Developing amyloid- β chaperone lipocalin-type prostaglandin D synthase protein as a magnetic resonance active early diagnostic tool

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**DEVELOPING AMYLOID- β CHAPERONE
LIPOCALIN-TYPE PROSTAGLANDIN D SYNTHASE
PROTEIN AS A MAGNETIC RESONANCE ACTIVE
EARLY DIAGNOSTIC TOOL**

BHARGY SHARMA

SCHOOL OF BIOLOGICAL SCIENCES

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BHARGY SHARMA

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological University in
partial fulfilment of the requirement for the degree of Doctor of
Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Assoc. Prof. Konstantin Pervushin

Authorship Attribution Statement

This thesis contains material from 1 paper(s) published in the following peer-reviewed journal(s) / from papers accepted at conferences in which I am listed as an author.

Table 1.1 is published in Kannaian, B., Sharma, B., Phillips, M. et al. Abundant neuroprotective chaperone Lipocalin-type prostaglandin D synthase (L-PGDS) disassembles the Amyloid- β fibrils. Sci Rep 9, 12579 (2019) doi:10.1038/s41598-019-48819-5.

K Bhuvaneswari performed the experiments and wrote in the manuscript.

I prepared the L-PGDS conjugates and performed experiments on the conjugates and wrote the manuscript.

Dr Margaret Phillips captured TEM images and wrote the manuscript.

Dr Malathy and Dr Gerhard Gruber performed and analyzed SAXS experiments.

Dr Sunil and Dr Siu K. Sze assisted with proteomics and analyzed the data.

Justin and Dr Mu Yuguang performed the molecular dynamic simulations.

Dr Ambrish and Dr Sierin Lim provided the ferritin samples.

Dr Konstantin Pervushin devised the experimental plan and supervised the study and manuscript preparation.

06-Dec-2019

Date

Bhargy Sharma

Dedicated to my parents...

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Abbreviations

3xTg	Triple transgenic Alzheimer's Disease
α Syn	α -synuclein
AD	Alzheimer's Disease
ADDL	Amyloid β - derived diffusible ligand
Ala	Alanine
ALS	Amyotrophic Lateral Sclerosis
APOE	Apolipoprotein E
APP	Amyloid Precursor Protein
Asn	Asparagine
ATP	Adenosine triphosphate
A β	Amyloid β
BACE	β -site APP cleavage enzyme
BBB	Blood-brain barrier
CA	Contrast Agent
CJD	Creutzfeldt-Jacob disease
CNR	contrast-to-noise ratio
CNS	Central Nervous System
COOH	Carboxyl
CPMG	Carr-Purcell-Meiboom-Gill
CSF	Cerebrospinal fluid

CT	Computed Tomography
Cys	Cysteine
DLS	Dynamic Light Scattering
DMSO	Dimethyl sulfoxide
DNP	Dopamine coated iron oxide nanoparticles
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EGCG	Epigallocatechin gallate
FAD	Familial Alzheimer's Disease
FDA	Food and Drug Administration
FLASH	Fast Low Angle Shot
FTD	Frontotemporal dementia
ftnAA	Ferritin nanocages
GF	Gel filtration
Gln	Glutamine
Glu	Glutamic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hsp	Heat shock protein
htt	Huntingtin
IAPP	Islet amyloid polypeptide
IMAC	Immobilized metal affinity chromatography
IONP	Iron-oxide nanoparticle

kDa	kilo Dalton
LA	LPGDS-ftnAA conjugate
LD	LPGDS-DNP conjugate
LM	LPGDS-MNS conjugate
L-PGDS	Lipocalin type prostaglandin D2 synthase
LS	LPGDS-SMNP conjugate
MAPT	Microtubule- associated protein tau
MES	2-(N-morpholino) ethane sulfonic acid
MNS	PEG-coated Magnetic Nanostructures
MR	Magnetic Resonance
MRI	Magnetic Resonance Imaging
MSME	Multi-Slice Multi Echo
MTT	3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
NaCl	Sodium Chloride
NMR	Nuclear Magnetic Resonance
NTA	Nitrilotriacetic acid
OD	Optical density
PBS	Phosphate-buffered Saline
PD	Parkinson's Disease
PEG	Polyethylene glycol
PET	Positron Emission Tomography

PGD2	Prostaglandin D2
PGH2	Prostaglandin H2
Phe	Phenylalanine
PLGA	Poly D, L- lactic-co-glycolic acid
PrP	Prion protein
PrP ^C	Cellular prion protein
PrP ^{Sc}	Scrapie isoform of the prion protein
PS1	Presenilin 1 gene
PS2	Presenilin 2 gene
RARE	Rapid Acquisition with Relaxation Enhancement
RF	Radiofrequency
ROI	Region of interest
SDS	Sodium Dodecyl Sulfate
SE	Spin echo
SEC	Size exclusion chromatography
sHsp	Small heat shock protein
SMNP	Silica-coated magnetic nanoparticles
SOD	Superoxide dismutase
SPECT	Single Photon Emission Computed Tomography
sulfo-NHS	N– hydroxy sulfo succinimide
T2*	T2 star

TCEP	Tris (2-carboxyethyl) phosphine
TE	Echo time
TEM	Transmission Electron Microscopy
ThT	Thioflavin T
TR	Repetition time
Tyr	Tyrosine
UPS	Ubiquitin proteasome system
US	Ultrasound
Val	Valine
WT	Wild type

Abstract

Lipocalin-type prostaglandin D synthase (L-PGDS) is an endogenous brain protein, shown previously as a significant chaperone for amyloid β (A β) peptide. It binds to monomeric A β as well as mature fibrils and is capable of inhibiting their aggregation. We recently showed that L-PGDS could successfully breakdown mature A β fibrils (manuscript submitted), which opens avenues for its use as a therapeutic agent in cases of Alzheimer's disease (AD).

This thesis is aimed at utilizing the theranostic (therapeutic + diagnostic) potential of L-PGDS in AD by early detection of A β using MRI. For this purpose, we covalently conjugated the recombinant L-PGDS protein with iron oxide-based nanoparticles with different outer coatings. Four different nanoparticles were compared for their T2 contrast enhancement. The functionality of the conjugated protein, its inhibition activity towards A β , the effect on cellular viability and the tendency to form aggregates were compared to choose the most efficient composition. For this purpose, we used a multi-disciplinary approach using chemical and biophysical assays, spectrophotometric techniques, structural and morphological studies, cellular assays and tissue histology, and pre-clinical mouse imaging to develop an L-PGDS-based theranostic molecular probe. Based on the results of *in vitro* assay, the biological probes designed by covalent conjugation of magnetic resonance active ferritin protein nanocages conjugated with L-PGDS were used for studies in AD mice. Injections in diseased mice showed hypointensity in mouse brain areas correlated with the presence of amyloid-rich structures compared to age-matched healthy mice.

All MRI data were acquired using Bruker spectrometers, and Paravision 6.0 software was used for processing and analysis. T2 relaxation curves were prepared for nanoparticles to compare relaxivity differences in the biological buffer. Differences in T2-star weighted image intensities were identified in the brains of healthy mice after non-invasive intranasal administration of conjugated L-PGDS probes. L-PGDS conjugated with ferritin nanocages injected in the ventricular chambers were seen to disperse towards the amyloid-rich brain regions in AD mice. To this end, we have shown here that L-PGDS protein has great outlook as a diagnostic agent for early identification of AD hallmarks in disease-prone populations, and potential as a therapeutic intervention.

Chapter 1

1. Introduction

According to the Alzheimer's Disease International, a new person develops dementia every 3 seconds. Early detection of dementia before its clinical manifestation can play a significant role in prevention and treatment of the related neurodegenerative disorders. In this chapter, a general background is provided for the rationale of this thesis work. It begins to establish the phenomenon of amyloidosis and its role in neurodegeneration. This is followed by a brief description of the Alzheimer's disease which comprises 70% of dementia cases and the role of amyloid β in its progression. Morphological and pathological changes in amyloid structures upon aggregation are delineated in further detail to relate the importance of its regulation by chaperone proteins to protect against disease pathology. A comprehensive understanding of the endogenous brain protein Lipocalin-type Prostaglandin D Synthase which acts as an extracellular chaperone for amyloid β is presented. A detailed review of the current detection modalities and advancements in the therapeutics is also provided in this context. It is a preamble to our detailed work for the development L-PGDS as a diagnostic tool with potentially therapeutic outlook.

1.1. Amyloidosis

Amyloids are misfolded or partially folded state of proteins which form elongated unbranched fibres with a cross- β fibre diffraction pattern, first seen by William Astbury in an egg white fibre [1]. Named as such due to misidentification as polysaccharides by Rudolph Virchow in 1854, the predominantly protein composition in the amyloids was later demonstrated by Friedrich and Kekulé. In eukaryotic biological systems, many amyloids are functional proteins which often contribute to the biofilm production, biogenesis of melanosomes, binding and storage of peptide hormones, long-term memory formation, sperm selection, and generation of the innate immune response to viral infections [2]. These proteins attain their amyloid state when the free energy barrier favours the formation of hydrogen bonds between the exposed amino groups and carboxyl groups, either by protein denaturation, improper folding, cleavage of properly folded peptides, or by the overproduction of intrinsically disordered proteins [1]. Amyloid formation or ‘Amyloidosis’ begins with the process of nucleation whereby few molecules come together with their amyloid-forming regions exposed at enough concentration for bond formation to generate a template for the structure of fibril backbone. This process usually requires a specific volume to begin and introducing a nucleus or ‘seed’ externally to fasten this process. Following this nucleation, individual molecules subsequently attach to the growing fibril, thus leading to a more rapid growth phase. The protofilaments usually form a dry steric zipper where the side chains in adjacent sheets interact tightly and render the interface devoid of water. A protofilament typically comprises up to six parallel or anti-parallel β -sheets with an inter-sheet distance of roughly 10Å [3]. They can be arranged in a twisted pattern, as flat ribbons, cylinders, and even as pseudo-crystalline sheets [4]. The amyloid production in mammals is usually associated with ageing and related proteinopathies.

1.1.1. Aggregation of amyloid proteins

Though compositionally different, amyloids are usually unbranched structures which can extend up to micrometre in length and few nanometres in diameter, with hydrogen bonds stabilizing the fibrillar structures. These aggregated species are insoluble in detergents, resistant to protease and bind to specific dyes like Thioflavin and Congo red. Different types of amyloid structures are produced

during the course of aggregation, whether on pathway to fibril formation or off-pathway amorphous aggregates (**Figure 1.1**).

Figure 1.1. Amyloid structures in the aggregation pathway. Various aggregates are produced during the kinetic process of fibrillation of monomeric amyloid peptides. The misfolded monomers interact to form small amyloidogenic oligomers which give rise to amyloid seeds on-pathway to mature fibril formation. These seeds catalyse the formation of filaments, fibril elongation, and bundling of mature fibrils. Unstructured amyloid aggregates are formed off-pathway due to the non-specific aggregation of denatured monomers and globular aggregates. These aggregates may also contribute to seed formation to promote the fibrillation process. Figure from David Talaga [5].

Nucleation is a stochastic process which causes the amyloids to behave differently even under similar experimental conditions [1]. The fibrillation process can be kinetically separated into a lag phase, log phase, and saturation phase. In nucleation-dependent fibril formation, the kinetic plot resembles a sigmoidal curve. In nucleus independent fibrillation, the kinetics is represented as a simple exponential increase which can be used to mathematically derive the rate of aggregation [3]. The nucleation dependent polymerization involves a lag phase in which the amyloid monomer converts into unstable misfolded species, or a kinetically favourable multimeric nucleus. Following the formation of this ‘nucleus’, a rapid polymerization process leads to the formation of aggregates in the growth phase [6]. The process of seeding accelerates the aggregation process

by addition of new nuclei to already formed oligomers, thus avoiding the lag phase.

1.1.2. Amyloids in human diseases

Nearly fifty different peptides and proteins are capable of forming disease-associated amyloid fibrils in the human body [1]. These amyloid fibrils share a typical architecture which comprises a cross- β quaternary structure within each protofilament, wherein the β -strands align perpendicular to the fibril axis with an approximate 4.7-4.8Å repeat along the axis [4]. The production of amyloids under laboratory conditions usually involves partial denaturation either with a destabilizing agent, change in pH, high temperature or by agitation [1]. Amyloid fibrils from different proteins are associated with clinical neurodegenerative disorders: Amyloid- β (A β) and microtubule-associated protein tau (MAPT) in cases of Alzheimer's Disease (AD) and Frontotemporal Dementia (FTD), Prion protein (PrP) in transmissible spongiform encephalopathies such as Creutzfeldt-Jacob disease (CJD), polyglutamine protein huntingtin (htt) in case of Huntington's disease, α -Synuclein (α Syn) in Parkinson's disease (PD), superoxide dismutase (SOD) and others in Amyotrophic Lateral Sclerosis (ALS) [4]. Moreover, the human Islet Amyloid Polypeptide (IAPP) or amylin aggregation is associated with non-neuropathic Type II Diabetes. AD pathology results from an imbalance between A β production versus its clearance, which leads to its accumulation in the body in an unusually high amount. In non-demented control brains, the most abundant A β species in the cerebrospinal fluid (CSF) is the peptide fragment containing the first 40 residues, herein mentioned as A β (1-40), which accounts for 50% of A β in the brain, followed by 16% of A β (1-38). The most aggregation species, A β (1-42) contributes 10% to the total content, while the remaining fraction comprises other A β species with 34, 37, 39 or 43 residues or those with 17-20 residues [7]. A β content in AD mainly includes N-terminally truncated A β , with cleavage sites at residues 2 to 11, which readily form aggregates and contribute to neuronal toxicity. The molecular structure of A β (1-40) fibrils found in AD brains comprise either two-fold or three-fold symmetry along the fibril axis (**Figure 1.2**) [7, 8]. Some amyloidogenic proteins can form structurally different polymorphic fibres through packing

polymorphism, segmental polymorphism, or formation of heterosteric zippers [1].

Figure 1.2. Molecular structure of A β (1-40) fibrils. A) 3-fold symmetry about fibril growth axis in A β fibrils isolated from the brain of an AD patient (PDB: 2M4J); B) 2-fold symmetry about fibril growth axis in A β (1-40) fibrils bearing Osaka deletion mutation E22 Δ related to early-onset AD (PDB: 2MVX) [7, 8].

In PD, the self-replicating α Syn protein forms Lewy bodies in the cytoplasm, which lead to degeneration of dopaminergic neurons [9]. α Syn protein aggregates belong to three major groups based on whether they are primarily disordered, rich in α -helices, or rich in β -sheet structures. In α Syn on-pathway oligomers rich in β -sheet structures, the residues 40-80 are protected in the hydrophobic core. Aggregates rich in α -helical content are formed in the presence of lipid membranes, and sometimes in early stages of α Syn aggregation [10]. N-terminal fragments of htt protein are prone to aggregation into a β -sheet rich fibrillar structure owing to trinucleotide repeat in the first exon of the encoding gene. Above the threshold of 35 glutamine residues, the polyQ expanse endows pathogenicity to the aggregated protein, which contributes to neuronal damage and dysfunction [11].

The cellular prion protein (PrP^C) is a cell surface glycoprotein present on the neurons which functions as an antioxidant to support cellular survival [12]. The scrapie isoform of PrP (PrP^{Sc}) is a protease-resistant insoluble disease-associated isoform which causes transmissible neurodegeneration by propagating as a β -sheet rich aggregated protein by imposing its conformation onto the PrP^C of the

host [13]. The gene involved in Huntington's disease contains a CAG trinucleotide repeat which leads to polyglutamine stretch, conferring a gain-of-function phenotype to the expressed protein to attain cross- β steric zipper which renders neuronal toxicity [14]. Aggregated polyglutamine proteins form polar zippers composed of antiparallel β -sheets. Mutations in copper-dependent protein SOD1 account for approximately 20% cases of ALS [14].

1.1.3. Alzheimer's disease

The neuropathological lesions observed by Alois Alzheimer in the post-mortem brain of his demented patient Auguste Deter, which was the first-ever record of this disease over a century ago in the year 1907, brought the extent of drastic degenerative effects of AD into common understanding [15]. The hallmarks of this malady are senile plaques which are extracellular depositions of $A\beta$, intraneuronal neurofibrillary tangles comprising hyperphosphorylated tau as well as neuritic loss and synaptic damage in brain regions including the hippocampus, mediotemporal cortex, and neocortical structures. Accepted pathophysiological causes of AD include $A\beta$ and tau aggregation, neurotransmitter deficits, inflammation, oxidative damage, and neuronal degeneration. The progression of AD is accompanied by memory defects, and impediment of cognitive and intellectual abilities, including executive function, recognition, language, and praxis. AD is clinically separated into two types depending on its genetic basis- (a) Early-onset AD or familial AD, and (b) Late-onset AD or sporadic AD, which makes up most cases. Familial AD pathology is mediated by mutations in APP gene, or the Presenilin protein I and II encoding genes Presenilin 1 (PS1) and Presenilin 2 (PS2) respectively, while a $\epsilon 4$ allele of the only confirmed susceptibility gene Apolipoprotein E (APOE) is known to confer typical late-onset type of AD [14]. Soluble $A\beta$ levels in the brain correlate more strongly with AD progression compared to insoluble plaques. It is known that the development of $A\beta$ plaques can precede the onset of cognitive decline and incidence of tau pathology by over two decades. Characteristic lesions in AD comprise of extracellular amyloid plaques which may be diffuse or neuritic and intracellular tau tangles. In the case of sporadic AD, the underlying cause may be a complex amalgamation of genetic, epigenetic, and environmental factors.

Depending on the age at onset and the duration of disease, AD has three major categories- typical AD, Posterior cortical atrophy, and early-onset AD. For the typical AD, the age of onset nears 65 years and duration of disease extends over a decade. On the other hand, posterior cortical atrophy AD onsets between ages 50 and 65. In the early-onset AD, the clinical symptoms for the patients occur as early as in their 40s and 50s, sometimes as a result of genetic predisposition known as familial AD. In all these cases, there are three recognized stages of disease progression, namely, preclinical stage followed by mild cognitive impairment, which ultimately escalates to dementia with functional impairments.

A significant attempt to explain AD was the amyloid cascade hypothesis which states “deposition of A β protein, the main component of the plaques, is the causative agent of Alzheimer's pathology and that the neurofibrillary tangles, cell loss, vascular damage, and dementia follow as a direct result of this deposition” as postulated by John Hardy and Gerald Higgins in 1992 [16]. According to this hypothesis, the point mutations in genes such as APP, PS1 or PS2 leads to overproduction and accumulation of A β , further causing its oligomerization and deposition as plaques. It emphasizes the critical role of A β aggregation in disease progression and pathology [17]. The A β aggregates have detrimental impact on the synapses leading to neuritic injury, prompt activation of microglia and astrocytes, alter neuronal homeostasis and cause oxidative stress. Furthermore, these amyloids alter the activity of enzyme kinases which lead to the formation of tau tangles. However, many researchers challenge this central role of A β and posit that the primary culprit could be tau or the collapse of homeostatic network [18]. Even considering the part of A β , some speculate that the soluble amyloid oligomers or plaques mediate neuronal damage through a gain-of-toxicity, while others note that the loss-of-function phenotypes resulting from mutations in native proteins such as TDP-43, which convert into amyloidogenic structures due to improper folding, are the principal perpetrators of the cellular toxicity [2].

1.2. Amyloid β

A β peptide is produced when the Amyloid Precursor Protein (APP) is cleaved by two aspartyl proteases, namely, α -secretase and β -secretase, followed by subsequent action of γ -secretase that generates A β peptides with 34-50 amino

acids found in most cases of sporadic AD [19]. α -secretase function usually leads to non-amyloidogenic peptide chains mediated by cleavage between residue positions 16 and 17 within the A β domain. The amyloidogenic β -secretase pathway involves the membrane-bound β -site APP cleavage enzyme (BACE1) cleaving APP at the Aspartic acid residue at the position 1 of A β peptide, which may be followed by additional cleavage between residues 10 and 11 [19]. γ -secretase is an intramembrane multi-subunit complex of multiple proteins which mediate cleavage within the transmembrane domain of APP, thus forming A β peptides which are imprecisely truncated at C-terminus to generate a sequence of 37-43 residues and released into the CSF.

A β peptide were first sequenced nearly four decades ago from the meningeal blood vessels in AD patients [20]. A β (1–42) is a more potent A β variant for fibrillation in the human brain. However, its concentration accounts for only about 5–10% of A β (1–40) produced [21]. Accumulation of A β (1–42) in the brain, and by extension, the relative ratio of A β (1–42): A β (1–40) can be used as a biomarker for AD with lower ratio in the plasma indicative of cerebral deposition of A β fibrils [21-23]. With AD progression, the levels of A β (1-42) decreases in the CSF, whereas the amount of A β (1-40) stays constant, their ratio being a valuable indicator of the parenchymal A β aggregation. A β (1–40) fibrils are less toxic physiologically whereas A β (1–43) fibrils are most cytotoxic [2]. Additionally, A β peptides truncated at the N-terminus (A β (n–42), where $n \in (2-11)$) are highly toxic and usually present in A β deposits in AD brain [24]. Molecular interactions of oligomeric species with other molecular complexes, metal ions, and cellular membranes also impact the extent of their toxic effects in the brain. Annular oligomeric aggregates can enhance their toxic impact by binding to lipid membranes and disrupting their integrity, a key observation in support of the amyloid pore hypothesis [25].

1.2.1. Change in A β morphology during aggregation

Since their first detection, amyloid structures have been studied in exquisite detail with significant structural insights from techniques such as solid-state nuclear magnetic resonance (NMR), electron microscopy, X-ray diffraction, crystallography, and micro-electron diffraction. Molecular models of amyloids have been proposed using results of these techniques in conjunction with site-

directed spin labelling and scanning mutagenesis methods. The amyloid fibrils of monomeric proteins within molecular weight range 3-30KDa usually acquire unbranched filamentous morphology with a diameter ranging within 5-15nm [3]. Standard features include a repeating β -sheets parallel to fibril axis, with near perpendicular branches. These sheets are usually in-register so that the strands align with each other whether they run parallel or antiparallel [26]. The separation between identical side-chains is 4.8 Å for parallel sheets and roughly 9.6Å for antiparallel sheets [1]. Tyrosine (Tyr), Asparagine (Asn) and Glutamine (Gln) are critical residues involved in steric zipper formation in the fundamental spine structure. Moreover, an identical amino acid sequence can form different steric zippers under varied incubation conditions (**Figure 1.3**).

The number of protofilaments in an amyloid assembly and their spatial arrangement determines the overall morphology of the fibril. Although both A β (1-40) and A β (1-42) fibrils acquire an S-shaped conformation, the structurally homogenous fibrils from seeded A β (1-42) acquire a stable triple parallel- β -sheet conformation [27]. Oligomers produced on-pathway obtain different structures based on the chemical environment of A β (1-42). Those formed in the presence of the small catechin molecule Epigallocatechin gallate (EGCG) have C-terminal β -sheet comprising of residues 22-39 and an unstructured N-terminal [10]. In the presence of detergents like Sodium Dodecyl Sulfate (SDS), A β (1-42) attained dimeric structures with parallel β -strands by residues 34-40 appending from a hairpin loop which was formed due to antiparallel strands formed by residues 18-23 and 28-33. These dimeric units comprised A β (1-42) globulomers. Under similar conditions, it was also observed that structural order increased towards the C-terminus with the residues 25-40 forming antiparallel β -strands [10].

Figure 1.3. Polymorphism in amyloid fibril assemblies. Different arrangements of two identical β -sheets can lead to morphology in an amyloid fibril. The two sheets can be arranged in parallel or anti-parallel aligned ‘face-to-face’ or ‘face-to-back’ to form different classes of steric zippers. The orientation of the sheets can be ‘up-up’ if the edge β -strands in both the sheets is in same direction, or ‘up-down’ if the edges face opposite direction. Figure adapted from [26].

1.2.2. Pathological effects of A β aggregation

It was first hypothesized in 1984 by George Glenner and Caine Wong that protein aggregation could be the main causative factor for AD pathology. A β was designated to be this protein at the advent of 90s [28]. Notwithstanding the

controversy of the amyloid cascade hypothesis, multiple studies have shown that the cerebral accumulation of A β is a key influence in the progression of AD [17]. A β deposits are biochemically as well as histopathologically heterogeneous. It was previously argued that the toxicity of amyloids is a consequence of mass action of aggregated protein in any organ. However, it is now universally agreed that oligomeric A β species are more toxic than the monomers and plaques. Studies have disproved the neurotoxic nature of amyloid plaques, while some have also argued that these protein aggregates may be neuroprotective [25]. A β (1-42) peptides are the earliest forms to aggregate and later get truncated or modified by phosphorylation or isomerization processes. Off-pathway products of soluble A β , including the low-n oligomers, A β -derived diffusible ligands (ADDLs), and protofibrils are among major toxic contributors towards AD pathology [29]. This theory that ADDLs might be the key drivers of amyloid-induced synaptic dysfunction is known as the revised amyloid hypothesis [30]. A β toxicity causes synaptic dysfunction, inhibition of long-term potentiation, interfere with neurotransmitter interactions, membrane disruption, neuritic dystrophy, gliosis, and disturb neuronal networks. Smaller soluble oligomers may mediate more damaging pathological effects due to their ease of diffusion through tissue compartments.

There are different pathologically relevant A β compositions: A β (1-40) and A β (1-42), A β truncated at N-terminal residue 3, 11 or 17, or A β with post-translational modifications such as phosphorylation, nitration, and isomerization. Peptides with truncations at positions 3 or 11 are resistant to proteolytic degradation and tend to undergo pyroglutamylation and prominent components of A β deposits [31]. Pyroglutamylated N-terminal truncated A β and peptides with additional functional groups on the side chain residues show more toxicity compared to the native protein. The fibrillar structure of A β with different amino acid content differ broadly.

Post-translational modifications of the A β peptides also contribute to their enhanced toxic effects. Among the three potential phosphorylation positions on serine and tyrosine residues, phospho-serine-8 A β has been found in AD and contributed to the formation of oligomeric aggregates which act as seeds for fibrillation [19]. Pyroglutamylation at positions 3 and 11 also contribute to the

A β induced neurotoxicity. Glycosylation modifications are present on the N-terminus truncated A β and up to 20 residue long species and have been found in the CSF of AD patients. Generation of nitrated A β as a by-product of oxidative stress and di-tyrosine formation at the residue 10 have been detected in AD plaques. These modified species have been shown to promote more aggregation and suppress long term potentiation in the hippocampus of AD mice. Non-enzymatic isomerization of aspartyl bonds causes a change in the secondary conformations of A β peptides towards those with a higher propensity to aggregation. The presence of racemized D-forms of the seryl and aspartyl residues in amyloid plaques also implies a higher tendency of this type of isomerization in A β (1-40) [19]. However, some of the modifications induced due to AD pathological effects may offer some protection. For example, the Methionine residue at position 35 is most prone to oxidative changes as a result of enhanced oxidative stress during AD; this Met35-oxidized A β has been demonstrated to delay the formation of fibrillar aggregates [32].

1.3. Regulation of amyloid aggregation

Proteostasis is maintained by the interplay between molecular chaperones and protein degradation machinery. Higher organisms employ an unfolded protein response to control protein folding through intracellular signalling [33]. Cellular protein regulation machinery takes care of protein homeostasis beginning at the biogenesis of the proteins through their translation, stabilization, folding, organelle targeting to their degradation. Housekeeping chaperones comprising heat shock proteins (Hsps), which constitutively express with the nascent protein and overexpress under stress conditions, bind the unfolded proteins and protect their exposed hydrophobic regions in amyloid protein from aggregation, thus promoting proper folding. Furthermore, protein disaggregases can restore the native protein function by reversing the formation of toxic oligomers and fibrils [9, 34]. Another critical factor associated with progression of neurodegenerative disorders is the dysregulation of repair enzymes such as chaperones.

1.3.1. Cellular chaperone machinery

There are three protein degradation pathways that a misfolded protein or aggregate may go through in a healthy cell [25]. The Ubiquitin proteasome

system (UPS) breaks down soluble misfolded proteins through an enzyme cascade which leads to passage through a narrow opening in the proteasome, breaking them into short peptides. In the second pathway of chaperone-mediated autophagy, a protein with exposed (Lys-Phe-Glu-Arg-Gln)-like regions gets recognized by the heat shock cognate 70 protein which binds and delivers them to be degraded by lysosomes and associated enzymes [25]. Proteins which escape these two pathways get degraded by macroautophagy when they are engulfed by the autophagosomes, which then fuse with lysosomes where the protein degradation takes place.

1.3.2. Amyloid chaperone proteins

The molecular chaperones in the cell provide the first line of defence against unfolded or improperly folded proteins. Many of these chaperones, such as Hsps, get overexpressed as a response to stress stimuli mediated by endogenous or exogenous stress factors. The Hsps are categorized into five groups based on their sequence homology, similar functional domains, and cellular localization: Hsp100s, Hsp90s, Hsp70/100, Hsp60, and small Hsps (sHsp) [25]. The Hsps get upregulated under stress conditions to aid in de novo protein folding, refolding, protein assembly, targeting, and degradation of disordered proteins. These proteins mainly utilize ATP as an energy source to induce protein folding or degradation in conjunction with non-ATP dependent small Hsps which act as ‘holdase’ for the action of larger Hsp complexes [35].

There are a few endogenous proteins which are secreted extracellularly and do not directly contribute to the cellular proteostasis machinery. However, due to their conformation and traditional roles within the body, these proteins might act as elusive extracellular chaperones to regulate amyloid aggregation. Continuous oxidative stress and shear pressure on the extracellular proteins due to hydrodynamic forces in interstitial matrix may lead to possible unfolding [36-38]. A comparison between chaperone activities of extracellular proteins is drawn in **Table 1.1**. The RNA expression levels for these proteins have been included based on the information available at the Human Protein Atlas available from www.proteinatlas.org [39].

Table 1.1. Extracellular proteins which act as amyloid chaperones.

Protein	Inhibit Primary Nucleation	Inhibit Secondary Nucleation	Inhibit Fibril Elongation	Disaggregate	Delay fibril growth	ATP dependency	Part of a complex	RNA Expression in Brain (TPM)	Reference
L-PGDS	Y	Y	N	Y	Y	N	N	1224.7	[34]
Clusterin (ApoJ)	Y	Y	Y	Y	Y	Y	Y	10875.4	[40]
α B-crystallin (sHsp)	Y	Y	Y	-		Y	Y	1888.7	[41, 42]
Hsp60	Y	Y	Y	-	Y	Y	Y	141.6	[43]
Hsp 70/40	Y	Y	Y	N	Y	Y	Y	5.0	[44]
Hsp 90- α	Y	N	Y	N	-	Y	Y	1021.5	[44]
Pyruvate kinase	Y	-	-	N	Y	Y	N	749.0	[45]
Transferrin	N	Y	Y	-	-	N	N	385.9	[46]
Transthyretin	Y	Y	Y	-	Y	N	Y	6.9	[47]
α 2-Macroglobulin	Y	-	N	N	Y	N	N	172.0	[48]
Catalase	N	-	-	Y	Y	N	Y	47.2	[45]
Lysozyme	N	Y	-	N	Y	N	N	22.5	[49]
Brichos domain containing proteins	N	Y	N	-	Y	N	N	7.0	[50, 51]
Haptoglobin	Y	-	N	N	Y	N	N	1.8	[52]
Albumin	N	Y	Y	-	Y	N	N	1.0	[53]
Apolipoprotein A1	-	Y	Y	-	-	N	N	0.1	[54]

The presence of chaperone proteins in the extracellular space is crucial to ensure systemic proteostasis. These extracellular proteins help regulate the fibrillation of pathological proteins in ageing and amyloidosis-related diseases. Proteins such as Clusterin, α 2-macroglobulin, Haptoglobin, Albumin, L-PGDS, and others play an additional role as molecular chaperones to maintain the stability of protein [36, 55].

1.4. Lipocalin-type Prostaglandin D Synthase

Lipocalin-type prostaglandin D₂ synthase (L-PGDS), is involved in several biological pathways and is a widely popular CSF biomarker [56]. It is a secreted protein found in organs like brain, heart, kidneys, testis, and associated tissues. As a protein of brain origin and the second most abundant protein in CSF, it plays an essential role in the sleep-wake cycle, nociception, vasodilation, regulation of inflammation, etc. [57]. The structure of L-PGDS exhibits a very flexible C-terminus and single eight-stranded β -barrel with a broad calyx that can bind to many lipophilic as well as some hydrophilic small molecules (**Figure 1.4**) [58, 59].

Figure 1.4. Crystal structure of wild-type human Lipocalin-type Prostaglandin D Synthase. 2.09Å resolution structure obtained through X-ray diffraction and NMR for L-PGDS bound with PEG MME 2000. (PDB: 4IMN) [60].

L-PGDS acts as a catalyst for the isomerization of PGH₂ to PGD₂ with cysteine 65 (Cys) as a critical residue at the active centre and acts as an extracellular transporter for lipophilic ligands [60, 61]. L-PGDS binds and solubilizes lipophilic molecules and poorly water-soluble drugs such as dipyridamole, diazepam, telmisartan, SN-38, and NBQX [59].

1.4.1. L-PGDS protein in the human body

L-PGDS is produced in the ventricular choroid plexus and secreted into the CSF. It is the most abundant intrathecally produced protein with its CSF concentration only second to albumin. L-PGDS is crucial to many biological processes, including nociception, vasodilation, sleep regulation, etc. It belongs to the family of lipocalins and has dual functions as a transporter of small lipophilic molecules as well as an enzyme catalysing isomerase reaction to convert a 9,11-endoperoxide group of prostaglandin H₂ (PGH₂) into prostaglandin D₂ (PGD₂) with 9-hydroxy and 11-keto groups (**Figure 1.5**) [62].

On the cellular level, it is involved in inflammation, cell differentiation, cell cycle, apoptosis, and cell survival [63]. L-PGDS is synthesized in the endoplasmic reticulum of arachnoid cells [64]. Native L-PGDS has a propensity for post-translational modifications including N-glycosylation and phosphorylation, oxidation of cysteines and signals peptide cleavage [65].

Figure 1.5. Isomerization of prostaglandin H₂ to Prostaglandin D₂ catalysed by L-PGDS. L-PGDS functions as an enzyme in a GSH-independent way to isomerize 9,11-endoperoxide group in PGH₂ to 9-hydroxy and 11-keto groups in PGD₂.

Owing to its abundance in the brain and extensive N-glycosylation, structural abnormalities in L-PGDS can affect functioning in Central Nervous System (CNS) [66]. Detectable levels of L-PGDS secreted by glial regions into human CSF can be about 8 µg/ml [66]. Presence of moiety NxS/T in the sequence may explain heavily glycosylated isoforms expressed in brain [66]. On account of its ubiquitous presence, it has been implicated in regulation of many neurological processes, such as regulation of sleep-wake cycle [67], insulin regulation [68 Eguchi, & Ragolia, 2013], reproduction [69] and many other processes. Protective role of L-PGDS after neurodegeneration post-stroke has been

repeatedly reported [70, 71]. Possible roles in mammalian testis and ovarian cells have also been suggested [72, 73]. We recently showed that recombinant human L-PGDS can disaggregate the pre-formed A β fibrils *in vitro* [34]. L-PGDS binds to the hydrophobic C-terminal of A β (1-40) in a 1:1 stoichiometric ratio as seen by Small Angle X-ray diffraction and Matrix-Assisted Laser Desorption/Ionization mass spectrometric techniques [34]. Molecular dynamics along with HSQC NMR spectrum show that the binding of A β (1-40) to L-PGDS is different from the substrate binding site within the protein to form an encounter complex. As a result, the A β (1-40) remain solvated in their monomeric conformations and do not form further aggregates.

1.4.2. L-PGDS as an extracellular chaperone of A β

Kanekiyo et al. conducted the first detailed study of the chaperone activity of L-PGDS towards A β in 2007. They studied the co-localization of L-PGDS with A β plaques in the AD brains. L-PGDS was shown to bind immobilized A β (1-40), inhibit A β aggregation, and co-localize with amyloid plaques in the frontal cortex of an AD patient [74]. The CSF free of L-PGDS could prevent 36% A β aggregation compared to 90% inhibition in presence of L-PGDS containing CSF. From this study, the authors concluded that L-PGDS functions as a major A β -chaperone in human CSF (**Figure 1.6**).

1.4.3. L-PGDS levels in different neurological disorders

L-PGDS is widely used in clinical diagnostic testing as a biomarker to assess CSF leak, which might be an indication of traumatic brain injury [75]. The cut off values in such testing are considered to be 0.25-6.0 mg/l. Proteomic and clinical studies have also shown some correlation between reduced levels of L-PGDS and disease progression in AD patients [76, 77]. A change in L-PGDS expression level in the brain have been observed in several neuropathologies. However, such study has been challenged by others [78]. Our recent work on the effects of L-PGDS as a chaperone on monomeric A β (1-40) and a disaggregase on pre-formed A β fibrils emphasizes the neuroprotective role of this protein [34].

Figure 1.6. Role of L-PGDS as an amyloid β -chaperone. L-PGDS prevents conversion of unstructured A β monomers into β -sheet rich oligomers and into soluble fibrils. Detection of L-PGDS co-localized with amyloid fibrils in mice brain, as shown by Kanekiyo *et al.* [74].

1.5. Early detection of amyloidosis

Current diagnostics for AD include labelling of nascent APP proteins in vivo, CSF biomarkers, or brain imaging for amyloids. Soluble A β aggregates are isolated from post-mortem human AD brain for characterization by homogenization in detergent micelles followed by differential ultracentrifugation are purified through immunoprecipitation with specific antibodies [79]. While the study of these toxic aggregates is crucial to foster a better understanding of AD, there is a dire need for new clinically translatable methods which can help to detect and potentially delay the progression of the disease. In addition to the amyloid tracers used in imaging, specific CSF and plasma biomarkers have been

investigated for early diagnosis of AD, well before the appearance of plaques or tangles [80]. Moreover, developing prognostic biomarkers for high-resolution imaging modalities can further help in therapeutics through detection of amyloid-related imaging abnormalities (ARIA).

1.5.1. Animal models of Alzheimer's disease

Transgenic mouse models genetically engineered to develop AD either overexpress human amyloidogenic proteins or their mutant forms which are usually associated with familial forms of AD. These models have been successfully used to evaluate pre-clinical amyloid modifying therapies and understand the underlying mechanisms which contribute to amyloid pathology and tauopathy. The blood-brain barrier (BBB) acts as the primary physical barrier to the influx of molecules into the brain. The endothelial cells of the BBB comprise extensive tight junctions which mainly restrict hydrophilic molecules to enter the brain. The other cellular components include the astrocyte end-feet and pericytes [30]. Notwithstanding, the ability to cross the BBB is most crucial for AD therapeutics administered systemically.

1.5.2. Different mouse models of Alzheimer's disease

Several AD mice models have been developed over the years to capture the genomic alterations that lead to AD phenotype. These models simulate either a single mutation or multiple genetic changes which lead to cognitive or functional changes in the phenotype [81]. Some models simulate both A β and tau mutations to study their cumulative impact on disease phenotype [82]. For example, the triple transgenic AD mice (3xTg) develop A β plaques, followed by the formation of tau tangles [83]. Additionally, they acquire plasticity in neuronal synapses and exhibit long term potentiation. These models show required phenotype on C57BL/6 background strain [81]. Presenilin gene mutations in PS1 and PS2 subunits also generate autosomal dominant mutants. In addition to these mutations, there are other genes such as APOE which act as risk factors for AD, and their variations may give rise to AD phenotype exhibiting some of the disease symptoms as in case of Familial Alzheimer's Disease (FAD) cases. 5xFAD mice are APP/PS1 double transgenic mice expressing five FAD-related APP mutations such as Swedish, Florida (I719V), and London, and PS1 mutations M146L and

L286V [84]. These mice almost exclusively overexpress A β (1-42) very rapidly and show pathological symptoms such as neuronal loss and cognitive decline within 4-5 months. A list of AD mouse models for the occurrence of disease lesions and symptoms have been tabulated in **Table 1.2** [85].

Table 1.2. Commonly used Alzheimer's disease mouse models.

Model	Gene mutations	Allele type	Promoter	Lab
3xTg	APP(Swe), tau(P301L); PS1(M146V)	Transgenic; Knock-in	Thy1	LaFerla [83]
5xFAD	APP(SweF Δ Lon); PS1(M146L; L286V)	Transgenic	Thy1	Vassar [84]
APP/PS1	APP(Swe); PS1dE9	Transgenic	Prnp	Borchelt [86]
Tg2576	APP(Swe)	Transgenic	Prnp	Hsiao [87]
J20	APP(SweInd)	Transgenic	PDGFB	Mucke [88]
PDAPP	APP(Ind)	Transgenic	PDGF	Games [89]
APP23	APP(Swe)	Transgenic	Thy1	Sturchler-Pierrat [90]
APPPS1	APP(Swe)	Transgenic	Thy1	Jucker [91]
Tg-SwDI	APP(SweDutIowa)	Transgenic	Thy1	Van Nostrand [92]

AD mice models with APP gene modifications generate autosomal dominant phenotype such as Swedish (K670N/M671L) mutant with higher β -secretase cleavage and an increase in A β production, London (V717I) and Indiana (V717F) mutants which favoured production of A β (1-42) compared to A β (1-40), Dutch (E693Q) and Arctic (E693G) mutations which promote fibril formation and hinder proteolysis (**Figure 1.7**).

Figure 1.7. Genetic mutations in Alzheimer’s disease transgenic mice. Amino acid mutations in APP protein sequence identified in familial AD cases used for designing AD mouse models, as mentioned next to each mutation. (Adapted from [93]).

1.5.3. Modalities to detect amyloids in mice brain

Since the gold standard for AD diagnosis is still histopathology on the post-mortem tissue, there is a general consensus to find other methods to confirm disease pathology. Amyloid tracers for positron emission tomography (PET) such as ^{11}C -PiB and ^{18}F -FDG are now widely used to detect fibrillar amyloid deposits in AD brain. Multi-photon optical microscopy is also gaining popularity for the study of brain lesions through topical or systemic injections of fluorophores. However, such microscopy can reliably detect only the structures present on the surface of the exposed mice brains. Novel formulations for nanoparticle probes are finding a new avenue in early diagnosis applications using non-invasive imaging [94].

1.5.4. Bio-probes for amyloid detection in AD mice

Several antibodies and sensitive probes have been developed over the years for amyloid detection in mice brain using several imaging techniques. A key restriction for their application is the poor brain targeting when administered through intravenous injections and other systemic pathways. An intranasal way of drug administration is recommended in recent times for targeting neurotherapeutic agents [95]. This route bypasses the BBB, by utilizing the olfactory system, the rostral migratory system, and trigeminal pathway to reach to regions within the brain.

1.5.5. Magnetic resonance imaging in biological studies

Anatomical studies play an essential role to help us enhance our understanding of the overall organization of our body. Complete structural knowledge of tissues

and organs can help understand how small changes can manifest as disorders or abnormalities. There has been continuous progress in terms of using various techniques to study details of animal bodies and developing those techniques to achieve high resolutions to characterize even the minutest anatomical features. Molecular imaging techniques can be effectively employed for the diagnosis of functional abnormalities in different types of organs and tissues before they lead to pathophysiological changes which contribute to the development of diseases. Among the available techniques for clinical and preclinical imaging, non-invasive procedures have gained popularity in recent years due to a growing interest in diagnosing the disease abnormalities before they become unavoidable. Magnetic resonance imaging (MRI) is one such non-invasive technique which has continuously been improved upon, to achieve high resolutions to study anatomical structures and dynamic biochemical and physical processes. MRI has been used as an established diagnostic tool in medical practice since the early 1980s [96]. The magnets, as well as the technique, have been continuously developed since then, and efforts to make use of it for high resolution anatomical as well as functional imaging have been promoted since then [97, 98]. These advancements have made MRI as a powerful tool for preclinical imaging. In addition to being non-invasive, it does not expose the subjects to any radioactive materials, which makes it highly desirable for translational studies as well.

Several techniques have been developed to be used as ways to achieve imaging of microstructures in the body both in clinical as well as preclinical setups. A Computed Tomography (CT) works through the reconstruction of images obtained by X-ray beam through the tissues to get a 3D structure projection of a tissue or organ. PET and Single Photon Emission Computed Tomography (SPECT) scans rely on the signal from radioactive tracers to get quantitative measurements for the physiological process in different body tissues and organs [99]. Ultrasound (US) imaging utilizes high- frequency sound waves to characterize tissues, as well as the amount of blood flow through vessels. Optical imaging techniques have also been continuously developing for imaging structures from microvasculature to whole-body animal imaging. While most of these studies have improved upon our overall understanding of mammalian bodies, they are still restricted with the types of information which can be derived

from them. As a single modality, they can provide us either with structural or functional information, but not both. For achieving complete information, it is often mandatory to use two or more of these properties as multi-modal imaging techniques.

MRI is unique in the way that it can be utilized for structural studies to provide accurate information about internal structures of our body as well as for functional studies to help quantify different physiological processes. MRI uniquely provides simultaneous modalities which combine both structural as well as dynamics information, which can be exploited to follow dynamic changes in blood rheology and effects of biochemical alterations on convoluted microstructures. There are many modalities of MRI regularly used in translational research. Diffusion-Weighted Imaging can be used for detection of edema or tumor based on the change in the diffusion rates of water molecular in nearby regions. Magnetic Resonance Angiography is used to produce detailed images of arteries and veins in the body without the use of external contrast agents. Slight manipulation of the pulse sequence and signal characteristics can provide differently “weighted” imaging, which provides much different information within the same anatomical areas [100]. Functional MRI is the variant of MRI intended to measure brain activity and connectivity, often using the blood oxygenation level-dependent contrast technique. Although MRI is probably least hazardous among current radiology techniques at clinical stages, its use is restricted in high resolution clinical imaging mainly due to the untested and unknown effects of exposure to high magnetic fields. Fruitful ultra-high field MRI studies on murine animals have provided us with suitable alternatives to enhance our understanding of other structures and their role in the normal functioning of the body.

1.5.6. Use of MRI for amyloid detection in mice brain

MRI is a preferred imaging modality over soft tissue contrast imaging using CT and PET scans as it does not use ionizing radiation for generating contrast and therefore, isn't correlated with possible renal diseases. A β plaques can show up on MRI scans employing a combination of high magnetic field strength, advanced pulse sequences, and longer scan times [101]. MRI can be a useful non-invasive tool to quantify the effects of these anatomical or functional changes in the organs

based on differences in blood volume, fat-to-tissue ratios, consequences of injuries or tumors, as well as biological metabolic processes.

Although there have been a multitude of post-mortem studies to see the extent of amyloid load in AD brain samples, there is a lack of non-invasive techniques to study disease progression. MRI is one such technique which can be used to analyze the structural and spatial changes in these amyloid structures, which gives rise to the disease pathologies. Gadolinium-based contrast agents have been used as clinical contrast agents for MRI for over two decades now. However, possible toxic effects of such contrast agents, especially causing the onset of nephrogenic systemic fibrosis, have proved a need for searching suitable alternatives as MRI contrast agents [102]. Iron-based contrast agents (CA) have been explored for their roles in improving the contrast of images obtained through MRI [103, 104].

Previous studies targeting amyloid plaques in the AD brain employed strategies to permeate the blood-brain barrier (BBB) by using A β (1-42) peptide-functionalized with polyethylene glycol (PEG) and simultaneous intra-carotid or femoral injection of mannitol [105]. Similarly, A β (1-40) peptides bound to gadolinium-diethylenetriaminepentaacetic acid and monocrySTALLINE iron-oxide nanoparticles (IONP) were used to detect plaques in AD mice through intravascular injections along with mannitol [106]. Use of functionalized contrast agents such as superparamagnetic nanoparticles with iron oxide cores has also gained momentum in the past years.

1.6. Therapeutics for Alzheimer's disease

The Existing therapeutics targeting AD can be divided into four categories, based on different AD pathology hypotheses [107]. Inhibition of A β production is mediated by β -secretase/BACE1 inhibitors or γ -secretase modulators, and metal protein attenuating compounds impede further aggregation. A β directed active and passive immunotherapy has shown potential in the removal of plaques and prevention of synaptic damage. Anti-A β drugs used in clinical treatments are enhancers of acetylcholine response, which mainly include Acetylcholine esterase inhibitors such as Donepezil, Rivastigmine and Galantamine, Tacrine, and some $\alpha 7$ nicotinic acetylcholine receptor agonists [107]. They alleviate AD symptoms by improving synaptic functions. Antagonists for glutamatergic

receptors such as N-methyl-D-aspartate receptors, such as Memantine, have also shown functional and cognitive benefits in moderate to severe AD. Despite the discontinuation of the humanized monoclonal antibody Bapineuzumab and the very recent discontinuation of phase III trials of Aducanumab, more anti-A β molecules like Solanezumab, Gantenerumab, Crenezumab, Ponezumab, and GSK933776 are being tested for their therapeutic potential in different clinical trial phases [107]. Tau-targeted immunotherapy is also under investigation. Drugs targeting tauopathy are inhibitors of the glycogen synthase kinase 3- β , which regulates the process of tau phosphorylation. Anti-inflammatory agents and metal protein attenuating compounds have also been studied as potential therapeutic agents for AD treatment.

There are advancements made in the pursuit of reducing amyloid-mediated toxicity with the search for small molecule drugs to inhibit amyloid formation, modifying the organization of proteostasis machinery to enhance amyloid degradation by proteasomes and autophagy, and engineering protein disaggregases capable of solubilizing toxic amyloid aggregates [2]. There is an increased interest in developing anti-A β antibodies much earlier in the course of AD treatments, at the prodromal and preclinical stages [108].

1.6.1. Protein chaperones

Proteostasis is a universal biological process which modulates the protein machinery in an organism by mediating protein synthesis, their folding, trafficking and targeting for downstream processes, as well as degradation of unfolded or toxic protein products. The process of conversion of a nascent unfolded protein into its fully folded functional conformational is a fast and reversible process. However, in some cases, the intermediate conformations linger for more time and partially fold into aggregates in irreversible off-folding pathways [109]. Molecular chaperones associate with nascent polypeptide chain releasing from the cellular ribosomal chamber as soon as it is translated. For cytosolic protein targeted to Endoplasmic Reticulum, these folding-assisting chaperone enzymes or ‘foldases’ help the proteins attain a functional 3D conformation [110]. Small heat shock proteins act as ‘holdase’ chaperones, which bind partially folded proteins and stabilize them in an ATP-independent manner to facilitate their proper folding or targeting to protein degradation systems [109].

1.6.2. Anti-A β antibodies

Monoclonal antibodies are developed as therapeutics targeting the amyloid oligomers and other aggregated forms to solubilize them into non-toxic species.

Table 1.3 tabulates the main anti- A β antibodies which progressed to clinical studies and their current testing status.

Table 1.3. Anti-A β antibodies in clinical trials of Alzheimer's Disease

Name	Details	Action	Testing Stage	Ref
AN1792	Full length A β 42 with QS21 adjuvant	Active	Discontinued	[108]
CAD106	Copies of A β (1-6) coupled to a bacteriophage coat protein carrier	Active	Phase 2 and 3	[111]
Bapineuzumab	Humanized IgG1 mAb; binds 5 N-terminal residues; soluble A β and fibrils	Passive	Discontinued after Phase 3	[112]
Solanezumab	Humanized IgG1 mAb; binds residues 16-26; clearance of monomers	Passive	Discontinued after Phase 3	[113]
Gantenerumab	Fully Human IgG1 mAb; binds conformational epitope on A β fibrils involving residues 3-12 and 18-27	Passive	Endpoints modified after Phase 2/3	[114]
Crenezumab	Engineered on IgG4 backbone; binds mid-domain of A β residues 13-24; monomers, oligomers, fibrils	Passive	Phase 3 trials with modified endpoints failed	[113]
Ponezumab	Humanized IgG2; targets C-terminus 30-40 residues of A β 40	Passive	Discontinued after Phase 2	[115]
Aducanumab	Fully human IgG1 mAb; selectively binds A β aggregates; N-terminus residues 3-6 and conformational epitope	Passive	Discontinued after Phase 3	[116, 117]
BAN2401	Humanized IgG1; binds soluble A β protofibrils	Passive	Trial ongoing	[118]

The isotypes Human IgG1 and mouse IgG2a have a higher affinity to Fc γ receptors, are therefore preferred for mediating A β clearance through phagocytosis and production of cytokines [80]. Some of these antibodies

recognize an epitope on the N- or C-terminus, or a stretch of residues within the peptide chain, while others recognize specific secondary structure conformations in the aggregates. For example, NU1 and NU4 antibodies recognize A β oligomers and fibrils, whereas 9D5 and NAB61 were developed against modified A β monomers [10]. Active vaccines such as AN-1792, CAD106, ACC-001 have shown potential to lower A β load along with the removal of plaques and reduced tau. However, due to significant side-effects observed during the Phase I trials, further investigations for most of these vaccinations have been discontinued. [107].

1.6.3. Engineered protein disaggregases

Proteins disaggregases are can dissolve amyloid protofibrils and toxic oligomers to recover soluble functional protein structures. Cellular Hsp complexes, especially Hsp110, Hsp70, and Hsp40 synergistic combination in humans and metazoan, as well as Hsp104 in yeast, Hsp90 and other Adenosine triphosphate (ATP)-dependent proteins like Nicotinamide mononucleotide adenylyl transferases, show disaggregase activity towards amyloids [2, 9]. As ATP-independent alternatives to the Hsp proteins, trimeric human chaperone HtrA1 has also been modified to enhance its disaggregase activity towards A β , tau and other amyloid peptides [2]. Similar disaggregase activity towards toxic amyloid aggregates have been shown by exogenous bacteriophage gene 3 protein as well [9].

1.6.4. Intervention against amyloid conditions

Biological and clinical interventions include inhibiting amyloid precursors as well as expression of amyloidogenic proteins, preventing undesirable post-translational modifications on the expressed peptides, and developing anti-A β immunotherapy and vaccinations [1]. Chemical interventions against amyloidosis have been developed to stabilize the soluble forms and maintain the structure in non-amyloidogenic mode. They can help screen for small molecule drugs targeting the oligomers and fibrillar structures and facilitate the protein self-aggregation to inhibit the growth of fibres. Chemical methods of designing peptides are used to cap fibrillar ends to block further amyloid aggregation [1].

1.7. Objectives and specific aims

This thesis aims to explore the early detection of amyloid structures by the development of protein-based molecular probes, which can help highlight certain regions and vascular structures in mice brain by enhancing the contrast. We use the L-PGDS protein as the targeting agent in these probes since it has already been known to bind monomeric or oligomeric amyloid structures and possibly prevent further aggregation. Four possible contrast agents have been conjugated with the L-PGDS protein and tested for their stability and biocompatibility. These conjugates can help report the diffusion of soluble amyloid structures through the brain regions at different stages of oligomerization and can be developed as possible theranostic (therapeutic + diagnostic) tools for early diagnosis. We compare the conjugates designed by different biophysical assays, cellular, and animal studies assisted by imaging studies and histochemical tests.

To achieve favourable outcomes for abovementioned objectives, we needed to differentiate between diagnostics and therapeutics, choose the detection mode of choice, figure out a way to prepare suitably functional biological probes, and study them in biological systems to study their biocompatibility.

The main aspects of this work are:

- a. Conjugation of L-PGDS protein with MRI contrast agent to develop suitable molecular probes,
- b. Functionality and biocompatibility tests for the designed conjugates,
- c. MRI experiments to study the diagnostic efficiency of these probes in live Alzheimer mice, and
- d. Histological analyses to corroborate MRI results.

For our proposed studies on mouse models of Alzheimer's disease, magnetic resonance imaging can be employed as a non-invasive imaging technique to study effects of anti-A β proteins on the progression of amyloidosis in the brains of AD mice. With this objective, our plan was to covalently conjugate protein molecules to iron-based MRI contrast agents. These conjugates will be administered to AD mice and studied for their localization with amyloid structures and their movement across the brain regions with disease progression. Upon completion, the results of this thesis can be instrumental in tracking the diffusion patterns of

amyloid entities in the mammalian brain during their aggregation, and thus help in developing oligomer-specific targeted therapeutics. Based on previous studies on L-PGDS interactions with A β using recombinant protein as well as CSF samples, and biochemical studies in our lab, we hypothesized that L-PGDS protein is capable of restricting the aggregation of the amyloid peptide into toxic oligomers [60, 74].

1.8. Organization of the dissertation

Chapter 1 is an overall introduction to the thesis and provides a background for the rationale behind this work. The literature supporting our hypothesis has been reviewed in detail and each component of this thesis is carefully overviewed.

Chapter 2 focuses on the first specific aim of this study, which is to develop an efficient way of preparing protein-based stable conjugate probes that can be used as potential theranostic agents. The chapter gives a brief introduction and provides the methodology for the specific conjugation mechanism and particle properties which allow for the preparation of such probes. The chapter concludes with results and discussion of the conjugation process.

Chapter 3 provides an account of the functional and biological assays which were conducted to test the suitability of using the conjugate probes in mouse models. Beginning with a brief introduction of the biological assays particularly used in this study, the chapter provides the methodology of these tests and explains the results which show the biocompatibility of these biological probes.

Chapter 4 provides a detailed explanation of the magnetic resonance-based detection studies done for this thesis. It explains the basic magnetic resonance imaging concepts which have been used in the diagnostic assays and provides the interpretation of contrast-enhanced experiments, which allowed for detection of conjugate probes due to presence of iron in the conjugates. With detailed methodology and results, the chapter ends with brief discussion of the *in vitro* and *in vivo* imaging experiments.

Chapter 5 details the histological studies done on the excised mouse brain samples, which provide supportive evidence to the imaging experiments. There

is a brief introduction to the staining procedures used, followed by the methodology, results and discussion.

Chapter 6 concludes the thesis work and provides an outlook for the future directions whereby this project can be steered.

Chapter 2

2. Development of covalently conjugated L-PGDS probes with magnetically active nanoparticles

There is a constant advancement of novel strategies to develop useful diagnostic probes for molecular and medical imaging. Chemical crosslinking allows for surface functionalization of the nanoparticles with specific proteins or other biomolecules to contrive multi-functional probes with desired characteristics. Conjugation of the L-PGDS protein on magnetic nanoparticles can help in the detection of brain amyloids using MRI. This chapter details the basic principles and methodology behind successful covalent conjugation of L-PGDS protein with iron oxide nanoparticles and ferritin nanocages. In this chapter, we focus on the carbodiimide crosslinking which covalently links the free amino groups of lysines and arginines on L-PGDS to the carboxyl groups on the surface of nanoparticles.

2.1. Introduction

The initial step in designing a theranostic probe is to identify a reliable mechanism to prepare the desired agents. The fundamental properties we accessed are whether the protein-based probes are active, their shelf-life and stability under physiological conditions, binding properties, stoichiometric ratios of probe components, any toxic effects, engineering the detection capabilities for required imaging modalities, and possible therapeutic impact on AD models. Nanoparticles find much use in biomedical applications owing to their smaller size and high surface-to-volume ratios. They are easy to functionalize and conjugate, to be used as an interface, interact readily with biological systems, and can be efficiently engineered to respond in a specific way to physical or chemical stimuli. The critical properties of nanoparticles relevant to MRI studies are high magnetic susceptibility and saturation magnetization, which can confer a faster and stronger response to the presence of the external magnetic field.

2.1.1. Covalent conjugation of proteins

Crosslinking chemistry involves the techniques to covalently conjugate two proteins or a protein with a small biomolecule to generate a unit with desired properties. Often, a cross-linker is used to facilitate this covalent bond formation without insertion of any extra atoms. Such reagents are mostly used for the formation of covalent linkages between primary amine and a carboxylic acid group to form an amide bond, between primary amine with a phosphate group to form phosphoramidate linkage, or formation of an amine linkage by reductive alkylation of a carbonyl group into a primary or secondary amine [119]. Similarly, sulfhydryl groups can bind to active carboxyl groups to form thiol esters. However, more reactive bifunctional cross-linkers with different functional groups separated by a spacer can be used to interact with target molecules [120] selectively. 1-Ethyl-3-(3-Dimethylaminopropyl) Carbodiimide hydrochloride (EDC) is a zero-length crosslinker, which mediates the formation of an amide bond between carboxyl functional groups and free amines with no additional atoms [119]. It reacts initially with the carboxyl group and forms an O-acylisourea intermediate. Molecules like N-hydroxy sulfo succinimide (sulfo-NHS) contribute to converting this reactive O-acylisourea ester intermediate into semi-stable amine-reactive NHS ester which

remains stable for few hours [121]. EDC is one of the most widely used linkers for preparation of protein conjugates.

2.1.2. Mechanism of carbodiimide conjugation

In this study, the EDC carbodiimide crosslinking facilitated by the addition of sulfo-NHS was used to form the conjugates, which were studied through Ninhydrin colorimetric assay and dynamic light scattering (DLS). EDC is water-soluble yet labile and needs to be freshly prepared for every reaction. Isourea is formed as a by-product (**Figure 2.1**). There is a trade-off between the yield of amide bond formation, which is the highest within the range of pH 4-6, to the highest activation of carboxyl groups which occurs between the pH 3.5-3.5 range [119]. Also, EDC is stable in solution only around pH 6.5 and above. In general, pH 4.5 to pH 7.5 have been used as suitable conditions for EDC-mediated cross-linking in proteins [119]. Taking all these factors into consideration, we used 0.1M 2-(N-morpholino) ethane sulfonic acid (MES) buffer at pH 6.0 for activation of nanoparticles and initiation of amide bond formation. Use of sulfo-NHS increases the efficiency of carbodiimide reactions and the yield of derived protein conjugate.

2.1.3. Magnetically active nanoparticles suitable for conjugation

In addition to the iron oxides such as magnetite, maghemite and hematite, the nanostructures composed of ferrites, which are mixed oxides of iron with fractions of transition metals such as manganese, nickel, zinc, copper, and cobalt, exhibit high magnetic susceptibility and can act as a superparamagnetic contrast agent. The critical properties of these nanostructures used for biomedical applications crucial to their feasibility for in vivo use are their biocompatibility, biodegradability, cellular toxicity, retention time, and magnetism, which are indeed synthetically engineered in pursuit of a suitable bio-imaging probe for diagnostic and therapeutic studies. Magnetic contrast agents currently used in biological applications of MRI include gadolinium, superparamagnetic small (50-200nm) and ultra-small (5–10 nm) superparamagnetic iron oxides, carbon nanotubes doped with gadolinium, as well as embedded paramagnetic micellar structures and liposomes, etc. [122].

Figure 2.1. Mechanism of EDC-NHS crosslinking. EDC reacts with active carboxyl groups on the surface of nanoparticles to form an active O-acylisourea intermediate. Sulfo-NHS binds to this unstable intermediate to form a dry-stable amine-reactive ester which gives rise to the stable conjugate product upon addition of primary amine in the reaction mixture. Image modified from [123].

Functionalization of magnetic nanoparticles is an essential step to utilize them for biological and medical applications. This process may include chemical or electrostatic addition of polymeric groups on the surface layer of these nanoparticles with capabilities to interact with specific biological molecules. Although still a lot of research is being done to improve their characterization, these magnetic nanoparticles can be very efficiently conjugated with suitable biological molecules and tracers, and thus have a tremendous potential to be further developed as biological probes for use in multi-modality imaging applications long with MRI, including CT, PET, photoacoustic tomography and near-infrared fluorescence imaging.

Addition of selected coating materials confers additional properties to the core nanoparticles. For example, combining the superparamagnetic IONPs with chitosan

functionalized with catechol moieties resulted in higher thermal stability, tolerance to pH changes in the solution, and enhanced response to stimuli [124].

2.1.4. Iron oxide nanoparticles

Superparamagnetic iron oxide nanoparticles (IONPs) are being increasingly used as MR contrast agents for diagnostic purpose. They are comparatively chemically inert, biocompatible, have lower toxicity, and potential to be chemically and magnetically utilized for biomedical applications such as imaging and drug delivery. Iron compositions commonly used in MRI applications are Fe_3O_4 known as magnetite, $\alpha\text{-Fe}_2\text{O}_3$ known as hematite, and $\gamma\text{-Fe}_2\text{O}_3$ known as maghemite [122]. IONPs are relevant for biomedical applications on account of potential electron hopping due to the presence of vacancies in the octahedral and tetrahedral arrangement of divalent ferrous ions and trivalent ferric ions in various polymorphs. Engineering the surface of IONPs to added different biocompatible layers is currently one of the most active fields of research. The commonly used materials for coating which find great use in biomedical applications include organic materials like polysaccharides, phospholipids, fatty acids, peptides and surfactants including polymers, as well as inorganic molecules such as silica and gold [30]. The limitations of IONPs are the loss of dispersity due to the aggregation of particles in solution, which causes a significant reduction in their shelf lives and the decline in their magnetic susceptibility due to oxidation upon exposure to air. However, it is still challenging to determine stoichiometry of such combinations due to the non-standard preparation process, quantity limitations for biological molecules, and fewer techniques to control and accurately monitor such interactions. IONP preparations such as Ferumoxtran-10 (Sinerem/Combixen) in Europe, US-Food and Drug Administration (FDA) approved Ferumoxytol (Feraheme) in North America, and Resovist in Japan are sold as non-stoichiometric solutions to be used clinically [122, 125]. Compared to their gadolinium counterparts, these IONPs show higher relaxivity and undergo much slower renal clearance.

The superparamagnetic IONPs are typically used as T2 contrast agents that provide negative contrast as a result of their massive magnetic moment. However, with modulating their relaxometry properties by changes in the thickness of coating layer and outer composition, or sometimes upon conjugation with other paramagnetic MR contrast agents such as gadolinium, they may also work as T1 contrast

enhancement agents. IONPs of hydrodynamic diameter size higher than 50nm can be actively phagocytosed by hepatic Kupffer cells [125]. IONPs with diametric size lesser than 50nm are preferred for imaging applications since they have a longer life in the systemic circulation. The plasma half-life of ultra-small IONPs is estimated to be 24-36 hours in humans and approximately 2 hours in mice [105]. Following the digestion of outer the coating, the iron core of these nanoparticles is metabolized by the body over 6-12 weeks. Previous clinical use of ultra-small IONPs has led to anaphylaxis and other immune reactions upon intravenous administration [125]. Functionalization of IONPs with 1,1-dicyano-2-[6-(dimethylamino) naphthalene-2-yl] propene carboxyl derivative has been used previously to target amyloid targets in AD mice brain [126].

2.1.5. Ferritin nanocages

Iron storage protein ferritin is composed of 24 heavy chain and light chain subunits leading to a 474kDa spherical oligomeric unit. Holo ferritin protein can be used as an MR contrast agent owing to the feasibility of enclosing the inorganic iron oxide within the core [127]. The capability of modulating both $r1$ and $r2$ relaxivity values renders ferritin nanocages a better contrast agent than the traditionally used gadolinium [128]. The magnetic susceptibility of iron in the form ferrihydrite, which exists in mammalian ferritin is two orders of magnitude lower compared to magnetite [30]. Thus, synthetically enhancing its magnetic properties is prerequisite to develop it as a comparable MR contrast agent.

2.1.6. Conjugation of L-PGDS to nanoparticles

We have explored the possibility of utilizing three of such iron-based CAs for our proposed studies in mice brain. Four types of contrast agents have been used to cross-link with L-PGDS for MRI studies: (i) Magnetic Nanostructures (MNS) samples were provided by Prof. Vinayak P Dravid at Northwestern University. (ii) Silica-coated magnetic nanoparticles (SMNP) and Dopamine- coated iron-oxide nanoparticles (DNP) provided by Assoc. Prof. Xing Bengang at School of Physical and Mathematical Sciences at NTU. (iii) Ferritin nanocages (ftnAA) provided by Assoc. Prof. Sierin Lim at the School of Chemical and Biomedical Engineering at NTU (**Figure 2.2**). Herein, MNS are iron oxide nanoparticles coated with nitro-

Dopamine linked and polyethylene glycol (PEG) as a functional group on the outer surface.

Figure 2.2. Iron oxide nanoparticles (IONPs) used in this study. A) Magnetic nanostructure (MNS) with iron oxide core covered with a layer n-DOPA before outer PEG coating. B) Silica-coated magnetic nanoparticle (SMNP) with iron oxide core coated with silica layer. C) Ferritin nanocages (ftnAA) with iron atoms loaded into hollow protein nanocages comprising 24 subunits. D) Dopamine coated iron oxide nanoparticles (DNP) with an outer layer of dopamine. All nanoparticles have carboxylic groups on the surface available for binding.

The DOPA-Fe³⁺ coordination bonds are known to form a dense crosslinking which also provides the mechanical strength to these nanostructures under experimental conditions and helps to maintain their structural integrity [124]. The PEG coating allows for proton diffusion from the water surrounding the MNS, thus maximizing the r_2 relaxivity [129]. It helps in better dispersion in biological media and improves the blood retention time, which is useful for intravenous injections. Also, the carboxyl group of PEG is readily available to bind free amino groups on the protein structure [130]. Ferritin nanocages form when 24 subunits of *Archaeoglobus fulgidus* ferritin AA mutant come together to create spherical cages encapsulating metal ions like Fe³⁺ [128]. The free carboxyl groups on the surface of these nanocages are also available for conjugation with free amino groups of target

proteins. Silica coating can be modified to have either free hydroxyl groups or free carboxyl groups exposed to interact with other molecules. Hydroxyl groups on the surface of SMNPs can bind to the cysteines of the protein [131]. Silica is biocompatible, hydrophilic, can be negatively charged above pH 2 – all these properties mean that silica-coated nanoparticles find major roles in biomedical application. However, the critical limitation emerges due to the non-biodegradable nature of silica [132].

The primary concern in nanoparticle-protein interactions is the possible loss of protein function due to surface changes conferred by the nanoparticles in their vicinity. Hence, the biocompatibility of the final conjugates and their functional activity are vital parameters to test before proceeding to diagnostic studies. The crosslinking of the L-PGDS protein with each of these nanoparticles utilizes the carbodiimide chemistry to form covalent bonds (**Figure 2.3**).

Figure 2.3. Schematic for covalent conjugation of L-PGDS with carboxyl groups in IONPs. In the presence of zero-length linkers like EDC, the carboxylic groups on the outer layer of IONPs form active intermediates which bind to lysines and arginines with free amine groups in L-PGDS.

Additionally, the superparamagnetic nature of the nanoparticles also allows for using magnetic separation as a step during the process of conjugate preparation (**Figure 2.4**).

Figure 2.4. Schematic for conjugation process. Magnetic separation is a common way to isolate IONPs within a solution and for separation of conjugates from non-bound protein molecules. The accelerated aggregation of magnetically separated conjugates and difficulty in isolating unbound nanoparticles pose a hindrance in this method of conjugate separation.

2.2. Materials and Methods

2.2.1. Chemicals

Media components for large scale cell culture:

Rosetta™ 2(DE3) Singles™ Competent Cells – Novagen

Bacto™ Tryptone - BD (Sparks, MD, USA)

Yeast Extract - BD (Sparks, MD, USA)

Glycerol, Molecular Biology grade – Promega (Madison, WI, USA)

Potassium dihydrogen phosphate - Merck (Darmstadt, Germany)

Di-phosphate hydrogen phosphate – Merck (Darmstadt, Germany)

IPTG – Thermo scientific (Rockford, IL, USA)

Buffers and reagents:

HEPES – MP Biomedicals (Solon, OH, USA)

Tris base, molecular biology grade – Promega (Madison, WI, USA)

Calbiochem Protease inhibitor cocktail – Merck (Darmstadt, Germany)

TCEP-HCl – Goldbio (St. Louis, MO, USA)

NaCl – Merck (Darmstadt, Germany)

Ni²⁺-NTA resin - Qiagen (Hilden, Germany)

Superdex 75 (10/300L) - GE Healthcare

BupHTM MES Buffered Saline – Thermo scientific (Rockford, IL, USA)

EDC – Thermo scientific (Rockford, IL, USA)

Sulfo-NHS – Thermo scientific (Rockford, IL, USA)

Ninhydrin – Sigma Aldrich (St. Louis, MO, USA)

Phosphotungstic acid hydrate – Sigma Aldrich (St. Louis, MO, USA)

40% Acrylamide/Bis Solution 37.5:1 – Bio-Rad (China)

Bradford Dye Reagent – Bio-Rad (USA)

SDS – Hoefer (San Francisco, CA, USA)

APS – Bio-Rad (USA)

TEMED – Bio-Rad (USA)

2.2.2. Instruments

Shaking incubator - Beckmann

Spectrophotometer - Beckmann

Centrifuge - Beckmann

Sonicator - VWR

Microfluidizer – LM200

AKTApure - GE

SDS-PAGE apparatus – GE

ND 1000 Spectrophotometer - Nanodrop

Weighing balance - Setra

Zetasizer Nano- Malvern

JEM 1400Flash Electron Microscope - Jeol

2.2.3. Expression of L-PGDS in E.coli

Native human L-PGDS (HGNC: 9592) sequence was chosen (UniProt: P41222) for recombinant protein expression in E.coli Rosetta™ 2(DE3) Singles cells: [MAPEAQVSVQPNFQQDKFLGRWFSAGLASNSSWLREKKAALSMCKSVV APATDGGGLNLTSTFLRKNQCETRTMLLQPAGSLGSSYSRSPHWGSTYSVS VVETDYDQYALLYSQGSKGPGEDFRMATLYSRTQTPRAELKEKFTAFCK AQQFTEDTIVFLPQTDKCMTEQAHHHHHH].

Based on the results from protein identification on the ExPASy server, the molecular weight of this protein construct is 19723.15 Daltons, with pI as 8.34 [133]. Under reducing conditions in aqueous buffer, the extinction coefficient at 280nm (ϵ) is 26930 M⁻¹ cm⁻¹. Recombinant human L-PGDS was cloned into a pNIC-CH2 protein expression vector with a C-terminal 6xHis tag, which is expressed in Escherichia coli Rosetta™ 2(DE3) Singles cells. The NTU Protein Production Platform provided this as a glycerol stock stored in -80°C. Starter culture was prepared by incubating a swab of this glycerol stock in 5ml autoclaved Luria Bertani (LB) broth, with the addition of 32µg/ml Chloramphenicol and 50µg/ml Kanamycin. This culture shaken with a speed of 180rpm at 37°C overnight till Optical density (OD) at 600nm of 0.8 was achieved to incubate the large scale growth medium.

2.2.4. Large-scale purification of L-PGDS

For large scale production of protein, the E. coli cells were grown in Terrific Broth (TB) medium. Cells were grown at 37 °C while shaking at 200 rpm till the OD_{600nm} reached 0.8-1.2. The cells were then induced with 0.5mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). Induced cells were allowed to grow at 18°C overnight before harvesting. The cells were harvested by centrifugation at 6000xg at 4°C. Cell pellets were lysed in buffer containing 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 300mM sodium chloride (NaCl), 1mM of reducing agent Tris (2-carboxyethyl)phosphine (TCEP) at pH 7.8 using sonicator followed by microfluidization. Lysed cells were pelleted at 18000xg at 4°C. The supernatant containing soluble protein fraction was passed through an Immobilized

Metal Affinity Chromatography (IMAC) column charged with Ni²⁺ ions. Non-specifically bound fractions were removed by washing the column twice with three column volumes of modified wash buffer consisting 20mM HEPES, 300mM NaCl at pH 7.8, with 20mM and 50mM of Imidazole, added in the first and second wash respectively. The protein was eluted at pH 7.8 in buffer containing 20mM HEPES, 300mM NaCl, and 500mM Imidazole. 2mM TCEP was added into the eluted solution. The eluted protein fraction was then concentrated using centricon tubes with 10,000Da cut-off filter units at 4000xg. The concentrated fraction is further purified by passing through a GE Superdex 75 size exclusion chromatography (SEC) or gel filtration (GF) column. Fractions collected under the chromatogram peak were run on a 12% Sodium dodecyl sulfate (SDS) - polyacrylamide gel. Protein fractions with a single band near 20KDa were used for further assays.

2.2.5. Characterization of nanoparticles used for conjugation

Transmission electron microscopy (TEM) was used for early quality test of nanoparticles before their conjugation with L-PGDS. Nanoparticle preparations were negatively stained using an equal volume of 1% phosphotungstic acid, and 5μl of this mixture was loaded on the carbon-coated copper TEM grids. The excess solution was wicked off using a filter paper from the edges of the grid, and the samples were either dried overnight or vacuum-dried for an hour before inserting the grids into the microscope. These grids were observed under JEM-1400 field-emission transmission electron microscope operating at 100kV. The images were acquired at 10,000x magnification using the user interface.

2.2.6. Carbodiimide crosslinking of L-PGDS and nanoparticles

To overcome the problem of self-polymerization, L-PGDS was only added to the activated solution after the contrast agents are activated using 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxy succinimide sulfo ester (sulfoNHS). As a general protocol, these reagents are dissolved in MES buffer at pH 6 just before the conjugation to give final concentrations of 2mM EDC and 5mM sulfoNHS in 50μl of CA samples. For 100 μl of starting CA concentrations, 100 μM EDC and 500 μM sulfoNHS were used in the reaction mixture. Upon activation, the CA are left to incubate in the dark at room temperature with occasional mixing for 30 minutes. The carbodiimide reaction is stopped by adding Tris at a final

concentration of 1mM. 150 μ l of L-PGDS at a final concentration of 100 μ M is added to each sample of activated CAs and left for overnight incubation with gentle shaking at room temperature. The incubated samples are tested for their size to confirm the extent of conjugation.

2.2.7. SDS-PAGE to test the efficiency of conjugation

The purified proteins and the conjugated samples were run in 12%-15% SDS-polyacrylamide gel electrophoreses at 100V. Coomassie R-250 dye was used for staining the protein samples in methanol-acetic acid solution followed by de-staining for an hour.

2.2.8. DLS study to check the conjugate size

The hydrodynamic sizes of the samples were compared using DLS before and after conjugation to establish successful conjugation. All the measurements were done on Malvern Zetasizer Nano ZS. Precision cells made of quartz with 10mm path length were used for measurements of 145 μ l of samples. Correlation curves were monitored to ensure no interference with dust particles. Size distributions by number were used to confirm the formation of conjugates.

2.3. Results and Discussion

2.3.1. Characterization of the iron oxide nanoparticles

Synthesis of IONPs can be achieved by the co-precipitation methods, microemulsion methods, hydrothermal methods including thermal decomposition, electrochemical deposition, or by sonochemical techniques [132]. Further details regarding the physical characterization of these iron nanoparticles such as colloidal stability, surface charge, percentage of surface coating, and measurement of their magnetization, magnetic susceptibility, and coercivity have been previously published by the respective groups and are beyond the scope of this thesis [130, 134-136].

Dynamic light scattering measurements of coated particles were taken to determine the hydrodynamic size of the four IONPs. FtnAA nanocages showed hydrodynamic diameter of 18.6 ± 2.5 nm, MNS showed 43.4 ± 14.6 nm. SMNP particles tended to settle at the base of sample tube when left unstirred for some time. Based on the DLS measurements, 89.5 % of SMNP particles showed 112.4 ± 14.6 nm, while

there was a larger peak observed 838.1 ± 124.0 nm representative of 10.5 % of SMNP sample. 98.5 % of the DNP particles showed a size of 75.8 ± 10.9 nm, 1.4 % had size of 328.8 ± 54.1 nm and 0.1% particles had hydrodynamic diameter of 1279.0 ± 192.1 nm based on the zetasizer readings (**Figure 2.5**).

Figure 2.5. Size measurement by number using DLS. The percentage of particle size distribution by number has been plotted on the y-axis against the hydrodynamic diameter size in nm on the x-axis. Based on the major peaks observed in DLS, the mean hydrodynamic size of ftnAA nanocages is around 19 nm, MNS is 43 nm, SMNP is 116 nm, and DNP is approximately 76 nm in diameter.

Transmission Electron Microscopy (TEM) was used to corroborate the results obtained from DLS studies. The spherical protein shells were clearly visible in ftnAA nanocages. The inner iron-oxide core of MNS was close to 10 nm in diameter. The aggregation tendency of the SMNP nanoparticles were clearly visible in TEM, where most of the grid was covered with groups of nanoparticles clumped together. Individual globular particles in the DNP sample with approximately 100 nm in diameter were clearly distinguished using TEM images (**Figure 2.6**).

Figure 2.6. TEM images for characterization of IONPs. A. TEM image for ftnAA nanocages (scale bar: 100nm); B. TEM image for MNS (scale bar: 10nm); C. TEM image for SMNP (scale bar: 500nm); D. TEM image for DNP (scale bar: 100nm).

2.3.2. Expression of L-PGDS in the soluble fraction of *E.coli* BL21 cells

Wild type (WT) L-PGDS was purified from *E.coli* grown in Terrific Broth at 37°C with shaking at 200 rpm for approximately 4 hours of incubation till the optical density (OD_{600nm}) reached 1-1.2. Following the IPTG induction, the temperature was brought down to 18°C for overnight shaking to modulate protein expression into the soluble fraction. The cell pellet post centrifugation was dissolved in binding buffer containing 20 mM HEPES and 300 mM NaCl at pH 7.8 and sonicated till for lysis, followed by micro-fluidization. The supernatant obtained after pelleting down the lysed cells was passed through a column containing Nickel-nitrilotriacetic acid (Ni-NTA) beads. The solution was incubated with Nickel NTA beads overnight for affinity binding at 4°C. 20 mg of L-PGDS was obtained from 2 L of

cell culture after IMAC. The wash buffer was used to remove any non-specifically bound proteins from the supernatant. The presence of protein in the eluate was confirmed using Bradford reagent. Post IMAC, the protein fractions were concentrated together and passed through GF which yielded approximately 8 mg of highly purified protein (**Figure 2.7**).

Figure 2.7. Purification of native recombinant L-PGDS. A) In SEC chromatogram, the pure fraction for L-PGDS gets eluted at 12 ml in a GE Superdex 75 10/300 column. B) In 12% SDS-PAGE gel is run, L-PGDS elutes around 20 kDa. The lanes indicate a. Protein ladder; b-d. IMAC fractions eluted at increasing imidazole concentrations of 10 mM, 40

mM, 70 mM respectively; e-g. IMAC fraction eluted with 500 mM Imidazole concentration; h. Sample injected in GF column; i-k. Fractions eluted from GF column under the peak shown in the SEC profile.

2.3.3. Quality check of purified L-PGDS using SEC and NMR

Due to the presence of 6x His tag, the L-PGDS protein-bound tightly to the beads. No significant protein elution was observed at increasing imidazole concentrations up to 100 mM. Therefore, 500mM imidazole was used to elute the protein. The buffer used for GF chromatography had lower salt concentration with 20 mM HEPES, 100 mM NaCl, and 2 mM TCEP at pH 7.4 The SEC column was equilibrated with this buffer before the addition of protein concentrate. Superdex 75 preparative columns which allow for separation of proteins within 3 – 70 kDa range were used for SEC. In the chromatography with a column volume of 25 ml, the single intensity peak for L-PGDS confirming that the purified monomeric protein of molecular weight 19.7 kDa eluted between 12-14 ml. Due to the propensity of disulfide bond formation, 2 mM TCEP was always added to the buffer at all the steps of elution.

The fractions under the main peak were collected and passed through the SDS page to confirm purity further. The fractions obtained after IMAC and SEC were run through SDS-PAGE gels to ensure that the highly purified protein fractions were used to proceed for further assays. A single band around 20 kDa was observed for the fractions collected under the peak in the protein eluted after SEC. In some batches, a less intense peak was observed upstream from the L-PGDS peak during the elution, which could represent the possible dimer formation. A higher band around 37 kDa appears after more extended shelf periods, which could be indicative of dimeric structure by disulphide bridge formation between Cys167 and Cys186. Pure monomeric fractions are used for all the biochemical and cellular assays. A simple 1D proton NMR of the pure protein showed chemical shift dispersion along nearly 4 ppm spread of amide signals from 6-10 ppm, resulting from the β -barrel conformation of the well-folded purified protein (**Figure 2.8**). For a disordered protein, the amide chemical shifts would concentrate within 8 - 8.5 ppm [137].

Figure 2.8. ^1H NMR for purified L-PGDS. Dispersion in amide region from 6-10 ppm represents the β -sheet rich folded structure; spectrum for SEC purified protein fraction in 20 mM HEPES buffer, pH 7.4 at 298K.

2.3.4. Carbodiimide conjugation of L-PGDS with nanoparticles can be achieved at physiological pH

The covalent conjugation of the carboxyl groups on the nanoparticle surface with the free amino groups on the lysines and arginines of L-PGDS, governed by the carbodiimide chemistry, was attained after overnight incubation with gentle shaking at room temperature. During the crosslinking reaction, EDC and sulfo-NHS stabilize the carboxyl groups on the surface of IONPs into an intermediary activated state in MES buffer at pH 6.0, conferring an affinity towards the incoming free NH_2 groups on the protein. EDC itself and its different combinations with different esters of NHS, have been previously used for such cross-linking whereas the combination of sulfo-esters of NHS appeared to be the most effective adjunct to facilitate the carbodiimide crosslinking [121]. Due to the extremely short half-life span of EDC in aqueous solution, the IONPs were incubated with EDC and sulfo-NHS and maintained in dark with gentle shaking for 120 minutes. After this duration, the surplus EDC molecules do not interact with other reaction components and the risk of self-conjugation is reduced. Once L-PGDS was added to the activated mix, the tubes were kept overnight at room temperature in the dark with gentle shaking. This

conjugation reaction was quenched by saturating the excess carboxyl groups with 100 mM Tris buffer after 12-14 hours of conjugation. The newly formed conjugates were soluble, and no precipitation was observed up to 24 hours incubation at 4°C.

The concentration of nanoparticles in each conjugate was identified based on the trends observed while comparing the contrast in MRI experiments. From 5.0 mg/ml stock solutions of SMNP and DNP, and 1.0 mg/ml Fe stock of MNS, 1/10 dilution of nanoparticles was taken to form equal volume solutions used for conjugation with the same volume of 100 mM L-PGDS protein. The concentration for comparable preparations of LA conjugates was chosen to be 2.5 mg/ml of ftnAA nanocages. While the contrast might be different for corresponding conjugate preparations due to aggregation propensity of IONPs, each conjugate use for further assays contained an equal amount of L-PGDS in the sample preparations. The preparation quality of LA conjugates could be identified more efficiently compared to other conjugates using SDS-PAGE. The success of conjugation was observed to depend on the concentration of EDC crosslinker added in the reaction mixture. Therefore, the amount of LA conjugates formed with different EDC concentrations was used as a measure to optimize the reaction conditions for crosslinking (**Figure 2.9**).

Figure 2.9. 7-18 % gradient SDS-PAGE for LA conjugates. a-h. Decreasing the concentration from 200 mM EDC to 50 mM EDC showed slight increase in conjugate production as observed by the thickness of bands at the separation of stacking and resolving gels since LA conjugates with ~ 500 kDa molecular weight were unable to cross into the separation.

Nanoparticles protein conjugation was tested using Ninhydrin reagent, which shows colorimetric change from yellow to blue upon binding with free NH₂ groups in the mixture (**Figure 2.10**). The yellow coloration of the Ninhydrin agent was retained in case of the conjugates, suggesting that the free NH₂ groups of L-PGDS were bound to the activated carboxyl groups on the IONPs. However, this technique could not be used for applying to the protein-protein conjugates of L-PGDS with ftnAA nanocages. Preparation of LA conjugates involved an additional step of SEC using Superose 6 resin column capable of eluting to get the conjugates (**Figure 2.11**). The Dynamic Light Scattering (DLS) studies were used to confirm the presence of conjugate in the final solution by size comparison.

Figure 2.10. Ninhydrin test to confirm IONP-LPGDS conjugation. Upon boiling at 100°C for 2 minutes, the yellow coloured ninhydrin turns blue in the presence of free primary amine groups. A) Blue coloration in free L-PGDS sample represents the availability of free primary amines. B) Yellow colouration in free IONP sample shows an absence of interaction with ninhydrin reagent. C) A heterogeneous mixture of IONP and L-PGDS without crosslinking showed blue coloration due to interaction with unbound amine groups. D) No change in coloration for the magnetically separated conjugated samples implying saturation of all lysines with IONP interactions. E) Blue coloration observed in unbounded L-PGDS after conjugation. F) No colour in wash shows no unbound L-PGDS.

Figure 2.11. SEC chromatogram for L-PGDS conjugation with ftnAA nanocages. In a Superose 6 column, 20 kDa L-PGDS sample eluted nearly at 20 ml elution volume, 480 kDa ftnAA nanocages eluted at 15 ml, the LA conjugates eluted before the nanocages as an indication of successful covalent conjugation.

Efficiency of the conjugation was estimated by Ninhydrin assay. Upon sample heating in presence of the Ninhydrin agent post conjugation and washing steps, the coloration of conjugates did not change from yellow to blue, indicating that there were no free amino acids on the L-PGDS in these conjugates. LA conjugates were passed through SEC to select the correct fraction, not used for confirmation by Ninhydrin assay due to presence of free amines on nanocages as well.

2.3.5. Increase in overall conjugation size and propensity for aggregation

DLS, also referred to as Quasi-Elastic Light Scattering, is based on the difference in the intensity of light scattered at a particular angle due to Brownian movement of particles and spatial restrictions in a limited volume of sample [138, 139]. It is a prevalent method for characterization of particle size in protein molecules and polymeric complexes. We have utilized this tool to confirm the completion of conjugation that led to the formation of protein-contrast agent complexes, using the change in their size distribution as the basis for the experiment.

Based on these size distribution experiments, the mean hydrodynamic diameter of L-PGDS was found to be 4.2 ± 0.1 nm with very slight variation among different batches. The roughly 18.0 nm size of ftnAA nanocages expanded to an average of 20.1 ± 1.5 nm upon the formation of LA conjugates. The mean hydrodynamic size increased to 68.3 ± 30.1 nm in case of LM conjugates, 118.6 ± 14.4 nm for LS, and to 100.8 ± 21.8 for LD samples. These conjugates remain stable in the solution up to 24 hours after preparation. However, studies were done on the conjugates stored at room temperature for two days, and at 4°C for a week, showed signs of aggregation due to the appearance of higher peaks in the spectra. Presence of larger molecules suggests that the conjugates tend towards aggregation. All the conjugates were freshly prepared for the following biological and cellular essays.

Figure 2.12. Dynamic Light Scattering profile for all IONP-LPGDS conjugates. The conjugated particles showed an increase in hydrodynamic size from control protein with a diameter of LA conjugates ~ 20 nm, LM conjugates ~ 70 nm, LS conjugates ~ 120 nm and LD conjugates ~ 100 nm.

As a general observation, the inorganic IONPs used in these experiments tend to be more polydisperse relative to the ftnAA nanocages. The confirmation of conjugation formation by the abovementioned methods has helped us optimize the amount of protein and different contrast agents required for the formation of the conjugates such that only a single species exists after overnight incubation of the activated contrast agents with L-PGDS at room temperature. These complexes are then used for further biocompatibility and functionality assays.

Based on the amino acid sequence of recombinant L-PGDS, there are ten lysines and eight arginines in each polypeptide chain. Therefore, a total of eighteen potential sites are available for covalent conjugation with activated carboxyl groups

from nanoparticles. A direct protein quantification approach by absorbance measurements was futile in our case since the nanoparticles also showed absorbance in the UV-visible range. Another protein quantification method previously used for nanoparticle coronas, surface plasmon resonance, posed additional difficulties due to lack of standard protocols concerning iron nanoparticles [140]. Isothermal titration calorimetry was also out of the question since the protein and nanoparticles did not bind through non-covalent interactions as observed by Ninhydrin colorimetric assay. Centrifugation to separate unbound IONPs aggravated particle sedimentation and led to the formation of very rigid mass which could not be re-solubilized. Therefore, the amount of protein in the conjugates was indirectly back calculated using initial concentrations added into the conjugate sample preparations.

DLS studies were able to confirm the formation of soluble conjugates in the sample. However, when the same samples were checked after a few weeks, the conjugates showed a propensity to form aggregates. Other biophysical techniques may also be employed to characterize the conjugates. For example, the characteristic peaks in the Fourier-transform infrared spectrum from Fe - O absorption bands around 500-600 cm^{-1} can be used for identification of iron oxide nanoparticles. The success of covalent conjugation can be determined by the presence of peaks in amide band around 1655 cm^{-1} and in carbonyl band around 1549 cm^{-1} [141]. Absorbance peak at 310 nm indicates the presence of ferric ions [128].

This unique way of formation of biological probes by covalently conjugating different molecules ensures compatibility with biological samples, including animal tissues. It provides more control over the targeting of these probes to a specific location and contrast enhancement in comparison with the surrounding environment. The conjugates were stable for up to 24 hours at room temperature in biological at pH 7.4. Thus, chemically these conjugates are compatible with biological systems and tend to have higher feasibility as a diagnostic tool to be used in living systems.

The SMNP and DNP stock preparations tended to sediment in the aqueous solution. Since both have a high concentration in the stock sample, these preparations were subjected to 5 min of sonicator bath at room temperature before conjugation

reaction, and always a diluted solution was used as the starting concentration of nanoparticles.

2.4. Summary

In this chapter, we characterized four magnetic nanoparticle candidates for covalent conjugation with L-PGDS to provide T2 contrast for MRI. Iron oxide nanoparticles with outer coatings of PEG, silica and dopamine, and ferritin nanocages were studied for their size and distribution using DLS and TEM. All the four contrast agents were soluble in water and physiological buffer, with silica coated nanoparticles with most propensity to aggregate. Recombinant protein was purified using IMAC by elution at 300-500 μ M imidazole concentrations in buffer. The eluted samples were further purified using size exclusion chromatography and the pure fractions eluted under the main peak were confirmed using SDS-PAGE. 1D NMR for purified protein showed a well-folded β -barrel structure. For covalent conjugation of the contrast agents to the protein, activation with 100 μ M EDC was carried out in MES buffer at slightly acidic pH. Upon activation, purified L-PGDS in physiological buffer was added and the conjugates after overnight incubation were tested for successful crosslinking by studying the change in size using DLS. Functioning and biocompatibility of these four conjugates with equal concentrations of L-PGDS will be detailed in the next chapter.

Chapter 3

3. Study of functional activity and biological compatibility of the conjugated L-PGDS

Biocompatibility of molecular probes is very important to gauge their efficacy as possible clinical therapeutic agents. Functionality of the L-PGDS protein in its conjugated form also indicates its ability to successfully mediate the chaperone activity towards amyloid aggregates. In this chapter, the functionality of the different L-PGDS conjugates is explored. Here we test the enzymatic activity of the L-PGDS within the conjugates. The inhibition of amyloid fibrillation in the presence of conjugates and the ability of the conjugates to breakdown the mature amyloid fibrils is studied here as a measure of their chaperone activity. The biocompatibility of the L-PGDS conjugates and their neuroprotective effects on neuroblastoma cells is also explored.

3.1. Introduction

Superparamagnetic IONPs have been developed for biological use due to their ease of synthesis, biocompatibility, and safety due to possible integration into haemoglobin metabolism [142]. While the reduction in particle size and surface modification enhance their target specificity, many of the MRI probes studied previously for AD diagnostics rely on the temporary opening of the BBB using mannitol injections, which limits their use for translation into the clinic. Poly D, L-lactic-co-glycolic acid (PLGA) is approved by the FDA as a biocompatible and degradable polymer suitable for drug delivery in case of brain diseases. In a recent study, PLGA molecules were conjugated with L-PGDS were targeted to the brain and successfully crossed the BBB without the need for any artificial opening [143]. It was noted that L-PGDS protein enabled these conjugates to enter the brain due to its lipophilic nature and presence of receptors in brain cells. Presence of biocompatible coatings such as PEG on the surface of the IONPs also facilitates the passage across the BBB. Here, we tested the functional ability of IONP-protein conjugates and their biocompatibility before their use in preclinical diagnostic imaging.

While the MRI contrast agents have been used for detection of cellular processes such as cellular apoptosis, gene expression, angiogenesis, and tumor formation, their use in disease detection has been somewhat limited till now due to lesser sensitivity of MRI compared to other imaging modalities [144]. However, conjugation of these contrast agents with suitable binding agents has also been explored previously for targeting organs in mice and humans for detection of disease pathology. Intravenous injections of superparamagnetic IONPs coated with 1,1-dicyano-2-[6-(dimethylamino)-naphthalene-2-yl] propene carboxyl derivative in rats and curcumin coated IONPs in Tg2576 mice showed binding of these particles to A β plaques in the rodent brains [145, 146]. Human H-chain ferritin conjugated with iron oxide nanocomposites were used for MRI labelling of macrophages [104]. Similarly, ultra-small IONPs functionalized with IgG4 antibody to target human activated platelets have been shown to successfully bind atherosclerotic plaques [147]. Researchers have also tried to inject A β (1-42)-conjugated ultra-small IONPs for targeting amyloid plaques in mice brain [148].

However, the possible damage due to the artificial injection of such potent self-aggregating amyloid is a crucial concern and has not been studied in any detail.

3.1.1. Effect of nanoparticle conjugation on protein function

Conjugation with nanoparticles can alter the protein behaviour through mediating protein instability which could lead to aggregation, loss of function, and by exposing new antigenic epitopes on the protein which could drastically diminish protein integrity within the biological systems [132]. These issues increase many folds in case of systemic injections where the nanoparticles come in contact with biological components in the bloodstream which can lead to non-specific binding of cellular proteins and small molecules giving rise to ‘corona’ around the injected nanoparticle probe. Therefore, we intended to avoid such unwanted non-specific interactions by incorporating intranasal administration of the protein-IONP conjugates and thereby bypassing the systemic route as well the BBB for direct entry into the brain regions through the olfactory pathway and trigeminal routes.

3.1.2. Assays to determine amyloid aggregation

A β (1-40) peptide acquires partially populated structural states where the residues 17-21 (Lys-Val-Phe-Phe-Ala) adopt a β -strand conformation. Small molecule fluorophores which bind to the cross- β -rich regions in amyloids are commonly used for detecting the aggregates. Thioflavin T (ThT), with unbound excitation maxima near 385 nm and emission at 445 nm, interacts with amyloid aggregates to emit fluorescence with a new excitation maximum at 450 nm and emission maximum near 484 nm [149]. Other commonly used dyes for amyloid detection are Congo red, and Thioflavin S. In addition to such colorimetric assays, techniques such as electron tomography and atomic fluorescence microscopy have also been used frequently to determine fibrillar structures and their interactions with lipid inclusion bodies within the cells [150, 151].

3.1.3. Mammalian cell cultures for the study of biocompatibility

The critical component of biocompatibility of a probe is to check for any potential toxicity through in vitro cellular assays. Cellular models for pathogenesis in case of AD are commonly observed in neuronal cells. Based on the specific study objectives, the effects on individual gene expression, protein activity, metabolic pathway or cellular activity can be monitored to identify any difference after

incubation with the probes [152]. For example, the Caspase activity as an indirect representative for cellular apoptosis is used for tissue cultures to study disease pathology [153]. Confocal and light microscopy are used to visualize the damage to the structural integrity in a cell using combinations of staining and immunohistochemistry. However, many histological stains provide a much easier tool to study any visible toxic effect of a specific treatment to the cells by the study of a number of viable cells after the said treatment.

3.1.4. Cell viability studies for neuronal cell cultures

Testing the toxicity effects of conjugated protein probes is pivotal to be determined before their practical use in biomedical applications. While using cellular toxicity assays for this purpose, potential points of false positives, such as modified cell dosage to aggregation, nanoparticle interference with colorimetric assays, or inaccurate fluorescence or absorbance readings due to overlap with nanoparticles, need to be adequately scrutinized.

MTT assay, a representative of cell proliferation, is widely used to access cell viability. It is a quantitative test which enables the measurement of cell growth by the direct relationship between the cellular activity and the absorbance observed at 570 nm due to the colorimetric product formation which converts the yellow dye into a purple product. Mitochondrial succinate dehydrogenase catalyses the enzymatic reduction of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to a purple product called formazan during the process of mitochondrial respiration (**Figure 3.1**). It is a commonly used and reproducible assay for adhesive cell types which grow into monolayers. One of the limitations of this assay is the insoluble nature of formazan product in aqueous buffer, which can be easily overcome by dissolving the purple crystals in the organic solvent Dimethyl sulfoxide (DMSO) or isopropyl alcohol. Soluble analogs of MTT, such as MTS reagent and XTT with soluble end-product, are also used for such cell viability tests. However, such analogs have lesser cellular uptake and may get reduced extracellularly, giving rise to conflicting assay results.

Figure 3.1. Reduction of MTT to formazan by mitochondrial enzymes. The yellow coloured dye MTT is easily absorbed into the cells and metabolized by cellular enzymes into purple coloured crystalline product formazan. Quantitative determination of formazan measured by absorbance provides an estimate of a number of viable cells in the assay.

The suitability of LPGDS-nanoparticles has been tested through various in vitro and in vivo studies done in neuronal cells. The chaperoning activity of conjugated probes and their effect on the viability of human neuroblastoma cells have been tested before initiating animal studies to confirm detection and therapeutic capabilities. Biophysical assays based on absorbance and fluorescence properties of the conjugated probes helped to detect their functionality and overall effect on cellular viability. The observations and results from such tests are detailed and discussed in this chapter.

3.2. Materials and methods

3.2.1. Chemicals and components

MTT – Biotium (Fremont, CA, USA)

DMSO – Sigma-Aldrich

Thioflavin T – Sigma-Aldrich

Amyloid β (1-40) lyophilized powder 98% purity – China Peptides (China)

HFIP - Sigma

Prostaglandin D2-MOX ELISA kit – Cayman Chemical (Ann Arbor, MI, USA)

Dulbecco's Modified Eagle Medium (DMEM)

Ham's F-12 growth media with 5% Fetal Bovine Serum (FBS) – Gibco

Phosphate-buffered Saline (PBS)

Triton-X – Bio-Rad (USA)

Formvar film-coated Cu grid 300 mesh – Inlab Supplies (Singapore)

Flat-bottom 96-well Black Polystyrene plates

Thermo Scientific Nunc Edge 96-well flat-bottom plates

Microplate adhesive film

3.2.2. Instruments

Freeze-dryer (Lyophilizer) - Virtis

Plate reader – Tecan Infinite M200Pro, Biotek Cytation 5 multi-mode plate readers

3.2.3. PGD2-MOX kit assay for the enzymatic study

To confirm the functionality of the conjugates, their capacity to isomerize the substrate prostaglandin H₂ (PGH₂) to prostaglandin D₂ (PGD₂), which is the primary reaction catalyzed by L-PGDS as an enzyme, was analyzed by an ELISA assay which recognizes PGD₂-MOX (Cayman Chemical, MI, USA). In this assay, the product PGD₂ is treated with methoxylamine (MOX) hydrochloride to give a stable derivative PGD₂-MOX. The steps in the test were carried out according to the manufacturer's instructions to use the kit. A standard curve was plotted between antibodies bound by tracer relative to maximum binding (%B/B₀) versus concentration of PGD₂-MOX produced during the assay.

3.2.4. Thioflavin T assay for amyloid inhibition

Small amounts of ice-cold 100 mM NaOH was added to lyophilized monomeric A β (1-40) peptide powder till it dissolved the white particles into a transparent film by gentle mixing in a centrifuge tube. This mixture was dissolved in 100% 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) and left for incubation at room temperature in the fume hood until the cloudy solution became transparent. The tubes were frozen at -80°C and the frozen samples were freeze-dried using lyophilizer overnight or until all the solvent got evaporated. The lyophilized powder or film was dissolved in the HEPES buffer to obtain 600-700 μ M peptide stock concentration just before setting the reaction and stored in ice until the assay. Thioflavin T (ThT) powder was dissolved in water and mixed thoroughly. The solution was filtered to remove any excess powder from the solution. This solution was sequentially diluted in HEPES

buffer until the stock concentration became 200 μM . The reaction samples used in Thioflavin T assay were added into flat-bottomed black 96-well polystyrene plates. 50 μM final $\text{A}\beta$ (1-40) concentration was used in the assay. ThT was added into every well to a final concentration of 20 μM . Final reaction volume in each well was 100 μl . The plate was covered with microplate adhesive film, and fluorescence values were obtained with excitation wavelength 440 nm and emission wavelength 484 nm.

3.2.5. TEM study to study disaggregation

For the study of the effect of L-PGDS conjugates on mature fibrils, 10 mg/ml of $\text{A}\beta$ 40 peptides were aggregated at room temperature for 72 hours with continuous shaking. The fibrils were equal volumes of negatively stained either with 2% phosphotungstic acid (PTA) or 2% uranyl acetate. This solution was dropped on formvar coated 300 mesh copper grids, and excess fluid was wicked out using filter paper. The samples were either vacuum dried for 1 hour or left overnight at room temperature before mounting into the microscope. 100 kV was used for image display with 40000x magnification at room temperature. High-resolution images were acquired by Dr Margaret Phillips for $\text{A}\beta$ (1-40) fibrils with the LA conjugates using Fei Technai T12 microscope to study the effect of LA conjugates on $\text{A}\beta$ (1-40) fibrils. The images for the LD conjugates were acquired using a 120kV Joel JEM 1400FLASH microscope.

3.2.6. MTT reduction assay for cell viability studies

The toxicity effects of all the conjugates were tested on SH-SY5Y human neuroblastoma cell line using [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) colorimetric assay. The cells were grown in 1:1 mix of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 growth media with 5% Fetal Bovine Serum (FBS) (Gibco) containing L-Glutamine and Phenol Red in 75 mm^3 T-75 flasks at 37°C in 5% carbon dioxide (CO_2) environment till they achieved 70-90% confluency. After 24 hours after transferring around roughly 2500 cells in each reaction well of 96 well transparent tissue culture plate, once the cells were stabilized, 10 μl of samples were added in each well containing 90 μl culture media. The samples were incubated for 24 hours to test their toxic effects before washing any toxic products using PBS. 90 μl of new media was added

to incubate with a colorimetric agent. MTT reagent stock was used in 5 mg/ml concentration. 10 μ l of this stock was added to each well-containing 90 μ l of growth media. After 4 hours of conversion into formazan product, the medium was removed, and Dimethyl sulfoxide (DMSO) was added to dissolve the purple crystals left in the dark for 1 hour before measurement of absorbance at 570 nm with 630 nm as a subtracted reference wavelength.

3.3. Results and Discussion

3.3.1. Isomerase activity of conjugated L-PGDS

We confirmed the functionality of the IONP-LPGDS conjugates by testing the extent of enzymatic activity of L-PGDS in each of the samples. Since the physiological role of L-PGDS as an enzyme is to isomerize the eicosanoid PGH₂ into PGD₂, this conversion was used as the basis for quantification of the function of recombinant human WT protein and in conjugation with IONPs. For this purpose, a commercial ELISA kit was used to estimate PGD₂ produced in the reaction by calculating the amount of a detectable methoxymated intermediate, herein mentioned as PGD₂-MOX [154]. The resulting product amounts were quantified by plotting a standard four-parametric curve as a percentage of absorbance plotted along the y-axis on a logarithmic graph with the x-axis representing the amount of PGD₂-MOX product formed in the units of pg/ml (**Figure 3.2**). Although the amount of PGD₂-MOX product formed was slightly lesser compared to the native protein, almost 70% activity was retained for LA conjugates. Normalizing with the amount of PGD₂-MOX formed by WT L-PGDS, conjugates with equimolar concentrations of protein showed 68.0 % yield for LA, 36.0 % for LM, 37.3 % for LS, and 30.7 % yield for LD complexes (**Figure 3.3**).

Figure 3.2. Standard curve for PGD2-MOX ELISA assay. The percentage ratio of the absorbance of a sample well (B) to that of the maximum binding well (B0) is plotted on the y-axis against the concentration of known amounts of Prostaglandin-D2-Methoxime product in pg/ml along x-axis. The enzymatic activity of the WT L-PGDS and the conjugates is calculated from the amount of PGD2-MOX product formed by each sample based on the four-parametric standard curve fit.

Figure 3.3. Enzymatic activity of IONP-LPGDS conjugates. Amount of PGD2-MOX product produced by L-PGDS conjugates as compared to the equimolar concentration of WT L-PGDS protein, calculated based on Standard MOX curve fit. As compared to L-PGDS, LA conjugates show 68.0%, LM show 36.0%, LS show 37.3% and LD conjugates show 30.7% enzymatic activity.

3.3.2. Inhibition of primary nucleation of A β (1-40) by L-PGDS conjugates

The aggregation kinetics of monomeric A β (1-40) peptides in the presence of WT human L-PGDS and its conjugates were followed for 60 h to observe the chaperone activity of L-PGDS. A β (1-40) control aggregation followed a sigmoidal curve for Thioflavin T dye fluorescence intensity, with a lag phase before the formation of seeds, a log phase of protofibril aggregation, followed by a stationary phase saturated with mature fibrils, where the inflection points are indicative of the primary and secondary nucleation processes respectively. In all the A β samples incubated with L-PGDS conjugates, an overall decrease in the fluorescent intensity of ThT was observed. As the normalized fluorescence intensity curves were plotted for all the conjugates, it was observed that compared to 100% fluorescence intensity for A β (1-40) control, the end-point fluorescence intensity was observed to range within 16-17% for A β treated with L-PGDS, 25% for treated with LA, 23-24% for LM, 15-17% for LS, and 25-27% for A β samples treated with LD conjugates (**Figure 3.4**). A reduction in the fluorescence intensity implies lesser cross- β amyloids structures in the solution, which is interpreted as inhibition of aggregate formation. Presence of WT L-PGDS and its conjugates with A β (1-40) delayed the

formation of such aggregates as well as reduced the total number of aggregates formed. Nanoparticles on their own did not inhibit the fibrillation of A β (1-40), yet presence of unbound nanoparticles interfered with the ThT signal giving erroneous fluorescence values.

Figure 3.4. Thioflavin T fluorophore intensity plot for A β (1-40) monomers in the presence of L-PGDS conjugates. Percentage fluorescence intensity at 485 nm is plotted on the y-axis as a function of time for 50 μ M A β (1-40) sample treated with 2.5 μ M of conjugates LA (blue), LM (cyan), LS (green), and LD (violet) with untreated 50 μ M A β (1-40) (black) and 2.5 μ M L-PGDS (red). Each sample contains 20 μ M ThT and were excited at 440 nm with reading taken every 30 min for a period of 72 h.

3.3.3. Inhibition of seeded nucleation of A β (1-40) by L-PGDS conjugates

For seeded nucleation, some preformed A β fibril seeds were inoculated to the A β (1-40) monomeric solution. The seeded aggregation begins with the secondary nucleation of already formed oligomeric aggregates present in the amyloidogenic sample. This seeded nucleation can fasten the fibrillation process bypassing the lag phase and prompting an exponential increase in the fluorescence intensity. The trend in delaying and decreasing the aggregation kinetics in the presence of L-PGDS conjugates was similar to that observed in the case of aggregation time it takes upon incubation with recombinant human WT L-PGDS. Reduction in the ThT fluorescence intensity was observed in all the A β samples incubated with the protein or conjugates. Compared to the fluorescence intensity curve normalized to 100 %

at the end-point for A β control, the maximum intensity values observed were ranging from 30-32 % for L-PGDS, 38-40 % for LA, 44-45 % for LM, 35-40 % for LS, and 22-23 % for LD conjugates (**Figure 3.5**).

Figure 3.5. Thioflavin T fluorophore intensity plot for seeded A β (1-40) in the presence of L-PGDS conjugates. Fluorescence intensity at 485 nm is plotted as a function of time for 50 μ M A β (1-40) sample treated with 2.5 μ M of conjugates LA (blue), LM (cyan), LS (green), and LD (violet) with untreated 50 μ M A β (1-40) (black) and 2.5 μ M L-PGDS (red). Each sample contains 20 μ M ThT and were excited at 440 nm with reading taken every 30 min for a period of 72 h.

It was inferred from the ThT fluorescence intensity curves that L-PGDS is capable of inhibiting both the primary and secondary nucleation of the A β peptide both in its native form and in conjugation with IONPs.

3.3.4. Breakdown of A β (1-40) fibrils by L-PGDS conjugates

TEM images of negatively stained A β (1-40) fibrils with L-PGDS protein molecules added to the solution just before loading onto the copper grid showed that the LA protein conjugates bind to the edges and lateral surface of mature A β (1-40) fibrils (**Figure 3.6**).

Figure 3.6. Transmission electron microscopy image for A β (1-40) fibrils in presence of LA conjugates. LA conjugates were used to visualize the binding of L-PGDS to A β (1-40) fibrils. L-PGDS binds to the lateral edges of the fibrils and accumulates near the tips of the fibrils.

Images acquired for samples of A β (1-40) fibrils co-incubated with LA conjugates and imaged after 30 minutes, show degradation of these fibrils into smaller species which could be monomers or small order oligomers, for which the images were acquired by Dr Margaret Phillips (**Figure 3.7**). Similar degradative effects were seen in case of LD conjugates, even in instances where the larger mature fibrils had acquired a crystalline morphology (**Figure 3.8**).

Figure 3.7. Breakdown of A β (1-40) fibrils by LA conjugates. The image panel shows different amyloid species observed in the grids after incubation of 2 week grown A β (1-40) fibrils for 30 min with LA conjugate samples (Images acquired by Dr Margaret Phillips).

Figure 3.8. Disaggregation function of LD conjugates on A β (1-40) fibrils. TEM images for A β (1-40) fibrils and nanoparticle-conjugated L-PGDS: a) Mix of DNP-LPGDS and

amyloid fibrils without incubation, b) Mix of DNP-LPGDS and amyloid fibrils upon 45 minutes incubation at room temperature, c) Amyloid fibrils after 40 hours, d) DNP-LPGDS.

3.3.5. Viability of neuronal cells in the presence of L-PGDS and conjugates

The protein and conjugate samples were tested for their biocompatibility in terms of their effect on the mitochondrial ability of human neuroblastoma SHSY-5Y cells to reduce MTT into formazan [155]. Protein concentrations up to 20 μM were tested for their impact on the cells after incubation of 24 hours. In a 96-well plate, 10 μl of each sample was added to 90 μl of the cells grown in DMEM media with roughly 2500 cells per well to begin. No significant cell death was observed in any of the wells (**Figure 3.9**). For conjugates with L-PGDS concentrations as high as 20 μM , more than 80% of cells were estimated to be viable based on the absorbance at 570 nm in the presence of LPGDS-IONP complexes (**Figure 3.10**). MTT-based cell viability tests show that the recombinant human L-PGDS protein is not toxic to the neuronal cells in its native form and in conjugation with IONPs.

Figure 3.9. Viability of SHSY-5Y neuroblastoma cells in the presence of L-PGDS. MTT cell-proliferation assay showed no significant cell toxicity for L-PGDS concentrations up to 20 μM .

Figure 3.10. Percentage cell viability in the presence of LPGDS-IONP conjugates. Cell-proliferation assay based on MTT absorbance was used to plot the percentage cell viability of human neuroblastoma SH-SY5Y cells in the presence of increasing concentrations of different L-PGDS conjugates up to 20 μM.

3.3.6. Impact on cell viability in the presence of Aβ (1-40)

An equal number of SHSY-5Y cells were incubated with 10 μl per well of 75 μM Aβ (1-40) monomeric samples allowed to grow for 48 hours at 37°C in a 96-well black plate. Increasing concentrations of different conjugated probes were added into respective wells. The absorbance of the formazan product at 570 nm was used to calculate the percentage of viable cells. It was observed that the wells with increasing concentrations of added L-PGDS conjugates show higher cell viability, indicating a protective effect of L-PGDS conjugates against the cytotoxicity induced by Aβ (1-40) aggregates. In comparison to the Aβ-treated cells without any conjugates, the absorbance was found to increase up to 10-folds in the wells which were treated with 20 μM conjugates for 24 hours (**Figure 3.11**). The absorbance increases with higher concentrations of the L-PGDS conjugates, thus reducing the toxic effects of Aβ (1-40) implying a potential outlook as neuroprotective probes.

Figure 3.11. Viability for SHSY-5Y cells incubated A β (1-40) in the presence of L-PGDS conjugates. A plot of the fold-increase in absorbance at 570 nm for increasing concentrations of L-PGDS conjugates based on MTT assay in the presence of 75 μ M A β in each well. The control represents the number of cells grown only in the culture medium.

3.4. Summary

The results in this chapter clearly show that the L-PGDS conjugates are functionally active and biocompatible. Also, these conjugates also act as inhibitors of A β aggregation and bind the growing fibrils to disaggregate them into smaller amyloid species. Thus, these conjugates are capable of preventing the aggregation of A β monomers, as well as breaking down the pre-formed fibrils in a bid of reversing the amyloidosis progression in disease pathology. The neuroprotective role of L-PGDS by improving the cell viability of neuronal cells in the presence of A β also supports the therapeutic benefit of these conjugate probes.

The activity of L-PGDS in the conjugates was found to be slightly lesser, which could be an indication of loss-of-function due to aggregation tendencies in the conjugates. Unlike their gadolinium counterparts, IONPs are not known to non-specifically distribute in the vasculature, probably due to their comparatively larger size and targeting properties. This property of IONPs makes them better agents for study in specific tissues to identify any abnormalities or tumors.

Chapter 4

4. Magnetic resonance study for contrast and detection differences between LPGDS-nanoparticle conjugates in mouse brain

Magnetic resonance imaging can be used for non-invasive early diagnosis of amyloids in the brain of Alzheimer's disease cases. The L-PGDS-conjugated magnetic nanoparticle probes provide sufficient MRI contrast and the specialized pulse sequences allow for their early detection in the brain. In this chapter, different magnetic nanoparticles are compared for their ability to impact the T2 relaxation and in turn the image contrast in MRI. We also show the enhancement in MRI contrast in the diseased mice brain compared to the healthy control after intraventricular injections of conjugated probes. Finally, we explore the possibility of using these conjugated probes for non-invasively targeting the brain regions through intranasal pathway, which we show here in control mice.

4.1. Introduction

The first clinical application of contrast agent in MRI took place in 1983 in the form of an injection of gadolinium chelate Gadopentetate Dimeglumine to a volunteer [125]. Following this instance, MR active contrast agents have found their use in MRI applications such as MR angiography, perfusion studies for tissue contrast enhancement, and imaging of the liver, lymph nodes, tumors, atherosclerotic plaques, and bone marrow. While the earliest contrast agents were gadolinium-based, their potential contribution to kidney damage in cases of nephrogenic sclerosis and accumulation in the brain has mostly unearthed a dire need for alternative biocompatible and less toxic MR contrast agents.

The relaxation of spins in a static magnetic field can be understood through a semiclassical vector model explained by the Bloch equations [156]. It governs the modulations in the bulk magnetic moment in the presence of an external magnetic field, whose axis is denoted to align with z-direction. When induced by a radio frequency (RF) pulse, the bulk magnetization undergoes precession about the z-axis with resonance frequency. The decay, otherwise known as relaxation, of the MR signal is governed by the rules of the Bloch model.

4.1.1. Image contrast in magnetic resonance imaging

Relaxation is the process through which magnetized proton spin systems regain equilibrium following an excitation RF pulse. The spin-lattice relaxation time constant T_1 or the longitudinal relaxation corresponds to the stabilization of spin populations and the transverse relaxation time constant T_2 or transverse relaxation is associated with the decay of coherences [157]. The spin-spin time constant T_2 represents the recovery of the transverse components of magnetization. Since the T_2 relaxation time for aqueous solutions can be very long and does not account for inhomogeneity in an external magnetic field, an apparent T_2 value, also known as T_2 star (T_2^*) is mostly preferred to calculate the signal-to-noise ratio (SNR). Hence, the signal decay is described by T_2^* rather than T_2 for stochastic magnetic field fluctuations.

$$\frac{1}{T_2^*} = \frac{1}{T_2 \text{ (natural)}} + \frac{1}{T_2 \text{ (inhomogeneity)}}$$

It has been noted that a combination of T1 and T2 effects of IONPs can help to determine the intracellular or extracellular location of the nanoparticles.

4.1.2. T2 and T2 star contrast in iron nanoparticles

T1 effects of IONPs tend to decrease in cellular compartments compared to the T2 effects, while the T2 star mostly remains unaffected [125]. Therefore, the IONPs have the diagnostic potential for use as molecular probes in MRI applications. As T2 contrast agents, IONPs shorten the relaxation time by proton diffusion-dependent dephasing or by the exchange of protons of IONP-bound water with the bulk leading to faster signal decay, whereas the more sensitive T2* relaxation value gets affected due to static dephasing as a result of magnetic field inhomogeneity [125]. Further enhancement of T2* effects can be achieved by using higher iron concentrations, gradient echo sequences, longer echo times, or by merely using higher magnetic fields for imaging.

4.1.3. Contrast agent quantification

The importance of quantifying the amount of protein conjugated to nanoparticle molecules is very intuitive for developing them into diagnostic tools for imaging. However, the essential presence of magnetic nanoparticles in the conjugates poses an immediate hindrance to accurate quantification of bound protein through varied spectrometric assays. Depending on the composition of surrounding coating and properties of the protein in question, UV-visible range spectrometric quantification of the protein absorbance may be affected in the commonly used Bradford assay or the bicinchoninic acid-based assay [158]. If the iron content in the nanoparticle is known, mathematical corrections can be used in case the shift is linearly correlated with the concentration of nanoparticles. With the exception of the specialized techniques such as radiolabelling and fluorescence measurements, the concentration of protein is still mostly estimated indirectly by comparing the initial protein concentration with the amount of residual protein determined in the unconjugated supernatant. This approach might provide limited accuracy in conjugation protocols such as ours, which involve multiple washing steps.

4.1.4. Carr-Purcell-Meiboom-Gill sequence

A Carr-Purcell pulse sequence can be represented as the equation:

$$(90^\circ x' \rightarrow \tau \rightarrow 180^\circ y' \rightarrow \tau \text{ (first echo)} \rightarrow \tau \rightarrow 180^\circ y' \rightarrow \tau \text{ (second echo)} \dots$$

Where the variation in τ allows for the calculation of T2 time.

It consists of a non-selective 90° pulse which is followed by a train of orthogonal non-selective 180° RF pulses with optional gradient spoilers. This sequence allows for non-localized T2 measurement through the determination of Mxy magnetization. The amplitude of the decaying spin echoes can be plotted against the time to give an exponential decay curve.

4.1.5. Gradient echo imaging

Gradient echo sequences can be used in-phase or out-of-phase to achieve a dark-bright contrast of the MR active nanoparticles to the surrounding fat content. GRE sequences utilize a combination of short repetition time (TR) and short echo time (TE) values allow for very rapid signal acquisition. Very low flip angles can be used in such sequences to improve the T2* sensitivity further. They are more suitable to detect T2* effects due to magnetic field inhomogeneity, which could be possibly caused by microscopic disturbances, including paramagnetic contrast agents, blood products, or iron deposits. In the case of T2*-weighted sequences, any lesions, structures, or areas of dephasing are seen as hypointense regions in the MR images.

4.1.6. Spin echo imaging

Spin echo (SE) sequence was introduced in 1950 by Erwin Hahn and is a fundamental program used for MRI since the earliest applications. SE sequence consists of a 90° excitation pulse which turns longitudinal magnetization completely into transverse magnetization, and a 180° refocusing pulse. The measured signal decays along with the transverse magnetization due to dephasing of the spins. Upon application of refocusing pulse, the spins rotate along an axis in a transverse plane which leads to re-phasing of these spins. This generates a spin-echo where the maximum signal occurs at the TE.

4.1.7. Mouse brain MRI

MRI studies in anaesthetized mice need to be completed in relatively shorter acquisition times, mostly less than 2 hours, which poses limitations for spatial resolutions within each scan as well as to the number of experiments which can be employed for the study. In case of AD mice, this time limit means that only a smaller percentage of amyloid deposits, the larger mature plaques (>50 μm), can be adequately visualized in the brain while the early deposits remain elusive [105].

Hence, the use of ultra-high magnetic field scanners is crucial for early diagnosis of amyloid deposition. The value of $T2^*$ as well as the resolution in the MR images are significantly improved with an increase in magnetic field strength, without any significant impact on the health of candidate mice. The specifications for the ultra-high field high-resolution MRI scanner used for imaging of contrast agents are mentioned in the **Table 4.1**. The effects of 2 hours exposure static magnet fields of 3.5-23.0 T on the physical health of mice has been shown to cause no severe long-term outcomes based on blood tests and organ weight while capable of reaching a spatial resolution of $\sim 18 \mu\text{m}$ at 21.1 T [159].

Table 4.1. Specifications of high-resolution MRI scanner.

Magnet	14 Tesla Bruker Ascend 600WB vertical bore
Probe	MicWB40
Gradient	Micro2.5 gradient system, up to 150G/cm at 60A
Software	Paravision 6.0.1
Coil diameter	25 mm, 30 mm
Pulse Sequence	Fast Low Angle Shot (FLASH)- gradient echo, RARE, MSME
Image Reconstruction	Dependent on experiment

The contrast enhancement upon administration of MR active biological probes can be a quantifiable measurement of $T2$ star values in specific regions of interest (ROIs). Such measures can also indicate the comparison for dispersion of the MR agents in AD versus wild type mice. In the case of ex vivo comparisons, voxel-based analysis is one of the previously used methodology for quantification [105]. Geometric models for brain structures can be used to detect morphological changes in case of disease conditions versus healthy individuals and across patients [160]. The early progression of AD is mostly asymptomatic in case of anatomical changes. The symptoms of brain atrophy in case of AD visible in the medial temporal lobe,

while the temporoparietal cortex is among the last brain regions to show visible atrophy on the imaging modalities. Indirect visibility of A β plaques on T2* MRI is attributed to the presence of iron deposits in these structures [161]. The spatial delineation of brain regions, also known as brain parcellation, is a critical step in the calculation of quantitative differences between samples.

4.1.8. Intranasal delivery for non-invasive administration

Intravenous administrations in animal models have shown that iron is retained in the choroid plexus epithelial cells and neuroglial cells. Hypotensive reactions and local discoloration have also been previously observed when the IONPs are injected intravenously [125]. Systemically administered formulations mostly accumulate in organs like liver and kidney, and only a tiny fraction ever reaches the brain and even lesser ever crosses the BBB. The intranasal injections allow brain targeting of these probes through olfactory lobes and hippocampus as they by-pass the BBB. It involves direct entry into the brain through the olfactory passage and trigeminal pathway by absorption through olfactory epithelium and trigeminal nerve (**Figure 4.1**). Another aim of this way of probe administration over systemic injections is to avoid the formation of nanoparticle corona through weak non-specific interactions with several proteins encountered during their passage through the bloodstream [132].

Figure 4.1. Schematic for an intranasal delivery pathway to the mouse brain. Alternative pathways through intranasal route for brain targeting without crossing the blood-brain barrier. The olfactory passage and trigeminal pathway allow for retention of conjugates into the brain. Image is taken from [162].

4.2. Materials and methods

4.2.1. Chemicals

Paraformaldehyde – Sigma Aldrich (Steinheim, Germany)

PBS – Vivantis (Malaysia)

Sucrose – Affymetrix/USB (Heysham, England)

Agarose – USB (Cleveland, OH, USA)

Fomblin – Sigma Aldrich (Germany)

4.2.2. Instruments

Bruker BioSpec 9.4T MRI scanner

Bruker Avance III HD 600 MHz (14.1 T) NMR spectrometer with the microimaging probe

The MRI measurements used a Bruker MicWB40 Probe in combination with the Micro2.5 Gradient System, which can provide a gradient strength of 150 G/cm (at 60 A). ImageJ and Matlab were used for image analysis.

4.2.3. Fast Low Angle Shot (FLASH)

FLASH method utilizes a single slice-selective radiofrequency pulse which generates a gradient echo. With short TE and TR, the steady-state signal can be acquired very fast independent of T1 and T2. Therefore, the FLASH methods were always run in the beginning as a localizer scan to get rapid pilot images ensuring correct positioning of the sample with respect to the centre of the magnet.

4.2.4. Rapid Acquisition with Relaxation Enhancement (RARE)

RARE allows for rapid imaging with refocussed echoes. It makes use of the CPMG sequence with selective RF pulses to generate multiple separately phase-encoded spin echoes. It was applied for accelerated high-resolution T2-weighted MR scans, where the TE = T2 and the repetition times were always greater than the T1 values. The scan times were reduced by modulating the RARE factor values which represent the number of different phase-encoded echoes. RARE scans with variable repetition time for saturation recovery allowed for the calculation of T1 and T2 using a single scan.

4.2.5. Multi-Slice Multi Echo scan (MSME)

MSME uses the CPMG sequence to generate multiple spin echoes with slice selective RF pulses. Each echo allows for a snap image with different echo time. It is used for T2 weighted MRI scan, which also allowed for T2 mapping. Relaxometry effects of iron-oxide based contrast agents in solution, agarose and mice brain were calculated with the help of T2 and T2* maps obtained through MSME scans.

4.2.6. Brain MRI studies on AD mouse models

Triple transgenic AD mice (3xTg-AD) were used as a disease model for MRI and histological studies. These animals have point mutations in three transgenes- presenilin (PS1), amyloid precursor protein (APP), and microtubule-associated protein tau gene. Methionine 146 residue is replaced with Valine in PS1, Proline 301 of tau mutated into Leucine, and Swedish double mutation in APP where Lysine and Methionine residues at locations 670 and 671 were changed into Asparagine and Leucine respectively [83].

4.2.7. Intranasal delivery for non-invasive administration

Anaesthetized mice were made to inhale 10 μ l of L-PGDS conjugates with 5 μ l solution per nostril. The mice were then set on the MRI scanner for scans for 4 h and 24 h post-administration scans.

4.2.8. Intraventricular injections for a longitudinal study

LA conjugates were administered in 3xTg AD mice through intraventricular injections with 2 μ l sample injected in each brain, with 1 μ l of conjugate sample injected over 10 min into either ventricle. This experimental setup was performed in Singapore Bioimaging Consortium by Ms Fransesca, and anaesthetized mice were imaged by Dr Joanes Grandjean. Ms Ling Yun helped with handling the anaesthetized mice and later in euthanizing the animals after MRI studies.

4.3. Results and Discussion

MR data acquisition and early processing were done using Paravision 6.0.1 software [163].

4.3.1. Contrast for IONPs in T2-weighted MR images

The enhancement in the image contrast was identified by comparison of the contrast-to-noise ratio (CNR) in T2-weighted MRI, which allowed us to qualitatively and quantitatively compare the improvement in the signal with increasing concentrations of the CAs. The stocks provided by the collaborators were directly used for contrast comparison with gentle mixing for SMNP particles to allow mixing into a homogeneous solution. 5 μ l of each stock solution was dissolved in 95 μ l of PBS to make the first concentration. This concentration was diluted into 1/10, 1/50, 1/100, 1/200, and 1/500 dilutions to form six phantom samples for contrast study using MRI. Each sample was added into a 200 μ l capillary glass tube and all six tubes were inserted into a 10 mm NMR glass tube filled with PBS for imaging in the vertical 14 T MRI scanner (**Figure 4.2**).

Figure 4.2. Contrast comparison for all contrast agents at serial dilutions. MRI phantom T2*-weighted RARE images showing different concentrations of contrast agent solutions in PBS buffer. 5 μ l of each contrast agent was dissolved in 95 μ l of buffer to get

the highest concentration. These samples were then serially diluted to make 1/10, 1/50, 1/100, 1/200, and 1/500 samples. The progression of contrast enhancement with increasing concentrations is evident.

4.3.2. T1 and T2 relaxation curves for analysis

For T2-weighted scans, the change in signal intensity was plotted against the echo times to obtain T2 relaxation curves. The value of relaxation time T2 is equal to the time required for the transverse magnetization to reduce to $1/e$ or roughly 37% of its original value. These values were plotted against the dilutions of nanoparticle stocks to estimate their r_2 relaxivities. Likewise, the T1 relaxation time can be calculated as 63% of the maximum value of z-component of magnetization. These exponential decay- based relaxation curves allowed for calculation of relaxivities r_1 and r_2 for all four contrast agents (**Table 4.2**). T1 and T2 relaxation curves were obtained using RARE pulse program, which leads to the generation of multiple spin echoes with slice selective RF pulses at 14 T. In a single customized SE experiment, images were acquired using varying TR and TE values. (**Figure 4.3, Figure 4.4, Figure 4.5, Figure 4.6**).

Figure 4.3. Relaxation curves for ftnAA contrast agent. Relaxation graphs for calculation of T2 and T1 calculations for the ftnAA contrast agent as generated from RARE image processing using Paravision 6.0. The signal intensity on the y-axis is unscaled and the echo time for a) and recovery time for b) are in ms.

Figure 4.4. Relaxation curves for MNS contrast agent. Relaxation graphs for calculation of T2 and T1 calculations for the MNS contrast agent as generated from RARE image processing using Paravision 6.0. The signal intensity on the y-axis is unscaled and the echo time for a) and recovery time for b) are in ms.

Figure 4.5. Relaxation curves for SMNP contrast agent. Relaxation graphs for calculation of T2 and T1 calculations for the SMNP contrast agent as generated from RARE image processing using Paravision 6.0. The signal intensity on the y-axis is unscaled and the echo time for a) and recovery time for b) are in ms.

Figure 4.6. Relaxation curves for DNP contrast agent. Relaxation graphs for calculation of T2 and T1 calculations for the DNP contrast agent as generated from RARE image processing using Paravision 6.0. The signal intensity on the y-axis is unscaled and the echo time for a) and recovery time for b) are in ms.

MR relaxation is sensitive to the thermodynamic properties of the different phases—solid, liquid, or gaseous phase of the sample under study [164]. Hence, we studied the relaxation properties in agarose phantoms as well due to their similarity to biological tissue composition. Similar CPMG sequences as above were used to scan nanoparticles embedded in 1% agarose gel as phantoms for mouse brain tissue. T1, T2, and T2* relaxation times were calculated, as mentioned in the **Table 4.2**. Spatial heterogeneity can also be observed in T2 relaxation MRI. The T1 relaxation values

were calculated by plotting the signal intensity as a function of TR while the TE was fixed. Alternatively, T2 was calculated through the plot of signal intensity as a function of TE with a fixed TR.

Table 4.2. Relaxivity values for all four contrast agents at 14 T.

Relaxivity (mM⁻¹ s⁻¹)	FtnAA	MNS	SMNP	DNP
<i>r</i> 2	159.01	105.10	2.08	10.01
<i>r</i> 1	0.42	4.45	0.20	0.73

4.3.3. Differences in T2 contrast after intranasal delivery

The concentrated L-PGDS conjugates were administered as 5µl in each nostril of anaesthetized BL6 control mice as a droplet using a pipette. The mice brain scans were acquired 4 hours and 24 hours post administration. No specific regions of hypointensity were observed likely due to lack of specificity in control mice. However, the total change in T2* contrast of the whole brain was compared for the two-time intervals.

In T2* weighted FLASH images acquired for mice brain after intranasal administration of L-PGDS conjugates, the contrast differences and susceptibility effects were noticeable. In the frontal brain sections, there was an increase in the T2* contrast and the inhomogeneity artefacts due to the presence of iron in the conjugates were more prominent (**Figure 4.7**).

Figure 4.7. Differences in T2* contrast upon intranasal administration of LA conjugates. The left and right panels represent the same slice in T2*-weighted FLASH images taken before and 24 hours after the intranasal delivery of LA conjugates in the healthy mice. The T2* susceptibility effects result in hypointensity in the brain regions with a high concentration of conjugates and an increase in susceptibility artefacts in the surrounding tissues seen in the images as distorted wiggles in the scan.

4.3.4. T2 contrast post intraventricular injections in healthy versus AD brain

Due to logistic limitations on the number of available age-matched 3xTg AD mice and lack of any specific binding through intranasal administration in control mice brain, intraventricular injections of 2 μ l LA conjugates were administered in healthy control and an age-matched AD mouse brain. The mice were scanned before injections, as well as 24 hours and 48 hours post injections to observe the diffusion of conjugates from the ventricular chambers to different amyloid-rich brain regions. The T2* signal hypointensity was observed after the injections (**Figure 4.8**).

A lack of signal intensity was observed in the brain regions surrounding the ventricles such as the fimbria, the dorsal thalamus and the pallidum and diffused to other sections of these tissues throughout 24 hours. The accumulation of injected L-PGDS conjugates was also observed in the anterior commissure region of the olfactory limb, which comprises the olfactory nerve. Hypointensity was observed in the amygdala, the medial septal nucleus and CA3 hippocampal region compared to baseline images. The corpus callosum and cerebral nuclei regions such as caudoputamen showed consistent T2 signal enhancement in the weighted images. The somatosensory and somatomotor areas, retrosplenial and anterior singulate region were the key cortical regions affected in T2-weighted images. This spatial pattern of hypointensity on T2-weighted images is consistent with previously identified amyloid-rich brain regions [165-167].

Figure 4.8. Accumulation of L-PGDS conjugates in amyloid-rich brain regions. Comparison of contrast upon intraventricular injections of LA conjugates in 1-year old healthy mouse versus age-matched 3xTg mouse. The accumulation of LA in brain regions is highlighted by the increase in hypointensity in the tissue as marked by red arrows in the image panel.

4.3.5. T2* contrast change after intraventricular injections in AD mouse

The comparison of MRI images for AD mice before and after the intraventricular injections of LA conjugates also showed enhancement in hypointensity in brain regions and some susceptibility artefacts due to the paramagnetic properties of LA conjugates (**Figure 4.9**). Brain regions including the choroid plexus, corpus callosum, nuclei in the thalamus region, and outer cortical layers were affected over

time. As previously mentioned, the diffusion of the LA conjugates to these areas correlates with the presence of amyloid structures.

Figure 4.9. Self-comparison of T2* contrast enhancement in AD mouse after injection of LA conjugates. Compared to the baseline, hypointensity and susceptibility artefacts seen in the images 24 h, 48 h, and 72 h post intraventricular injection images have been marked with red arrows. The scans were acquired at 11.4 T with image size was 200 x 106 pixels, with field of view 17 x 9 mm, 60° flip angle, TE 10 ms, TR 1500 ms with 3 averages each for 38 slices through the mouse brain.

4.3.6. Change in contrast over time post intraventricular injections of LA

The enhancement in T2* contrast after 24 hours if intraventricular injections were retained and even increased at 48 hours post injections. The brain regions with high amyloid content, especially the thalamus, the fimbria and the cerebral nuclei showed more loss of signal over time, indicating the localization of LA conjugates in these regions (**Figure 4.10**). The area near dentate gyrus and CA1 hippocampal field showed an increase in hypointensity at 48 hours as compared to 24 hours post injections. The change in hypointensity pattern over the regions of thalamus also indicates that the LA conjugates diffuse over time in the brain to the amyloid-rich areas.

Figure 4.10. T2 contrast comparison 24 h and 48 h post injections in AD mice. Left side of the panel shows T2-weighted MRI images post 24 h of intraventricular injection and the right side shows same slices post 48 h. Retention of hypointensity in amyloid-rich regions is marked by white arrows throughout the images.

4.4. Summary

The contrast comparison of the IONPs is a crucial step in determining the combination in the ideal conjugate probe. Based on the unambiguity in the change in contrast with increasing concentrations of the IONP and the homogeneity of higher IONP concentrations in aqueous solution, ftnAA were much superior than the other IONPs. LA conjugates were transported to the mice brain through intranasal administration to a higher extent as observed from the MRI images in healthy mice and further corroborated through histological studies explained in later chapter. Due to conjugate homogeneity, the LA conjugates were easy to administer to 3xTg AD mice during the intraventricular injections, whereas significant

backflow was observed during the attempts to administer LD conjugates through intraventricular injections. Over time, the LA conjugates were seen to diffuse to amyloid-rich brain regions. Vascular contrast enhancement by IONPs can last for several days and enhancement of contrast in the tissues up to several weeks. This extended lifespan can be utilized for longitudinal studies to identify the possible therapeutic effect of the conjugates on disease progression.

Chapter 5

5. Histological Study of Mouse Brain

Histological analysis of biological tissues can help in identifying various anatomical details at microscopic level. It provides a corroborative evidence for the in vivo imaging by other modalities such as MRI. In this chapter, we use histology on the chemically preserved brain slices to detect the presence of the conjugated probes in the brain after intranasal administration. There are special dyes and stains to detect presence of neuropathological amyloid depositions in vitro. Additionally, the magnetic nanoparticles can be identified by other stains which bind specifically to iron. We also detail the histology on the mouse brain slices excised after intraventricular injections to visualized co-localization of the probes in amyloid-rich brain regions. We show here that the results from in vivo imaging using MRI correlate well with the in vitro histological study of the sliced brain.

5.1. Introduction

Histochemical sectioning provides a basis for further confirmation of the observations made through the MRI experiments. Preparation of a histological staining experiment involves careful preparation of tissue sections dependent on pre-staining procedures involving perfusion of the small animal, preparation of compatible slides, and a suitable way of tissue sectioning prior to the actual treatment with the staining dye.

5.1.1. Tissue sectioning

Based on the type of staining, there are two types of sectioning- cryo-sectioning and paraffin-wax sectioning. For small animal organs such as the pancreas, fixing in PBS and embedding in the paraffin wax and mounting onto the slides in a heated water bath is the preferred method of preparation of tissue sections of 5-10 μm thickness, followed by storage in ethylene glycol cryo-protectant [168]. Cryo-sectioning of the brain tissue is preferred for easy preparation of 25-100 μm thick sections for full tissue staining.

5.1.2. Mouse brain regions

Isocortex shows the earliest signs of amyloid aggregation in the APP/PS1 and Tg2576 mice [167]. The emergence of amyloid plaques follows in other brain areas including the olfactory, hippocampal and cortical subplate regions. However, this pattern is not consistent for other amyloid-based AD mouse models. For example, the hAPP-J20 mice show the most density of plaques in the retroplenial cortical region [167]. Brain regions such as basal ganglia, olfactory bulb, hippocampus, cerebellum etc. are associated with degeneration in AD cases. 3xTg mice show age-dependent tau and amyloid pathology with the development of A β plaques in their hippocampus and cortex at 12 months of age [169].

5.1.3. Mouse brain histology

The amyloid plaques and tau tangles are often identified using immunostaining with specific antibodies, or stains which bind to the proteinaceous aggregates. Additionally, mouse monoclonal antibodies and rabbit polyclonal antibodies are often used for immunohistochemical staining of amyloids and pre-amyloid deposits [168]. Diaminobenzidine tetrahydrochloride is commonly used as a chromogen in addition to hydrogen peroxide to enhance the staining intensity when using

antibodies. Here, we use A β specific stains to identify the presence of amyloids in specific brain regions. A combination of stains for anatomically could identify the presence of amyloids and localization of chaperone proteins with the plaques in one and half years old 3xTg AD mice [170].

5.1.4. Amyloid stain

Congo red and Thioflavin S dyes recognize the cross- β structures in amyloid oligomers. Therefore, these are popularly used for histological staining of AD brain tissue sections [168]. Other histological stains for brain amyloid detection include iodine, silver methenamine, Bielschowsky stain, Cross, Bodian and Gallyas stains, which are less common now. Congo Red is the sodium salt of benzidinediazo-bis-1-naphthylamine-4-sulphonic acid ($C_{32}H_{22}N_6Na_2O_6S_2$) which gives an orange-red appearance under light microscopy. It is dichroic and shows an apple-green birefringence upon cross-polarization under a fluorescent microscope. It binds parallel to the fibrillar axis in an anti-parallel fashion to the β -sheets in roughly 80% of amyloid fibrils and protofibrils, while antiparallel to the fibril axis at the end of protofibrils in the remaining 20% cases [171].

5.1.5. Iron stain

Iron containing components can be stained blue in the presence of hydrochloric acid and potassium ferrocyanide. It forms a blue colloidal complex of ferric ferrocyanide, known as Prussian Blue [172]. Iron oxide nanoparticles are stained using Prussian Blue as a colorimetric tool to identify and quantify their presence in a specific tissue. A pink or red counterstain such as nuclear fast red or safranin O can be used.

5.2. Materials and methods

5.2.1. Chemicals

O.C.T.TM - Tissue-Tek

Paraformaldehyde (PFA) – Sigma-Aldrich (for 4% solution in PBS)

Sucrose – Bio-basic (for 30% solution in PBS)

Prussian Blue Iron Stain kit (250 ml of each: 4% Potassium Ferrocyanide, 4% Hydrochloric Acid, 1% Nuclear Fast Red) – Polysciences, Inc

Congo Red – Sigma-Aldrich

Hydrochloric acid

Liquid Nitrogen

Dry ice

Ethanol

PBS

5.2.2. Instruments

Light microscope – Nikon Eclipse *Ci*

Cryostat – Leica

Aluminium cryomolds

Forceps

Knife blade

Brush

Glass slides

5.2.3. Brain extraction from euthanized mice

The anaesthetized mice treated with L-PGDS conjugates were euthanized using transcardial perfusion with PBS, followed by injection of 4% PFA to fix the tissues in the body. Once the tissues were fixed, the head was sliced, and the skin surrounding the skull was cut. Gentle cuts were made at the top of the scalp till the hard tissue gave way to mice brain and was gently removed using forceps. This step was done by Ms Francesca in SBiC.

The extracted brains were stored in 4% PFA overnight. The samples were then washed with PBS. Before staining, the brain samples were soaked in a falcon tube in 5% sucrose solution until the soluble tissue sunk at the bottom. The brain was then transferred to 15% sucrose. After it descended, the brain was transferred into 30% sucrose solution. It was stored in this solution for 24 hours till it got sunk entirely at the bottom of the tube. This brain infused with 30% sucrose was frozen for cryo-slicing.

5.2.4. Frozen brain slicing

Excised mouse brain tissue was embedded in the tissue-freezing OCT medium in a small mould made from aluminium foil. This mould was uniformly frozen by gently funding with liquid nitrogen. Once all the OCT surrounding the brain tissue turned white, the moulds were stored at -80°C until cryosectioning. The aluminium casing and excess OCT surrounding the frozen brain tissue was removed using a blade to set up the tissue for mounting on Leica cryostat. OCT was used to fix the mould in the cryostat, and 25-50 µm tissue slices were collected on charged glass slides. These glass slides were stored in -20° C until staining.

5.2.5. Congo red staining

In the brain slices of AD mice, the amyloid structures were stained using a Congo red stain solution. The stock solution was prepared by adding 2.5 g to 250 ml of 50% ethanol. The undissolved material was filtered through a Whatman filter paper. The glass slides with brain slices were dipped in this stock solution for 10 minutes. The slide was rinsed under running water. The slide was dipped in 70% ethanol to remove excess stain. 100% ethanol was used to dry the slides. Slides were stored at -20°C before viewing under the microscope.

5.2.6. Prussian blue staining

Histological staining of iron present in the conjugates was achieved by incubation with Perl's agent as per the product instructions. Equal amounts of solution A, 4% Potassium Ferrocyanide and solution B, 4% Hydrochloric Acid, were mixed for the working solution. The stain was given 2 changes of the solution at 10 minutes each at RT. Excess stain was rinsed in several changes of distilled water. The slides were kept in the counterstain solution C, Nuclear Fast Red, for 2 minutes. The slides were rinsed in running tap water and dehydrated through ascending alcohols. Slides were stored at -20°C before viewing under light microscope.

5.3. Results and Discussion

5.3.1. Detection of LA conjugates in healthy mouse brain

Prussian blue staining of the brain tissue slices excised post-mortem from the healthy mice after intranasal conjugate delivery showed the blue coloration indicating the presence of IONP-LPGDS conjugates, compared to only pink

background of nuclear fast red observed in the slice from an untreated control mouse. The regions of blue coloration coincide with the areas surrounding or connected to olfactory pathway (**Figure 5.1**).

Figure 5.1. Prussian blue staining detects the LA conjugates in healthy mice brain after intranasal delivery. Histological images on the right show the same regions scanned using MRI on the left. Three consecutive slices show different brain regions showing presence of L-PGDS conjugates. Scale bars: 1 mm.

5.3.2. Detection of LS conjugates in healthy mouse brain

LS conjugates were detected near the amyloid-rich brain regions of corpus callosum, hippocampal CA1 and CA3 regions near the ventricle, near the cerebral nuclei in thalamus region, and some accumulation near the cortex in the healthy mice brain (**Figure 5.2**). Blue coloration was observed around the dentate gyrus which is associated with memory formation and processing. The presence of

Figure 5.2. Prussian blue staining detects the LS conjugates in healthy mice brain after intranasal delivery. Histological images on the right show the same regions scanned using MRI on the left. Localization of dye near the hippocampal region is clearly visible. The lower image panel shows a zoomed section into the corpus callosum to show spread of conjugates. Scale bars: 500 μ m.

5.3.3. Histological staining of LA conjugates in 3xTg mouse brain

In the 3xTg mouse brain slices excised post 72 h of intraventricular injections, the LA conjugates were found in AD-associated brain regions. We detected LA conjugates in the hippocampus, cortical regions including the entorhinal cortex and retrosplenial cortex, and corpus callosum [169]. Within the hippocampus, LA conjugates mostly populated the CA1 and CA3 regions, and accumulated around the dentate gyrus. Additionally, these conjugates were also found near the sensory cortex, motor cortex and entorhinal cortical regions (**Figure 5.3**).

Figure 5.3. Presence of LA conjugates in AD associated mouse brain regions. Histology in the mouse brain slices excised after intraventricular injections of show detection of amyloids using Congo Red staining (Left) in the same brain regions as presence of LA conjugates using Prussian Blue staining (Right) in AD associated brain regions- A.

Hippocampus, B. Corpus callosum, C. Isocortex, and D. Cerebral cortex. Scale bar: 500 μm .

5.3.4. Co-localization of LA conjugates in amyloid-rich regions

Staining the hypointense regions in T2* MRI images from 3xTg mice with Congo Red and Prussian Blue helped us detect the co-localization of LA conjugates with A β in the mouse brain (**Figure 5.4**).

Figure 5.4. Histological staining of 3xTg mouse brain slices. Regions of hypointensity in T2* MRI images are stained with Congo red and Prussian blue staining of mouse brain slices of different regions after excision from 3xTg mice 72 h post intraventricular injection with LA conjugates. White arrows indicate the regions of co-localization in amyloid-rich brain regions.

5.4. Summary

Iron oxide nanoparticles can catalyse the oxidation of chromophore substrate in the presence of peroxidase; this property can be used for detection of peroxidase substrates such as 3,3',5,5'-tetramethylbenzidine to generate a blue-coloured oxidized product or di-azo-aminobenzene to give brown colour in the tissue [127]. Histological studies on the excised brains previously imaged through MRI allows for a confirmation of the imaging experiments. While we mainly relied here on the dye-based staining assays, immuno-histochemical studies which utilize anti-A β antibodies or L-PGDS markers can provide additional confirmation to the co-localization tests for L-PGDS and A β , as well as a measure of their interactions.

Chapter 6

6. Conclusions and Future Directions

Previous chapters have detailed the development of functional and biocompatible diagnostic tools by conjugating L-PGDS protein to magnetic contrast agents. This chapter provides a brief conclusion and summary of the work done through this thesis and the overall findings. This chapter also outlines the possible course of direction to develop this work and possible outlook for its expansion.

6.1. Thesis conclusions

This thesis is a proof-of-concept study aimed to design and test a suitable L-PGDS protein-based molecular probe for detection of amyloids in the brain of AD mice and confer therapeutic intervention to amyloidosis dependent disease pathology.

We used the endogenous human brain protein L-PGDS in this study due to its A β chaperone function and protective role in many neurological disorders [70, 74, 173-176]. We have also shown recently that L-PGDS also acts as an amyloid disaggregase in addition to its chaperone activity [34]. Here we posit L-PGDS can be very efficiently used with functionalized MR-active contrast agents for early detection A β in AD brain. To begin the process of developing an endogenous protein as a diagnostic and therapeutic agent, the key requirement is to ensure it can be visualized using presently available detection modalities. We chose to work with MRI detection modality due to its comparative radiation safety and low physiological impact on animals [159]. For contrast-enhanced detection, the protein should be bound to an imaging agent, the IONP in our case, and should be capable of to higher amyloid species including oligomers.

In this study, four contrast agents with iron oxide core and different outer layers were functionalized to bind amyloid- β chaperone L-PGDS protein. Here, non-invasive MRI able to provide macroscopic imaging of the mice brain is used as a tool to detect conjugated diagnostic agents which can bind to amyloid- β . For this purpose, L-PGDS protein was chemically conjugated with these nanoparticles, which can provide T2 relaxation-based contrast during MRI scans.

The suitability of each of the contrast agents was compared by T2-weighted MRI using CPMG pulse sequences. Following the biophysical and cellular assays to test the stability and biocompatibility of the conjugates, MRI experiments were used to detect their localization in the amyloid-rich regions in the brain of anaesthetized mice. Enhancement of T2 contrast due to the presence of conjugates led to hypointensity in the brain regions.

We also categorized the properties of L-PGDS conjugates as diagnostic agents which were tested using MRI and TEM studies. The therapeutic property of these conjugates was studied using in vitro aggregation dependent fluorescence assays and cellular toxicity tests (**Figure 6.1**).

Figure 6.1. Concluding schematic of theranostic study of L-PGDS conjugates.

We hope that the results from this study will encourage further research in the development of alternative brain-targeted biological probes. The comparison between IONPs in this study provides a streamlined platform about which more MR active agents can be compared for risk versus benefit analysis. The surface composition of the nanoparticles as well as the amino acid sequence of the targeting protein, in this case, L-PGDS, can be deliberately mutated to alleviate immune response elicited upon in vivo administration. Passage of IONPs through blood circulation can cause the formation of coronas whereby endogenous proteins can bind while these nanoparticles reach their target. This property can be utilized by choosing suitable agents for further functionalization or conjugation to aid the transport across barriers, for example by enhancing the interaction with apolipoprotein which improves the transport of nanoparticles across the BBB [125].

6.2. Future directions

One of the probable routes is to express the protein in a more compatible eukaryotic system such as insect cells to see if any physiologically relevant post-translational modifications can improve the protective effects of L-PGDS [177]. Use of L-PGDS extracted from brain CSF would provide a more comparable sample for making any conclusions in favour of potential translatable therapeutic effects of L-PGDS as a chaperone and disaggregase [174, 178].

Identification of more functional L-PGDS mutants can also enhance the chaperoning action of the protein [179]. While the previous study has hypothesized that the catalytic residue Cys65 in the recombinant human L-PGDS is critical for chaperone activity, the work done in our lab suggests otherwise. The C65A mutant of recombinant L-PGDS also showed significant chaperoning activity. This work should be further explored to more L-PGDS mutations which can enhance its chaperoning activity while decreasing the potential inflammatory impact of the protein in biological systems. Testing the suitability of this method to develop other amyloid chaperone proteins into theranostic tools. The results of this study can be expanded to proteins such as clusterin, serum albumin, α B-crystallin and Brichos domain-containing proteins. Use of amyloid-specific affibody proteins can provide a more concise probe development strategy. We have tested the Z $\text{A}\beta$ 3 affibody for its purification efficiency in bacterial cells [180, 181]. Easy soluble expression of affibody on large scale using specialized protein tags can provide for a much tuneable alternative to large chaperone proteins. Modulation of iron oxide nanoparticles to allow for binding higher protein concentrations possibly through encapsulation strategies to use the nanoparticles as delivery cargo [182, 183].

Cohort studies with larger mouse populations will bridge the gap towards deriving concrete conclusions from the results of this study, which can be directly translated towards clinical interventions. Using a more expanded animal population will provide an opportunity to make statistically significant conclusions from the observations in this study. In addition to the existing observations, more longitudinal studies on mice populations are needed to fully understand the diffusion pattern of drug probes towards their target, as well as to understand the elimination mechanism employed by the biological systems to sequester out any excess or remaining probe vehicles.

Appendix

7. Appendix

Table 7.1. T2 relaxation time calculations

Model	AA-T2					
Equation	$y = A1 * \exp(-x/t2) + y0$					
Plot	B	E	H	K	N	Q
y0	0.14331 ± 0.12623	-0.06537 ± 0.07914	0.18005 ± 0.18771	0.22624 ± 0.05712	0.33222 ± 0.19147	0.72938 ± 0.04424
A1	25.1416 ± 0.13252	25.44526 ± 0.07824	25.09698 ± 0.27663	24.85494 ± 0.05716	25.14437 ± 0.1999	30.17087 ± 0.95499
t2	155.20049 ± 2.72993	161.06846 ± 1.69476	129.87171 ± 4.05155	159.82586 ± 1.25173	155.7073 ± 4.14098	39.68528 ± 1.25217
Reduced Chi-Sqr	0.00641	0.00225	0.02357	0.0012	0.01461	0.00663
R-Square (COD)	0.99992	0.99997	0.99969	0.99998	0.99981	0.99979
Adj. R- Square	0.99986	0.99995	0.99948	0.99997	0.99969	0.99966

Model	MNS-T2					
Equation	$y = A1 \cdot \exp(-x/t2) + y0$					
Plot	B	E	H	K	N	Q
y0	0.54732 ± 0.09513	0.27313 ± 0.07596	0.161 ± 0.08545	0.3532 ± 0.03804	0.56913 ± 0.09395	0.72045 ± 0.03375
A1	24.56519 ± 0.16492	25.51436 ± 0.0954	25.14787 ± 0.10535	24.86552 ± 0.04734	24.08475 ± 0.25663	22.95451 ± 0.26754
t2	120.05085 ± 2.10121	140.69034 ± 1.61332	142.04018 ± 1.84145	141.3515 ± 0.82898	96.34447 ± 2.14783	57.79959 ± 0.9245
Reduced Chi-Sqr	0.00741	0.0031	0.00381	7.66E-04	0.01178	0.00306
R-Square (COD)	0.99989	0.99996	0.99995	0.99999	0.99981	0.99991
Adj. R-Square	0.99982	0.99993	0.99992	0.99998	0.99968	0.99985

Model	SMNP-T2					
Equation	$y = A1 \cdot \exp(-x/t2) + y0$					
Plot	B	E	H	K	N	Q
y0	0.15956 ± 0.13686	0.32663 ± 0.15358	0.02441 ± 0.23839	0.38429 ± 0.0458	0.5581 ± 0.13068	0.58895 ± 0.0624
A1	24.8876 ± 0.1556	25.23055 ± 0.19728	25.75157 ± 0.23848	25.26464 ± 0.04891	23.64444 ± 0.19184	24.22911 ± 0.09169
t2	148.31124 ± 2.98394	139.07107 ± 3.29775	159.84951 ± 5.04175	153.62859 ± 0.98504	130.11715 ± 2.99379	130.05178 ± 1.39495
Reduced Chi-Sqr	0.00863	0.01308	0.0209	8.70E-04	0.01136	0.00259
R-Square (COD)	0.99989	0.99983	0.99974	0.99999	0.99983	0.99996
Adj. R-Square	0.99981	0.99972	0.99957	0.99998	0.99972	0.99994

Model	DNP-T2					
Equation	$y = A1 \cdot \exp(-x/t2) + y0$					
Plot	B	E	H	K	N	Q
y0	0.68136 ± 0.0841	0.52027 ± 0.04259	0.29384 ± 0.11397	0.22603 ± 0.11753	0.58829 ± 0.0678	0.44846 ± 0.0658
A1	20.73134 ± 0.31578	23.69084 ± 0.11507	24.69461 ± 0.15115	25.04533 ± 0.14851	24.2539 ± 0.08746	24.06798 ± 0.1083
t2	82.34435 ± 2.28278	96.86149 ± 0.9893	136.84106 ± 2.49997	140.24797 ± 2.54264	138.77512 ± 1.51445	123.08387 ± 1.48216
Reduced Chi-Sqr	0.01244	0.0024	0.00753	0.00748	0.00256	0.00333
R-Square (COD)	0.99969	0.99996	0.9999	0.9999	0.99996	0.99995
Adj. R-Square	0.99948	0.99993	0.99983	0.99984	0.99994	0.99992

Table 7.2. T1 relaxation time calculations

Model	AA-T1					
Equation	$y = A1 \cdot \exp(-x/t1) + y0$					
Plot	B	E	H	K	N	Q
y0	24.36003 ± 0.11912	24.61959 ± 0.13051	23.40099 ± 0.10279	24.26449 ± 0.06288	24.56661 ± 0.10567	13.98147 ± 0.05242
A1	- 24.61241 ± 0.22291	-24.76459 ± 0.23714	-23.25202 ± 0.18085	-24.52716 ± 0.11627	- 24.69452 ± 0.19352	-13.746 ± 0.11416
t1	2723.647 8 ± 66.06486	2783.8658 9 ± 72.33099	2850.8462 7 ± 61.02334	2748.1145 3 ± 35.07581	2767.696 1 ± 58.64557	2430.1033 3 ± 50.55068
Reduced Chi-Sqr	0.03051	0.0354	0.02113	0.00839	0.02342	0.00694
R-Square (COD)	0.99966	0.99961	0.99974	0.99991	0.99974	0.99974
Adj. R- Square	0.99953	0.99946	0.99964	0.99987	0.99964	0.99964

Model	MNS-T1					
Equation	$y = A1 \cdot \exp(-x/t1) + y0$					
Plot	B	E	H	K	N	Q
y0	23.60127 ± 0.13405	24.76401 ± 0.13509	24.67411 ± 0.14881	22.96289 ± 0.19686	19.35259 ± 0.23448	12.27423 ± 0.55717
A1	-24.0003 ± 0.25572	-24.64467 ± 0.22596	-24.47321 ± 0.24427	-22.88649 ± 0.34452	-19.58938 ± 0.51178	-13.84812 ± 2.69514
t1	2685.104 99 ± 75.9759	2958.731 84 ± 76.33755	2999.865 37 ± 84.95359	2861.968 56 ± 118.8462 2	2426.150 33 ± 158.6166 1	1313.311 64 ± 454.7983 9
Reduced Chi-Sqr	0.0395	0.03426	0.04058	0.07699	0.13928	1.3038
R-Square (COD)	0.99954	0.99963	0.99956	0.99903	0.99746	0.93394
Adj. R- Square	0.99935	0.99948	0.99938	0.99864	0.99644	0.90752

Model	SMNP-T1					
Equation	$y = A1 \cdot \exp(-x/t1) + y0$					
Plot	B	E	H	K	N	Q
y0	24.17598 ± 0.15315	24.4227 $\pm$ 0.14273	24.2063 $\pm$ 0.16911	24.08143 ± 0.15688	21.90632 ± 0.22054	20.90892 ± 0.20198
A1	-24.17595 ± 0.27494	-24.41214 ± 0.25207	-24.60334 ± 0.32429	-24.33314 ± 0.29829	-21.90488 ± 0.41342	-22.00048 ± 0.49597
t1	2808.7349 9 $\pm$ 87.1296	2842.9248 1 $\pm$ 80.65368	2674.5825 9 $\pm$ 93.40047	2691.5599 1 $\pm$ 87.74644	2720.1802 7 $\pm$ 137.38999	2215.9075 1 $\pm$ 118.73937
Reduced Chi-Sqr	0.04805	0.04092	0.06322	0.05389	0.1048	0.11525
R-Square (COD)	0.99945	0.99954	0.99929	0.99939	0.99853	0.99826
Adj. R- Square	0.99923	0.99936	0.99901	0.99914	0.99794	0.99757

Model	DNP-T1					
Equation	$y = A1 \cdot \exp(-x/t1) + y0$					
Plot	B	E	H	K	N	Q
y0	13.88655 ± 0.39931	19.03174 ± 0.25706	23.292 $\pm$ 0.11609	23.66733 ± 0.07068	23.70535 ± 0.11279	22.2324 $\pm$ 0.09824
A1	-15.3007 ± 1.70558	-19.7391 ± 0.62289	-23.69141 ± 0.21919	-23.86691 ± 0.12968	-23.88184 ± 0.20421	- 22.47204 ± 0.1842
t1	1440.6052 3 $\pm$ 301.44921	2238.8135 3 $\pm$ 168.89853	2705.8146 5 $\pm$ 66.78211	2763.9152 5 $\pm$ 40.57307	2791.1434 8 $\pm$ 64.85848	2719.755 9 $\pm$ 59.65571
Reduced Chi-Sqr	0.6372	0.18452	0.02928	0.0105	0.02632	0.0208
R-Square (COD)	0.97449	0.99657	0.99965	0.99988	0.99969	0.99972
Adj. R- Square	0.96429	0.9952	0.99951	0.99983	0.99957	0.99961

Table 7.3. Calculations for relaxivity values.

Concentration (mM)	T1 (s)	R1 (s ⁻¹)	T2 (s)	R2 (s ⁻¹)
FtnAA				
1.2E-4	2430.10333	0.000411505	39.68528	0.02519826
1.2E-5	2850.84627	0.000350773	129.87171	0.007699906
2.4E-6	2723.64788	0.000367155	155.20049	0.006443279
1.2E-6	2767.6961	0.000361311	155.7073	0.006422306
6E-7	2748.11453	0.000363886	159.82586	0.00625681
2.4E-7	2783.86589	0.000359213	161.06846	0.00620854
MNS				
9.3115E-5	1313.31164	0.000761434	57.79959	0.017301161
9.3115E-6	2426.15033	0.000412176	96.34447	0.010379423
1.8623E-6	2685.10499	0.000372425	120.05085	0.008329804
9.3115E-7	2958.73184	0.000337983	140.69034	0.007107809
4.656E-7	2861.96856	0.00034941	141.3515	0.007074562
1.8625E-7	2999.86537	0.000333348	142.04018	0.007040261
SMNP				
4.47665E-4	2215.90751	0.000451282	130.05178	0.007689245
4.47665E-5	2720.18027	0.000367623	130.11715	0.007685382
8.9533E-6	2842.92481	0.00035175	139.07107	0.007190568
4.47665E-6	2808.73499	0.000356032	148.31124	0.006742577
2.23835E-6	2691.55991	0.000371532	153.62859	0.006509205
8.9533E-7	2674.58259	0.00037389	159.84951	0.006255884
DNP				
4.47665E-4	1440.60523	0.000694153	82.34435	0.012144124
4.47665E-5	2238.81353	0.000446665	96.86149	0.01032402
8.9533E-6	2719.7559	0.00036768	123.08387	0.008124541
4.47665E-6	2705.81465	0.000369574	136.84106	0.007307748
2.23835E-6	2791.14348	0.000358276	138.77512	0.007205903
8.9533E-7	2763.91525	0.000361806	140.24797	0.007130228

Figure 7.1. Unmarked images showing T2 difference comparison in healthy (Left) versus 3xTg AD mouse brain (Right) after intraventricular injection of LA conjugate. The images were acquired at 11.4 T using T2_TurboRARE sequence with image size was 200 x 106 pixels,

with field of view 17 x 9 mm, RARE factor: 4, TE 15.56 ms, TR 3500 ms with 38 slices of thickness 0.4 mm each through the mouse brain, scan time: 3 min 2 sec, motion averaging, flip back, fat suppression.

Table 7.4. T2* values for select brain regions of interest before and 72 h after intraventricular injection of LA conjugates in 3xTg AD mouse.

0 h	72 h	Brain Region
36.14	36.77	Olfactory bulb
37.21	36.69	Neocortex
36.14	35.08	Corpus callosum
36.02	35.70	Corpus callosum
38.03	36.01	Hypothalamus
36.54	33.41	Thalamus
35.42	34.63	Hippocampus
33.24	32.85	Cerebral aqueduct
34.45	33.73	Cerebellum

T2* values (ms) in the table were calculated from selected circular regions of interest in T2 map_MSME images with TR: 2200 ms, echo spacing: 8.16 ms, image 80 x 80, FOV: 20 x 20 mm, slice thickness: 0.5 mm, no. of echo images: 9, flip back, excitation 90 degree, refocusing 180 degree.

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