where the amyloid protein is deposited only in a particular type of tissue or organ[8]. Accumulation of these toxic amyloid deposits can lead to neurodegenerative diseases as well as non-neurological diseases.

1.3. Protein misfolding in Non-Neurological diseases

Misfolded protein aggregates accumulate in the peripheral tissues and lead to conditions such as corneal dystrophy, Sick cell anemia, type 2 diabetes, cystic fibrosis, inherited cataracts, some forms of atherosclerosis, and systemic amyloidosis. The protein associated with each disease is different (Table 1.1). However, all these diseases share the common mechanism of aggregation, which leads to the formation of sticky amyloid fibrils. There are several possible causes for aggregation. They include hyperphosphorylation of proteins, self-catalytic conformational conversion and mutations that cause protein instability. Deficiency in proteasomes, the cellular machinery to degrade aging proteins, may result in increased concentration of these amyloidogenic proteins, which leads to aggregation. Improper autophagy also causes amyloid aggregation [3].

Disease	Aggregating protein/peptide	Distribution
Systemic amyloidosis	Immunoglobulin light chain	Systemic
Sickle cell anemia	Hemoglobin	Systemic
Type 2 diabetes	Islet amyloid polypeptide (amylin)	Localized
Cataract	Crystallins	Localized
Corneal dystrophy	Transforming growth factor β induced	Localized
Hemodialysis-associated amyloidosis	β 2- microglobulin	Systemic
Retinitis pigmentosa	Rhodopsin	Localized

Reactive amyloidosis (or) Amyloid A amyloidosis	Serum amyloid A	Systemic
Mallory bodies	Keratin intermediate filament proteins	Localized
Cystic fibrosis	Cystic fibrosis transmembrane conductance regulator (CFTR) protein	Systemic

Table 1.1: List of Proteins/peptides that undergo aggregation in various non-neurological amyloid diseases and their distribution in the body.

1.3.1. Type 2 diabetes

Type 2 diabetes is a non-insulin dependent, widespread chronic metabolic disease characterized by increased blood glucose levels. In 2014, the total number of people living with diabetes was estimated to be approximately 422 million. Type 2 diabetes is mainly characterized by insulin resistance, the body's inability to use up insulin [9]. Through the early phases of type 2 diabetes, the pancreatic β cells produce more insulin to overcome the systemic insulin resistance. However, in the later stages, the β cells lose the capacity to produce insulin which leads to hyperglycemia.

Amylin or IAPP (Islet amyloid polypeptide) aggregation to amyloid fibrils is a hallmark pathology associated with type 2 diabetes [10]. Together with insulin, pancreatic β cells secrete amylin, a 37 amino acid long amyloidogenic peptide hormone, in response to high levels of glucose. Amylin is secreted in both diabetic and healthy individuals. However, amylin protein plaques were found in the β cells of type 2 diabetic patients, which were absent in healthy controls. This peptide aggregates in a concentration-dependent

manner and leads to membrane disruption and cell toxicity, whereas the monomeric form is unstructured and non-toxic [11].

Like A β peptides, amylin plaques also have the characteristic β sheet structure and amylin binds to the same receptor as A β and gets degraded by the same protease, insulin degrading enzyme (IDE). While amylin is mainly produced and secreted by the β -cells in the pancreas, its expression occurs in other locations such as the gut and in the sensory nervous system. Amylin readily crosses the blood brain barrier (BBB) and mediates several activities which includes improving glucose metabolism, relaxing cerebrovascular structure, modulating inflammatory reaction and perhaps enhancing neural regeneration. Abundant A β in the AD brain could block or interfere with the binding of amylin to its receptor and hinder its functions [12]. Studies using animal models for AD demonstrate that amylin and its analog, pramlintide reduce the AD pathology in the brain and improve cognitive impairment in AD [13].

1.3.2. Systemic amyloidosis

Systemic amyloidosis is a type of amyloid disease where the amyloidogenic protein enters the blood circulation and eventually accumulates as amyloid fibrils in many target organs [7]. The widely prevalent form of systemic amyloidosis is light chain amyloidosis (AL). In AL amyloidosis, the plasma cells (fully differentiated B cells) proliferate abnormally in the bone marrow and secrete an excess of light chain (LC) into the bloodstream where the proteins misfold, aggregate and finally deposit the amyloid fibrils in various target organs leading to organ dysfunction, organ failure and death [14] (Fig. 1.3). Similar to other amyloid diseases, the amyloid fibrils formed by the light chain also

adopt the stable, intramolecular cross β -sheet structure that can be stained using ThioflavinT and Congo red dyes [15].

Figure 1.3: Representation of the molecular mechanism underlying AL amyloidosis.

The light chain produced by the plasma cells in the bone marrow misfolds and aggregate to form amyloid fibrils, which are eventually deposited in various target organs leading to organ dysfunction and death. Figure adapted and modified from [16].

1.3.3. Cataract

The lens is an integral part of the eye that focusses light into the retina. Maintaining the solubility of the crystallin proteins in the lens is crucial to maintain the transparency of the eye lens, which is essential for vision [17]. The highly concentrated crystallin proteins arrange in a well-ordered manner to retain the transparency of the lens. Disrupted arrangement of the crystallin proteins, because of aggregation or precipitation results in impaired lens light refraction and potential cataract formation and eventually, loss of vision [18]. The aggregates formed by the crystallin proteins can be amorphous, ordered amyloid fibrils, or a combination of both. α -Crystallin is the major lens protein that belongs to the

family of small heat shock proteins (sHsp). α -Crystallin, which is composed of two subunits α A and α B-crystallin, exhibits chaperone activity by preventing the aggregation of all the crystallin proteins, including itself [18]. Both α A-crystallin and α B-crystallin retain their chaperone activity to a significant degree even after the formation of amorphous aggregates and amyloid fibrils [19]. α B-crystallin is present extensively outside the lens, where it functions as a chaperone.

1.3.4. Corneal dystrophy

Corneal dystrophies are the group of progressive, bilateral, genetic disorders that occurs in the cornea, the transparent, avascular tissue. The five different layers of the cornea are the epithelium, Bowman's layer, the stroma, Descemet's membrane, and the endothelium with the stroma making up around 90% of the cornea's thickness. An equilibrium of cellular and molecular processes retains the transparency of the cornea, which is essential for normal, clear vision.

Accumulation of aggregated mutant proteins in the cornea affects the transparency and ultimately, the vision. Mutations in transforming growth factor beta-induced (TGFB1) gene is mainly associated with the stromal and Bowman's membrane corneal dystrophy [20-22]. Till date, 66 different mutations have been reported in the TGFB1 protein that alter the proteolytic processing of the mutant, which results in abnormal aggregation [23, 24].

Depending on the type of mutation, the protein deposits in the cornea could be amyloid fibrils, amorphous aggregates, or a combination of both [22]. Long lattice lines or amyloid fibrils are observed in lattice corneal dystrophy (LCD) (Fig. 1.4A), whereas amorphous protein aggregates are observed in granular corneal dystrophy I (GCD type 1)

(Fig. 1.4B). Both amyloid fibrils and amorphous aggregates are evident in GCD type 2 otherwise known as combined granular-lattice dystrophy (Fig. 1.4C) [25-27].

Figure 1.4: Types of Corneal dystrophy

(A) Lattice corneal dystrophy displays long lattices or amyloid fibrils (B) Granular corneal dystrophy 1 (GCD type 1) displays granular or amorphous protein aggregates (C) Combined granular-lattice dystrophy (GCD type 2) displays both granular aggregates and amyloid structures. Figure adapted and modified from [27].

Recent proteomics studies showed that the fragment 611–642 in the 4th fascilin-like domain-1 (FAS-1) domain of TGFBIp is enriched in the amyloid deposits of patients with LCD. Based on the amino acid substitution type and position, clinically related mutations in this fragment (M619K, N622K, N622H, G623R and H626R) lead to changes in the cytotoxicity, amyloid kinetics, and thermal stability of fibrils [20]. E⁶¹¹PVAEPDIMATNGVVHVITNVLQ⁶³³ peptide from the WT-TGFBIp and the mutants N622K, N622H, G623R were taken in our preliminary study to check the inhibitory activity of L-PGDS (data not shown). Among these, G623R is highly amyloidogenic and aggregates faster than the other peptides.

1.4. Protein misfolding in Neurodegenerative diseases

Neurodegeneration is a process in which the structure and function of the neurons get affected and resulting in neuronal cell death. Aggregation of misfolded proteins in the

brain is the common underlying mechanism in neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Amyotrophic lateral sclerosis (ALS) and Frontotemporal lobar degeneration (FTLD) [28]. The aggregated proteins might be deposited outside the cell (extracellular) or in the cytoplasm. Unlike the non-neurological amyloid diseases, protein deposition in neurodegenerative amyloid diseases is localized in the brain region. The major proteins/peptides involved in each disease, the location of aggregate deposition and the affected brain regions are tabulated in Table 1.2.

Disease	Proteins/peptides involved	Localization of aggregates	Brain region affected
Alzheimer's disease	Amyloid- β , tau	Extracellular and intracytoplasmic	Cerebral cortex, hippocampus
Parkinson's disease	α -synuclein, DJ-1, parkin, PS1 and PS2, PINK1, HTRA2, LRRK2	Cytoplasmic	Substantia nigra, hypothalamus
Amyotrophic lateral sclerosis	SOD1, TDP-43, FUS	Cytoplasmic	Motor cortex, brain stem
Corticobasal degeneration	Tau	Neuronal	Cerebral cortex, basal ganglia
Familial amyloid polyneuropathy	Transthyretin	Extracellular	Peripheral nervous system

Huntington's disease	Huntingtin	Nuclear	The striatum, cerebral cortex
Frontotemporal lobar degeneration	TDP-43, tau, FUS	Cytoplasmic	Frontal and temporal lobes
Spinocerebral ataxia	Ataxin-3	Neuronal	Cerebellum and spinocerebellar
Prion diseases	Prion	Extracellular	Brain (grey matter), peripheral nervous system

Table 1.2: A comprehensive list of few neurodegenerative diseases and proteins involved in the pathology with the localization of aggregate deposition and the affected brain region.

Ageing is the primary risk factor associated with neurodegenerative diseases. Ageing or mutation disturbs the delicate balance between protein synthesis, folding, and degradation of misfolded proteins and results in the accumulation of aggregated proteins [3].

1.4.1. Parkinson's disease (PD)

Parkinson's disease is a highly devastating neurodegenerative disorder characterized by the progressive loss of neuronal cells that produce dopamine in the Substantia nigra [29]. Intraneuronal accumulation of α -synuclein fibrils that form the Lewy bodies (LB) is a pathological hallmark associated with PD [30]. α -synuclein protein is encoded by the *SNCA* gene. A30P and A53T mutants of α -synuclein contribute to familial PD [29]. The common symptoms of PD include tremors, slowness of movement

(bradykinesia), postural instability, and rigidity. Apart from these characteristic motor symptoms, the PD patients might also have experienced some non-motor symptoms, like problems in sleep, cognitive dysfunction, or autonomic dysfunction several years before the diagnosis. Although the average onset of this progressive disorder is over 60 years, the disease process might have started decades ahead. Almost 70% of the dopaminergic neurons would have been affected by the time the symptoms are observed. Administration of dopamine to compensate the loss is one of the current treatment options carried out for PD [31].

1.4.2. Huntington's disease (HD)

The repeat of polyglutamine (poly Q) coding trinucleotide (CAG) in the first exon of the Huntingtin (HTT) gene on chromosome 4 is the prime cause of the progressive neurodegenerative disorder, Huntington's disease. The polyQ repeats in the N-terminus of the huntingtin protein (HttQn) contribute to the aggregation resulting in the formation of β -sheet amyloid fibrils in the neuronal intranuclear inclusions [32, 33]. In the cell, the mutant huntingtin leads to neuronal dysfunction and death through several mechanisms, such as disruption of protein homeostasis, mitochondrial function, and transcription [34]. In the HD patients, the number of CAG repeats increases from an average of 16-20 repeats to >35 repeats. The autosomal dominant inherited neurodegenerative disease, HD, is a rare condition that affects nearly 5–10 people per 100,000 [35]. The common symptoms of HD include dementia, depression, loss of self-awareness, anxiety, and motor and cognitive impairments. The current treatment focuses on suppressing chorea, the involuntary and irregular movements of the limbs. However, currently, there are no disease-modifying treatments available.

1.4.3. Frontotemporal lobar degeneration (FTLD)

Frontotemporal lobar degeneration (FTLD), formerly called as Pick's disease is a distressing age-related neurodegenerative disorder that predominantly affects the social behaviors, cognition, personality, language and causes other clinical abnormalities [36]. FTLD is recently recognized as the usual form of early-onset dementia before the age of 65 [37]. Many pathological, clinical, and molecular pieces of evidence confirm the close relationship between FTLD and amyotrophic lateral sclerosis (ALS). The clinical FTLD shows heterogeneous neuropathology, with the common feature being the frontal and temporal lobe degeneration [38, 39]. The inclusion bodies that are either TAR DNA-binding protein 43 kDa (TDP-43)-positive (FTLD-TDP) or tau-positive (FTLD-TAU) are the major pathologies associated with FTLD (Fig. 1.5) [40].

The ubiquitously expressed RNA- binding protein, TDP-43, mostly resides in the nucleus. However, in the diseased neurons, the expression of TDP-43 is significantly increased and localized in the cytosol suggesting that the loss of function of TDP-43 in the nucleus contributes to the initiation or progression of the disease. Genetic mutations in TDP-43 results in some familial and sporadic ALS further emphasizing the perception that FTLD and ALS are closely related conditions referred to as TDP-43 proteinopathies [42]. Mutations in the progranulin, which is located on chromosome 17, is another cause of FTLD without tau pathology. The precise mechanism of FTLD instigated by a deficiency in progranulin remains elusive. However, tau-negative and ubiquitin-positive neuronal inclusions containing TDP-43 and its fragments are pathological hallmarks of FTLD [38, 39].

Figure 1.5: Pathology associated with Frontotemporal lobar degeneration.

(A) Neuronal loss, gliosis, and microvacuolation are observed in the superficial cortical layers
 (B) Hyperphosphorylated tau-positive cytoplasmic inclusions inside the neurons called Pick bodies (arrow head) and branched astroglial processes. (C) Cytoplasmic TDP-43 inclusions in dentate gyrus granule cells. Figure adapted and modified from [41].

1.4.4. Amyotrophic lateral sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS), otherwise known as Lou Gehrig's disease, is a deadly motor neuron disease, characterized by the loss of neurons in the brain and the spinal cord [29]. Some of the primary symptoms of ALS include difficulty speaking (dysarthria), difficulty breathing (dyspnea), difficulty swallowing (dysphagia), muscle atrophy, and muscle spasticity [43, 44]. The two major types of ALS include the familial, which is hereditary and the sporadic, which is non-hereditary [45]. ALS is a rare, genetically

heterogeneous, neurodegenerative disease affecting 1 in 100,000 population. Sporadic ALS is the most common form, whereas the familial form accounts for only 5–10% of the total cases [44].

TDP-43 was identified as a critical component of the insoluble ubiquitinated inclusions of both sporadic ALS and FTLD [39]. Shortly after that, mutations in fused in sarcoma (FUS), another RNA binding protein (RBP), were recognized in some patients with familial ALS and sporadic ALS [46]. Deposition of misfolded copper/zinc Superoxide dismutase (SOD) in the neural tissue is another pathology associated with ALS [47]. More than 100 different SOD mutants are associated with familial ALS [48]. A subset of patients with ALS also develops frontal lobe dysfunction or frontotemporal lobar degeneration (FTLD), indicating that other non-motor cortical structures may also be involved.

1.5. A brief overview of Alzheimer's disease (AD)

The German Psychiatrist, Dr. Alois Alzheimer first described the condition called Alzheimer's disease (AD), an aggressive form of dementia, in 1907 [49]. AD is a chronic, irreversible, progressive disorder associated with synaptic dysfunction and neurodegeneration. The most critical risk factor associated with AD is aging. However, it can also occur in middle age, which is the early-onset AD [50]. AD is the massive cause of dementia accounting for 60–70% of cases. Loss of memory, disorientation, mood swings, complications with language, inability to perform day to day activities, loss of motivation, and psychological and behavioral issues are some of the symptoms associated with AD [51]. Such symptoms arise due to the degeneration of neurons in the brain parts responsible for learning, thinking, and cognitive function (memory). As the disease develops, the nerve cells in other brain regions are also affected. AD patients are bed-ridden and require day-

and-night care during the final stages of the disease. AD is believed to start 20 years before the signs and symptoms show up, with minor changes in the brain that are unnoticeable by the patients themselves [52].

The prevalence of dementia is expected to increase in the future in low and middle-income countries where the incidence of diabetes, hypertension, and cardiovascular disease is growing [53]. Till date, around 5.8 million people are affected by AD in the United States and it is the 6th major cause of death [52]. If the therapeutics remain inadequate, the number is expected to multiply by three times by 2050. Extracellular deposits of amyloid- β in the senile plaques (SP) and the intracellular neurofibrillary tangles (NFTs) formed by hyperphosphorylated tau are the two major pathological hallmarks associated with AD [54] (Fig. 1.6). The monomeric form of A β peptides is neuroprotective, whereas they form soluble oligomers or mature fibrils to become neurotoxic [55]. Monomeric amyloid peptides aggregate together to form low molecular oligomers proceeding to soluble higher-order oligomers then to spherical oligomers which then form protofibrils and finally the insoluble matured fibrils. It has been described in several studies that the spherical oligomers and protofibrils exhibit more toxicity compared to the mature fibrils [56].

The secondary structure of A β is a critical feature determining the neurotoxicity as the monomeric random coil adopts β -sheet fibril structure upon aggregation, which is highly toxic to the cells [58]. The structural transition of amyloid- β from random coil to β -sheet includes an intermediate α -helical structure. The A β plaques interfere with the communication between neurons at synapses leading to the neuronal death, whereas the neurofibrillary tau tangles obstruct the transportation of nutrients and other vital molecules

inside the neurons. Once the growth of A β plaques reaches a plateau, the tau tangles expand all around the brain [59].

Figure 1.6: Changes in the brain and the two main pathological hallmarks associated with AD

The cerebral cortex and the Hippocampus region are tremendously shrunk, and the ventricles are severely enlarged in case of AD compared to the healthy brain. The senile plaques are made up of A β fibrils whereas the neurofibrillary tangles are comprised of the hyperphosphorylated tau. Figure adapted and modified from [57].

Inflammation and atrophy are the other main changes in the AD brain. The immune cells in the brain, microglia are activated by the toxic A β and tau proteins. Microglia attempt to remove the toxic proteins in addition to the removal of debris from the dead cells. When the microglia are unable to clear all the debris, chronic inflammation is supposed to develop. The loss of neurons results in atrophy (shrinkage of the brain). Ultimately, the brain's ability to metabolize glucose gets reduced, affecting the normal function [52].

Like other neurodegenerative diseases, AD is genetically categorized into two forms, namely familial and sporadic. Familial Alzheimer's disease (FAD) is the early-onset AD (EOAD), which is extensively determined by genetic factors. The typical onset

age ranges between 30 to 65 years, accounting for nearly 1% to 6% of the total AD cases. Although the familial form is clinically indistinguishable from the sporadic form, FAD is generally diagnosed in families where multiple persons are affected in more than one generation. Mutations in one of these genes: amyloid precursor protein (APP), presenilin 1 (PSEN1), presenilin 2 (PSEN2) causes the FAD. People who have inherited the mutant APP and PSEN1 will develop AD for sure, whereas PSEN2 mutation inherited people have 95% chance to develop AD [60].

Sporadic AD, the late-onset type is the most common form of AD affecting more than 90% of the total cases. Usually, the late-onset age is above 65 years. Both genetic and environmental factors contribute to the complex, sporadic form of the disease [61]. The existence of the $\epsilon 4$ allele in Apolipoprotein E (APOE) is the only established genetic risk factor for sporadic AD. However, above 20 genetic risk factors were identified by Genome-wide association studies that are implicated in cholesterol metabolism, inflammatory, and endosomal vesicle recycling pathways [62].

Oxidative stress in the aging brain is another significant pathology associated with AD. Oxidative stress occurs due to the imbalance in the redox state, resulting in the reactive oxygen species (ROS) generation or impaired antioxidant system [63]. Redox metals such as Copper, Iron, and Zinc are found accumulated in the AD plaques. A β peptides interact with Fe^{3+} and Cu^{2+} and reduce them to produce H_2O_2 and other partially ROS, which leads to lipid peroxidation and disruption of protein/DNA structure [64, 65]. The oxidized M35 residue present in the amyloid plaques was found to be involved in the redox reduction reaction of Cu^{2+} to generate ROS [66].

1.5.1. Proteolytic processing of APP

The type 1 integral protein, APP is the larger precursor of A β peptides and plays a crucial role in the pathogenesis of AD. APP is expressed in several tissues, particularly in the synapses of neurons and is implicated in the regulation of synaptic formation and repair, iron export, and anterograde neuronal transport. APP has a large extracellular glycosylated N-terminus, a membrane-spanning domain, and a short cytoplasmic C-terminus [67]. The larger precursor molecule, APP is cut by β -secretases and γ -secretases to yield A β peptides of 37-49 residues in length, which are associated with the amyloid cascade hypothesis of AD [68]. The two different proteolytic pathways of APP processing are amyloidogenic and non-amyloidogenic (Fig. 1.7).

In the non-amyloidogenic pathway, α -secretase cuts the APP first and releases the sAPP α from the cell surface resulting in the formation of an 83-amino-acid α -C-terminal APP fragment (CTF α). The sAPP α production surges in reaction to the electrical activity and muscarinic acetylcholine receptor activation, proposing that α -secretase cleavage of APP is enlarged by the neuronal activity [69]. In the amyloidogenic pathway, APP is first cleaved by the β -secretase to release the sAPP β and leaves the membrane-tethered β -C terminal fragments (CTF β) of 99 residues. The γ -secretase further cleaves the CTF α and CTF β intramembrane, liberating the P3 (3 kDa) and A β (4 kDa) peptides, respectively [70, 71].

The γ -secretase can cleave the 99-amino-acid C-terminal fragment (CTF β or C99) at multiple sites to generate 43, 45, 46, 48, 49 and 51 residues fragments which are further processed to the primary forms of A β , the A β 40, and the A β 42, in the endocytic compartments [72, 73]. The γ -secretase cleaves the C99 and frees an APP intracellular

domain (AICD) which can be translocated to the nucleus to regulate gene expression and apoptotic gene induction. Caspases further cleave the APP/C99 to generate the neurotoxic peptide C31 [74]. The abundance of β -site APP cleaving enzyme in neurons expedite the amyloidogenic pathway in the brain and damage the neuronal survival.

Figure 1.7: Proteolytic pathways of APP

Non-amyloidogenic and amyloidogenic pathways of APP processing. Sequential cleavage of APP by α -secretase and γ -secretase release the P3 and APP intracellular domain (AICD) in the non-amyloidogenic pathway whereas the cleavage of APP by β -secretase and γ -secretase results in the production of AICD and Aβ peptides, which aggregates to form fibrils in the amyloidogenic pathway. Figure adapted from [67].

1.5.1.1. Aβ isoforms of varying length

The consecutive cleavage of the C-terminal fragment β (CTFβ) by γ -secretase at multiple positions generates peptides of different lengths, the most common forms being Aβ40 and Aβ42. The additional Ile-Ala dipeptide in the C-terminus increases the

hydrophobicity and the aggregation propensity of A β 42 compared to the A β 40 isoform [75]. Although the levels of A β 40 is higher than A β 42 in the cerebrospinal fluid (9:1(A β 40: A β 42)), A β 42 is highly deposited in the plaques indicating that A β 42 is more pathogenic than the A β 40 peptide. Both A β 40 and A β 42 spontaneously form fibrils, however A β 42 forms fibrils much faster than A β 40 [76]. The ratio of A β 42 : A β 40 is higher in some FAD, which probably causes the early-onset of the disease due to enhanced toxicity from A β 42 and less protection by A β 40 [77].

Notably, A β 42 is more neurotoxic and is significantly essential for the plaque formation, whereas A β 40 seems to be the most abundant peptide deposited in the cerebral vessels. Besides the total A β concentration, the relative level of A β 42 is a critical factor determining the rate of amyloidogenesis. The C-terminal length appears to be crucial for the neurotoxicity and oligomerization of the A β peptide [78]. However, when the freshly solubilized A β 40 or A β 42 was injected into rat brains, A β 40 formed fibril structures that are similar to the fibrils of AD while the A β 42 formed nonfibrillar amorphous material [79]. The rapid aggregation propensity of A β 42 makes it more challenging to work in experiments like NMR spectroscopy, which requires longer acquisition time. Besides A β 42, A β 40 is the other peptide that is neurotoxic and clinically implicated in AD. Thus, A β 40 has been chosen as the main peptide in this study.

The A β (25–35) peptide (G²⁵SNKGAIIGLM³⁵) is the shortest A β fragment processed by the brain proteases *in vivo* [80]. Thus, this peptide retains the toxicity of the full-length peptide and displays significant levels of molecular aggregation although the metal-binding sites are missing. In agreement with this finding, A β (25–35) peptide has been proposed to represent the biologically active region of A β [81]. A β (25-35), the fast

aggregating peptide, has shown increased oxidative stress and neuronal damage in rats [82-84]. Recent studies have shown that the monomeric and oligomeric form of A β (25–35) are also capable of crossing the cellular plasmatic membranes inducing intracellular mechanisms of toxicity [85, 86]. Considering these facts, A β (25–35) is chosen as the second peptide of interest in this study to establish the chaperone effect of L-PGDS.

1.5.1.2. A β aggregation

The A β peptides thus formed from the APP undergoes nucleation-dependent aggregation to form amyloid fibrils. There are two phases in the process of fibril formation. First is the lag phase (or) nucleation phase, the slow, rate-limiting step in the aggregation process that is thermodynamically unfavorable. Primary nucleation occurs in the lag phase and involves only monomers without any contributions from already formed aggregates. In this process, monomers associate into small aggregates that can reach sizes at which they grow faster by monomer addition compared to the rate by which they dissociate. Primary nucleation may occur in bulk solution (homogeneous nucleation) or at a surface (heterogeneous nucleation).

Second is the growth phase (or) elongation phase in which more monomers are further added to the existing nucleus and drive the aggregation process rapidly to form amyloid fibrils. This is a rapid phase and is thermodynamically favorable. Soon after the elongation phase, the secondary nucleation events occur [87]. Secondary nucleation is the process whereby transient binding to a fibril surface accelerates aggregation by promoting the formation of nuclei on the fibril surface. In the presence of a molecular chaperone, the kinetics of aggregation may be altered as a result of the interactions between the molecular chaperone and one or more of the protein species present in the system, which can lead to fundamental changes in the microscopic events such as primary nucleation, elongation and

secondary nucleation in the aggregation process. Different chaperone proteins target various microscopic events to control the aggregation (Fig. 1.8.A, B).

Figure 1.8: Mechanism of aggregation and chaperone interaction at different steps

(A) A schematic representation of the microscopic events occurs in the aggregation of amyloid peptides. The chaperone proteins can interact at any stage of aggregation. Chaperone mediated inhibition of various microscopic events in aggregation such as (B) primary nucleation, (C) elongation, and (D) secondary nucleation. Figure adapted from [88].

1.5.1.2.1. Thioflavin T assay for monitoring aggregation

Thioflavin T (ThT) assay is the most commonly used technique for studying the amyloid fibrillation. ThT forms micelles in aqueous buffers at usual working concentrations (10-20 μ M). The benzothiazole dye, ThT, binds along the length of the amyloid fibrils and exhibit enhanced fluorescence at 482nm when excited at 450nm (Fig. 1.9). The central carbon-carbon bond connecting the benzothiazole and amino benzene rings in ThT undergoes rotational immobilization to enhance the fluorescence [89].

Figure 1.9: ThT binding to fibrils

A schematic representation of ThT binding to the length of the fibrils and the structure of ThT.

1.5.2. Amyloid cascade hypothesis

The most established theory of AD, the amyloid cascade hypothesis was proposed by Hardy and Allsop in 1991 (Fig. 1.10). It suggests that the pathological buildup of A β peptides, generated by the proteolytic processing of APP, is the prime pathological process in AD driven by the imbalance between the A β production and clearance. The development of neurofibrillary tangles and the resultant neuronal dysfunction and neurodegeneration, possibly facilitated by inflammation, are believed to be the downstream processes [90].

Genetics strongly supports the crucial role of A β . Mutations in the three genes coding Amyloid precursor protein (APP), Presenilin 1 and 2 lead to increased accumulation of A β peptides, which are believed to be pathogenic in AD conditions [54]. Under normal circumstances, almost 90% APP is cleaved by α - and γ -secretases leading to the non-amyloidogenic pathway [91]. A missense mutation in APP (A673T) leads to a lifetime decline in APP cleavage by β -secretase granting a minimized clinical risk of AD [92]. ApoE is involved in the clearance of amyloid, in sporadic AD.

Figure 1.10: Sequential pathological events resulting in AD projected by the amyloid cascade hypothesis.

The blue arrow specifies that the Aβ oligomers might directly induce the neuritic, synaptic damage, and hyperphosphorylation of tau besides the activation of inflammatory cascades. Figure adapted from [93].

People with Down syndrome have an increased chance of developing AD. Down syndrome patients carry three copies of chromosome 21 instead of two. The gene encoding APP, the protein from where A β fragments are produced, is located on Chromosome 21. Thus, the extra copy of chromosome 21 might increase A β production in the brain contributing to AD [52]. While the A β fibrils within the senile plaques were believed to be crucial for the AD development; it is now proposed that the soluble A β oligomers might be the significant pathological forms. These toxic oligomers or amyloid-derived diffusible ligands (ADDLs) interact with the neurons through a surface receptor and alter the structure of the synapse, thereby disturbing the neuronal communication [94].

When the A β oligomers, extracted from the AD brain, were applied to the neurons, *in vitro*, the long-term potentiation is inhibited, leading to synaptic dysfunction, dendritic spine damage, and death of neurons [95, 96]. Plaques act as a reservoir from which the oligomeric A β diffuse, or might even act as a protective mechanism sequestering the toxic A β series until a physiological saturation point is reached [93]. Unavailability of reliable biomarkers for early diagnosis and lack of efficient preventive strategies and treatments remain as the significant challenges in AD [97, 98]. Thus, more details on the AD pathogenesis is necessary for the progress of enhanced diagnostic and therapeutic procedures.

1.5.3. A β toxicity

The toxicity of A β are facilitated through several mechanisms such as mitochondrial diffusion, oxidative stress, alterations in membrane permeability, inflammation and synaptic dysfunction through its interaction with some neurotransmitter's receptors. A β peptides interact with the redox metals such as copper, iron and zinc resulting in the

formation of Reactive oxygen species (ROS), thus leading to the oxidative stress [99]. A β peptides also exhibit toxicity by interacting with the membranes. It is well established that the cellular exposure to A β results in increased levels of intracellular calcium, which is closely associated with the processes of damage and cell death. In the presence of calcium, A β can create transition pores into the mitochondrial membrane through which cytochrome C can be released and, therefore, proapoptotic signaling pathways can be started resulting in mitochondrial dysfunction.

High levels of cholesterol also lead to increased A β synthesis and facilitate its interaction with the membrane. Also, A β directly interacts with the lipid components of the cell membrane, forming pores or ionic channels that help with Ca²⁺ entering the cell. It has been shown that A β (25–35) fragment forms nonspecific channels in lipid membranes more efficiently compared to the full peptides [100]. The end effect of pore formation is a loss of membrane potential, and loss of solutes resulting in cell death. Maintaining the lipid rafts, and the membrane proteins associated with them, is very important for *in vivo* cell survival [101].

1.5.4. Role of Tau in Alzheimer's disease

Tau, the microtubule-associated protein, is the major constituent of neurofibrillary tangles in Alzheimer's disease (AD) and of various other forms of intracellular inclusions in frontotemporal dementias (FTDs). The tau protein can be divided into four regions such as N-terminal region, proline-rich domain, microtubule-binding domain (MBD), and C-terminal region. Tau contains 85 potential threonine (T), serine (S) and tyrosine (Y) phosphorylation sites. Under physiological conditions, tau regulates the assembly and maintenance of the structural stability of microtubules and the cytoskeletal structure is

maintained by phosphorylation [102]. In the diseased brain, tau becomes abnormally hyperphosphorylated, which ultimately causes the microtubules to disassemble, and the free tau molecules aggregate into paired helical filaments. This soluble hyperphosphorylated tau is, however, clearly distinct from aggregated fibrillary tau in neurofibrillary tangles, despite both being implicated in tau toxicity [103]. A large body of evidence suggests that tau hyperphosphorylation results from perturbation of cellular signaling, mainly through imbalance in the activities of different protein kinases and phosphatases [103]. In AD, increased levels of intracellular soluble A β cause abnormal phosphorylation of tau protein and its subsequent release from microtubules. A study reported that tau can undergo liquid–liquid phase separation (LLPS), similar to several other neurodegenerative disease-associated proteins such as FUS, TDP43, hnRNPA1 and suggested that this observation may provide a biological mechanism for tau aggregation in neurodegenerative diseases [104]. APOE4, the strongest genetic risk factor for late-onset AD, affects tau pathogenesis, neuroinflammation, and tau-mediated neurodegeneration independent of A β pathology [105].

1.6. Molecular chaperones

Chaperones are the ubiquitous class of endogenous proteins or protein complexes which aids the nascent polypeptide to fold into a functionally active three-dimensional structure. Some chaperones are also involved in preventing the aggregation and unfolding of misfolded proteins. ATP binding and hydrolysis is involved in chaperone-mediated protein folding and unfolding. Molecular chaperones might interact with other chaperone molecules of the same group or another group, or even with co-chaperones and co-factors to form functional complexes. Hence, these protein complexes represent machines that

perform the chaperoning process. Besides the primary role in *de novo* protein folding, molecular chaperones are the key players in protein degradation, trafficking and general stabilization [1, 2, 106]. Because of their stabilizing ability, the expression of many molecular chaperones is upregulated in degenerative diseases that involves protein misfolding and aggregation, such as, AD, PD, and Creutzfeldt–Jakob. In the cell, the well-characterized chaperone proteins such as HSP40, HSP60 and HSP70, are closely involved in regulating the correct folding of some proteins in the crowded environment of the cell. The small heat-shock proteins (sHSPs) are another class of ATP-independent, intracellular molecular chaperones that are not directly involved in protein folding. Instead, they are the major proteins associated with stabilizing other proteins under stress conditions (e.g. heat, oxidation, etc.) to prevent aggregation. In this process, the partly folded, stressed protein interacts via predominantly hydrophobic interactions with the sHSPs to form a complex [107].

Chaperone are involved in the proteostasis network to fold newly synthesized proteins, remodel misfolded states and to disaggregate aberrant structures with the protein degradation facilitated by the Ubiquitin-proteasome system (UPS) and the autophagy system (Fig. 1.11). Cells must invest nearly 180 different chaperone components and their regulators, 600 UPS components, and 30 different autophagy components to maintain the proteome integrity and provide adaptation to environmental changes [1]. Prevention of aggregation is the primary effort of the chaperone system, although the disaggregation machinery for the aggregated proteins has been described in bacteria and fungi, comprising the oligomeric AAA+ proteins like HSP104 and ClpB, the *E. coli* molecular chaperone

protein, which cooperates with HSP70 [108]. Chaperones do not have highly specific substrates, like enzymes [109].

Figure 1.11: Molecular chaperones in Proteostasis network

The chaperones facilitate the nascent polypeptide to fold into the native structure and remodel the misfolded state. Besides, they also recognize the oligomers, amorphous aggregates, and the fibrils for disaggregation or degradation through UPS and autophagy systems. Figure adapted from [1].

Though the steric property of the backbone of the protein constrains the range of protein shapes, that are recognized by the rigorous quality control mechanisms, the client proteins expose some features like unpaired cysteines, immature glycans, hydrophobic shapes, and specific sequence motifs, for chaperone recognition and association [110]. In fact, molecular chaperones can recognize the misfolded proteins when the hidden hydrophobic domains or specific sequences are exposed [111, 112]. The molecular chaperones might stabilize the unstable conformers of nascent polypeptides through this association to prevent aggregation and enable correct folding or assembly. Many

chaperones shift between high and low-affinity states for unfolded and partially folded proteins in a fashion controlled by ATP binding and hydrolysis. The chaperones stabilize the unfolded protein substrates transiently and mediate folding or assembly. The folded protein is released from the chaperones after folding.

Figure 1.12: Protein folding mediated by molecular chaperones through kinetic partitioning

In biological environments, the unfolded protein collapses into a partially folded intermediate. Chaperones shift between high and low-affinity states for unfolded and partially folded proteins in a fashion controlled by ATP binding and hydrolysis. When the rate constant for folding (K_{fold}) is larger the chaperone binding (K_{on}) and aggregation (K_{agg}) rate constants, folding occurs. Binding or rebinding of the aberrant protein to chaperone allows kinetic partitioning, and thereby inhibits aggregation and supports folding. When K_{on} is larger than K_{fold} , the chaperone acts as a holdase and holds the protein in a nonaggregated state for translocating to other chaperones or for degradation. During stress, K_{agg} is larger than K_{on} , leading to aggregation. K_{off} is the rate constant for protein release from chaperone, and P_i is inorganic phosphate. Figure adapted from [2].

Protein folding and refolding is stimulated by kinetic partitioning in the ATP-dependent chaperone mechanism (Fig. 1.12). When the folding rate constant is higher than the rate constants of aggregation or chaperone rebinding, efficient folding is achieved. In case of slow folding, the protein might be transported to a chaperone with distinct mechanistic properties or to the degradation machinery. Aggregation happens when the concentration of the folding intermediates surpasses the chaperone capability. In such circumstances, the cellular stress response is induced which increases the stress-regulated chaperone levels.

1.6.1. Heat shock proteins

Heat shock proteins (HSPs) are the largest group of chaperones which are produced in response to high temperature or oxidative stress. These proteins are named as HSP10, HSP40, HSP60, HSP70, HSP90, and HSP100 based on their molecular weight. Another class of HSP with the molecular weight ranging between 12-42 KDa, the small heat shock proteins (sHSPs), lack ATP binding site unlike HSPs and aids in folding along with HSPs. sHSPs typically associate early with the misfolded proteins. They hold proteins in a reversible state that helps facilitate refolding or degradation by other chaperones and co-factors. These sHSPs interactions require dynamic oligomerization state. There are ten human sHSPs (*E. coli* has two), of which the most populous and widespread are α B-crystallin (HSPB5) and HSP27 (HSPB1) [113]. Heat shock proteins act as a chaperone by binding to the sticky surfaces of unfolded protein to fold correctly, thereby preventing the aggregation and also to the damaged protein after stress to refold into native form. They act as monitors and carry misfolded proteins to the proteasome for degradation. HSP70 identifies KFERQ motif in proteins and transports them to the lysosomal compartments for

degradation, a classic example for chaperone-mediated autophagy. HSP110 functions as a disaggregase to solubilize the preformed fibrillar aggregates [5]. These proteins are mainly located in the cytosol and organelles such as endoplasmic reticulum, nucleus, and mitochondria. These stress proteins are produced rapidly by transcription and translation [114]. In addition to the chaperone function, they are also involved in immunity, apoptosis, and cardiovascular diseases.

1.6.2. Chaperonin

All molecular chaperones carry out similar activities, but structurally, they are classified into two groups, namely chaperones and chaperonins. The first group consists of monomers with molecular weight ranging between 70–100 kDa. The second group contains chaperonins, which are a complex oligomeric structure with a molecular weight of ~800 kDa [115]. Chaperonins are the huge protein complexes associated with the correct folding of newly synthesized proteins using ATP as energy, thereby preventing aggregation.

Additionally, chaperonins tag the misfolded proteins for degradation. They are ubiquitously present in all living cells. They are the large cylindrical double-ring assemblies containing hydrophobic inner part called Anfinsen's cage to which the partially unfolded protein attaches for folding. There are two main classes of chaperonins. Group I are homo-oligomeric, that have seven-fold symmetry in bacteria, chloroplasts and mitochondria and collaborate with HSP10 proteins (and GroES in bacteria), which form the lid of the folding cage. HSP60s in eukaryotes and GroEL in bacteria are the well-known group I chaperonins. The group II chaperonins are hetero-oligomeric, that contains eight to nine subunits per ring and are found in archaea and the eukaryotes [1].

GroEL/GroES is a well-characterized chaperonin required for folding of many proteins in *E. coli*. GroEL interacts with nearly 250 proteins in the cytosol with molecular weight ranging between 20 and 50 kDa. The apical domains in the GroEL provide the hydrophobic residues for substrate binding to the centre of the ring. Consequent folding depends on the encapsulation of the substrate by GroES. GroEL is the large protein to which the unfolded protein binds along with ATP. This induces a conformational change upon which the cap GroES binds. The individual subunits in the chaperonin are arranged in such a way to form a hydrophilic chamber favorable for protein folding. The protein enclosed in this chamber undergoes folding for ~10 seconds, the time required for hydrolysis of ATP in the GroES-bound ring. The folded protein is released upon GroES dissociation, which is allosterically activated by binding of ATP in the opposite ring (Fig. 1.13) [1, 116].

Figure 1.13: Process of protein folding in GroEL-GroES chaperonin system

Substrate binds to GroEL and might initiate local unfolding. A conformational rearrangement in the GroEL apical domains is triggered by ATP binding. Then GroES binds to form the cis complex and encapsulates the substrate for folding. Simultaneously, ADP and GroES detach from the opposite (trans) GroEL ring and allow the substrate to release. The new substrate fold in the encapsulated environment within ~10 s, in the newly formed cis complex. ATP and GroES binds to the trans ring and open the cis complex. Figure adapted and modified from [1].

TCP-1 Ring complex (TRiC) is a well-known eukaryotic chaperonin protein containing eight to nine subunits. Eukaryotic chaperonins also consist of the same three domains, namely apical, intermediate, and equatorial domain. Unlike bacterial chaperonins, TRiC can perform protein folding without any cap proteins like GroES. However, the reaction cycle in TRiC is much slower than the GroEL cycle and provides an extended period of protein encapsulation and folding. TRiC interacts with nearly 10% of newly synthesized proteins in the cytosol, which includes actin and tubulins. Folding of some cytosolic proteins cannot be replaced by other chaperones, which indicates that the evolved structural and mechanistic features of TRiC are different from other chaperones. Initially, TRiC was believed to fold only a few cytoskeletal proteins, but now it is found to be involved in folding of several other proteins [117]. Interestingly, TRiC also prevents the accumulation of toxic aggregates by Huntington's disease protein. Although both the HSP70 and the chaperonins function by utilizing ATP, they differ basically in that the HSP70 releases the substrate protein into the bulk solution for folding, while the chaperonins allow the folding of single protein enclosed inside a cage [1].

1.7. Lipocalin-type prostaglandin D synthase

Prostaglandins are the eicosanoids derived from Arachidonic acid, which plays a significant role in inflammation [118]. Cyclooxygenases and prostaglandin synthases catalyze the production of Prostaglandins. Cyclooxygenases are the main target for most Non-steroidal anti-inflammatory drugs (NSAIDs).

Lipocalin-type prostaglandin D synthase (L-PGDS) or β -trace is a 26 KDa, heavily glycosylated protein, majorly expressed in the brain, male genital organs, placenta, endothelial cells, and heart tissue. Two different types of PGDS, the hematopoietic-

prostaglandin D synthase (H-PGDS) and lipocalin-type prostaglandin D synthase (L-PGDS) catalyze the same reaction [119]. However, they have different catalytic properties, amino acid sequences, tertiary structure, and gene structure. H-PGDS is a cytosolic, GSH-requiring enzyme majorly expressed in spleen and mast cells [120].

L-PGDS, an essential protein in the prostaglandin pathway, is a dual functioning protein performing both as an enzyme and as a transporter [121]. It is the only protein found to have enzymatic activity in the lipocalin family. It catalyzes the isomerization of 9, 11-endoperoxide group of PGH₂ (substrate) to 9 α -hydroxyl, 11-keto group of PGD₂ (product) (Fig.1.14) [122]. Reducing agents such as DTT, TCEP, or β -mercaptoethanol are required for the catalysis. C65 is found to be the critical residue involved in the enzymatic reaction, as the mutation of C65 residue to alanine/serine leads to the complete loss of activity in the recombinant human, rat and mouse enzymes [123].

Human L-PGDS has four cysteine residues in the positions 65, 89, 167 and 186 among which C65, C89, and C186 are conserved among all species and C89 and C186 form a disulfide bridge. Non-mammalian species like chicken and Zebrafish lack C65 and have weak or no enzymatic activity and acts as a lipophilic ligand transporter [125].

Mutation studies on L-PGDS showed that K59A and Y149A mutants showed increased activity while M64A, L79A, F83A, L131A mutants showed reduced activity, and C65A mutation leads to a complete loss of action [126]. It also acts as a transporter for various lipophilic ligands such as heme, bilirubin, biliverdin, retinoic acid, thyroid hormones, and gangliosides [127, 128].

Figure 1.14: L-PGDS as an enzyme in the Prostaglandin pathway

L-PGDS catalyzes the isomerization of 9, 11-endoperoxide group of PGH2 to 9α-hydroxyl, 11-keto group of PGD2 (product). Figure adapted and modified from [124].

Figure 1.15: Crystal structures of Human wild type L-PGDS.

(A) Crystal structure of human WT- L-PGDS in the apo form (PDB ID: 4IMN) (Resolution: 2.09 Å). The key Cys 65 residue is highlighted in yellow. (B) Crystal structure of human WT-L-PGDS in complex with the substrate analog (U44069) (PDB ID: 4IMO) (Resolution: 1.88 Å). The substrate analog is shown in yellow. The critical Cys 65 residue is highlighted in blue. (C) The three-dimensional structure of L-PGDS (PDB ID: 3O2Y) with the hydrophobic and hydrophilic residues highlighted in blue and red, respectively. The hydrophobic cavity is highlighted by the broken line. Figure 1.15C is adapted from [129].

It binds with heme and its metabolites, Bilirubin, and biliverdin at a K_d value of 20.9 nM, 17.0 nM, and 19.5 nM, respectively [130]. Kume et al. reported that Trp 43 is involved in binding with the lipophilic ligands although the residues Trp 54 and Trp 112 forms an aromatic bridge demonstrating that the ligands bind deep inside the hydrophobic pocket of L-PGDS. It has been widely accepted that the lipophilic ligands like heme,

bilirubin and biliverdin interact with the hydrophobic cavities of L-PGDS (highlighted in Fig. 1.15C) and other lipocalins using hydrophobic interactions [129, 130].

The crystal structure of L-PGDS reported by S. Lim et al. showed the typical lipocalin fold with eight stranded, antiparallel beta-barrel structure with two α -helices with one helix near the substrate-binding pocket. The eight beta sheets named A-H form the core barrel and are connected by the intersheet loops, namely CD, EF, GH, and Ω loop which connects to the helix 2 [121] (Fig. 1.15A, B).

1.7.1. Role of L-PGDS in brain

Apart from the functions of L-PGDS as an enzyme or lipophilic ligand transporter, it is also shown to have chaperone activity for the pathogenic A β peptide. β -trace (or) L-PGDS is the second most abundant protein after albumin in the human cerebrospinal fluid (CSF), with the estimated concentrations of 26 mg/l [131, 132]. L-PGDS regulates many neurological processes and its altered expression levels in the brain results in various pathological conditions [133, 134]. It has been reported that L-PGDS plays a protective role after an ischemic stroke, scavenges the reactive oxygen species (ROS) using the thiol group of C65 and stimulates chemotactic migration of microglial cells and recruitment of astrocytes at the site of injury [135-137].

The *ptgds* gene expression was found to be upregulated in AD phenotypes and is clearly associated with the amyloid plaques [138, 139]. The expression of L-PGDS in the prefrontal cortex of the brain is linked with the clinical and pathological traits of AD, where its level of expression is higher compared to ALS and PD [139, 140]. L-PGDS was proposed to be a major endogenous A β chaperone capable of inhibiting A β 40 and A β 42 aggregation within its physiological range. Additionally, L-PGDS inhibited the seed-

dependent aggregation of A β . It was also mentioned that the C65 mutant does not have chaperone activity indicating the importance of C65 [58]. Histological studies of Tg2576 AD mice confirmed the co-localization of L-PGDS with A β . Remarkably, these Tg2576 mice are prone to insulin resistance and type II diabetes [141]. The well-established molecular links between diabetes and AD suggests that possibly L-PGDS plays a central role in AD than previously suspected [142, 143].

It has been reported that the recombinant human L-PGDS binds with both the monomeric and fibril form of A β 40 with the K_d value of 20 nM and 31 nM respectively. L-PGDS binds with A β (1-28) (D¹AEFRHDSGYEVHHQKLVFFAEDVGSNK²⁸), A β 42 (D¹AEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA⁴²), and also A β (25-35) (G²⁵SNKGAIIGLM³⁵) at similar high affinity comparable to A β 40. L-PGDS interaction with the hydrophobic residues (25-28) in A β might inhibit the conformational change (Fig. 1.16).

On the whole, L-PGDS acts as an endogenous chaperone in CSF to prevent aggregation of A β , which leads to neuropathological conditions [58]. Clusterin is also an ubiquitous, extracellular chaperone, which inhibits the formation of A β fibrils at sub-stoichiometric levels [144]. Like sHSPs, clusterin is a chaperone with the ability to stabilize partly unfolded stressed proteins with extremely broad substrate specificity [145]. However, the concentration of clusterin in CSF is found to be 2 μ g/ml, which is several folds lesser than L-PGDS [144]. This again highlights the importance of L-PGDS as a brain chaperone.

In our study, we demonstrate that the extracellular protein L-PGDS has a unique ability to disaggregate the pre-formed fibrils of A β *in vitro* besides its protective role as a chaperone. ThT assay and TEM images confirmed the disaggregation of A β 40 and A β (25-

35) fibrils by L-PGDS. WT-L-PGDS primarily localizes at the growing tips or fractures of A β 40 fibrils and thereby disintegrates them. Through proteomics study, we proved that the recombinant human WT-L-PGDS could efficiently solubilize several crucial proteins (that were usually upregulated in AD) from the highly insoluble proteins aggregates collected from the human AD brain.

Figure 1.16: Proposed mechanism for inhibition of A β aggregation by L-PGDS.

L-PGDS prevents the aggregation of A β by inhibiting the conversion of monomers to β -sheet structure and the subsequent seed-dependent fibrillogenesis of A β . Figure reconstructed from [58].

Additionally, we establish the formation of L-PGDS-A β 40 complex in 1:1 stoichiometry through MALDI-TOF MS. Our NMR results confirm that the binding site and mode of interaction of L-PGDS with monomeric A β 40 peptide is distinctly different from the catalytic substrates (investigated previously) [121]. We report the first model structure of L-PGDS-A β 40 complex. The detailed analysis of ThT assay revealed the possible mechanism for inhibition of A β 40 aggregation by L-PGDS. L-PGDS inhibits the

primary nucleation by binding to A β 40 monomers and inhibits the secondary nucleation by binding to fibril surfaces, thereby reducing the final fibril content. Our work demonstrates the molecular basis of the inhibitory and disaggregase activities of L-PGDS supporting its role as a major amyloid- β chaperone in AD.

CHAPTER 2

MATERIALS AND METHODS

2.1. Protein expression and purification

The gene encoding Human Wild-type L-PGDS was cloned into a pNIC-CH vector containing a C-terminal hexa-histidine tag. 1- 22 amino acids encoding the signal peptide was chopped off from the gene sequence. The plasmid was transformed into Rosetta 2 DE3 singles *E. coli* cells (Novagen) for expression. Protein expression conditions were initially optimized by growing cells at 37°C and 18°C and were induced with 0.5 and 1mM IPTG. Terrific broth containing high levels of tryptone and yeast extract was used to produce unlabeled L-PGDS. The cells were induced with 1mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) when the OD₆₀₀ reached 0.8-1.0. The cells were then incubated at 37°C for 3-4h and then harvested at 7500 rpm for 20 min. 1g/L ¹⁵N labeled ammonium chloride was used in M9 media to produce single labeled L-PGDS. To produce labeled protein, cells were grown in 4L of terrific broth until OD₆₀₀ reached 0.6 and then the cells were harvested at 4000rpm [146]. The cells were resuspended in 1L of labeled M9 media and incubated for an hour before inducing with 0.5mM IPTG followed by incubation at 18°C overnight.

The unlabeled and labeled L-PGDS cells were lysed using microfluidizer and the lysate was passed through the Ni-NTA resin for immobilized metal affinity chromatography (IMAC) purification. Protein collected from IMAC was denatured completely with 8M Urea to get rid of any hydrophobic junks attached to the hydrophobic pocket of L-PGDS. The protein was then refolded by dialysis at 4°C and was further

purified by Superdex 75 10/300 GL Gel filtration column in AKTA Fast performance liquid chromatography (FPLC) in 20mM HEPES, 150mM NaCl, 2mM TCEP (pH-6.5). 2mM TCEP was maintained throughout the purification procedure to keep the C65 of L-PGDS in a reduced state to be functionally active. The fractions were loaded in an SDS-PAGE, and the pure fractions were pooled and concentrated.

LPGDS sequence:

**MAPEAQVSVQPNFQQDKFLGRWFSAGLASNSSWLREKKAALSMCKS
VVAPATDGGLNLTSTFLRKNQCETRTMLLPAGSLGSYSYRSPHWGS
TYSVSVVETDQYALLYSQGSKGPGEDFRMATLYSRTQTPRAELKE
KFTAFCKAQGFTEDTIVFLPQTDKCMTEQAHHHHHH**

2.2. A β 40 purification

pET 28a vector carrying A β 40 gene (without His-tag) was transformed to Rosetta 2 DE3 singles *E. coli* cells (Novagen) [147]. Unlabeled and uniformly ^{15}N labeled cells were grown in Terrific broth and M9 media, respectively. 0.5mM IPTG was added when OD₆₀₀ reaches 0.6-0.8 to overexpress the peptide. The cells were incubated at 37°C for 4 h after induction to get the peptide in inclusion bodies. For labeled peptide samples, the cells were initially grown in 4 L of unlabeled media until it reaches the log phase after which the peptide expression was carried out in ^{15}N labeled M9 media.

Inclusion bodies need to be extracted from the cells to purify the peptide. The cells were lysed using a sonicator for several cycles to remove soluble proteins. After several washes in the buffer, the inclusion bodies were dissolved in urea and dialyzed against water. The precipitate was lyophilized and was further purified by C18 column in *Waters*, High-performance liquid chromatography (HPLC) at a flow rate of 2 ml/min with a linear

gradient of Acetonitrile/water containing 0.1% Trifluoroacetic acid (TFA). The purity of the peptide was checked by mass spectrometry.

A β 40 sequence:

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV

2.3. Enzymatic assay

The enzymatic reaction was carried out with a fixed concentration of L-PGDS (enzyme) and varying concentration of PGH2 (substrate) in a 50 μ l reaction mix containing 20mM HEPES, 150mM NaCl and 2mM TCEP (tris (2-carboxyethyl) phosphine) pH-6.5. TCEP is the reducing agent to keep the Cys65 in a reduced state suitable for catalysis. The reaction mix was incubated at 25°C for 3 min and then stopped by adding an equal volume of acetone. The product formed was methoximated by adding equal volume of Methoxyamine hydrochloride (MOX-HCl) and incubated at 60°C for 30 minutes to make it stable as the product itself is very unstable. The amount of PGD2-MOX formed is estimated using the commercial PGD2-MOX ELISA kit from Cayman Chemicals, Ann Arbor, MI, USA.

2.4. Preparation of monomeric peptides

Synthetic A β (25-35) and A β 40 were purchased from China Peptides (Suzhou, China). Lyophilized A β peptides were treated with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and incubated at room temperature to break any preformed aggregates. The excess HFIP was then removed by lyophilization, and the dried peptide film was dissolved in a small volume of 100 mM NaOH and subsequently diluted with the buffer containing 20 mM HEPES, 100 mM NaCl, 2 mM TCEP (pH-7.5).

2.5. Thioflavin T assay

Inhibition and disaggregation of A β aggregation by L-PGDS was studied using Thioflavin T (Sigma), a fluorophore that specifically binds to the β -sheet structure of amyloids and shows enhanced fluorescence. To study inhibition, monomeric peptides were prepared in buffer containing 20 mM HEPES, 100 mM NaCl, 2 mM TCEP (pH-7.5) at a final concentration of 50 μ M A β 40 or A β (25-35) and 20 μ M ThT with or without 1 μ M and 5 μ M of WT-L-PGDS or C65A mutant. The disaggregase activity of L-PGDS was investigated by adding 5 μ M of WT-L-PGDS or C65A mutant to the preformed fibrils of A β 40 and A β (25-35). To compare the effect of L-PGDS with mechanical breakage of fibrils, A β 40 fibrils was sonicated in a bath sonicator for 2-3 min. Experiments were carried out in triplicates in NUNC 96 well black plate with incubation at 37°C in TECAN infinite M200 Pro microplate reader with orbital shaking before each measurement. The plate was sealed completely to prevent evaporation and measurements were read with an excitation wavelength at 440 nm and emission at 485 nm with 9 and 20 nm bandwidth respectively. IC50 curve fitting was performed in GraphPad prism7 software.

2.6. Mass Spectrometry

10 μ l of the L-PGDS-A β 40 sample was added to 10 μ l of matrix solution containing 10 mg/ml sinapic acid (Sigma Aldrich) in 0.1% TFA/50% Acetonitrile before spotting onto a 384-well stainless steel MALDI target plate (Applied Biosystems). The linear mid mass mode on the ABI 4800 Proteomics Analyzer MALDI TOF/TOF mass spectrometer (Applied Biosystems) was used to acquire the data of the spotted samples.

2.7. Fluorescence microscopy

To study the inhibitory effect of L-PGDS, 50 μ M of A β 40 with or without 1, 5, and 10 μ M WT-L-PGDS was incubated at 37°C for 60 h with 20 μ M ThT in the buffer containing 20 mM HEPES, 100 mM NaCl, 2 mM TCEP (pH 7.5). Aggregation was monitored in TECAN infinite M200 Pro microplate reader. 10 μ l of each sample was applied on a glass slide, and images were acquired by a fluorescence microscope (Olympus microscope with Cool SNAP ^{HQ2} camera) with 10x magnification.

2.8. Transmission Electron Microscopy

To establish the disaggregase activity of L-PGDS, 50 μ M of A β 40 and A β (25-35) was incubated for 60 h at 37°C in buffer containing 20 mM HEPES, 100 mM NaCl, 2 mM TCEP (pH-7.5). A β samples treated with and without L-PGDS were applied on copper-rhodium 400 mesh grids with 15 nm carbon coating (thickness) (prepared in-house) followed by negative staining with 2% uranyl acetate and then air dried. The samples were then viewed under FEI T12, 120 kV Transmission electron microscope equipped with a 4K CCD camera (FEI) between 48000X to 68000x magnification under low dose conditions.

2.9. HPLC and DLS analyses of fibril disaggregation

To quantify the amount of soluble A β 40 in the untreated and L-PGDS treated A β 40 fibrils, the samples were injected into Agilent Zorbax 300SB-C8 column connected to Agilent tech 1260 Infinity system and run at the flow rate of 2 ml/min with a linear gradient of Acetonitrile/water containing 0.1% v/v TFA. The area under the curve was measured by manual peak integration. Dynamic Light Scattering (DLS) analysis was used to determine the size distribution of untreated and L-PGDS treated A β 40 fibrils. The measurements and data analysis were carried out using a Malvern Zetasizer Nano series instrument.

2.10. NMR titration

NMR spectroscopy experiments were carried out in Bruker Avance 700 MHz with triple resonance z-axis gradient cryoprobe and Bruker DRX 600-MHz spectrometer equipped with a cryoprobe at 298 K. Uniformly ^{15}N labeled L-PGDS, and A β 40 samples were prepared in 20 mM HEPES, 150 mM NaCl, 2 mM TCEP (pH-6.5) at a concentration of 0.25mM and 0.2mM respectively with 5% D $_2$ O. Two dimensional ^1H - ^{15}N HSQC spectra of ^{15}N L-PGDS and unlabeled A β 40 were recorded at molar ratios of 1:0 and 1:4 to identify the key residues of L-PGDS involved in A β 40 binding. Similarly, to detect the important residues on A β 40, we recorded ^1H - ^{15}N HSQC spectra of ^{15}N A β 40 with unlabeled L-PGDS at molar ratios of 1:0 and 1:0.5. Spectra were referenced concerning to 4, 4- dimethyl-4-silapentane-1-sulfonic acid (DSS) and then overlapped to check if the cross peaks showed any shift or change in signal intensity. Freshly prepared monomeric A β 40 was used immediately in both the titrations to ensure that the majority population is monomers. Data were processed in Topspin 3.5 (Bruker Corporation) and then analyzed using Computer-aided resonance assignment (CARA) (www.nmr.ch)[148]. Changes in ^1H and ^{15}N chemical shifts were calculated using the equation:

$$\Delta\omega = \{[\Delta\omega(^1\text{H})^2] + [0.25 \times \Delta\omega(^{15}\text{N})]^2\}^{1/2}$$

2.11. Conjugation of L-PGDS to ferritin nanocages

Conjugation of L-PGDS to ferritin nanocage is essential to visualize the L-PGDS under TEM since L-PGDS is a small molecule that cannot be seen under TEM. 1.6 mg/ml magnetoferritin nanocages (kindly provided by Dr. Sierin Lim, NTU) were activated for carbodiimide conjugation by adding a 1:2.5 molar mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) (EDC) and N-

hydroxysulfosuccinimide (sulfo-NHS) in MES buffer and incubated at room temperature while shaking for 90 min in the dark. 50 μ M L-PGDS in 20 mM HEPES buffer with 100 mM NaCl, 2 mM TCEP (pH 7.5) was added to this mixture in equal volume and left for overnight incubation in the dark with gentle shaking. Crosslinking reaction was quenched by adding Tris (pH 7.5) at a final concentration of 150 μ M. These conjugates were passed through IMAC and PD10 desalting columns to remove excess unbound ferritin nanocages.

2.12. Small-angle X-ray scattering (SAXS)

The SAXS data were collected with a BRUKER NANOSTAR SAXS instrument equipped with a Metal-Jet X-ray source (Excillum, Germany) and VÅNTEC 2000-detector system [149]. The scattering patterns were measured using a sample at a detector distance of 0.67 m and a wavelength of $\lambda = 1.3414$ Å, which covered a range of momentum transfer of $0.016 < q < 0.4$ Å⁻¹ ($q = 4\pi \sin(\theta)/\lambda$, where 2θ is the scattering angle). The SAXS experiments were carried out at 15°C with a sample volume of 40 μ l in a vacuum-tight quartz capillary. Radiation damage for the protein sample was monitored with six 5 min exposures, and no radiation effect was observed. The data were normalized to the intensity of the transmitted beam. The scattering of the buffer was subtracted, and the difference curves were scaled with protein concentration. All the data processing steps were performed using the program PRIMUS [150] from the ATSAS package. The experimental data obtained for all protein samples were analyzed for aggregation using the Guinier region. The forward scattering, $I(0)$, and the radius of gyration, R_g , were computed using the Guinier approximation. These parameters were also computed from the extended scattering patterns using the indirect transform package GNOM [151], which provided the distance distribution function, $P(r)$, the maximum particle dimension, D_{max} , and the radius of

gyration, R_g . The qualitative particle motion was inferred by plotting the scattering patterns in the normalized Kratky plot [152] and Porod-Debye plot [153]. *Ab initio* low-resolution models of the proteins were built by the program DAMMIN which considers low angle data ($q < 2 \text{ nm}^{-1}$) [154]. 20 independent *ab initio* reconstructions were performed for each protein and then averaged using DAMAVER [155]. The averaged and filtered *ab initio* model was superimposed with the atomic model using SUPCOMB [156]. The theoretical scattering curves from the atomic structures were generated and evaluated against the experimental scattering curves using CRY SOL [157]. Rigid body modeling was performed using CORAL [158] by docking the individual domains of the high-resolution structures against the experimental data. The oligomeric state of the protein was confirmed from the molecular mass calculation based on $I(0)$, Porod volume (V_p), excluded volume (V_{ex}) and the volume of correlation (V_c) [159, 160]. SAXS experiments were carried out in collaboration with Prof. Gerhard Gruber and Dr. Malathy Sony. Bhuvaneswari prepared the samples and assisted while recording the experiments and analyzing the data.

2.13. Molecular dynamics simulation

2.13.1. Model Building

The initial structure of L-PGDS was taken from Lim et al., 2013 (PDB ID 4IMN)[121]. The structure of Anticalin US7 in complex with A β 40, (PDB ID 4MVI) [161] contained residues 16-28 of A β 40 resolved, was used to construct the model of L-PGDS in complex with A β 40. Since L-PGDS contains the lipocalin fold, which is structurally similar to Anticalin US7, it is reasonable to assume that A β 40 would bind in a structurally similar manner towards L-PGDS as that of Anticalin US7. The structure of the complex of Anticalin US7 with A β (16-28) (PDB ID: 4MVI) was aligned to the structure of L-PGDS

(PDB ID: 4IMN), and coordinates of L-PGDS (from 4IMN) and A β (16-28) (from 4MVI) were extracted to build the initial model of L-PGDS with A β (16-28). The unresolved residues of A β 40, i.e., segments D1-Q15 and G27 to V40, were modeled in an arbitrarily extended conformation using Discovery Studio 4.1. The L-PGDS-A β 40 complex was subjected to steepest descent energy minimization in-vacuo using the Gromacs 5.1.2 package [162] and the charmm36M force field [163] until a force convergence of 1000.0 kJ/mol/nm was achieved.

2.13.2. Simulation setup

To study the binding mode of A β 40 in complex with L-PGDS, we performed classical molecular dynamics simulations using the Gromacs 5.1.2 package. The CHARMM36m force field was used, whereby the parameter sets have been recently optimized for simulations of intrinsically disordered and folded proteins. The system was solvated with CHARMM-modified TIP3P [164] water in a cubic box with a distance of 1.2 nm from the solute to the box edge. Na⁺ and Cl⁻ counterions were added to neutralize the system and to achieve a salt concentration of 0.15 M. Bonds containing hydrogen atoms were constrained using the LINCS [165] algorithm, to enable a time step of 2fs. Particle Mesh Ewald [166] was used with a cutoff of 1.2 nm for electrostatics, and a cutoff of 1.2 nm was used for van der Waals interactions. Steepest descent energy minimization was performed until a force convergence of 1000.0 kJ/mol/nm is reached to remove any initial bad contacts. An equilibration of 1ns in the NVT ensemble was performed before the start of production simulation. The temperature of the system was maintained at 300 K using the V-rescale [167] thermostat. 5 sets of classical molecular dynamics simulations were

performed with different random initial velocities for 300ns. Trajectory frames were saved every 2 ps.

2.13.3. Analysis of Simulation

The last 200ns of each simulation repeat was combined to produce a 1 μ s trajectory of sampling. The analysis was conducted using built-in Gromacs tools. The A β 40 radius of gyration was computed using the gmx gyrate tool. To identify the representative conformations of the L-PGDS-A β 40 binding, we performed geometric clustering of the simulation frames using the Gromos algorithm. The clustering group was selected as the A β 40 peptide, using a cutoff of 0.8nm. Clustering was performed for every fifth frame to minimize statistical correlation. MD simulation was performed in collaboration with Dr. Mu Yuguang and Mr. Justin Ng Tze Yang. Bhuvaneswari provided all the NMR chemical shift for the residues involved in interaction.

2.14. Extraction of aggregated proteins from human brain tissue

To check the disaggregase role of L-PGDS, we extracted aggregated proteins from post-mortem brain tissue samples of a 69-year-old male diagnosed with subarachnoid hemorrhage and dementia, which was obtained from the brain bank of the Choku Medical Institute of the Fukushima hospital (Toyohashi, Aichi, Japan). The scientific use of human material, like a brain tissue sample, was conducted following the Declaration of Helsinki, and informed approval was obtained from the guardians of the patients. All procedures were approved and executed following the ethical guidelines of the Nanyang Technological University ethics board. Brain tissue was first dissected, and the large blood vessels were removed. Then, the tissue was cut into small pieces and homogenized in the buffer comprising 2% SDS, 50 mM ammonium acetate (pH 6.0). The homogenized sample

was then spun at 3000 x g, at 10 °C for 10 min to remove the cell debris. The supernatant was collected and ultra-centrifuged at 112000 × g, at 10 °C for 2 h. The resultant pellet was considered as the aggregated proteins since these proteins were insoluble in 2% SDS and 50 mM ammonium acetate buffer (pH 6.0). Then, the pellet was washed with the buffer containing 20mM HEPES, 100mM NaCl, 2mM TCEP (pH-7.5), and the supernatant was discarded. Thus, all the buffer soluble proteins were removed before treating the aggregates with WT-L-PGDS in a buffer containing 20mM HEPES, 100mM NaCl, 2mM TCEP (pH-7.5), Hexafluoroisopropanol (HFIP), and formic acid (FA). Formic acid is a common solvent used to dissolve protein aggregates from the brain and HFIP is a universal solvent used to break aggregates in β -sheet peptides. So, HFIP and formic acid treated samples act as positive controls in this study to compare the effect of L-PGDS.

2.15. Sample preparation and LC-MS/MS analysis

The soluble fractions were loaded on to the SDS-PAGE and separated at 100 V. Coomassie brilliant blue staining was performed to visualize the protein bands. The gel lane was cut and processed for in-gel digestion after reduction with 10 mM DTT and alkylation with 55 mM iodoacetamide as described earlier. The gel pieces were subjected to sequencing grade modified trypsin (Promega, Madison, WI) digestion at 37 °C overnight. The tryptic peptides were extracted using 50% acetonitrile/5% acetic acid, vacuum centrifuged to dryness and subjected to LC-MS/MS analysis. LC-MS/MS analysis was performed using QExactive mass spectrometers (Thermo Fisher, MA) coupled with a Dionex RSLC nanoLC system. A 5 μ l sample was injected into an acclaim peptide trap column via the auto-sampler of the Dionex RSLC nanoLC system. Mobile phase A (0.1 % FA in 5 % acetonitrile) and mobile phase B (0.1 % FA in acetonitrile) were used to form a

60 min gradient. The peptides were analyzed on a Dionex EASY-spray column (PepMap C18, 3 μ m, 100° A) using an EASY nanospray at an electrospray potential of 1.5 kV. The MS was recorded at 350–1600 m/z at resolution 70,000 m/z . Maximum accumulation time was set at 100 ms and dynamic exclusion was 30 s. MS/MS spectra resolution was 35,000 at m/z 200; ACG was 1×10^6 for the full MS and 2×10^5 for the MS2 scan. For Higher-energy collisional dissociation (HCD), 10 most intense ions above 1000 count threshold were chosen and 120 ms was set for maximum ion accumulation. The collision energy was 28 and an isolation width of 2 Da for the MS2 scan. Single charged and unassigned ions were excluded from MS/MS. For HCD, normalized collision energy was fixed to 28. The underfill ratio, as 0.1 %.

2.16. Data Analysis

Proteome Discoverer (PD, Thermo Scientific, San Jose, USA) ver 1.4 software was used to analyze MS data acquired by QExactive. The MS/MS spectra were deisotoped and deconvoluted using the MS2 spectrum processor. Database search was conducted using a Mascot (version 2.2.06, Matrix Science, Boston, MA) search engine against an in-house built database and UniProt human database (Released on 7/25/2016, 70,849 sequences, 23,964,784 residues). The peptide precursor mass tolerances of 10 ppm and 0.02 Da mass tolerance was used during data search. The fixed modification was carbamidomethylation (+57.021 Da) of cysteine residues, while deamidation (+0.984 Da) of asparagine and glutamine residues and oxidation (+15.995 Da) of methionine residues were variable. The resultant search output was exported to an excel file for further analysis. Proteomics work was carried out in collaboration with Dr. Newman Sze and Dr. Sunil Adav. Bhuvaneswari carried out the sample preparation with Dr. Sunil (Extraction of protein aggregates from

AD brain and treatment with L-PGDS and other solvents, sample preparation for LC-MS)
and analysis of the Mass spec data.

CHAPTER 3

PROTECTIVE ROLE OF L-PGDS AS A CHAPERONE

3.1. Overview of the protective role of chaperones

In neurodegenerative diseases, molecular chaperones play an essential role in proteostasis by interacting with the target protein and thereby preventing their aggregation. Several intracellular and extracellular chaperones were found to carry out these functions. The two main categories of chaperones include ATP-dependent chaperones, which require ATP as energy to facilitate protein folding, and ATP-independent chaperones, which inhibit the aggregation of proteins under stress. Extracellular chaperones function without consuming ATP since the extracellular levels of ATP are low [1]. Chaperone proteins are indispensable in eukaryotes to ensure the proper folding of the nascent polypeptides and refolding of misfolded protein aggregates. The stress-induced proteins such as HSPs or heat shock cognates (Hscs) are found in the ER, mitochondria, cytoplasm, or extracellular space [1, 168]. The stress-inducible chaperone HSP70 have shown potentiality to protect the brain from both global and focal ischemia *in vivo* and in cell culture models of ischemia/reperfusion injury *in vitro*. Chaperones protect the brain from ischemia and ischemia-like injury by involving antinecrotic, anti-apoptotic, and anti-aggregation mechanisms [169].

In many studies, chaperones have shown a protective role in neurodegenerative disease models; however, the molecular basis of this protection is not clear. Since the major role of chaperones is to recognize misfolded proteins, it is rational to study the effects of chaperones on amyloid assembly [170]. With this objective, the impacts of chaperones in

an *in vitro* model of huntingtin aggregation were analyzed. HSP70 and HSP40 mitigate the spherical and annular oligomer formation of mutant huntingtin and enable the buildup of fibrillar and amorphous aggregates. HSP70 and HSP40 showed concomitant stabilization of monomeric huntingtin conformation in biochemical assays. Consistent with these results, fluorescence resonance energy transfer (FRET) also showed that HSP70 and HSP40 prevent an intramolecular conformational change in a mutant huntingtin fragment [171]. On the whole, HSP70 and HSP40 assist the misfolded monomers in undergoing ON-pathway aggregation to form fibrils while reducing the promotion of spherical and annular oligomers formation, that are important for neurotoxicity, which might be OFF-pathway for fibril formation (Fig. 3.1).

Figure 3.1: Proposed activities of HSP70 and HSP40 on amyloid assembly

A native monomer can misfold to form several different conformations, such as an annular oligomer, a spherical oligomer, an amorphous aggregate or a fibril. HSP70/HSP40 (red sphere) might inhibit the intramolecular transition that leads to the formation of spherical and annular oligomers by stabilizing the native monomer and concurrently stabilize a conformation that promotes inclusion body formation. Figure adapted from [170].

The molecular chaperones facilitate neuroprotection by targeting several levels that might not be exclusively connected to protein aggregation. For instance, chaperone-mediated autophagy clears the toxic misfolded assemblies, and proteasomal degradation helps in the prevention of aberrant interactions with critical proteins like transcription factors and signaling molecules. Chaperones assist intracellular trafficking, including synaptic transmission, to prevent neuronal dysfunction. Additionally, molecular chaperones can also interfere with oxidative stress and block apoptotic signalling pathways. Inside the cell, chaperones are involved in the transportation of the unfolded proteins to lysosomes or proteasomes for degradation [172]. Proteasomes, for example, ubiquitin-specific protease USP14 and 26S help in the degradation of amyloids [173, 174]. The extracellular chaperones bind to the misfolded proteins and bring them to the scavenger receptors which prompt the intracellular degradation [175]. Insulin degrading enzyme (IDE), high-temperature requirement A (HTRA1), neprilysin, some serine and cysteine protease, and zinc metalloproteins/peptides are some of the well-established Amyloid degrading enzymes (ADE) [176, 177]. Clusterin, $\alpha 2$ macroglobulin, and haptoglobin are the extracellular chaperones which directly target the misfolded proteins and mediate their degradation [178].

Under normal conditions, A β is secreted into the human cerebrospinal fluid (CSF) [179] and some unknown extracellular chaperones control their aggregation in the healthy brain. In this context, the pathological aggregation and accumulation of A β in sporadic AD, could be the result of deficiencies in the specific amyloid- β chaperones conferring the neuroprotective function [180, 181]. Kanekiyo *et al.* showed that L-PGDS could potentially inhibit A β aggregation [58]. However, the mechanism of chaperone function of L-PGDS

still remains elusive. Thus, in this chapter, we explore the ability and possible mechanism of the major human CSF protein, L-PGDS, to inhibit the aggregation of A β 40 and A β (25-35).

3.2. Results

3.2.1. Optimization of L-PGDS expression and purification

The cells grown at varying temperature with varying IPTG concentration showed different expression profiles. The protein expression was found to be higher in cells grown at 37°C compared to 18°C. Localization experiment done from these cells, revealed that protein is expressed in soluble fraction not in inclusion bodies even after growing the cells at 37°C for 4h after induction (Fig. 3.2).

Figure 3.2: SDS-PAGE for expression of L-PGDS

The lanes loaded with induced cells show overexpression of protein at 20KDa. The expression is very high at 37°C with 0.5mM and 1mM IPTG compared to the cells grown at 18°C. The cells grown at 37°C and induced with 1mM IPTG showed slightly higher expression levels compared to 0.5mM.

The cells were grown in terrific broth at 37°C for 4h after induction, to produce unlabeled L-PGDS whereas, for ¹⁵N labeled protein production, cells were grown at 18°C

overnight. The cells were lysed using a sonicator in a buffer containing 50mM sodium phosphate, 300mM NaCl (pH-7.5) and the protein was purified using Ni-NTA column chromatography, which binds explicitly the His tagged proteins. SDS-PAGE of purification profile shows that eluate has many other impurities and so it was further purified by Size exclusion chromatography (SEC), which separates proteins based on their molecular weights. SEC profile shows a single peak around 12ml and purity of L-PGDS protein was analyzed by SDS-PAGE (Fig. 3.3A, 3.3B). The final purification of L-PGDS led to a monomeric protein of mass ~19.7 kDa.

Figure 3.3: Purification profile of L-PGDS

(A) SDS-PAGE showing purification profile of L-PGDS after Ni-NTA column chromatography with an intense band in the eluate containing 500mM imidazole (B) Size exclusion chromatography showing pure fraction of L-PGDS at 12ml elution volume (Inset: SDS-PAGE gel image showing purity of L-PGDS).

3.2.2. Expression and purification of A β 40

The Rosetta BL21 cells transformed with the plasmid containing the gene encoding A β 40, expressed the peptide in inclusion bodies without any solubilization tag. The cells

were lysed and washed for several cycles to isolate the pure inclusion bodies which was then dissolved in 8M urea to extract the peptide. The purification profile on SDS-PAGE shows intense band in inclusion bodies (Fig. 3.4). The peptide was then dialyzed against water to remove urea before injecting the sample into HPLC for further purification. HPLC purifies peptides based on polarity and the chromatogram shows one peak at retention time 25 min with a linear gradient of Acetonitrile/water containing 0.1% Trifluoroacetic acid (TFA) at a flow rate of 2 ml/min (Fig. 3.5A). The fractions were collected from the HPLC and a small aliquot was sent for MALDI-TOF MS to analyze the molecular weight. MALDI spectrum shows single peak at 4.5 KDa indicating A β 40 which was then lyophilized (Fig. 3.5B).

Figure 3.4: Purification profile of A β 40

A β 40 expressed in an oligomeric state with bands in higher molecular weight than the expected 4.3KDa. Lane 7 showing a high concentration of A β 40 peptide in inclusion bodies.

Figure 3.5: Purification of A β 40.

(A) HPLC chromatogram of A β 40 at 220nm (B) MALDI spectrum showing pure monomeric A β 40 with a molecular weight ~4.5KDa.

3.2.3. Catalysis of L-PGDS

Enzymatic reaction was carried out to check if the recombinantly expressed L-PGDS retains its basic functional activity. The PGD₂ product formed from the reaction mix was methoximated to make it stable and was analyzed using the PGD₂-MOX kit. The standard graph was plotted, and equation was obtained by linear regression. The amount of PGD₂-MOX from the reaction mix was analyzed from the standard curve. Michaelis-Menten kinetic parameters were calculated by plotting the substrate concentration versus rate of reaction. The Michaelis-Menten constant, K_m was calculated to be 13.88 μ M and V_{max} was found to be 0.72053 μ M/min, thus showing the catalytic activity of L-PGDS (Fig. 3.6).

Figure 3.6: Michaelis-Menten catalysis of L-PGDS

0.2 μ M of L-PGDS was used constantly with varying substrate concentration from 0.3906 μ M to 50 μ M for 3 min in 20mM HEPES, 150mM NaCl, 2mM TCEP (pH-6.5).

3.2.4. Specific binding interaction between L-PGDS and A β 40

In order to specifically identify the residues in the binding interface, 2D ^1H - ^{15}N Heteronuclear single quantum coherence (HSQC) spectrum was recorded for ^{15}N labeled L-PGDS with unlabeled A β 40 and vice versa. ^1H - ^{15}N HSQC is a highly sensitive experiment that displays one peak for each unique proton attached to the nitrogen. Chemical Shift Perturbation (CSP) arises in a HSQC spectrum as a result of changes in the environment of a nuclei when a ligand is titrated to a protein. These changes in chemical shifts can be used to determine the binding interface, ligand affinity or possibly the complex structure [182]. Firstly, HSQC spectrum of free ^{15}N labeled L-PGDS was recorded and then titrated with unlabeled monomeric A β 40 peptide at a molar ratio of 1:4 (Protein:Peptide)

(Fig. 3.7). The resonance assignment for L-PGDS was transferred from Sing Mei Lim et al, [121].

Upon interaction with A β 40, residues D37, L40, S63, L77, L84, A129, K137, R144 and F179 showed changes in chemical shift whereas cross peaks stemming from L55, E57, T73, G76, A99, G100, S104, T115, D126, F163, T188 and Q190 showed major reduction in signal intensity. Additionally, cross peaks from residues A49, N51, S67, L79, T82, T91, S114, V121, T123, Y128, L130, K137, G140, M145, A146, T147, K160, I177 and D184 showed complete disappearance as a result of interaction. In the CSP plot, residues which showed $CSP > 0.03$ are highlighted in red (Fig. 3.8A) and residues which showed intensity ratio (I/I_0) < 0.4 are highlighted in red in the intensity ratio plot (Fig. 3.8B). The residues which showed $CSP > 0.03$, attenuated signal intensity and complete disappearance upon A β 40 binding are mapped on the MD simulation model of L-PGDS-A β 40 complex (3.9A, C, and D).

In contrast, resonances from residues D37, F39, W43, A49, W54, R56, E57, S67, M64, C65, W112, Y116, Y128, G140, D142, R144, T147 and L148 showed chemical shift perturbation upon binding with the substrate analog SA U44069 in addition to the residues T73, G75, G76 and L77 that showed significant enhancement of signal intensities [121]. This clearly demonstrates that catalytic and proximal residues in L-PGDS are not affected by A β 40 binding and that the binding mode is different from substrate binding. Residues showing chemical shift perturbation and increased signal intensity upon substrate analog binding are mapped on the crystal structure of L-PGDS-substrate analog complex (Fig. 3.9B). The excessive signal broadening or loss of signal intensity observed in the spectrum of L-PGDS-A β 40 complex could possibly be due to the intermediate exchange regime

Figure 3.8: Plot for chemical shift perturbation and intensity ratio

(A) Chemical shift perturbation plot for L-PGDS showing residues with $CSP > 0.03$ highlighted in red (B) Bar graph showing changes in intensity ratio (I/I_o) comparing L-PGDS alone and L-PGDS- $A\beta 40$ complex. Residues showing, $I/I_o < 0.4$ are highlighted in red. I_o is the intensity of free protein and I is the intensity of the complex.

Figure 3.9: Mapping of binding interface on L-PGDS structure

(A) MD model of L-PGDS-A β 40 complex with L-PGDS shown in green and A β 40 shown in yellow. Residues which showed CSP > 0.03, major signal attenuation and complete disappearance upon A β 40 binding are highlighted in red, light blue and dark blue respectively. Residues which showed CSP > 0.02, and complete disappearance in A β 40 are highlighted in hot pink and magenta respectively. (B) Crystal structure of L-PGDS-substrate complex in green with residues showing CSP and increased intensity highlighted in red and blue respectively. Substrate is shown in yellow. Possible binding pocket is highlighted in (A) and (B) with a grey circle. (C) Surface representation of L-PGDS-A β 40 complex with the cartoon overlaid on it. Interacting residues highlighted in red and blue in L-PGDS and A β 40 respectively. (D) Zoomed view of the surface representation of L-PGDS-A β 40 complex.

Figure 3.10: ^1H - ^{15}N HSQC NMR titration of labeled monomeric $\text{A}\beta 40$ with unlabeled L-PGDS. Overlaid ^1H - ^{15}N HSQC spectra of free $\text{A}\beta 40$ (blue) titrated with unlabeled L-PGDS (red) at a molar ratio of 1:0.5. Resonance assignments of monomeric $\text{A}\beta 40$ spectrum was obtained from Biological magnetic resonance data bank (BMRB) entry 11435.

In addition, reverse NMR titration was performed for ^{15}N labeled monomeric $\text{A}\beta 40$ and unlabeled L-PGDS to identify the residues in $\text{A}\beta 40$ that are involved in interaction. ^1H - ^{15}N HSQC was recorded for ^{15}N -labeled monomeric $\text{A}\beta 40$ peptide in the absence and

presence of unlabeled L-PGDS at a molar ratio of 1:0.5 (Fig. 3.10). The resonance assignment for the ^1H - ^{15}N HSQC spectrum of monomeric A β 40 was transferred from biological magnetic resonance data bank (BMRB) entry 11435. As observed for ^{15}N L-PGDS-A β 40 titration, many cross peaks have disappeared in the reverse titration. Chemical shift perturbation (> 0.02 ppm) was observed for cross peaks stemming from the residues R5, D23, V24, L34 and M35 while significant reduction in signal intensity was observed for residues V18, F19, F20, A21, E22, D23, V24, G25, S26, K28, G29, A30, I31, I32, G33, L34, M35, V36, G37, G38, V39 and V40.

Figure 3.11: CSP and intensity ratio plot

(A) Chemical shift changes between the free A β 40 peptide and L-PGDS-A β 40 complex. Residues with CSP > 0.02 are colored in orange (B) Bar plot representing the attenuation of cross-peak intensity ratio (I/I_o) of free peptide (I_o) and the peptide-protein complex (I). Residues with $I/I_o < 0.2$ are colored in orange.

Residues that showed CSP > 0.02 ppm and $I/I_o < 0.2$ are highlighted in orange in the CSP plot and intensity ratio plot respectively (Fig. 3.11A, 3.11B). Changes in chemical shift and line broadening or signal disappearance observed in L-PGDS and A β 40 indicates the complex formation. NMR titration results of ^{15}N -labeled monomeric A β 40 peptide with

unlabeled L-PGDS reveals that the hydrophobic C-terminus of A β 40 (residues 18–40) is involved in interaction with L-PGDS. This observation agrees well with the SPR results reported earlier in which all A β fragments containing residues 25-42 showed high affinity binding while A β (1-16) did not bind to L-PGDS [58]. Three-dimensional structure determination of L-PGDS-A β 40 complex was not feasible because of the extensive line broadening observed in the HSQC spectrum upon binding.

3.2.5. L-PGDS binds to A β 40 at 1:1 stoichiometry

In our previous studies, we presented the crystal structure of L-PGDS in complex with substrate analogs which showed that two molecules of substrate analogs bind to L-PGDS at catalytic and peripheral sites with different binding affinities [121].

Figure 3.12: MALDI-TOF MS spectrum of L-PGDS-A β 40 complex

The peak at 19.8 kDa represents L-PGDS, and the peak at 24.1 kDa represents the 1:1 complex of L-PGDS-A β 40 (19.8 kDa + 4.3 kDa). No peak for 1:2 (L-PGDS: A β 40) complex is detected.

To determine the binding stoichiometry of L-PGDS-A β 40 complex, Matrix-assisted laser desorption/ionization–Time of flight Mass spectrometry (MALDI-TOF MS) was used. The results revealed that a small percentage of L-PGDS forms a complex with A β 40 at 1:1 stoichiometry and the complex is adequately stable to endure the desorption/ionization condition in the experiment. In the MALDI-TOF MS spectrum, a peak at 24.1 KDa was observed in addition to the peak at 19.8 KDa which corresponds to L-PGDS. The molecular weight of L-PGDS is ~19.8 KDa and of A β 40 is ~4.3 KDa and so the peak observed at 24.1 KDa corresponds to the L-PGDS-A β 40 complex at 1:1 stoichiometry (19.8 KDa +4.3 KDa = 24.1 KDa). No additional peaks were observed corresponding to L-PGDS-A β 40 complex at 1:2 or higher stoichiometry (Fig. 3.12). Thus, the MALDI-TOF MS spectrum confirms the formation of L-PGDS-A β 40 complex at 1:1 stoichiometry. Alternatively, the peak at 24.1 KDa might have resulted due to a transient interaction between L-PGDS and A β 40 as it occurs for the ATP-independent molecular chaperone, α B-crystallin with α -synuclein [185] and β 2-microglobulin [186].

3.2.6. A β 40 interaction with L-PGDS adds an extra domain

Small angle X-ray scattering (SAXS) was carried out for L-PGDS-A β 40 complex in solution at 1:1 stoichiometry in order to determine the overall shape change in L-PGDS caused by A β 40 binding. SAXS gives information on particle size (D_{max}), radius of gyration (R_g), conformation, assembly state and low-resolution shape. To check if the protein undergoes aggregation at higher concentrations, SAXS of L-PGDS was carried out at concentrations 1.0, 2.5 and 4.0 mg/ml. Guinier plot ($\ln I(q)$ vs. q^2) for these samples appeared linear, which indicates that there is no aggregation or concentration-dependent surge in particle size (Fig. 3.13A). At a protein concentration of 2.5 mg/ml, the

experimental R_g value for L-PGDS calculated from the slope of the linear fit is found to be 17.91 ± 0.32 Å (Fig. 3.13B) (Table 3.1). The distance distribution function ($P[r]$) was obtained by the indirect fourier transformation of the scattering curve. $P[r]$ is a histogram of distances between all possible atoms pairs in a particle.

Figure 3.13: Scattering curves and Guinier plot for L-PGDS

(A) Solution X-ray scattering pattern ($\circ$) of L-PGDS at 1.0 mg/ml (black), 2.5 mg/ml (green) and 4.0 mg/ml (orange) concentration. (Inset) Guinier plots show linearity at all concentrations used, indicating no aggregation. (B) Experimental scattering pattern ($\circ$) and calculated scattering profile of the crystal structure (—; red) of L-PGDS (black) and in complex with A β 40 peptide (blue). (Inset) Guinier plots show linearity. The scattering profiles are offset for clarity by applying arbitrary scale factors.

A slightly right skewed distribution with single local maximum observed in the $P[r]$ of L-PGDS indicates the presence of a slight elongation in the globular protein (Fig. 3.14A). The D_{max} and R_g value of L-PGDS was calculated to be 57 ± 5 Å and 18.04 ± 0.17 Å respectively. The R_g value calculated from the $P[r]$ function agrees well with R_g value calculated from the Guinier plot (Table. 3.1). The normalized kratky plot [152] derived from the scattering curve exhibited a standard globular proteins profile for L-PGDS (Fig.

3.14B). The Porod-Debye plot ($q^4 \cdot I(q)$ vs q^4) produced from the scattering data exhibited a plateau which is characteristic for a compact molecule (Porod exponent being 3.9) (Fig. 3.14C). The molecular weight of L-PGDS calculated based on the volumes obtained from the higher angle of scattering data and the excluded volumes along with the volume of correlation shows that L-PGDS exists in monomeric state in solution (Table. 3.1).

Data collection parameters		
Instrument (source & detector)	Bruker NanoStar equipped with MetalJet eXcillum X-ray source and VANTEC-2000 detector	
Beam geometry	100 μm slit	
Wavelength ($\text{\AA}$)	0.134	
q range ($\text{\AA}^{-1}$)	0.016 – 0.4	
Exposure time (min)	30 (6 frames x 5 min)	
Temperature (K)	288.15	
Protein sample	LPGDS	LPGDS + A β 40
Concentration range (mg ml^{-1})	2.5	2.5
Structural parameters		
$I(0)$ (arbitrary units) [from P(r)]	32.03 ± 0.27	38.76 ± 0.34
R_g ($\text{\AA}$) [from P(r)]	18.04 ± 0.17	19.13 ± 0.22
$I(0)$ (arbitrary units) (from Guinier)	32.09 ± 0.37	38.82 ± 0.38
R_g ($\text{\AA}$) (from Guinier)	17.91 ± 0.32	18.91 ± 0.29
D_{max} ($\text{\AA}$)	57 ± 5	65 ± 5
Porod volume estimate (V_p) ($\text{\AA}^3$)	~ 23000	~ 25900
DAMMIN excluded volume (V_{ex}) ($\text{\AA}^3$)	~ 32452	~ 34251
Dry volume from sequence ($\text{\AA}^3$) ‡	~ 22710	~ 27928
Molecular mass determination		
Calculated monomeric MM (kDa) [from sequence*]	~ 19	~ 23
MM from Porod invariant ($V_p/1.6$) (kDa)	14 ± 2	16 ± 2
MM from excluded volume ($V_{ex}/2$) (kDa)	16 ± 2	17 ± 2
MM from volume of correlation (V_c) (kDa)	20 ± 2	22 ± 2
Software employed		
Primary data reduction	BRUKER SAS	
Data processing	PRIMUS	
<i>Ab initio</i> analysis	DAMMIN	
Validation and averaging	DAMAVR	
Computation of model intensities	CRY SOL	
Rigid body modeling	CORAL	
3D graphics representations	PyMOL	

* http://web.expasy.org/compute_pi/

‡ <http://www.basic.northwestern.edu/biotools/proteincalc.html>

Table 3.1: Data collection and parameters derived from the scattering for L-PGDS and L-PGDS-A β 40 complex.

Figure 3.14: $P(r)$ distribution, Kratky plot and Porod-debye plot for L-PGDS and L-PGDS-A β 40 complex

(A) Pair-distance distribution function $P(r)$ of L-PGDS (black), overlayed with the L-PGDS-A β 40 complex (blue). L-PGDS-A β 40 complex showed an extended tail (represented by the blue arrow). (B) Normalized Kratky plot of L-PGDS (●; black) compared to its complex and the compact globular lysozyme (●; grey) with a peak (---; grey), representing the theoretical peak and assuming an ideal Guinier region of a globular particle. (C) Porod-Debye plot of L-PGDS (○; black) and L-PGDS with A β 40 (○; blue). The presence of Porod-Debye plateau in the plot suggests both proteins are compact in solution.

Using CRY SOL [157], the theoretical scattering curve for the crystal structure of L-PGDS (PDB ID: 4IMN) was calculated. This theoretical curve fitted well with the

experimental scattering data with 0.35 discrepancy (χ^2) (Fig. 3.13B). *Ab initio* reconstruction of L-PGDS yielded ten low-resolution shapes with the normalized spatial discrepancy (NSD) of 0.52 ± 0.02 . These ten low-resolution models were averaged and filtered, and the final model was then overlaid with the crystal structure of L-PGDS with the NSD of 1.05 (Fig. 3.15A).

The low-resolution shape of L-PGDS consists of a globular and a shorter unoccupied domain. The extra density observed in the bead model (indicated by arrow) of L-PGDS suggests that the flexible N and C-terminal residues (which were not found in the crystal structure) might occupy this density. Rigid body modeling was carried out to add the missing residues in N and C-terminus of L-PGDS. The model reconstructed using CORAL, fitted better to the experimental data with a discrepancy (χ^2) of 0.27 than the crystal structure ($\chi^2=0.35$) (Fig 3.15B). The CORAL model was then superimposed on to the low-resolution shape of L-PGDS with the NSD of 1.09.

Upon addition of A β 40, a stable L-PGDS-A β 40 complex was formed without any aggregation as validated by the Guinier plot (Fig. 3.13B; inset). However, the R_g -value was increased to 18.91 ± 0.29 Å, indicating a 1 Å increase in the particle size (Table. 3.1). Moreover, the D_{max} of L-PGDS-A β 40 complex was calculated as 65 ± 5 Å, which indicates 8 Å increase in the overall length. The $P[r]$ distribution of L-PGDS- A β 40 complex showed a long tail with a small bump which signifies the presence of an additional domain (Fig. 3.14A). Additionally, a slight shift observed for the L-PGDS-A β 40 complex from free protein in the normalized Kratky plot is suggestive of elongated shape (Fig. 3.14B). The porod-debye plot of L-PGDS-A β 40 complex, similar to the free L-PGDS, exhibited a

plateau with porod exponent of 3.9. This indicates that the complex is a compact molecule (Fig. 3.14C).

Figure 3.15: CORAL model overlaid with L-PGDS shape and fitting

(A) The averaged and filtered envelope of L-PGDS (grey) from ten independent *ab initio* reconstructions using DAMMIN superimposed (top) onto the cartoon representation of the crystal structure (green; PDB ID: 4IMN) and (bottom) with the CORAL model (cyan). The flexible N- and C-terminal residues in L-PGDS are shown in red. The unoccupied density is represented by an arrow. Front (left) and side (right) views are displayed. (B) Fitting of the CORAL model (—; red) to the experimental scattering pattern (○) for L-PGDS (black) and the L-PGDS-Aβ40 complex (blue).

A big globular domain and an elongated protrusion were observed in the *ab initio* low-resolution shape of L-PGDS-Aβ40 complex with an additional density corresponding to the Aβ40 peptide. A CORAL model of L-PGDS-Aβ40 complex was generated using Anticalin-Aβ40 complex (PDB ID: 4MVI) as a template. Anticalins are the novel type of neutralizing protein reagent engineered based on the human lipocalin scaffold. They are structurally very similar to L-PGDS [161]. This high-resolution structure contains only residues from 16-28 of the Aβ40 peptide and the N- and C-terminal residues are not visible in the electron density map, suggesting they might be flexible. Moreover, the NMR titration

performed above on the ^{15}N labeled A β 40 also showed that the N-terminal 16 residues of A β 40 are not primarily involved in the interaction. Taken together, since L-PGDS is very similar to Anticalins (r.m.s. deviation of 0.97 Å), a model of A β 40 peptide was generated for residues 16-28 in L-PGDS positioning it as in the anticalin structure. The N- and C-terminal residues of the L-PGDS (7 and 15 residues in N- and C-terminals) and A β 40 peptide (15 residues in N-terminus and 12-residues in C-terminus) were allowed to be flexible during model generation. Anticalin is a close homolog of L-PGDS. This CORAL model showed a better fit to the experimental data ($\chi^2 = 0.31$) and is superimposed to the shape calculated from the scattering curve with the NSD of 0.9.

Figure 3.16: The CORAL model of L-PGDS-A β 40 complex overlaid with the shape calculated for the complex.

The averaged and filtered ab initio low-resolution shape of L-PGDS with A β 40 (blue) superimposed (top) onto the cartoon representation of the crystal structure (green; PDB ID: 4IMN) and (bottom) with the CORAL model (cyan). The flexible N- and C-terminal residues in L-PGDS are shown in red and for A β 40 in magenta.

In the CORAL model, the N and C-terminal residues of L-PGDS and the C-terminus of A β 40 were placed inside the larger globular domain whereas the N-terminus of A β 40 is extended occupying the extra elongated density (Fig. 3.16). The normal $P(r)$ distribution, a plateau in the Porod-Debye plot, a peak at the theoretical value in the normalized Kratky plot, a smaller NSD value between the *ab initio* shapes and a good fit to a CORAL model indicate the formation of a compact and rigid L-PGDS-A β 40 complex and hence a single structure was derived for the complex.

3.2.7. Molecular Dynamics simulation of L-PGDS-A β 40 complex

Classical molecular dynamics simulations were carried out with five repeats to reconstruct a representative model for the L-PGDS-A β 40 complex. Random different initial velocities were taken to increase the sampling of the conformational space. Throughout the five repeats of classical molecular dynamics simulations, the plot for the radius of gyration (R_g) of A β 40 displayed a diverse range and dynamic variation between 1.0 to 2.4 nm (Fig. 3.17). The difference in radius of gyration proposes that the A β 40 peptide has the conformational flexibility and is an intrinsically disordered peptide.

Structures corresponding to the A β 40 conformations that showed radius of gyration > 1.95 nm during simulations were extracted. A total of 7 clusters were obtained from the geometric clustering carried out for A β 40 with a 0.8nm clustering cutoff. Among these, the top 5 clusters corresponds to more than 99% of the conformations. Cluster 2 comprises of $\sim 12\%$ of the conformations. The representative structure extracted from cluster 2 is well in agreement with experimental data (Fig. 3.18A).

Figure 3.17: Radius of gyration plot for A β 40

5 simulations of 300ns with random initial velocities were performed for the L-PGDS-A β 40 complex. The last 200ns of each simulation repeat was extracted and combined for radius of gyration calculation.

In the structure shown in Fig. 3.17A, 1-16 residues of A β 40 are not interacting with L-PGDS. The N-terminus of A β 40 is extended as shown in the SAXS model for L-PGDS-A β 40 complex (Fig. 3.16). The residues 18-26 of A β 40 are bound to the entrance of the calyx of L-PGDS whereas the C-terminal residues of A β 40, 29-40 were packed transiently around the shallow groove of L-PGDS, displaying significant conformational changes in packing. The crystal structure of L-PGDS-substrate complex and L-PGDS-A β 40 complex model clearly shows that catalytic and proximal residues in L-PGDS are not affected by A β 40 binding which demonstrates the difference in the mode of binding to L-PGDS (Fig. 3.18A, 3.18B).

Figure 3.18: Model of L-PGDS in complex with Aβ40 and L-PGDS-substrate analog complex

(A) MD simulation model of L-PGDS-Aβ40 complex obtained from molecular dynamics simulation. Structure of L-PGDS is represented in cyan and Aβ40 is represented in yellow. Residues which showed chemical shift perturbation or line broadening upon binding as shown by NMR are highlighted in red and blue in L-PGDS and Aβ40 respectively (B) X-ray structure of the L-PGDS-substrate analog complex from Lim et al. L-PGDS structure is shown in cyan and the substrate analog is represented in yellow. Residues that showed chemical shift perturbation or signal broadening upon binding with the substrate analog are highlighted in red [121].

Average heavy atom distances between the residues of L-PGDS and Aβ40 were used to generate a contact map with a range of 0 (white) to beyond 10 Å (dark blue), in order to apprehend the interaction of L-PGDS with Aβ40. Several light-colored regions, which indicate that L-PGDS and Aβ40 residues are in close proximity, were identified on the contact map (Fig. 3.19). NMR chemical shift perturbation (CSP) identified residues and the residues which showed signal attenuation or complete disappearance highlighted in red for L-PGDS and highlighted in yellow for Aβ40.

Figure 3.19: Contact map of average heavy atom residue distances between L-PGDS and A β 40, with a range from 0 (white) to beyond 10 Å (dark blue).

Distances were calculated using the Gromacs gmx distance tool over simulation frames belonging to the cluster for which the representative model is derived. NMR identified L-PGDS residues are highlighted in red, while NMR identified A β 40 residues are highlighted in yellow. For clarity purposes, NMR identified residues of L-PGDS with average simulation calculated distances of more than 10 Å towards all A β 40 residues are not highlighted, and vice versa. Simulation model identified additional contact sites between L-PGDS and A β 40 are highlighted in Magenta.

For clarity purposes, NMR identified residues of L-PGDS with average simulation calculated distances of more than 10 Å towards all A β 40 residues are not highlighted, and vice versa. It is clear that there are intersections between the NMR identified residues and regions of the contact map where simulation calculated average distances between L-PGDS and A β 40 are low (light colored regions on the contact map), thus showing that our model agrees with experimental NMR data, showing a direct binding between L-PGDS and A β 40 (Fig. 3.18A, 3.19). Moreover, our simulation model is able to propose additional contact sites between L-PGDS and A β 40 (Magenta) (Fig. 3.19). It is important to note that in addition to direct binding, CSP or signal disappearance can also arise as a result of conformational changes in the protein that were induced during ligand binding [182]. Several residues of L-PGDS in which CSP or peak disappearance were detected to be deep within the L-PGDS calyx (Fig. 3.18A). We postulate that the presence of such chemical shifts and the broad loss of NMR resonances are because of the potential allosteric changes in L-PGDS upon A β 40 binding.

3.2.8. L-PGDS inhibits A β aggregation

3.2.8.1. Thioflavin T assay

Thioflavin T (ThT) is a benzothiazole dye, which binds to the amyloid fibrils and emits enhanced fluorescence. It explicitly recognizes the beta-sheet structure of amyloids for binding and so shows low fluorescence with the unstructured monomeric A β 40. ThT assay was carried out for 50 μ M A β 40 and A β (25-35) peptides in the absence and presence of L-PGDS at 37°C to study the inhibitory effect of L-PGDS in A β aggregation. C65A mutant of L-PGDS is also taken for this assay since it is enzymatically inactive and was believed to show no chaperone effect. A characteristic sigmoidal curve involving primary nucleation, secondary nucleation, and fibril elongation [187] was observed for both A β 40 and A β (25-35) peptides indicating the process of aggregation (Fig. 3.20A, 3.20B).

Figure 3.20: Inhibition of A β 40 and A β (25-25) aggregation by L-PGDS

(A) Thioflavin T fluorescence plot for 50 μ M A β 40 (black) treated with 1 μ M (red), 5 μ M WT L-PGDS (blue), 1 μ M (green), 5 μ M C65A mutant (purple) (B) Thioflavin T curve for 50 μ M A β (25-35) (black), treated with 1 μ M (red), 5 μ M WT L-PGDS (blue), 1 μ M (green), 5 μ M C65A mutant (purple). (Inset: Zoomed view).

For A β 40, the fluorescence intensity increased after 8h of lag time and then reached a plateau after 14h of incubation whereas, the curve for A β (25-35) showed a surge in fluorescence intensity after 4h and the plateau was reached after 7h. The fluorescence intensity for A β (25-35) was much higher than the A β 40, suggesting that more fibrils were formed with extended hydrophobic surfaces for better fluorescence. A β 40 peptide incubated with 1 and 5 μ M WT- L-PGDS and C65A mutant displayed significantly reduced fluorescence compared to the untreated control (Fig. 3.20A). The curve for A β (25-35) shows that 5 μ M WT-L-PGDS and 5 μ M C65A mutant is sufficiently enough to completely inhibit the aggregation for at least 20 h (Fig 3.20B).

Figure 3.21: Percentage inhibition of A β aggregation.

Inhibition of A β 40 and A β (25-35) in the presence of 5 μ M WT-L-PGDS (red) and C65A (blue).

The percentage inhibition of A β 40 aggregation by 5 μ M WT-L-PGDS and C65A mutant are ~80% and ~60% respectively. Both WT-L-PGDS and C65A mutant showed 100% inhibition for A β (25-35) aggregation (Fig 3.21). It was earlier reported by *Kanekiyo et al.* that C65A mutant does not inhibit the aggregation of A β 40 emphasizing C65 as a

critical residue in L-PGDS responsible for chaperone activity [58]. However, our result shows that the chaperone activity is still retained in the C65A mutant, although the WT-L-PGDS showed better inhibitory activity than C65A mutant. A β 40 peptide incubated with 1 μ M WT- L-PGDS and C65A mutant (Protein: Peptide (1:50)) also showed ~40% inhibition of aggregation and even at such low concentration, both WT-L-PGDS and C65A inhibited the aggregation of A β (25–35) by 100%.

Figure 3.22: Amylofit plot

The curves acquired for 50 μ M A β 40 in the absence (grey), and presence (blue) of 0.75 μ M WT-L-PGDS fitted using the multistep secondary nucleation dominated model with a mean residual error of 0.00158 in Amylofit. The fluorescence data were normalized before fitting.

The curves acquired for A β 40 incubated with and without L-PGDS were taken for analysis in Amylofit [88] to study the effect of WT-L-PGDS in the microscopic events of A β 40 aggregation. The multistep secondary nucleation dominated model was used for curve fitting. The data showed a proper fitting with a mean residual error of 0.00158 (Fig. 3.22). From the curve, the rate constants for primary nucleation, secondary nucleation, and elongation for untreated A β 40 control were found to be $4.36 \times 10^{-4} \text{ M}^{-1} \text{ h}^{-1}$, $376.9 \text{ M}^{-1} \text{ h}^{-1}$,

and $3.77 \times 10^3 \text{ M}^{-1} \text{ h}^{-1}$ respectively whereas for L-PGDS treated A β 40, the rate constants were calculated to be $3.03 \times 10^{-5} \text{ M}^{-1} \text{ h}^{-1}$, $59.85 \text{ M}^{-1} \text{ h}^{-1}$, and $3.6 \times 10^3 \text{ M}^{-1} \text{ h}^{-1}$ respectively.

The primary and secondary nucleation rate constants calculated for L-PGDS treated A β 40 showed a fourteen-fold and six-fold reduction, respectively compared to the untreated A β 40. The values for elongation are similar implying that L-PGDS does not affect the rate of A β 40 fibril elongation. This result demonstrates the inhibition of primary and secondary nucleation of A β 40 aggregation by L-PGDS. The conclusion proposes that L-PGDS targets the monomers, oligomers, and fibril surface leading to reduced fibril content compared to the untreated control.

Figure 3.23: IC_{50} of L-PGDS for A β 40

(A) Fluorescence plot for 50 μM A β 40 incubated with increasing concentrations of WT-L-PGDS. This data has been used for IC_{50} calculation (B) IC_{50} fitting curve for L-PGDS on A β 40 aggregation ($IC_{50} = 0.98 \pm 0.09 \mu\text{M}$) (S.E.M calculated for $n=3$). The fluorescence values were normalized and then taken for IC_{50} calculation.

ThT assay was carried out for A β 40 with different concentrations of WT-L-PGDS to calculate the IC_{50} (Fig. 3.23A). The end-point fluorescence intensity was taken for IC_{50}

calculation, and the data were normalized. Non-linear curve fitting (Inhibitor vs. normalized response - Variable slope) was used to fit the curve in Graphpad prism 7. The IC₅₀ of WT-L-PGDS for A β 40 aggregation was calculated to be $0.98 \pm 0.09 \mu\text{M}$ (Fig. 3.23B).

3.2.8.2. Bilirubin binding with L-PGDS studied by absorbance scan

The lipophilic-ligand carrier activity of L-PGDS is well established. L-PGDS has been found to bind to heme and heme metabolites with high affinity and acts as a scavenger in the human brain [58, 129, 130, 188]. Higher levels of bilirubin and biliverdin were found in the cerebrospinal fluid of AD patients [189]. It has been hypothesized that these small molecules bind to L-PGDS and possibly affect its A β chaperone activity [58]. Hence, to further investigate if the lipophilic ligand binding affects the chaperone activity of L-PGDS, aggregation of A β 40 and A β (25-35) peptides was monitored in the presence and absence of free WT-L-PGDS protein and WT-L-PGDS-bilirubin (L-PGDS-BR) complex.

Figure 3.24: Bilirubin binds with L-PGDS

UV/Vis absorption spectrum of free bilirubin and L-PGDS-BR complex. Increased intensity and shifted peak maxima observed for L-PGDS-BR in comparison to the free bilirubin control confirms the formation of L-PGDS-BR complex.

Firstly, WT-L-PGDS-BR complex was made, and the binding was confirmed by the absorbance spectrum of bilirubin. The absorbance scan between the wavelength ranges of 350-650 nm is shown for both free bilirubin and L-PGDS-BR complex (Fig. 3.24). The absorbance scan showed maximum absorbance (0.09535) at 436 nm for free bilirubin whereas the L-PGDS-BR complex showed maximum intensity (0.2164) at 446 nm. The spectrophotometric analysis showed that the absorbance increased (hyperchromicity) and a 10nm red shift in the peak maxima was observed for the L-PGDS-BR complex compared to the free bilirubin. Previous reports have also presented increased absorbance of bilirubin at 430 nm and a redshift upon binding to the binding partners [190, 191]. Thus, the formation of WT-L-PGDS-BR complex is confirmed.

3.2.8.3. Inhibition of A β aggregation by L-PGDS-BR

The curve for A β 40 control showed increased fluorescence intensity after ~10h of incubation and reached a plateau after ~20h whereas for A β 40 peptide incubated with free WT-L-PGDS and WT-L-PGDS-BR complex the fluorescence intensity increased after ~40h and ~30 h of incubation and reached a plateau after ~50h and ~40h respectively (Fig.3.25A). For A β (25-35) peptide, the fluorescence intensity increased after ~4h of lag time and reached a plateau after ~8h (Fig. 3.25B). The final fluorescence intensity for A β 40 incubated with WT-L-PGDS and WT-L-PGDS-BR complex was extensively reduced compared to the untreated A β 40 control. The end point fluorescence intensity in each sample was taken for calculating the percentage inhibition. Both WT-L-PGDS and WT-L-PGDS-BR complex showed ~80% inhibition of A β 40 aggregation (Fig. 3.25C). Similarly, for A β (25-35), both WT-L-PGDS and WT-L-PGDS-BR complex showed ~100% inhibition of aggregation (Fig. 3.25C). Both A β 40 and A β (25-35) incubated with free

bilirubin (control) did not show any inhibition. This result indicates that the chaperone activity of L-PGDS is not affected by the bilirubin binding.

Figure 3.25: Inhibitory activity of L-PGDS-bilirubin complex

(A) ThT curve for Aβ40 peptide in the absence (black) and presence of WT-L-PGDS (red), BR alone (green) and L-PGDS-BR complex (blue) (B) curve monitoring the aggregation of free Aβ (25-35) peptide and the samples incubated with WT-L-PGDS (red) and L-PGDS-BR complex (blue) (C) Percentage inhibition of Aβ40 and Aβ (25-35) aggregation by free WT-L-PGDS (blue) and WT-L-PGDS-BR complex (Cyan).

3.2.8.4. Inhibition of Aβ40 aggregation studied by fluorescence microscopy

Fluorescence microscopy images of untreated and WT-L-PGDS treated Aβ40 were acquired to study the effect of L-PGDS on the morphological changes of Aβ40. Untreated

50 μM A β 40 control and 50 μM A β 40 samples incubated with 1, 5, and 10 μM WT-L-PGDS were taken for the study. The fluorescence microscopy images displayed long fibrils for the untreated A β 40 control (Fig. 3.26 (left)) whereas larger amorphous structures were found in the A β 40 sample incubated with 1 μM WT-L-PGDS. And, small amorphous structures were observed in the A β 40 peptide sample incubated with 5 and 10 μM WT-L-PGDS (Fig. 3.26 (right)).

The conversion of A β 40 fibrils into amorphous aggregates in the presence of 1 μM L-PGDS is a common occurrence when molecular chaperones interact with amyloids. For instance, α B-crystallin treated α -synuclein fibrils and β_2 -microglobulin also showed large amorphous aggregates in electron micrographs[185] [186]. This conversion probably occurs because, *in vivo*, amorphous aggregates are more readily broken down by the cellular degradative pathways than the fibrillar ones, and that amorphous aggregates are not cytotoxic.

Figure 3.26: Inhibition of A β 40 studied by fluorescence microscopy

Fluorescence microscopy images (Scale bar: 50 μm) of untreated 50 μM A β 40 (control) and A β 40 incubated with 1 μM , 5 μM and 10 μM WT- L-PGDS (left to right).

These fluorescence microscopy images were used to quantify the length of aggregates in the untreated A β 40 control, and A β 40 peptide samples incubated with varying

concentrations of L-PGDS. The amyloid length was measured using the Image j software. From each sample, more than 100 amyloid structures were taken for investigation. The average length of amyloids in the untreated control and 1, 5, and 10 μM WT-L-PGDS treated A β 40 were measured to be $61.94 \pm 4.48 \mu\text{m}$, and 35.41 ± 3.3 , 10.17 ± 0.69 , and $3.96 \pm 0.71 \mu\text{m}$ respectively (Fig. 3.27A). This confirms that the inhibition of A β 40 aggregation by L-PGDS is concentration-dependent.

The average length of amyloids calculated from each sample is presented as a normal distribution. The normal distribution was plotted using Origin pro-2018 (Fig. 3.27B). The interpretations made from the ThT assay and Fluorescence microscopy images propose that L-PGDS affects both the aggregation kinetics and the morphology of A β aggregates.

Figure 3.27: Quantification of aggregate size using fluorescence microscopy images

(A) Quantification of the average size of the amyloids from untreated A β 40 and 1 μM , 5 μM and 10 μM WT- L-PGDS treated samples (S.E.M calculated for $n \sim 120$). (B) Normal distribution for the length of amyloids formed for 50 μM A β 40 (orange) incubated with 1 μM (green), 5 μM (blue) and 10 μM WT- L-PGDS (magenta) calculated using fluorescence microscopy images.

Additional representative fluorescence images for untreated A β 40 control and A β 40 samples incubated with 1, and 5 μ M WT-L-PGDS are presented in (Fig. 3.28).

Figure 3.28: Fluorescence microscopy images of untreated and L-PGDS treated A β 40

Representative fluorescence images for A β 40 control and A β 40 incubated with 1 μ M and 5 μ M WT-L-PGDS.

3.3. Discussion

Molecular chaperones show a protective role by inhibiting or modulating protein aggregation in neurodegenerative diseases [5]. Among the few proteins studied for their

chaperone function, HSP60 showed inhibition of aggregation by targeting the early oligomeric species, which acts as potential seeds in the aggregation process [192]. Clusterin is an extracellular chaperone, which inhibits the formation of A β fibrils at sub-stoichiometric levels [144] in addition to the sequestration of A β oligomers. Clusterin is a biomarker for Alzheimer's disease and is a potent molecular chaperone and acts similar to that of sHSPs [193]. The eye protein, α B-crystallin is an intracellular small heat-shock protein that is found extensively outside the lens which inhibits the elongation and fragmentation of A β fibrils *in vitro*. Both α B-crystallin and clusterin may perform this function in the brain but this has not been determined experimentally [194, 195]. Human Brichos domains precisely target and prevent the secondary nucleation in the A β aggregation process and thereby controls the human A β 42 toxicity [196]. ApoE and transthyretin proteins have been shown to inhibit A β aggregation [197, 198]. Nevertheless, ApoE is not mainly produced in the CNS [199], while A β is a brain-specific protein [179, 200]. Although some of these proteins may act as major A β chaperones contributing to maintain the A β systemic homeostasis, the hunt for a key brain-specific chaperone provoked us to investigate the mechanism of L-PGDS as a chaperone for both inhibition of A β aggregation and disaggregation of preformed fibrils.

L-PGDS is an important and abundant protein in the brain. L-PGDS knockout mice displayed phenotypes of type II diabetes and symptoms of neurodegeneration [201]. The level of L-PGDS is upregulated in response to stress stimulus suggesting a possible stress protection mechanism of L-PGDS. Additional neuronal apoptosis was observed in the L-PGDS deficient mice which strongly indicates the importance of its protective function in neuronal cells and the surrounding oligodendrocytes [202]. It binds to heme and heme

metabolites with high affinity and specificity [130], potentially stabilizing the deregulated heme metabolism in AD. It is likely to be involved in redox reactions with cytochrome-C and A β -heme complex (to be published) as found in its close homolog lipocalin α 1-microglobulin [203]. It should be noted that some of the antidepressant drugs like diazepam (Valium), the long term usage of which has been linked to dementia [204], bind to L-PGDS at a high affinity [205], perhaps interfering with its neuroprotective function.

In our study, WT-L-PGDS inhibited the aggregation of A β 40 by 80% even at the sub-stoichiometric ratio. The rate constants calculated from the ThT curve using Amylofit shows that L-PGDS inhibits the primary nucleation of A β 40 by interacting with monomers and secondary nucleation of A β 40 by interacting with the fibril surface thus resulting in the reduced final fibril content. NMR titration, SAXS model and the MD simulation model consistently showed the specific interaction of the C-terminus of monomeric A β 40 with L-PGDS. G25-G28, which is a part of the C-terminus of A β 40, are the key residues contributing to the conformational change from monomeric, random coiled structure to the fibrillar, β -sheet structure of A β 40 [206]. Previous SPR results also indicated G25-G28 region in A β 40 as the interaction site for L-PGDS [58]. Here, we postulate that L-PGDS specifically interacts with these residues thereby preventing the conformational change of A β . Our MALDI-TOF MS result reveals that a small percentage of stable L-PGDS-A β 40 complex is formed at 1:1 stoichiometry. However, the A β peptide in the L-PGDS-A β 40 complex holds a substantial degree of conformational flexibility, possibly adopting various conformations which includes extended beta-hairpin as seen in anticalin/A β 40 complex or short helices simulated by our Hamiltonian replica-exchange MD simulations (data not shown).

Our results exhibit efficient inhibition of A β 40 and A β (25-35) aggregation by L-PGDS even at a molar ratio of 1:10, which is consistent with the previous studies [58]. The concentration of L-PGDS under normal condition (26 μ g/ml) in CSF is sufficient to inhibit aggregation and maintain the monomeric state of A β 40 (1.6–17.1 ng/ml in CSF) [131, 207]. However, studies have shown that in AD patients, the levels of β -trace/L-PGDS in the CSF is significantly ($p < 0.05$) reduced compared to the healthy individuals. Normalized quantity of β -trace obtained from 2D gel electrophoresis in healthy control is 14006 ± 5764 whereas in AD, the level is 8879 ± 2197 (mean $\pm$ S.D) [208, 209]. Although several other chaperone proteins like Hsp60, Brichos, and α B-crystallin have shown inhibition of A β aggregation [192, 210, 211], the highly abundance of L-PGDS in the CSF compared to the above mentioned proteins and its ability to inhibit aggregation at substoichiometric ratios, elevate its importance in the A β homeostasis [131, 212].

CHAPTER 4

L-PGDS DISAGGREGATES A β FIBRILS

4.1. Overview on Disaggregases

Considering the significant amount of energy and time required for *de novo* protein synthesis, the reactivation of aggregated proteins is more important for cell survival than the removal of aggregates [213]. A special group of chaperone machineries, named disaggregases, disaggregate the preformed aggregates and restore the protein's native structure, as a defense mechanism of PQC [5, 214]. Thus, the loss-of-function or toxic gain-of-function triggered by the misfolded protein aggregates can be eliminated [215].

The molecular machines, HSP104 and the caseinolytic peptidase B (ClpB) belongs to the AAA+ superfamily are known to disaggregate the insoluble protein aggregates utilizing ATP [216]. They are crucial for cell survival under stress. Additionally, assembly and disassembly of prions are also regulated by HSP104. HSP104 and ClpB collaborates with HSP70 or DnaK chaperones for most of their protein remodeling activities. For instance, through crowbar activity, the larger aggregates are broken down to smaller ones by HSP104/ClpB and subsequently HSP70/DnaK act on the smaller aggregates (Fig. 4.1A).

HSP104/ClpB catalyze the disaggregation and reactivation probably by extracting the unfolded proteins from the aggregates via translocation through the HSP104/ClpB central cavity (Fig. 4.1B). The translocated protein is directly taken over by HSP70/DnaK, to prevent the reaggregation of the extracted polypeptide. Then the polypeptides are released into the cell for spontaneous or chaperone-mediated refolding. The crowbar

activity and the translocation activity of HSP104/ClpB might happen either simultaneously or sequentially [214, 216].

Figure 4.1: Possible mechanisms of disaggregation by HSP104/ClpB

(A) Larger aggregates broken by HSP104/ClpB through crowbar activity (B) HSP104/ClpB extract the unfolded proteins from the aggregates by via translocation activity. Figure adapted from [214].

Although the ClpB/HSP104 system displays an interesting activity, their homologs are not found in systems other than yeast and bacteria. Several studies specify that the maintenance of proteostasis in mammalian cells rely on the cellular programs to control the harmful effects of protein aggregation [217]. Mammalian HSP110 and small heat shock proteins collaborate with HSP70 and HSP40 homologues and facilitate the remodeling of protein aggregates in an ATP-independent manner [218]. These interpretations propose that

the higher eukaryotic cells have evolved different approaches and mechanisms to rescue protein aggregates.

In yeast systems, HSP104 collaborates with HSP70 and its co-chaperone complex with HSP40, and HSP110 to reactivate the aggregated fibrils in yeast, especially prion proteins utilizing ATP as an energy source [219, 220]. The same co-chaperone complex without HSP104 affects the amyloid solubilization in metazoa [33, 221]. Antibodies that showed degradation of misfolded amyloids into smaller units, which can be sequestered out of body, have been examined for their therapeutic potential as disaggregases in AD [222]. Monoclonal antibodies (mAbs) disrupt the fibrillar structure and deteriorate the A β fibril morphology. Antibodies which interact with the N-terminal residues are more effective than those binding to the central (13-28) or C-terminal region [222]. Nevertheless, equimolar amounts of these antibodies are generally essential to observe any significant effect on amyloid morphology [222]. Unfortunately, the antibody Aducanumab failed to treat AD in the clinical trial recently [223]. Taken together, the general mechanism for amyloid disaggregation requires ATP as energy or disruption of non-covalent interactions through non-ATP driven processes.

An example for ATP-independent disaggregase system is a 38-kDa subunit of chloroplast signal recognition particle (cpSRP43) [224]. cpSRP43 is efficient in disaggregating the Light-harvesting chlorophyll a/b-binding (LHC) protein aggregates in an ATP-independent fashion and without the aid of any co-chaperones [224, 225].

In our study, we found that L-PGDS is capable of disassembling A β fibrils even at sub-stoichiometric ratios (1:10 protein:peptide), unlike the antibodies mentioned above. Interestingly, L-PGDS disaggregates the A β fibrils in an ATP-independent manner. In this

chapter, we have demonstrated the unexplored function of L-PGDS as a potential A β disaggregase further reinforcing its role as a major amyloid- β chaperone.

4.2. Results

4.2.1. Thioflavin T assay

Thioflavin T assay was performed for 50 μ M A β 40 and 50 μ M A β (25-35) to establish the potential role of L-PGDS in the disaggregation of A β fibrils. The fluorescence of the amyloid peptides A β 40 and A β (25-35) incubated for spontaneous aggregation reached a plateau at the end of 22 h and 15 h respectively suggesting the formation of fibrils (Fig. 4.2A and 4.2B).

Figure 4.2: Disaggregation of A β 40 and A β (25-35) fibrils by L-PGDS

(A) Thioflavin T fluorescence plotted against time for 50 μ M A β 40 control (black) treated with 5 μ M WT L-PGDS (red) or 5 μ M C65A mutant of L-PGDS (blue), or upon sonication (green) after 22 h (black arrow) of aggregation. (B) Fluorescence plot for A β (25-35) fibrils obtained from 50 μ M monomers over 20 h. The curves represent A β (25-35) fibrils (black), fibrils with the addition of 5 μ M WT-L-PGDS (red) or 5 μ M C65A mutant of L-PGDS (blue) at 15th hour (black arrow) of incubation. The shift in the lag phase onset of the aggregation might be caused by the presence of smaller aggregates which remained even after the solubilization in HFIP.

A sudden reduction in fluorescence intensity was observed for both A β 40 and A β (25–35) peptides upon addition of 5 μ M WT-L-PGDS and 5 μ M C65A mutant of L-PGDS at 22 h and 15 h respectively. Additionally, the fluorescence intensity was reduced further over time and then reached a plateau. The preformed A β 40 fibrils sonicated for a few minutes also showed a similar effect as shown by WT-L-PGDS and C65A mutant. This observation suggests that the breakage of A β 40 fibrils is as effective as the mechanical fracturing of the filaments (Fig. 4.2A). The untreated A β 40 and A β (25–35) peptide controls showed no change in fluorescence intensity. These interpretations suggest that the morphology of A β 40 and A β (25–35) fibrils are modified by L-PGDS. The curve shows that the C65A mutant is equally active as the WT-L-PGDS in dismantling both the fibrils of A β 40 and A β (25–35).

Both WT-L-PGDS and C65A mutant disaggregated the A β 40 and A β (25–35) fibrils within a few minutes of addition, suggesting a rapid disaggregation process. Overall, our results exhibit that the morphology of A β 40 and A β (25–35) fibrils can be altered by both WT-L-PGDS and C65A mutant by fragmenting or disassembling the fibrils.

4.2.2. Transmission electron microscopy

The transmission electron microscopy (TEM) images were acquired for untreated, and WT-L-PGDS treated fibrils of A β 40 and A β (25–35) to study the effect of L-PGDS on the morphology of preformed fibrils. Longer fibrils were observed in the TEM micrographs of untreated A β 40, and A β (25–35) controls, which is typical for these peptides (Fig.4.3A and 4.3B [left]). The preformed fibrils of 50 μ M A β 40 and A β (25–35) were incubated with 5 μ M WT-L-PGDS for few minutes and then coated on the TEM grids to visualize under the microscope. The WT- L-PGDS treated A β 40, and A β (25–35) fibril samples displayed

smaller amorphous structures as seen in (Fig. 4.3C and 4.3D [right]). This observation indicates that WT-L-PGDS disaggregates both A β 40 and A β (25-35) fibrils more efficiently and rapidly.

Figure 4.3: Transmission electron microscopy images

(A) Transmission electron micrographs of mature A β 40 fibrils alone (left) and upon treatment with WT-L-PGDS (right) (B) TEM micrographs showing A β (25-35) fibrils alone (left) and A β (25-35) fibrils treated with WT-L-PGDS (right).

The electron micrographs were further analyzed in the Image j software to quantify the aggregate size in both untreated and WT-L-PGDS treated fibrils of A β 40 and A β (25-35). The average length of the A β 40 and A β (25-35) fibrils with and without L-PGDS treatment were measured and plotted. The average length calculated for untreated A β 40 and A β (25-35) fibrils is 299.95 ± 6.98 nm and 507.91 ± 23.18 nm, respectively. But the average length of L-PGDS treated A β 40, and A β (25-35) fibrils have reduced to 35.70 ± 2.17 nm and 191.55 ± 17 nm, respectively (Fig. 4.4). More than 150 fibrils were taken for analysis.

Normal distribution of the fibril length calculated for the untreated and L-PGDS treated samples of A β 40 and A β (25-35) is depicted in Fig. 4.5A and 4.5B respectively. The decrease in fibril length confirms the disaggregation of A β 40 and A β (25-35) fibrils by L-PGDS. More representative TEM images are shown in Fig. 4.6.

Figure 4.4: Quantification of fibrils using TEM images

Analysis of average length of the A β 40 fibrils (blue) and A β (25-35) (cyan) fibrils from TEM images in the presence or absence of L-PGDS ($n \sim 175$). S.E.M. were plotted.

Figure 4.5: Normal distribution using the calculated average length

Distributions of fibril length for 50 μ M A β 40 and A β (25-35) (orange) and after disintegration by 5 μ M WT- L-PGDS (green) calculated using transmission electron microscopy images.

Figure 4.6: Representative TEM images

Representative TEM images for (A) Aβ40 control (B) Aβ40 fibrils treated with 5 μM WT-L-PGDS (C) Aβ (25-35) control (D) Aβ (25-35) fibrils treated with 5 μM WT-L-PGDS. (Scale bar: 100nm).

4.2.3. Localization of L-PGDS on A β 40 fibrils

TEM micrographs of A β 40 fibrils with and without L-PGDS were captured to identify the site of binding of L-PGDS on A β 40 fibrils. L-PGDS is ~4 nm in size as determined by Dynamic light scattering (DLS) and so might not be visible under TEM. Therefore, L-PGDS was conjugated with magnetoferritin nanocages for easy visualization. Then, the size of the conjugates was measured using DLS. Data were acquired in Malvern Zetasizer Nano series equipment. Free L-PGDS shows a peak at 4nm, whereas the Ferritin conjugated L-PGDS shows a peak at 20 nm, thus confirming the efficient conjugation process (Fig.4.7).

Figure 4.7: Size distribution of L-PGDS and L-PGDS-Ferritin conjugate

The difference in the size of free L-PGDS and L-PGDS-ferritin conjugate shown by Dynamic Light scattering (DLS). Size distribution by a number indicates that peak at 4nm for L-PGDS is moved to 20nm in case of the conjugate.

Furthermore, the chaperone activity of the L-PGDS-Ferritin conjugate for A β 40 was checked by the ThT assay to test the functionality of the conjugate. The ThT results confirmed that the L-PGDS-ferritin conjugate is functionally active and inhibits the A β 40 aggregation. The L-PGDS-ferritin conjugation procedure and the functionality test for the

conjugates were carried out by Bhargy Sharma. The TEM images of the A β 40 fibrils incubated with L-PGDS-ferritin conjugates displayed that more conjugates primarily bind to the growing tips of A β 40 fibrils (Fig. 4.8A) and thereby obstruct the fibril growth and perhaps contribute to the disintegration of the fibrils. The A β 40 fibrils incubated with ferritin alone acted as a control. The micrographs of the control showed that the ferritin alone did not bind to the fibrils or affect the morphology of the fibrils (Fig. 4.8B). When the incubation with L-PGDS-ferritin conjugates is prolonged, the fibrils are broken down (Fig. 4.8C).

Figure 4.8: Localization of L-PGDS on A β 40 fibrils

TEM micrographs (Scale bar: 100 nm) to identify the localization of L-PGDS to A β 40 fibrils. (A) L-PGDS conjugated with ferritin nanocages can be seen localized to the tips of fibrils whereas (B) Ferritin nanocages alone do not interact with the fibrils. (C) With longer incubation time, L-PGDS-ferritin complex breaks the fibrils.

4.2.4. Disaggregation of A β 40 fibrils by L-PGDS studied using High-performance liquid chromatography (HPLC)

The efficiency of L-PGDS to disaggregate the preformed fibrils of A β 40 was further confirmed using standard High-performance Liquid Chromatography (HPLC). The HPLC chromatogram at A₂₇₄ was used for the quantification of the soluble fraction in the untreated

A β 40 fibril control, and the L-PGDS treated A β 40 fibrils. The L-PGDS treated sample was expected to show more soluble fraction (monomers) compared to the untreated control because of the effect of disaggregation by L-PGDS.

Figure 4.9: HPLC chromatogram of untreated and L-PGDS treated A β 40 fibrils

HPLC chromatogram for showing the monomer peak in (A) A β 40 fibril control and (B) A β 40 fibrils treated with WT-L-PGDS.

Firstly, the untreated A β 40 fibril sample was injected into the HPLC, and then the L-PGDS treated A β 40 fibril sample was injected. The HPLC chromatogram showed few peaks, and the samples were collected from each peak, and the molecular weight was measured using a MALDI-TOF MS. The HPLC chromatogram for the untreated A β 40 fibril control showed a monomer peak with $A_{274}=170$ mAU, whereas the L-PGDS treated A β 40 sample displayed a monomer peak with $A_{274}=275$ mAU (Fig. 4.9A, 4.9B).

Moreover, the area under the curve calculated for the monomer peak of the untreated and L-PGDS treated A β 40 samples were found to be 3880 and 4068, respectively. Both the absorbance intensity and the area under the curve for the monomer peak is higher in the L-PGDS treated A β 40 sample compared to the untreated A β 40 control. Our HPLC results

evidently report that the number of monomers in the L-PGDS treated A β 40 sample is higher than the untreated A β 40 control because of the disaggregation property of L-PGDS.

4.2.5. Disaggregation of A β 40 fibrils by L-PGDS studied using Dynamic light scattering (DLS)

Dynamic light scattering (DLS) experiments were carried out for the untreated A β 40 fibril control, and the L-PGDS treated A β 40 fibrils to quantify the size of the aggregates in each sample. All data were acquired in Malvern Zetasizer Nano series equipment. All data obtained from the DLS were an average of three repeats.

Figure 4.10: DLS for untreated and L-PGDS treated A β 40 fibrils

Overlay of the size distribution by intensity for untreated A β 40 fibrils (red) and A β 40 fibrils treated with L-PGDS (blue). The peak at 4nm corresponds to L-PGDS.

The size distribution by intensity for the untreated A β 40 fibrils showed an intense high peak at ~2000 nm, and low-intensity peaks at ~750 nm and ~340 nm representing the presence of more fibrils and fewer oligomers (Fig. 4.10). Nevertheless, the size distribution by intensity for the L-PGDS treated A β 40 fibril sample displayed peaks at ~970 nm, ~270 nm, and ~4 nm (Fig. 4.10). The peak at ~4 nm corresponds to the size of L-PGDS in the L-

PGDS treated sample. The major peak in the untreated A β 40 fibril sample (~2000 nm) has reduced several folds and shifted to ~1000 nm, and ~270 nm in the L-PGDS treated samples. Thus, the DLS results confirm that the A β 40 fibrils are broken down to small oligomers by L-PGDS.

4.2.6. Solubilization of key proteins from the protein aggregates of AD patient brain by L-PGDS

Insoluble protein aggregates were extracted from the human AD brain and treated with L-PGDS to study the disaggregase role of L-PGDS. Firstly, the human AD brain tissue was homogenized in an SDS containing buffer and centrifuged at low speed to remove the cell debris. Then the collected supernatant was spun at high speed using an ultracentrifuge to extract the aggregated proteins which was again washed with the buffer containing 20mM HEPES, 100mM NaCl, 2mM TCEP (pH-7.5).

The resulting protein aggregates are highly insoluble since all the SDS soluble and buffer soluble proteins are already removed in the previous steps. An equal amount of protein aggregates were weighed out and treated with L-PGDS, Hexafluoroisopropanol (HFIP) and formic acid. All the samples were then centrifuged and only the soluble proteins in the supernatant were taken to the next step of proteomics study.

The insoluble protein aggregates treated with L-PGDS resulted in the solubilization of several proteins. HFIP and formic acid treated samples are positive controls. The proteomic analysis of the L-PGDS-treated sample identified around 187 proteins, whereas the HFIP and formic acid treated samples showed 191 and 365 proteins, respectively.

Protein name	Musunuri et al, 2014	Schonberger et al, 2001	Lujian Liao et al, 2004	Our study (Proteins extracted by LPGDS)	Our study (Proteins extracted by HFIP)	Our study (Proteins extracted by Formic acid)
α -crystallin B chain	X	✓	X	✓	✓	✓
α -enolase	✓	✓	X	✓	✓	✓
Glyceraldehyde 3-phosphate dehydrogenase	✓	✓	X	✓	X	✓
Hemoglobin	X	✓	X	✓	✓	✓
Succinyl CoA: 3-ketoacid-coenzyme A transferase	X	✓	X	✓	X	✓
Synaptotagmin	X	✓	X	✓	X	X
Ferritin	✓	X	X	✓	✓	✓
Malate dehydrogenase, cytoplasmic	✓	X	X	✓	X	X
Serum albumin	✓	✓	X	✓	✓	✓
Transketolase	✓	X	X	✓	X	✓
Acetyl-CoA acetyltransferase, mitochondrial	✓	X	X	✓	X	X
Pyruvate kinase	✓	X	X	✓	X	X
L-Lactate dehydrogenase B chain	✓	X	X	✓	X	X
14-3-3 Protein ζ/δ	✓	X	X	✓	X	X
14-3-3 Protein β/α	✓	X	✓	X	X	X
Cathepsin D	✓	X	✓	X	✓	✓
Heat shock protein HSP 90- α	✓	X	X	✓	X	✓
Heat shock 70 kDa protein	✓	X	X	X	✓	✓
Glial fibrillary acidic protein	✓	X	✓	X	✓	✓
Dynamin 1	X	X	✓	✓	X	✓
Dynein , heavy chain 1	X	X	✓	✓	X	X
Profilin-2	✓	✓	X	X	X	X
Phosphofructokinase	X	X	✓	✓	X	X
Clathrin heavy chain	✓	X	✓	✓	X	✓
Dihydropyrimidinase-related protein 2	✓	✓	X	✓	X	✓
Cofilin-1	✓	X	X	✓	✓	✓

Table 4.1: Proteomic analysis of L-PGDS, Formic acid and HFIP treated protein aggregates

Comparison of proteins extracted by L-PGDS, Formic acid, and HFIP from insoluble protein aggregates of AD brain to proteins commonly found in AD brain published in the literature.

L-PGDS was efficient in dissolving few essential proteins like synaptotagmin, 14-3-3 Protein ζ/δ , 14-3-3 Protein β/α , Malate dehydrogenase, Pyruvate kinase, Acetyl-CoA acetyl transferase, L-Lactate dehydrogenase, Profilin-2, Dynein, and phosphofructokinase. Nevertheless, formic acid and HFIP could not solubilize these proteins. 14-3-3 ζ is an abundant protein in the brain which has the chaperone activity to prevent protein aggregation under stress conditions *in vitro* and *in vivo*. Its expression is induced by heat-shock factor-1 [226].

L-PGDS was able to extract out the protein, hemoglobin whose level is found to be significantly increased in AD brain, where it binds to A β fibrils and co-localizes with amyloid plaques [227]. Additionally, proteins such glyceraldehyde 3-phosphate dehydrogenase, L-lactate dehydrogenase B chain, and α -enolase, which were earlier found to be significantly elevated in AD brain [228] were also found in the proteomic analysis of L-PGDS treated sample. Moreover, L-PGDS has solubilized some glycolytic and energy metabolic enzymes such as Acetyl-CoA acetyltransferase, transketolase, serum albumin, phosphofructokinase, and malate dehydrogenase which are also upregulated in the AD brain [228, 229]. In addition, chaperones like HSP 90 protein, α -crystallin B chain that are generally rich in AD in the area of senile plaques [230]; membrane trafficking proteins including dynein, clathrin, and dynamin; and signal transduction proteins such as profilin-2, 14-3-3 β/α , synaptotagmin, 14-3-3 ζ/δ , and Dihydropyrimidinase-related protein 2 were also identified in the L-PGDS-treated samples (Table 4.1).

4.3. Discussion

Our ThT assay, TEM images, HPLC, DLS and proteomics data exhibit the role of L-PGDS as a disaggregase by dismantling the preformed fibrils of A β 40 and A β (25–35).

Disaggregases are the molecular chaperones which aid in disaggregation of the pre-formed fibrils [213]. HSP104 is a well-established protein disaggregase in yeast. It was shown to disaggregate the pre-formed fibrils of α -synuclein which is involved in Parkinson's disease (PD) [215]. The ATP-dependent chaperone, HSP104 collaborates with HSP70 and HSP40 to extricate the unfolded polypeptide chain from the aggregates.

A well-established ATP independent disaggregase that performs without the aid of co-chaperones is the highly conserved serine protease, high-temperature requirement A (HTRA1). HTRA1 has the combine activity of disintegration of aggregates and their degradation. But, the proteolytically inactive variant of HTRA1 (HTRA1^{S328A}) disintegrates the preformed fibrils of tau protein without the aid of any co-chaperones in an ATP-independent manner, unlike HSP104 [28]. In the sedimentation assay, HTRA1^{S328A} disintegrates the preformed fibrils of 4R tau when treated at an equimolar ratio [177]. Quantification of fibrils from the AFM image of the untreated tau fibrils and HTRA1^{S328A} treated fibrils showed that the number of fibrils in the HTRA1^{S328A} treated samples reduced by fourfold compared to the control. However, the AFM images still shows residual fibrils or aggregates [177]. CpSRP43, a disaggregase in plants, reverses the aggregation of LHCP without utilizing ATP by establishing a highly specific interaction with its substrate. As a result of binding of the first CpSRP43 molecule, the aggregated LHCP undergoes a conformational change which supports the stronger binding of the second molecule thereby remodeling the aggregates.

Similar to HTRA1, L-PGDS also doesn't require any co-chaperones or ATP as energy source for performing the disaggregase activity. L-PGDS disaggregates the preformed fibrils of A β 40 and A β (25-35) fibrils rapidly even at sub-stoichiometric ratios

(1:10 (protein:peptide)). HPLC and DLS results further corroborated the disaggregase activity of L-PGDS for A β 40. Furthermore, several key proteins commonly found in AD brain proteome were efficiently solubilized by L-PGDS when added to the insoluble protein aggregates extracted from the AD brain tissue. Proteomic analysis of L-PGDS solubilized plaque proteins resulted in the identification of 187 proteins, which includes chaperone proteins and proteins of different pathways including glycolysis, energy metabolism and signal transduction. The comparison of literature survey as presented in Table. 4.1 and our proteomic data indicates the identification of some novel proteins other than those like glial fibrillary acidic protein, heat shock protein HSP 90- α , heat shock 70 kDa protein and many more, which were identified in AD amyloid plaque by other researchers. Based on this, it can be interpreted that L-PGDS has stronger disaggregation property. These results further reinforce the disaggregase role of L-PGDS.

In this chapter, we hypothesize the possible molecular mechanism by which L-PGDS disintegrates the preformed fibrils of A β . The region G25-G28 in A β is crucial for the conformational change from monomers to fibrils, and these residues are exposed in the fibrils, that contain the bend which connects two β -sheets [206]. Our NMR titration results and the previous SPR study [58] showed that L-PGDS specifically interacts with these residues in the C-terminal of A β . Thus, we postulate that through this interaction, L-PGDS possibly disrupts the non-covalent intermolecular interactions between the fibrils and thereby disassembles them. In the ThT assay, sonication and L-PGDS treatment of A β 40 fibrils showed similar effects suggesting that L-PGDS directly interacts with the amyloid fibrils and results in fibril remodeling instead of passively shifting the monomer/fibril equilibrium by sequestering the monomers. L-PGDS can disaggregate A β 40 fibrils through

specific binding interactions, without the aid of co-chaperones and ATP consumption, a mechanism similar to the molecular chaperone CpSRP43 [224].

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

5.1. Summary

Inhibition of A β aggregation and disintegration of preformed A β fibrils might be an excellent way to control or treat AD. In our study, the brain-specific protein, L-PGDS have shown both the inhibitory and disaggregation activities for A β aggregation. Although several proteins were found to have similar functions, L-PGDS has certain unique properties that differentiate it from the other proteins. Unfortunately, the levels of L-PGDS in CSF is significantly reduced in AD patients compared to healthy control [209].

The reduced estradiol level in the postmenopausal depression, a risk factor for AD, provokes reduced expression of L-PGDS [231]. Additionally, the C-terminal exon 6-truncated L-PGDS found in AD is believed to be yet another risk factor for AD [58]. The deletion of C-terminus results in the formation of unfolded or inactivated L-PGDS which might possibly affect the chaperone activity [232]. Thus, it is rational to propose that the disturbances in the function of L-PGDS might result in the sporadic AD. High-affinity recombinant L-PGDS or drugs that increase the affinity of L-PGDS for A β can be designed using the high-resolution crystal structure of L-PGDS as a template. Fascinatingly, the APP transgenic mice showed enhanced expression of L-PGDS and decreased deposition of A β in an enriched environment compared with standard animal housing conditions [233].

Till date, 17 important human proteins and protein complexes have exhibited a possible neuroprotective function as amyloid- β chaperones (Table. 5.1). They exhibit a different mode of action such as targeting primary or secondary nucleation, disaggregating

amyloid bundles, delaying fibril growth and so on. Some of the chaperones are intracellular and some are secreted. Some are ATP-dependent while some are ATP-independent for their action. The levels of expression of these chaperones in various brain areas such as cerebral cortex, caudate, hippocampus, and cerebellum [234] vary significantly, reflecting their relative contribution to the neuroprotection.

Protein	Inhibit primary nucleation	Inhibit secondary nucleation	Inhibit Fibril elongation	Dis-aggregate bundle of fibres	Delay fibril growth	ATP dependency	Part of a large complex	RNA expression levels in Brain (TPM)	Location	Cerebral cortex	Hippocampus	Caudate	Cerebellum
L-PGDS	Y	Y	N	Y	Y	N	N	1224.7	intracellular, secreted	high in neuronal cells, high in endothelial cells	medium in glial cells, low in neuronal cells	low in neuronal cells, low in glial cells	medium in cells in molecular layer, low in cells in granular layer, low in purkinje cells
Clusterin (ApoJ)	Y	Y	Y	Y	Y	Y	Y	10875.4	intracellular, secreted	medium in neutrophils	medium in neuronal cells	not detected	not detected
α B-crystallin (sHsp)	Y	Y	Y	-	-	Y	Y	1888.7	intracellular, membrane	high in glial cells, high in neutrophils	high in glial cells	high in glial cells	medium in cells in granular layer
Hsp60	Y	Y	Y	-	Y	Y	Y	141.6	intracellular	low in neuronal cells, low in glial cells	low in neuronal cells, low in glial cells	low in neuronal cells, low in glial cells	low in purkinje cells
Hsp 70/40	Y	Y	Y	N	Y	Y	Y	5	intracellular	low in neuronal cells, low in endothelial cells, low in glial cells	low in neuronal cells, low in glial cells	low in neuronal cells, low in glial cells	low in cells in molecular layer, low in cells in granular layer, low in purkinje cells
Hsp 90- α	Y	N	Y	N	-	Y	Y	1021.5	intracellular secreted	low in neuronal cells	not detected	not detected	low in purkinje cells
Pyruvate kinase	Y	-	-	N	Y	Y	N	749	intracellular	medium in neuronal cells, medium in neutrophils	low in neuronal cells, low in glial cells	medium in neuronal cells	medium in cells in molecular layer, low in cells in granular layer
Transferrin	N	Y	Y	-	-	N	N	385.9	intracellular secreted	low in neutrophils	not detected	low in neuronal cells	not detected
Transthyretin	Y	Y	Y	-	Y	N	Y	6.9	secreted	not detected	not detected	not detected	not detected
α 2-Macroglobulin	Y	-	N	N	Y	N	N	172	secreted	low in neuronal, high in endothelial cells	low in neuronal cells	not detected	low in purkinje cells
Catalase	N	-	-	Y	Y	N	Y	47.2	intracellular	not detected	not detected	not detected	not detected
Lysozyme	N	Y	-	N	Y	N	N	22.5	secreted	not detected	not detected	not detected	not detected
Brichos domain containing proteins	N	Y	N	-	Y	N	N	7	intracellular, membrane	not detected	not detected	not detected	not detected
Haptoglobin	Y	-	N	N	Y	N	N	1.8	intracellular secreted	not detected	not detected	not detected	not detected
Serum Albumin	N	Y	Y	-	Y	N	N	1	intracellular secreted	low in endothelial cells	low in glial cells	not detected	low in molecular layer
Apolipoprotein A1	-	Y	Y	-	-	N	N	0.1	intracellular secreted	not detected	not detected	not detected	not detected

Table 5.1: Comparison of chaperone activity for traditional chaperones and non-chaperone proteins that affect amyloid- β aggregation. RNA expression levels are Courtesy of Human Protein Atlas, www.proteinatlas.org, (Y) - Yes, (N) - No, (-) Not known.

L-PGDS is a unique chaperone that is ubiquitously expressed in various parts of the brain including cerebral cortex, hippocampus, caudate, and cerebellum where amyloid deposits are also concentrated. L-PGDS effectively inhibits the primary and secondary nucleation by targeting the monomers and fibril surface of A β . L-PGDS inhibits the aggregation of A β 40 by 80% even at the sub-stoichiometric ratios. L-PGDS specifically interacts with G25-G28 of A β 40 and thereby prevents the conformational change of A β . Moreover, the high abundance of L-PGDS in the CSF compared to other chaperone proteins uplift its influence on the A β homeostasis[131, 212] [131, 212].

Additionally, L-PGDS also disintegrates the preformed fibrils of A β through specific interaction without the help of any co-chaperones in an ATP independent manner. The disaggregation of preformed A β 40 and A β (25-35) fibrils is rapid even at sub-stoichiometric ratios. On the whole, the novel disaggregase property and the inhibitory activity of L-PGDS described in this thesis explains the mechanism for its previously suggested role as a major endogenous A β chaperone. Thus, our results offer more understanding of the AD pathogenesis and also a possible therapeutic approach for AD.

5.2. Future directions

Some of the future directions of this work can be:

1. The precise molecular mechanism underlying the disaggregation of A β fibrils by L-PGDS must be explored using the Solid-state NMR studies of untreated and L-PGDS treated fibrils.
2. Additionally, Cryo-EM can be used to determine the structure of the A β 40 fibrils in the presence and absence of L-PGDS.

3. Additional NMR experiments (e.g. Relaxation experiments) to determine the backbone dynamics of ^{15}N labelled L-PGDS in the presence and absence of A β 40 would be useful to nail down the interaction site(s) on the former with A β 40. Potentially, the same experiments could be undertaken with ^{15}N -labelled A β 40 to determine its interaction site(s) with L-PGDS.
4. Synergistic effect of L-PGDS with other extracellular chaperones (e.g. clusterin) can be studied for inhibition and disaggregation of A β 40 fibrils, and other amyloids.
5. The chaperone activity of L-PGDS has to be established for other amyloidogenic peptides such as Tau (another major peptide involved in AD) and shorter amyloidogenic peptides from TGFBIp (implicated in corneal dystrophy). As a preliminary study, we chose the Wild type (TGFBIp-E⁶¹¹PVAEPDIMATNGVVHVITNVLQ⁶³²) which is enriched in LCD, and the mutants N622K, N622H, and G623R peptides for ThT assay. L-PGDS showed the capability to effectively inhibit all four peptides in sub-stoichiometric ratios.

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