Role of Biomolecule Cues in Alzheimer's disease

by

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List of Abbreviations

AD	Alzheimer's Disease
Aβ	Amyloid-beta
NFTs	Neurofibrillary tangles
MT	Microtubules
PGs	Proteoglycans
GAGs	Glycosaminoglycans
Hep	Heparin
HS	Heparan Sulfate
DS	Dermatan Sulfate
HA	Hyaluronan
TG	Transglutaminase
LOX	Lysyl Oxidase
ECM	Extracellular Matrix
CSF	Cerebrospinal Fluid
Tf	Transferrin
HSA	Human Serum Albumin

Abstract

The master's thesis study investigates the role of biomolecules in Alzheimer's disease. Alzheimer's disease is caused by amyloid plaques and neurofibrillary tangles. The major cause of amyloid plaques is the aggregation of 1-42 amino acid peptide (abeta) fragment of the amyloid precursor protein. Neurofibrillary tangles on the other hand, are caused predominantly by the aggregation of microtubule binding hexapeptide (VQIVYK) region of the tau protein. The plaques and tangles ultimately cause neuronal cell toxicity, leading to Alzheimer's disease. Several factors are known to cause abeta and tau aggregation. Here in this study, we investigate the effect of biomolecules on the aggregation formation of abeta and tau peptides, and test the potential of macromolecules, small molecules, and nanotherapeutics on the inhibition of abeta and tau peptides aggregation. The objective of the thesis study is achieved by investigating the role of (i) glycosaminoglycans (ii) extracellular enzymes transglutaminase and lysyl oxidase, and (iii) cerebral proteins transferrin and human albumin in Alzheimer's causing peptides abeta and tau aggregation and toxicity. Characterization tools used for this study include biochemical assays, transmission electron microscopy, confocal microscopy, atomic force microscopy, dynamic light scattering, and cellular assays. The thesis study reveals that, glycosaminoglycans, and extracellular enzymes promote the aggregation of tau and abeta peptides, while cerebral proteins prevent the aggregation. Further, results show that the effect of biomolecules mediated Alzheimer's peptide aggregation and could be inhibited in the presence of macromolecules, small molecules, and nanoformulated cerebral proteins.

Chapter 1: Introduction

1.1 Introduction

Alzheimer's disease (AD) is a neurological disorder that causes dementia; it affects 10% of the population age over 60 [1-2]. It is characterized by the presence of two abnormal proteins; extracellular amyloid plaques and intracellular neurofibrillary tau tangles in the brain [3-6]. Tau proteins consist of 441 amino acids with a microtubule binding region, that contains four repeated peptide units [7]. The hexapeptide repeats VQIVYK and VQIINK are found in the carboxyl region of the tau protein; they form beta sheets, aggregates, and fibril structure that resemble tau aggregation in vitro [8-11]. Amyloid-β (Aβ) is formed by cleavage of amyloid precursor protein (APP) by β-secretase and Υ-secretase that are deposited in the cerebrospinal fluid (CSF) and blood [12]. The two main components of the Aβ peptide are Aβ (1-40) and Aβ (1-42) depending on their sequence.

Several factors contribute to Alzheimer's disease progression. Among them, extracellular biomolecules like glycosaminoglycans (GAGs) and enzymes like transglutaminase (TG) and lysyl oxidase (LOX), HSA been shown to co-localize and promote Aβ aggregation by catalyzing the crosslinking of Aβ and tau proteins leading to AD [13-14]. On the other hand, some plasma and cerebrospinal fluid proteins like transferrin (Tf) and human serum albumin (HSA) has been known to bind to Aβ in blood to prevent the deposition of Aβ plaques in brain (15-16). Due to the critical role of abeta and tau aggregation in AD, over the years several therapeutic approaches have been used to inhibit tau and Aβ aggregations [17-19]. Approaches include the application of small

molecules components [20-21], macromolecules [22-24], ligands based nanoparticles [25-26], and Aβ vaccines [27].

Although previous studies report the role of biomolecules in Alzheimer's disease, Alzheimer's causing protein aggregation studies in the presence of biomolecules in vitro, hasn't been explored in detail yet. In this thesis study, role of biomolecules glycosaminoglycans, extracellular enzymes, and cerebral protein on Alzheimer's protein aggregation in vitro, is studied in detail. In addition, macromolecules, small molecules, and nanotechnological mediated therapeutic approaches for the inhibition of biomolecules mediated protein aggregation also studied briefly. The characterization tests that are used for this study include thioflavin-T and thioflavin-S, turbidity assays, dynamic light scattering, confocal microscopy, transmission electron microscopy, atomic force microscopy, and cellular assays. The thesis study could help new avenues for treating Alzheimer's by targeting the biomolecules that promote protein aggregation during the disease.

1.2 Research Objectives

The overall goal of the thesis study is to investigate the role of biomolecuels on Alzhimer's protein aggregation. The research objectives of this thesis are described in the following specific aims:

Specific Aim1: Study the role of Glycosaminoglycans in Alzheimer's protein aggregation.

Specific Aim2: Study the role of extracellular enzymes in Alzheimer's disease.

Specific Aim 3: Study the role of cerebral proteins in Alzheimer's disease.

1.3 Thesis Organization

This thesis is organized into seven chapters. Chapter 2 is a general background of Alzheimer's disease and protein aggregation, as well as approaches used to characterize the aggregation. Potential drug therapeutics approaches for treating Alzheimer's are also briefly reviewed. The intention of the background is to provide the context for the motivation and goals of this work and

to include an outline of the specific objectives of this work. Chapter 3 investigates the role of glycosaminoglycans on tau peptide aggregation, and Chapter 4 is a paper published in biopolymers that addresses the effect of cationic polymers on tau aggregation. Chapter 5 is a study on the role of extracellular enzymes on Alzheimer's protein aggregation, while Chapter 6 investigates the role of cerebral proteins on abeta aggregation. Finally, Chapter 7 includes general conclusions and a summary of the contributions of this work, and future directions.

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Chapter 2: Background and Literature Review

2.1 Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disorder that causes dementia because of the continuous neuronal loss in brain [1]. It was statistically shown that 35.6 million people have AD; also, this estimation is said to double every 20 years [1, 2]. The treatments for AD are limited, short-term, and expensive ($604 billion in 2010) [3-7]. Moreover, the extracellular amyloid-beta (Aβ) plaques and intracellular hyperphosphorylated neurofibrillary tau tangles (NFTs) are the two hallmarks of AD [8, 9]. Also, the soluble building blocks of the Aβ and tau plaques and tangles respectively influence each other [10]. It was shown by experimental studies that injecting a mouse with Aβ into the brain increased the formation of tangles [11]. Another experiment conducted on mice with tau deficiency reveals that the effect of Aβ plaques was ameliorated, indicating the interplay between the two proteins [12]. Figure 2.1 depicts the signaling between abeta and tau.
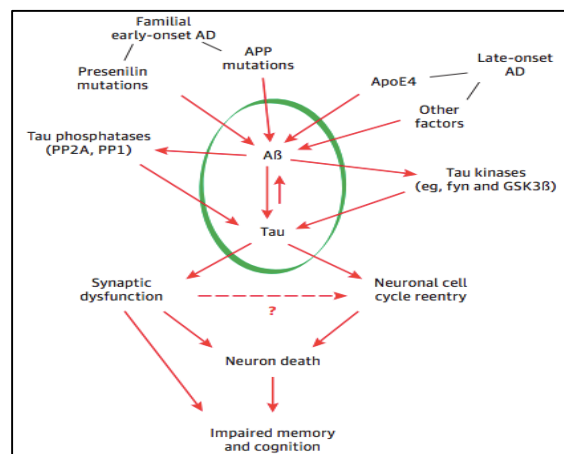
Figure 2.1: Signaling from Aβ through Tau in AD [10]

2.2 Alzheimer's Disease-causing proteins

2.2.1 Tau protein aggregation and AD

Tau protein is composed of microtubules (MT) binding domain that hold the carboxy- terminal (C-terminal), the proline region, and the acidic amino acid region [13, 14]. The microtubules binding domain in the tau protein stabilizes microtubules in neurons of healthy people [13, 14]. Whereas in AD patients, tau proteins hyper phosphorylate, aggregate, and misfold into neurofibrillary tangles during AD [14, 15]. These aggregates destabilize microtubules, which lead to deficiency in the metabolism of the proteins and synaptic malfunctioning [16, 17]. NFTs can't be discarded and degraded by the neurons, so they will continuously come together and increase in the cytoplasm. It was proven that NFTs go through a specific pathway in AD patients [18-20]. First, tau proteins detach from MT for different reasons (phosphorylation, dephosphorylation, and tau gene mutation) [14]. Then, in the pretangle phase (pathogenic phase), tau proteins misfold and form nonfibrillary tau deposits (soluble). Additionally, these pretangles can't be detected by β sheet dyes [21-23]. The second phase is the NFT formation, tau proteins start to aggregate which will cause the dendrites to shrink (shorten). Neuronal cells can survive for years before they get completely affected by tau aggregation and die. Also, the number of NFTs is connected to the severity or level of AD (dementia) [24]. Lastly, the ghost tangles phase, where the tangles become extracellular after cell death of neurons and the neuronal abrasions become more visible [15, 24, 25]. Figure 2.2 illustrates the aggregation of tau and abeta during AD.
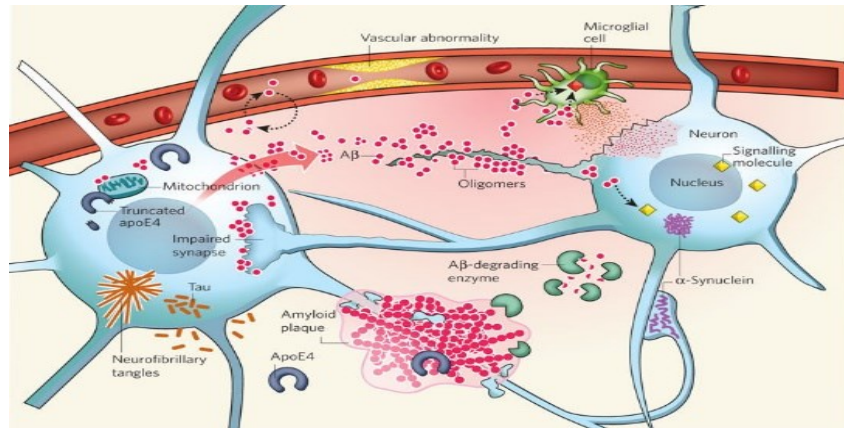
Figure 2.2: Aggregation of Tau and Aβ proteins in AD [25]

2.2.2. Amyloid-beta and Alzheimer's disease

Senile plaques found in the brain of AD patients are composed of 39-42 residues of Aβ peptides [26-28]. Normally, Aβ peptides are the products of amyloid β protein precursor (APP) metabolism reaction [29, 30]. Whereas in AD patients, APP is cleaved by β-secretase and Υ-secretase (proteolysis) which will increase the production of Aβ peptides [26, 27, 31-33]. Also, several beta fragments are generated during proteolysis process (Aβ 1-42 and Aβ 1-40), but it has shown that Aβ (1-42) peptides are found at higher concentrations in Aβ plaques [26, 34]. Then, Aβ peptides aggregate and fold into Aβ fibrils [35-37] that accumulate into Aβ plaques [38]. Furthermore, it was previously shown that Aβ fibrils are cytotoxic to cells [39, 40] and lead to neuronal disturbance (main cause of dementia) [37, 39, 41, 42]. Figure 2.3 illustrates the abeta formation in AD patients.
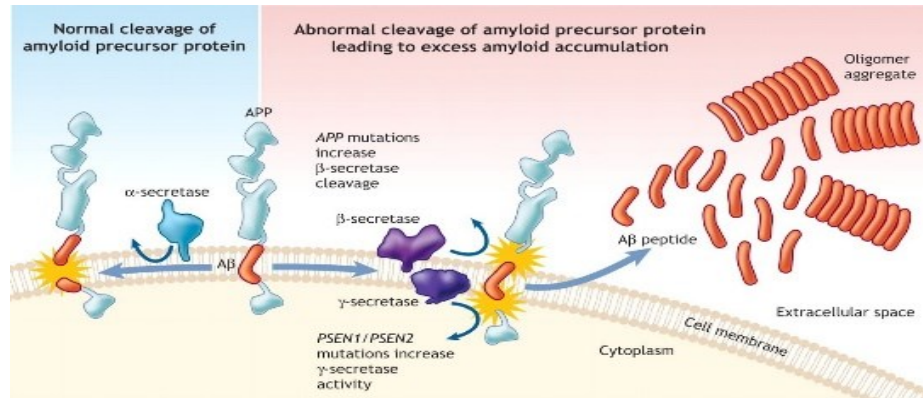
Figure 2.3: Formation of Aβ plaques in AD patients [43]

2.3. Extracellular biomolecules and Alzheimer's disease (AD)

2.3.1 Glycosaminoglycans (GAGs) and AD

Proteoglycans (PGs) are proteins that are composed of GAGs side chains [44]. GAGs are long, linear, and heterogeneous polysaccharides found in the extracellular matrix (ECM) [45]. There are several forms of GAGs depending on their structure and degree of sulfates. The major GAGs that are found to influence AD are: Heparan sulfate (HS), Heparin (Hep), Dermatan sulfate (DS), Chondroitin sulfate (CS) and Hyaluronan (HA) [46, 47]. Some functions of GAGs/PGs are maintaining homeostasis, balance growth factor activities, balance signal transduction, and help in cell-to-cell interactions [45, 48-50]. It was also proven that AD can be influenced by GAGs [51] by affecting tau protein aggregation [52] and Aβ peptide aggregation [53]. Sulfated GAGs share the same location of Aβ plaques, so it was proven that GAGs bind to Aβ to induce fibrilization [53-57]. Additionally, GAGs induce NFTs by increasing phosphorylation of tau (hyperphosphorylation) depending on the degree of sulfation [52, 53, 58]. It has been reported that tau assemble into filaments in the presence HS [59]; also, it was proved that tau changes are induced in the presence of Hep [60, 61].

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2.3.2 Extracellular enzymes and AD

Aβ aggregation has been proven to be stabilized by several extracellular enzymes, including transglutaminase (TG) and lysyl oxidase (LOX) [62-64]. One of the most studied TG member of the calcium-activated enzymes is the tissue transglutaminase (tTG) that was proven to be associated with AD [65]. It was previously shown that tTG enzymes help neurofilaments proteins like Aβ [66], APP [67, 68], and tau [69, 70] in cross-liking the ECM during diseases [71-73]. They are found in different areas of the brain like the cytoplasm of neurons [74], the nuclei, and the ECM (where Aβ aggregation occur) [75]. LOX are copper dependent amine oxidase enzymes that convert generated enzyme aldehyde residues by catalyzing lysine side chains (amines) on the ECM proteins [76] mainly collagen and elastin [77, 78]. These ECM proteins especially collagen (heparan sulfate) are associated with Aβ aggregation and the development of senile plaques [79]. It was experimentally proven that LOX increased by 30% in AD patients [76].

2.3.3 CSF proteins and AD

AD detection is limited [80, 81]; it was suggested to study the biomarkers and proteins in the cerebrospinal fluid (CSF) to understand more the pathology of AD [82]. Some of these molecules are Transferrin (Tf) and Human Serum Albumin (HSA); it was proven that Tf and HSA inhibit Aβ aggregation [83-86]. Tf are iron transport proteins that are used for iron homeostasis [87]; any mutation or interference in iron homeostasis will lead to AD due to neurotoxicity [87, 88]. Additionally, HSA was shown to bind to 90-95% of Aβ in blood plasma [89] which proves that Aβ plaques are found in the exterior of the brain [90]. Also, it was experimentally proven that HSA doesn't bind to Aβ monomers, it selectively binds to Aβ fibrils (β-structures) [84].

2.4. Therapeutic Approaches to treat tau and Aβ aggregation

Several therapeutic approaches have been used to inhibit tau proteins and peptides aggregation [91-93]. One of the approaches used is using small molecules components that were shown to have anti-tau aggregation properties [94, 95]. Also, previous studies showed that cationic small molecules and urea inhibit tau aggregation [96-98]. Another approach proved to inhibit tau aggregation is using macromolecules [99-101]. Some already used macromolecules that affect tau aggregations are D-enantiomer peptide [101], eight amino acid peptide Davunetide [100], and D-amino acid inhibitor peptide [101]. One of the methods to treat AD is to inhibit the overproduction of Aβ to clear them from the brain. The clearance of Aβ peptides/aggregates from brain using anti-Aβ molecules is challenging due to the blood brain barrier (BBB). There are three previously tested approaches to target Aβ aggregation pathways. First approach was done using ligands that directly affect Aβ like PEG [102] and lipid [103] based nanoparticles. Another approach was tested using small molecules like curcumin, melatonin [104, 105], and Aβ vaccines [106]. Also, strategies to target APP or β-secretase and ϒ-secretase enzymes have been investigated [107, 108].

2.5. Methods

2.5.1 Aggregates Formation

2.5.1.1   Aβ 1-42 aggregation

Each of the Aβ forms (aggregated, unaggregated, fibrils, plaques) has its own preparation method [109]. In this study, we aggregated Aβ by dissolving 0.5 mg in 250 µl HFIP. Then, the Aβ-HFIP solution was aliquoted into five vials (100 µg/vial) and kept in a chemical hold until HFIP completely evaporates. Then, the vials are stored at -20 °C until usage. The aggregation is either formed in PBS buffer or DMSO/10 mM HCl depending on the application.

2.5.1.2   Tau peptides aggregation

Tau peptides were first dissolved in water at 1 mM concentration, and stored at -20°C until usage. Tau aggregation was formed in MOPS buffer at pH 7. For the tau aggregation initiation, heparin is mainly used in the study.

2.5.2   Nanoparticle preparation

Poly (lactic-co-glycolic acid) (PLGA) is a biodegradable polymer used to develop polymeric nanoparticles [110]. It is widely used because of its characteristics; it is biodegradable, biocompatible, FDA approved, adaptable to different drug types [110]. First, the PLGA nanoparticle was formed by emulsion. Then the particles were surface modified with EDC/NHS chemistry to render protein functionalization. In this study, we coated Tf and HSA with PLGA-NP to inhibit Aβ 1-42 aggregation.

2.5.3 Characterization Techniques

2.5.3.1 Thioflavin T (ThT) fluorescence

ThT is a dye that fluoresce at 449 nm excitation and 482 nm emission when it binds to amyloid fibrils [111]. In our experiment ThtT was used to detect Aβ 1-42 fibrils under different conditions.

2.5.3.2 Thioflavin S (ThS) fluorescence

ThS is a benzothiazole dye that fluoresce at 440 nm/ 485 nm when bind to protein aggregation [112]. In our study, the ThS was used to detect tau aggregation in the presence and absence of GAGAs.

2.5.3.3 Turbidity

Turbidity is the effect of the incident beam by light scattering which can be measured directly (transmission) by using spectrophotometer/plate reader and indirectly static light scattering) [113]. Here, we measured tau peptide aggregation at 400 nm (absorption).

2.5.3.4 Dynamic Light Scattering (DLS)

DLS instrument is used to predict the size of the samples. Dynamic light scattering detects the particle size in the nanometer range, by utilizing Brownian motion principles. We used it to predict the size of abeta, and tau aggregation with and without biomolecules, drugs, as well as to predict the size of nanoparticles.

2.5.3.5 Confocal Microscopy

Confocal microscopy uses special optical components to form high resolution and contrast images. We use it to image cells after immunofluorescence staining (zo-1 staining for Aβ 1-42 with TG, LOX, and B/LP), and for cellular uptake of nanoparticles characterization.

2.5.3.6 Atomic Force Microscopy (AFM)

AFM is a scanning force microscope that takes very-high-resolution images in an order of nanometer. In our study, we used AFM to image the topography of our samples tau proteins with and without GAGs, and Aβ 1-42 with the extracellular enzymes and the CSF proteins, by spotting them on a mica and imaging them in taping mode using a Hitachi AFM in the lab.

2.5.3.7 Transmission Electron Microscopy (TEM)

TEM is an electron microscopy technique that forms an image of the samples by transmitting a beam of electron through it. The samples were spotted on a holy carbon copper grid, and then stained with 2% phosphotungstic acid.

2.5.3.8 Cell Assays

2.5.3.8.1 Cell viability

Cell viability assay was performed using XTT assay to measure cell viability and metabolism. XTT measurements was used in all our studies is determined by, the formation of formazan dye by measuring the absorbance at 470 nm using a spectrophotometer, and then calculating the percentage of viability based on the difference in absorbance between control cells to that of cells treated with aggregates and drugs.

2.5.3.8.2 Oxidative Stress

The DCFH-DA assay is used to measure the oxidative stress associated with an increased production of reactive oxygen species. Severe oxidative stress can cause cellular death. Quantifying oxidative stress by 2,7-dichlorodihydrofluorescein (DCFH) based fluorescent probes has been widely reported [114, 115]. We studied oxidative stress of Aβ 1-42 peptides in presence of extracellular enzymes with and without small molecule drugs, at 485/535 nm excitation/emission.

2.5.3.8.4 Immunofluorescence

Immunofluorescence is used to detect the cellular markers due to changes and influences of the samples on the cells by utilizing antibody-antigen binding. The cells are stained with the cellular

target of interest with both primary and secondary antibodies with fluorescent dyes, and then

imaged using confocal microscope.

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Chapter 3: Role of Glycosaminoglycans in Tau Peptides Aggregation

3.1 Introduction

Tau protein aggregation and subsequent neurofibrillary tangle formation are hallmark of Alzheimer's disease [1, 2]. Tau protein consists of 441 amino acids with microtubule binding region that contains four repeat peptide units [3, 4]. They are composed of hexapeptide domains VQIVYK, and VQIINK, and are believed to be the major drivers of tau pathological assembly [5-9], and are widely used as a model to study tau aggregation in vitro.

Several factors are known to influence the tau aggregation [5, 10-12]. Among them, polyanions, glycosaminoglycans (GAGs) and their sulfated forms, and RNA have shown to induce Alzheimer's like changes in tau protein and believed to play a major role in tau aggregation [13, 14]. Further, sulfated glycosaminoglycans have shown to promote tau aggregation and have been found to be present in Alzheimer's disease affected brain [13, 15]. Previously, it has been shown that GAGs influence the aggregation of amyloid beta peptide ($A\beta_{42}$) aggregation in vitro [16]. Tau protein also has been shown to assemble into filaments like morphology in the presence of sulfated heparin [17], and heparin induced conformational changes in tau protein have been observed [18, 19]. In addition, tau peptide fragments aggregation in the presence of heparin has been investigated before [20]. But, the role of other glycosaminoglycan molecules on tau hexapeptides, and their extracellular mimetic conditions haven't been explored in detail.

Here in this thesis chapter we study the effect of GAG molecules (in solution and on surface) heparin (Hep), heparin sulfate (HepS), dermatan sulfate (DS), chondroitin sulfate (CS), and hyaluronan (HA) on the aggregation of tau peptides. The peptides used are, hexapeptide domain ^{306}VQIVYK^{311} (Tau V) segment from the microtubule binding region of tau protein, and the tau mutant peptide fragment GKVQIINKLDL (Tau N), widely found in taupathy. The aggregation of the peptides was studied utilizing the biochemical, biophysical, and cell toxicity assays. The thesis study revealed that the heparin, chondroitin sulfate, and dermatan sulfate in solution, significantly promote tau peptides aggregation and fibril formation, while surface coated GAGs did not promote significant aggregation.

3.2 Materials and Methods

3.2.1 Materials

Tau peptides were custom synthesized from Genscript. Low molecular weight heparin, heparin sulfate (GAG-HS01), and dermatan sulfate (GAG-DS01) were purchased from Galen Laboratory Supplies. Chondroitin sulfate (C4384), hylaluronic acid (53747), and all other chemicals, and reagents were purchased from Sigma Aldrich. ELISA 96 well Clear Amino Surface Plate was purchased from Galen laboratory supplies.

3.2.2 ThS measurements

First stock solutions of tau peptides (1 mM) and GAGs (1 mM) were prepared by dissolving in deionized water. ThS was freshly prepared at a concentration of 0.5 mg/ml in 20 mM MOPS buffer, pH 7. For the ThS measurement, 10 μl of peptide was dissolved in 2.5 μl GAG stock solution, 10 μl ThS, and MOPS buffer to make the final 100 μl working solution. A M3 spectramax spectrophotometer was used to measure the ThS fluorescence at 440/490 nm, excitation and

emission. Aggregation kinetic measurements were performed every two minutes for 30 minutes. Three independent experiments were carried out and the average readings were obtained.

3.2.3 DLS

A Malvern zetasizer instrument in the lab was used to measure the size of the aggregation of the peptides with GAGs. Samples were prepared under similar conditions without the ThS, and the size measurements were performed. Three independent size measurement experiments were performed for each GAGs condition.

3.2.4 Turbidity

The peptides aggregation was measured by the turbidity of the samples at 400 nm. Samples were prepared under similar conditions without the ThS, and the turbidity measurements were performed using a spectramax M3 spectrophotometer in the lab. Three independent size measurement experiments were performed for each GAGs condition.

3.2.5 TEM

TEM images were obtained using the JEOL JSM 1400 TEM at UM-Ann arbor electron microscope facility. The peptides were aggregated with GAGs for 1 hour, spotted on holy carbon copper grids, and then stained with 1% uranyl formate.

3.2.6 Cell toxicity assay

The toxicity of tau peptides aggregates with GAGs on human neuroblastoma SH-SY5Y cells was studied by the XTT assay. Cells were obtained from American Type Culture Collection (ATCC), and cultured according to the protocol. For the study, 2×10^4 cells/well, were cultured in 96 well

plates overnight. Peptides (32 µM) with or without GAGs of concentrations (8 µM) were aggregated for 24 hours, and subsequently incubated for 48 hours.

3.2.7 GAGs surface coating and aggregation assays

To study the influence of surface coated GAGs on peptide aggregation, first GAGS were coated on GAGs binding plates. For GAGs binding, 100 µl solution of 0.01 M PBS containing 25 µM of GAGs (Hep, CS, DS, HepS, and HA) were added to a GAGs binding plate and incubated at room temperature according to manufacturer's protocol. After 24 hours, the PBS/GAGs solution was aspirated and the wells were washed three times with PBS buffer. For aggregation studies on GAGs coated plates, 15 µl of 1 mM Tau N, 15 µl ThS (0.5mg/ml), and 120 µl MOPS (20 mM) solution was added into each well. The turbidity of the peptide aggregates was measured at 400 nm wavelength after 30 min incubation. AFM was to characterize the morphology.

3.2.8 AFM

Hitachi AFM 5100N was used to obtain the AFM images for tau peptides samples with GAGs. For preparation, 2 µl of the aggregated sample solutions were spotted on freshly cleaved mica, and kept to dry before imaging. AFM tips (Applied Nanostructures Inc.) with an average resonance frequency of 300 kHz, was used to image samples using tapping mode. The length and the width of the cantilever are 125 µm and 30 µm respectively, and the average force constant is 37 N/m.

3.2.9 Statistical Analysis

Each experiment was conducted three or more times, and they are presented as mean ± standard error of the mean (SEM). P-values were determined from the results of at least 3-independent experiments. Statistical significance was computed using analysis of variance (ANOVA), followed by Tukey's HSD post-hoc analysis test. **$p < 0.01$, *$p < 0.05$ are considered as significance.

3.3 Results

In this chapter we studied the influence of GAGS on tau peptides aggregation, and cellular toxicity. For the GAGS induced aggregation study, two different peptides to GAGs molar ratios 4:1 or 20:1 were used. First, we used thioflavin to study the aggregation. Thioflavin S measurements show that the GAGs influence both Tau V, and Tau N peptides aggregation in vitro. Aggregation of the peptides was enhanced in the presence of chondroitin sulfate, heparin, and dermatan sulfate (Figure 3.1 A, B). Sulfated heparin and hyaluronan on the other hand exhibited minimal aggregation effect. At both 4:1, and 20:1 molar ratios, chondroitin sulfate seems to influence the aggregation of Tau V peptide (Figure 3.1A, Figure 3.2A). Tau N aggregation is enhanced in the presence of chondroitin sulfate at lower molar ratios (Figure 3.2B), while at higher GAGs content, heparin seems to have more influence on the Tau N aggregation (Figure 3.1B). Also, it is important to note that, compared to heparin, the sulfated form of heparin did not exhibit significant aggregation.
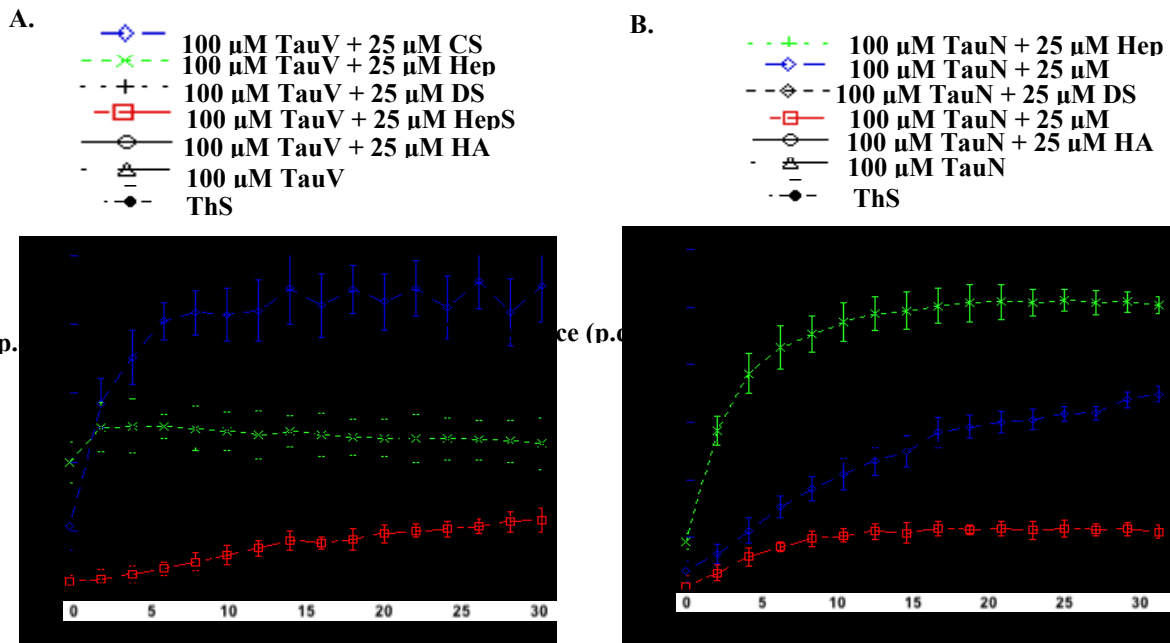
Figure 3.1. A). Tau V peptide (100 μM) aggregation kinetics was studied with GAGs (25 μM). **B).** Tau N peptide (100 μM) aggregation kinetics was studied with GAGs (25 μM). ThS fluorescence, show the aggregation is influenced by the presence of glycosaminoglycan used.

A.

Legend:
- —◇— 100 μM TauV + 5 μM CS
- --×-- 100 μM TauV + 5 μM Hep
- ···+··· 100 μM TauV + 5 μM DS
- —○— 100 μM TauV + 5 μM HA
- —□— 100 μM TauV + 5 μM HepS
- △
- ●

B.

Legend:
- —◇— 100 μM TauN + 5 μM CS
- ···+··· 100 μM TauN + 5 μM DS
- --×-- 100 μM TauN + 5 μM Hep

ThS fluorescence (p.d.)
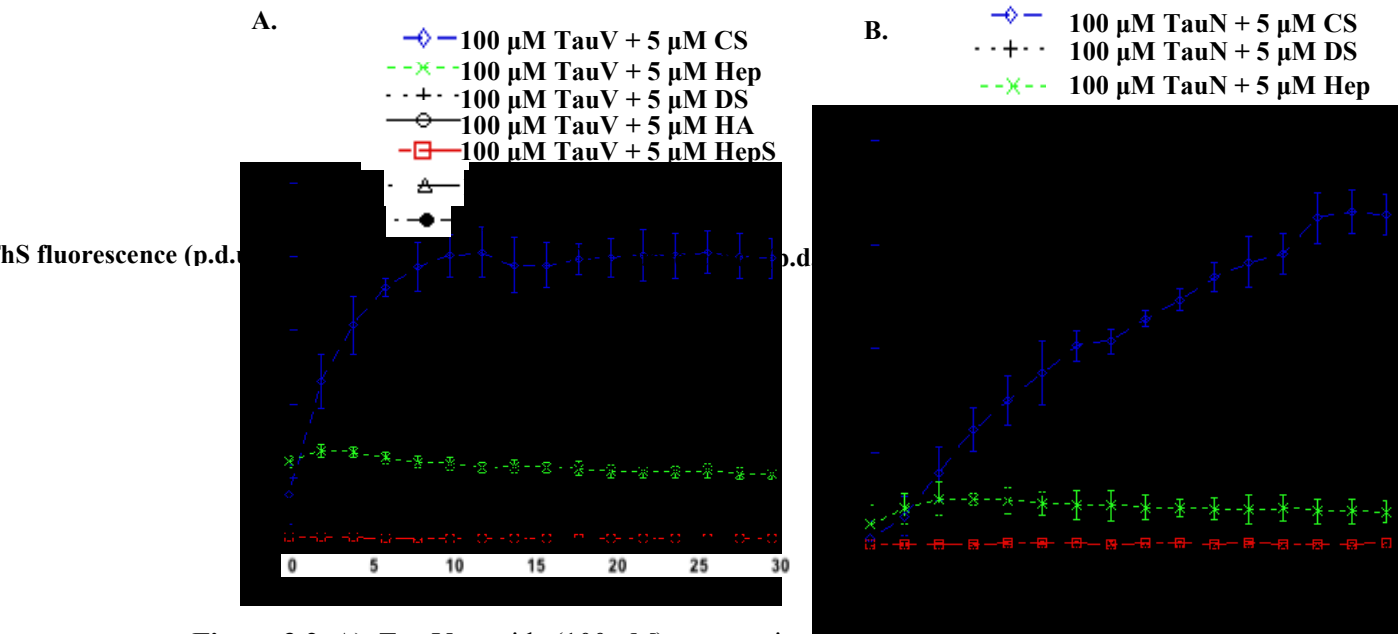
Figure 3.2. A). Tau V peptide (100 μM) aggregation kinetics was studied with and without GAGs (5 μM). **B).** Tau N peptide (100 μM) aggregation kinetics was studied with and without GAGs (5 μM).

DLS, and turbidity measurements were then used to measure the size distribution, and turbidity of the peptide aggregates, with and without GAGs. As can be seen from Figure 3.3(i), chondroitin sulfate, heparin, and dermatan sulfate treated Tau N peptides, exhibited larger sizes, indicating the presence of aggregation or fibrillar structures. As for the Tau V peptide aggregation size, significant increase in all the GAGs treated peptides was observed compared to Tau V peptide alone (Figure 3.3 (ii)).

(i).

Legend:
- TauN
- TauN + HA
- TauN + HepS
- ...ep
- ...S
- ...S

Intensity

Size (nm)

(ii).

Legend:
- TauV
- TauV + HA
- ...V + HepS
- ...V + Hep
- ...V + DS
- ...V + CS

...sity (p...

Size (nm)
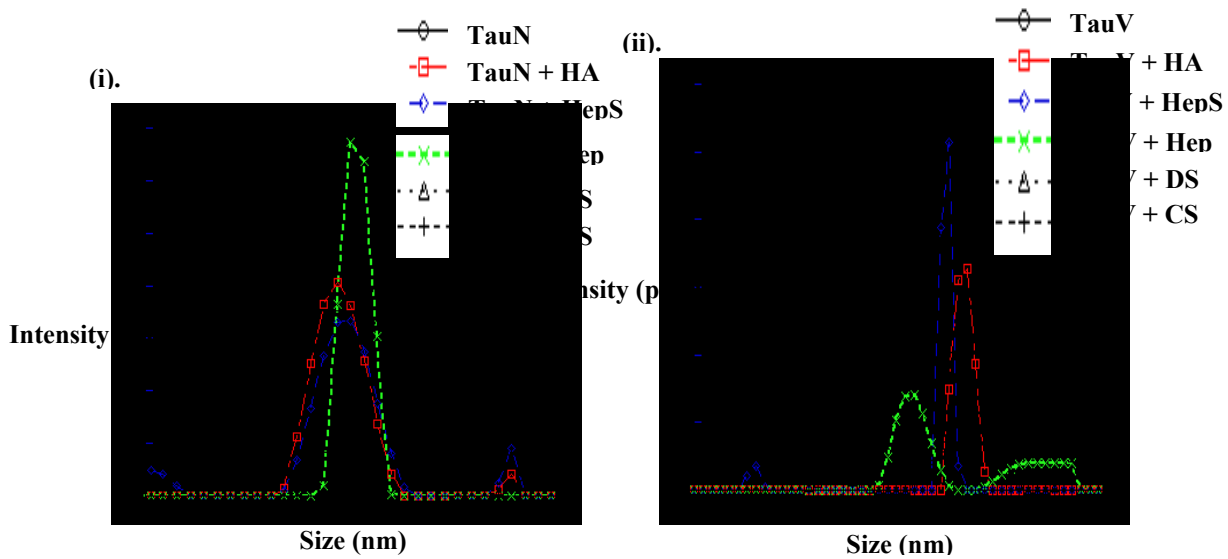
Figure 3.3 A. (i). Dynamic light scattering measurements of the Tau N peptide (100 μM) aggregates with GAGs (25 μM). **(ii)** Tau V peptide (100 μM) aggregation with and without GAGs (25 μM), assessed by turbidity measurements.

As Tau N showed significant aggregation in the presence of GAGs revealed by ThS measurements, next we tested the morphology of the Tau N aggregates by TEM (Figure 3.4). Images reveal, that the Tau N peptides treated with heparin, and chondroitin sulfate exhibited higher amount of fibril like structures, in agreement with the ThS measurements. Dermatan sulfate treated peptides exhibited aggregates like morphology. Also in agreement with the ThS measurements, the TEM images of heparin sulfate, and hyaluronan treated samples showed less aggregated structures compared to other GAGs treated samples.
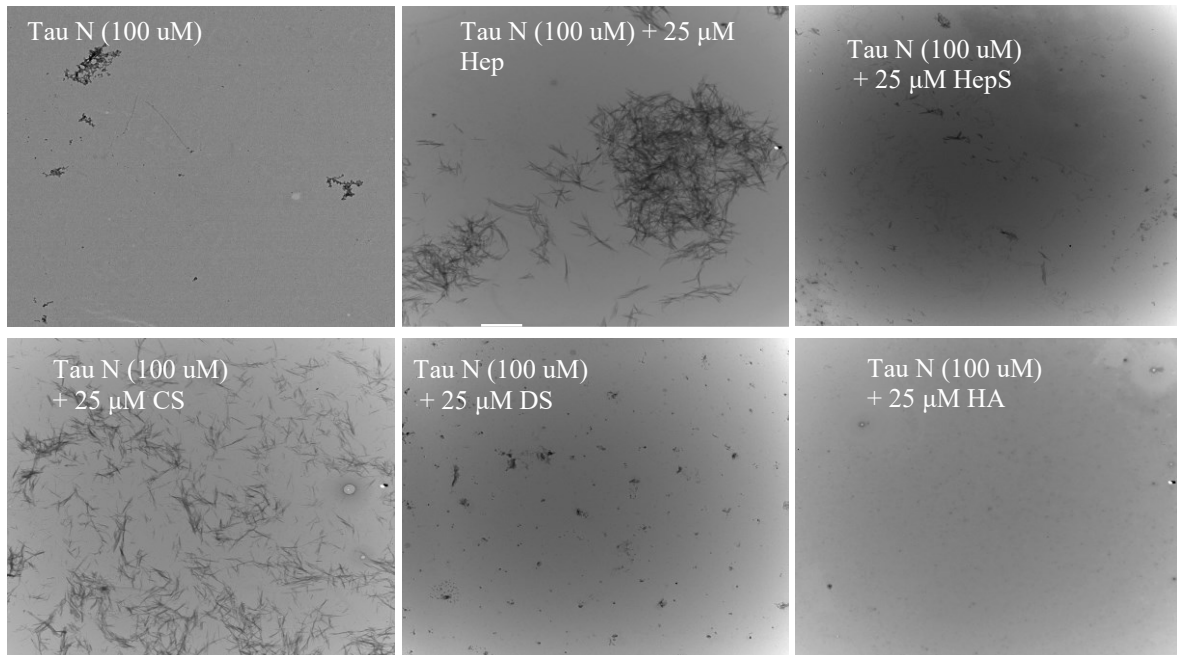
Figure 3.4. A). TEM images of Tau N peptide aggregation in the presence of GAGs. Scale bar 1 μm.

Next, we tested whether aggregation tendencies were observed under extracellular mimetic conditions. As stated before, since Tau N exhibited significant aggregation in solution in the presence of GAGs as revealed by ths (Figure 3.1), we tested their aggregation tendency due to surface coated GAGs. For this, GAGs were coated on special GAGs binding plates, and then Tau N peptides solutions were subsequently incubated on the surface. As can be seen from AFM images in Figure 3.5, all the GAGs exposed peptides showed some aggregation tendency. However, the amount of aggregates seems to be less, compared to the aggregation formed due to the presence of GAGs in the solution form. To further confirm this, we did turbidity measurements. As can be seen from Figure 3.6, there is no significant difference among different GAGS treatment compared to untreated GAGs. Although chondroitin sulfate showed more aggregation/fibril formation revealed from both AFM and turbidity, it was not statistically significant. The data

suggests, that the intracellular GAGs may have major role in tau aggregation compared to extracellular GAGs.
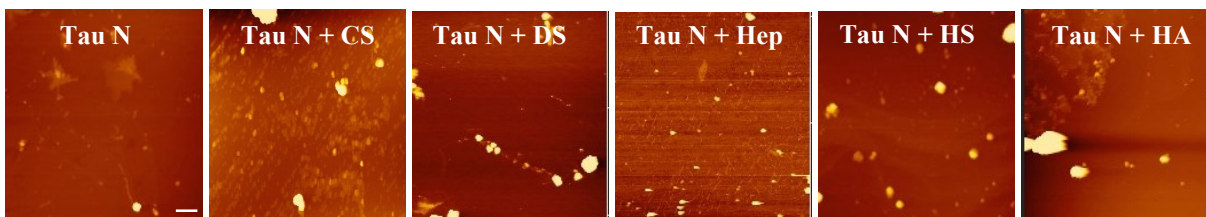
Figure 3.5. AFM images of Tau N peptide aggregation in the presence of GAGs coated surfaces. Scale bar 500 nm.
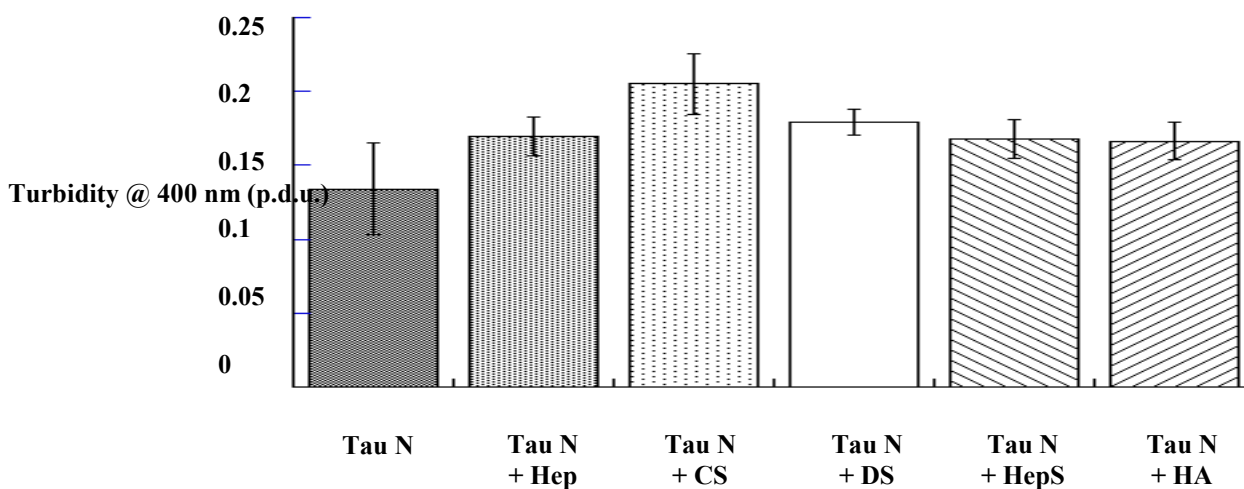
Figure 3.6. Tau N peptide aggregation with and without GAGs coated surfaces, assessed by turbidity measurements.

To assess the toxicity effects of GAGs on tau peptide aggregation, we performed cellular toxicity studies of Tau N and Tau V aggregated samples in the presence of GAGs. For the study, we used SH-SY5Y human neuroblastoma cells, that is widely used as a model cell line in neurodegenerative disease studies to assess the toxicity effects [21]. Cells were cultured in 96 well plates and incubated with Tau peptides and GAGs for 48 hours and XTT assay was performed to assess the cell viability. As can be seen from the Figure 3.7, all the samples treated with GAGs exhibited toxicity effects. While dermatan sulfate treated samples show a slight increase in

toxicity, statistically it is not significantly different from other GAGs treated samples indicating the importance of all the GAGs in peptide aggregation mediated toxicity. The enhanced toxicity in the presence of dermatan sulfate may be due to its influence in the formation of aggregates like morphology compared to fibrillar structures as observed from the TEM images. Finally, we have tested the toxicity effect of GAGs alone. Cell viability assay reveal, none of the GAGs exhibited toxicity to SH-SY5Y cells (Figure 3.8).
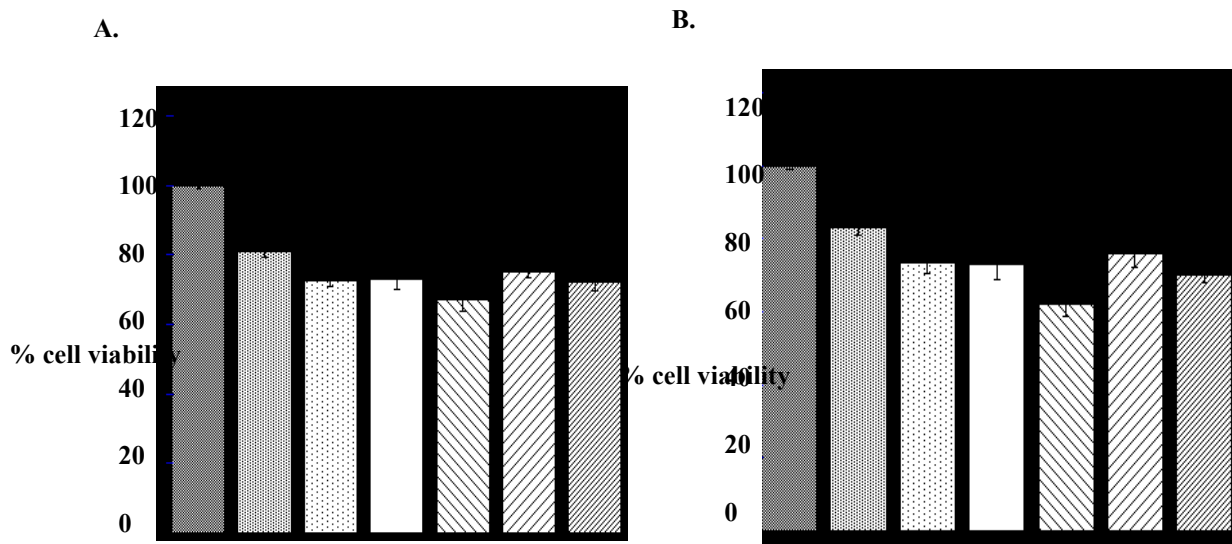
Figure 3.7. Toxicity effect of Tau peptides aggregates with GAGs on SH-SY5Y cells. **A)** Tau N with GAGs **B)** Tau V with GAGs. Peptides in the presence of GAGs exhibited significant toxicity, *p< 0.05.
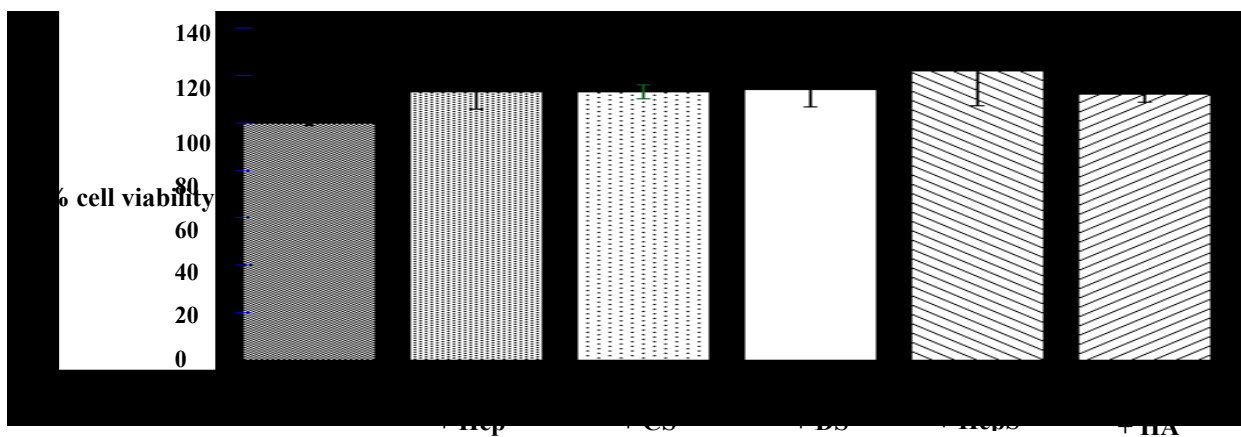
Figure 3.8. Effect of GAGs on SH-SY5Y cells viability. The toxicity data show no significant toxicity of GAGs at the tested concentrations (8 μM) relevant to the study.

3.4 Discussion

Glycosaminoglycans have shown to play a key role in Alzheimer's disease. A recent study suggest that N-glycans may play an important role in Alzheimer's disease [22]. In another study, it is stated that GAGs have a scaffolding role on amyloidogenic proteins [23]. Further, it has been reported that glycosaminoglycan heparin, which is anionic in nature, believed to play a key role in the tau aggregation by interacting with the microtubule binding region [13, 18]. Here we show that GAGS, both in solution and on surface, have the capability to influence the tau peptides aggregation, fibrillization and toxicity in vitro. As far as GAGs are concerned, chondroitin sulfate, dermatan sulfate, and heparin seem to influence the peptide aggregation more compared to other GAGs. Although it has been reported that the sulfated form of heparin is found in the amyloid plaques, it did not promote significant aggregation of tau peptides under in vitro conditions. Hyaluronan showed the least aggregation promoting effects among the GAGs studied. Further, the GAGs in solution seems to have more influence on the aggregation compared to the surface coated GAGs.

3.5 Conclusion

From this thesis chapter study, we show that GAGs influence the tau peptides aggregation under in vitro conditions. The kind of glycosaminoglycan, and the sulfate modifications, and the physical representation also seems to influence the aggregation mechanism. This study could open further opportunities for the investigation of polyanions associated tau aggregation mechanisms and therapeutic interventions.

3.6 Acknowledgment

to thank the University of Michigan Microscope and Imaging (MIL) facility for the use of TEM and confocal microscopes.

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Chapter 4: Tau peptides and tau mutant protein aggregation inhibition by cationic polyethyleneimine and polyarginine

Keerthi Nadimidla, Tania Ismail, and Mathumai Kanapathipillai

4.1 Abstract

Tau protein plays a major role in Alzheimer's disease. The tau protein loses its functionality by self-aggregation due to the two six-amino acid sequences VQIVYK, and VQIINK of the protein. Hence it's imperative to find therapeutics that could inhibit the self-aggregation of this tau peptide fragments. Here we study the inhibitory potential of a cationic polymer polyethyleneimine (PEI), and a cationic polypeptide arginine (Arg) on the aggregation of VQIVYK, and GKVQIINKLDL peptides, and tau mutant protein (P301L), found frequently in taupathy. Various characterization methods are employed including thioflavin T, transmission electron microscopy (TEM), and dynamic light scattering (DLS) to study the aggregation/inhibition process *in vitro*. Results show, PEI and Arg significantly inhibit tau peptides and protein aggregation. The study could be applied to understand tau protein aggregation mechanism in the presence of cationic polymers.

Key words: Aggregation, tau peptide, polyethyleneimine, polyarginine, inhibition, cation.

4.2 Introduction

Tau protein plays an important role in the regulation of microtubules in the brain. During Alzheimer's and related pathological conditions, tau has been shown to dissociate from the microtubule, self-aggregates, and form neurofibrillary tangles [1, 2]. Cell to cell transmission of the aggregates and fibrillar structures results in neuronal cell death and subsequent progression of the Alzheimer's [3, 4]. The hexapeptide repeats VQIVYK, and VQIINK found in the carboxyl region of the tau protein, believe to be the drivers in the formation of inter-tau beta sheets, and subsequent aggregation and fibril structures [2, 5, 6]. The hexapeptide repeats are well characterized, and have shown to form beta-sheets, and subsequent fibrillar assembly with cross beta sheet structure, *in vitro* [6, 7]. They are widely used as a model to study tau aggregation, and for the investigation of therapeutic interventions of this pathologic aggregation [4, 6, 8].

Several therapeutic modalities for the inhibition of tau peptide/protein aggregation have been reported [9, 10]. Among the approaches, the use of small molecule compounds has recently gained a lot of interest. Several small molecules have shown to exhibit anti-tau aggregation properties against tau peptides and protein [11, 12]. However, there have been only few studies reported on the effect of macromolecules on tau aggregation mechanism and in tau pathology [13-15]. Recent study by Dammers and others show the modulation of tau aggregation mechanism by D-enantiomer peptides [13]. Other macromolecules that have been shown to have effect on tau pathology are, an eight amino acid peptide Davunetide [14], and a D-aminoacid inhibitor peptide [15]. By studying the macromolecules-tau interaction, further insights on tau aggregation mechanism and inhibition could be unraveled, and could aid in the development of tau aggregation inhibitory molecules, and drug delivery strategies. Previous studies have shown that cationic small molecules, and cationic osmolyte urea have inhibitory potential on tau aggregation [8, 16, 17].

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However, effect of cationic polymers on tau peptide aggregation inhibition hasn't been explored in detail yet. We are particularly interested in studying the cationic polyethyleneimine and polyarginine polymers on their effect on tau aggregation.

Here in this paper we investigated the inhibitory potential of the cationic polymers PEI and PR on the aggregation of tau hexapeptide domain [306] VQIVYK[311] (Tau V) segment from the microtubule binding region of tau protein, and the peptide fragment GKVQIINKLDL (Tau N), which is widely found in taupathy. The peptides are well characterized and have been used as models to study the tau aggregation [6, 8]. In addition, to have a physiologically closer mimic, we also investigate the aggregation inhibition of a mutant tau protein (P301L), found in adult brains affected by taupathy [18, 19]. The aggregation with and without the cationic polymer, and polypeptide/protein were studied utilizing the standard peptide aggregation characterization methods thioflavin S, TEM, and dynamic light scattering (DLS). The results show that the cationic PEI and Arg, exhibited significant inhibitory effect on the tau peptides and protein aggregation. The study demonstrates for the first time that cationic polymers could inhibit tau peptide and protein aggregation, and could be used as model compounds to study the tau aggregation mechanism *in vitro.*

4.3 Materials and Methods

4.3.1 Materials

Tau peptides, and corresponding mutant peptides were custom synthesized from Genscript. Tau mutant protein, P301L was obtained from rPeptide. Low molecular weight heparin was purchased from Galen laboratory supplies. All other chemicals, and reagents were purchased from Sigma Aldrich. The polyethyleneimine-branched (Mw 25,000, cat no: 408727), and poly-l-arginine

hydrochloride (Mw 5,000-15,000, cat no: P4663) were obtained from Sigma Aldrich, and was used as obtained.

4.3.2 Thioflavin S (ThS) fluorescence measurement

Tau peptides stocks of 1 mM were prepared by dissolving in deionized water. ThS of 0.5 mg/ml was freshly prepared with 20 mM MOPS buffer, pH 7. 1 mM Heparin stock solutions were prepared by dissolving in deionized water. For the ThS assay, 10 μl of peptide was dissolved in 2.5 μl heparin, 10 μl ThS, and MOPS buffer with or without the cationic inhibitors to make the final 100 μl working solution. ThS fluorescence was measured at 440/490 nm excitation and emission using a spectrophotometer in the lab. Kinetic measurements were performed with 2 minutes interval for 30 min. The experiments were repeated for three independent experiments.

4.3.3 DLS

DLS measurements were performed using a Malvern zetasizer instrument in the lab. Samples were prepared under similar conditions without the ThS, and diluted 10 times, and the size measurements were performed. The experiments were repeated for three independent experiments.

4.3.4 TEM

For TEM imaging, the peptides, after 2 hours of aggregation were spotted on holy carbon copper grids, and then stained with 1% uranyl formate. The samples were then be imaged using the JEOL JSM 1400 TEM at UM-Ann arbor electron microscope facility.

4.3.5. Cell toxicity assay

The toxicity of PEI and Arg on normal human neuroblastoma SH-SY5Y cells was studied by the XTT assay. Cells were obtained from the American Type Culture Collection (ATCC), and were

cultured according to the manufacturers protocol. We have also tested the toxicity effect of Tau N on these cells. For the study, $2x10^4$ cells/well, were cultured in 96 well plates over night. For PEI and Arg toxicity, PEI concentrations of 50-200 nM, and Arg concentrations of 1- 4 µM were incubated for 48 hours. For Tau N toxicity study, Tau N concentrations of 10, 20, and 40 µM were tested with and without PEI and Arg. XTT assay was performed at 470 nm, and the viability was determined according to manufacturer's protocol.

4.3.6 Statistical Analysis

Data were collected from three or more replicates for each experiment, and they are presented as mean ± standard error of the mean (SEM). P-values were determined from the results of at least 3-independent experiments for statistical significance unpaired T-test was used.

4.4 Results and Discussion

The approach used for the study is depicted in Figure 4.1. The goal of the study is to test the potential of cationic polymer PEI and cationic peptide Arg on the inhibition of tau peptide/protein aggregation, and subsequent fibril formation. First, we performed thioflavin S measurements to probe the aggregation kinetics of the tau peptides with and without PEI and Arg. The thioflavin S measurements show that the cationic polymers successfully inhibit both Tau V, and Tau N peptides aggregation *in vitro*. The kinetic study show, faster aggregation for peptides without any inhibitors. PEI with hundred times lower molar concentrations was able to inhibit both peptides aggregation, while Arg with about ten times lower molar concentrations was able to inhibit the peptides Tau N (Figure 4.2), and Tau V (Figure 4.3) aggregation. To confirm, that the aggregation of Tau N, and Tau V is sequence specific, we did scrambled peptides VKYVIQ, and GKIVQNIVLKKLD

aggregation. As can be seen from Figure 4.4, the scrambled peptides did not show significant

aggregation, indicating the importance of hexapeptides domain in tau aggregation.
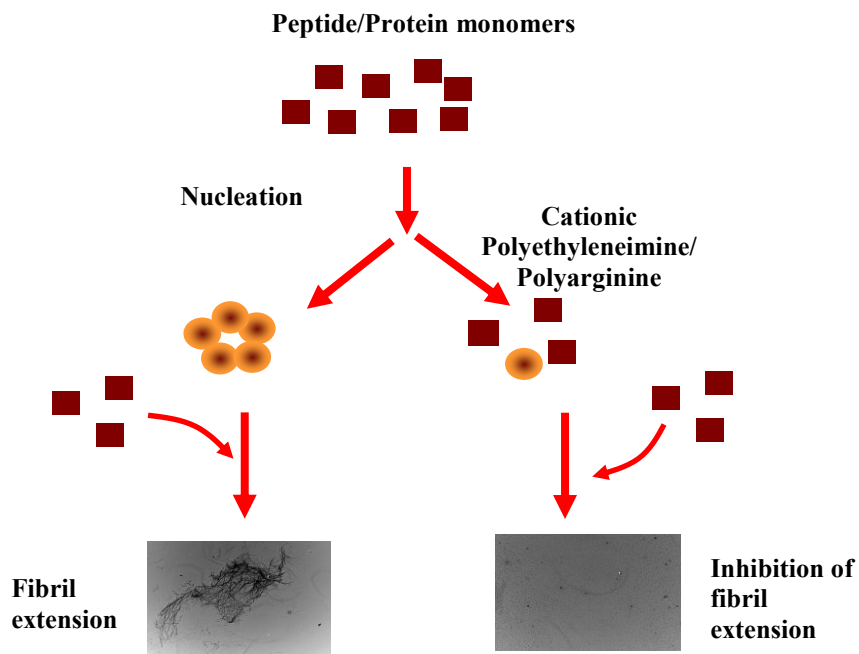
Figure 4.1. Schematic of the approach used. Proposed tau peptides aggregation inhibition by cationic polymer polyethyleneimine and cationic polypeptide polyarginine
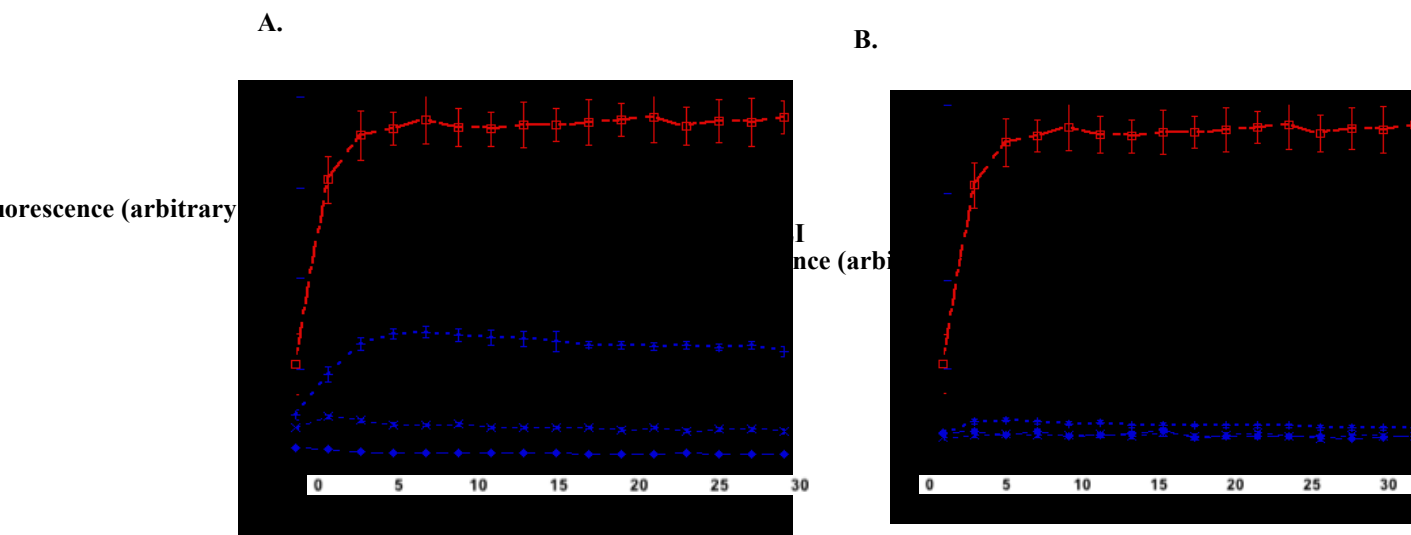
Figure 4.2. Tau N peptide (100 uM) aggregation kinetics was studied with and without PEI and Arg at concentrations depicted in the figure. Thioflavin S fluorescence show significant inhibitory effect of both PEI (**A),** and Arg **(B)** on Tau N aggregation.
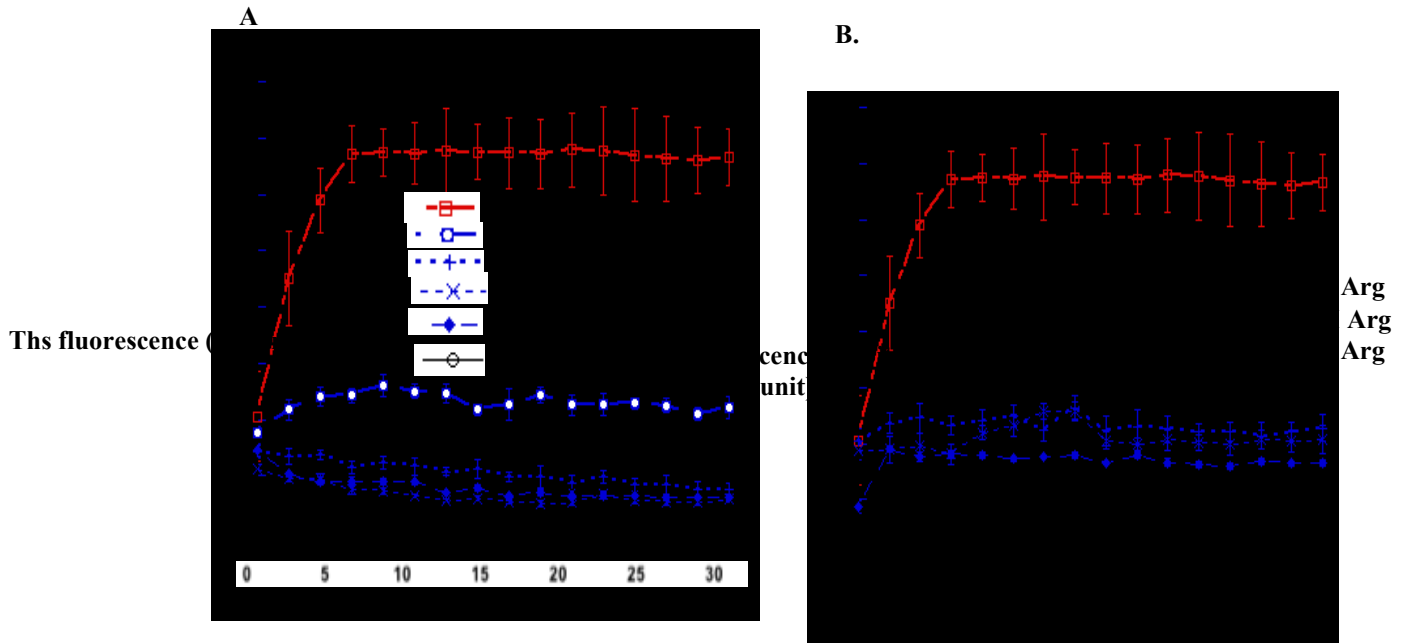
Figure 4.3. Tau V peptide (100 uM) aggregation kinetics was studied with and without PEI and Arg at concentrations as depicted in the figure. Thioflavin S fluorescence show significant inhibitory effect of both PEI **(A)**, and Arg **(B)** on Tau V aggregation
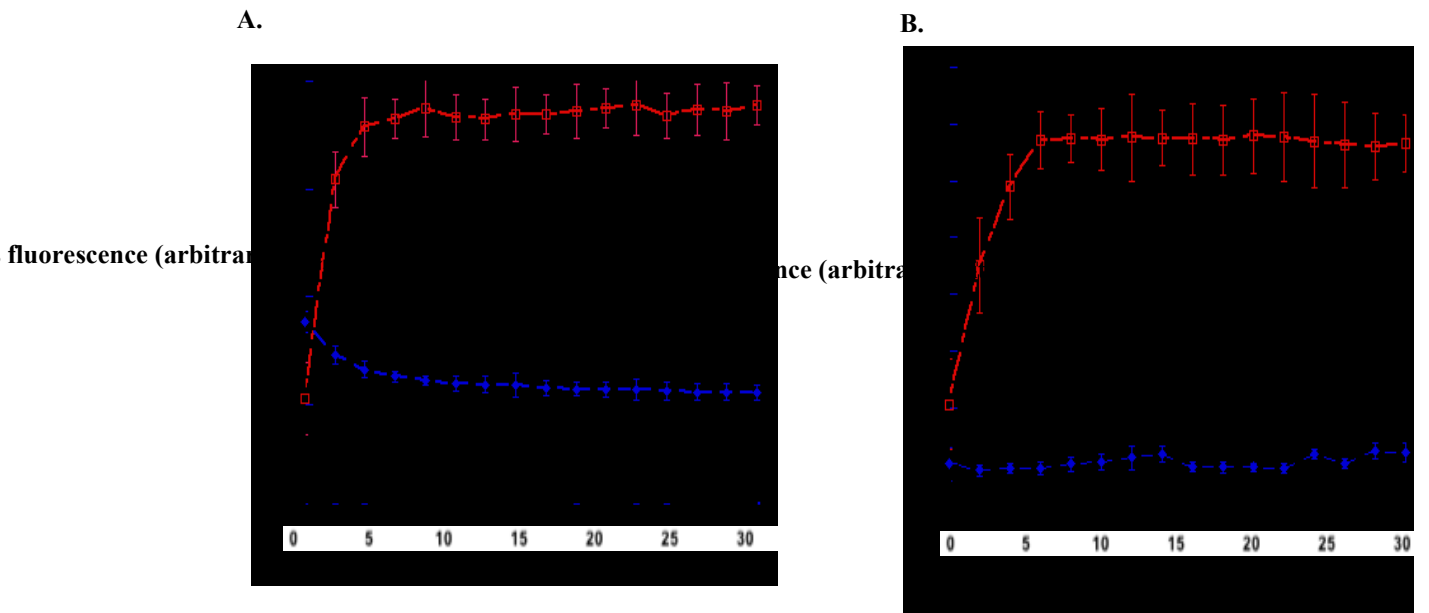
Figure 4.4. Scrambled peptides aggregation kinetics was studied with Thioflavin S binding assay. Thioflavin S fluorescence, indicate the scrambled peptides did not exhibit significant peptide aggregation.

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We then tested the morphology of the Tau N and Tau V aggregates with and without PEI and Arg using TEM. Images reveal, that the tau peptide Tau N fibers formed more fibrils compared to the Tau V aggregates, corroborating the ThS data. PEI, and Arg in few micromolar concentrations were able to effectively inhibit the aggregation of 100 μM peptides as seen in Figure 4.5, and is in agreement with the ThS data. Further, as a complementary technique, we performed dynamic light scattering measurements to study the size of the peptides aggregation with and without PEI and Arg. The size measurements for GKVQIINKLDL (Tau N) aggregation show, that the aggregation size is reduced in the presence of polyethyleneimine or polyarginine, in corroboration with the ThS and TEM measurements (Figure 4.6).

A.
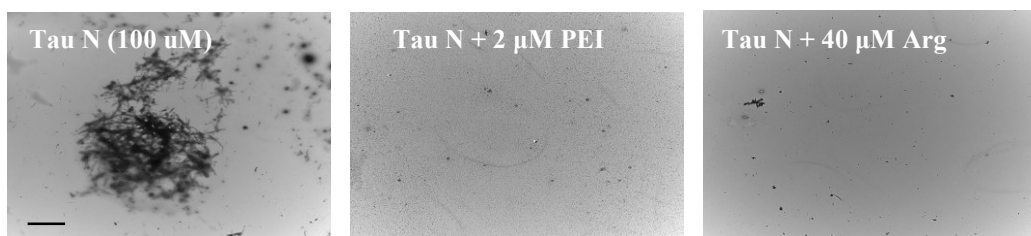
B.
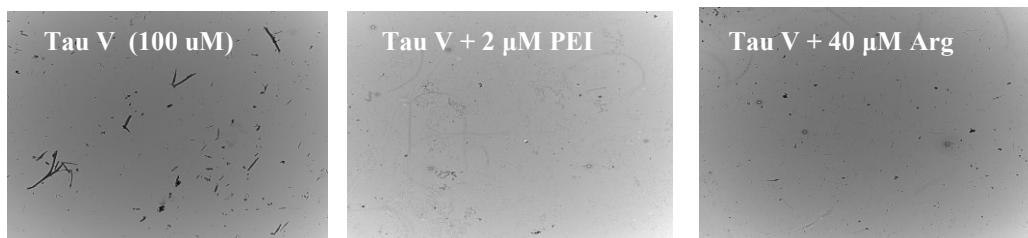
Figure 4.5. TEM images show the tau peptides aggregation inhibition in the presence of PEI and Arg. **(A)** Tau N, with and without PEI or Arg, **(B)** Tau V, with and without PEI or Arg. Scale bar 1 μm.

A.   **B.**

Volume %   Volume %

Size (nm)   Size (nm)
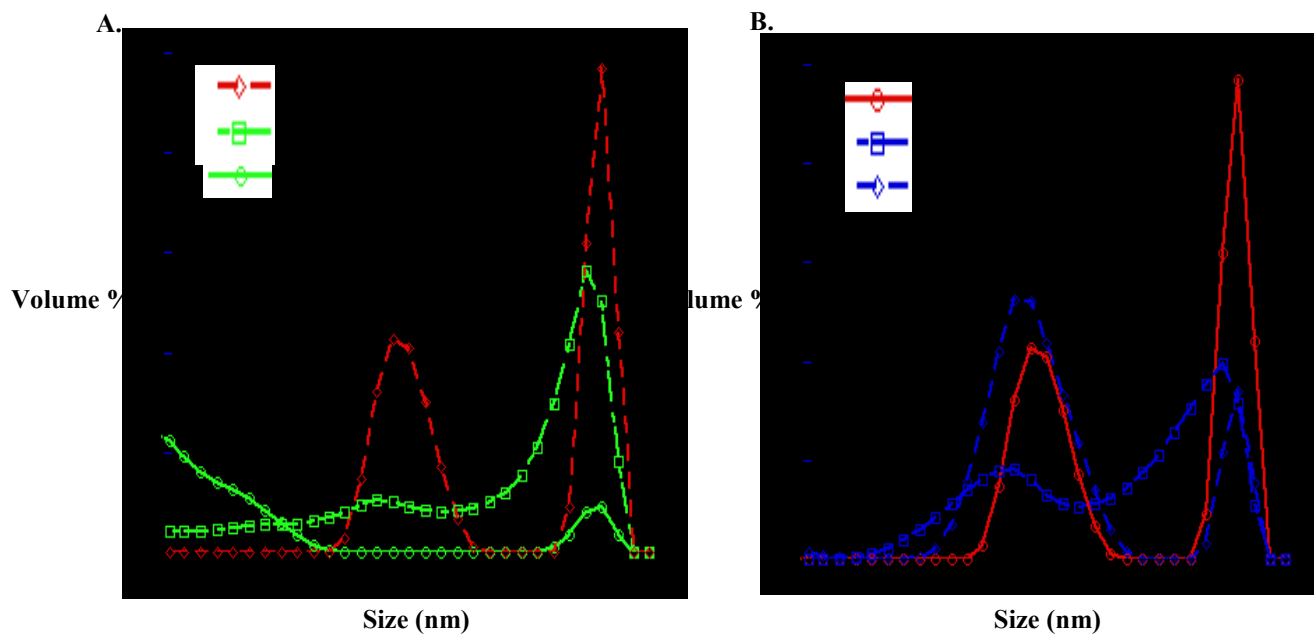
Figure 4.6. Dynamic light scattering measurements of the Tau N peptide aggregation with and without PEI **(A)** or Arg **(B)**, indicating the reduction in aggregation size in the presence of PEI or Arg.

We then studied the inhibitory effect of the cationic polymers on tau mutant protein aggregation. The aggregation inhibition of a mutant tau protein (P301L), found in adult brains affected by taupathy was studied. To test the effect of aggregation inhibition, we performed aggregation kinetics of tau mutant protein aggregation with and without PEI and Arg similar to the peptide aggregation experiments described earlier. As can be seen from ThS measurements (Figure 4.7A), DLS (Figure 4.7B), and TEM images (Figure 4.7C), the cationic polymers are able to inhibit the protein aggregation.
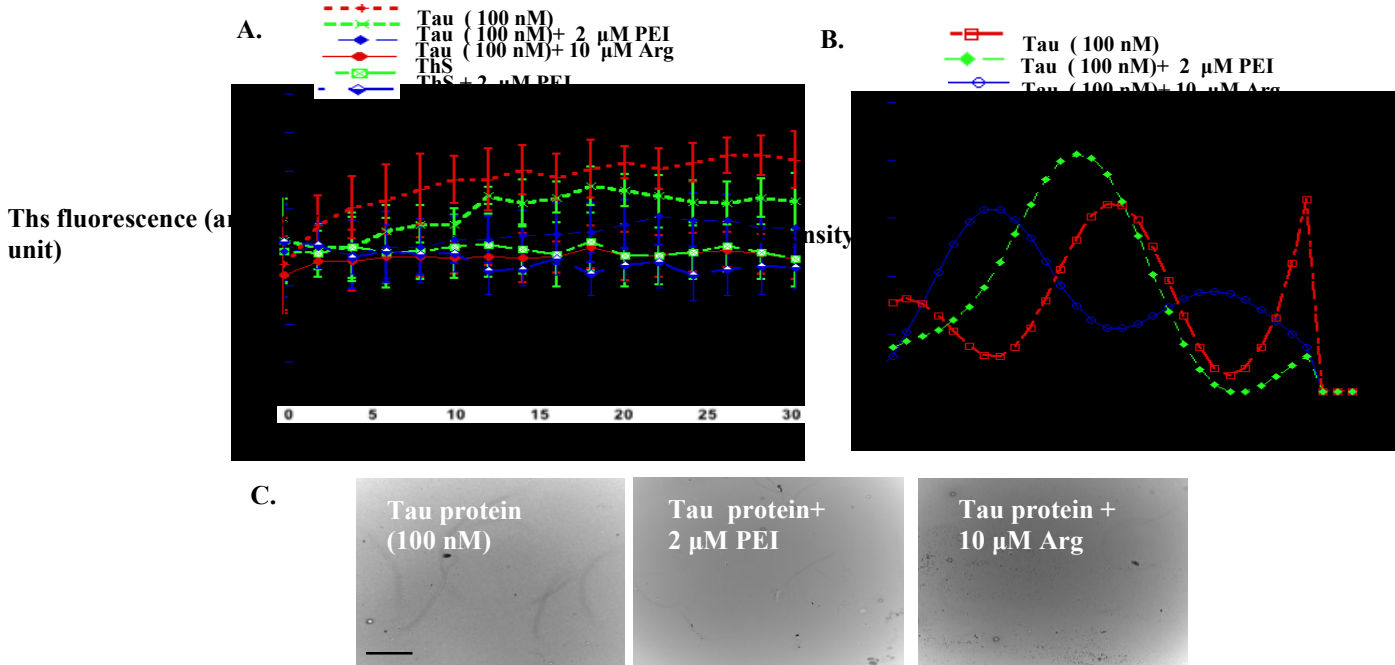
Figure 4.7. Tau mutant protein (100 nM) aggregation inhibition studies show that PEI and Arg were able to inhibit protein aggregation **A)** Thioflavin S, **B)** Dynamic light scattering, and **C)** TEM images. Scale bar 1 μm.

Finally, to assess whether the cationic peptides have toxicity effects in normal cells, we performed cellular toxicity studies on normal cells in the presence of PEI and Arg with varying concentrations. For the study, we used SH-SY5Y human neuroblastoma cells, which is also used as a model cell line in neurodegenerative disease studies [20], and hence would be a suitable for our study. Cells were cultured in 96 well plates and incubated with PEI and Arg for 48 hours and XTT assay was performed to assess the cell viability. As can be seen from the Figure 4.8, no significant toxicity was observed from PEI or Arg up to a few micro molar concentrations. We have also tested the toxicity effects of Tau N on SH-SY5Y cells (Figure 4.9). Tau N exhibited toxicity around 40 μM of peptide concentration, while the lower concentrations showed no toxicity (Figure 4.9A). Tau N treated with PEI and Arg did not exhibit cellular toxicity at the tested

concentrations (Figure 4.9B, C). Hence, the cationic polymers, could have therapeutic potential against tau aggregation.

A.

	No treatment
	50 nMPEI
	100 nM PEI

B.

	No treatment
	1 μM Arg
	2 μM Arg
	3 μM Arg
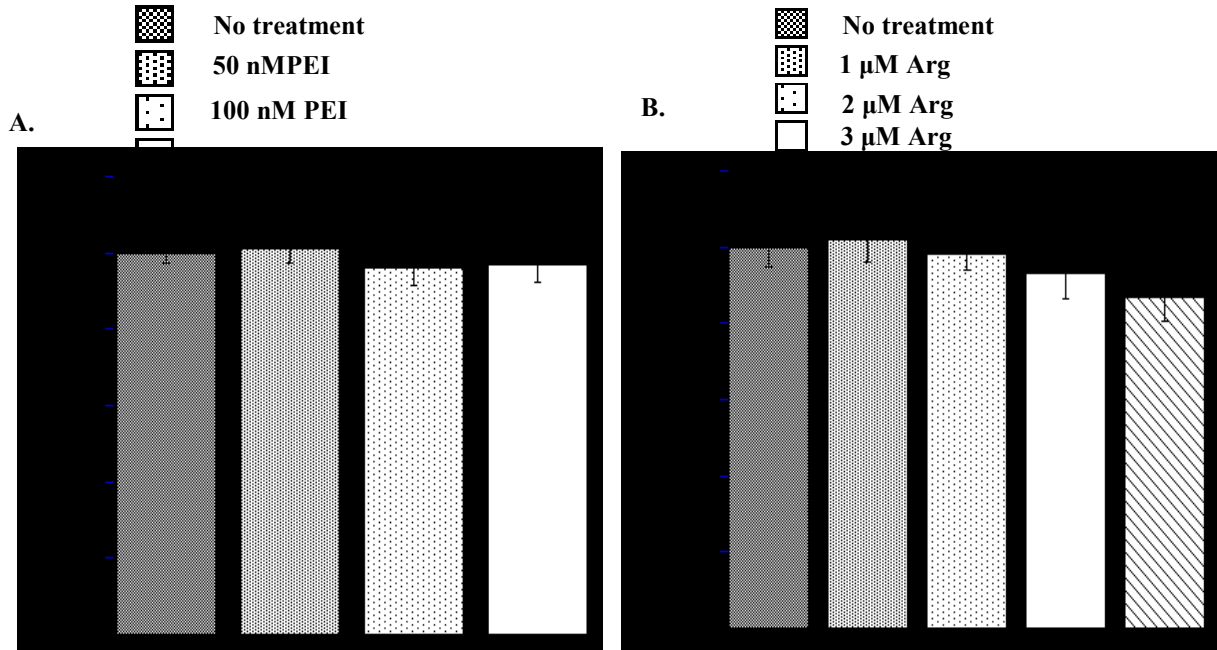
Figure 4.8. XTT assay show no significant decrease on the cell viability of SH-SY5Y cells in the presence of **(A)** PEI or **(B)** Arg at few micromolar concentrations.

A.

	No treatment
	Tau N (10 μM)
	Tau N (20 uM)
	Tau N (40 uM)

B.

	No treatment
	Tau N (10 μM) + PEI 200 nM
	Tau N (20 μM) + PEI 200 nM
	Tau N (40 μM) + PEI 200 nM

C.

	No treatment
	Tau N (10 μM) + Arg 2 μM
	Tau N (20 μM) + Arg 2 μM
	Tau N (40 μM) + Arg 2 μM
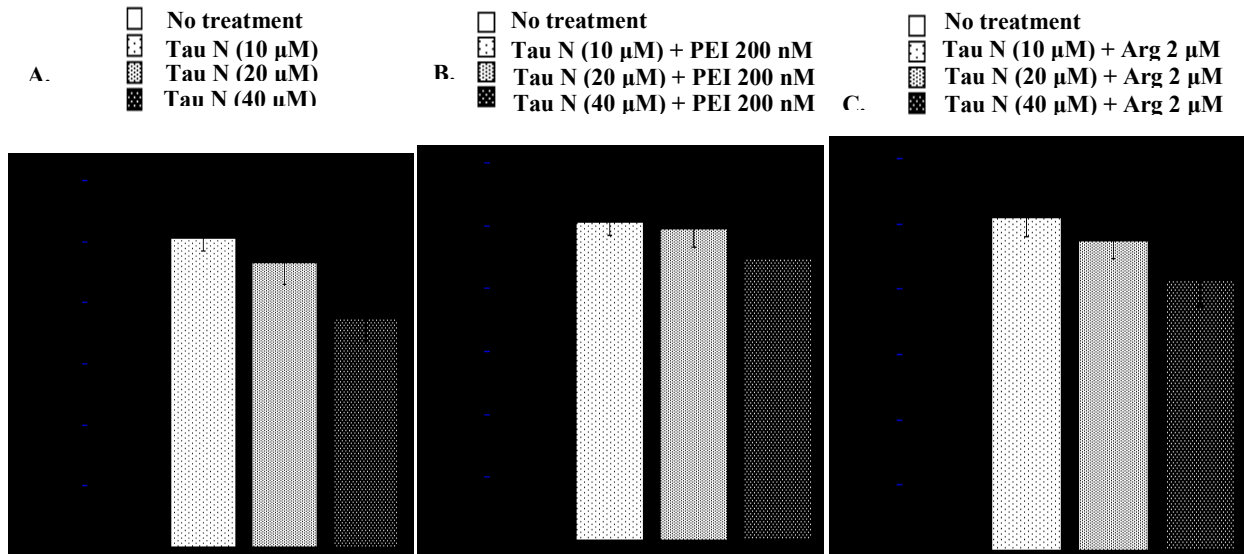
Figure 4.9. Tau N toxicity effect on SH-SY5Y cells. **A)** Tau N alone **B)** Tau N with 200 nM PEI **C)** Tau N with 2 μM Arg. * $p < 0.05$. Data are expressed as mean ± SEM.

Here we show that cationic polymers have the capability to inhibit tau mutant fibrillization *in vitro*. It is widely known that microtubules and polyanions contribute to the neurofibrillary tangle formation [21, 22], and glycosaminoglycans, which are anionic in nature, believed to play a key role in the tau aggregation [23, 24]. Hence, by interacting with the glycosaminoglycan heparin that is used in the study, the cationic PEI and Arg could have prevented the tau peptides and protein aggregation. Previously, it has been reported that several cationic small molecule compounds such as, cyanine, phenothiazine, and arylmethine in micromolar concentrations, exhibited inhibitory effects by interacting with the nucleation process mediated by the tau peptides [16]. Another recent study report the inhibitory effect of urea, a cationic osmolyte on tau fibrillization [8]. These studies further indicate the influence of cationic molecules on tau aggregation inhibition, and the current study would provide new insights and future investigation on the influence of cationic macromolecules on tau aggregation.

4.5 Conclusion

The cationic polymers PEI and Arg, exhibited inhibitory effects on tau peptides, and protein aggregation *in vitro*, and could aid in understanding the mechanism of tau aggregation inhibitory process. Further, they could to be used as drug carriers for treating tau aggregation. PEI and Arg have shown potential in gene delivery [25-28], and hence could be used in Alzheimer's gene therapy. Arg is also known for its cell penetrating capabilities [29, 30], and could be used as a drug depot for effective intracellular delivery of therapies for tau aggregation, which is mainly observed inside the cells [4, 19]. From this proof of concept study, we show that the cationic polymeric compounds may serve as potential inhibitors for tau peptide aggregation, and could be applied to study the mechanism of tau protein aggregation.

4.6 Acknowledgment

4.7 References

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Chapter 5: Role of extracellular enzymes in Alzheimer's peptides aggregation

5.1 Introduction

Alzheimer's disease is characterized by senile plaques and neurofibrillary tangles in the brain tissue [1-4]. Several extracellular matrix factors play a role in promoting and stabilizing abeta and tau aggregation [5-8]. Among them extracellular enzymes are one of the key constituents found co-localized with abeta and tau aggregates [6, 8]. Extracellular enzymes transglutaminase (TG), and lysyl oxidase (LOX) are known to crosslink the extracellular matrix and modulate the ECM during diseases [6, 8, 9]. Recent studies report that both TG, and LOX have been observed in Alzheimer's disease brain tissues [9-12]. Transglutaminase is believed to contribute to Alzheimer's pathology by either directly binding to abeta and tau, or modulating the protein aggregation cascade and neurotoxicity by crosslinking the ECM [13, 14]. The result is the production of neurotoxic and protease resistant abeta and tau. Lysyl oxidase, which is known to play a major role in collagen crosslinking also believe to contribute to abeta pathology in a similar manner [9].

Here in this chapter, we study the effect of extracellular enzymes transglutaminase and LOX peptide on the aggregation of Aβ (1-42) and tau peptide (VQIVYK) that are found predominantly in Alzheimer's disease patients. In addition, we study the potential of natural antioxidants betaine and l-proline in inhibiting transglutaminase and lysyl oxidase mediated aggregation and

fibrillization. Proline and betaine are known to influence protein solvation, prevent aggregation and misfolding [15]. Further, recently it has been shown they have inhibitory potential on human serum albumin, and insulin aggregation/fibril formation [16, 17]. Hence, they could have inhibitory effect on extracellular enzyme mediated abeta and tau aggregation. The studies were carried out using thioflavin T (ThT), atomic force microscopy (AFM), cell toxicity assay (XTT), and oxidative stress assays. The results show that transglutaminase (TG), and lysyl oxidase peptide (LOX) promote the aggregation and fibrillization of Aβ (1-42), and oxidative stress. Further, betaine (B) and l-proline (LP) inhibited the effect of TG and LOX induced Aβ (1-42) fibrillization, and oxidative stress. However, at the tested conditions, tau peptide aggregation was not promoted in the presence of transglutaminase or lysyl oxidase.

5.2 Materials and Methods

5.2.1 Materials

Abeta peptide (1-42), ultra-pure (1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) treated), is purchased from rPeptide. Tau peptides were custom synthesized from Genscript. Transglutaminase (TG) was purchased from either modernized pantry LLC or Ajinomoto. Human Lysyl oxidase peptide (LOX- (EDTSCDYGYHRRFA)) was custom synthesized from Genscript. L-proline (LP) and betaine (B), and all other chemicals were purchased from Sigma Aldrich.

5.2.2 Stock Solutions Preparation

1mg of Beta-Amyloid (1-42) was dissolved in 500µl HFIP. Then, the solution was aliquoted into ten vials (100µg/vial), and was kept in a chemical hood for complete evaporation of HFIP overnight. One vial of Aβ (1-42) was dissolved in 500µl HCl (10mM) and 10 µl of DMSO to get a final concentration of 48µM (stock). For transglutaminase (TG) 5% of solution was freshly

prepared by dissolving 50mg/ml in water. Betaine (B) and L-proline (LP) were dissolved in HCl (10mM) and DMSO to a final concentration of 10M (stock). Tau peptides stocks (1mM) were prepared by dissolving in water.

5.2.3 ThT Fluorescence Measurement

For the aggregation study, 3.2 mg/ml concentration of Tht was prepared in Tris buffer, pH 8. For Tht measurement, 50µl of 48µM Aβ (1-42), 1.1 µl of 5%, 2.5% or 1% TG or 1.25 ul or 2.5 ul of 10 mM LOX, and 0.5ul of 10M B or LP were dissolved in HCl (10mM) and 2% DMSO to make the final working solution. The samples were kept to aggregate for 24 hours in an incubator at 37°C. For the tau peptides aggregation, 10 µl of peptide was dissolved in 10 µl ThS, and MOPS buffer with or without TG or LOX to make the final 100 µl working solution. Spectramax M3 spectrophotometer was used to measure the Tht fluorescence at 440/482 nm excitation/emission. Abeta aggregation end points were measured at 24, and 48 hours. Tau aggregation was measured at 440/490 nm, every 2 minutes interval for 30 min. Three independent experiments were done, and the average readings were obtained.

5.2.4 AFM

AFM images were obtained using Hitachi AFM5100N in the lab. Abeta peptide was aggregated in the presence of TG or LOX, and with and without the small molecule drugs betaine and l-proline, similar to thioflavin t sample preparations. After 24 hours of incubation, 5µl of the solutions were spotted on a freshly cleaved mica and kept to dry before imaging. Tapping mode was used to image the samples, and AFM tips (Nanosensor, CA), with a resonance frequency of 45-115 kHz was used. The average length and width of the cantilever is 225 and 28 micrometers, and the force constant of the tip is 0.5-9.5 N/m. A scan area of 1 x 1 or 5x 5 um is imaged for at least 2 different samples of each condition.

5.2.5 DCFH-DA assay

The effect of oxidative stress of abeta peptide, in the presence of extracellular enzymes, and small molecule drugs on human Brain Endothelial Cells (hBMVEC) was studied by DCFH-DA assay. Quantifying oxidative stress by 2,7-dichlorodihydrofluorescein (DCFH) based fluorescent probes has been widely reported [18, 19]. For the study, hBMVEC cells were used. The cells were kindly provided by Dr. Kalyan Kondapalli at UM-Dearborn, and were cultured in medium containing M-199, supplemented with 10% FBS, and 5% PenStrep according to the standard protocol. For this experiment, $2x10^4$ cells/well were cultured in a 96 well plate for 24 hours overnight. Cells were then treated with final concentrations of abeta peptide (2μM), TG (0.005%) or LOX (0.0005 %), and the small molecule osmolyte drugs (5mM), and subsequently were incubated for 48 hours. DCFH-DA assay fluorescence was performed at 485/535 nm, excitation and emission. Three independent DCFH-DA experiments were performed, and the average readings were obtained.

5.2.6 Cell toxicity assay

The toxicity of Aβ (1-42) fibrils in the presence of transglutaminase and drugs on human brain microvascular endothelial cells (hBMVEC) was studied by XTT assay. Cells were cultured at $2x10^4$ cells/well in a 96 well plate overnight. Aβ (1-42) was dissolved in 200 ul PBS and 0.6 ul ammonium hydroxide (20-30%) to a final concentration of around 19.2 μM. For transglutaminase (TG) 10% solution was freshly prepared by dissolving 100 mg/mL in water, and Betaine (B) and L-proline (LP) were dissolved in water to get a final concentration of 10M. Cells were then treated with final concentrations of, abeta peptide (2 μM), TG (0.005%), and with and without small molecule drugs (5 mM), and subsequently were incubated for 48 hours. The XTT assay was analyzed by the formazan production at 470 nm, using a M3 spectrophotometer according to the manufacturer's protocol.

5.2.7 Immunofluorescence

hBMVEC cells were cultured in 8 well chambers at 2×10^4 cell density, and abeta with and without extracellular enzymes, and osmolytes were added to the wells, similar to the XTT experimental conditions. Cells were then incubated for 48 hours. Immunostaining of ZO-1 (purchased from cell signaling technology) was performed following standard staining protocol. Immunofluorescence images of tight junctions were obtained using a Nikon A-1 spectral confocal microscope at the UM-Ann Arbor microscopy image analysis laboratory (MIL), and images were obtained for 2 different samples of each condition.

5.2.8 Statistical Analysis

In this study, each experiment was conducted three or more times, and they are presented as mean ± standard error of the mean (SEM). P-values were determined from the results of at least 3-independent experiments. Statistical significance was computed using analysis of variance (ANOVA), and Tukey's HSD post-hoc analysis test was performed. **$p < 0.01$, and *$p < 0.05$ were considered as significance.

5.3 Results and Discussion

First in this chapter study, we tested whether TG and LOX promote aggregation of the peptides. The aggregation studies were analyzed by thioflavin T, thioflavin S, and AFM measurements. The thioflavin T measurements of abeta aggregation show that the both TG and LOX significantly promote abeta aggregation (Figure 5.1 A, B). On the other hand, no significant aggregation was observed with tau peptides treated with TG or LOX (Figure 5.2 A, B). Since abeta exhibited significance aggregation in the presence of TG and LOX, we focused our rest of the study on abeta. We then tested the effect of l-proline and betaine on abeta aggregation in the presence of TG or

LOX. The thiolfavin-T measurements reveal, inhibition of TG and LOX mediated aggregation in the presence of B and LP (Figure 5.3A, B). AFM images corroborate the findings. From Figure 5.4, it can be seen that TG and LOX promote fibrillar morphology of abeta. When aggregated in the presence of l-proline less fibril or aggregation was observed (Figure 5.4).
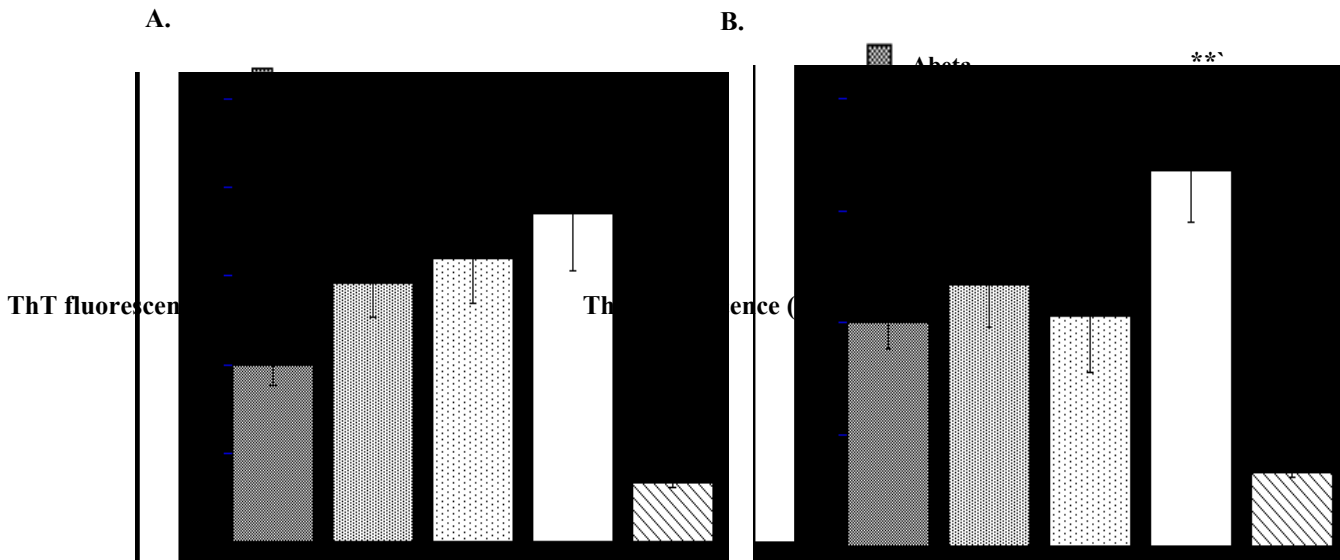
Figure 5.1. Thioflavin-T fluorescence measurements of abeta peptide aggregation. **A).** Abeta peptide (45 μM) aggregation in the presence of 0.1%, 0.05%, and 0.01% transglutaminase **B).** Abeta peptide (45 μM) aggregation in the presence of 0.01%, 0.005%, and 0.0025% of lysyl oxidase. ThT fluorescence measurements indicate that the abeta aggregation is promoted in the presence of extracellular enzymes. Data with significance compared to abeta is represented as **p<0.01.
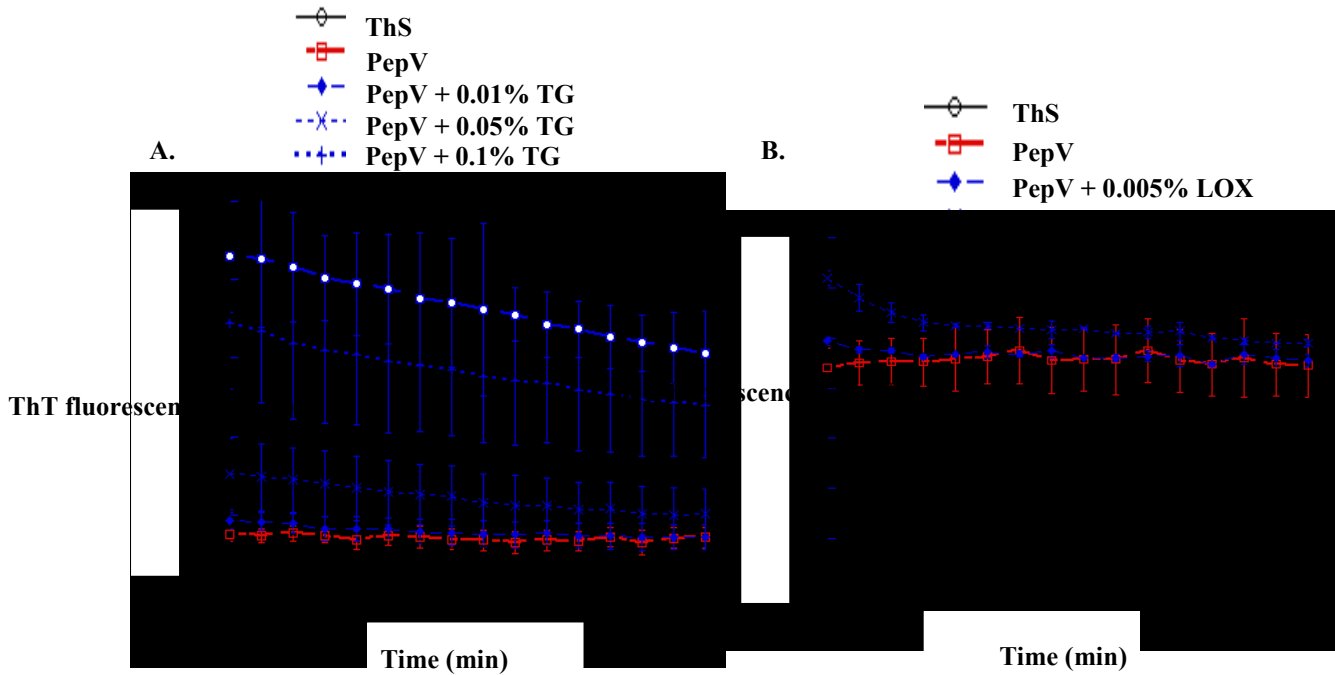
Figure 5.2. Thioflavin-S fluorescence measurements of tau peptide aggregation kinetics. **A).** Tau peptide VQIVYK (100 μM) aggregation in the presence of 0.1%, 0.05%, and 0.01% transglutaminase **B).** Tau peptide (100 μM) aggregation in the presence of 0.01%, and 0.005% of lysyl oxidase. Ths fluorescence measurements indicate that the tau aggregation is not promoted in the presence of extracellular enzymes alone.
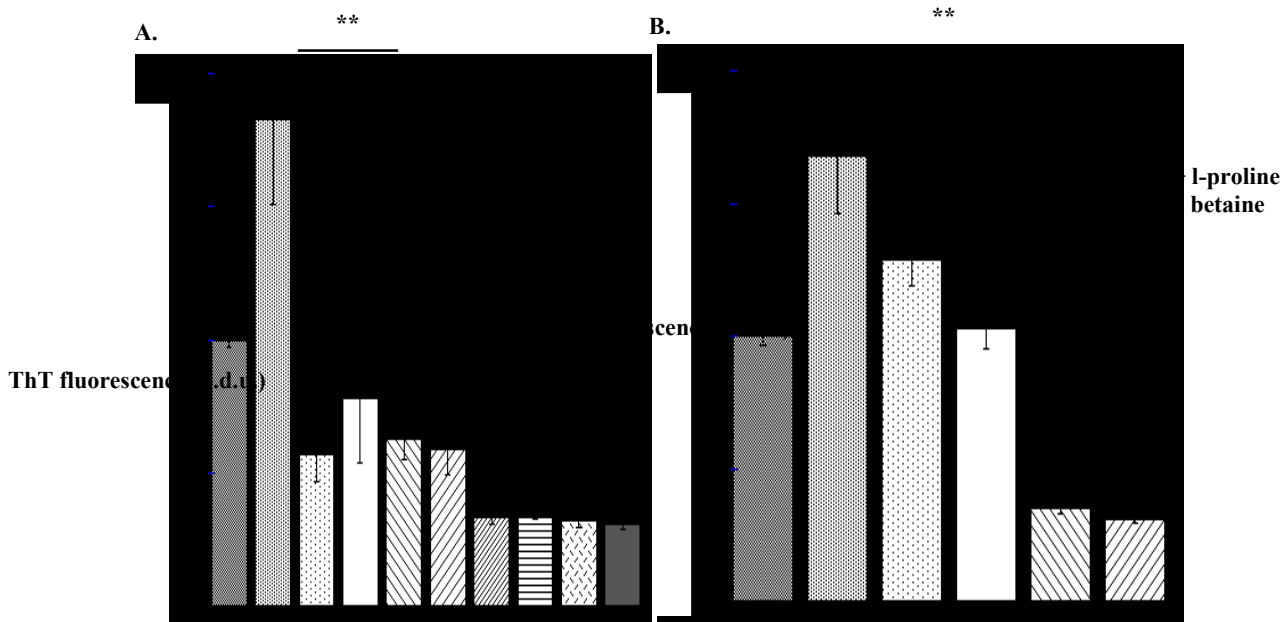
Figure 5.3. Thioflavin-T fluorescence measurements of abeta peptide aggregation. **A).** Abeta peptide (45 μM) aggregation in the presence of 0.1% transglutaminase and 5 mM of osmolytes l-proline and betaine **B).** Abeta peptide (45 μM) aggregation in the presence of 0.01% of lysyl oxidase, and 5 mM of osmolytes l-proline or betaine. ThT fluorescence measurements indicate that the abeta aggregation is modulated by the presence of extracellular enzymes and osmolytes. Data with significance is represented as **p$<$0.01.

A.

Abeta42	Abeta42 + TG	Abeta42 +TG +LP	Abeta42 +TG +B	TG

B.

Abeta42	Abeta42 + LOX	Abeta42 + LOX + LP	Abeta42 + LOX + B	LOX
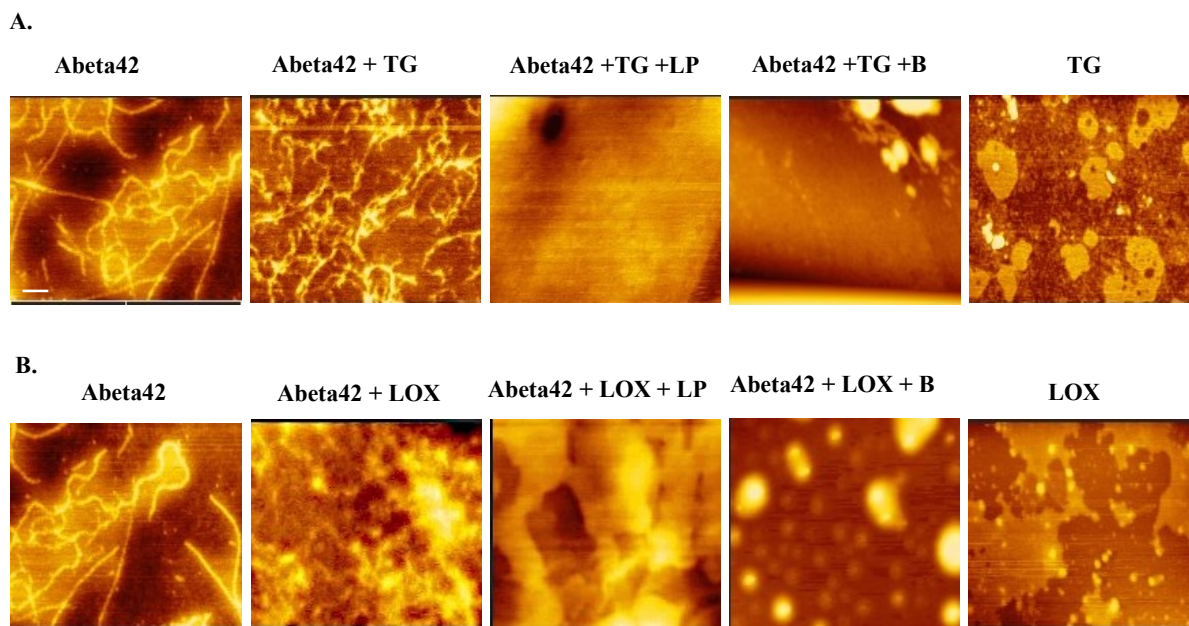
Figure 5.4. Morphology of abeta peptide (45 µM) aggregation in the presence of TG, LOX, and osmolytes l-proline and betaine, imaged by atomic force microscopy. The images reveal abeta aggregation/fibrillization is promoted in the presence of TG and LOX, and inhibited in the presence of l-proline and betaine. Scale bar 100 nm.

Next oxidative stress measurements were performed to determine whether abeta, with and without the extracellular enzymes and small molecules exhibit oxidative stress. For the study human brain microvascular endothelial cells (hBMVEC) were used. First, we tested the effect of transglutaminase induced oxidative stress. As can be seen from Figure 5.5A, transglutaminase indeed promote oxidative stress of abeta aggregates. The oxidative stress effects were significantly inhibited in the presence of l-proline and betaine (Figure 5.5A). The oxidative stress measurements were then performed with lysyl oxidase treated samples. Cells treated with abeta and lysyl oxidase exhibited enhanced oxidative stress. When treated with the osmolytes significant inhibition in the oxidative stress was observed (Figure 5.5B).
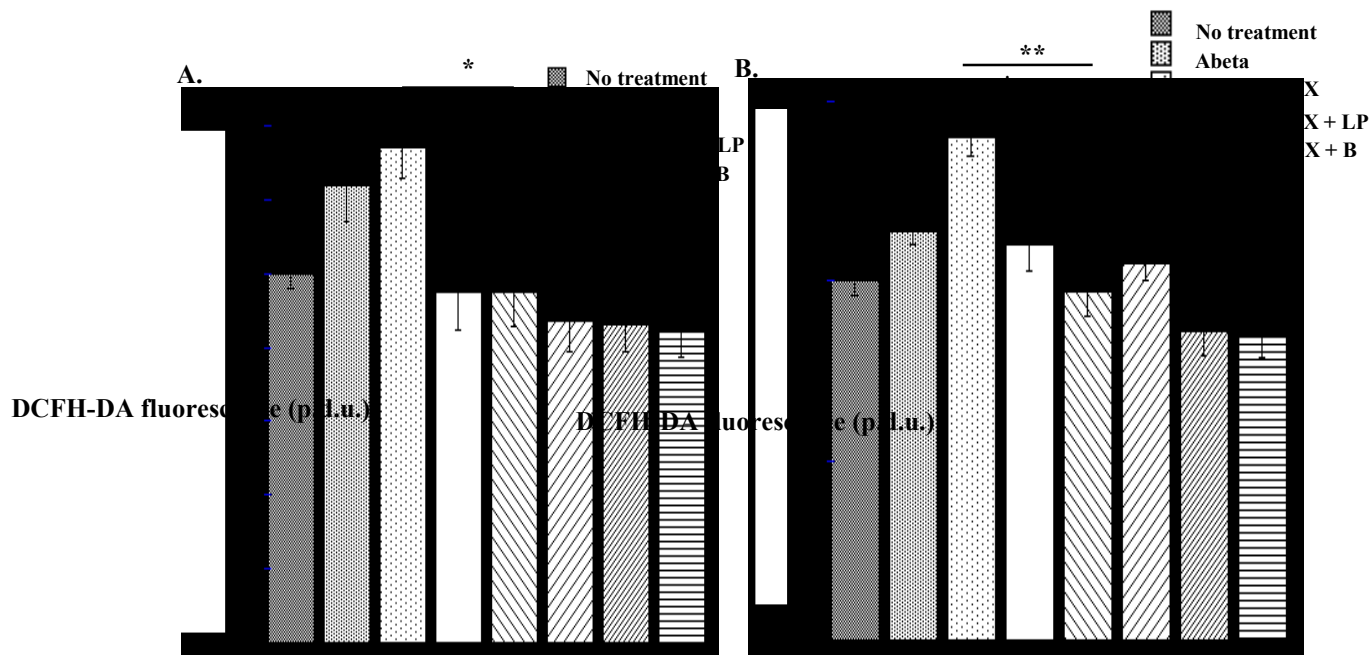
Figure 5.5. Effect of abeta induced oxidative stress on hBMVEC with and without extracellular enzymes and small molecules **A).** Oxidative stress induced by Abeta peptide (2 μM) in the presence of transglutaminase, and osmolytes l-proline and betaine **B).** Oxidative stress induced by Abeta peptide (2 μM) in the presence of lysyl oxidase peptide and osmolytes l-proline and betaine. Oxidative stress is significantly reduced in the presence of osmolytes. Data with significance is represented as **p<0.01, *p< 0.05.

To assess whether abeta and extracellular enzymes play a role in blood brain barrier damage, we performed immunofluorescence staining of ZO-1. The cytoplasmic accessory protein ZO-1, under normal conditions distribute continuously around the cell membrane [20]. However, in the presence of abeta, it has been shown that the tight junctions were disrupted, and broken ZO-1 links were observed [20]. Similarly, in this study as can be seen from Figure 5.4, when the cells were treated with abeta or abeta with TG or LOX, the ZO-1 staining is diffused compared to the control cells without any treatment. Moreover, when the cells were co-treated with osmolytes betaine or l-proline, significant restoration of cell junctions were observed (Figure 5.6). This finding is in corroboration with that of oxidative stress findings, where increased and decreased oxidative stress were observed in the presence of extracellular enzymes, and small molecule drugs.

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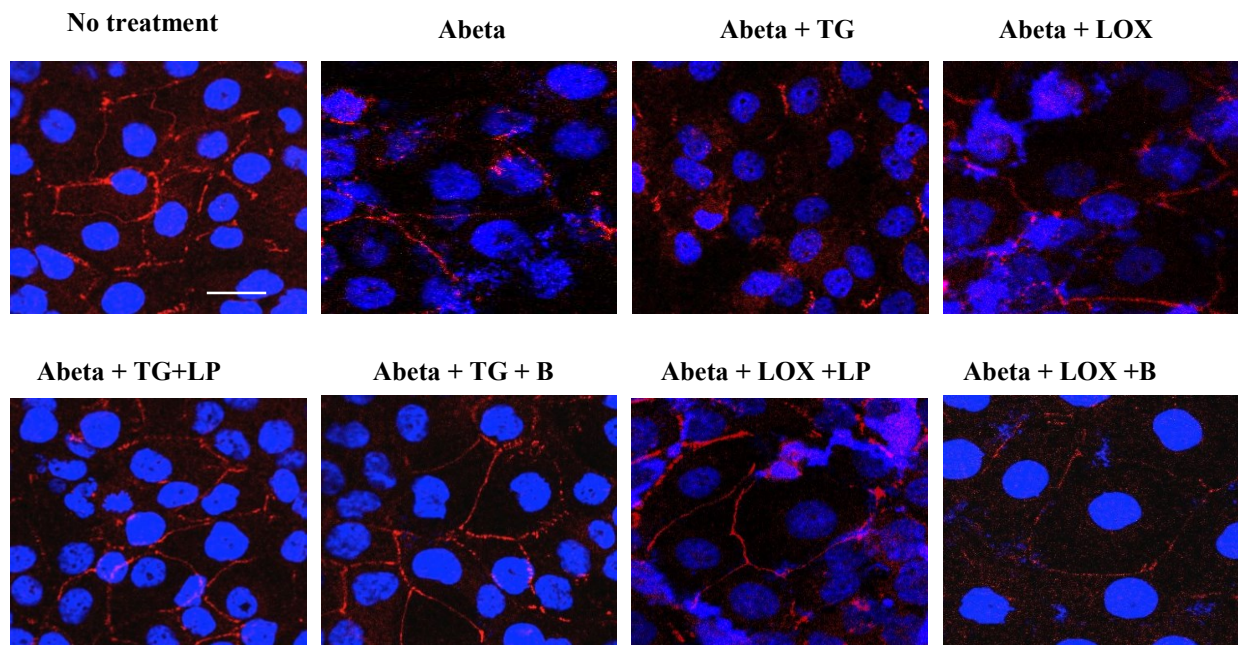
**Figure 5.6.** Blood brain barrier damage induced by abeta peptide (2 μM) aggregation in the presence of transglutaminase and osmolytes l-proline and betaine, visualized by tight junction protein ZO-1. Tight junction is significantly restored in the presence of osmolytes. Scale bar 10 μm.

Finally, we tested the toxicity effects of abeta with and without extracellular enzymes and small osmolyte molecules. For the toxicity study we used human brain microvascular endothelial cells (hBMVEC). Cells were treated with abeta and combination of the enzymes and drugs for 48 hours, and XTT assay was performed. However, as can be seen from Figure 5.7, at the tested concentrations, no significant toxicity was observed with all the treatment combinations of abeta, extracellular enzymes, and small drug molecules.
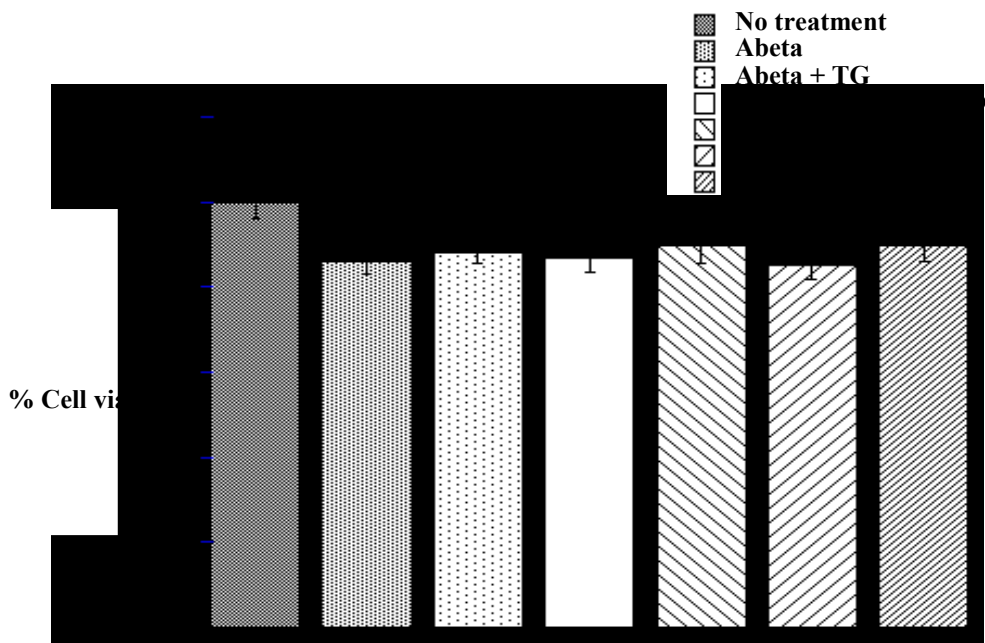
Figure 5.7. Assessment of toxicity effect of abeta peptide aggregates (2 μM) in the presence of transglutaminase (0.005%) and osmolytes (5 mM) in human brain microvascular endothelial cells. No significant difference in XTT absorbance is observed at the tested conditions.

5.4 Conclusion

In this chapter, we show that extracellular enzymes have the capability to influence the abeta peptides aggregation, fibrillization, and oxidative stress in vitro. As far as tau peptides aggregation is concerned, unlike glycosaminoglycans, which are known to promote tau aggregation significantly [21, 22], extracellular enzymes alone seem to be not enough to promote the aggregation. Extracellular enzymes have been shown to colocalize with amyloid plaques, and subsequently crosslink them and prevent them from degradation [9]. This in turn leads to excessive oxidative stress and neuronal death. In this study, we show, in the presence of osmolytes, the aggregation, and oxidative stress effects could be minimized. Hence, osmolytes could be applied to inhibit or prevent extracellular enzymes mediated abeta aggregation/fibrillization and oxidative stress, and mitigate potential long-term toxicity effects.

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Chapter 6: Role of cerebral fluid proteins in abeta peptide aggregation

6.1 Introduction

During Alzheimer's disease, increase in abeta concentration has been shown to be present in the blood and in brain [1]. The large amount of abeta is believed to contribute to the disease either by crossing the blood brain barrier and facilitates aggregation formation in the brain or by preventing abeta clearance from the brain to the circulation. One way to prevent this large amount of abeta, is to sequester the excess abeta in the blood, and in the brain. Cerebrospinal fluid (CSF) proteins transferrin and human serum albumin has been shown to inhibit abeta aggregation. The proteins believed to bind to the abeta oligomer, and prevent abeta monomer binding to free abeta oligomers, and hence subsequent aggregation and plaque formation [2]. Hence there is growing interest in utilizing cerebral proteins to treat Alzheimer's. Hence a cerebral protein drug depot that can capture the excess abeta in the blood or in the brain could prevent unwanted abeta aggregation, oxidative stress, and subsequent cell toxicity leading to Alzheimer's disease.

Transferrin is a 79 kDa glycoprotein found in plasma, human serum, cerebrospinal fluid (CSF), and brain. Recent studies show that transferrin has protective role in abeta aggregation formation. Transferrin has been shown to bind the abeta oligomer preferentially, and prevent abeta oligomers nucleation and hence subsequent aggregation and plaque formation. In addition, transferrin is an iron transport protein that is used for iron homeostasis [3]. Abundant free ions have been also

known to induce toxic forms of abeta, promoting aggregation. Hence transferrin has major inhibitory role in preventing abeta toxicity. It was shown that any failure and malfunction in iron homeostasis will lead to neurotoxicity which will in turn lead to AD [3-6].

Another CSF protein that exhibit abeta protein aggregation inhibitory effects is human serum albumin. When AD patients treated with plasma replacements containing abeta-free human serum albumin, decreased abeta accumulations in the brain and cognitive impairment was observed. Further, HSA promoted neuronal survival by preventing amyloid entering into brain neurons. HSA binds to 90-95% of Aβ in blood plasma [7, 8]. The concentration of HSA was found to be higher in blood plasma (640μM) [9] compared to low concentration of HSA (3 μM) in CSF [10]. This proves why Aβ plaques are found in the exterior space of the brain [11]. Normally, Aβ clearance mechanism is formed when Aβ bounds to albumin and then transported from brain into blood along Aβ concentration gradient [12, 13]. Also, it was shown that Aβ-albumin complexes decreased in blood, which showed that Aβ transport from brain to blood also decreased in AD patients [14].

In this chapter we study how HSA and transferrin proteins decrease Aβ (1-42) aggregation under different conditions. Free proteins or proteins coated on poly (lactic-co-glycolic acid) (PLGA) nanoparticles to enhance their specificity in targeting Aβ, and to cross blood brain barrier (BBB) easily were used for the study. The aggregation studies were characterized using thioflavin T (ThT), atomic force microscopy (AFM), confocal microscope, and dynamic light scattering (DLS).

6.2 Materials and Methods

6.2.1 Materials

Abeta peptide (1-42), ultra-pure (1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) treated), was purchased from rPeptide. Transferrin (77-81 kDa), and Human Serum Albumin (66.4 kDa) were purchased from Sigma Aldrich.

6.2.2 Nanoparticles preparation

Standard emulsion method was used to prepare nanoparticles. Briefly, PLGA polymer was dissolved in ethyl acetate and emulsified with 1% PVA using probe sonicator. The emulsion was then stirred overnight until the solvent is evaporated. The particles were then centrifuged washed, and finally reconstituted in water and stored at 4°C until it is used. The particles were then surface coated with t Tf or HSA by using EDC/ NHS chemistry, using standard protocol. The amount of protein conjugated was then quantified using protein assay. Three different coating densities of Tf and HSA was achieved. For the Tf coated particles, the final concentrations of Tf in 5 mg/ml of PLGA particles were found to be of 3mg/ml (25 uM), or 30 ug/ml (250 nM) or 60 ug/ml (500 nM). For the HSA coated particles, the final concentrations of HSA in 5 mg/ml of PLGA particles were found to be of 2.5 mg/ml (25 uM), or 25 ug/ml (250 nM) or 50 ug/ml (500 nM).

6.2.3 ThT

A 3.2 mg/ml concentration of ThT was dissolved in Tris buffer (pH 8). For free Tf and HSA inhibitory studies, 45 µM of Aβ (1-42) was aggregated with 5 or 10 µM of Tf or HSA for 24 hours. For nanoparticle coated Tf samples, 20 µl of 19 µM Aβ (1-42) was added to 5 µl of PBS buffer for 2 hours. Then, NP (water was added instead of proteins) and NP/ Tf and NP/HSA with Tf or HSA concentrations of (5 µM, 50 nM, 100 nM) or 5 µM of free Tf or HSA were

added and aggregated for 24 hours at 37°C. Three independent experiments were performed, and ThT fluorescence was measured using Spectramax M3 spectrometer at 440/482 nm fluorescence endpoint.

6.2.4 DLS

The size of the aggregated samples was measured using a Malven zetasizer instrument in the lab. The same sample conditions used for the ThT measurements were used for the DLS study. Also, the size of NP, NP/ Tf, and NP/HSA, were measured for NP size characterization. Three independent size measurement experiments were performed.

6.2.5 AFM

HITACHI AFM 5100N in lab was used. The AFM tip (Nanosensors, CA) was used for the imaging. Images were performed in tapping mode, with force constant is 0.5-9.5 N/m, resonance frequency 45-115 KHz, average length is 225 µm, and the average width is 28 µm. Some of the ThT samples were sonicated and 1 µl of the solutions were spotted on mica. Then, the samples were imaged after drying. Also, nanoparticles were imaged using the tapping mode, and the size of the particles was determined. All sample conditions were imaged three times at a scan area of 6x6 µm.

6.2.6 Nanoparticle binding assay

Samples were prepared to similar protocol used for the ThT measurements. Then the samples were centrifuged, and the supernatant were imaged by confocal microscope, to assess the abeta aggregation in solution. Confocal images were performed with Leica confocal microscope in the department (funded by NSF-MRI grant to Dr. Gargi Ghosh (PI)).

6.3 Results and discussion

First, we tested the ability of transferrin and human serum albumin on inhibiting abeta peptide aggregation. For that, we tested the aggregation of abeta peptide with and without t Tf and HSA by thioflavin assay. As can be seen from the tht measurements in Figure 6.1, the CSF proteins were able to inhibit the aggregation of abeta peptide. We further confirmed this by testing the aggregation size by DLS. In the presence of Tf or HSA, the aggregation was significantly reduced (Figure 6.2).
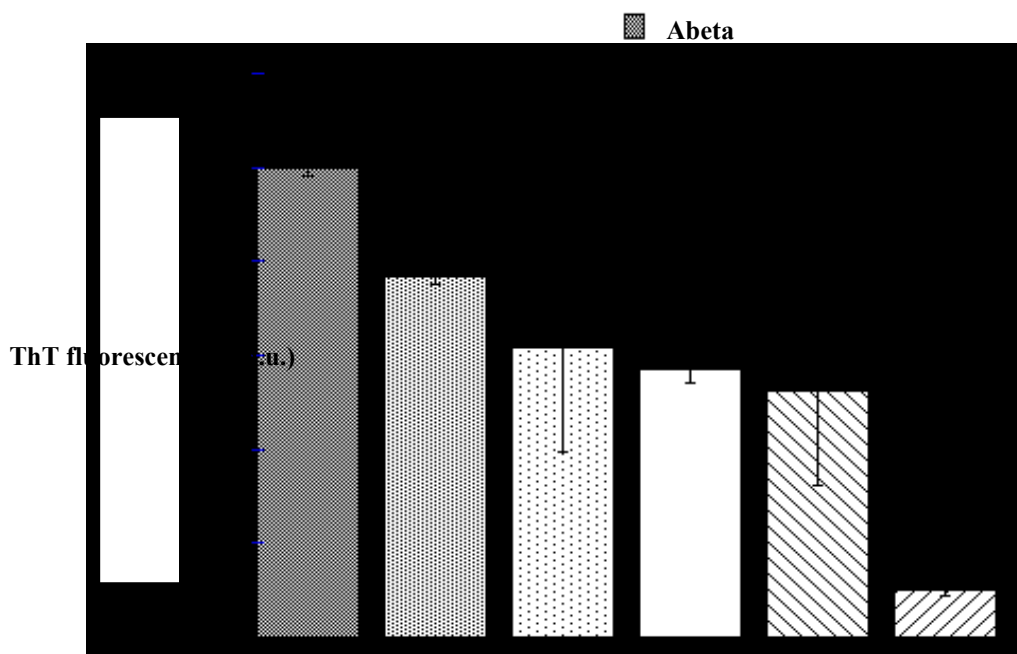

Figure 6.1. Thioflavin-T fluorescence measurements of abeta peptide (45 μM) aggregation with and without Tf and HSA. The ThT measurements indicate the inhibitory potential of Tf and HSA.
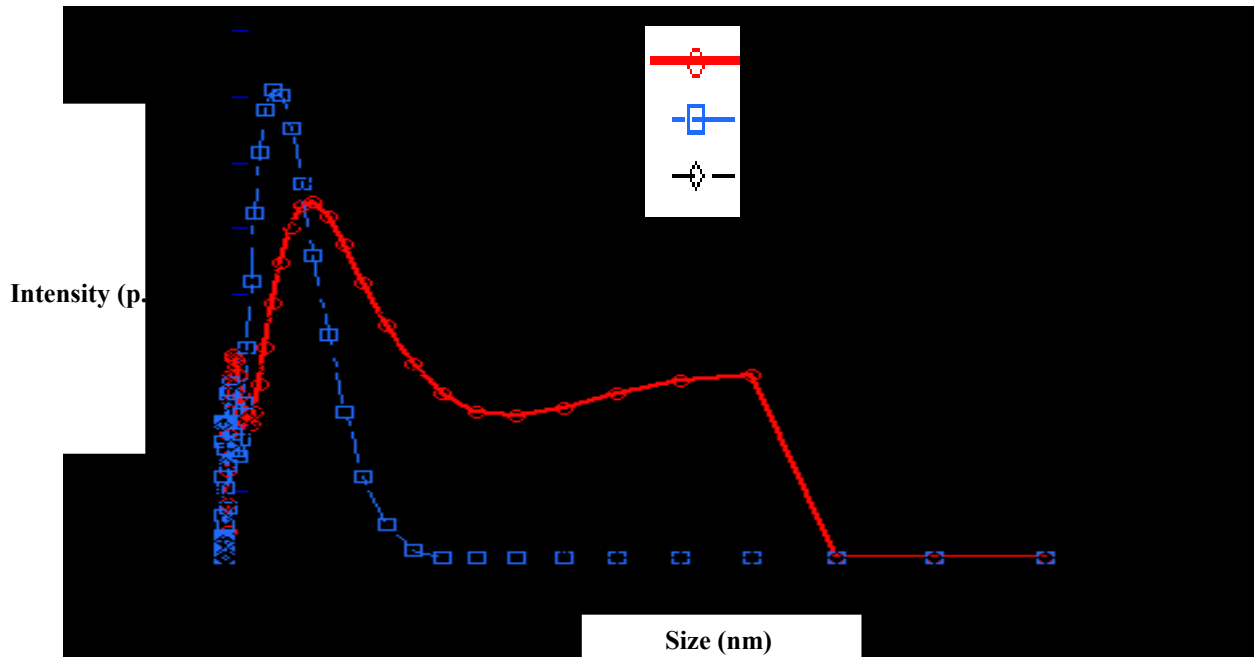
Figure 6.2. DLS measurements show the size of abeta peptide (45 μM) aggregation with and without Tf and HSA (10 μM) concentrations.

We then used nanotechnology to deliver Tf and HSA to test their ability to capture abeta in solution and to prevent aggregation. Nanoparticles have the ability to deliver the proteins with increased therapeutic efficacy compared to systemic delivery. Further, they can increase the circulation of the proteins in the blood. Moreover, the technology could be applied to capture abeta in the blood or in the brain parenchyma to reduce the monomeric and oligomeric abeta, and to prevent aggregation. First, we produced PLGA nanoparticles with around 200 nm using standard emulsion technique, and subsequently surface coated with and HSA proteins. We calculated the amount of the coating by protein assay. The nanoparticle size and morphology were characterized by TEM and DLS. Particles around 200 nm size were obtained. The coating of Tf and HSA did not significantly increase the size of the particles (Figure 6.3).
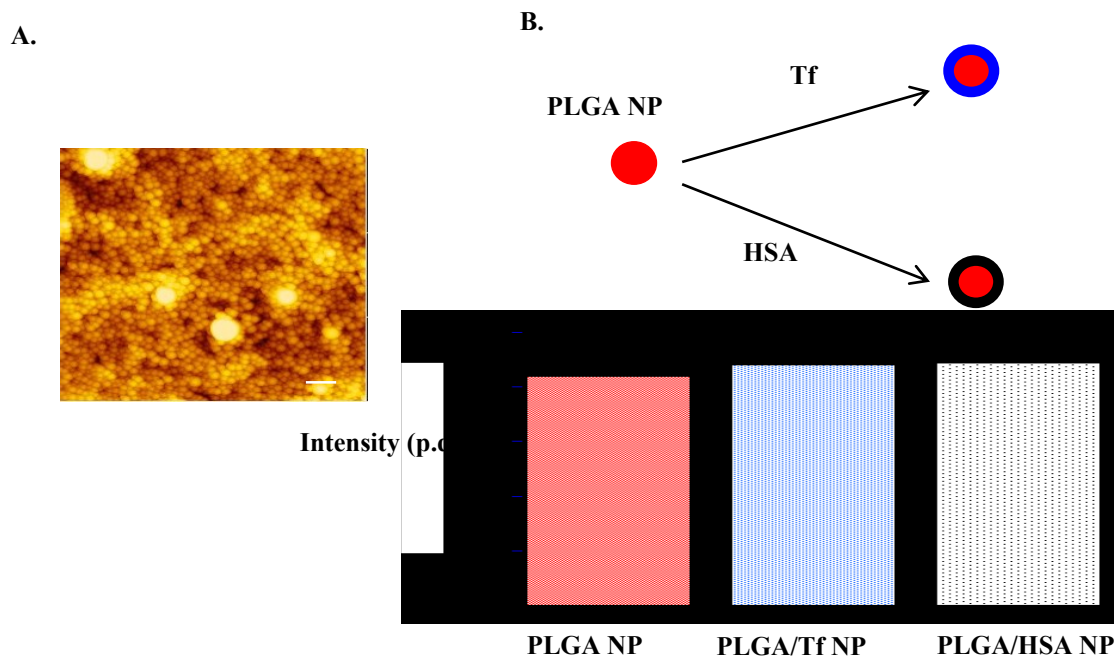
Figure 6.3. Nanoparticle characterization (A) PLGA nanoparticle morphology observed by AFM. (B) Surface coating of PLGA nanoparticles with Tf or HSA, and size characterization by DLS. Scale bar 500nm.

We then tested the potential of the particles in abeta aggregation inhibition by thioflavin-T. We tested different Tf and HSA coating amounts on abeta aggregation. From the studies, we found that when coated with 100 nM of Tf and HSA, the aggregation of the abeta is inhibited (Figure 6.4). The aggregation inhibition was further studied with confocal microscopy, and AFM. Abeta aggregation was labeled with ThT, and the amount of abeta in the supernatant after 24 hours' is observed by confocal microscopy. As can be seen from the images, the abeta alone samples increase the amount of ThT fluorescence in the supernatant compared to coated nanoparticles, indicating most of the abeta could be bound to Tf /HSA coated nanoparticles (Figure 6.5). The supernatant of nanoparticles showed less aggregated size, indicating inhibition. The AFM images corroborate the findings, where more bound abeta aggregates in the coated nanoparticles compared to nanoparticles alone where scattered aggregation can be observed (Figure 6.6).
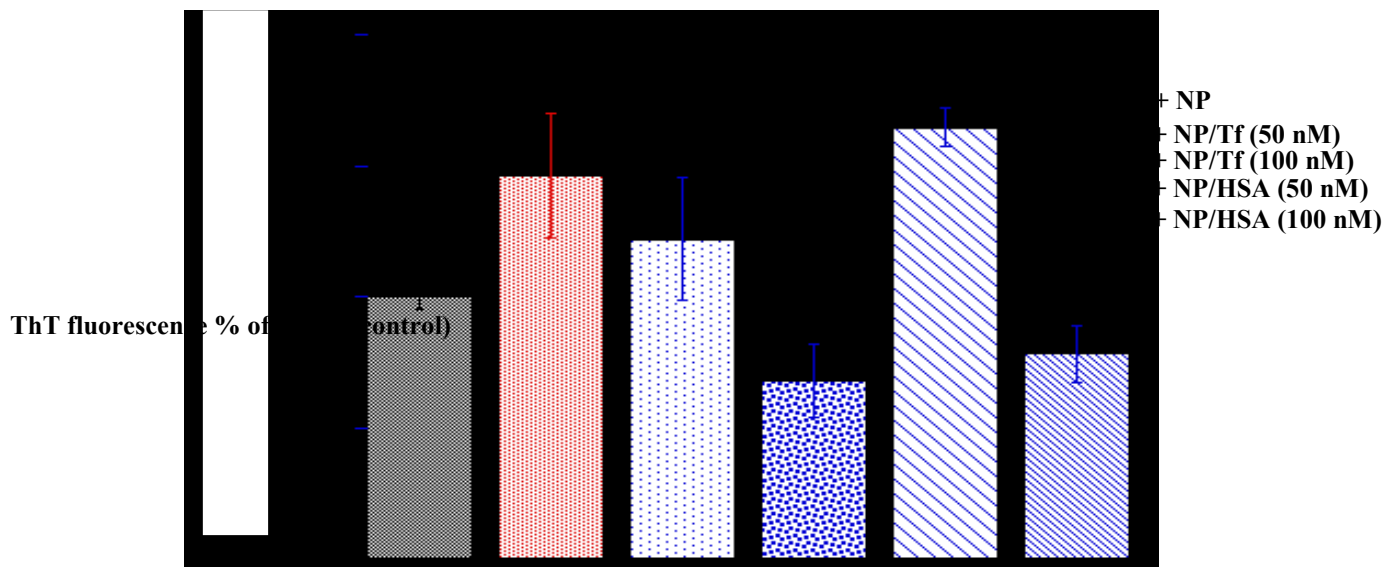
Figure 6.4. Thioflavin-T fluorescence measurements of abeta peptide (15uM) aggregation with and without Tf and HSA coated nanoparticles.

| Abeta | Abeta-NP | Abeta-NP/Tf | Abeta-NP/HSA |
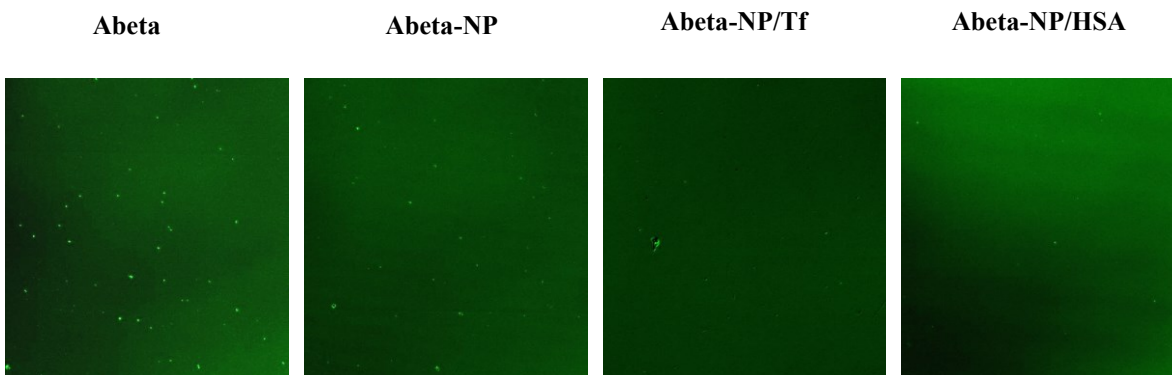
Figure 6.5. Confocal images of the supernatant of the abeta peptide aggregation with and without Tf and HSA coated NP.

| Abeta | Abeta-NP | Abeta-NP/Tf | Abeta-NP/HSA |
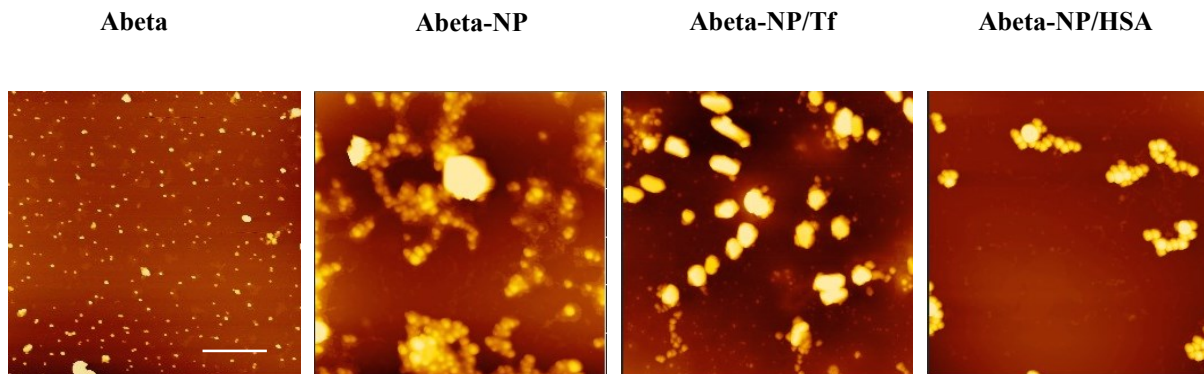
Figure 6.6. AFM images of abeta peptide aggregation with and without Tf and HSA coated nanoparticles ted nanoparticles. Scale bar 500 nm.

6.4 Conclusion

In this chapter, we show that transferrin, and human serum albumin coated nanoparticles bind abeta, and significantly prevented abeta aggregation. Further, the amount of abeta in solution is significantly reduced in nanoparticles coated samples. Hence, nanoformulated cerebral proteins could be applied to reduce the amount of abeta in AD patients. Further, they could be further modified to successfully cross the blood barrier and capture the unwanted abeta in the brain, and could have potential as a nanotherapeutic approach for treating Alzheimer's disease.

6.5 Reference

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Chapter 7: Conclusion

7.1. General Conclusions

Although several studies have been reported on understanding the biomolecule cues associated with Alzheimer's disease, the role of extracellular biomolecules in Alzheimer's causing peptides aggregation and potential novel drug targets studied in this thesis, hasn't been explored in detail. In this thesis study, the role of glycosaminoglycans, extracellular enzymes, and cerebral proteins in Alzheimer's causing peptides abeta peptide, and tau peptide aggregation has been studied in detail. Thioflavin-T, TEM, AFM, DLS, and cellular characterization methods were used to study the aggregation process. The thesis study shows that glycosaminoglycans promote the aggregation and toxicity of tau peptides, and the effects could be mitigated by the application of cationic polymers, polyethyleneimine and polyarginine. Extracellular enzymes transglutaminase and lysyl oxidase on the other hand, promoted the aggregation and oxidative stress of abeta peptides, and small molecule osmolytes l-proline and betaine show potential in inhibiting the aggregation of the abeta peptides. In addition, the thesis study reveals that the abeta aggregation process could be inhibited by cerebral proteins transferrin, and human serum albumin nanoformulations.

7.2. Future directions

This thesis studies the role of biomolecule cues and their inhibition by potential drug candidates in detail. However, the mechanism of action of the inhibitory drugs on peptide aggregation in the presence of biomolecules has yet to be studied. Further, the major challenge in Alzheimer's drug

delivery is the blood brain barrier (BBB). Hence, the potential drug molecules identified in this thesis and in the literature could be nanoformulated to cross the BBB effectively, and target the affected region for effective drug delivery. The detailed characterization, and mechanism of action of the nanoformulation needs to be studied in vitro. Finally, the effective nanoformulations could be tested in vivo, in Alzheimer's disease model to realize its effectiveness.