

# **Effects of Chemical and Physical Perturbants in Aggregation and Nanoscale Assembly of Peptides**

*Thesis submitted in partial fulfilment of the requirements  
for the award of the degree of*

**Doctor of Philosophy**

in

**Biosciences and Bioengineering**

by

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*Under the supervision of*

**Dr. Vibin Ramakrishnan**



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**APRIL, 2019**





***Dedicated to***

***My Family***

It is from their love, care and support, I derive my strengths...





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*“No one can whistle a symphony. It takes a whole orchestra to play it.”*

– H. E. Luccock

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April 8, 2019

Gaurav Pandey





# Declaration

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I certify that

- The work contained in this thesis is original and has been done by myself and under the general supervision of my supervisor(s).
- The work reported herein has not been submitted to any other Institute for any degree or diploma.
- Whenever I have used materials (concepts, ideas, text, expressions, data, graphs, diagrams, theoretical analysis, results, etc.) from other sources, I have given due credit by citing them in the text of the thesis and giving their details in the references. Elaborate sentences used verbatim from published work have been clearly identified and quoted.
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April 8, 2019

  
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## Certificate

This is to certify that this thesis entitled "**Effects of Chemical and Physical Perturbants in Aggregation and Nanoscale Assembly of Peptides**" submitted by **Gaurav Pandey**, in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy, to the Indian Institute of Technology Guwahati, Assam, India, is a record of the bonafide research work carried out by him under my guidance and supervision at the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Assam, India. To the best of my knowledge, no part of the work reported in this thesis has been presented for the award of any degree at any other institution.

Date: April 8, 2019

Place: Guwahati

Dr. Vibin Ramakrishnan

(Thesis Supervisor)





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## List of Abbreviations

|              |                                                                           |
|--------------|---------------------------------------------------------------------------|
| <b>AD</b>    | Alzheimer's disease                                                       |
| <b>AFM</b>   | Atomic force microscopy                                                   |
| <b>BBB</b>   | Blood-brain barrier                                                       |
| <b>CD</b>    | Circular dichroism                                                        |
| <b>CPP</b>   | Cell-penetrating peptide                                                  |
| <b>DIPEA</b> | N,N-diisopropylethylamine                                                 |
| <b>DMEM</b>  | Dulbecco's modified Eagle's medium                                        |
| <b>DMF</b>   | Dimethylformamide                                                         |
| <b>DLS</b>   | Dynamic light scattering                                                  |
| <b>EDTA</b>  | Ethylenediaminetetraacetic acid                                           |
| <b>EF</b>    | Electric field                                                            |
| <b>FBS</b>   | Fetal bovine serum                                                        |
| <b>FITC</b>  | Fluorescein isothiocyanate                                                |
| <b>Fmoc</b>  | Fluorenylmethyloxycarbonyl chloride                                       |
| <b>FESEM</b> | Field emission scanning electron microscopy                               |
| <b>FETEM</b> | Field emission transmission electron microscopy                           |
| <b>FTIR</b>  | Fourier-transform infrared spectroscopy                                   |
| <b>HBTU</b>  | N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-yl)uranium hexafluorophosphate |
| <b>HCCA</b>  | $\alpha$ -Cyano-4-hydroxycinnamic acid                                    |
| <b>HFIP</b>  | 1,1,1,3,3,3-Hexafluoro-2-propanol                                         |
| <b>HOBt</b>  | 1-hydroxybenzotriazole hydrate                                            |

## List of Abbreviations

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|                  |                                                              |
|------------------|--------------------------------------------------------------|
| <b>HPLC</b>      | High performance liquid chromatography                       |
| <b>MALDI-TOF</b> | Matrix-assisted laser desorption ionization-time of flight   |
| <b>MTT</b>       | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| <b>NFTs</b>      | Neurofibrillary tangles                                      |
| <b>PHFs</b>      | Paired helical filaments                                     |
| <b>SAXS</b>      | Small-angle X-ray scattering                                 |
| <b>TFA</b>       | Trifluoroacetic acid                                         |
| <b>ThT</b>       | Thioflavin T                                                 |
| <b>TFE</b>       | Trifluoroethanol                                             |
| <b>XRD</b>       | X-ray diffraction                                            |

# Abstract

Interactions between proteins in a transient manner are central to many biological processes that happen with high precision in biological systems. Self-assembly of peptides or proteins is a spontaneous process resulting in very stable ordered structures. In biology, the concept of self-assembly was popularized by cross-beta spines of amyloid fibrils. From instigating dementia in Alzheimer's disease (AD) to causing type 2 diabetes (T2D), amyloid diseases are some of the most progressively debilitating malady in human system. Currently, there are no known therapies for averting amyloid diseases, and so far, attempts to discern anti-amyloid cure have been mostly unproductive. Modulating or inhibiting the amyloid aggregation may be a useful therapeutic intervention to overcome the multifaceted pathology allied with amyloid diseases. The cross- $\beta$  structure of amyloids consists of insoluble fibrous  $\beta$ -sheet aggregates, and is considered thermodynamically to be the most stable biological structure. Developing an effective way to control the organization of these self-assembling fibrils will not only be of great value to the armamentarium of anti-amyloid therapeutics, but will be useful in harnessing the remarkable stability of amyloid fibrils for fabricating application oriented materials at the molecular level.

The work detailed in this dissertation is an attempt to understand the critical aspects of amyloidogenic-assembly of steric-zipper peptides derived from broader segments of proteopathic peptides/proteins. We will discuss the effects of electric and magnetic fields of varying strengths on the assembly of the smallest self-assembling peptide, diphenylalanine. We have synthesized a series of short steric-zipper peptides, to employ them as model systems in our study. We have examined the consequence of electric field-mediated stress on the aggregation kinetics of reported aggregating peptides and tested their efficacy as non-invasive anti-aggregation therapeutic. We have designed and synthesized over 20 peptide-based perturbants, to target the core-hydrophobic motifs of amyloid- $\beta$  peptide

and tau protein, which are hallmarks of AD pathology. The efficacy of the designed molecular perturbants was investigated on the model peptide systems using several biophysical as well as cell-viability assays. We have also performed a comparative analysis of aggregation kinetics of our model peptides and studied the geometric and energetic rationale for their differential aggregation rates. Further, a prototype design of a futuristic non-invasive therapeutic device based on electric-field mediated modulation of amyloidogenesis has also been proposed. The thesis is divided into six working chapters describing the design and characterization of physical and molecular perturbants of aggregation.



# CHAPTER 1

## INTRODUCTION, RESEARCH DESIGN AND OBJECTIVES

*The call for the development of a cure for amyloid-mediated disorders like Alzheimer's disease has gained momentum in the past two decades. The use of peptides as modulators influencing the self-assembly is a fruitful option due to the various advantages of peptides over other small molecule-based approaches. The biocompatibility, high chemical diversity, high selectivity, low toxicity, good tolerance, high chemical and biological diversity, low accumulation in tissues, the possibility of rational design, and highly developed techniques for analyzing their mode of action, and blood-brain barrier (BBB) permeability, provide peptides an edge over other molecules. Non-invasive therapeutics have also emerged as potential alternatives to conventional molecular methods to combat aggregation. In the course of this study, we will attempt to assess the efficacy of electric field and de novo designed peptides as aggregation modulators.*





## **1.1 INTRODUCTION**

Man's quest to understand life is a fascinating affair – its intricacy and sensitivity are exclusive, and despite ages of human inquisitiveness, many facets remain enigmatic to the human mind. Life is grounded on a fine-tuned molecular organization of an array of biological and biochemical processes. Decoding and comprehending these processes have been closely allied to improvements in the general health status of humanity aided by advancements in molecular medicine, surgery, and diagnostic tools. Diverse levels of these complex processes have historically shaped the purviews of natural sciences viz. physics, chemistry, and biology. The quest to achieve an advanced conception about how the processes on a molecular scale affect the landscape of life has sparked the development of disciplines such as biotechnology, biophysics, biochemistry, and biomedicine. There is no stern differentiation among these disciplines, and their scopes often overlap. This thesis belongs to this broad research zone.

The cell is the basic unit of structure and function in all living organisms. A cell consists of various essential biomolecules which enable its biological functioning. Protein and nucleic acids are two of the most important biomolecules. Nucleic acids function to store and transmit the genetic information through the nucleic acid sequence. Proteins, in contrast, perform a myriad of various functions ranging from serving as a fuel source to enzyme catalysis and immune response [1, 2]. The proteomes of human cells typically encompass between 10,000 and 20,000 different proteins [3]. They comprise of chains of amino acids linked by peptide bonds forming polypeptide chains. The polypeptide chains fold into a three-dimensional structure, through a mechanism secured by another protein known as chaperones. Under abnormal conditions, proteins misfold and self-assemble to form either amorphous aggregates or unbranched, elongated fibrillar structures, referred to as amyloid fibrils [4].

## Chapter 1

### Introduction, Research Design And Objectives

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The significance of protein aggregation and amyloid formation has progressively gained interest in the last two decades, due to the findings showing the products of protein aggregation, the cross- $\beta$  amyloid fibrils are linked to biological dysfunction in diseases like neurodegenerative disorders, type 2 diabetes, and prion diseases. Atomic X-ray derived model of amyloid-related structures have been employed to decipher the features of cross- $\beta$  structures of amyloid fibrils. Cross- $\beta$  structures have a motif called steric zipper, in which the inter-digitation of their side chains tightly binds a pair of  $\beta$  sheets. This steric zipper is supposed to be the core of seeding amyloid by stacking the same units repeatedly [5].

The abnormal protein aggregation triggers more than 50 diseases, collectively known as proteopathies. Alzheimer's disease (AD) is the most prevalent of them all. AD affects millions of people worldwide. Currently, in the absence of any cure, this number is increasing at a breakneck pace. As life expectancy is growing, the proteopathies jointly impose an increasing burden on the aging population. The available FDA approved therapeutic options only provide symptomatic relief. Understanding the causal mechanism of the disease and designing optimal treatment approach is the prime focus of the ongoing research. More than 100 disease-modulating agents encompassing small-molecules, peptides, antibodies, cell, and gene-based therapies are currently in AD therapeutic pipeline [6,7]. Therapeutic inhibition using peptide-based modulators holds much significance for future AD therapy. The previous failure of molecular therapies has also elicited the development of non-invasive treatment alternatives using physical modulators like light, ultrasound, electric/magnetic stimulations and electric field.

The concentration in protein aggregation is, though, not limited to its health repercussions. Many more proteins than those identified to have associated with debilitating diseases were found to possess the ability of fibrillization, suggesting that most, if not all, amino acid sequences might be able to misfold into similar ordered assemblies and aggregation may thus be a generic feature of a polypeptide

chain [8, 9]. This evidence puts protein misfolding into a much broader perspective. Several studies have shown that protein aggregation is not always hazardous and may have a functional role in cellular systems like melanosomes [10, 11]. The self-assembling property of amyloids has inspired the application of its mechanistic principles towards the development of templates to serve as building blocks for a variety of applications [12-14]. One example of such an application is the production of conductive nanowires from genetically engineered yeast prion protein [15, 16].

Studies suggest that the morphological features, as well as the mechanism of poisoning, remain conserved in different amyloidogenic proteins [17]. Such patterns give us hope that, if we design a strategy to perturb the self-assembly process of amyloidogenic peptides, the same approach may in principle be extended to modulate and develop peptide-based self-assemblies for a wide range of nanotechnology applications.

## **1.2 RESEARCH DESIGN**

Design and development of effective modulatory agents targeting the aggregation of disease-associated peptide segments for therapeutic as well as application fronts is the primary goal of this thesis. It is centered around a hybrid approach employing structure-based designed peptides (molecular) along with non-invasive electric field (physical) as perturbants of amyloid aggregation.

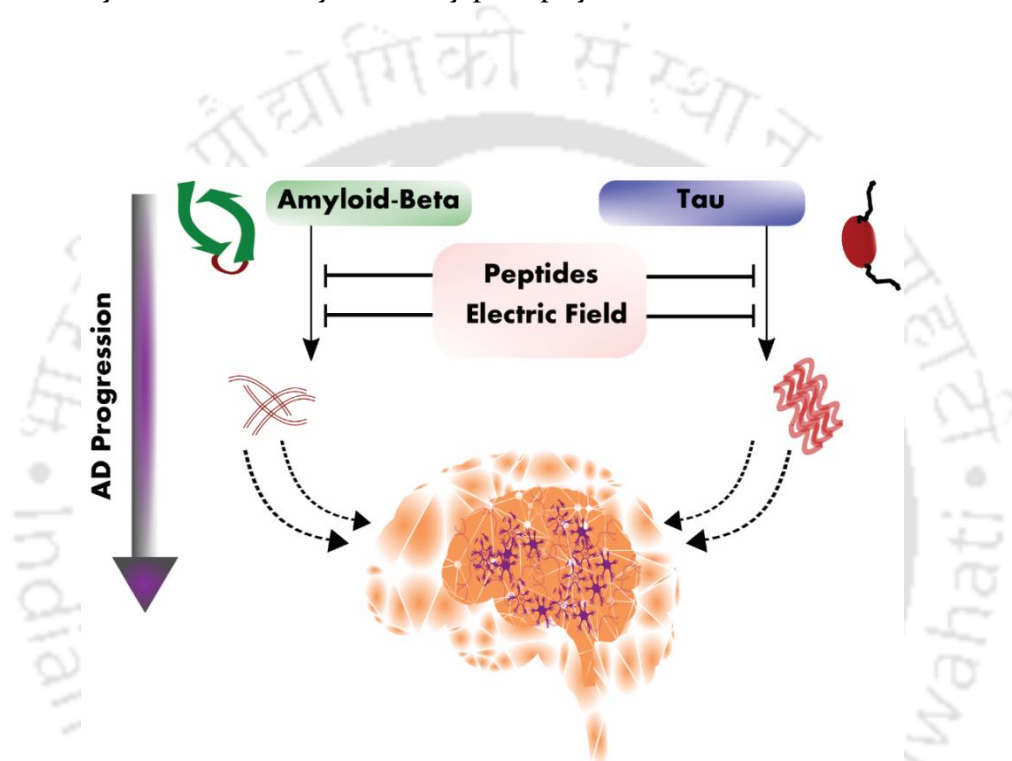
In the subsequent chapters, we will discuss the development of structure-based designed peptides as molecular, and electric/magnetic fields as physical modulators of self-assembly. The peptide-based perturbants were designed to target core-recognition motifs of amyloid- $\beta$  peptide and tau protein, two pathological hallmarks of AD. We fabricated an electric field device to assess the effects of electric field on the AD-associated model peptide systems. Electric field

## Chapter 1

### Introduction, Research Design And Objectives

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experiments aimed to ascertain the efficacy of electric field not only as an anti-amyloid agent having a therapeutic potential but also as self-assembly modulator which may aid in generating application oriented nano-materials without any chemical additives. A series of biophysical techniques were applied to study the self-assembly and its modulation. Cell viability studies were performed to test the reduction in amyloid-induced cytotoxicity post physical or chemical modulation.



**Figure 1.1** A schematic illustration of our hybrid approach.

Further, we propose a design for a future non-invasive therapeutic device employing electric field as a modulator of aggregation. We further investigated the geometric origins of differential aggregation kinetics of a set 9 short steric-zipper peptides. Distinctly different rates of aggregation, observed from the objective analysis of peptides with similar sequence length, directed us to further investigate the specific roles of geometry and chemistry on their self-assembly kinetics. This thesis is divided into six working chapters describing the design and

characterization of physical and molecular perturbants of aggregation, followed by a study on aggregation kinetics of steric zipper peptides and the design of an electric field-based device prototype for amyloid disorders.

### **1.3 THESIS OBJECTIVES**

With the aim to bridge the stated knowledge lacunae, the present study has been designed to address the following objectives:

- (1) Design and fabrication of two laboratory devices to investigate the role of the electric and magnetic fields, as physical modulators of peptide aggregation.
- (2) Evaluate the effects of physical modulators (electric and magnetic fields) on the nanoscale assembly of the shortest self-assembling peptide, diphenylalanine (FF).
- (3) Design, synthesis, and characterization of reported steric zipper aggregating peptides.
- (4) Evaluate the impact of physical modulators on the amyloidogenicity of steric zipper peptides.
- (5) Design, synthesis, and characterization peptide-based molecular perturbants targeting the core recognition motifs of disease-associated amyloidogenic peptides.
- (6) Examine the effects of physical perturbants on the self-assembly of amyloidogenic peptides.
- (7) To study the comparative aggregation kinetics of reported steric zipper peptides and investigate the geometric origins of their differential rates of aggregation.

## Chapter 1

### Introduction, Research Design And Objectives

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## CHAPTER 2

# PROTEIN AGGREGATION DISEASES: INVASIVE AND NON-INVASIVE THERAPEUTICS

*This chapter is intended to provide an overview of protein aggregation diseases and the state of the art in therapeutic options available for treating them, both invasive and non-invasive.*



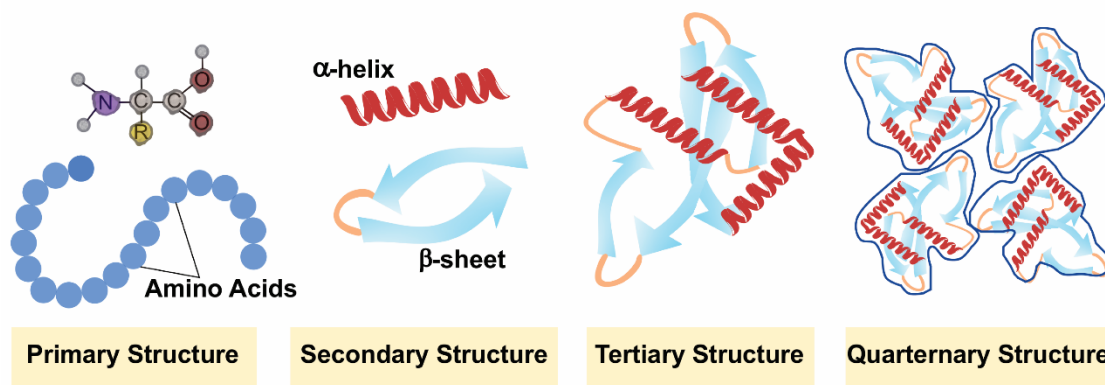


## 2.1 PROTEINS AND PEPTIDES

Proteins are the workhorses of the cell; performing essentially every activity of life. Our body harbors over a million distinct types of proteins, and more than half the dry weight of a cell is constituted by proteins [1]. They serve a broad range of functions, central to the maintenance of life and have always attracted the attention of researchers.

Proteins are heteropolymers of amino acids linked by peptide bonds [2]. Polymers of amino acids with roughly 2-50 amino acids are generally referred to as peptides. The linear chain of amino acids constituting a polypeptide chain is referred to as the primary structure of a protein. These polypeptide chains driven by hydrogen bonding, organize themselves to have varying spatial arrangements like  $\alpha$ -helices or  $\beta$ -sheets, forming the secondary structure of a protein. The secondary structures are driven by non-specific hydrophobic interactions, further arrange themselves to create the functional three-dimensional domain, called as the tertiary structure of a protein. The domains comprising of the tertiary structure associate by ionic, salt bridges, disulfide and other non-covalent interactions like van der Waals forces between their amino acid residues, thus forming the quaternary structure of a protein (Figure 2.1). The structural organization is directed by the amino acid chains constituting the primary structure.

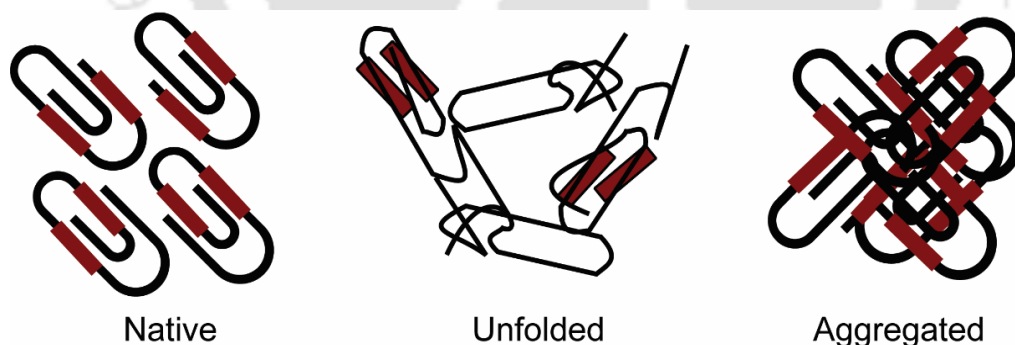
### Structural Organization of Proteins



**Figure 2.1** A schematic representation of four levels of protein structure organization (adapted from reference 2).

## 2.2 PROTEIN FOLDING, MISFOLDING AND AGGREGATION

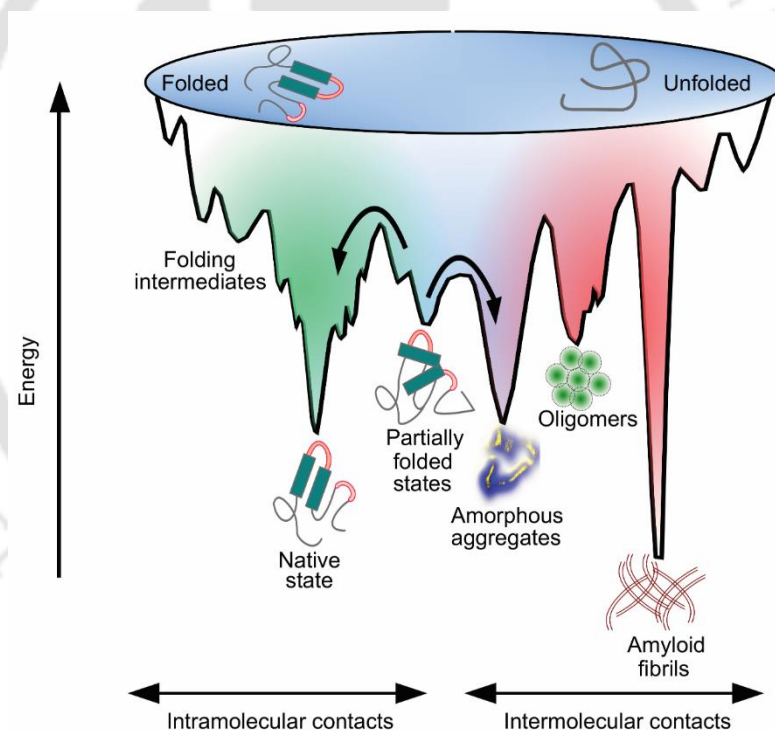
A protein can exist in either folded, unfolded or aggregated state (Figure 2.2). Protein folding is a complex process that involves a polypeptide chain folding into its 3D native state, from an unfolded state under physiological conditions. Due to the possibility of multiple combinations of the 20 amino acids, the question arises how an apparently complex, correctly folded functional structure can be formed within milliseconds. The Levinthal paradox points to this fundamental question stating that given the number of all possible conformations of an unfolded polypeptide chain, it would not be possible for the protein to reach its native state in a physically meaningful time [3]. However, recent studies provide another insight based on energy landscape theory and folding funnel hypothesis [4-11]. The protein folding process allows the protein to reach a thermodynamically stable state, where its free energy decreases and reaches the minimum. Although, during the process, the protein can also be entrapped at an intermediate state by reaching some local energy minima, slowing the folding process [12-17].



**Figure 2.2** A graphical illustration folded, unfolded and aggregated protein.

The plot of free energy vs. the conformational sampling generates the folding energy landscape. The energetically favorable intermolecular interactions, enables the polypeptide chain to sample a series of local minima in a folding funnel,

eventually reaching the global minimal energy conformation (GMEC) [1, 17] (Figure 2.3). Proteins with a large number of amino acids often form non-native conformations (intermediate states) which are energetically favorable, low energy states. This leads to a partially folded/misfolded protein formation [17, 18]. Another model by Fersht known as the nucleation-condensation model proposes that during the transition state of the folding process, there exists a folding nucleus formed by the hydrophobic residues [10, 19, 20]. The nucleation is followed by the simultaneous formation of the secondary and tertiary structures, and the entire protein shapes up around the nucleus to give rise to the native structure.



**Figure 2.3** A graphical illustration energy landscape for protein folding and aggregation (adapted from reference 11).

In a cellular environment, protein synthesis occurs in the ribosome. Majority of the proteins fold with the assistance of molecular chaperones inside the cytoplasmic and endoplasmic reticulum [21, 22]. The molecular chaperones

prevent the non-specific aggregation by binding to the unfolded or partially folded protein [18, 22-25]. Cytosolic enzymes and proteasomes generally degrade the misfolded proteins [26, 27].

The instigation of protein misfolding has been attributed to diverse factors including:

- (a) Events resulting in alterations of the primary protein sequence, such as errors in translation [28] or transcription [29], and genetic mutations [30].
- (b) Environmental factors like changes in pH or temperature, triggering the destabilization of native protein structure and causing it to misfold fold or unfold [31-35].

Misfolding of a protein leads to its malfunction and causes diseased conditions [36-38]. In a partially folded or misfolded protein, the hydrophobic or the buried amino acid residues are exposed, resulting in the formation of amorphous aggregates. These aggregates have a propensity to form  $\beta$ -sheet rich oligomers and amyloid fibrils [39-43]. Some of the diseases associated with protein misfolding and aggregation include diseases like Alzheimer's, Parkinson's, Huntington's, tauopathies, type II diabetes, etc. [44].

The formation of amyloid aggregates is a three-step process following a nucleation-dependent pathway [45] (Figure 2.4).

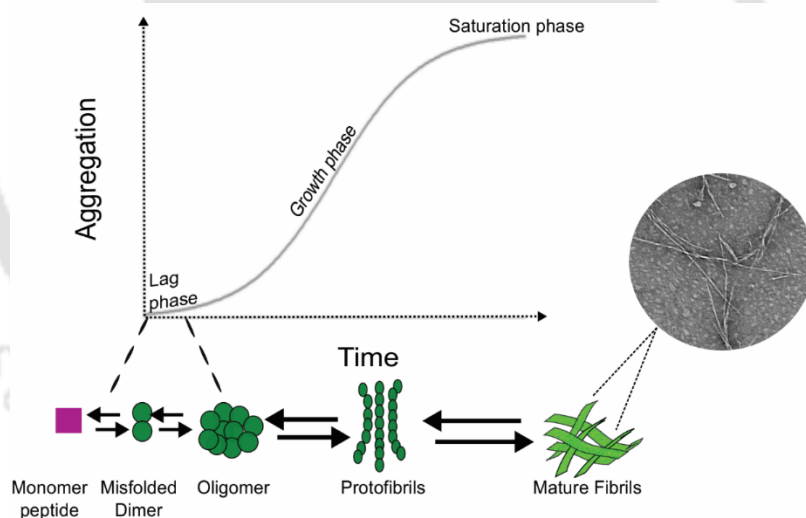
It involves:

- 1) Lag phase: Also known as the nucleation phase in which pre-fibrillar oligomers are formed. Lag phase is the slowest step and is considered as the rate-limiting step.
- 2) Elongation or growth phase: Here the oligomeric nuclei formed to act as templates leading to fast fibrillar growth and thereby formation of mature

insoluble  $\beta$ -sheet rich amyloid fibrils. These amyloids are found as extracellular deposits in various neurodegenerative diseases like Alzheimer's.

3) Stationary phase: When maximum fibril growth is reached a plateau, is achieved at the steady state.

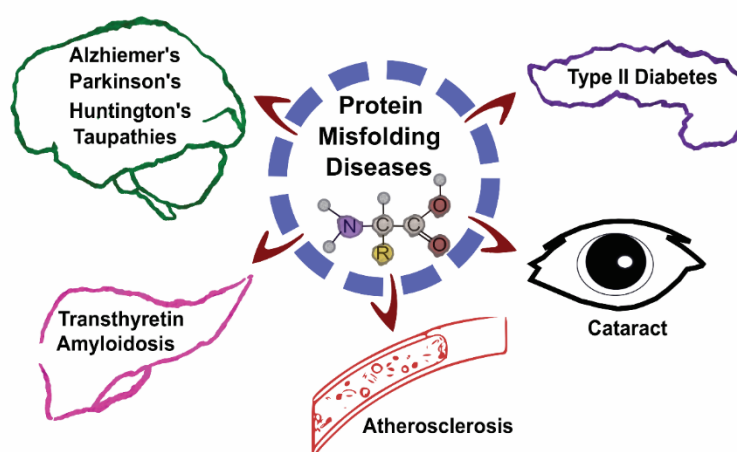
Studies show that, it is the prefibrillar aggregates and not the mature amyloid fibers that are toxic to the cell [46, 47]. However, the underlying mechanism of amyloid toxicity is still not completely known and remains an area of research interest [48, 49]. Although protein folding and aggregation have distinct processes, it has been suggested that for a given polypeptide chain the two events compete with one another, depending on the environmental conditions. So at a time, one is favored over the other [50].



**Figure 2.4** Schematic representation illustrating the kinetics of amyloid formation (adapted from reference 45).

### 2.3 PROTEIN AGGREGATION DISEASES

Even though all the information required for proteins to fold is encrypted in their amino acid composition, there are numerous other factors involved *in vivo*. Nascent polypeptide chains, present in a cellular environment, packed with surrounding interacting proteins, confront a formidable task in finding the precise interactions that produce a folded and a functional protein. Though the human body is equipped with an innate defense which averts the native proteins from misfolding, yet a substantial number of diseases owe their origin to the abnormal folding/aggregation of proteins [1, 51-57]. The abnormally folded proteins either lose their normal physiological function or become toxic or both [58]. Several misfolded proteins malfunction, and thereby result in the disease condition, due to loss of their function. They self-assemble to form toxic species, disrupt the cells, and cause many diseases, due to their toxic gain of function [58] (Figure 2.5).



**Figure 2.5** A graphical illustration of protein misfolding diseases with their target organs.

Under disease conditions, proteins and peptides undergo structural transitions from their native state into ordered  $\beta$ -sheet conformation, having high propensity to self-assemble into insoluble deposits. These insoluble self-assemblies are termed as “amyloids.” The term “amyloid” was coined by the physician and



## Chapter 2

### Protein Aggregation: Invasive and Non-Invasive Therapeutics

biologist Rudolph Virchow, in the year 1854, to denote a macroscopic cerebral tissue abnormality, that showed positive iodine staining reaction [59], commonly exhibited by starch, (*amylon* in Greek, *amylum* in Latin). Some of the diseases associated with protein misfolding are listed in Table 2.1.

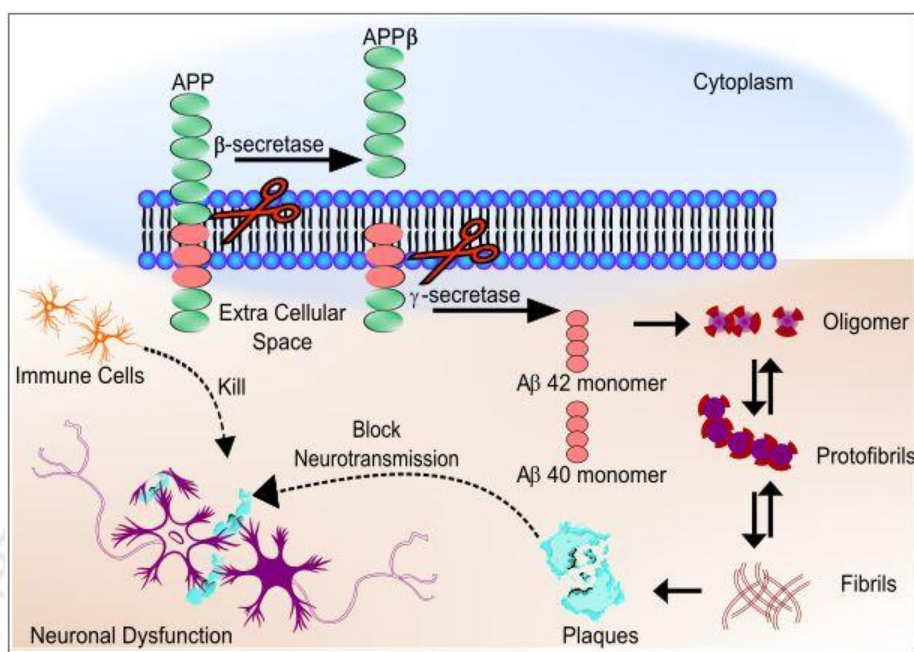
**Table 2.1** List of protein aggregation diseases and the misfolded proteins involved in the disease conditions [60, 61].

| Disease                           | Aggregation protein or peptide              |
|-----------------------------------|---------------------------------------------|
| Hypercholesterolemia              | Low-density lipoprotein receptor            |
| Cystic fibrosis                   | Cystic fibrosis trans membrane regulator    |
| Phenylketonuria                   | Phenylalanine hydroxylase                   |
| Amyotrophic lateral sclerosis     | Superoxide dismutase 1                      |
| Huntington's disease              | Huntington                                  |
| Marfan syndrome                   | Fibrillin                                   |
| Osteogenesis imperfecta           | Procollagen                                 |
| Sickle cell anaemia               | Haemoglobin                                 |
| $\alpha$ 1-Antitrypsin deficiency | $\alpha$ 1-Antitrypsin                      |
| Tay-Sachs disease                 | $\beta$ -Hexosaminidase                     |
| Scurvy                            | Collagen                                    |
| Alzheimer's disease               | Amyloid $\beta$ -peptide/Tau                |
| Parkinson's disease               | $\alpha$ -Synuclein                         |
| Spongiform encephalopathies       | Prion protein                               |
| Familial amyloidoses              | Transthyretin/Lysozyme                      |
| Retinitis pigmentosa              | Rhodopsin                                   |
| Cataracts                         | Crystallins                                 |
| Amyloid light chain amyloidosis   | Immunoglobulin light chain or its fragments |
| Cancer                            | P53                                         |
| Type 2 diabetes mellitus          | Islet Amyloid PolyPeptide (IAPP)            |
| Dialysis-related amyloidosis      | $\beta$ 2-microglobulin                     |
| HIV infection                     | Semen-derived amyloidogenic peptides        |

Alzheimer's disease (AD) is the most common of all aggregation-mediated disorders and affects around 50 million people worldwide [62]. This number is expected to triple by 2030 in the absence of any cure. AD is characterized by the presence of dense, insoluble deposits of amyloid-beta ( $A\beta$ ) peptides outside and around neurons. These deposits often referred to as "plaques," are a result of the abnormal aggregation of  $A\beta$  peptide into toxic aggregates. Although the exact mechanism of the disease is still poorly understood, several hypothesis have been proposed to explain this disease [63], from various contributing factors. The most accepted of them are  $A\beta$  or the amyloid cascade hypothesis [64] and the tau hypothesis [65, 66].

The amyloid cascade hypothesis asserts that amyloid plaques formed by  $A\beta$  peptide aggregation are a result of abnormal proteolytic cleavage of a transmembrane glycoprotein in the central nervous system, known as the amyloid precursor protein (APP). Enzyme  $\beta$ -secretase first splits the APP between Met-671 and Asp-672, followed by further cleaving by  $\gamma$ -secretase to yield  $A\beta_{(1-40)}$  and  $A\beta_{(1-42)}$  segments in a 9:1 ratio [67-69]. The  $A\beta_{(1-40)}$  and  $A\beta_{(1-42)}$  peptides subsequently aggregate to form toxic oligomers, which further assemble to form protofibrils and fibrils (Figure 2.6). The toxic  $A\beta$  aggregates interact with neurons and glial cells causing the activation of mitochondrial dysfunction and increased oxidative stress [70]. The plaques formed from  $A\beta$  fibrillar aggregates block the communication between the neurons, impeding the synaptic signaling, which induces neuronal apoptosis and immune cell-mediated cell death. The loss neurons result in impairment of memory and cognition.

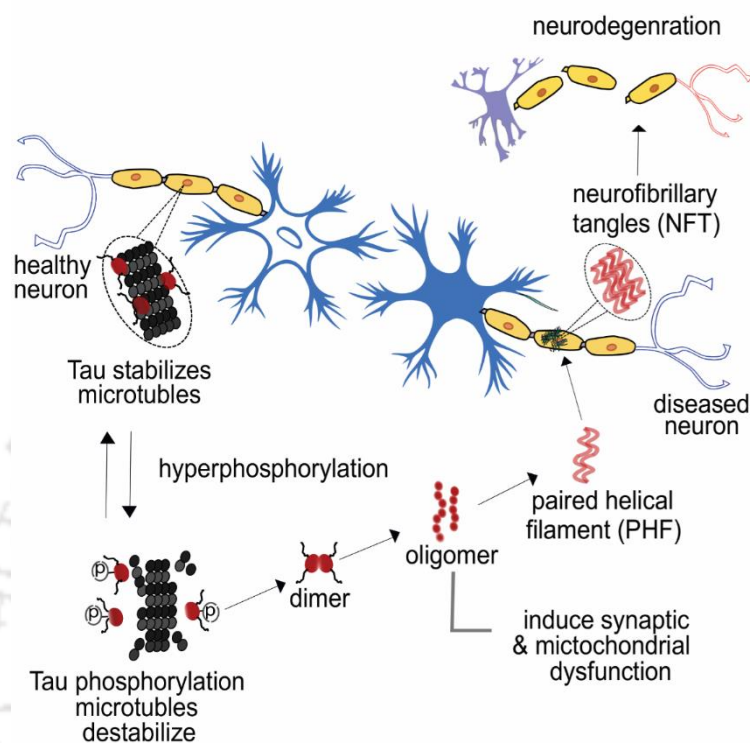




**Figure 2.6** A schematic illustration of the amyloid cascade hypothesis based on amyloidogenic pathway (adapted from reference 70).

The tau hypothesis postulates that abnormal hyperphosphorylation of a microtubule-associated protein tau (MAPT), causes it to aggregate into paired helical filaments (PHFs) and neurofibrillary tangles (NFTs). MAPT is a normal condition, involved in the stabilization of microtubules [65, 71]. As per this hypothesis, the aggregated tau pathology precedes the A $\beta$  aggregation and is the principal cause of neurodegeneration in AD (Figure 2.7). Although recent reports have stressed upon the role of tau pathology to have better correlation to loss of cognition and memory impairment in AD [71-73], yet several studies persist in backing the central role of A $\beta$  in AD [74, 75] and regard that the changes in Tau are a consequence of A $\beta$  pathology [76, 77]. Moreover, the role of hyperphosphorylation, driving tau aggregation has also been opposed [77]. The

debate is still inconclusive whether tau or A $\beta$  is the primary causative reason to AD, and the discussion is still open.



**Figure 2.7** A schematic illustration of tau hypothesis directed neurodegeneration in Alzheimer's disease (adapted from reference 66).

In the absence of complete understanding of the mechanism, protein misfolding diseases like Alzheimer's continue to proliferate and remain as the leading cause of death which cannot be prevented, cured or even slowed. With millions of people already suffering, a conclusive understanding of the disease mechanism and the development of an effective therapy is important.

**2.4 MOLECULAR-BASED THERAPIES FOR ALZHEIMER'S DISEASE**

The accumulation of amyloid fibrils in the human body has been associated with many debilitating diseases, consequently making this area of research timely and significant.

**2.4.1 Disease Diagnosis**

The early diagnostic assessment of the disease will assist in predicting the onset of disease and also aid in developing preventive therapies. In addition to primary physical examination by a clinician for assessing the overall neurological health of the patient, the diagnostic strategies under study include, (a) identification of potential disease biomarkers like blood [78] and cerebrospinal fluid [79], (b) brain imaging using positron emission tomography scanning, magnetic resonance imaging (MRI), and single photon emission computed tomography (SPECT) [78-81].

**2.4.2 Available Drugs**

Currently, there are no drugs available to cure, stop or slow the progression of AD. The existing drug and non-drug options only provide symptomatic relief to AD patients [82-84]. U.S. Food and Drug Administration (FDA) has classified the treatment regimen for AD in the following two categories [85-87]:

- (a) Acetylcholinesterase inhibitors, which include drugs namely, rivastigmine, galantamine, tacrine, and donepezil. As the name says, these drugs inhibit acetylcholinesterase and a resulting increased concentration of a neurotransmitter acetylcholine, which in turn facilitates better communication between nerve cells [87-89]. The common side-effects of these drugs include nausea, vomiting, increased frequency of bowel movements and loss of appetite [89, 90].

(b) N-methyl-D-aspartate receptor (NMDA) receptor antagonist, known as memantine. Memantine is employed in cases of moderate-to-severe AD. The drug functions by controlling the activity of glutamate, a neurotransmitter neuronal communication and information processing. Glutamate plays a crucial role in learning and memory by activating the NMDA receptors to allow a precise amount of calcium into a neuron, which aids in information processing and storage [91, 92]. Surplus NMDA receptor activation results in excess calcium into the neurons, which causes the death of the nerve cells [93]. The side effects of these drugs include constipation, headache, dizziness, and confusion [94].

The combination of (a) and (b) known by its commercial name, namzaric [95] is the latest FDA approved drug. While this drug has helped in improving cognition, it has also shown severe adverse side-effects like nausea, vomiting, loss of appetite, slow heartbeat, seizures, and acidity [96, 97].

### **2.4.3 Therapeutic methods under development**

Scientists have identified various novel therapeutic strategies for AD that focus primarily on lowering or eliminating the amyloid oligomers. Approaches currently under development are the following:

#### **2.4.3.1 Methods targeting Amyloid- $\beta$ peptide**

##### **Methods targeting Amyloid- $\beta$ peptide by averting $A\beta$ formation**

Following approaches have been proposed to reduce or restrict the production of  $A\beta$ , the principal malefactor in AD (Figure 2.8).

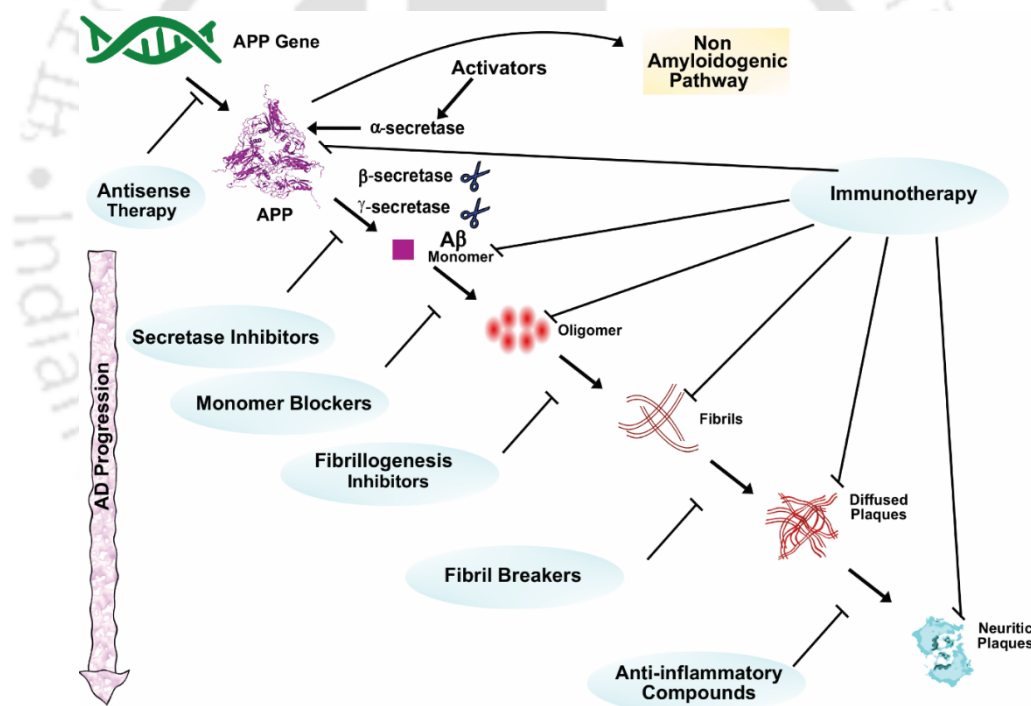
##### *(a) Inhibition of gamma ( $\gamma$ )-secretase or $\beta$ -secretase*

AD is characterized by toxic  $A\beta$  peptides generated by  $\beta$ - and  $\gamma$ -secretase-mediated abnormal cleavage of the amyloid precursor protein (APP).

Inhibiting the activity of these proteolytic enzymes can potentially avert the AD pathogenesis. A number of  $\gamma$  and  $\beta$ -secretase inhibitors have been developed to block these proteases [98-102].

*(b) Activation of alpha ( $\alpha$ ) secretase*

Reverting APP processing towards non-amyloidogenic route by enhancing  $\alpha$ -secretase activity, marks a reduction in the production of full-length  $A\beta$  and a consequent diminution in the possibility of AD [103]. Therefore, the synthesis of activators of  $\alpha$ -secretase or  $\alpha$ -secretase mimetics is deemed as a potentially useful therapeutic option against toxic  $A\beta$  [104, 105].



**Figure 2.8** A schematic representation of therapeutic strategies targeting the intervention of  $A\beta$  aggregation (PDB id 3KTM was used for representing APP structure).

### **2.4.3.2 Methods targeting Amyloid- $\beta$ peptide by impeding A $\beta$ self-assembly**

A $\beta$  fragments can self-assemble to form toxic soluble oligomers. These misfolded oligomers, also known as seeds have been reported to possess the ability to catalyse a chain reaction triggering the aggregation of non-toxic, non-aggregated A $\beta$  molecules to toxic oligomeric state. Blocking the self-assembly of A $\beta$  has been one of the most sought after potential strategies for AD treatment [106, 107]. Following are some of the modulatory approaches employed to impede A $\beta$  aggregation:

#### *(a) Small molecule-based modulators*

Small molecule-based potential therapeutic schemes target the progression of the amyloidogenic pathway and operate by isolating the monomeric form of amyloid- $\beta$ , impeding its self-assembly [108, 109], or inhibiting the A $\beta$  oligomerization [109-114] altering the toxic A $\beta$  oligomers [115]. The small molecule-based modulators comprise of low molecular weight organic compounds, capable of binding to the monomeric A $\beta$  peptide [116, 117]. Several polyphenol-containing compounds inhibit A $\beta$  oligomerization and reduce cognitive deterioration [106, 118-122]. Oleuropein, is such a polyphenol derived from olive oil, that has shown to have hypolipidemic [123], antioxidant [124], and anti-inflammatory [125] potential.

#### *(b) Immunotherapy*

Immunotherapy activates the host immune system to identify and arrest of A $\beta$  or triggers the production of antibodies that target the removal of A $\beta$  oligomers or plaques to avert plaque accumulation. Both active, as well as passive A $\beta$  immunization may be employed as a therapeutic option directed at the inhibition of A $\beta$  oligomerization. The epitopes of A $\beta$  are exposed and accessible for the antibody-



mediated arrest of the soluble or oligomeric form of the peptide. Anti A $\beta$  antibodies stimulate the microglial clearance amyloid plaques and reallocate the A $\beta$  peptide from the brain to the systemic circulation [126]. Numerous anti A $\beta$  immunotherapeutics have been cleared for clinical trials in the past few years and some of these are binding to specific A $\beta$  epitopes. Among them, BAN-2401 [127], aducanumab [128, 129], gantenerumab [130, 131], crenezumab [132], solanezumab [132, 133], and intravenous immunoglobulin G (IVIG) [134, 135], are currently the Phase III clinical trials [136-139]. A recent study has also reported the efficacy of FDA-approved immunotherapy-based cancer drugs known as programmed death 1 (PD-1) inhibitors, in removing A $\beta$  plaques and enhancing cognitive performance in a mouse model of Alzheimer's disease [140]. The progress in research and clinical science have equipped the field of immunotherapy towards promising disease-modifying therapy for AD.

*(c) Peptide-based modulators*

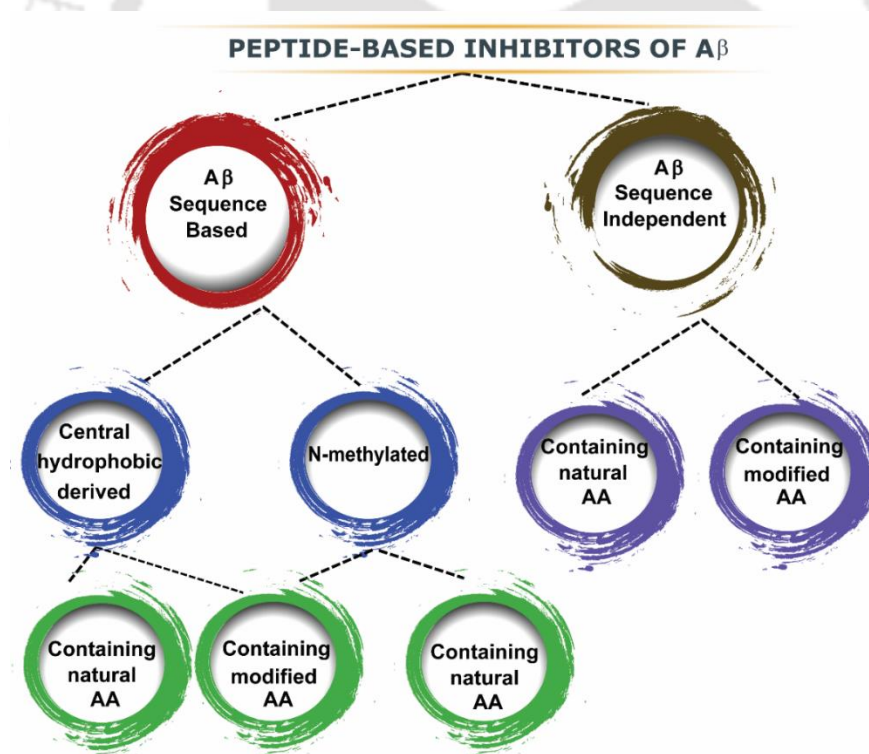
The use of peptides as modulators of A $\beta$  aggregation is a practical option and offers several advantages like biocompatibility, low toxicity and high specificity, low accumulation in tissues, chemically diverse design space and better probability of BBB permeation [70]. The use of peptides as perturbants has been hampered due to their vulnerability to proteolytic cleavage. However, modified peptides having un-natural and d-amino acids have shown decreased permeability to proteases [141-143].

A growing number of short peptides have been shown to have inhibitory effects on amyloid aggregation [67]. These peptide-based modulators of A $\beta$  can be classified into several categories [64] (Figure 2.10).

The two most common, sequence-dependent strategies are:

Peptides based on core recognition motif of A $\beta$

Tjernberg and co-workers termed the short peptide segment “KLVFF” (A $\beta$ <sub>16-20</sub>), as the core recognition motif for designing potent inhibitors of A $\beta$  self-assembly [144, 145]. The peptide-based perturbants binding to this region could also bind to full-length A $\beta$  and blocked its fibrilization (Figure 2.11) [146]. Since then, numerous peptides targeting the core A $\beta$  region have been developed to prevent the aggregation of A $\beta$  into  $\beta$ -sheet-rich fibrils; these peptides are hence referred to as  $\beta$ -sheet breakers [26, 147-154].

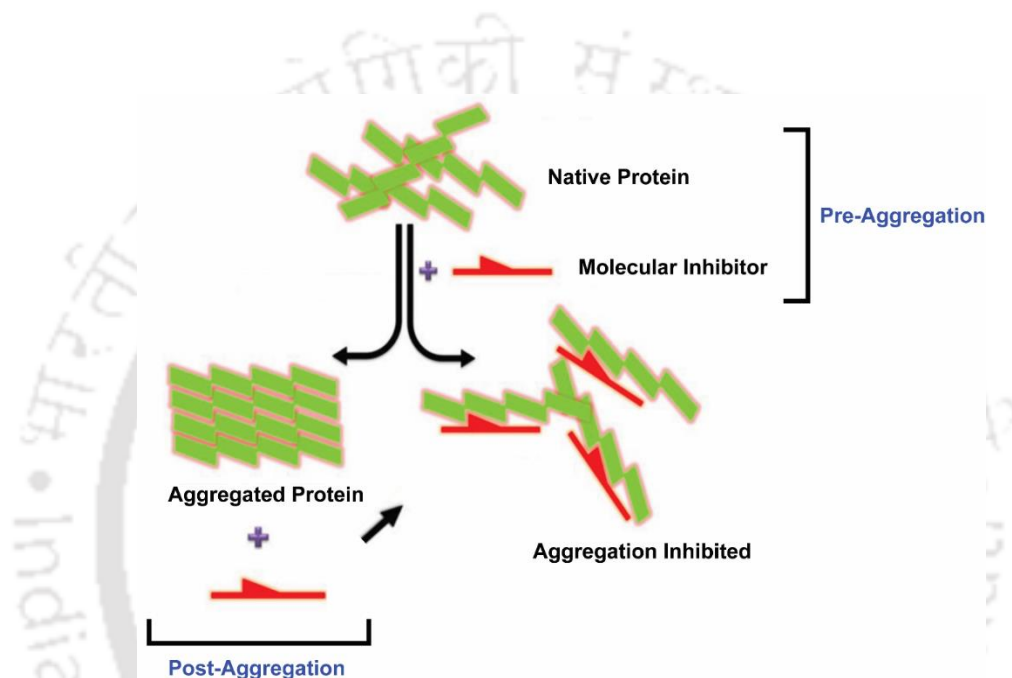


**Figure 2.9** A schematic illustration showing the classification of peptide-based inhibitors of A $\beta$  aggregation.



### N-methylated peptides

The steric effect of N-methylation is considered to decrease hydrogen bonding between amyloidogenic proteins [155]. Consequently, many N-methylated peptides have been employed to prevent amyloid fibrillization [26, 156-159].



**Figure 2.10** A schematic illustration showing the mechanism of peptide-based inhibition of toxic A $\beta$  self-assembly.

#### (a) Metal ion-based chelators

The metal ion hypothesis stress upon the role of metal ions ( $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Fe}^{2+}$ ) homeostasis in the brain. The disruption of metal ion homeostasis has been considered to play an essential role in the neuropathogenesis of AD [160] and has thus stimulated a lot of interest in their therapeutic application. Consequently, many metal-ion

chelators have been evaluated for their efficacy in the intervention of A $\beta$  self-association [161-164].

#### **2.4.3.2 Therapies targeting Tau protein**

Modulatory approaches targeting pathological aggregation of tau protein in AD and associated tauopathies have shown a lot of promise in animal models. Recent studies on tau, suggest that tau pathology has a better correlation with the severity of dementia than amyloid- $\beta$  plaque burden [165-167]. Hence, the impeding tau aggregation or removing the aggregated forms of tau may be clinically more effective as a therapeutic strategy, than clearing A $\beta$  aggregates. Therapies based on tau protein modulation are still in the stage of infancy, with phenothiazine methylene blue (an inhibitor of tau protein) being the only option currently under phase III clinical trials [100]. The critical bottlenecks in tau-based therapeutic research include:

- (a) Tauopathies: tau pathology is linked with over 30 neurological disorders, hence developing AD specific tau inhibitor is challenging,
- (b) Lack of suitable tau-based animal models: the mouse models under development fail to replicate the tau pathology,
- (c) No mutation in tau has yet been associated to AD [168], and
- (d) Ambiguity over the role of tau hyperphosphorylation in AD pathology.

Nonetheless, the possibility of using a tau aggregation inhibitor for AD is now being evaluated in a global phase 3 programme [169]. Following is the list of molecular methods currently under development for targeting tau:

##### **(1) Immunotherapy**

The immunotherapeutic approaches are based explicitly on the stage of the disease since the profile of tau epitope varies throughout the disease [93].

The targets of antibodies include (a) hyperphosphorylated tau, (b) total tau, (c) precise tau, (d) multimeric tau, or (e) fragments of tau [170-176]. Clinical trials of a good number of active and passive tau immunotherapy are in progress [171].

## **(2) Small-molecule-based modulators of Tau**

Small molecular modulators pursuing inhibition of tau aggregation, operate by preventing its oligomerization or blocking its fibrillization [177]. Such small molecular weight compounds include the following:

### **(a) Polyphenols**

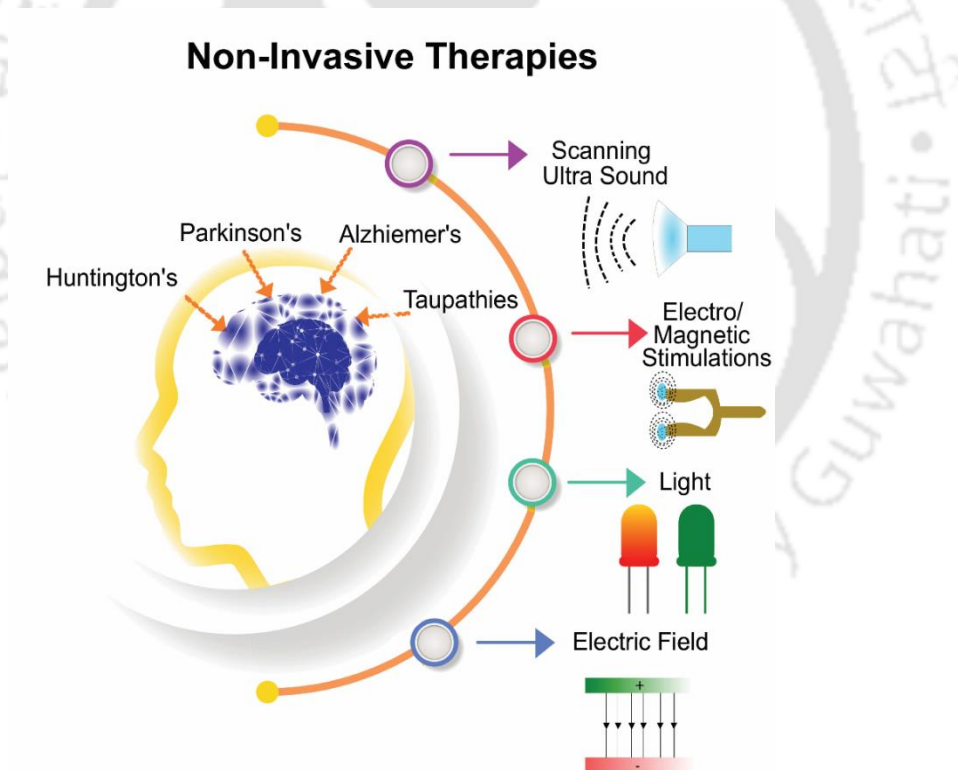
Polyphenols like phenothiazines, porphyrins, benzothiazoles, cyanines, phenylthiazole-hydra-zides, N-phenylamines, thioxothiazolidinone, aminothienopyridazines, and anthraquinones [169, 177-179]. Natural polyphenols like epigallocatechin gallate (derived from green tea), oleocanthal, oleuropein (both found in olive oil), etc. are covalent inhibitors of tau and they mainly target tau monomers [179].

### **(b) Peptides targeting the core recognition motif of Tau**

Two hexapeptide segments VQIVYK and VQIINK, present in the “repeat domain” of tau, have been reported to be crucial for tau aggregation into neurofibrillary tangles (NFTs) [179-183]. These segments are thus regarded to form the core recognition motif for tau protein and as serve an ideal model for developing structure-based inhibitors of tau self-assembly [73, 181, 184-189]. A series of peptide-based modulators have already been reported. They serve as a roadmap for further development of novel peptide-based therapeutics for AD.

## 2.5 NON INVASIVE THERAPIES FOR ALZHEIMER'S DISEASE

Researchers have made remarkable breakthroughs in understanding molecular and cellular aspects of the disease, yet AD continues to be idiopathic, and the therapies required to slow, stop or delay the onset of disease progression are still unavailable [190]. The failure of molecular therapeutic approaches has triggered a shift towards physical, non-invasive therapies, which avoid most of the bottlenecks of the molecular counterpart (Figure 2.11). Though the evolution of non-invasive therapies is still in infancy, positive reports on their efficacy are indeed very optimistic.



**Figure 2.11** A graphical illustration of potential non-invasive therapeutic approaches for neurodegenerative diseases.

Following are some of the non-invasive methods developed as potential non-invasive treatment for AD:

**(a) Scanning ultrasound**

Scanning ultrasound (SUS) is a noninvasive strategy that has lately been shown to reduce the plaque load and restore memory in an AD mouse model [191, 192]. SUS operates by transiently opening the blood-brain barrier (BBB) and promotes the internalization of the amyloid plaques into microglial lysosomes via phagocytosis, thus reduces the plaques. The ability of SUS to open the BBB can also be utilized for drug delivery purpose [193]. In addition to SUS, non-thermal focused ultrasound coupled with an intravenous injection of microbubbles has also been demonstrated to have the potential to open the BBB for therapeutic applications [194]. The short and long term effects of SUS have also been evaluated, and the experimental results propose that ultrasound can be used to avert an age-linked loss of dendrites present in a neuron without damaging the neuronal excitability [195].

**(b) Light Therapy**

Light alone as well in combination with molecular modulators is effective in arresting neuronal death by intervening the amyloid aggregation and have been proposed to have a safe and effective therapeutic potential [196-198]. Several studies have reported the positive effects of near infrared (NIR) light-based treatment on  $\beta$ -amyloid or tau transgenic mouse models [199-203]. These studies have shown that treatment with NIR light not only facilitated a decrease in levels of amyloid plaques, neurofibrillary tangles, oxidative stress, and inflammation. It also improved mitochondrial functioning and increased the ATP levels [196]. A study by Lee et al. showed visible light in combination with xanthene dyes, induced inhibition of A $\beta$

self-assembly by preventing its conformational conversion from monomeric form to  $\beta$ -sheet form. The inhibition of self-assembly also resulted in the reduction of A $\beta$ -mediated cytotoxicity [204]. Although in its early stages, with the most of results still at the “proof of concept” stage, light-based therapy has the promise to mature into a non-invasive and effective neuroprotective treatment for Alzheimer's patients.

### **(c) Noninvasive Brain Stimulation**

Noninvasive Brain Stimulation (NBS), mainly transcranial magnetic stimulation (TMS) and transcranial direct current stimulation (tDCS), propose a promising alternative approach to non-invasive pharmacological interventions for AD [205-207]. TMS employs Faraday's electromagnetic induction principle to electric currents in specific regions of the brain. Several studies have shown the efficacy of TMS techniques in improving the memory [208] and executive function [209] of AD patients. Although various groups have reported conflicting results, employing different protocols and thus TMS treatment is regarded to offer a symptomatic relief only [210]. The tDCS moderates brain excitability through low-amplitude direct current via electrodes attached to the scalp of the patient [211, 212]. Recent reports show that tDCS can improve visual, word-recognition memory, selective attention, working memory, and word recall in AD patients [213-217]. The efficacy of pre-clinical NBS methods, prompt a need for controlled, multiple-site randomized clinical trials to assess their potential as therapeutics for AD.

### **(d) Electric Field**

A few studies examining the effects of an external electric field (EF) on amyloidogenic peptide/protein have reported an irreversible change in their structure mediated by the EF. The applied EF effect resulted in



permanent or induced dipole formation along the direction of the applied field, modulating the native  $\beta$ -sheet conformation of amyloids [218, 219]. Molecular dynamics simulation studies in the presence of external electric fields on A $\beta$  amyloid peptides and insulin have conclusively predicted the effect of an external field in modulating structure formation [220, 221]. A recent study, by Andrij Baumketner, explored the possibility of using an external electric field to disaggregate amyloid fibrils, by inducing folding into an  $\alpha$ -helical state reducing their  $\beta$  sheet conformation [222]. A theoretical study by Ojeda-May and Garcia predicted that a shift from a  $\beta$ -sheet into a helix-like structure could be induced by a constant electric field [223]. They attributed that electric fields can produce rotational motion of molecules with a dipole moment, at the amide planes and align the dipole in the direction of the field vector. A separate study by D. Porchke described that a cooperative change in the structure of bacteriorhodopsin could be induced by applying a reasonably low strength [ $150 \text{ Vcm}^{-1}$ ] electric field [219]. An experimental study by Sen et al. has reported the effect frequency-dependent alternating current (AC) electric field induced oscillation to dissociate preformed amyloid fibrils [224]. Table 2.2 and 2.3 list various theoretical and experimental studies aimed at exploring the effects of electric field on amyloidogenic peptides/proteins.

The use of electric fields as a therapeutic has not been explored to its full potential on experimental fronts. The results of theoretical studies are contradictory. Electric fields of low magnitudes, do have the potential to serve as an effective, non-invasive tool to combat AD and other amyloidogenic diseases. The domain, though requires more experimental examinations for efficacy and safety to eligible as a potential therapeutic option.



**Table 2.2** *List of theoretical reports on application of electric field-based modulation.*

| Article                     | Conclusions                                                                                                                                                                              |
|-----------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Kelly, et al., 2015 [221]   | The acceleration of ordered FF aggregate formation can be induced by weak electric fields, through the alignment of individual molecular dipole moments.                                 |
| Baumketner, 2014 [222]      | Electric field can induce a conformational transition from $\beta$ -sheet into $\alpha$ -helical conformation in amyloidogenic proteins and can be employed as an aggregation inhibitor. |
| May, et al., 2010 [223]     | Constant external constant electric field can modify the secondary structure of a protein and facilitating a shift from a $\beta$ -sheet into $\alpha$ -helical conformation.            |
| Lu, Yan, et al., 2018 [225] | The transmembrane electric field (TMEF) can stabilize the rare conformations of amyloid peptides.                                                                                        |
| Dong, et al., 1996 [226]    | The effect of very strong electric field ( $>10^8$ V/m) on bovine pancreatic trypsin inhibitor induced structural changes by modulating the dipole moment.                               |

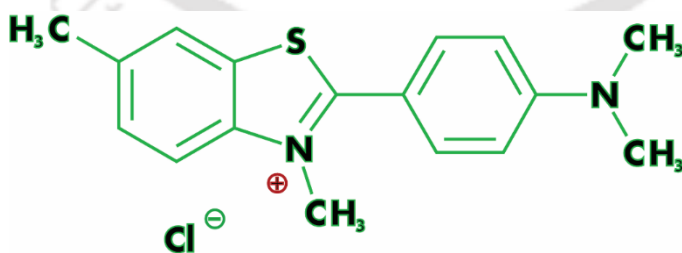
**Table 2.3** List of experimental reports on application of electric field-based modulation.

| Article                             | Conclusions                                                                                                                                                                                                                                                   |
|-------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Porschke, 1987</b><br>[219]      | A cooperative change in structure of bacteriorhodopsin could be induced by applying electric field having low field strength of $150 \text{ Vcm}^{-1}$ .                                                                                                      |
| <b>Sen, et al., 2017</b><br>[224]   | The application of frequency-dependent alternating current (AC) electric field can dissociate the preformed amyloid fibrils.                                                                                                                                  |
| <b>Wei, et al., 2009</b><br>[227]   | The secondary structure of lysozyme changed from $\alpha$ -helical to $\beta$ -sheet and random coil conformation due to the effect of thermal stress induced by pulsed electric field of high strength ( $3.5 \times 10^6 \text{ Vm}^{-1}$ ).                |
| <b>Bekard, et al., 2014</b> [228]   | Frictional energy dissipation as a result of prolonged exposure of proteins (lysozyme and bovine serum albumin) to an electric field, could result in protein to misfold by perturbing the hydrogen bonding involved in the stabilization of their structure. |
| <b>Pandey, et al., 2014</b> [229]   | Application of the static electric field of suitable strength can dissociate preformed fibrils of human serum albumin (HAS) protein.                                                                                                                          |
| <b>Zheng, et al. 2015</b><br>[230]  | The exposure of human insulin protein to non-uniform AC electric fields results in acceleration of insulin fibrillization. The process of monomer to fibril conversion is direct without the formation of oligomeric precursors.                              |
| <b>Pandey, et al. 2017</b> [231]    | Electric field arrested the self-assembly of diphenylalanine peptide and self-assemblies formed post electric-field exposure, showed difference in their morphology, physical properties and crystal habit.                                                   |
| <b>Pandey, et al. 2019</b> [232]    | Electric field arrested the aggregation core peptide segments of tau protein. EF treatment also reduced the aggregation induced cytotoxicity.                                                                                                                 |
| <b>Saikia, et al. 2019</b><br>[233] | Electric field arrested the aggregation of $A\beta_{16-22}$ and $A\beta_{1-42}$ peptides and triggered a conformational shift from $\beta$ to non- $\beta$ conformation. EF treatment also reduced the aggregation induced cytotoxicity.                      |

## 2.6 BIOPHYSICAL APPROACHES TO CHARACTERIZE AMYLOID FORMATION

The discovery of amyloids was followed by a search for methods to characterize and validate their presence. During the initial years, observation under light microscope aided by a variety of histologic dyes was employed to observe the fibrils. Currently, a wide range of techniques is used to characterize and study the aggregation phenomenon. The main aim of such techniques includes the visualization, quantification and structural determination of various amyloidogenic species. The following is a list of biophysical approaches frequently used for studying amyloids:

- i. *Thioflavin T (ThT) fluorescence assay*: Thioflavin T is a benzothiazole-based fluorescent dye widely used for quantitative estimation of amyloids *in vivo* or *in vitro*. ThT binds to amyloid fibrils, gives an enhanced fluorescence emission. Upon binding to fibrils, ThT also undergoes a shift in its excitation and emission spectrum from 385 nm to 450 nm and 450 to 482 nm respectively [234]. The fluorescence intensity of the ThT added aggregated peptide solution is directly proportional to the amount of fibrils present in the solution. ThT dye comprises a benzylamine and a benzathiole ring linked through a carbon-carbon bond (Figure 2.12).



**Figure 2.12** Structure of Thioflavin T molecule.

When the dye is in solution in unbound form, these two rings can rotate freely, and their rotation results in quenching of any photon excitation generated an excited state. Conversely, when ThT binds to amyloid fibrils, it can maintain its excited state, due to the binding mediated immobilization of two rotational plane of the two benzylamine and benzathiole rings [235]. ThT assay can be employed to investigate the modulatory effects of external elements like aggregation inhibitors on the fibrillization. The enhancement of fluorescence signifies increased fibrillization while decrement of the same indicates the arrest of fibrillar self-assembly [236].

ii. *Ultraviolet (UV) absorption*

UV absorption assay is useful for estimating the extent of aggregation in peptides having a tryptophan (Trp) or a tyrosine (Tyr) in their primary sequence. The aggregated peptide solution is centrifuged at 12000g for 20 minutes, and then the Tyr/Trp absorbance (at 280 nm) of the supernatant is recorded. The dense aggregates settle down upon centrifuging, leaving the only the soluble peptide in the supernatant [237]. The measured absorbance is inversely proportional to the magnitude of aggregation and can be compared with the initial absorbance of un-aggregated peptide solution of the same concentration, to determine the percentage of aggregation using the following formulae:

a)  $C = \frac{A}{\epsilon l}$  (Beer-Lambert's Law)

b) % percentage aggregation =  $\frac{C_i - C_f}{C_i} \times 100$

Where C is the estimated peptide concentration, A is the absorbance at 280 nm,  $\epsilon$  is the molar extinction coefficient of Trp/Tyr at 280 nm,  $C_i$  and  $C_f$  are the initial and final peptide concentrations estimated using Beer-Lambert's law (a).

iii. *Static light scattering at right angle Assay*

Static light scattering at right angle assay utilizes the laser light and a photodetector to measure the intensity of light having an excitation wavelength of 450nm, reflected at a right angle by the peptide solution [238]. The scattered light at the same wavelength (450 nm), is measured by the photodetector. The scattering intensity is directly proportional to the amount of fibrillation. This method can be used to assess the extent of aggregation in the peptide solution semi-quantitatively.

iv. *Dynamic Light Scattering (DLS)*

The key distinction between static light scattering and dynamic light scattering is that the former measures the average scattering intensity over time, whereas the latter calculates the fluctuations in the scattering light intensity over a period of time [234]. The fluctuations are instigated by movement of particles (aggregates) and therefore called the diffusion coefficients [239, 240]. The diffusion coefficients are converted to particle size. The particle size measured using DLS is used to estimate the fibrillization kinetics of aggregation and also for determining the effect of an aggregation inhibitor.

v. *Field-Emission Transmission Electron Microscope (FETEM)/Field Emission Scanning Electron Microscopy (FESEM)/Atomic Force Microscopy (AFM)*

Microscopic techniques like FETEM, FESEM, and AFM, are used to obtain structural information on self-assemblies and study the amyloid morphology. An AFM comprises of a probe (a cantilever with a sharp tip) that interacts with the sample through a scanning motion. The scanning motion of the probe is monitored by a laser beam that reflects off the cantilever. A position sensitive photo-detector (PSPD) working in a feedback loop, detects the reflected beams to record a three-dimensional

image of the surface topography of the amyloidogenic sample [234, 241]. FETEM microscopy provides high-resolution imaging of the amyloid sample dried on a carbon-coated copper grid as a thin film. FETEM allows the sample to interact with a focused beam of electrons [242] while FESEM delivers high-resolution imaging by focusing a high-energy electron beam on the gold/platinum coated dried amyloid sample film and detecting the generated secondary electrons from the sample [243].

vi. *Fourier Transform Infra-Red spectroscopy (FTIR)*

FTIR spectroscopy is used to determine the secondary structure conformation of the sample by measuring the asymmetric stretching frequencies of amide I band lying between  $1600\text{ cm}^{-1}$ - $1700\text{ cm}^{-1}$  and attributed to C=O stretching vibration. The conformations are assigned based on specific signature bands specific to the secondary structure of a protein (Table 2.4) [244].

**Table 2.4** FTIR-based secondary structure assignment.

| Amide I Frequency ( $\text{cm}^{-1}$ ) | Secondary Structure                   |
|----------------------------------------|---------------------------------------|
| 1675-1695                              | Antiparallel, parallel $\beta$ -sheet |
| 1660-1670                              | $3_{10}$ -helix                       |
| 1648-1660                              | $\alpha$ -helix                       |
| 1640-1648                              | Unordered                             |
| 1625-1640                              | $\beta$ -sheet                        |

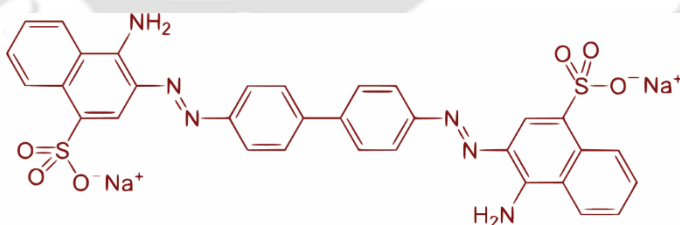
vii. *Circular Dichroism (CD)*

CD is a light absorption spectroscopy, allows real-time monitoring of the secondary structure of the peptide/protein in a solution [245]. CD computes the change in absorbance of right- and left-circularly polarized light allowed to pass through a peptide/protein solution and provides a characteristic signature band, representative of their structures. The  $\alpha$ -helices have negative bands at 222 nm and 208 nm and a positive band at 195 nm [246].

The antiparallel  $\beta$ -sheets show negative bands at 218 nm and a positive band at 195 nm [247]. The disordered proteins show a shallow band above 210 nm and the negative band near 195 nm [248]. The CD instrument must be accurately calibrated, and the exact concentration of the sample should be known, to obtain reliable data. Buffers absorbing in light in the far-UV region can interfere with the measurements, and hence CD is not compatible with solutions having high concentrations of buffer components like dithiothreitol, histidine, imidazole, etc. [234, 249]. However, transparent phosphate, Tris, and borate buffers do not intervene with the measurements [249].

viii. *Congo Red birefringence*

Congo red is the sodium salt of 3,3'-([1,1'-biphenyl]-4,4'-diyl)bis(4-aminonaphthalene-1-sulfonic acid) (Figure 2.13). Amyloids bind to Congo red dye and when viewed under an optical microscope using cross-polarized light, show a characteristic green-gold birefringence [61, 242]. The absorption maxima of Congo red shifts under cross-polarized light from a wavelength of 490 nm to 540 nm [250].

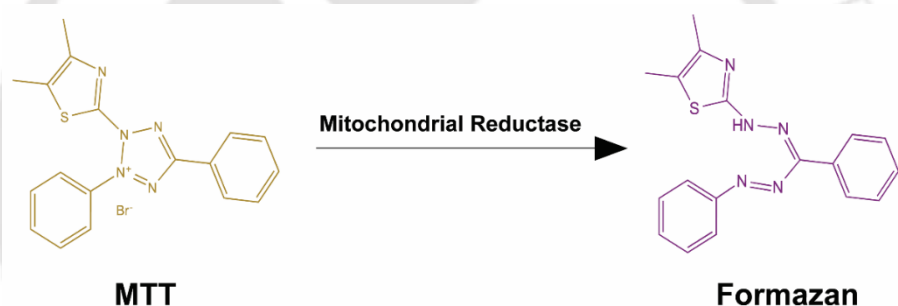


**Figure 2.13** Structure of Congo red molecule.



ix. *MTT assay*

MTT or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, is a tetrazolium dye. The assay measures the reduction of yellow tetrazole (MTT) by mitochondrial reductase enzyme to, purple colored formazan [251] (Figure 2.14). The amount of formazan formed is estimated spectrophotometrically. As MTT reduction can only happen in metabolically active cells, the level of formazan (product) is a measure of cellular viability. MTT assay is a calorimetric assay, and hence extensively used to assess *in vitro* cellular toxicity of treated and untreated amyloidogenic peptides/proteins.



**Figure 2.14** Reaction showing reduction of MTT to formazan by the action enzyme mitochondrial reductase.

## 2.7 SELF-ASSEMBLING PEPTIDES

Self-assembly refers to the spontaneous organization of components of a system into an ordered functional unit. This phenomenon is reversible and can be the result of direct (specific interactions between the individual components) or indirect (environment mediated, for example, temperature, pH) interactions. It is driven by minimization of the free energy of the system, achieved by minimization

of repulsive and maximization of attractive molecular interactions. The current interest in this field arises from several reasons:

- i. it is considered that information on the molecular basis of protein self-assembly and aggregation may help to elucidate the physiochemical features of protein folding,
- ii. self-assembly is one of the few practical strategies for making ensembles of nanostructures and,
- iii. studying self-assembly is also expected to shed light on the molecular basis of diseases mediated by abnormal aggregation.

The intermolecular forces like van der Waals interactions, hydrogen bonding, hydrophobic interactions, weak polar interactions,  $\pi$ - $\pi$  interactions as well as electrostatic interactions, hold the components into well-defined stable structures, such that the assembled structure is in equilibrium with the environment as well as the individual components.

There are two main types of self-assembly: static and dynamic [252]. Static self-assembly comprises of systems that are at global or local equilibrium, do not dissipate energy and may require energy (for example, globular proteins are formed by static self-assembly) to form the assembly while dynamic self-assembly involves energy dissipation to create ordered structures.

Peptide-based self-assemblies are extensively studied for their potential applications in the fields of bio-nanotechnology and nano-material fabrication (Table 2.5). These self-assemblies are highly ordered nanostructures with a high degree of symmetry.

**Table 2.5** List of some of the reported self-assembling peptide sequences.

| Amyloid Sequence                                                                                                                                                                                                             | Article                                          |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------|
| FGAIL; TNVGSNTY; QRLANFLVH; KLVFF;<br>LVFFA; LPFFD; KLVFFAE; NFGSVQFV;<br>SFNNGDCCFILD; SFFSFLGEAFD;<br>PHGGGWGQ; PQGGYQQYN; GNNQQNY                                                                                         | Gazit, 2002 [253]                                |
| DFNKF; DFNK; FNKF; DFN                                                                                                                                                                                                       | Reches, et al., 2002 [254]                       |
| FLVHS; NFLVH; SNNFGAILSS; NFGAIL;<br>NFGAILSS; TNVGSNTY; ATQRLANFLVHSS;<br>NFLVHSSNN; NFLVHSS; FLVHSS                                                                                                                        | Mazor, et al., 2002 [255]                        |
| FD; FF                                                                                                                                                                                                                       | Reches, et al. 2003 [256]                        |
| Alpha Synuclein                                                                                                                                                                                                              | Galvagnion, et al., 2015 [257]                   |
| Ph-GG-Ph; NFGAIL; NFLVH; NFGSVQ;<br>DFNKF; DFNK; Boc-Ala-Aib-Val-OMe; Boc-<br>Ala-Aib-Ile-OMe; Boc-Ala-Gly-Val-OMe                                                                                                           | Reches, et al., 2006 [258]                       |
| Alpha-synuclein(NACP gene)                                                                                                                                                                                                   | Porat, et al., 2006 [259]                        |
| IAPP                                                                                                                                                                                                                         | Bakou, et al., 2017 [260]                        |
| NFGAILSS; NWGAILSS; NFGSVQ; DFNKF;<br>DFNK                                                                                                                                                                                   | Gazit, 2007 [261]                                |
| KLVSFFAE; NFGAI; NFGSV; DFNK; STVIIE; (P) <sub>1</sub> -<br>(PKRHW) <sub>2</sub> -(VLSWFNQ) <sub>3</sub> -(ILTYWFN) <sub>4</sub> -(FIY)-<br>(PKRH); NH <sub>2</sub> -D-Phe-D-Phe-COOH; NH <sub>2</sub> -L-Phe-<br>L-Phe-COOH | Colombo, G. et al., 2007 [262]                   |
| Tat CPP                                                                                                                                                                                                                      | Gazit, 2008 [263]; Macchi, et<br>al., 2017 [264] |
| A6D, A6K, V6D <sub>2</sub> , L6K <sub>2</sub> , KA <sub>4</sub> K, KA6K, KA8K<br>KGAVK                                                                                                                                       | Qiu, et al., 2018 [265]                          |
| (Ala) <sub>6</sub> Asp; (Val) <sub>6</sub> Asp; (Gly) <sub>8</sub> Asp <sub>2</sub> ; Lys(Val) <sub>6</sub> ;<br>Fmoc-Leu <sub>2</sub> ; Fmoc-Leu <sub>3</sub> ;                                                             | Scanlon, et al., 2008 [266]                      |
| LL, LF, FL, FF                                                                                                                                                                                                               | Castillo., et al., 2011 [267]                    |

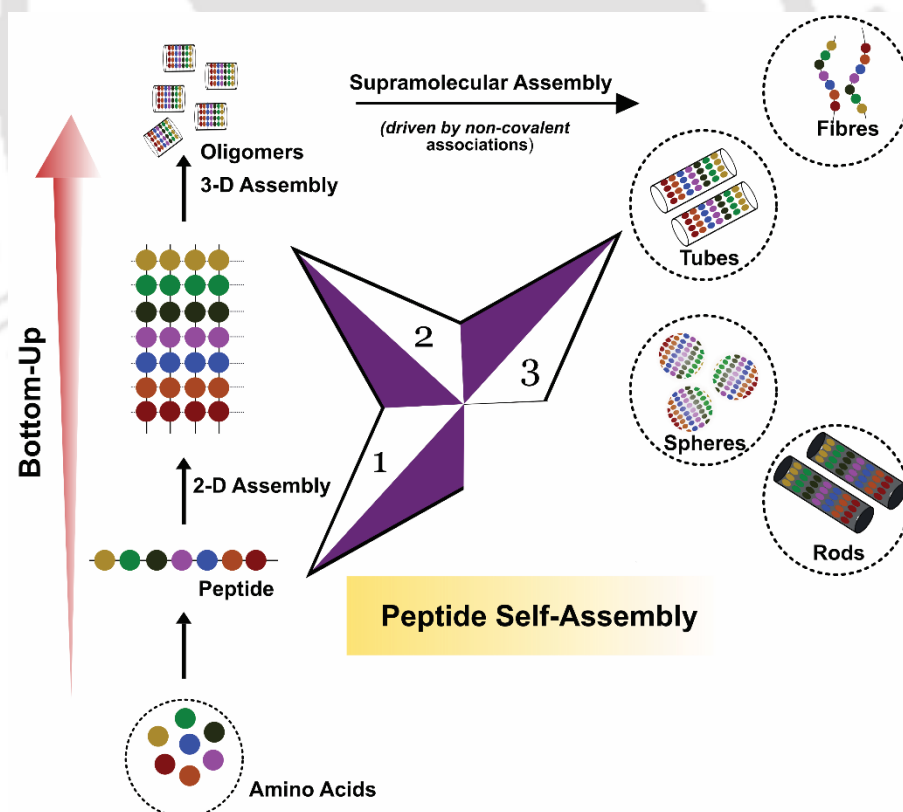
## **2.8 FUNCTIONAL MATERIALS FROM AGGREGATION**

Over the past three decades, the focus of nanotechnology has been towards the development of novel nanostructures and exploration of their potential applications such as materials for nano-electronics, catalysts for bioprocessing and sensors for biomedical technology [268, 269]. The “bottom-up” fabrication strategy, involving the arrangement of one unit at a time have been widely used for the construction of functional materials with tailor-made features [270] (Figure 2.15). In the field of chemistry and biology, the process of self-assembly of small molecules, spontaneously organizing into bigger structured blocks have been crucial to create and functionalize the self-assembled structures [271].

Amongst all the small biomolecules like DNA, proteins, cellular organelles, etc. proteins and peptides stand a choice to be used as building blocks for functional materials [272, 273]. The combination of naturally occurring 20 amino acids, along with unnatural and modified amino acids offers a wide diversity of building blocks. The designed sequences can be either chemically synthesized or produced from microorganisms. The sequence-specific self-assembly makes the designed peptides to take the desired conformations ( $\alpha$ -helix,  $\beta$ -sheets) which could be fine-tuned to form 3 dimensional building blocks for supramolecular structures [274, 275].

Proteins and peptides have different physicochemical properties depending on their conformation and stereochemical configuration of the amino acids. This makes them sensitive to environmental changes like pH, temperature, salt content, external stimulus, etc. Hence, they can be used as versatile biocompatible smart materials for the de-novo design of functional materials [276, 277] (Figure 2.16). Protein and peptide supramolecular structures have been conjugated with various other molecules to produce hybrid conjugates, peptidomimetics which finds applications in material science [278].

One of the most widely studied molecular system is diphenylalanine (FF) self-assembly which is a part of the core fragment of Alzheimer's amyloid beta ( $A\beta$ ) protein. FF self assembles to form ordered nanotubes, microtubes, nanowires, and nanocrystals under different conditions [256, 279-283]. Reches and Gazit showed casting metal wires using diphenylalanine nanotubes as moulds [256]. Diphenylalanine peptide nanotubes have also been fabricated to form trilayered coaxial nanocables using metal nanoparticles, and can have unique electromagnetic properties [284]. In another study, amyloid protein fiber from *Saccharomyces cerevisiae* Sup 35 self-assembly have been used to form conducting nanowires [285]. An investigation by del Mercato et al. showed the electrical conductivity in intrinsically fluorescent amyloid-like fibril of polypeptide poly(ValGlyGlyLeuGly) [286].



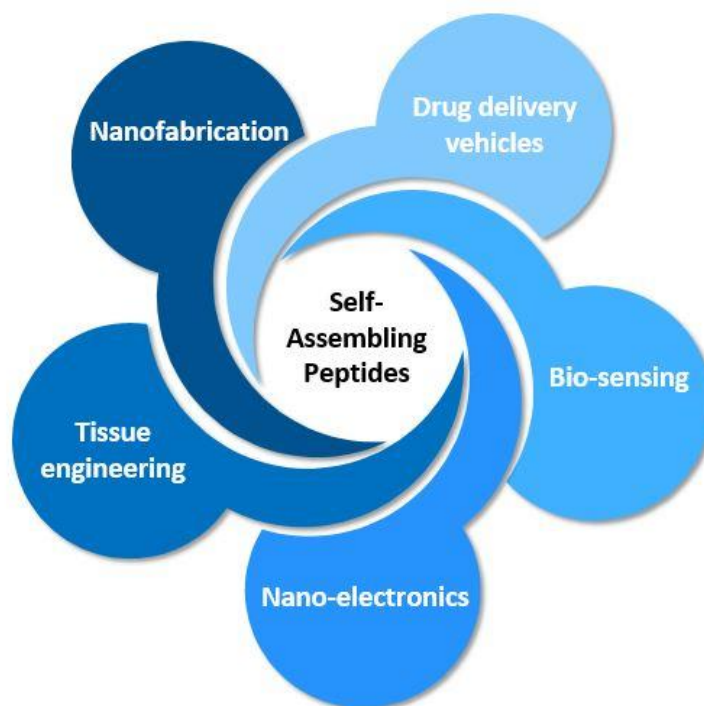
**Figure 2.15** Diagrammatic illustration of bottom-up approach of peptide self-assembly.

Proteins and peptide self-assembled structures have been utilized as biosensors also. Insulin amyloid fibrils have been functionalized with conducting and luminescent polymers as electrochromic and electrochemical transistors, providing a single multifunctional device in biological sensing [287]. Self-assembled FF nanowires have been modified with a conductive polymer and used as a biosensor and in cell culture for the growth of PC-12 and Hela cells [288]. Research by S. L. Gras et al. showed amyloid fibers to be functionalized for biological cell adhesion via the RGD motif and thus can be used as biomaterials [289]. In another study, insulin amyloid fibers in conjugation with glucose oxidase using glutaraldehyde have been utilized as a scaffold for enzyme immobilization [290]. Protein amyloid fibrils from fusion protein made up of Cytochrome b<sub>562</sub> with an SH<sub>3</sub> dimer sequence have been functionalized to bind with metalloporphyrins [291]. Moreover, conjugation of photochromic compounds with polypeptides and peptide nanostructures have been shown as potential photo modulating biosensors [292].

While mostly inorganic nanostructures have been known to have optical properties, recent studies on protein/peptide-based nanostructures have been functionalized like quantum dots. Self-assembly of tertbutoxycarbonyl-Phe-Phe-OH (Boc-FF) peptide nanospheres has been shown to exhibit optical characteristics of quantum dot [293]. Self-assembled nanostructures and amyloid fibrils have also been conjugated with polymer complex to be used as polymer light-emitting diodes [25, 294].

Another very promising material is the formation of gels like hydrogels, organogels, and aerogels from both amyloidogenic and non-amyloid peptides self-assembled structures [295-297]. Hydrogel formation has been reported to form short Fmoc-dipeptides some of which are suitable for three-dimensional cell culture [298-300].





**Figure 2.16** *Diagrammatic representation of prospective applications of peptide self-assembly.*

Krysmann et al. reported the formation of hydrogel from the amyloidogenic fragment of  $\beta$ -amyloid, KLVFF [301]. An excellent review by Fichman et al. also shows the varied technological applications of short peptide based gels [302]. Presence of network-like structure and their property to trap small molecules, several peptide-based hydrogels have widely been used as drug entrapment and release vehicles or as scaffolds in cell culture [303, 304].

Apart from amyloidogenic protein sequences, designed peptide amphiphiles also gives various superstructures [305, 306]. An example includes the work by Stup and co-workers where peptide amphiphiles have been modified with cell binding sequences to form hydrogels capable of growth and differentiation of neuronal cells [307]. Apart from tissue engineering and cell culture, functional peptide-



based structures have also been shown to have potent antibacterial activities. Pioneering work by Schneider et al. on  $\beta$ -turn containing MAX1 sequence which proved inherent antibacterial properties against both gram-positive and gram-negative bacteria [252, 308].

The above examples provide only a miniature view among the many reports available on different applications of functionalized materials from protein and peptide-based aggregation structures. With the option of various design based strategies and promise of tailor-made end products, self-assembly of proteins and peptides have thus gained increasing attention from researchers across fields of biology, chemistry, and materials science over the last few decades.

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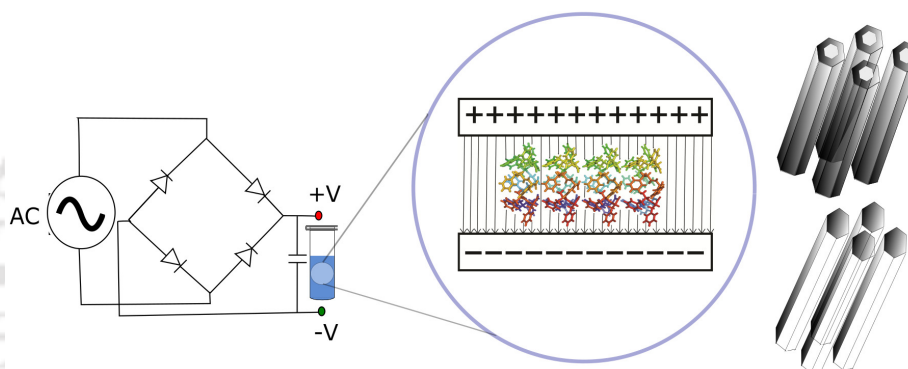


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## CHAPTER 3

# MODULATION OF PEPTIDE BASED NANO-ASSEMBLIES WITH ELECTRIC AND MAGNETIC FIELDS



*Peptide based nano-assemblies with their self-organizing ability has shown lot of promise due to their high degree of thermal and chemical stability, for biomaterial fabrication. Developing an effective way to control the organization of these structures is crucial for fabricating application-oriented materials at the molecular level. This chapter discusses the impact of electric and magnetic field-mediated perturbation of the self-assembly phenomenon, upon the chemical and structural properties of diphenylalanine assembly.*

An article based on this chapter: Gaurav Pandey, et al., Scientific Reports 7.1 (2017): 2726.





### 3.1 INTRODUCTION

Ability to design, control morphology, and tune up physical and chemical properties at nanoscale characterize the heart of nanotechnology. Generation of nano-level architecture should ideally have a design phase at the molecular scale. Morphology of such systems can be better controlled, if they assemble further-on to micro dimensions. Nanotechnology research mostly focuses on the latter half by way of their imminent utility while fabricating materials at larger dimensions. Application of physical agents for tailoring the nanostructure morphology can be very useful for nanostructure fabrication.

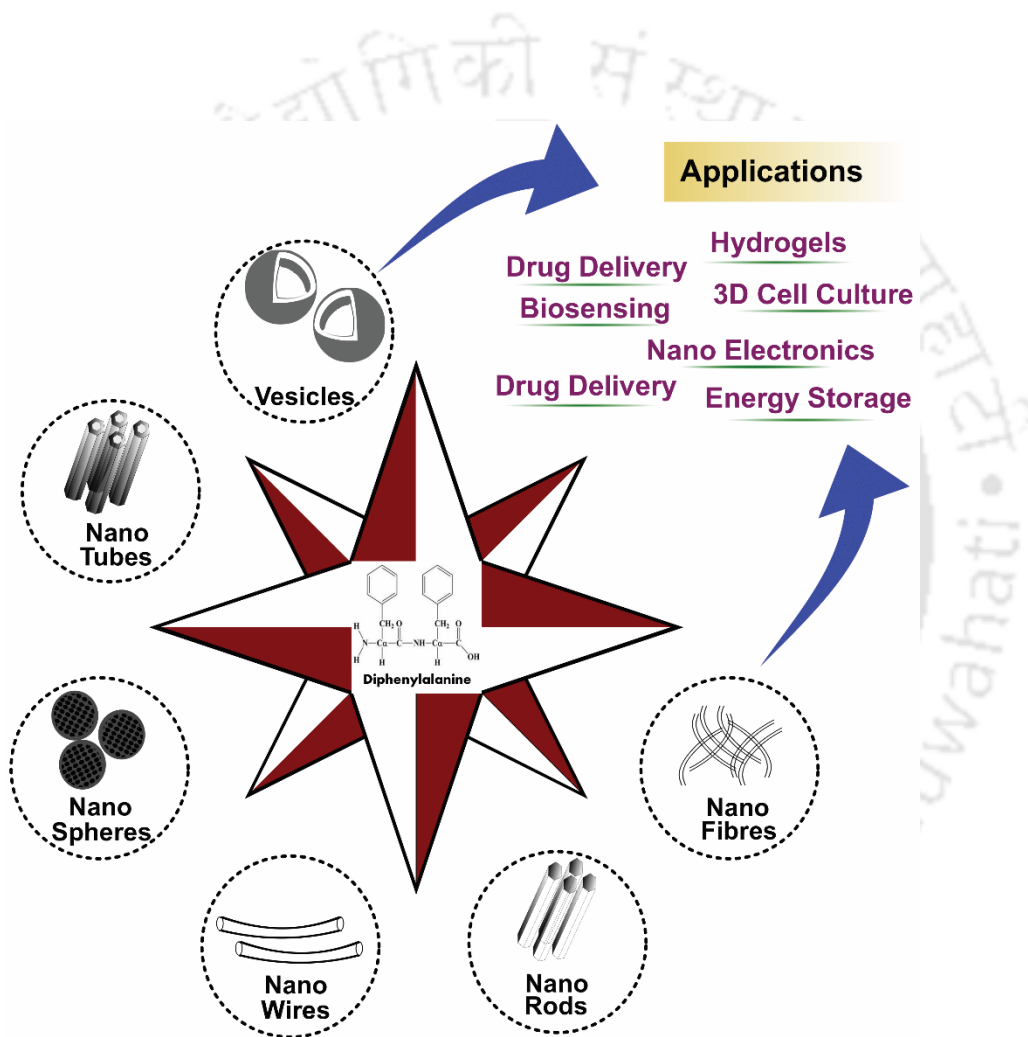
In the last decade, there has been an increased focus on organic and bio-organic nano-assemblies. Peptide nanotubes, their physical properties, and assembly morphologies are extensively studied due to their excellent biocompatibility as well as functional and structural diversity. Many ordered supramolecular structures have been constructed using peptides as the building blocks. The most extensively utilized peptide-based building block is diphenylalanine (Phe-Phe or FF), which is the shortest bio-molecule known to self-assemble into ordered nanostructures. FF incidentally is also the core recognition motif of the  $\beta$ -amyloid polypeptide, a peptide associated with Alzheimer's disease [1]. It can self-assemble into a variety of structures like microtubes, nanotubes [2], microcrystals, nanofibers [3], nanorods [4, 5] and nanowires [6].

The potential of these supramolecular structures have been utilized in diverse fields including nanofabrication, drug delivery vehicles [7], bio-sensing [8], energy storage devices, and hydrogels for tissue engineering [9, 10] (Figure 3.1). The crystal structure of FF exhibits a non-centrosymmetric hexagonal space group (P6<sub>1</sub>) [1, 11], which allows it to possess properties like piezoelectricity [12, 13], optical activity [14, 15], and ferroelectricity [16]. Due to its low dimensional highly ordered structure, it also exhibits quantum confinement [17], forming quantum dots [18].

### Chapter 3

## Modulation of Peptide Based Nano-Assemblies with Electric and Magnetic Fields

Self-assembled structures formed by analogues of FF such as Ac-Phe-Phe-NH<sub>2</sub>, NH<sub>2</sub>-Phe-Phe-NH<sub>2</sub>, NH<sub>2</sub>-(*p*-nitro-Phe)-( *p*-nitro-Phe)-COOH, NH<sub>2</sub>(4-phenyl-Phe)-(4-phenyl-Phe)-COOH, PEGylated tetra-phenylalanine (L6-F<sub>4</sub>), β-AspFF etc. have also resulted in tubular, fibrillar and squared plate structures respectively [10, 19, 20].



**Figure 3.1** Schematic illustration of diverse nanostructures formed by diphenylalanine-based building blocks and their prospective applications.

One of the key challenges in the field of supramolecular chemistry has been controlling the self-assembly of molecules into ordered functional units.

Previously, a number of strategies including pH mediated control [21, 22], solvent mediated control [23], covalent modifications [24], vapor deposition [25, 26], temperature [27], surface [28, 29], relative humidity [30] symmetry [31] and magnetite coating on the surface of nanotubes [32] have been employed to regulate the architecture of diphenylalanine self-assemblies. But studies with fairly high magnetic fields have not yielded the level of response as expected, though a response was forced, at a magnetic field range of 12 T or more [33]. Recent theoretical investigations predict measurable response to applied electric field, but practical demonstration has not been reported so far, with the best of our knowledge. The present study aims to investigate the impact of electric and magnetic field mediated perturbation, upon the chemical and physical properties of peptide nano assemblies, with diphenylalanine as the model molecular system. Electric field studies on biomolecules (like DNA and proteins) have shown permanent or induced dipole formation along the direction of applied field, indicating that electric field interaction could modulate their dipole moments [34, 35]. Molecular dynamics studies in the presence of external electric fields on A $\beta$  amyloid peptides and insulin have conclusively predicted the effect of an external field in modulating structure formation [12, 36] Andrij Baumketner in a recent study, explored the feasibility of using external electric field to disaggregate amyloid fibrils, by inducing folding into an  $\alpha$ -helical state reducing their  $\beta$  sheet conformation [37]. This is especially important because FF is the core recognition motif of  $\beta$ -amyloid segment. Here in this study, we attempt to confirm the effect of electric field on diphenylalanine self-assembly using experimental approach.

Protein crystals have been reported to show alignment in the presence of strong magnetic field. This alignment was believed to be the result of diamagnetic anisotropy of the peptide bonds and aromatic amino acids in protein molecules [38]. These aromatic side chains play an important role in directing the amyloid fibril self-assembly through  $\pi$ - $\pi$  stacking interactions [39]. Magnetic field has been

previously employed to align  $\beta$ -amyloid fibrils for X-ray fiber diffraction studies [40-43]. So far various groups have reported studies involving magnetic field exposure of self-assembled diphenylalanine assemblies post formation [32, 33]. We, however use electric or magnetic field, concurrently while formation of nano-assemblies. This is achieved by starting the experiment at a temperature (95 °C) that do not support assembly. The system is then cooled to facilitate assembly under electric or magnetic field of chosen strength at desired optimum concentrations for assembly. Our results indicate that field induced perturbation approach can be a promising tool for controlling nano-assembly, thus generating novel architectures. Modulation of nano-level architecture of peptide based model systems with external stimulus, points to a potentially rewarding strategy to re-work proven nano-materials to expand their application spectrum.

### **3.2 EXPERIMENTAL APPROACHES**

#### **3.2.1 Materials**

The FF peptide was purchased from Sigma-Aldrich. Deionized water was used for all the experiments.

#### **3.2.2 Preparation of Peptide Solution**

Fresh solution of diphenylalanine peptide was prepared by dissolving the lyophilized form of the peptide in deionized water (at 95 °C) to a concentration of 2 mg/ml [27]. The vial containing the sample was kept in a water bath (Grant sub aqua 26 plus) set at 95 °C, allowing peptide to dissolve completely. Fresh stock was prepared for each experiment to avoid any pre-aggregation. Peptide nano/micro tubes were formed while cooling to room temperature (25 °C).

### **3.2.3 Pre-aggregation test**

To ensure the absence of any pre-aggregation in peptide stock solution a right angle scatter was recorded using a spectrofluorometer (Jasco FP 8500) at 450 nm. The slit width was set at 2.5 nm [44].

### **3.2.4 Electric Field Experiments**

The experiment was performed with FF at a concentration of 2 mg/ml at room temperature (25 °C) for a duration of 5 hours, at three different DC and AC electric fields: 0 Vcm<sup>-1</sup> (control), 150 Vcm<sup>-1</sup> and 300 Vcm<sup>-1</sup> (Figure 3.2A). Two parallel aluminum electrodes separated by a distance of 1 cm were anchored to a plate to produce a horizontal DC electric field (Figure 3.2B). These plates were connected to a full-wave bridge rectifier. A single-phase variable autotransformer was used to regulate the voltage and to produce a horizontal electric field between the two parallel plates. To generate an AC electric field a similar setup was built without a full-wave bridge rectifier. A 2 ml of FF peptide solution (at 95 °C) was taken in a microcentrifuge tube and allowed to cool in the presence of electric field for a duration of 5 hours. No voltage was applied between the electrodes in the case of control experiment. All the experiments were done in parallel, to ensure that sample allowed to cool under electric field (AC as well as DC), magnetic field and control (no field) conditions at same rate.

### **3.2.5 Magnetic Field Experiment**

Similar to electric field experiment, a magnetic field experiment was also performed with 2mg/ml FF solution at room temperature for a duration of 5 hours. Peptide solution (95 °C) was taken in a microcentrifuge tube and allowed to cool in the presence of magnetic field measuring 0.6 T (Figure 3.2C).



### **3.2.6 Static Right Angle Light Scattering Assay**

Static right angle light scattering was recorded to estimate the extent of self-assembly in the field exposed peptide solution, using a spectrofluorometer (Jasco FP 8500) at 450 nm. The slit width was set at 2.5 nm. Post electric annealing, field-exposed peptide solution was taken in a fluorescence cuvette (Helma, Sigma-Aldrich) of 1 cm path length, and its static light scattering was recorded immediately for 12000 seconds. For control as well as magnetic field sample which already comprised dense FF aggregates, static light scattering was recorded only for 1000 seconds. The term “post incubation/annealing” refers to the time period after 5 hours, when both electric as well as magnetic field were switched off.

### **3.2.7 Field Emission Scanning Electron Microscopy (FESEM)**

FESEM analyses were performed using a Zeiss Sigma FESEM at 2-3 kV. Field exposed peptide samples were loaded on a glass slide for analyses and air dried. Samples were coated with gold for enhancing conductivity.

### **3.2.8 Melting Point Estimation**

Melting points for the diphenylalanine self-assemblies formed under different experimental conditions were recorded using Optics Technology Digital Melting Point Apparatus. The solid samples (FF self-assemblies) were packed in thin glass capillaries and loaded in the apparatus. For each sample, the temperature at which the first drop of liquid appears was recorded as the melting point of that sample.

### **3.2.9 Raman Spectroscopy**

Raman measurements of dried FF self-assemblies were performed using a high-resolution micro-Raman spectrometer (Jobin Horiba, LabRam HR800); 514 nm laser was used for all the experiments.

### **3.2.10 Powder XRD**

X-Ray diffraction (XRD) patterns were obtained from a Rigaku X-ray diffractometer (Model: TRAX III) powder diffractometer operating at 50 kV and 100 mA using Ni-filtered Cu-K $\alpha$  radiation ( $\lambda = 1.54 \text{ \AA}$ ). The diffractogram were recorded in the  $2\theta$  range of  $0-30^\circ$ .

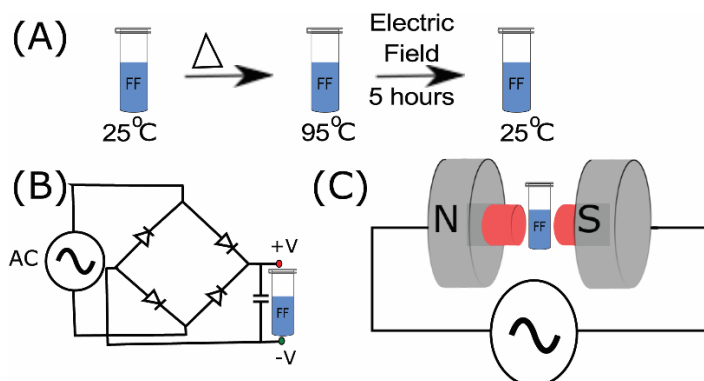
### **3.2.11 Photoluminescence Assay**

The photoluminescence measurements were recorded post FF self-assembly formation (in each of the sample) using Jasco FP 8500 spectrofluorometer (excitation  $\lambda = 256 \text{ nm}$  slit width  $2.5 \text{ nm}$ , emission  $\lambda = 260-320 \text{ nm}$  slit width  $2.5 \text{ nm}$ ) to investigate the effect of field exposure on optical properties of FF.  $600 \mu\text{l}$  of peptide solution (consisting of FF self-assemblies) was taken in a fluorescence cuvette (Helma, Sigma Aldrich) of  $1 \text{ cm}$  path length and its photoluminescence was recorded.

## **3.3 RESULTS AND DISCUSSION**

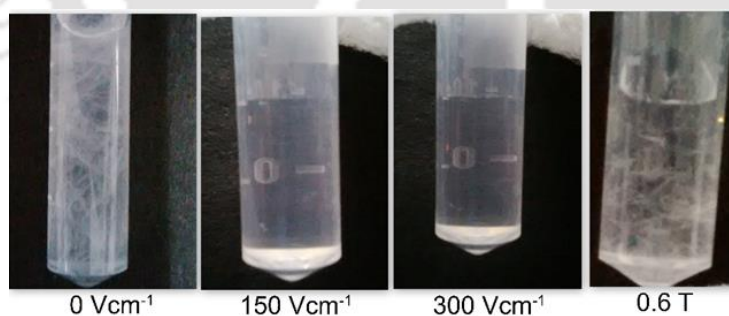
### **3.3.1 Electric and magnetic field effects on nano-assembly**

The experimental system containing the peptide sample was allowed to cool in the presence of electric ( $150 \text{ Vcm}^{-1}/50 \text{ Hz}$ ,  $300 \text{ Vcm}^{-1}/50 \text{ Hz}$  AC electric field, and  $150 \text{ Vcm}^{-1}$ ,  $300 \text{ Vcm}^{-1}$  DC) fields, magnetic field and ambient conditions continuously for 5 hours till the system cools and gets equilibrated at room temperature ( $25^\circ \text{C}$ ) completely (Figure 3.2).



**Figure 3.2** Schematic representation of experimental setup. (A) Dissolved peptide was allowed to cool down from 95 °C to room temperature while incubating in the presence of electric field. (B) electric field set up and (C) magnetic field setup.

Interestingly, there was no visible self-assembly as long as the sample was under electric field exposure. The control sample with no field, and the sample cooled under magnetic field (0.6 T), showed visible nano-assemblies (Figure 3.3), which was further confirmed by light scattering and FESEM experiment.

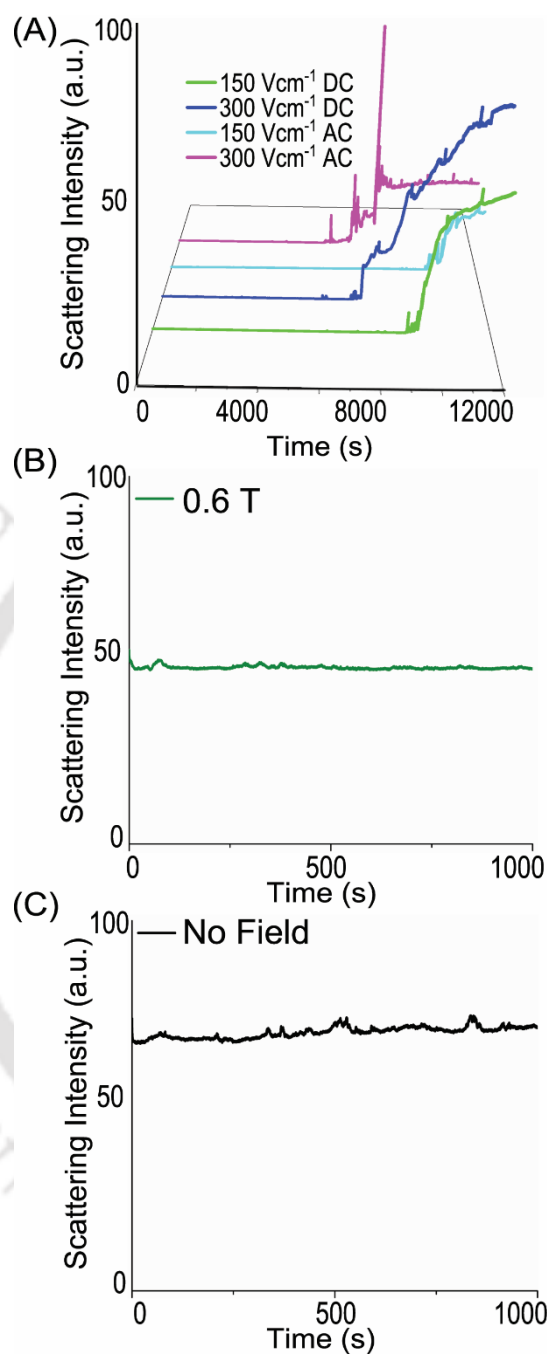


**Figure 3.3** Post annealing under various field conditions: Images were taken after cooling the FF solution for 5 hours. Electric field (150 Vcm<sup>-1</sup> and 300 Vcm<sup>-1</sup> clearly has a prohibitive effect on aggregation and nano-assembly of FF; whereas magnetic fields at 0.6 T and 0 V (no field) show a dense growth of visible self-assembled FF aggregates. Images were taken after cooling the FF solution for 5 hours. Electric field (150 Vcm<sup>-1</sup> and 300 Vcm<sup>-1</sup> clearly has a prohibitive effect on aggregation and nano-assembly of FF.

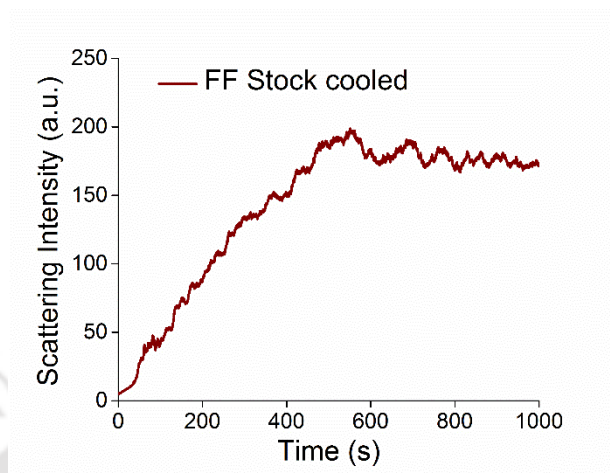
These observations suggest that both AC as well as DC electric fields could inhibit an undirected aggregation and a directed self-assembly process.

Previously, it has been shown that FF tubes align in the presence of a very high magnetic field (12 T) [33], perhaps magnetic fields as low as 0.6 T was not robust enough to perturb the forces involved in the self-assembly process. Static light scattering was recorded at right angles for each of the sample to quantify the aggregation of FF annealed under an electric and magnetic field in comparison to the control sample (Figure 3.4). The right angled static light scatter of the samples exposed to AC and DC electric field showed an increase in scattering intensity with time, indicating spontaneous initiation of peptide self-assembly.

In contrast, the control and magnetic field exposed samples showed very high scattering intensity at zero time signifying the presence of pre-formed self-assemblies (or aggregates) at appreciable dimensions (Figure 3.4 B, C). For comparing this phenomenon, peptide solution was cooled from 95 to 25 °C and its right angled static light scatter was recorded as a control experiment. This experiment also showed a pattern similar to electric field exposed samples, and the right-angled static light scattering showed an increase with time, as a result of the spontaneous initiation of peptide self-assembly upon cooling (Figure 3.5).



**Figure 3.4** Effect of the electric and magnetic field on FF nano-assembly. The graphs represent static right angle scatter plots post incubation at (A) electric field, (B) magnetic field and (C) no field mentioned conditions in arbitrary units (a.u.).

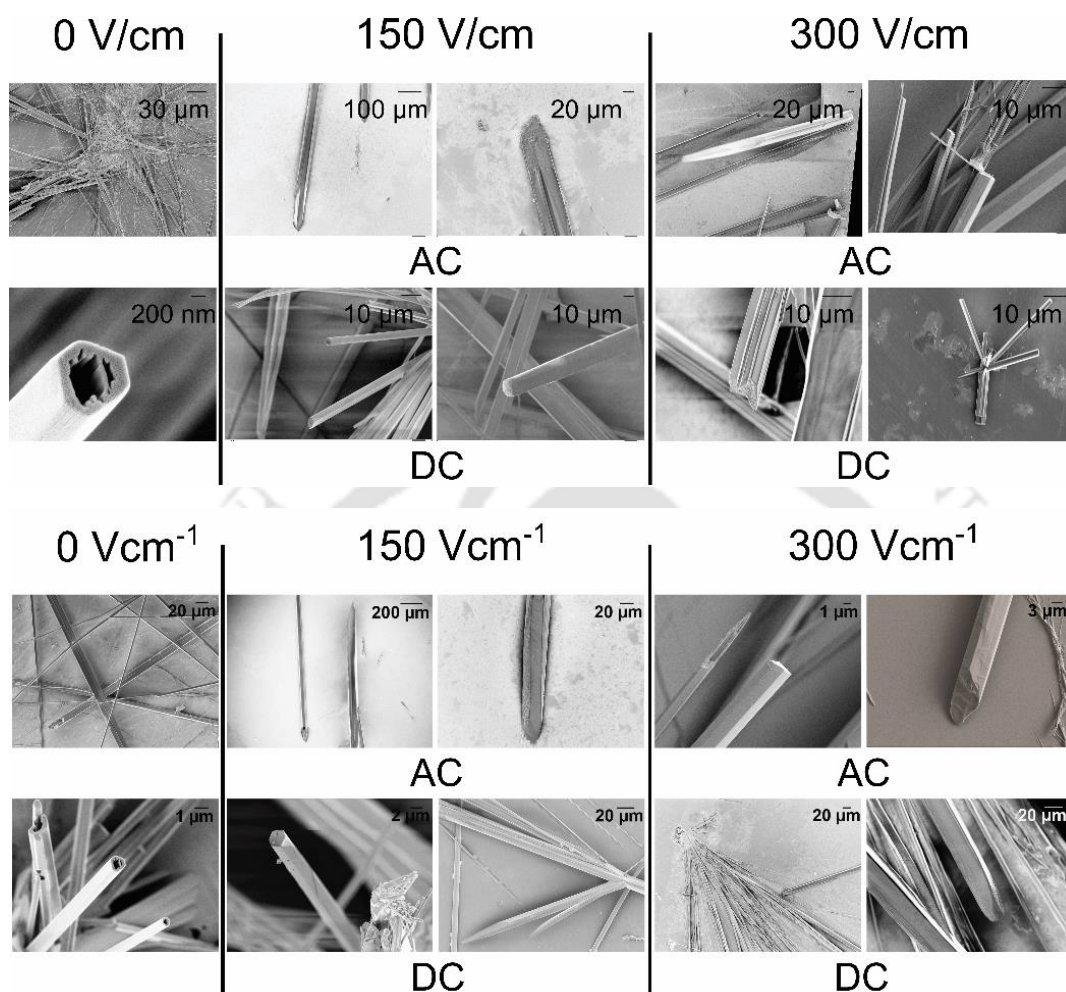


**Figure 3.5** FF nano-assembly formation. Dissolved peptides were allowed to cool down from 95 °C to room temperature and concurrently static right angle scatter was recorded. The graph represents static right angle scatter plot in arbitrary units (a.u.).

### 3.3.2 Effect of the electric field in modulating nanotube morphologies

The samples were analyzed using FESEM to gain an insight into the effect of electric field on the peptide self-assemblies, (Figure 3.6). Morphologically the self-assemblies formed post AC, and DC electric field exposures were more aligned and showed the presence of branched structures. They also showed a morphological shift to rod-like structures, though tubes were also observed. The self-assemblies formed in the control sample ( $0 \text{ Vcm}^{-1}$ ) formed an exclusive tube-like morphology and were randomly oriented in comparison to the field samples. The results indicate the possibility that electric field exposure modulates the formation of the peptide self-assemblies, though the magnitude of this influence may vary with sample molecular systems.





**Figure 3.6** Scanning electron micrographs of FF self-assemblies formed post annealing at room temperature conditions: The nanorods and nanotubes formed after five experimental conditions reported ( $0 \text{ V cm}^{-1}$ ,  $150 \text{ V cm}^{-1}$  DC and AC electric field;  $300 \text{ V cm}^{-1}$  AC and DC electric field) are shown. Field induced assemblies have greater tendencies of alignment and a morphological shift to rod like structures (compared to tubes) in the overall population of nano-micro dimensional structures.

To investigate the effect of electric field exposure upon the physical properties of peptide self-assemblies, melting points of each sample were measured. The

samples showed the difference in their melting points, further confirming, field induced modulation in the assembly architecture of FF nanostructures (Table 3.1).

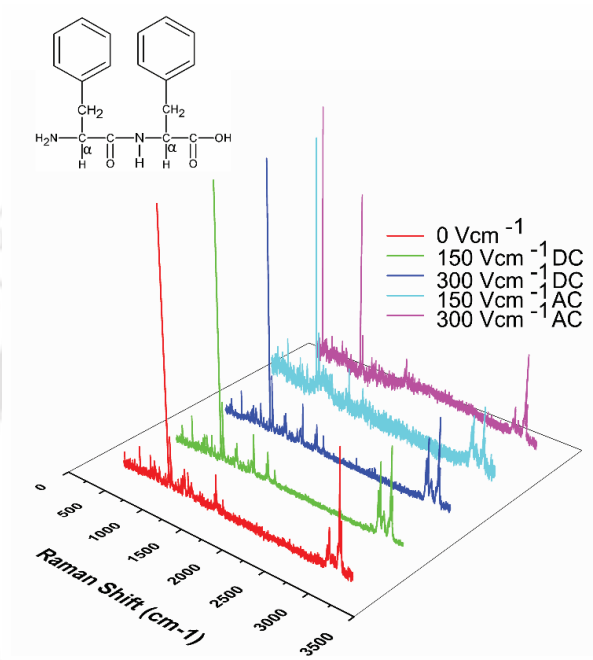
**Table 3.1** *Melting points for diphenylalanine nano-assembled tubes/rods at specific electric fields applied.*

| Sample                | Melting Point ( °C) |
|-----------------------|---------------------|
| 0 Vcm <sup>-1</sup>   | 288-291             |
| 150 Vcm <sup>-1</sup> | 284-285             |
| 300 Vcm <sup>-1</sup> | 278-280             |
| FF powder (sigma)     | 291-292             |

### 3.3.3 Effect of external stimulus on the chemical constitution

High-resolution micro-Raman spectroscopy is a non-invasive technique that can be employed to examine the functional group composition of the material. This technique was performed on dried diphenylalanine self-assemblies, from each of the experimental conditions to probe the impact of field exposure on the chemical composition of the peptide. The Raman spectra of the self-assemblies formed in control and field exposed samples are shown in Figure 3.7. The Raman bands correspond to aromatic rings of FF are at 1004 cm<sup>-1</sup>, 1034 cm<sup>-1</sup>, 1590 cm<sup>-1</sup>, 1429 cm<sup>-1</sup> and 1608 cm<sup>-1</sup>. Peak at 1688 cm<sup>-1</sup> band can be attributed to C=O stretching while small peaks detected between 1154 to 1300 cm<sup>-1</sup> can be assigned to the acyclic C-C stretching [45, 46]. Table 3.2 lists the Raman shifts of chemical functional groups of diphenylalanine. Similar characteristic bands were observed in each of the samples, and there was no remarkable difference; means molecule is not affected

by the field induced perturbation. Therefore, the difference in assembly, morphology and melting points may be attributed to the modulatory effect of electric field on assembly formation.



**Figure 3.7** Chemical constitution from Raman spectra. Raman spectra were recorded at five different experimental conditions (0 Vcm<sup>-1</sup>, 150 V cm<sup>-1</sup> DC and AC electric field; 300 Vcm<sup>-1</sup> AC and DC electric field). Spectral analysis suggests that chemical constitution of all five samples are identical, indicating that field effect is modulating only the assembly formation at nano-level.

**Table 3.2** Raman peak assignments for diphenylalanine self-assembled nanostructures.

| Functional Group                        | Raman Spectra Peak (cm <sup>-1</sup> ) |
|-----------------------------------------|----------------------------------------|
| C-C<br>acyclic, aliphatic<br>stretching | 1154,1300                              |
| Benzene ring                            | 768,818,1004,1034,1590,1429,1608       |
| C=O stretching                          | 1688                                   |
| CH stretching (aromatic)                | 3054                                   |

To confirm the phase purity and examine any possible difference in crystal structure on the self-assemblies formed with and without electric field exposure, powder X-ray diffraction (PXRD) measurements were performed with Cu-K $\alpha$  radiation ( $\lambda = 1.54 \text{ \AA}$ ). Le-Bail profile fitting has been performed by assuming hexagonal crystal structure symmetry with P6<sub>1</sub> space group [47]. A pseudo-Voigt (P-V) function was used to fit the profile by using Full Prof Suite [48]. P-V is an approximation of Voigt function, which is a linear combination of the Gaussian profile and Lorentzian profile [49, 50]. This function allows to accommodate asymmetries in the X-ray diffraction peaks. The scattered points in Figure 3.8 shows the experimentally observed diffraction data and the red colour solid line represent the Le-Bail profile fitting of diphenylalanine polycrystalline samples exposed to electric field.

These X-ray diffraction patterns are in-agreement with hexagonal structure proposed by Gorbitz and the resultant crystallographic data is consistent with the published FF crystal structure (CCDC 163340) [1]. No spurious peaks were observed in the X-ray diffraction pattern which confirms the formation of pure diphenylalanine.

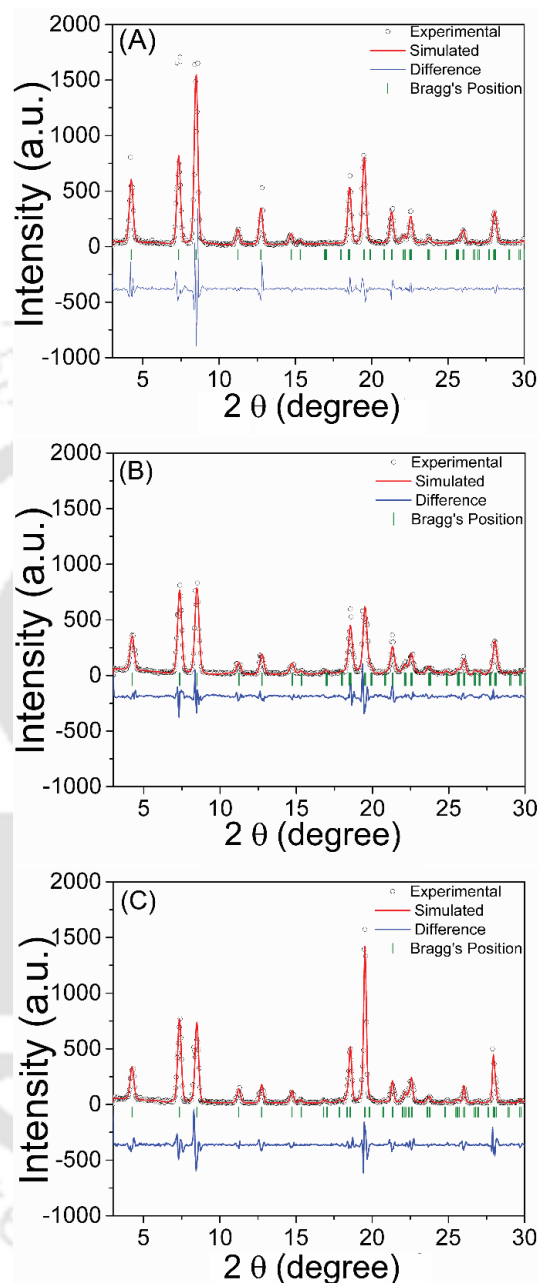
The Le-Bail profile fitting of X-ray diffraction patterns also showed that all three samples differ with respect to their lattice parameters. Average crystallite size 'P' and micro strain 'η' play a significant role in the peak broadening [51]. In order to estimate the individual contribution of 'P' and 'η' on peak broadening, the Williamson Hall (W-H) analysis was employed.

The W-H analysis is governed by the following relation with which we can estimate the contribution of crystallite size 'P' and lattice strain 'η' on X-ray peak broadening:

$$\beta \cos \theta = \frac{K\lambda}{P} + \eta \sin \theta$$

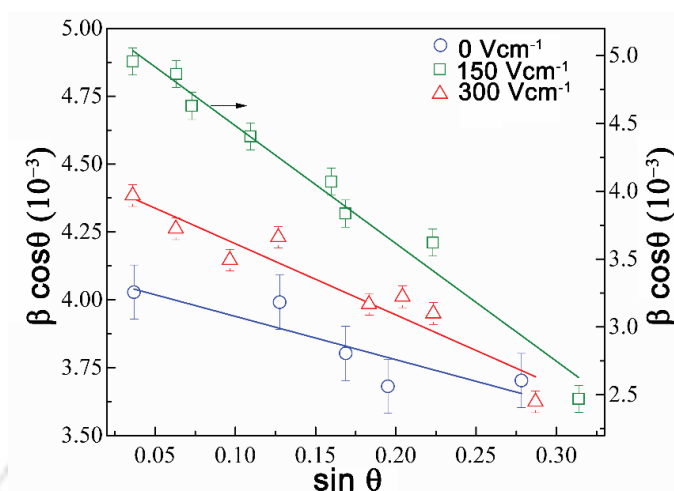
where,  $\beta$  is the full-width half maximum of diffraction peak intensity, K (~0.89) is the shape factor, and  $\lambda$  is the wavelength of Cu-K $\alpha$  X-ray radiation.

Crystallite size ( $P \sim 322\text{-}342 \text{ \AA}$ ) of the particles gives a rough estimation of the domain size which diffract coherently. It is mostly not the same as particle size due to the formation of polycrystalline aggregates [52]. Lattice strain on the other hand quantifies the distribution of lattice constants originating from imperfections in the crystals, such as dislocations inside the lattice. Figure 3.9 shows the W-H plot ( $\beta \cos \theta$  versus  $\sin \theta$ ) for FF under different electric fields. From the slope and intercept of these plots, the magnitude of 'P' and 'η' was estimated. The self-assemblies formed post-electric field exposure differ significantly on average crystallite size as well as lattice strain. All the crystallographic parameters for the FF obtained from the profile fitting are listed in Table 3.3.



**Figure 3.8** X-ray powder diffractogram of FF self-assemblies at (A) ambient conditions, post DC electric field incubations at (B) 150 Vcm<sup>-1</sup> and (C) 300 Vcm<sup>-1</sup>. Hollow dots: measured powder diffraction pattern after background subtraction; solid red curve: simulated profile; green vertical tick marks: Bragg's positions; and the solid blue curve: residual between the experimental and simulated profiles.



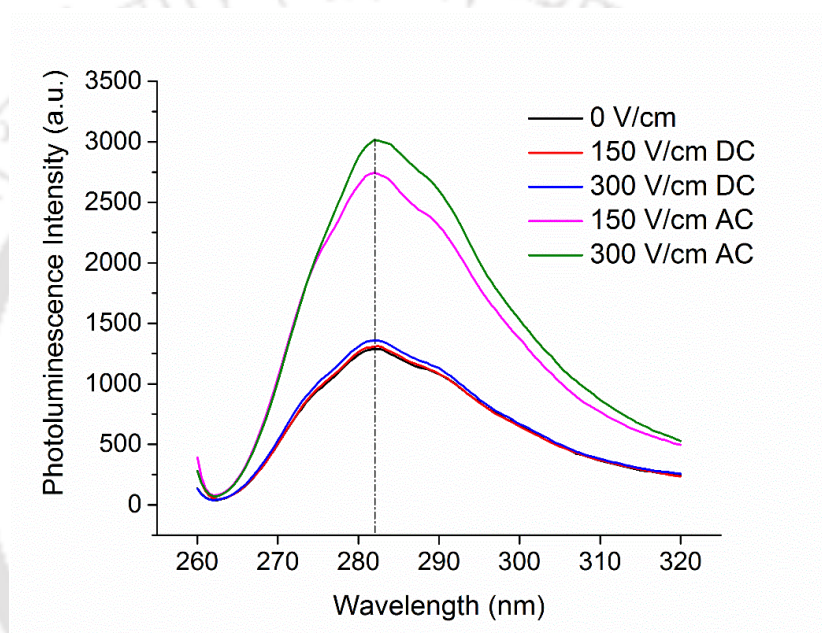


**Figure 3.9** The Williamson-Hall plots ( $\beta \cos\theta$  versus  $\sin\theta$ ) of FF self-assemblies formed at ambient conditions. The hollow blue, olive and red color symbols represent the post-DC electric field incubation of 0  $\text{Vcm}^{-1}$ , 150  $\text{Vcm}^{-1}$  and 300  $\text{Vcm}^{-1}$ , respectively.

**Table 3.3** Lattice parameters derived from Le-Bail profile fitting and W-H analysis of X-ray diffraction patterns of diphenylalanine self-assembled structures.

|                            | 0 $\text{Vcm}^{-1}$  | 150 $\text{Vcm}^{-1}$ | 300 $\text{Vcm}^{-1}$ |
|----------------------------|----------------------|-----------------------|-----------------------|
| <b>a</b>                   | 24.069124            | 24.069912             | 24.052418             |
| <b>b</b>                   | 24.069124            | 24.069912             | 24.052418             |
| <b>c</b>                   | 5.407554             | 5.402272              | 5.454072              |
| <b><math>\alpha</math></b> | 90°                  | 90°                   | 90°                   |
| <b><math>\beta</math></b>  | 90°                  | 90°                   | 90°                   |
| <b><math>\gamma</math></b> | 120°                 | 120°                  | 120°                  |
| <b>P</b>                   | 322 Å                | 403 Å                 | 343 Å                 |
| <b><math>\eta</math></b>   | $2.2 \times 10^{-3}$ | $6.2 \times 10^{-3}$  | $4 \times 10^{-3}$    |

The photoluminescence spectra for the FF self-assemblies formed under the three different experimental conditions were recorded to examine electric field influence on the optical properties of the peptide self-assemblies. The spectra were found to show a similar pattern, indicating that the field induced morphological changes did not influence the inherent optical properties of the material (Figure 3.10).



**Figure 3.10** Photoluminescence spectra of FF structures assembled after 5 experimental conditions reported (0 V cm<sup>-1</sup>, 150 V cm<sup>-1</sup> DC and AC electric field; 300 Vcm<sup>-1</sup> AC and DC electric field) are shown.

### 3.4 CONCLUSIONS

We demonstrated that electric field could arrest the hierarchical self-assembly of diphenylalanine, and self-assembly was initiated only after (at least 10 minutes) the withdrawal of external electric field whereas magnetic field at the experimental (0.6 T) range has no significant effect on assembly formation. FF is the core recognition motif of amyloid  $\beta$  polypeptide. Electric field effect is in agreement with the earlier observation made by Andrij Baumketner, that external electric field can disaggregate amyloid fibrils by inducing folding [37]. Self-assembly of diphenylalanine peptide after annealing in the presence of electric field showed the distinct morphological difference. Hill and co-workers earlier studied the little effect of magnetic field, and the results are in full agreement with our observation. The electric field exposure also modulated the stability of the self-assembled structures without affecting the overall chemical constitution of the material structure as well the inherent optical properties of the material. Martin Garcia and Ojeda May have predicted that an external constant electric field can even modify the secondary structure of a protein, by inducing a transition from  $\beta$ -sheet to  $\alpha$ -helical like conformation [53]. Furthermore, we demonstrated that the self-assemblies formed post electric-field exposure showed the difference in their crystal habit. W-H analysis of the X-ray diffraction patterns revealed the differences in the peptide self-assemblies formed post electric field exposure of varying magnitudes, with respect to their lattice parameters as well as crystallite size. The microstrain associated with self-assembled structures also varied with changes in the magnitude of the electric field. We infer that our strategy in employing electric field mediated perturbation of electrostatic interactions directing the peptide self-assembly, can be used to generate nanostructures with distinct morphologies from the same material. Further, in line with the theoretical investigations discussed in the earlier sections, this experiment suggests that electric field can modulate the physical properties of nanostructures and therefore

has the potential to be deployed as a fabrication tool for a myriad of application-oriented materials without any chemical additives.

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### Chapter 3

## Modulation of Peptide Based Nano-Assemblies with Electric and Magnetic Fields

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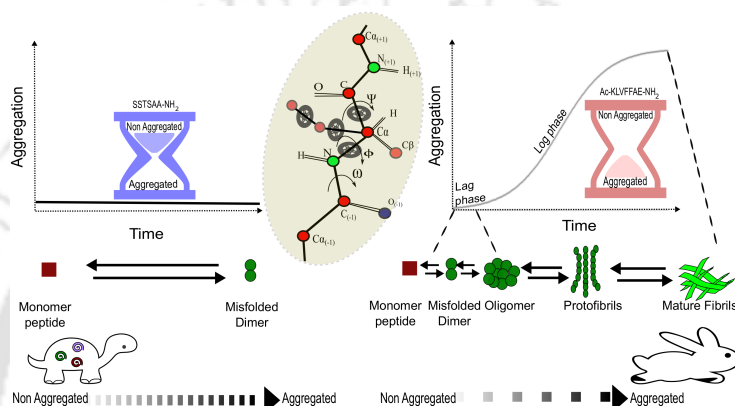
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## CHAPTER 4

## GEOMETRIC ORIGINS OF AGGREGATION

## KINETICS



The steric zipper is a term coined for recently reported crystal structures of short peptide segments of amyloidogenic proteins. These steric zipper peptides comprising of a pair  $\beta$ -sheet layers tightly bound by the inter-digitation of their side-chains have been proposed to represent the fundamental structural unit of amyloid fibrils. A comprehensive understanding of factors regulating the kinetics of amyloidogenesis of these steric zippers may serve to sculpt the folding and aggregation energy landscapes in vivo to enable folding of a mis/unfolded conformations to a functional form while precluding aggregation. In this study, we have attempted to achieve this objective, by using biophysical techniques like thioflavin T fluorescence, IR spectroscopy and electron microscopy to compare and characterize the aggregation kinetics of nine steric zipper peptides along with a rigorous statistical and computational analysis to investigate the geometrical rationale for the differential aggregation rates of our model peptide systems.



## **4.1 INTRODUCTION**

Protein folding is a rapid process by which a polypeptide chain undergoes conformational changes which are energetically downhill towards the biologically active native 3D structure. This phenomenon is depicted in the funnel-shaped energy landscapes. Proteins are soluble in their native form. The phenomenon of amyloidogenesis involves the conversion of normally soluble peptides or proteins into insoluble amyloid plaques. Assembly of peptides or proteins into ordered fibrils is a systematic process instigated by changes in their conformation resulting in a series of intermediate states, culminating in non-functional amyloid plaques.

The pathology of amyloid-associated disorders remains elusive. Recent, structural characterization via X-ray diffraction studies have revealed the presence of a packed and dehydrated structural motif, designated as ‘steric zipper’ which is formed by tight interlocking of a pair of complementary  $\beta$  sheets. These steric zippers have been reported to constitute the common structural motif of more than 30 peptides associated with a series of amyloid-induced diseases, stressing upon the molecular level similarity of amyloids. Nucleation-dependent aggregation is one of the most putative models for the formation of amyloid fibrils [1-5]. According to this model, the process of fibrillogenesis involves a rate-determining nucleation phase wherein the monomeric peptide, misfold to form an oligomer.

These oligomers are referred to as “nuclei”, are rich in  $\beta$ -sheets and grow further to form mature fibrils [6]. The nucleation and growth phase are followed by a breaking and static phase [7]. The aggregation phenomenon is dependent upon the  $\beta$ -sheet propensity of peptide and small changes in sequence can lead to an alteration in the aggregation pathway [8]. Hydrophobic interactions are the driving force in the nucleation phase [6]. The mechanism of aggregation is affected by a series of factors, including temperature, pH, ionic strength [6], initial protein concentration [7], surfactants [9], filament breakage [10], and macromolecular

crowding [11]. A slight alteration of the free energy profile is enough to cause a wide shift in the fibril formation mechanisms [5].

Aggregation is a self-replicating process [12]. Controlling the self-assembly is challenging post its onset. Kinetics of aggregation is concerned with the route by which a monomeric protein or peptide aggregates to mature amyloid fibrils and the rate at which this phenomenon of fibrillogenesis (conversion of a monomeric peptide to mature fibrils) proceeds. In the past few decades, deciphering the mechanism and kinetics of aggregation has been a topic of fundamental interest across a wide range of scientific disciplines fueled by its association with a series of ageing-associated disorders. Studying the path traversed during fibrillogenesis, and kinetics of the pathway is very crucial to understand the mechanism of disease and thus devise potential strategies based to prevent fibril formation or replication. The available biophysical techniques and assays including Thioflavin T (ThT) fluorescence estimation [13], dynamic light scattering (DLS) [13], quartz crystal oscillator [14], Fourier transfer infrared spectroscopy (FTIR) [2], circular dichroism (CD) [15], small-angle X-ray scattering (SAXS) [16], isothermal titration calorimetry (ITC) [13], nuclear magnetic resonance (NMR) [17], transmission electron microscopy (TEM) [18], and X-ray diffraction [18], have been useful in aiding the qualitative examination of amyloid growth but it has been extremely challenging to link these qualitative results to the causative factors directing the route and rate of protein/peptide self-assembly [19, 20].

The theoretical approaches focused at elucidation of self-assembly kinetics have utilized molecular dynamics [8, 19, 20], asymptotic analysis [7], thermodynamic analysis involving mass and non-mass action equations [21-24], and logistic equations [25]. Numerous growth models for the self-association of amyloid-forming systems have been proposed [10, 26-28]. The models currently available in literature have emphasized on the factors governing monomer association [22], pre-nucleation, primary and secondary nucleation [23, 27, 29], role of  $\beta$  sheet

propensity [19], initial protein concentration [10, 21], electrostatic interactions [30] energy barriers [31], surface affinity [32], and role of water [33].

Eisenberg et al. have reported a series of atomic structures derived from disease-causing naturally occurring aggregation-prone peptides [12, 34-38]. These crystal structures comprised of a pair of self-complementary, elongated  $\beta$ -sheets stacked in parallel or anti-parallel fashion, forming a tight and dry interface (to form a so-called “steric zipper”). These small steric zipper peptides forming the aggregation core, are recognized *in vitro* model systems for amyloid fibril formation and have been reported to demonstrate all essential properties of disease causing full-fledged proteins sequences.

We have used nine of these steric zipper peptides as model systems for our investigation on their comparative aggregation kinetics. These systems are subsequences of larger disease-related (Alzheimer’s and type-II diabetes) as well as non-disease causing (fungal prions) peptide sequences. To gain an insight into their aggregation behaviour, these peptides were synthesised by solid-phase peptide synthesis method and were characterized by standard Thioflavin T fluorescence assay, scattering assay, FETEM microscopy and FTIR spectroscopy. We have allegorically modelled our molecular systems as a machine with 4 rotors  $\phi$ ,  $\psi$ ,  $\chi_1$  and  $\chi_2$ , with its propensity to aggregate is directed by combinations of its rotor directions [39]. We observe that each peptide system shows differential rotor motions due to the difference in their amino acid composition. Based on this model, we have proposed substitution matrices (MIDMAT) by calculating the statistical basin-associated free energy of protein folding for each amino acid. In this work, we employ these matrices to investigate the role of dihedral driven geometry on the aggregation kinetics of short peptide sequences. Each amino acid comprises a unique geometrical composition, which explains the differential rates of their aggregation. Understanding the correlation between aggregate growth mechanism and the amino acid composition will be very helpful for understanding



the peptide aggregation phenomenon and will also aid in developing sequence-based strategies for therapeutic intervention.

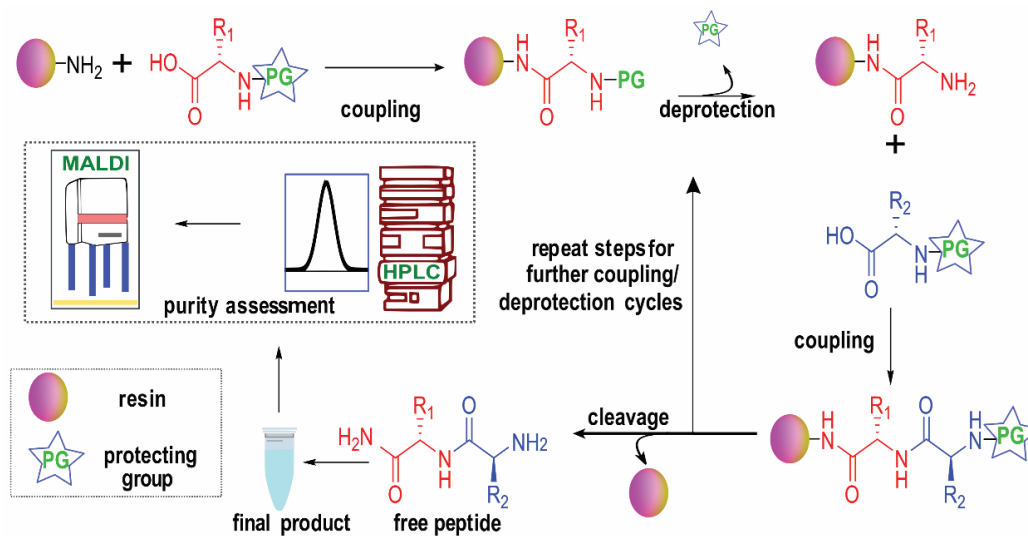
## **4.2 EXPERIMENTAL APPROACHES**

### **4.2.1 Materials**

All the chemical reagents were obtained from commercial suppliers and used without any further purification unless otherwise mentioned. Amino-acids and  $\alpha$ -cyano-4-hydroxycinnamic acid matrix were purchased from Sigma Aldrich. Rink amide resin, Thioflavin T dye, ethanedithiol, Diethyl ether, Trifluoroacetic acid, phenol, thioanisole, and ethanedithiol were procured from Merck. Deionized water was used for all experiments.

### **4.2.2 Peptide Synthesis and Characterization**

Peptides were synthesized by solid-phase peptide synthesis using Rink amide resin (Novabiochem) and standard Fmoc chemistry. The synthesized peptide was cleaved from the resin and deprotected using a mixture containing TFA/m-cresol/thioanisole/ethanedithiol in a ratio of 20:2:2:1, respectively, for 12 hours at room temperature. The peptide was precipitated in ice-cold diethyl ether. The peptide was dissolved in deionized water and its purity was verified on Shimadzu Prominence HPLC system using analytical C-18 reverse phase column. The purity of peptide was further characterized using MALDI-TOF mass spectrometry in the Laser Desorption positive mode, using  $\alpha$ -cyano-4-hydroxycinnamic acid matrix on a MALDI-TOF mass spectrometer (Bruker autoflex speed). Figure 4.1 shows a schematic illustration of the methods used for peptide synthesis and their characterization.



**Figure 4.1** Schematic illustration of solid-phase peptide synthesis and characterization.

#### 4.2.3 Preparation of Peptide Solution

The fresh peptide solution was prepared by dissolving the dried form of the peptide in deionized water (at 95 °C) to a 3 mM concentration. The vial containing the samples was kept in a water bath (Grant sub aqua 26 plus) set at 95 °C, allowing peptides to dissolve completely. The fresh stock was prepared for each experiment to avoid any pre-aggregation.

#### 4.2.4 Thioflavin T Fluorescence Assay

ThT binding assay was performed by combining 20  $\mu\text{L}$ , 3 mM peptide (at 95 °C) to a solution of 10  $\mu\text{M}$  ThT in 50 mM sodium phosphate buffer at pH 7.4. Fluorescence spectra were recorded on a spectrofluorometer (Jasco FP 8500) at 25 °C using a 1 cm path length quartz cuvette (Helma, Sigma-Aldrich). The excitation wavelength was set at 450 nm (slit width = 2.5 nm), and emission was monitored at 460-540 nm (slit width = 5 nm). The readings were taken up to 120h.

#### **4.2.5 Static Right Angle Light Scattering Assay**

Static light scattering was recorded using spectrofluorometer (Jasco FP 8500) at 450 nm, for comparing aggregation pattern of the synthesized aggregating peptide sequences. The fresh peptide solution was prepared by dissolving the lyophilized form of the peptide in deionized water (at 95 °C) to a 3 mM concentration. The peptide solution (at 95 °C) was taken in a fluorescence cuvette (Helma, Sigma-Aldrich) of 1 cm path length, and its static light scattering was recorded immediately for 60 seconds till 72h.

#### **4.2.6 Fourier transform infrared spectroscopy (FTIR)**

Fourier transform infrared (FTIR) spectroscopy is used to detect the presence of the secondary structure of proteins. An increase in  $\beta$ -sheet secondary structure indicates amyloid fibril formation. The peptide solution was prepared with double distilled water or suitable buffer (pH 7.4). 10-20  $\mu$ L of 0.5 mM peptide solution mixed with small amount of KBr and pulverized to form into a disk-shaped pellet. All spectra were recorded in the frequency range 500-4000  $\text{cm}^{-1}$ , at a resolution of 2  $\text{cm}^{-1}$ , with a scanning speed of 2 mm/sec and 30 scans per sample. The average data is plotted.

#### **4.2.7 Field-emission transmission electron microscopy (FETEM)**

FETEM images were recorded for the dried peptide samples using a JEM-2100F (JEOL, Japan) transmission electron microscope at the Central Instruments Facility, IIT Guwahati to probe into the morphology of the peptide structures. 10  $\mu$ L of the peptide sample was deposited on a carbon-coated 200 mesh sized copper grid. After 2 minutes, the excess sample was blotted out from the periphery of the grid and was subsequently stained with a negative stain of 2% uranyl acetate solution. The dye was then blotted out after 30 seconds, and the sample was allowed to air dry.

#### **4.2.8 Basin Energy Calculations**

Our statistical analysis is based on a set of more than 24,000 non-redundant protein structures available in the PDB database. The dataset was downloaded from PDB, and dihedral angles were calculated for each PDB structure. The frequency dihedral distributions for each amino acid were mapped. This frequency distribution was used to calculate statistical basin-associated free energy for each amino acid. The free energy associated with aggregation ( $\Delta G_A$ ) was calculated by summation of free energies associated with the dihedrals of steric zipper PDBs. The free energy of folding ( $\Delta G_F$ ) was calculated by summation of the lowest energy basins of  $\Phi$ ,  $\Psi$ ,  $X_1$ , and  $X_2$  dihedral distributions for each amino acid.  $\Delta G_A$  and  $\Delta G_F$  for each peptide sequence were divided by peptide length for normalization. The difference in free energy of folding and aggregation was calculated by subtracting normalized  $\Delta G_A$  from normalized  $\Delta G_F$ .

### **4.3 RESULTS AND DISCUSSION**

#### **4.3.1 Synthesis and Characterization of Peptides**

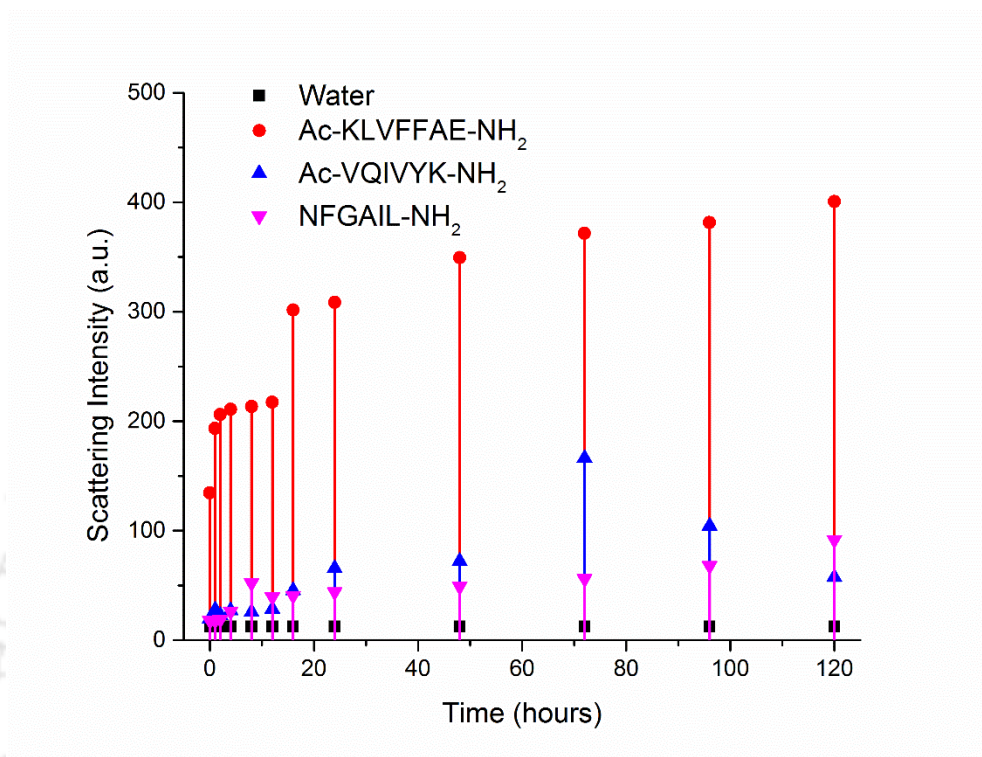
We synthesized the 9 model peptide systems, purified using high-performance liquid chromatography (HPLC) and verified its purity using MALDI-TOF mass spectrometry (Appendix Table A1). Table 4.1 shows the list of synthesized peptide sequences already reported to exhibit aggregation. To test our postulation, we dissolved Ac-KLVFFAE-NH<sub>2</sub>, Ac-VQIVYK-NH<sub>2</sub> and NFGAIL-NH<sub>2</sub> peptides in water and allowed them to aggregate.

**Table 4.1** Summary of synthesized aggregating peptide sequences.

| Id | Sequence                   | PDB ID           | Source                         | Associated Disease  |
|----|----------------------------|------------------|--------------------------------|---------------------|
| A1 | Ac-KLVFFAE-NH <sub>2</sub> | 3OW9<br>(KLVFFA) | Amyloid $\beta$ (16-22)        | Alzheimer's disease |
| A2 | MVGGVVIA-NH <sub>2</sub>   | 2Y3K             | Amyloid $\beta$ (35-42)        |                     |
| A3 | Ac-VQIVYK-NH <sub>2</sub>  | 2ON9             | Tau protein                    |                     |
| A4 | NKGAIL-NH <sub>2</sub>     | 3Q2X             | Amyloid $\beta$ (27-32)        |                     |
| A5 | LSFSKD-NH <sub>2</sub>     | 3LOZ             | Tau protein                    |                     |
| A6 | SSTNVG-NH <sub>2</sub>     | 3DG1             | Islet Amyloid Polypeptide      | Type 2 diabetes     |
| A7 | NFGAIL-NH <sub>2</sub>     | 1KUW             | Islet Amyloid Polypeptide      |                     |
| A8 | GNNQQNY-NH <sub>2</sub>    | 1YJP             | Yeast prion protein (sup35)    | Fungal prion        |
| A9 | SSTSAA-NH <sub>2</sub>     | 2ONW             | Bovine Pancreatic Ribonuclease |                     |

#### 4.3.2 Initial Assessment of Differential Aggregation Kinetics via Right Angled Scattering Light Scattering Assay

To ascertain the differential aggregation behaviour of the chosen peptide steric zipper peptide systems, we allowed peptides A1, A3 and A7 to aggregate under identical conditions and concurrently estimated the extent of aggregation using intermittent static light scattering measurements. The intensity of static light scattering is directly proportional to the degree of aggregation. We observed that scattering light intensities increased with time for all three peptides. However, this increase was differential, indicating their differential rates of aggregation (Figure 4.2).



**Figure 4.2** Static light scattering at right angle. The graph shows differential aggregation kinetics of A<sub>1</sub> (Ac-KLVFFAE-NH<sub>2</sub>), A<sub>3</sub> (Ac-VQIVYK-NH<sub>2</sub>) and A<sub>7</sub> (NFGAIL-NH<sub>2</sub>) peptides, measured in terms of scattering intensities recorded for a duration of 120 hours.

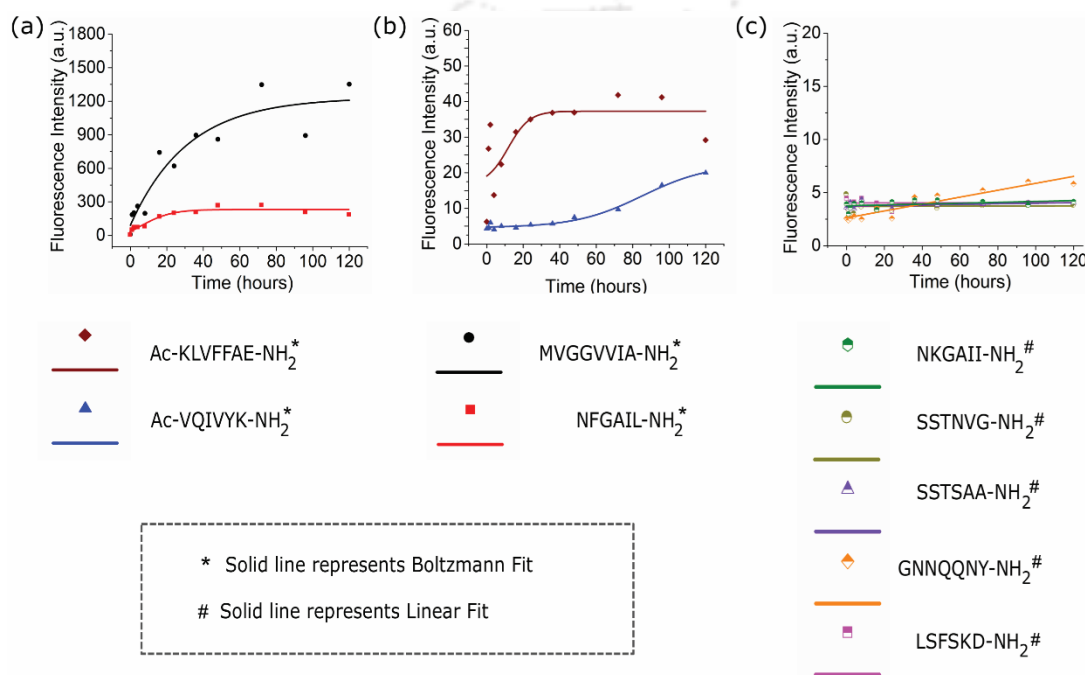
#### 4.3.3 Aggregation Kinetics Estimation using ThT Fluorescence Assay

The preliminary results from this experiment directed us to compare the aggregation kinetics of all 9 model peptides. Thus, 9 peptides were allowed to aggregate under identical conditions, by dissolving in water. Thioflavin T (ThT) binding assay was performed at regular intervals, to quantify the aggregation in each of the peptide systems.

The intensity of ThT fluorescence emission is directly proportional to the extent of aggregation. Based on their rate of aggregation, derived from the change in ThT



fluorescence intensities with time, the peptides may be divided into 3 clusters: (a) fast, (b) medium, and (c) slow aggregating peptides as shown in Figure 4.3. The ThT fluorescence assay data of slow and medium aggregating peptides were fitted linearly, and in the case of fast aggregating peptides, sigmoidal fitting has been performed using the Boltzmann function.

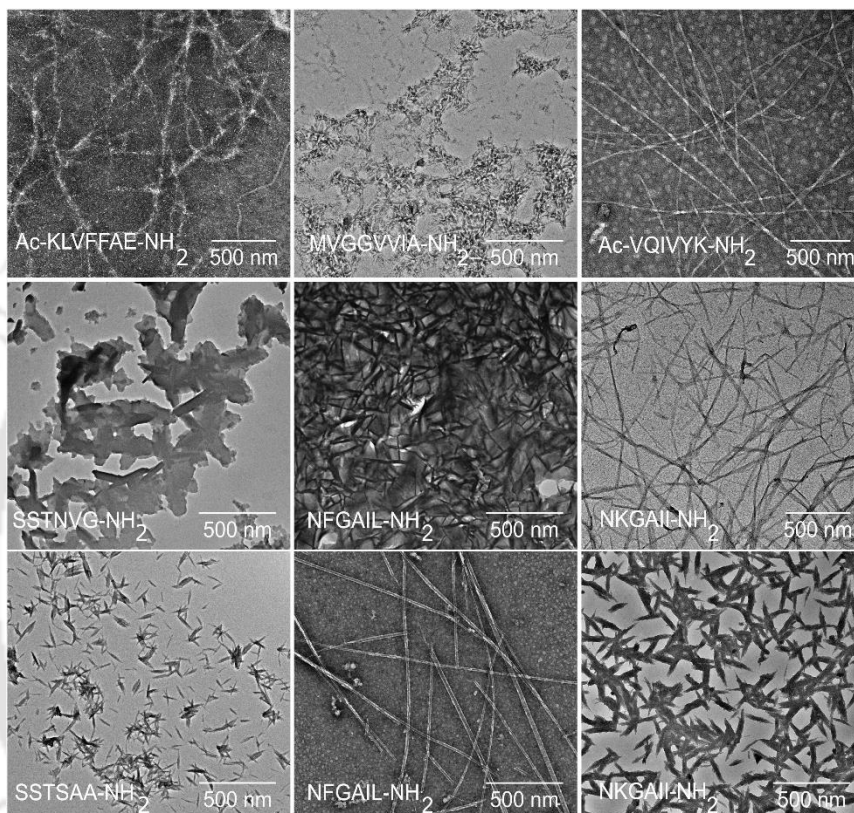


**Figure 4.3** ThT fluorescence plots of aggregating peptides: showing their kinetics of aggregating over a duration of 120 hours. The peptides have been divided into (a) fast (a) medium and (c) slow aggregating peptides.

#### 4.3.4 Morphology Examination by FETEM

Field-Emission Transmission Electron Microscope (FETEM) microscopy was used to confirm the propensity of peptides to form fibrillar morphologies. Owing to the difference in their rates of aggregation, the peptide samples were allowed to aggregate at varied time points, followed by morphological studies using electron

microscopy. The TEM micrographs of A1, A2, A3, A4, A7 and A8 samples showed the presence of long fibrillar self-assemblies. Peptides A5, A6, and A9 showed presence of thin proto-fibril like structures, upon TEM-based examination (Figure 4.4). Jain et al. have previously reported similar proto-fibrillar morphologies [40].

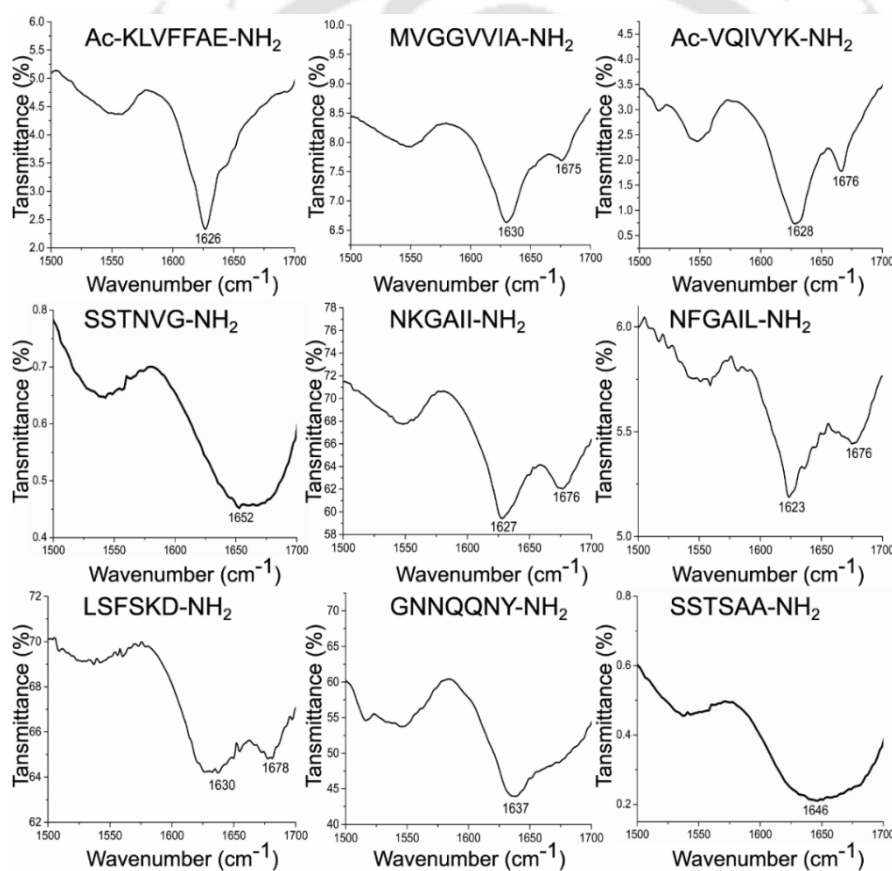


**Figure 4.4** FETEM micrographs of 9 models aggregating peptide systems allowed to aggregate for varied time duration, showing the classical fibrillar morphologies associated with amyloid forming systems.

#### 4.3.5 Conformational Characterization by FTIR

The peptides were dissolved in water by heating to 95 °C, and allowed to aggregate for 120 hours at room temperature. The conformational states of peptides were monitored by Fourier-transform infrared spectroscopy (FTIR). A1 and A8 displayed

a single transmittance band around  $1626\text{ cm}^{-1}$  and  $1637$  in the amide I region, suggestive of a parallel  $\beta$ -sheet structure [41-43]. Samples A2, A3, A4, A5, and A7 showed transmittance signal having major band around  $1623$ - $1630\text{ cm}^{-1}$ , followed by a minor band ranging from  $1675$  to  $1678\text{ cm}^{-1}$ , indicative of an anti-parallel  $\beta$ -sheet profile (Figure 4.5). The ratio of major and minor bands has been stated to be proportional to the percentage of anti-parallel arrangement of the  $\beta$ -strands in a  $\beta$ -sheet [41, 44].



**Figure 4.5** FTIR spectra of the steric zipper peptides.

FTIR spectra of A6 displayed a band around  $1652\text{ cm}^{-1}$ , while A9 showed a signal around  $1646\text{ cm}^{-1}$ . The spectra of both A6 and A9 fall in the intermediate zone, and

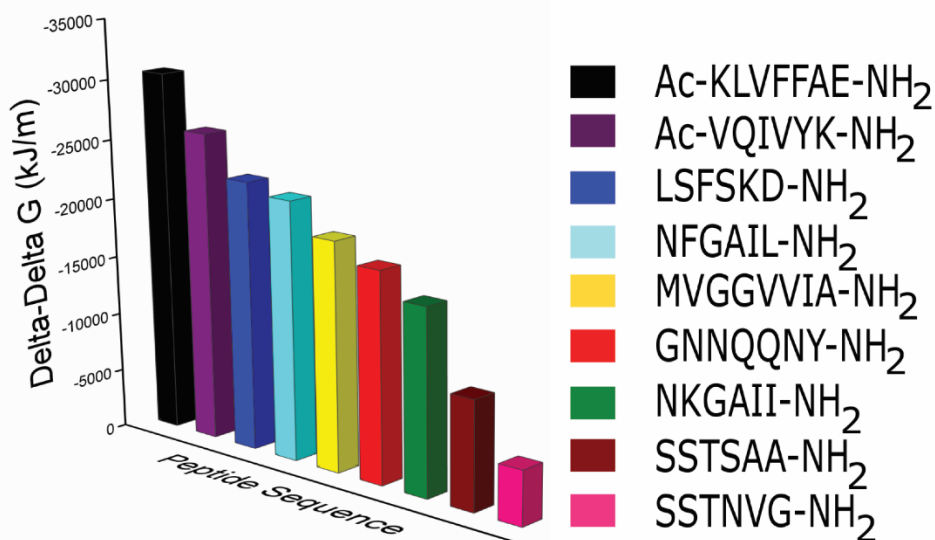
thus can be either assigned to an  $\alpha$ -helix or a random coil [41]. The presence non  $\beta$ -signature for peptides A6 and A9, further explains their slow aggregation rates.

#### **4.3.6 Geometrical Directives for Aggregation Kinetics**

In one of our previous studies, we have mapped the geometrical evolution of the protein folding structure from a set of more than 24,000 non-redundant protein structures available in the PDB database [39]. The rotational plane was divided into four basins and dihedral distributions for each amino acid were mapped. This frequency distribution of dihedral angles different rotational basins was used to generate three substitution matrices (MIDMATs) by calculating the statistical basin-associated free energy of protein folding for each amino acid. The basin-associated energy distributions in MIDMAT-1 were used for the calculation of basin-associated free energy for the aggregating peptides used in the study using their crystal structures from the PDB database. This basin-associated free energy for aggregation is henceforth mentioned as the “aggregation-associated free energy”. The aggregation-associated free energy was normalized for each aggregating peptide by dividing it with the length of the peptide to prevent biases arising from the length of different peptides. The ideal free energy of protein folding was calculated by summation of the lowest energy basins of  $\Phi$ ,  $\Psi$ ,  $X_1$ , and  $X_2$  dihedral distributions for each amino acid from MIDMAT-1 matrix, henceforth termed as “Free energy of protein folding”. This free energy of protein folding for each peptide was divided by peptide length for normalization.

An energy landscape for the protein aggregation and protein folding has been suggested by Patricia L. Clark [45]. The differences in various energy minima in such landscapes are often understood to be the driving force for a spontaneous protein folding/aggregation process. Therefore, it is hypothesized that a protein with the most plausible pathway would have the highest rate of aggregation. We begin our investigation by comparing the free energies of folding and aggregation of all nine steric zipper peptides synthesized and characterized by *in vitro*

experiments. We estimated the free energy of the coiled structure of each sequence, from the most stable conformational basin statistics of amino acid rotational isomers, adapted from Dunbrack library. The free energy of aggregated structure of the steric zipper has also been estimated from their respective crystal structures reported in protein data bank. Free energy difference between the coiled and aggregated conformations were calculated by employing the already reported, free energy calculations based on basin statistics [39]. We found that the kinetics of the set of peptides examined in this study was correlating well with their free energy estimates, except in the case of LSFSKD (Figure 4.6).



**Figure 4.6** Free energy difference plot of model aggregating peptide systems, obtained by subtracting aggregation-associated free energy from their respective random coil structure.

This result however, is inconclusive. To understand the geometric origins of aggregation kinetics, we undertook a detailed examination of structural evolution during aggregation. This rather cumbersome and demanding exercise could not be completed within the time of submission of this thesis, and hence not presented.



#### **4.4 CONCLUSIONS**

A comprehensive understanding of factors regulating the kinetics of amyloidogenesis of these steric zippers may serve to understand the folding and aggregation energy landscapes. This would enable possible interventions at molecular level, to modulate the folding of such sequences to a functional form, precluding aggregation. In this study, we have attempted to achieve this objective, by using biophysical techniques to characterize the aggregation kinetics of nine steric zipper peptides along with a rigorous statistical and computational analysis to investigate the geometrical rationale for the differential aggregation rates of our model peptide systems. The first part of the objective has been successfully completed, and the theoretical methods to compute the energetics are in progress. Therefore, it may be pre-mature to conclude this chapter with a clear understanding, and hence not attempted.

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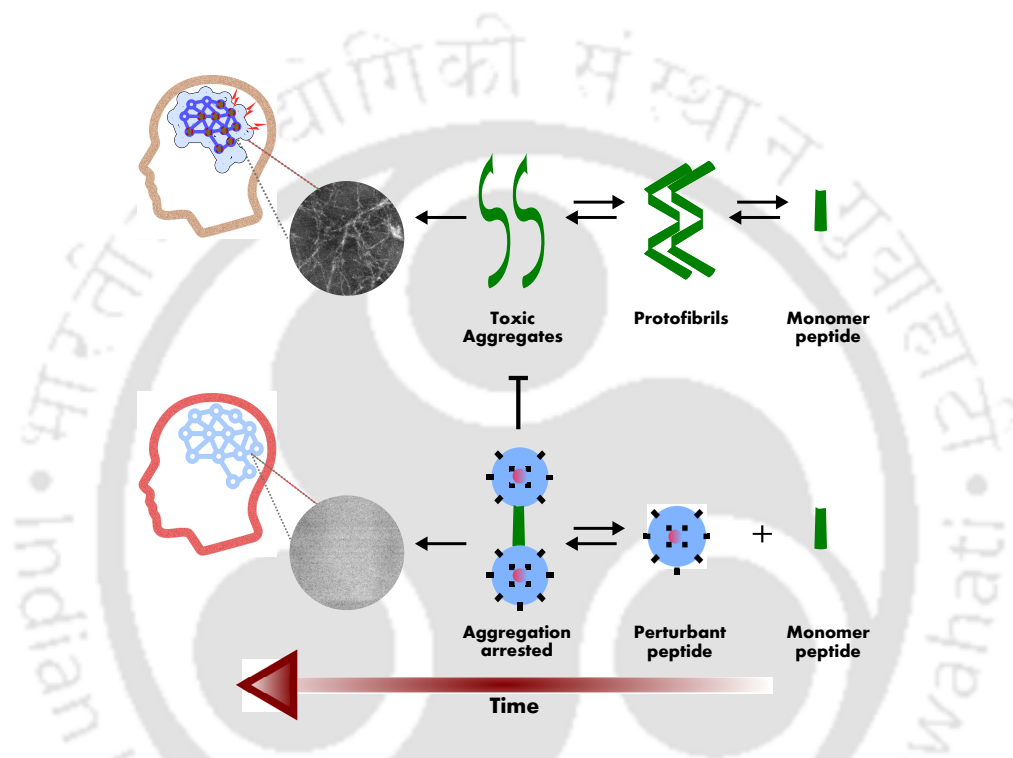
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## CHAPTER 5

### MOLECULAR PERTURBATION OF AMYLOID

#### SELF-ASSEMBLY



Neurodegenerative disorders are incurable, resulting in progressive loss of nerve cells, often devastating for the suffering patient. Progressive neurodegeneration causes difficulties with movement (called ataxias), or cognition and memory (called dementias). Dementias are accountable for the major burden of neurodegenerative diseases, with Alzheimer's representing approximately 60-70% of dementia cases. In the course of this study, we demonstrate the application of peptide-based perturbants explicitly designed to intervene in the aggregation of core recognition domain of amyloid-beta peptide, a significant malefactor in Alzheimer's disease.



## 5.1 INTRODUCTION

Dense plaques originating from amyloid-beta ( $A\beta$ ) self-assembly have been recovered in substantial amounts in the autopsied brain of Alzheimer's disease (AD) patients.  $A\beta$  fibrillogenesis is thus regarded as a crucial component in triggering the AD pathology [1-3]. The old and conventional amyloid cascade hypothesis has been questioned in modern research owing to the insufficient correlation between  $A\beta$  buildup in the brain and cognitive damage in the AD patients. At the same time, tau protein aggregation has been reported to show a better correlation to the extent of loss of cognition and memory impairment [4-6]. Several recent reports however, persist in backing the central role of  $A\beta$  in AD [7, 8]. Overproduction of  $A\beta$  is observed in almost all the familial kinds of AD [9, 10]. The overexpression of mutant amyloid precursor protein (APP) in animal models has been reported to elicit the development of phenotypes linked with AD [11, 12]. Besides, the  $A\beta$  oligomers have exhibited excessive neurotoxicity [13-15], thus supporting the view of having a crucial role in the disease onset.

Extensive studies have concentrated on developing inhibitors of  $A\beta$  toxicity as possible AD therapeutics [16-20]. As per the amyloid stretch hypothesis, short amino acid stretches offer the significant driving force necessary to initiate the amyloid self-assembly, in a protein [21] and serve as core regions seeding the self-assembly. Therefore, developing molecules that specifically attach to these core amyloid stretches and block their association, might aid in preventing the aggregation and the neurotoxicity of the corresponding full-length protein.

Amino acid segment LVFF located at position 17 to 20 in  $A\beta_{(1-42)}$  has been described to form the central hydrophobic core, critical for the self-assembly of  $A\beta$  peptide into  $\beta$ -sheets. The  $\beta$ -sheet content is reduced upon substitution of the LVFF fragment in  $A\beta_{(1-42)}$  [22]. The compounds that block the LVFFA domain arrest the  $A\beta$  fibrillation [23]. Moreover,  $A\beta_{(16-22)}$  (KLVFFAE) is one of the shortest sequences that self-assembles to form amyloid fibrils, similar to full-length  $A\beta$  at neutral pH



[24, 25]. A $\beta$  (16–22) having aggregation dynamics identical to its parent peptide [26], may thus serve as an ideal template for evaluating the efficacy of designed amyloid aggregation inhibitors.

In this chapter we have employed a structure-based design approach, to generate a series of peptides aimed at modulating the self-assembly of Ac-KLVFFAE-NH<sub>2</sub> peptide. The peptides have been referred to as “perturbant peptides.” We have evaluated the efficacy of these designed peptide-based perturbants, using a series of biophysical techniques.

## **5.2 EXPERIMENTAL APPROACHES**

### **5.2.1 Materials**

All the chemicals and reagents were procured as per the highest grade available from industrial suppliers and used without any further purification.

### **5.2.2 Peptide Synthesis and Characterization**

Solid phase peptide synthesis was carried out using the standard Fmoc chemistry on Rink amide resin as described in chapter 4, section 4.2.1.

### **5.2.3 Preparation of Peptide Solution**

The peptides were weighed, and stock solutions were prepared in deionized water to achieve a concentration of 500  $\mu$ M. The peptides were incubated at room temperature for 24 h in the presence and absence of designed molecular perturbants at equimolar concentration.

### **5.2.4 Thioflavin T (ThT) Fluorescence Assay**

ThT fluorescence assay was performed for the peptides at different incubation time on a Jasco FP 8500 spectrofluorometer. 50  $\mu$ M of peptide solutions were prepared in 50 mM phosphate buffer (pH 7.4) and 10  $\mu$ M ThT. ThT fluorescence intensity was monitored for 60 seconds at an excitation wavelength set to 450 nm and an

emission wavelength of 485 nm. The emission and excitation slit widths were fixed to 2.5 nm and 5 nm respectively.

#### **5.2.5 Dynamic Light Scattering (DLS)**

The size distribution of aggregates formed was determined by Dynamic Light Scattering. DLS experiments were performed on the Zetasizer Nano ZS (Malvern Instruments) equipped with a 633 nm He-Ne laser source at 25 °C using the 173 degrees non-invasive backscattering configuration. Measurements were taken for 24 h incubated peptide samples. For each of the sample, the representative intensity-weighted hydrodynamic diameters were plotted. The average hydrodynamic diameter was measured based on ten scans.

#### **5.2.6 Field-emission transmission electron microscopy (FETEM)**

FETEM images were recorded to examine the peptide morphology examination. The samples were prepared and analyzed as described in chapter 4, section 4.2.6.

#### **5.2.7 Fourier transform infrared spectroscopy (FTIR)**

FTIR spectra were recorded for the dried peptide samples on a Shimadzu IRAffinity-1S Fourier Transform Infrared Spectrophotometer using the Attenuated total reflection (ATR) module and equipped with a diamond crystal. 10 µL of the samples were deposited on the crystal surface and allowed to dry. All spectra were recorded at a resolution of 4 cm<sup>-1</sup>, and an average of 64 scans was plotted.

#### **5.2.8 Cell Viability Assay**

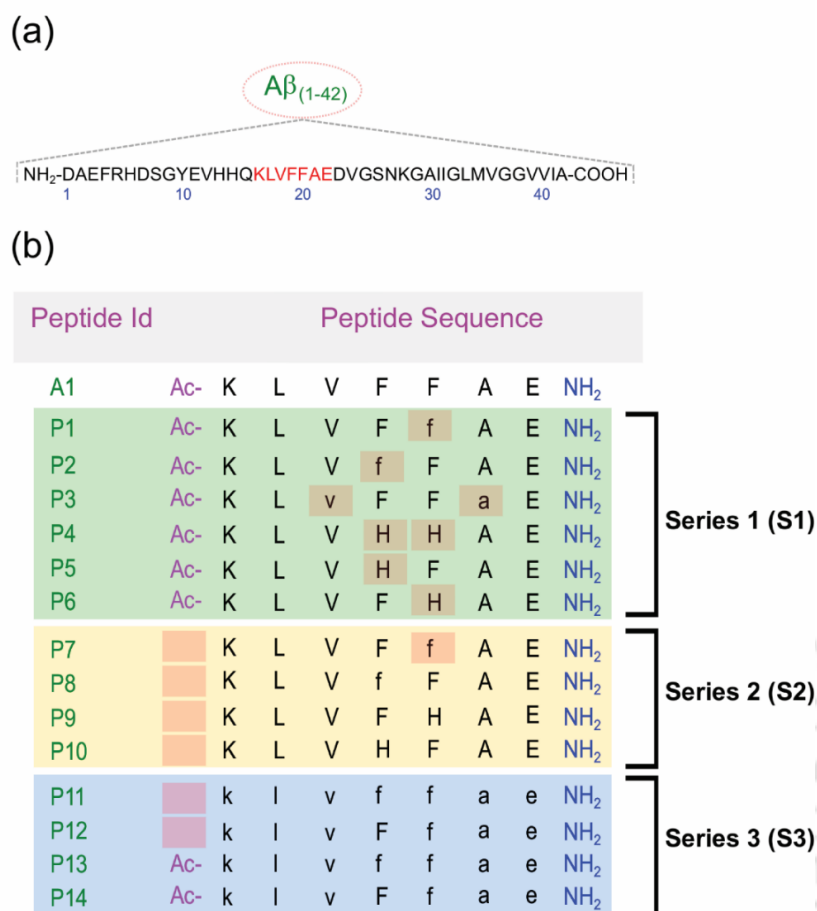
MTT assays were performed to assess the cytotoxicity of designed perturbant peptides, perturbant treated and untreated Ac-KLVFFAE-am and aggregates using neuroblastoma IMR-32 neuronal cell lines and HEK-293, human embryonic kidney cell lines purchased from the repository of the National Centre for Cell Science (NCCS) Pune, India. The IMR-32 and HEK-293 cells were maintained T25 culture

flask containing DMEM, medium. All the cells were supplemented with 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin at 37 °C under 5% CO<sub>2</sub> and were maintained for further assay by subsequent re-culturing. Before the MTT assays, each of the cells (80 µL) were cultured in 96-well plates at a density of  $5 \times 10^3$  cells/well for 24 h in a CO<sub>2</sub> cell culture box (Forma SteriCycle CO<sub>2</sub> Incubator 371, Thermo Scientific, USA). Then, the cells were treated with 20 µL control as well as treated peptide samples (25 µM) and for 24 h. Post incubation, 10 µL MTT solutions (5.5 mg/mL) were added into each well. The medium was replaced with 100 µL DMSO to dissolve the formazan crystals after 4h. Absorbance at 570 nm was measured by a multifunctional microplate reader (TECAN Austria GmbH, Grödig, Austria) to estimate the cell viability under respective treatments. Six replicates were performed, and the data were averaged.

## 5.3 RESULTS AND DISCUSSION

### 5.3.1 Design, synthesis and characterization of the peptides

The designed peptides were synthesized by solid phase peptide synthesis (SPSS) techniques. The synthesis scheme and purity assessment protocols employed were the same as discussed in chapter 4, section 4.2.1. Table A1 of appendix lists the mass of the synthesized peptides determined using MALDI-TOF. The amino acid and corresponding peptide id of the designed and synthesized peptide sequences are shown in Figure 5.1 (b). The perturbants have been divided into three series namely, S<sub>1</sub>, S<sub>2</sub> and, S<sub>3</sub>, highlighted in green, yellow and blue respectively.



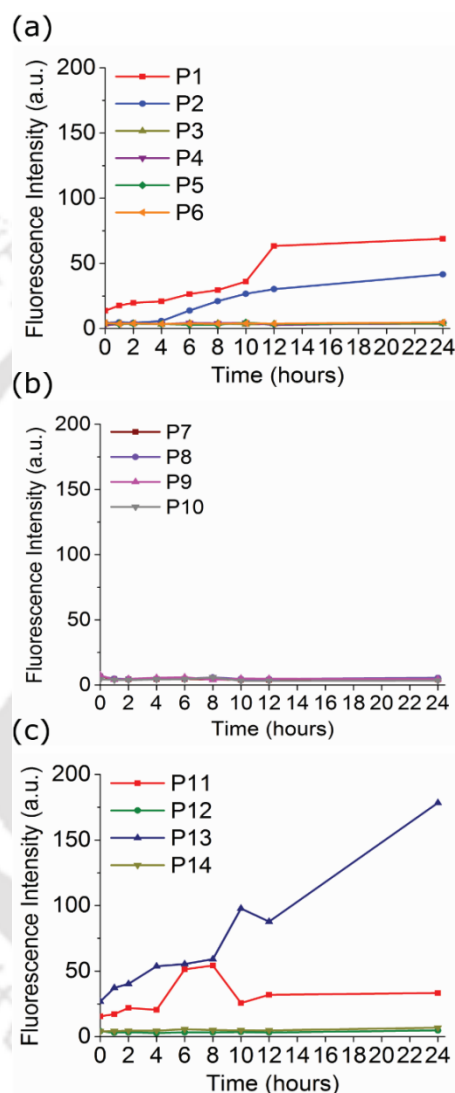
**Figure 5.1** Schematic representation of amino acid composition of  $A\beta_{(1-42)}$  peptide, with KLVFFAE segment as its core aggregation segment, (b) list of synthesized peptide sequences along with their respective id is shown classified into three distinct series.

### 5.3.2 Assessment of amyloidogenicity of the designed peptide-based perturbants

The amyloidogenicity of the designed peptides was monitored using spectroscopic and microscopic experiments followed by cell culture studies.

### 5.3.2.1 Thioflavin T fluorescence assay

The perturbant peptides were dissolved in water. The aggregation propensity was determined intermittently up to 24 hours, by adding ThT dye (Figure 5.2).



**Figure 5.2** ThT assay profiles of perturbant peptides. The data shows time-dependent ThT fluorescence measurements for  $S_1$ ,  $S_2$  and  $S_3$  perturbant peptides, which are allowed to aggregate under ambient conditions.

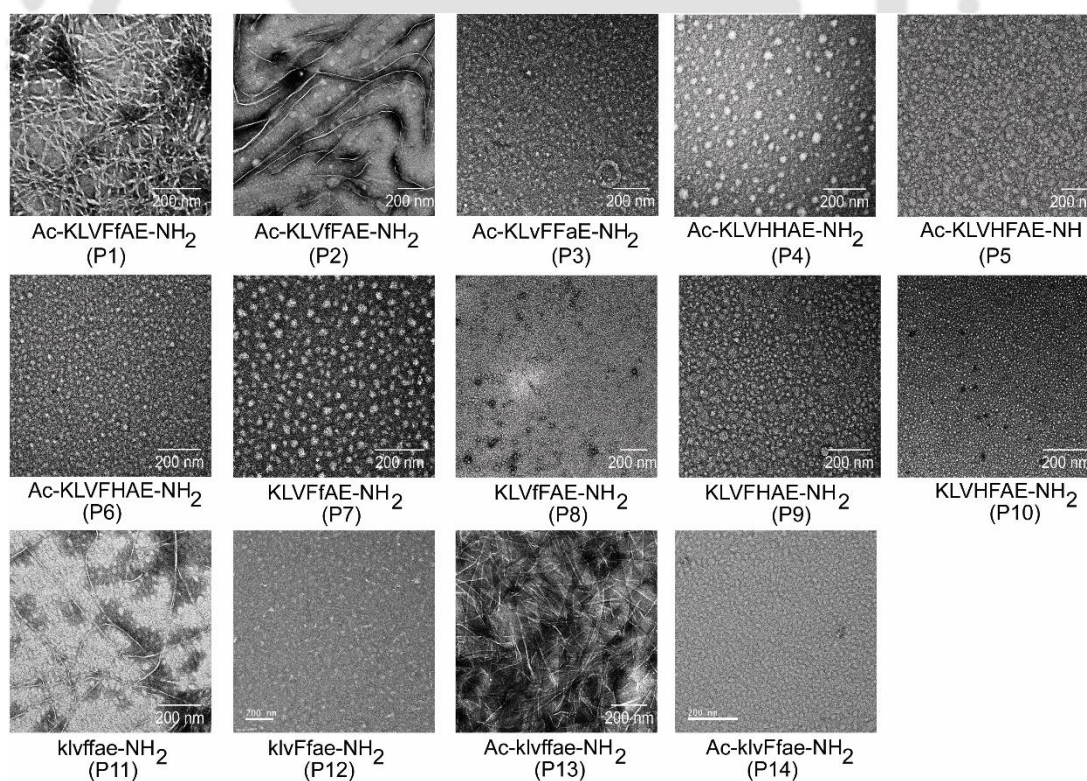
The experimental results show that for P1, P2, P11 and P13 samples, ThT fluorescence intensity increased with time, indicating the amyloidogenic character



of these peptides. In contrast, peptides P3-P6, all S2 series peptides along with P12 and P14 did not show any significant increase in fluorescence up to 24 hours, suggesting their non-amyloidogenic.

### 5.3.2.2 FETEM Analysis

To further investigate the self-assembling nature of our designed peptides, we drop casted the water dissolved peptides, previously incubated for 24 hours, at room temperature and recorded the FETEM images of the dried samples (Figure 5.3). Samples P1, P2, P11, and P13, showed the presence of dense fibrillar assemblies, an attribute of amyloidogenic peptides. FETEM results further confirmed the high aggregation propensity of P1, P2, P11, and P13 peptides. The FETEM micrographs P3-P6, S2 series, P12, and P14 peptides, corroborated with their ThT data and no fibrillar assemblies were observed for these samples.

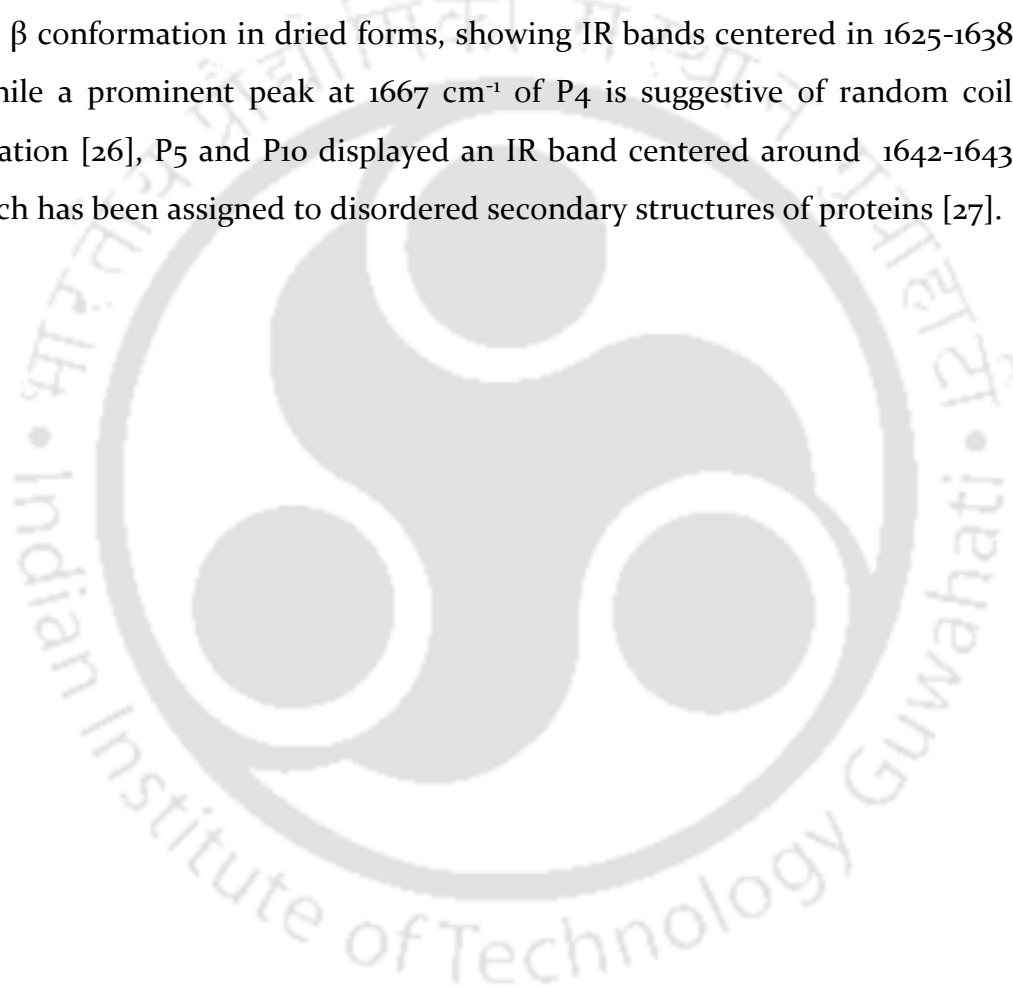


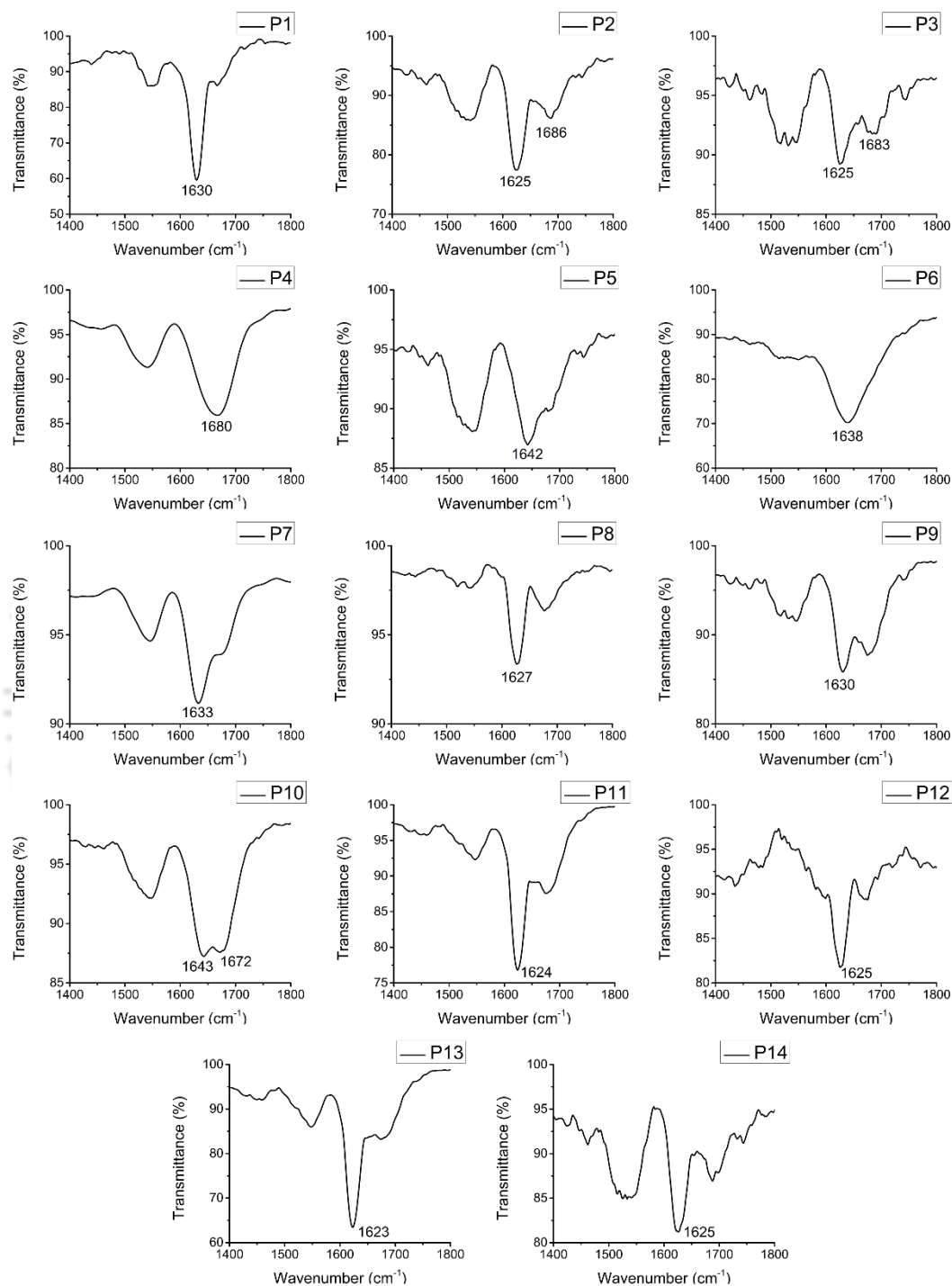
**Figure 5.3** FETEM micrographs of S1, S2 and S3 perturbant peptides.



### 5.3.2.3 FTIR Analysis

To probe into the secondary structural information of the perturbant peptides FTIR spectra of the dried films of the 24 h old peptide samples were recorded (Figure 5.4). The peptides P<sub>1</sub>, P<sub>2</sub>, P<sub>11</sub> and P<sub>13</sub> showed a characteristic amide I band of  $\beta$ -sheet (IR peaks centered around 1625-1630  $\text{cm}^{-1}$ ) which correlates with their enhanced ThT fluorescence. The peptides P<sub>3</sub>, P<sub>6</sub>, P<sub>7</sub>, P<sub>8</sub>, P<sub>9</sub>, P<sub>12</sub> and P<sub>14</sub> are found to retain  $\beta$  conformation in dried forms, showing IR bands centered in 1625-1638  $\text{cm}^{-1}$ . While a prominent peak at 1667  $\text{cm}^{-1}$  of P<sub>4</sub> is suggestive of random coil conformation [26], P<sub>5</sub> and P<sub>10</sub> displayed an IR band centered around 1642-1643  $\text{cm}^{-1}$  which has been assigned to disordered secondary structures of proteins [27].

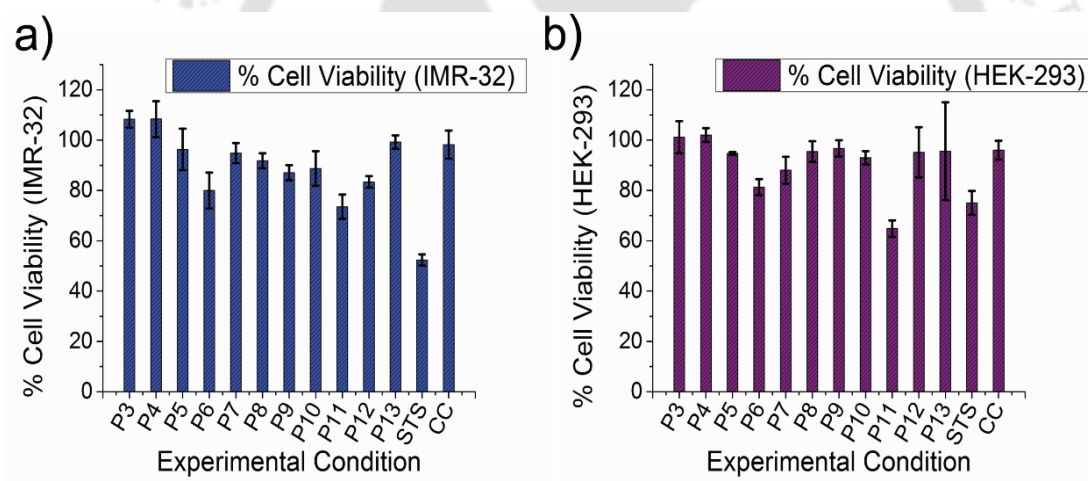




**Figure 5.4** FTIR spectra of *S*<sub>1</sub>, *S*<sub>2</sub>, and *S*<sub>3</sub> perturbant peptides.

### 5.3.2.4 Toxicity Estimation Study

The intrinsic toxicity of the perturbant peptides was analyzed by an *in vitro* cytotoxicity analysis on human neuroblastoma, IMR-32 neuronal and human embryonic kidney, HEK-293, cell lines. The peptide solutions were added to the cells and incubated for 48 hours. Samples added with staurosporine and untreated cells served as positive and negative control respectively. The toxicity was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. The IMR-32 and HEK-293 cell viability estimates are shown in figure 5.5 and table 5.1. The results of toxicity estimation studies show that each of the selected peptides except P11 showed less than 20% toxicity.



**Figure 5.5** Perturbant toxicity estimation. The inherent toxicity of designed peptide perturbants was estimated by adding them to (a) IMR-32 and (b) HEK-293 cells, and the cytotoxicity was determined using an MTT assay.

**Table 5.1** Summary of MTT assay data for IMR-32 and HEK-293 cell viability upon treatment with the designed peptide-based perturbants.

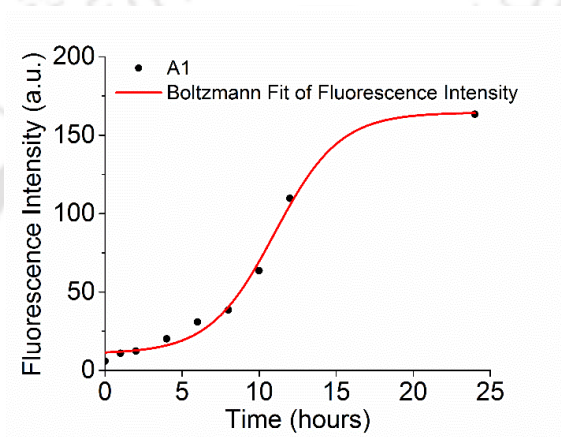
| Experimental Condition | % Cell Viability (IMR-32) | Std. Dev. | % Cell Viability (HEK-293) | Std. Dev. |
|------------------------|---------------------------|-----------|----------------------------|-----------|
| P3                     | 108.26316                 | 3.38769   | 101.14719                  | 6.39184   |
| P4                     | 108.35088                 | 7.1279    | 102.01299                  | 2.76031   |
| P5                     | 96.31579                  | 8.23798   | 94.69697                   | 0.58921   |
| P6                     | 79.98245                  | 7.11935   | 81.27706                   | 3.21434   |
| P7                     | 94.82456                  | 3.98416   | 88.0303                    | 5.3674    |
| P8                     | 91.80702                  | 2.98673   | 95.47619                   | 4.07404   |
| P9                     | 87.03509                  | 3.00339   | 96.68831                   | 3.23113   |
| P10                    | 88.7193                   | 6.87046   | 92.96537                   | 2.6074    |
| P11                    | 73.52631                  | 4.86863   | 64.82684                   | 3.28826   |
| P12                    | 83.38597                  | 2.29918   | 95.12987                   | 9.97784   |
| P13                    | 99.21053                  | 2.64837   | 95.58442                   | 19.44293  |
| STS                    | 52.36842                  | 2.23854   | 75.06493                   | 4.72422   |
| CC                     | 98.19298                  | 5.59391   | 96.01732                   | 3.7191    |

### 5.3.3 Effect of the designed peptides on model aggregating peptide

Next, we examined the modulatory effect of designed peptide perturbants on the aggregation kinetics of Ac-KLVFFAE-am (A<sub>1</sub>). The aggregating peptide, A<sub>1</sub> was allowed to aggregate in the presence and absence of equimolar concentration of each of the perturbant peptide. The effect of peptide treatment was checked using various biophysical and cytotoxicity assessment experiments.

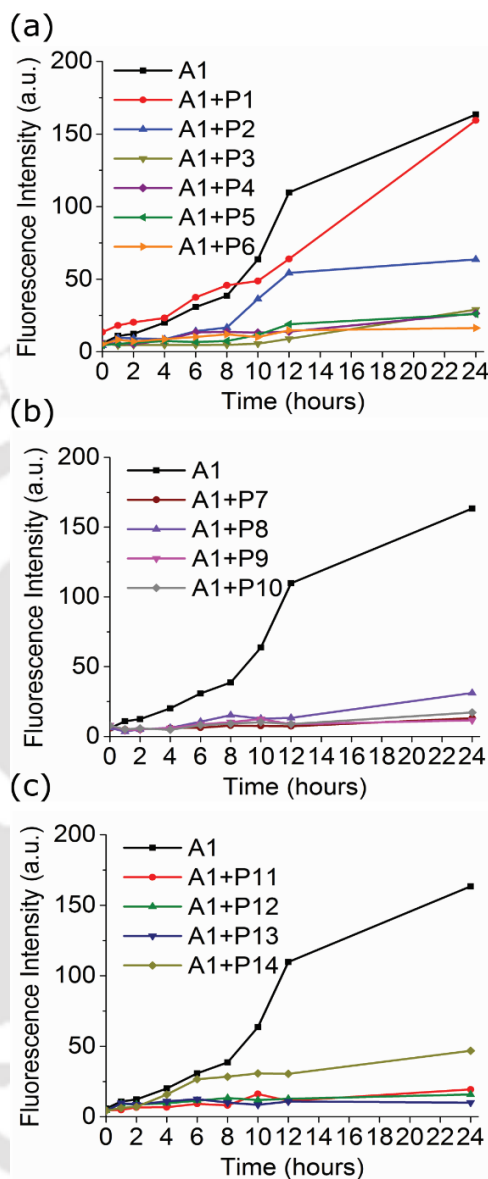
### 5.3.3.1 Examining the kinetics of self-assembly using Thioflavin T fluorescence assay

The quantitative estimation of self-assembly in treated and untreated of untreated A<sub>1</sub> peptide was tested by a time-dependent ThT fluorescence assay. The fluorescence intensity of untreated A<sub>1</sub> peptide increased with time, showing a characteristic sigmoidal curve (Figure 5.6).



**Figure 5.6** ThT fluorescence assay based aggregation kinetics profile of A<sub>1</sub> peptide. The fluorescence intensity increased with time. It was fitted using a Boltzmann function and showed a sigmoidal curve.

The A<sub>1</sub> peptide sample allowed to aggregate in the presence of S<sub>1</sub> peptides P<sub>3</sub>, P<sub>4</sub>, P<sub>5</sub> displayed around the 6-fold decrease in ThT fluorescence. P<sub>6</sub> treated peptides showed a much improved 10-fold reduction in ThT fluorescence, compared with the untreated A<sub>1</sub> peptide. On the contrary, the A<sub>1</sub> peptide allowed to aggregate in the presence of P<sub>1</sub> showed a negligible change in its aggregation kinetics profile, while P<sub>2</sub> treated peptide showed a 2.5-fold reduction in fluorescence signal (Figure 5.7 and Table 5.2).



**Figure 5.7** Effect of designed peptide perturbants on model amyloidogenic peptide. Fibrillation kinetics of A1 was assessed by ThT fluorescence emission in the presence and absence of (a) S<sub>1</sub>, (b) S<sub>2</sub>, and (c) S<sub>3</sub> perturbant peptides.

The A1 samples, treated with S<sub>2</sub> peptides P7, P8, p9 and P10 displayed around 12.5, 5, 14 and 10-fold decrease in the ThT fluorescence respectively (Figure 5.7 and Table 5.2). Moreover, A1 peptide showed 8.5, 10, 16, and a 3.5-fold decline in the ThT



## Chapter 5

### Molecular Perturbation of Amyloid Self-Assembly

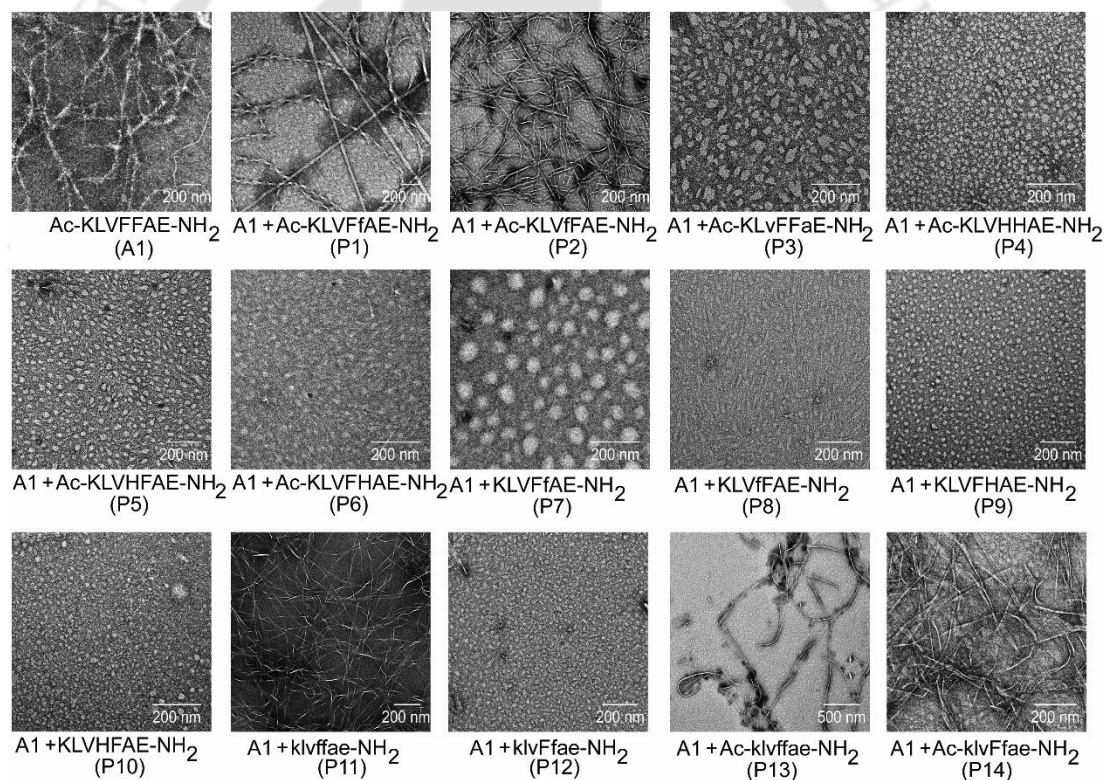
fluorescence upon treatment with P<sub>11</sub>, P<sub>12</sub>, P<sub>13</sub> and P<sub>14</sub>, S<sub>3</sub> perturbants (Figure 5.7 and Table 5.2).

**Table 5.2** Comparative thioflavin T fluorescence data for perturbant treated and untreated A<sub>1</sub> peptide samples.

| Time  |  | Fluorescence Intensity (a.u.) |           |          |          |          |          |          |              |
|-------|--|-------------------------------|-----------|----------|----------|----------|----------|----------|--------------|
| hours |  | A1                            | A1+P1     | A1+P2    | A1+P3    | A1+P4    | A1+P5    | A1+P6    |              |
| 0     |  | 5.92843                       | 13.53509  | 4.33789  | 6.15016  | 5.90935  | 4.58067  | 5.27976  |              |
| 1     |  | 10.88518                      | 18.1833   | 9.65815  | 4.33932  | 5.64473  | 5.42674  | 8.29963  |              |
| 2     |  | 12.36109                      | 20.31797  | 9.28343  | 4.70771  | 5.25117  | 6.2013   | 7.14381  |              |
| 4     |  | 20.07077                      | 23.32051  | 8.65533  | 4.64907  | 8.00232  | 7.39766  | 8.70642  |              |
| 6     |  | 30.84014                      | 37.45674  | 14.2252  | 4.72634  | 13.39754 | 6.82611  | 10.27702 |              |
| 8     |  | 38.63131                      | 45.73864  | 16.69394 | 4.77836  | 13.76839 | 7.40874  | 11.99588 |              |
| 10    |  | 63.65656                      | 48.79428  | 36.41931 | 5.58949  | 13.24308 | 11.60294 | 10.16035 |              |
| 12    |  | 109.70516                     | 63.92382  | 54.32083 | 8.91501  | 13.92064 | 19.01318 | 14.76147 |              |
| 24    |  | 163.3543                      | 159.38856 | 63.62716 | 29.07217 | 26.40886 | 25.98904 | 16.37056 |              |
|       |  | A1+P7                         | A1+P8     | A1+P9    | A1+P10   | A1+P11   | A1+P12   | A1+P13   | A1+P14       |
| 0     |  | 5.81579                       | 6.07333   | 7.91799  | 7.78984  | 4.58143  | 4.64217  | 3.99856  | 4.19374      |
| 1     |  | 5.0344<br>2                   | 3.54972   | 4.5486   | 5.11656  | 4.86869  | 9.11704  | 9.71359  | 6.32774      |
| 2     |  | 4.9495                        | 5.08634   | 4.98133  | 5.76326  | 6.66155  | 8.67003  | 8.66596  | 7.16213      |
| 4     |  | 6.07189                       | 6.12874   | 6.02488  | 4.77042  | 6.78586  | 9.64959  | 11.01921 | 15.78291     |
| 6     |  | 6.23558                       | 10.52863  | 8.63888  | 8.06287  | 9.20636  | 11.44005 | 12.53233 | 26.6039<br>1 |
| 8     |  | 7.7598                        | 15.25198  | 10.24225 | 9.12625  | 8.14227  | 13.31848 | 10.04013 | 28.41461     |
| 10    |  | 7.4887                        | 12.71701  | 12.52697 | 10.1686  | 16.16231 | 11.84812 | 8.44418  | 30.76316     |
| 12    |  | 7.30793                       | 13.26944  | 8.77838  | 8.86754  | 11.32643 | 12.7381  | 10.83571 | 30.4204      |
| 24    |  | 13.1086<br>6                  | 31.1547   | 11.59894 | 17.13359 | 19.39073 | 15.82892 | 10.05016 | 46.8653<br>5 |

### 5.3.3.2 Morphological examination using FETEM

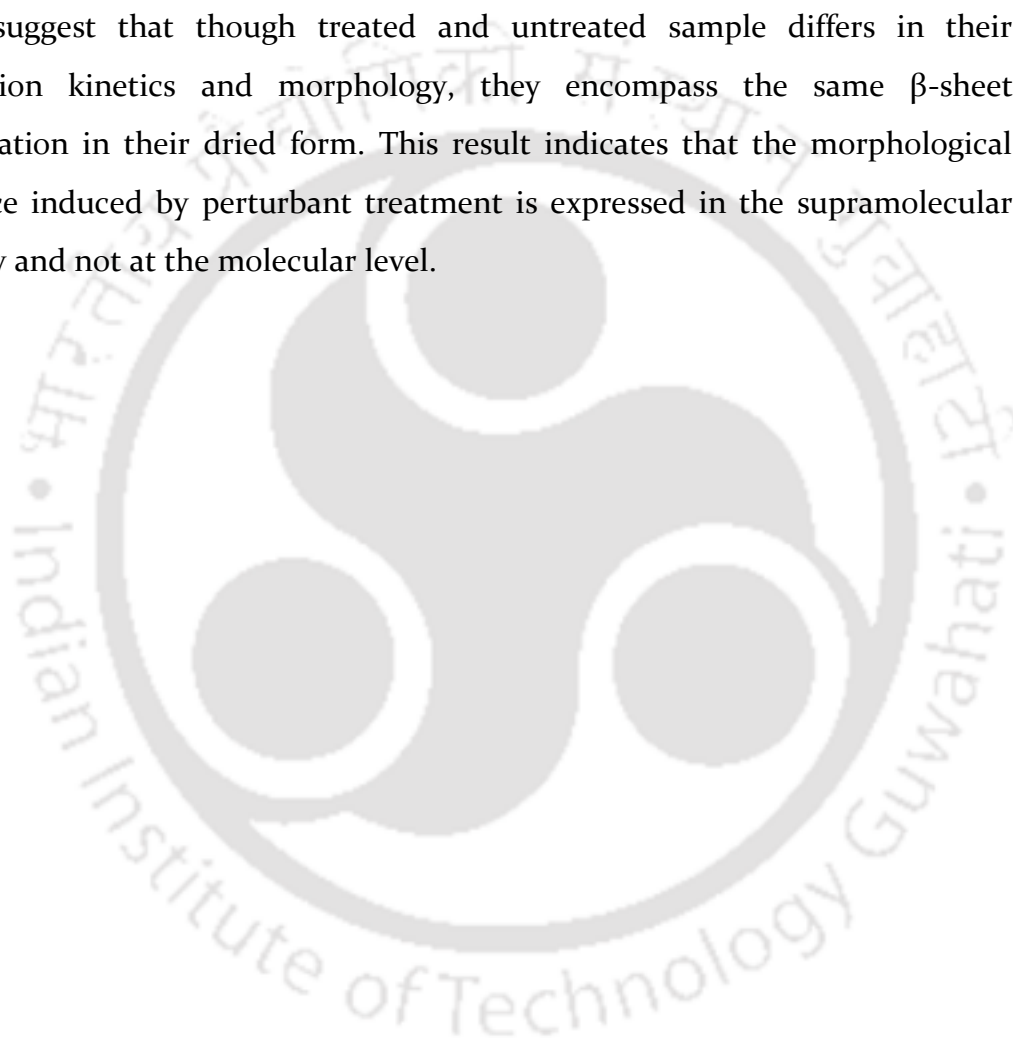
Field-emission transmission electron microscope (FETEM) images were recorded to study the effects of the designed molecular perturbants on the morphology of A<sub>1</sub> self-assembly. The untreated sample (A<sub>1</sub>), as well as the sample treated with peptides P<sub>1</sub>, P<sub>2</sub>, P<sub>11</sub>, P<sub>13</sub> and P<sub>14</sub>, displayed the presence of dense fibrils. Contrastingly, samples treated with peptides P<sub>3</sub>, P<sub>4</sub>, P<sub>5</sub>, P<sub>6</sub>, P<sub>7</sub>, P<sub>8</sub>, P<sub>9</sub>, P<sub>10</sub>, and P<sub>12</sub> showed spherical structures, and no fibrillar structures were observed indicating absence of the amyloid formation upon treatment (Figure 5.8).

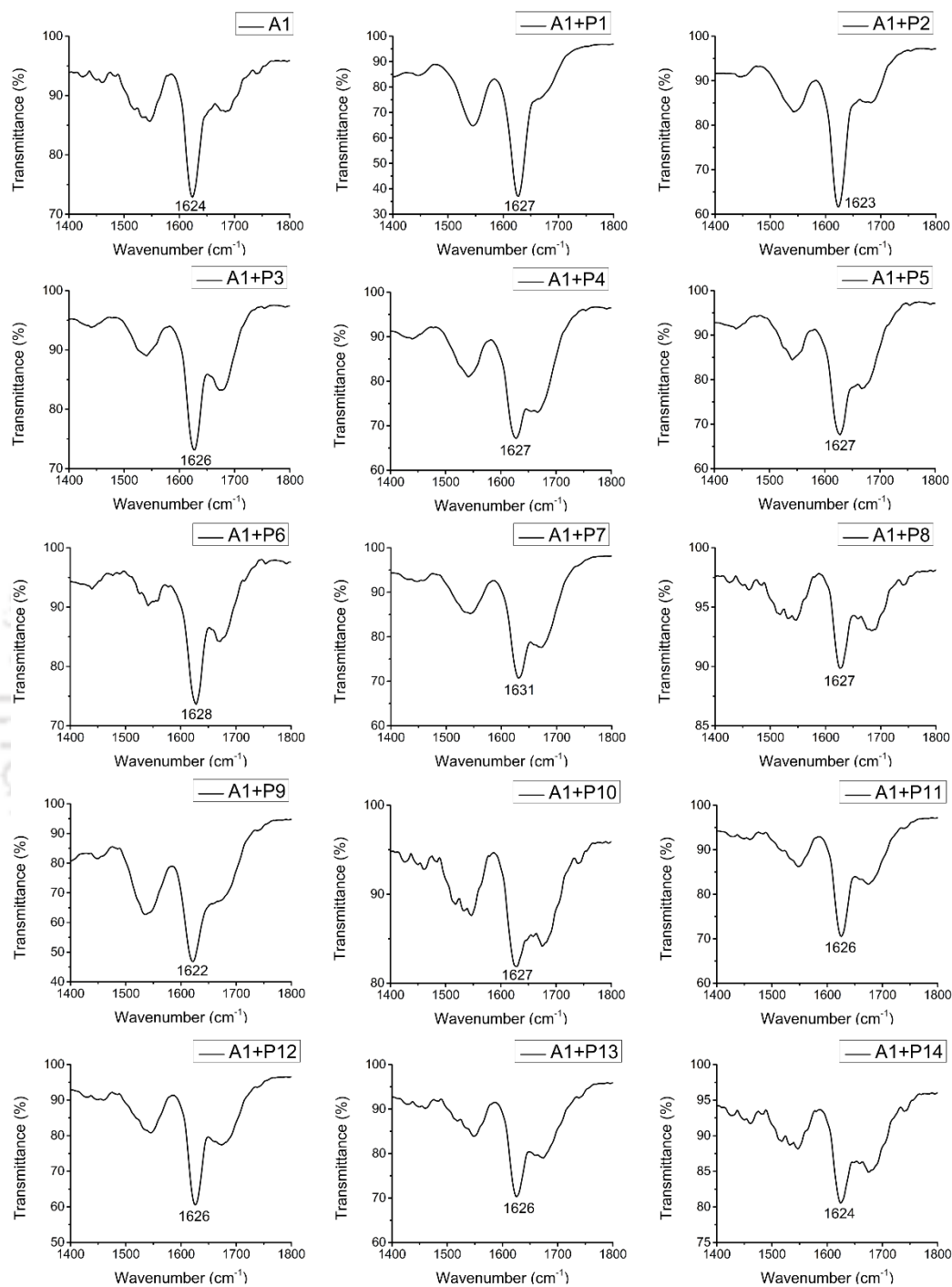


**Figure 5.8** FETEM analysis. FETEM micrographs of S<sub>1</sub>, S<sub>2</sub>, and S<sub>3</sub> perturbant treated and untreated A<sub>1</sub> peptide.

### **5.3.3.3 Secondary structure elucidation using FTIR**

The secondary structure conformations of the treated and untreated A<sub>1</sub> samples were determined by FTIR analysis. The dried films of both treated as well as untreated A<sub>1</sub> peptide samples showed the presence of the amide I band, a characteristic signature of  $\beta$ -sheet around 1622-1631 cm<sup>-1</sup> (Figure 5.9) [28, 29]. FTIR results suggest that though treated and untreated sample differs in their aggregation kinetics and morphology, they encompass the same  $\beta$ -sheet conformation in their dried form. This result indicates that the morphological difference induced by perturbant treatment is expressed in the supramolecular assembly and not at the molecular level.



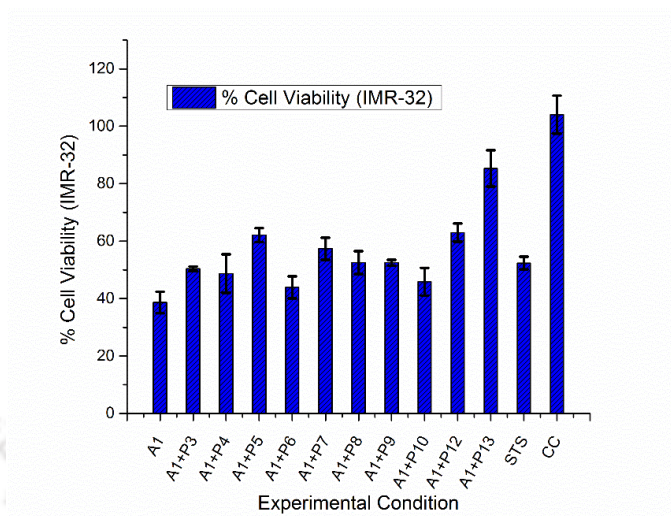


**Figure 5.9** Conformational analysis. FTIR spectra of *S3* perturbant treated and untreated *A1* peptide.

### **5.3.3.5 Cytotoxicity reduction analysis**

The transitions of a soluble protein into oligomers and further to fibrillar assemblies are essential phases in the amyloid-induced pathogenesis in AD. Molecules that can impede these transitions may be useful in the prevention or treatment of the disease. Thus, molecules capable of blocking these core amyloidogenic stretches like KLVFFAE may arrest not only the aggregation of the full-length protein, but also the amyloid-induced cytotoxicity. The deceleration of A $\beta$  self-assembly, by the designed molecular perturbants, suggests their potential efficacy for therapeutic applications in amyloidogenic diseases. Therefore, we estimated the enhancement in the cell viability as a result of perturbant treatment, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. A $\beta$  samples were allowed to aggregate in the presence (treated) or absence (untreated) of an inhibitor/accelerator perturbant peptide. Human neuroblastoma IMR-32 neuronal cell lines were treated with 25  $\mu$ M of perturbant treated and untreated A $\beta$  peptides solution. Staurosporine was added as a positive control, while untreated cells (denoted as cc) served as negative control. The test results show that the untreated amyloidogenic A $\beta$  peptide solution lowered the cell viability of IMR-32 cells to around 39%. The treatment with designed perturbant peptides, however, resulted in cell viability improvements from 5% to 46% in IMR-32 cell lines (Figure 5.10 and Table 5.3). Although the improvements in cell viabilities were significant except for P5, P12 and P13 peptides providing an increase of 22%, 23%, and 45% respectively, our design scheme arrested the aggregation of the chosen model peptide system and may need further optimizations to deliver improved cell-viabilities with more potent perturbants. The observations of low cell-viabilities for peptides blocking amyloid formation indicate that the arrested peptide may be in its toxic oligomeric form.





**Figure 5.10** Effect of peptide perturbants on the cellular toxicity of A<sub>1</sub> peptide. A<sub>1</sub> samples allowed to aggregate in the presence and absence of perturbant peptides were added to IMR-32 cells. The CC and STS denote untreated negative and staurosporine treated positive controls. The cytotoxicity effect was determined using an MTT assay.

**Table 5.3** Summary of MTT assay data for IMR-32 cell viability upon treatment with the designed peptide-based perturbant treated and untreated A<sub>1</sub> samples.

| Experimental Condition          | % Cell Viability (IMR-32) | Std. Dev. |
|---------------------------------|---------------------------|-----------|
| A <sub>1</sub>                  | 38.66264                  | 3.76495   |
| A <sub>1</sub> +P <sub>3</sub>  | 50.43992                  | 0.77675   |
| A <sub>1</sub> +P <sub>4</sub>  | 48.73052                  | 6.70008   |
| A <sub>1</sub> +P <sub>5</sub>  | 62.10407                  | 2.44982   |
| A <sub>1</sub> +P <sub>6</sub>  | 43.92911                  | 3.86515   |
| A <sub>1</sub> +P <sub>7</sub>  | 57.36551                  | 3.86796   |
| A <sub>1</sub> +P <sub>8</sub>  | 52.50126                  | 4.00575   |
| A <sub>1</sub> +P <sub>9</sub>  | 52.50126                  | 1.00781   |
| A <sub>1</sub> +P <sub>10</sub> | 45.85219                  | 4.8004    |
| A <sub>1</sub> +P <sub>12</sub> | 62.99648                  | 3.16031   |
| A <sub>1</sub> +P <sub>13</sub> | 85.33183                  | 6.35411   |
| STS                             | 52.36842                  | 2.23854   |
| CC                              | 104.03005                 | 6.613     |



## 5.4 CONCLUSIONS

Arresting the aggregation of amyloidogenic proteins and peptides holds much potential as a therapeutic approach for the treatment of proteopathies. The failure of small molecule-based amyloid modulators in clinical trials has raised questions on their efficacy. However, promising results from phase 1b clinical trials of a high-affinity anti-A $\beta$  antibody, aducanumab, have been encouraging [30-32]. Here, we designed three series of structure-based aggregation modulators for core-peptide segment of A $\beta$  <sub>(1-42)</sub> peptide by substituting the amino-acids in and around the hydrophobic domain of the peptide. This hydrophobic domain formed by two phenylalanine residues in the 19th and 20th positions of the A $\beta$  <sub>(1-42)</sub> peptide, has been reported to play a crucial role in the self-assembly of the polypeptide [33]. We demonstrated that most of our designed perturbant peptide showed minimal amyloidogenicity and cellular toxicity. The effect the designed peptides on the aggregation kinetics, morphology, secondary structure conformation and cytotoxicity of the A $\beta$  <sub>(16-22)</sub> peptide was also investigated. Our results showed that treatment with perturbants decelerated the aggregation kinetics of A<sub>1</sub> peptide, and prevented it to form the toxic fibrillar morphologies. The treated peptide still showed the  $\beta$ -sheet conformation in FTIR experiments, indicating that the modulatory effect of perturbants was limited to the assembly level. However, the results of MTT assay-based cytotoxicity studies showed that there were no significant improvements in the cell viability of the treated A<sub>1</sub> samples, indicating the perturbant mediated arrest may be happening at the oligomeric state of amyloid kinetics, and this oligomeric state is also considered to be cytotoxic. Our results offer new potential candidates for arresting amyloid self-assembly designed on the hydrophobic core of A $\beta$  <sub>(1-42)</sub> peptide as a template, though our design approach requires further optimizations and toxicity assays to improve their therapeutic potential.

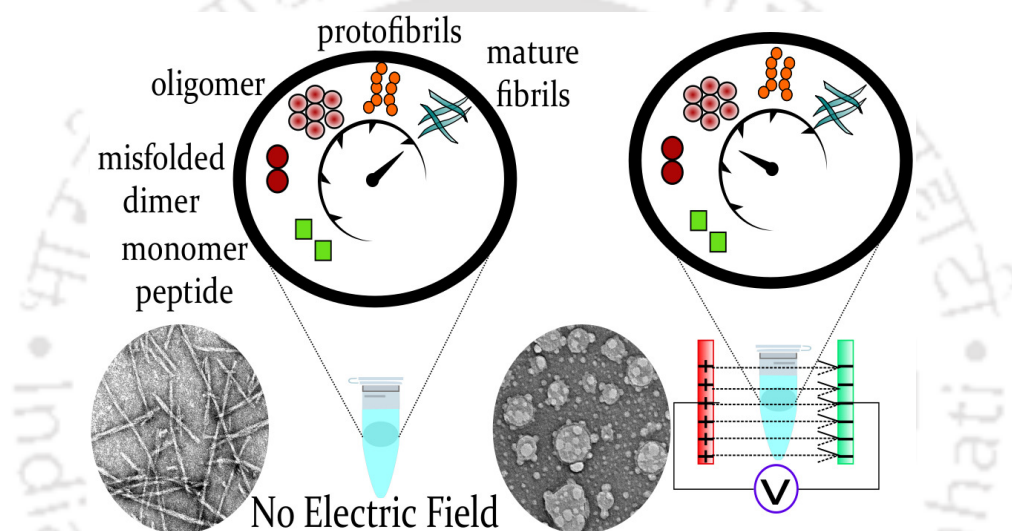
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## CHAPTER 6

# SCIENTIFIC ROADMAP FOR A POTENTIAL NON-INVASIVE THERAPY AGAINST TAUOPATHIES



Toxic aggregation of tau protein to neurofibrillary tangles (NFTS) is a central pathological event involved in tauopathies. Inhibition of tau protein aggregation can serve as a straightforward therapeutic strategy. However, tau-based therapeutic solutions are not very common. Phenothiazine methylene blue (tau protein inhibitor) is currently the only drug under phase III clinical trials. In this chapter, a non-invasive strategy is presented for modulating the aggregation of core peptide segments of tau protein (VQIVYK and VQIINK) by using electric fields of varying strengths.

An article based on this chapter is: Gaurav Pandey, et al., RSC Advances 9.9 (2019): 4744-4750.





## **6.1 INTRODUCTION**

Amyloid fibrils are naturally occurring nano-fibrils, associated with a large number of human diseases such as Alzheimer's disease (AD), Parkinson's disease, and type II diabetes mellitus. The phenomenon of amyloidogenesis involves the conversion of normally soluble peptides or proteins into insoluble amyloid plaques which eventually lead to progressive loss of neurons from specific regions of the brain.

AD is the most prevalent neurological disorder and affects approximately 50 million people worldwide [1]. Although this number is expected to increase to 132 million by 2050, there is no clinically approved therapy to cure or retard its progression [1]. Promising therapeutic strategies under research include native state stabilisation or fibrillation inhibition using small molecules [2-6], immunotherapy [7, 8], peptide or peptidomimetics [9-14], nanoparticles [15, 16], transient metals [17-20], supramolecular inhibition of fibrillation [21, 22], and sequestering the monomeric form of the peptide [23].

Nucleation-dependent aggregation is one of the most accepted models for the formation of amyloid fibrils [24-26]. As per this model, the process of fibrillogenesis involves a slow nucleation phase, wherein the monomeric peptide misfolds and undergoes self-assembly to form an oligomer. These oligomers serve as "nuclei" and are rich in  $\beta$ -sheets. The slow (rate-limiting) nucleation phase succeeds a rapid growth phase, which involves self-association of oligomers to yield protofibrils, and they further self-assemble to form mature amyloid plaques.

The small molecule-based therapeutic strategies to modulate aggregation have shown promise, while some have inhibited fibril formation, others have arrested fibrillar growth. Nonetheless, they show poor ability to penetrate the blood-brain barrier and are often highly toxic. Non-invasive therapies involving the application of scanning ultrasound [27, 28], light [29], transcranial stimulations [30, 31], etc. have also been found effective. Accumulation of misfolded proteins, beta-amyloid



(A $\beta$ ) and hyperphosphorylated tau, which aggregates and form amyloid plaques and tau tangles are regarded as the putative cause for AD onset. Although A $\beta$  has conventionally been considered as the major malefactor, the investigations in recent years have hinted a shift towards the crucial role, soluble oligomers and neurofibrillary tangles of tau play in the AD disease condition [32, 33]. The instigation of tau protein aggregation, linked with over 20 neurological disorders known as tauopathies, has been ascribed to the presence of two hexapeptide motifs “Ac-VQIVYK-am (PHF6) and Ac-VQIINK-am (PHF6\*)” [34]. These motifs are found in R<sub>2</sub> and R<sub>3</sub> repeat domains of tau protein and have been reported to be critical for conferring the strong aggregation tendency to tau protein [35]. Therefore, these peptide segments are regarded as the ideal model for assessing the potential therapies for the AD [36]. In this chapter, we report an externally applied electric field (EF) induced deceleration of aggregation kinetics of core peptide segments of tau protein VQIVYK and VQIINK.

Several theoretical studies have already reported the potential ability of the electric field, to induce conformational changes in the protein/peptide conformation by modulating the dipole alignments [37-41]. Molecular dynamics-based studies in the presence of electric field have predicted that a constant electric field could induce a conformational switch from a  $\beta$ -sheet to an  $\alpha$ -helix through a global reordering of the dipole moments at the amide planes [42]. The sheet-helix transitions mediated by external electric fields have also been reported to disaggregate amyloid fibrils [43].

Several recent experimental findings have also confirmed the possibility of electric field mediated fibrillar disruption [44, 45]. Porchke et al. reported a cooperative change in the structure of bacteriorhodopsin, induced at a remarkably low field strength of 150 Vcm<sup>-1</sup> [46]. We have already tested the effect of the electric field mediated modulation on the core recognition motif of the  $\beta$ -amyloid polypeptide, diphenylalanine (L-Phe-L-Phe, FF). FF is also the shortest molecule known to self-

assemble into ordered nanostructures [47]. These findings stimulated our interest towards experimenting further the impact of the electric field upon core peptide segments of tau protein.

## **6.2 EXPERIMENTAL APPROACHES**

### **6.2.1 Materials**

Fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids and *N,N,N',N'*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU) were purchased from Novabiochem (Darmstadt, Germany). *N,N*-diisopropylethylamine (DIPEA), piperidine, trifluoroacetic acid, ethanedithiol, thioanisole, acetic anhydride, ThT dye were procured from Sigma-Aldrich, Rink amide resin, *N,N*-dimethylformamide, diethyl ether, and *m*-cresol from Merck, 1-hydroxybenzotriazole hydrate (HOBt) from Sisco Research Laboratory and 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) from TCI Chemicals Pvt. Ltd. All other reagents used, were of the highest grade available.

### **6.2.2 Peptide Synthesis and Characterization**

The core tau peptide sequences Ac-VQIVYK-am (PhF6) and Ac-VQIINK-am (PhF6\*) sequence were synthesized by solid phase peptide synthesis using rink amide resin and standard Fmoc chemistry, as described in chapter 4, section 4.2.1

### **6.2.3 Monomerization of Peptide**

The peptide was monomerized by an overnight pre-treatment with hexafluoroisopropanol (HFIP), and the solvent was evaporated by air drying/ using a speed vac.

#### **6.2.4 Pre-aggregation Assays**

##### **6.2.4.1 Dynamic Light Scattering (DLS)**

To ensure the absence of any pre-formed aggregates, the size estimation assay was performed by dynamic light scattering method. Sample containing 500  $\mu\text{M}$  (100  $\mu\text{L}$ ) peptide (both EF treated and untreated) with incubation times of 0h and 16 h, were examined as discussed in chapter 5, section 5.2.5.

##### **6.2.4.2 Static Right Angle Light Scattering Assay**

To ensure the absence of any pre-aggregation in peptide stock solution a right angle scatter was recorded using a spectrofluorometer (Jasco FP 8500) at 450 nm. The slit width was set at 2.5 nm.

##### **6.2.4.3 Tyrosine Absorbance Assay**

The tyrosine absorbance was recorded at 280 nm, to determine the soluble concentration of PhF6 peptide initially.

#### **6.2.5 Electric field Experiments**

The peptide solution was exposed to electric field environment immediately after the addition of deionized water, allowing it to aggregate in the presence of an electric field. No voltage was applied in the case of the control experiment. The following electric field conditions were employed:

- i) 150  $\text{Vcm}^{-1}$  AC and DC
- ii) 300  $\text{Vcm}^{-1}$  AC and DC
- iii) 0  $\text{Vcm}^{-1}$  (control)

#### **6.2.6 Aggregation Kinetics Evaluation by Thioflavin T Fluorescence Assay**

Thioflavin T binds to amyloid fibrils, not to monomer peptides and gives an enhanced fluorescence emission [49]. The enhanced fluorescence can be observed

by fluorescence microscopy or by fluorescence spectroscopy. To assess the rate of aggregation ThT binding assay was performed by combining 15  $\mu$ L, 500  $\mu$ M peptide to a solution of 10  $\mu$ M ThT in 50 mM sodium phosphate buffer. Fluorescence spectra were recorded on a spectrofluorometer (Jasco FP 8500) at 25 °C using a 1 cm path length quartz cuvette (Helma, Sigma Aldrich). The excitation wavelength was set at 450 nm (slit width = 2.5 nm), and a time scan emission was monitored at 485 nm (slit width = 5 nm).

#### **6.2.7 Tyrosine Absorbance Assay**

The tyrosine absorbance was recorded at 280 nm, to determine the soluble concentration of PhF6 peptide in respective samples. Post 16 h, the electric field was turned off, peptide samples were centrifuged at 12000g for 20 min, and the supernatant was collected, and its absorbance was measured at 280 nm.

#### **6.2.8 Dynamic Light Scattering (DLS)**

To determine the size distribution of aggregates formed in control, as well as electric field, exposed samples, dynamic light scattering analysis, was performed, as described in the section 6.2.4.1, after an incubation time of 16 hours and 6 hours for PhF6 and PhF6\* respectively.

#### **6.2.9 Static Right Angle Light Scattering Assay**

To compare the extent of aggregation in the electric field exposed sample to the control sample a right angle scatter was recorded, same as described above, after an incubation 16 hours and 6 hours for PhF6 and PhF6\* respectively.

#### **6.2.10 Cell Viability Assay**

MTT assays were performed to assess the cytotoxicity of PhF6 and PhF6\* aggregates using neuroblastoma SH-SY5Y, IMR-32 neuronal cell lines and PC-12, rat pheochromocytoma cell lines procured from the repository of the National Centre for Cell Science (NCCS) Pune, India. The SH-SY5Y, IMR-32, and PC-12 cells

were maintained in DMEM/F12, DMEM, and RPMI 1640, respectively. All the cells were supplemented with 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin at 37 °C under 5% CO<sub>2</sub>. Prior to the MTT assays, each of the cells (80 µL) were cultured in 96-well plates at a density of  $5 \times 10^3$  cells/well for 24 h in a CO<sub>2</sub> cell culture box (Forma SteriCycle CO<sub>2</sub> Incubator 371, Thermo Scientific, USA). Then, the cells were treated with 20 µL control as well as field exposed peptide samples (25 µM) and incubated for another 24 h. After that, 10 µL MTT solutions (5.5 mg/mL) were added into each well. After 4 h incubation, the medium was replaced with 100 µL DMSO to dissolve the formazan crystals. Then, absorbance at 570 nm was measured by a multifunctional microplate reader (TECAN Austria GmbH, Grödig, Austria). Six replicates were performed, and the data were averaged.

#### **6.2.11 Field Emission Transmission Electron Microscopy (FETEM) analysis**

FETEM imaging was carried out as discussed in chapter 4, section 4.2.6.

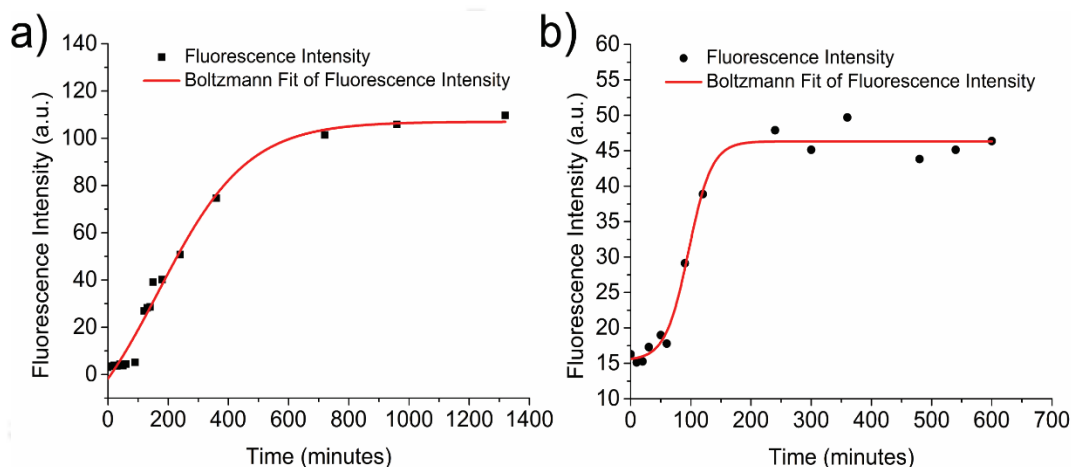
#### **6.2.12 Fourier-transform infrared spectroscopy (FTIR)**

Both control as well as field-exposed samples of both PhF6 and PhF6\* peptide were cast on potassium bromide (KBr) pellet immediately after taking out from the field. FTIR spectra were recorded on a Perkin-Elmer (Model: Spectrum Two) IR spectrometer at 1 cm<sup>-1</sup> resolution between 1200 cm<sup>-1</sup> to 2000 cm<sup>-1</sup> (amide I). The background scan was subtracted from the sample scans, and text files were plotted using OriginPro 8 software.

### **6.3 RESULTS AND DISCUSSION**

To test our hypothesis, we synthesised the model peptides (Ac-VQIVYK-am and Ac-VQIINK-am), purified using high-performance liquid chromatography (HPLC) and verified its purity using MALDI-TOF mass spectrometry (Appendix Table A1).

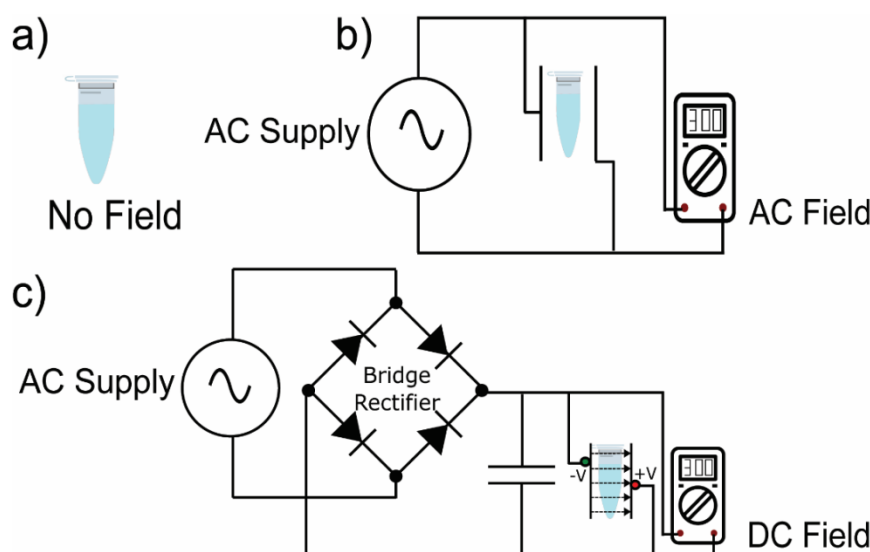
We performed an initial aggregation kinetics assay for each of the peptides to determine the time duration of our experiments (Figure 6.1).



**Figure 6.1** Aggregation Kinetics profile of (a) PhF6 and (b) PHF6\* peptide, determined by time dependent thioflavin T fluorescence assay.

Based on their aggregation kinetics profile, we allowed PhF6 and PhF6\* to aggregate for 16 hours and 6 hours respectively, in the presence and absence of alternating and direct current (AC and DC) electric fields of varying strengths by placing them in AC/DC field set-up (Figure 6.2).

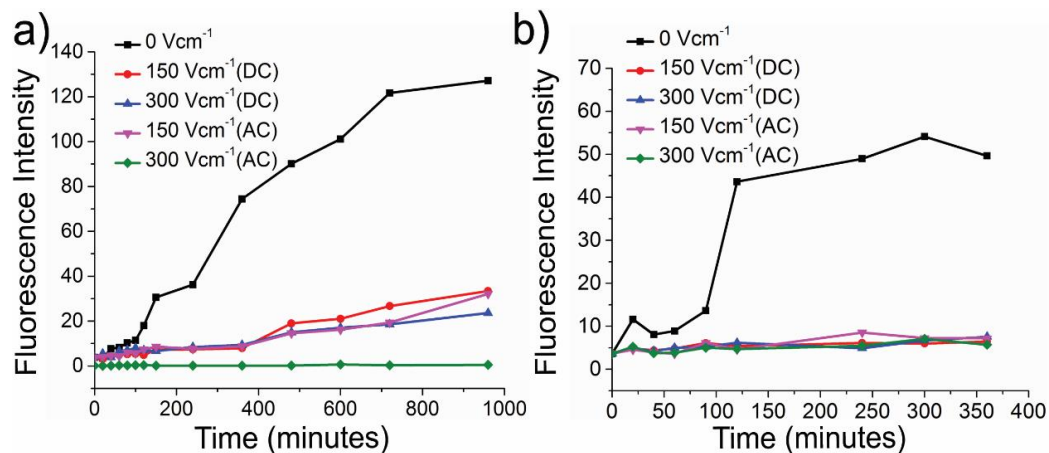




**Figure 6.2** Schematic illustration of the experimental setup. DC electric field setup comprises of horizontal plates connected to a full wave bridge rectifier circuit. Voltage is regulated using an autotransformer. AC field setup is similar to a DC set-up but without a bridge rectifier. Dissolved peptides were allowed to aggregate in (a) ambient conditions (0  $Vcm^{-1}$ ) and the presence of (b) AC and (c) DC fields.

### 6.3.1 Effect of Electric Field on the Aggregation

The kinetics of the amyloid formation was examined by a time-dependent thioflavin T (ThT) fluorescence assay. Thioflavin T is a dye that binds to amyloid fibrils and gives an enhanced fluorescence emission upon binding [44]. The fluorescence intensity of PhF6 and PhF6\* peptide increased with time, showing a typical sigmoidal curve, in the absence of an electric field. On the contrary, the peptide samples allowed to aggregate in the presence of electric field (both AC and DC) showed 6-fold lesser fluorescence intensity compared to the control, suggesting delayed aggregation kinetics (Figure 6.3 and Table 6.1 and 6.2)



**Figure 6.3** Retardation of aggregation by the electric field. Fibrillation kinetics of (a) PhF6 and (b) PHF6\* was examined by ThT fluorescence emission.

**Table 6.1** Comparative thioflavin T fluorescence data for treated (electric field) and untreated PhF6 peptide samples.

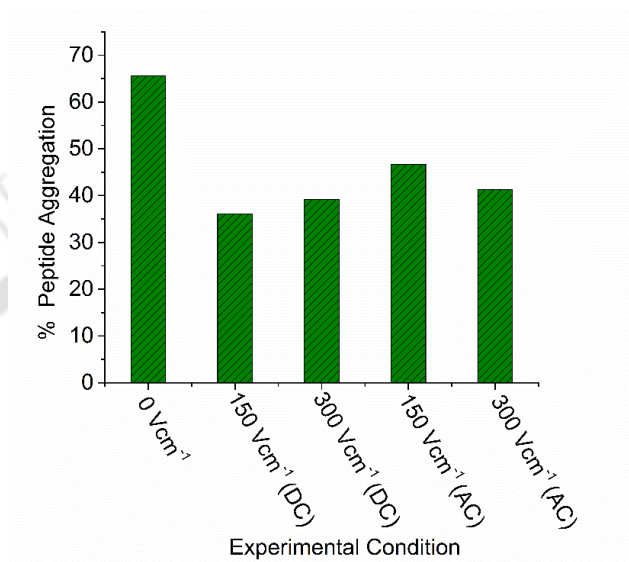
| Time (min.) | Fluorescence Intensity (a.u.)<br>0 V/cm | Std. Dev. | Fluorescence Intensity (a.u.)<br>150 V/cm (DC) | Std. Dev. | Fluorescence Intensity (a.u.)<br>300 V/cm (DC) | Std. Dev. | Fluorescence Intensity (a.u.)<br>150 V/cm (AC) | Std. Dev. | Fluorescence Intensity (a.u.)<br>300 V/cm (AC) | Std. Dev. |
|-------------|-----------------------------------------|-----------|------------------------------------------------|-----------|------------------------------------------------|-----------|------------------------------------------------|-----------|------------------------------------------------|-----------|
| 0           | 3.78466                                 | 0.11362   | 3.78466                                        | 0.11362   | 3.78466                                        | 0.11362   | 3.78466                                        | 0.11362   | 3.78466                                        | 0.11362   |
| 20          | 3.49173                                 | 0.0706    | 3.16595                                        | 0.06933   | 5.54196                                        | 0.09839   | 3.94184                                        | 0.07166   | 4.63786                                        | 0.07586   |
| 40          | 7.64945                                 | 0.32159   | 4.14102                                        | 0.16139   | 3.89939                                        | 0.11331   | 4.62679                                        | 0.09555   | 6.62668                                        | 0.08811   |
| 60          | 8.21572                                 | 0.17852   | 4.82133                                        | 0.11965   | 6.58798                                        | 0.08255   | 4.35337                                        | 0.24532   | 7.54103                                        | 0.27692   |
| 80          | 10.23876                                | 0.3023    | 5.17981                                        | 0.07746   | 7.02221                                        | 0.08574   | 5.96412                                        | 0.07929   | 7.37728                                        | 0.21763   |
| 100         | 11.39967                                | 0.51601   | 5.40583                                        | 0.14331   | 7.94105                                        | 0.08985   | 5.65875                                        | 0.12512   | 7.2354                                         | 0.33141   |
| 120         | 17.93171                                | 0.16115   | 4.83675                                        | 0.1694    | 6.93912                                        | 0.28923   | 7.361                                          | 0.0902    | 6.44631                                        | 0.3605    |
| 150         | 30.61325                                | 0.25589   | 7.17733                                        | 0.08934   | 6.63268                                        | 0.54475   | 8.55075                                        | 0.1079    | 8.78657                                        | 0.10024   |
| 240         | 36.1836                                 | 0.36474   | 7.38816                                        | 0.31358   | 8.42042                                        | 0.21639   | 7.66766                                        | 0.30546   | 11.63167                                       | 0.0989    |
| 360         | 74.44483                                | 0.32742   | 7.91511                                        | 0.42257   | 9.31676                                        | 0.39744   | 8.71079                                        | 0.17352   | 10.07957                                       | 0.14558   |
| 480         | 90.13458                                | 0.48547   | 18.95891                                       | 0.15037   | 14.97963                                       | 0.17174   | 14.58304                                       | 0.19087   | 20.59567                                       | 0.15189   |
| 600         | 101.0842                                | 1.68668   | 21.00824                                       | 0.34084   | 16.97724                                       | 0.11511   | 16.18648                                       | 0.85326   | 20.30977                                       | 0.62177   |
| 720         | 121.6417                                | 4.31804   | 26.6759                                        | 0.55251   | 18.54152                                       | 0.75316   | 19.32805                                       | 0.55964   | 24.71845                                       | 0.31217   |
| 960         | 127.1251                                | 2.36654   | 33.37058                                       | 0.29541   | 23.54854                                       | 0.33164   | 32.07641                                       | 0.68747   | 20.21911                                       | 0.48271   |

**Table 6.2** Comparative thioflavin T fluorescence data for treated (electric field) and untreated PhF6\* peptide samples.

| Time (min.) | Fluorescence Intensity (a.u.)<br>0 V/cm | Std. Dev. | Fluorescence Intensity (a.u.)<br>150 V/cm (DC) | Std. Dev. | Fluorescence Intensity (a.u.)<br>300 V/cm (DC) | Std. Dev. | Fluorescence Intensity (a.u.)<br>150 V/cm (AC) | Std. Dev. | Fluorescence Intensity (a.u.)<br>300 V/cm (AC) | Std. Dev. |
|-------------|-----------------------------------------|-----------|------------------------------------------------|-----------|------------------------------------------------|-----------|------------------------------------------------|-----------|------------------------------------------------|-----------|
| 0           | 3.65543                                 | 0.19374   | 3.65543                                        | 0.19374   | 3.65543                                        | 0.19374   | 3.65543                                        | 0.19374   | 3.65543                                        | 0.19374   |
| 20          | 11.61434                                | 0.1326    | 4.83019                                        | 0.07256   | 4.91645                                        | 0.29585   | 4.53997                                        | 0.5867    | 5.2249                                         | 0.2176    |
| 40          | 8.06531                                 | 0.16883   | 4.40173                                        | 0.66074   | 4.23282                                        | 0.88534   | 3.95734                                        | 0.40161   | 3.75338                                        | 0.20492   |
| 60          | 8.9155                                  | 1.17015   | 4.81716                                        | 0.21556   | 4.987                                          | 0.06713   | 3.61079                                        | 0.2402    | 3.83191                                        | 0.29964   |
| 90          | 13.65369                                | 0.95364   | 6.10656                                        | 0.27155   | 5.26822                                        | 0.78605   | 6.10953                                        | 0.52616   | 5.02986                                        | 0.45221   |
| 120         | 43.64246                                | 0.50713   | 5.35128                                        | 0.46102   | 6.16659                                        | 1.42783   | 4.59655                                        | 0.28731   | 4.70611                                        | 0.56348   |
| 240         | 48.95573                                | 0.81686   | 6.13732                                        | 1.27464   | 4.90916                                        | 0.3253    | 8.5425                                         | 0.3875    | 5.48153                                        | 0.41479   |
| 300         | 54.12469                                | 0.25612   | 5.98967                                        | 0.84064   | 6.70051                                        | 1.349     | 7.24982                                        | 1.49903   | 7.06711                                        | 1.26756   |
| 360         | 49.62951                                | 1.40278   | 6.46123                                        | 1.4182    | 7.56686                                        | 1.16942   | 7.19581                                        | 0.32818   | 5.70447                                        | 0.64689   |

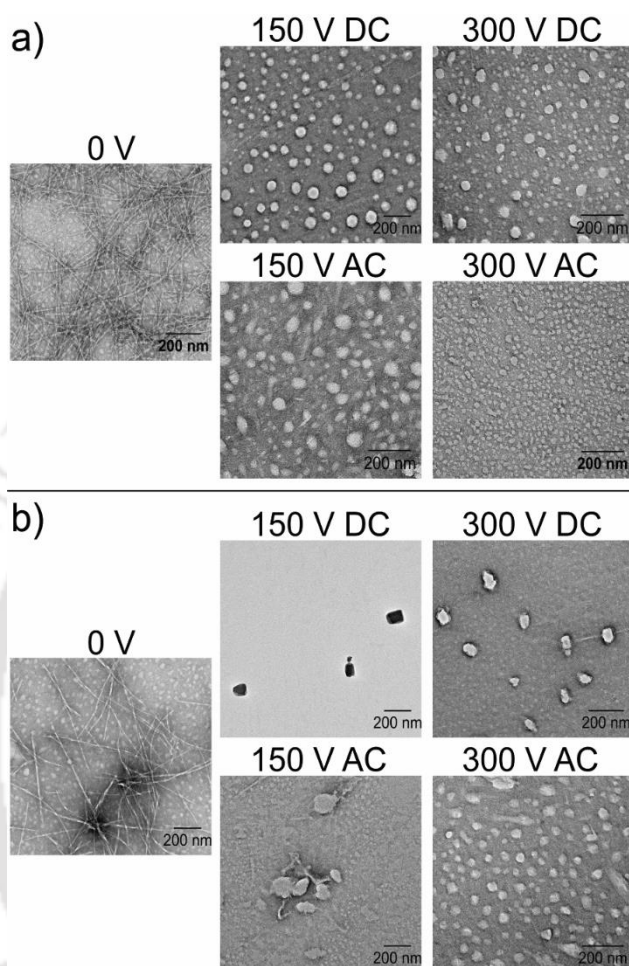
To compare quantitatively, we also examined the extent of aggregation by tyrosine absorbance assay. Each of the respective PhF6 samples was centrifuged at 12000g for 20 minutes, and then the tyrosine absorbance of the supernatant was recorded. The absorbance will be inversely proportional to the magnitude of aggregation. We observed that electric field exposed samples showed higher tyrosine absorbance, and hence less percentage aggregation compared to the control sample (Figure 6.4). The ThT and tyrosine absorbance data clearly show the retarding effect of the electric field. Several theoretical studies have reported the modulatory effect of electric field on the peptide-based amyloid forming systems [42, 43]. It has been proposed that electric field forced the peptide dipole to align parallel to its direction, thus modulating the self-assembly. Our experimental observations comply with the earlier theoretical reports, conducted by two different groups headed by Martin E. Garcia and Andrij Baumketner.

### 6.3.2 Effect of Electric Field on the Morphology and Secondary Structure Conformation



**Figure 6.4** Estimation of percentage aggregation using tyrosine estimation, by measuring the absorbance intensity of PhF6 peptide in solution. Absorbance at 280 nm is measured after an incubation period of 16 hours in presence and absence of electric field.

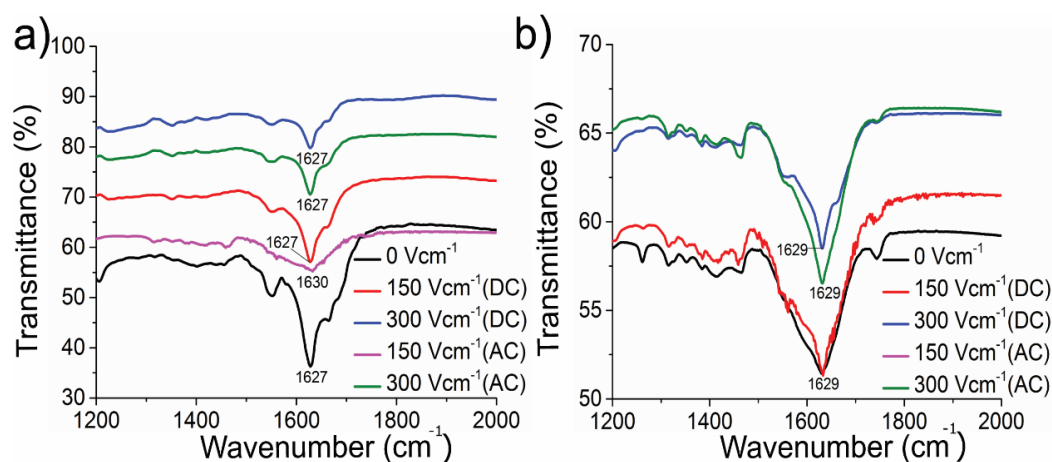
Field-emission transmission electron microscope (FETEM) analysis has been performed to investigate the effects of electric field on the morphology of PhF6 and PhF6\* aggregates. The control sample shows the presence of dense fibrils. Contrastingly, electric field samples show spherical structures and no fibrillar assemblies were observed. (Figure 6.5)).



**Figure 6.5** FETEM micrographs of (a) PhF6 and (b) PHF6\* samples. Both samples were allowed to aggregate in the presence and absence of DC and AC electric field of varying strengths.

The secondary structure conformation of each sample as dried films was determined by Fourier-transform infrared spectroscopy (FTIR) analysis. Both electric field treated as well as untreated PhF6 and PhF6\* samples showed the amide I band, a characteristic signature of  $\beta$ -sheet (Figure 6.6) [48, 49].





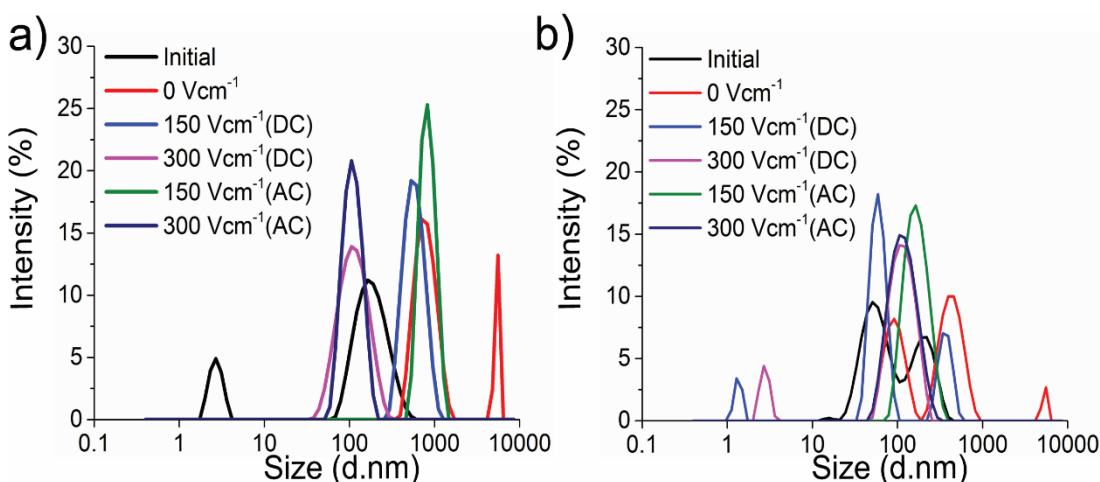
**Figure 6.6** Secondary structure determined by FTIR spectroscopy. Both (a) PhF6 and (b) PHF6\* samples were allowed to aggregate in the presence and absence of DC and AC electric field of varying strengths.

FTIR results indicate that though electric field and control sample differ in their aggregation kinetics and morphology, they have same  $\beta$ -sheet conformation. This result suggests that the morphological difference induced by EF is manifested in the supramolecular assembly levels and not at the molecular level.

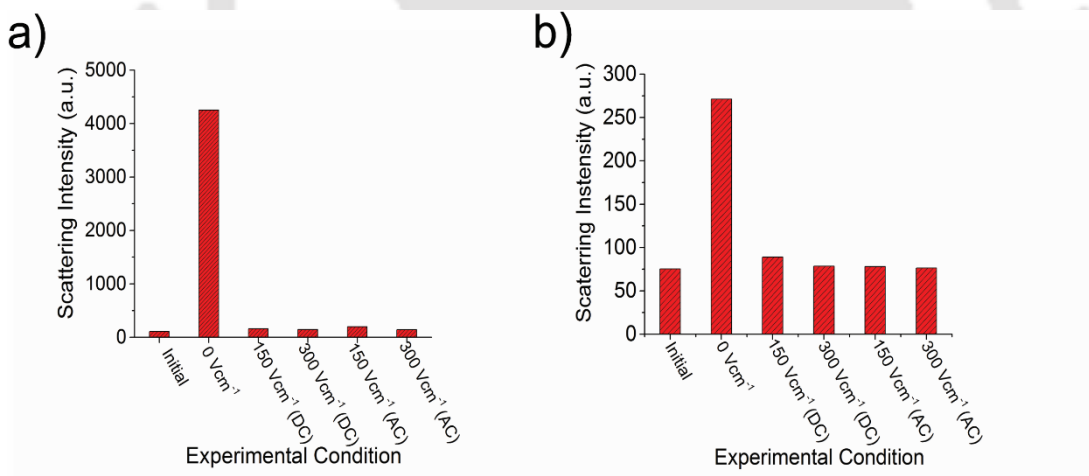
### 6.3.3 Effect of Electric Field on the Aggregate Size

The size of aggregates has been reported to increase with time [50]. To compare the size distribution of self-assemblies formed by electric field exposed and control sample, dynamic and static light scattering measurements were recorded. The DLS plot of control samples show an increase in size, with time. However, in the case of electric field samples, this increase is markedly reduced (Figure 6.7). Static light scattering experiment further supported this observation, wherein, the control samples having large-sized aggregates, showed significantly high scattering intensities compared to the electric field exposed samples (Figure 6.8).





**Figure 6.7** Size estimation using dynamic light scattering (DLS).

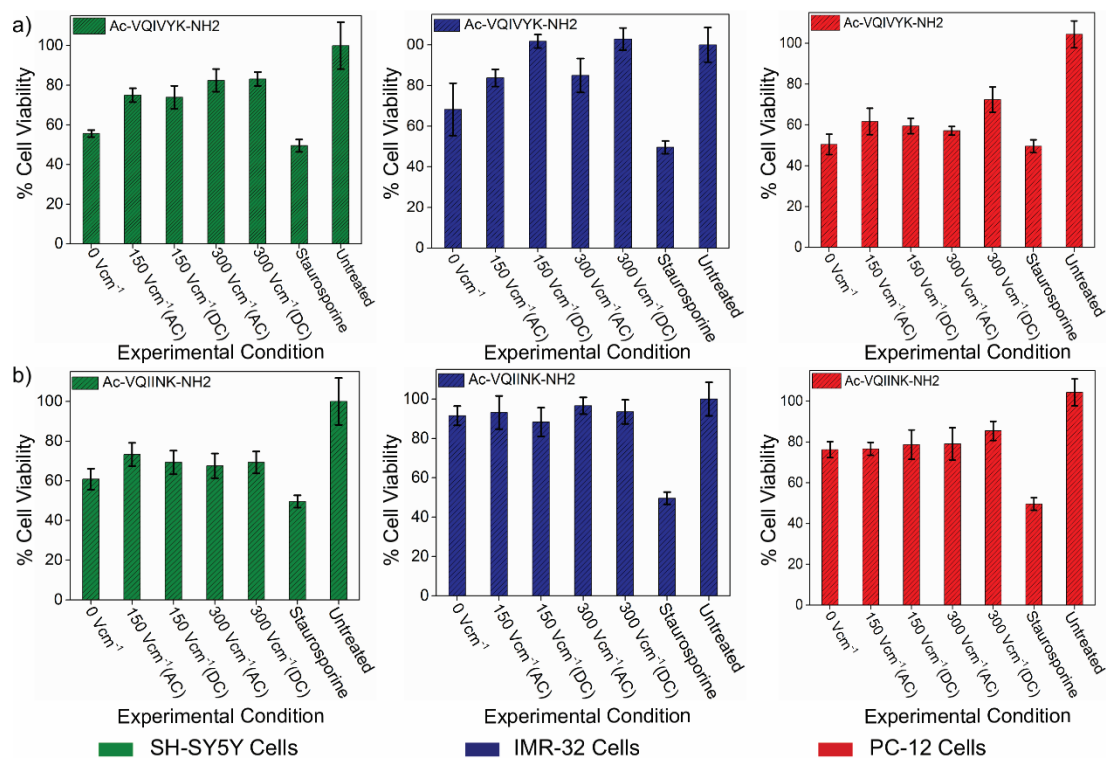


**Figure 6.8** Static right angle scatter plots. Graph represent comparative static light scattering by (a) PhF6 and (b) PhF6\* samples. The peptide samples allowed to aggregate under ambient conditions (o Vcm<sup>-1</sup>), AC and DC electric fields. The o Vcm<sup>-1</sup> showed a very high scattering intensity compared to field samples, thus indicating higher aggregation compared to treated samples.

#### **6.3.4 Effect of Electric Field on the Cellular Toxicity**

The cytotoxic self-assemblies of amyloid and tau protein aggregates are regarded as the hallmark of AD pathology and have been attributed to causing neuronal loss and synaptic dysfunction. Hence, intervening in the self-assembly phenomenon may serve as a complementary therapeutic option. The retardation of aggregation kinetics of our model peptides (PhF6 and PhF6\*), in the presence of an electric field, confirms the efficacy of the electric field for therapeutic applications in amyloidogenic diseases. Therefore, we assessed the improvement in the cell viability as a result of electric field treatment using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Human neuroblastoma SH-SY5Y and IMR-32 neuronal cell lines and PC-12, rat pheochromocytoma cell lines were treated with 25  $\mu$ M PhF6 and PhF6\* peptide solutions. The peptide samples were initially allowed to aggregate in the presence (treated) or absence (untreated) of an electric field.

The assay results show that the untreated aggregated PhF6 peptide solution reduced cell viability of SH-SY5Y, IMR-32 and PC-12 cells to 55%, 68%, and 50% respectively. Field treated samples, on the other hand, increases the cell viability from 18 % to 45 %, depending on the field strength and cell type (Figure 6.9). Similarly, in the case of PhF6\*, peptide samples were showing cell viability estimates at 60%, 91%, and 76%, for SH-SY5Y, IMR-32 and PC-12 cell lines respectively. Here also, the electric field treatment resulted in cell viability improvements up to 36% (Figure 6.9, and Table 6.3-6.5). These cell viability assay results support the neuroprotective behaviour of the electric field.



**Figure 6.9** Effect of DC and AC electric field on the cellular toxicity of PhF6 and PhF6\* peptide. Post incubation, peptide samples allowed to aggregate in the presence and absence of AC/DC electric field were added to SH-SY5Y, IMR-32 and PC-12 cells. The cytotoxicity effect was determined using an MTT assay. The electric field exposure reduced the toxicity of PhF6 and PhF6\* peptide is evident from the increased cell viability compared to the control.

**Chapter 6**

**Scientific Roadmap for a Potential Non-Invasive Therapy Against  
Tauopathies**

**Table 6.3** Summary of MTT assay data for SH-SY5Y cell viability in presence of treated and untreated PhF6 and PhF6\* peptide samples.

| Experimental Condition | % Cell Viability (SH-SY5Y)<br>Ac-VQIVYK-NH <sub>2</sub> | Std. Dev.<br>Ac-VQIVYK-NH <sub>2</sub> | % Cell Viability (SH-SY5Y)<br>Ac-VQIINK-NH <sub>2</sub> | Std. Dev.<br>Ac-VQIINK-NH <sub>2</sub> |
|------------------------|---------------------------------------------------------|----------------------------------------|---------------------------------------------------------|----------------------------------------|
| oV                     | 55.64322                                                | 1.768472                               | 60.80617                                                | 5.274733                               |
| 150 AC                 | 75.04288                                                | 3.484566                               | 73.27616                                                | 5.860087                               |
| 150 DC                 | 73.92796                                                | 5.787593                               | 69.26244                                                | 5.920028                               |
| 300 AC                 | 82.43568                                                | 5.734737                               | 67.49571                                                | 6.215325                               |
| 300 DC                 | 83.15609                                                | 3.480342                               | 69.25386                                                | 5.518031                               |
| Staurosporine          | 69.7084                                                 | 7.887985                               | 69.7084                                                 | 7.887985                               |
| Untreated              | 99.97141                                                | 11.8114                                | 99.97141                                                | 11.8114                                |

**Table 6.4** Summary of MTT assay data for IMR-32 cell viability in presence of treated and untreated PhF6 and PhF6\* peptide samples.

| Experimental Condition | % Cell Viability (IMR-32)<br>Ac-VQIVYK-NH <sub>2</sub> | Std. Dev.<br>Ac-VQIVYK-NH <sub>2</sub> | % Cell Viability (IMR-32)<br>Ac-VQIINK-NH <sub>2</sub> | Std. Dev.<br>Ac-VQIINK-NH <sub>2</sub> |
|------------------------|--------------------------------------------------------|----------------------------------------|--------------------------------------------------------|----------------------------------------|
| oV                     | 68.16654                                               | 12.90302                               | 91.57246                                               | 4.90684                                |
| 150 AC                 | 83.72123                                               | 4.24394                                | 93.13977                                               | 8.43164                                |
| 150 DC                 | 101.7585                                               | 3.33622                                | 88.30935                                               | 7.29277                                |
| 300 AC                 | 84.94957                                               | 8.32598                                | 96.61271                                               | 4.25072                                |
| 300 DC                 | 102.8704                                               | 5.46537                                | 93.51019                                               | 6.13001                                |
| Staurosporine          | 66.58273                                               | 5.74858                                | 66.58273                                               | 5.74858                                |
| Untreated              | 99.98715                                               | 8.50592                                | 99.98715                                               | 8.50592                                |

**Table 6.5** Summary of MTT assay data for PC-12 cell viability in presence of treated and untreated PhF6 and PhF6\* peptide samples.

| Experimental Condition | % Cell Viability (PC-12)<br>Ac-VQIVYK-NH <sub>2</sub> | Std. Dev.<br>Ac-VQIVYK-NH <sub>2</sub> | % Cell Viability (PC-12)<br>Ac-VQIINK-NH <sub>2</sub> | Std. Dev.<br>Ac-VQIINK-NH <sub>2</sub> |
|------------------------|-------------------------------------------------------|----------------------------------------|-------------------------------------------------------|----------------------------------------|
| oV                     | 68.16654                                              | 12.90302                               | 91.57246                                              | 4.90684                                |
| 150 AC                 | 83.72123                                              | 4.24394                                | 93.13977                                              | 8.43164                                |
| 150 DC                 | 101.7585                                              | 3.33622                                | 88.30935                                              | 7.29277                                |
| 300 AC                 | 84.94957                                              | 8.32598                                | 96.61271                                              | 4.25072                                |
| 300 DC                 | 102.8704                                              | 5.46537                                | 93.51019                                              | 6.13001                                |
| Staurosporine          | 66.58273                                              | 5.74858                                | 66.58273                                              | 5.74858                                |
| Untreated              | 99.98715                                              | 8.50592                                | 99.98715                                              | 8.50592                                |

## 6.4 CONCLUSIONS

Aggregation of the protein chain to the ordered and patterned functional or dysfunctional forms is principally guided by physical forces such as electrostatics, van der Waals and hydrogen bonding interactions. Formation of biological forms is ‘rooted in its physics’, is an observation by D’Arcy Wentworth Thompson in his book on growth and form at the beginning of the last century, while attempting to quantitatively assess the structural patterns and formations in biological systems [51]. Inspiration to modulate peptide assembly using a physical perturbant like the electric field is derived from such and similar observations. Our experimental results convincingly prove that the electric field exposure decelerated the aggregation kinetics of PhF6 and PhF6\* peptides. The structures formed by both the peptides, allowed to aggregate under field conditions were spherical and lacked the usual fibrillar morphology formed by the control peptide. The size of the

aggregates formed by EF treated samples was also smaller compared to the untreated ones. Both EF treated as well as untreated samples had  $\beta$ -sheet conformation, indicating that the EF-induced modulatory effect is at the assembly, and not at the molecular level. Both PhF6 and PhF6\* aggregates are toxic to neuronal cells. A comparative experiment with EF treated and untreated samples with two neuronal and one normal cell lines, showed that EF treated samples were 18 to 45 % less toxic, under various experimental conditions. Pathological aggregation of tau protein has been reported to have links with more than 20 neurodegenerative diseases, collectively referred to as tauopathies. Despite having a causative association with diseases affecting millions of people worldwide, limited tau-based therapeutic options are available. We infer that electric field modulation can effectively retard tau protein aggregation and supramolecular assembly of the core peptide segment of tau. Mechanistic investigations on the possible effects of the electric field using molecular dynamics simulations have been published by three different groups [42, 43, 52]. Our experimental observations qualitatively align more with the mechanistic predictions of Martin E. Garcia [42] and Andrij Baumketner [43]. However, toxicity estimation of EF has to be more rigorously performed, but recent positive reports on the efficacy of similar non-invasive therapies are very optimistic [27-31]. The electric field can, therefore, be referred to advanced phases of clinical examination, for potential use as a therapeutic option against tauopathies.

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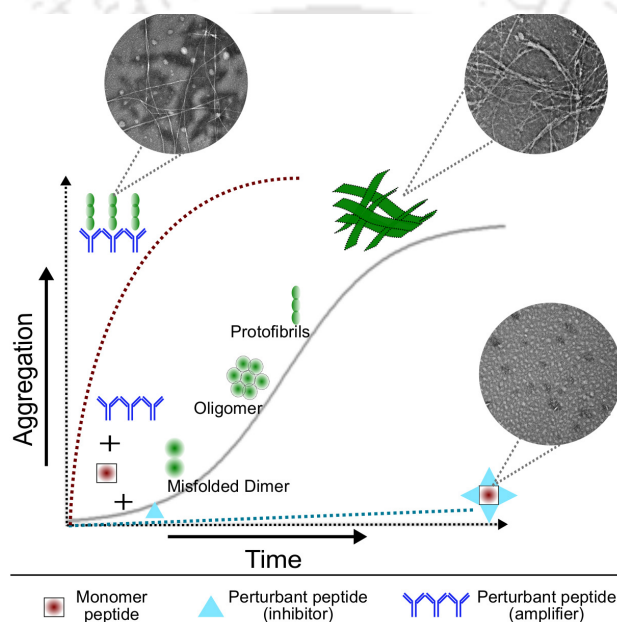
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## CHAPTER 7

### AGGREGATION MODULATORS FOR AMYLOIDOGENIC SEGMENT OF TAU



The abnormal assembly of tau into neurofibrillary tangles has been associated with over 30 debilitating disorders known as tauopathies. Tauopathies affect millions of people worldwide, yet no clinically approved solution for anti-tau aggregation is currently available. The design of tau aggregation inhibitors has been challenging owing to the dynamic nature of its self-assemblies. Here we employ the structure-based approach to design a series of short peptide-based perturbants of the core hydrophobic fragment of tau protein and examine their efficacy using various biophysical methods, toxicity and blood-brain barrier crossing ability assessment assays.





## **7.1 INTRODUCTION**

Tau is a constituent protein of the neurofibrillary tangles, one of the hallmarks of Alzheimer's disease (AD) [1]. Therefore, it invokes significant interest for decoding the disease mechanisms. Tauopathies is a collective term for a series of progressive neuropathologies characterized by the aggregation of abnormally phosphorylated microtubule-associated protein tau, into paired helical filaments (PHFs) and neurofibrillary tangles (NFTs) [2]. Under normal conditions, the primary function of tau protein is the stabilization of axonal microtubules by binding through its microtubules binding domain. The hyperphosphorylation of tau in diseased condition results in destabilization of microtubules leading to axonal transport inhibition [3].

The microtubule binding domain of tau comprises of a proline-rich region followed by repeat domains which vary from 4, 3 or 2 in number, depending upon the length of the isoform. These repeat domains interact with microtubules. Two hexapeptide motifs, 306VQIVYK<sub>311</sub> in the third repeat domain and 275VQIINK<sub>280</sub> in the second repeat region, said to be critical in driving the aggregation of the entire tau protein [4, 5] (Figure 7.1 (a)).

Modulation of these motifs (e.g., by mutations) prevents tau from aggregating, not only *in vitro* but also in cell and animal models [6-8]. These hexapeptides also aggregate to form fibrils similar to those developed by tau and are regarded as an ideal model for not only examining the mechanisms that boost tau fibrillation, but also as a screening platform for designing tau aggregation inhibitors [9].

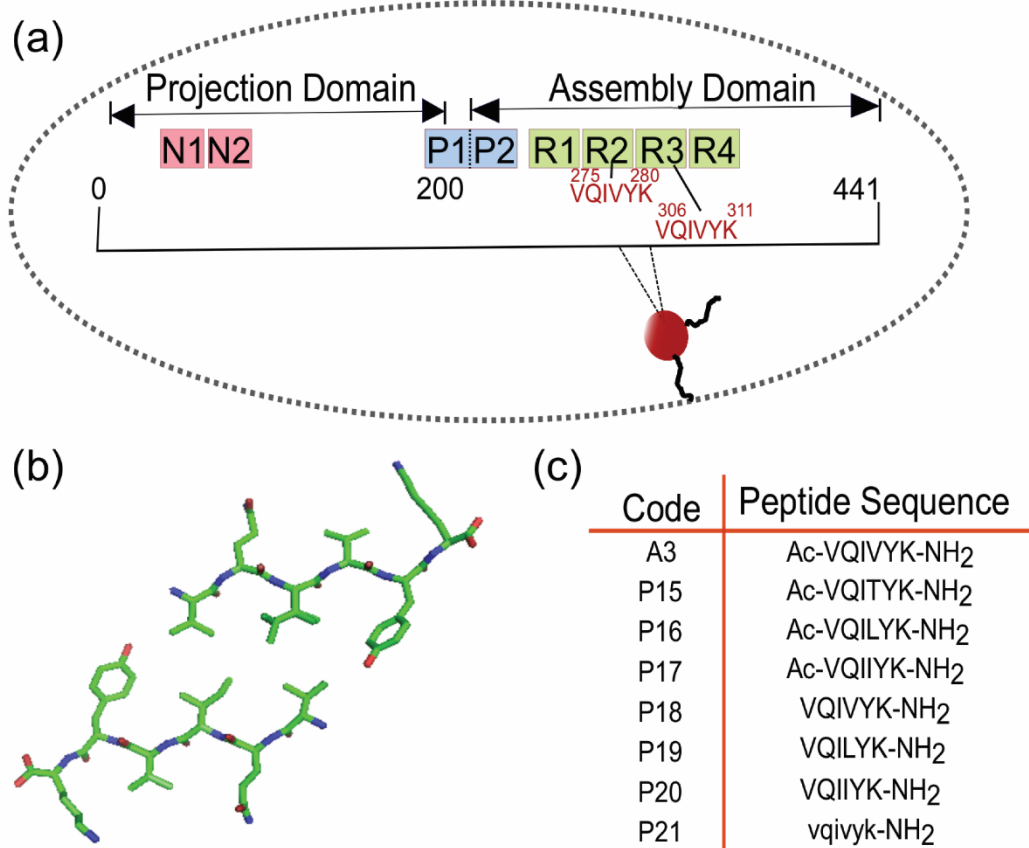
Designing specific inhibitors of tau aggregation can aid in determining the mechanism of tau aggregation mediated cognitive decline in AD and other tauopathies. Furthermore, inhibitors that arrest seeding by capping the ends of tau fibrils could prevent the progression of tau self-assembly. The crystal structure of the steric zipper form of VQIVYK segment (shown in Figure 7.1 (b)), from the R3

domain, has been utilized to design structure-based inhibitors of VQIVYK aggregation [10-13]. These experiments showed that VQIVYK aggregation inhibitors are highly effective in arresting the aggregation of 3-repeat (3R) tau isoforms *in vitro* [13].

Despite having a causative association with diseases affecting millions of people, with AD, the most prevalent of all, no disease-modifying therapies are currently available for tauopathies. The available FDA approved drugs (Donepezil, Galantamine, Tacrine, and Rivastigmine) provide symptomatic relief and also have several side-effects [14]. While most therapeutic strategies in the past have targeted amyloid beta, recent reports are stressing upon the crucial role of tau derived soluble oligomers and neurofibrillary tangles, have initiated a shift towards the design of therapeutic solutions targeting tau aggregation. Phenothiazine methylene blue (tau protein inhibitor) is presently the only drug under phase III clinical trials [15].

Failure of molecule-based remedies due to their inherent toxicity, poor proteolytic stability, high costs, or poor blood-brain barrier (BBB) permeability, have stimulated a push towards the development of non-invasive therapies such as scanning ultrasound [16, 17], electric fields [18-20], transcranial stimulations by magnetism or electric current [21, 22], and light [23] etc.

Peptide-based inhibitors offering better specificity, high selectivity, low toxicity, better BBB permeability, high design diversity, and low accumulation, can thus serve as a useful alternative. Currently, 140 peptide-based drugs are in clinical trials, and around 500 have been referred for preclinical assessment [15]. These findings inspired our pursuit towards designing short peptide-based perturbants, targeting the aggregation of core peptide segments of tau protein.



**Figure 7.1** (a) Schematic representation of domain organization of human tau protein, (b) steric-zipper structure of VQIVYK peptide (PDB id:2ON9), and (c) list of peptide sequences.

## 7.2 EXPERIMENTAL APPROACHES

### 7.2.1 Materials

All the chemical reagents were purchased from industrial suppliers and used without any further purification.

### 7.2.2 Peptide Synthesis and Characterization

Solid phase peptide synthesis was done using the standard Fmoc chemistry on Rink amide resin as described in chapter 4, section 4.2.1.

### **7.2.3 Preparation of Peptide Solution**

The stock peptide solutions were prepared in deionized water by weighing the dried form of the peptide and dissolving at a concentration of 1 mM.

### **7.2.4 Thioflavin T Fluorescence Assay**

Thioflavin T (ThT) binding assay was performed by combining 7.5  $\mu$ L, 1 mM peptide to a solution of 10  $\mu$ M ThT in 50 mM sodium phosphate buffer pH 7.4. ThT fluorescence was recorded at 482 nm (emission slit width = 5 nm) for 60 seconds at regular time intervals on a spectrofluorometer (Jasco FP 8500) at 25 °C using a 1 cm path length quartz cuvette (Helma, Sigma-Aldrich), with excitation wavelength set at 450 nm (slit width= 2.5 nm).

### **7.2.5 Dynamic Light Scattering (DLS)**

The size estimation assay was done by dynamic light scattering for 16 hour incubated peptide samples at 25 °C as discussed in chapter 5, section 5.2.5.

### **7.2.6 Field-emission transmission electron microscopy (FETEM)**

FETEM imaging was performed as discussed in chapter 4, section 4.2.6.

### **7.2.7 Fourier transform infrared spectroscopy (FTIR)**

FTIR spectra measurements were for 16 h of incubated peptide samples using the protocol described in chapter 5, section 5.2.7.

### **7.2.8 Cell Viability Assay**

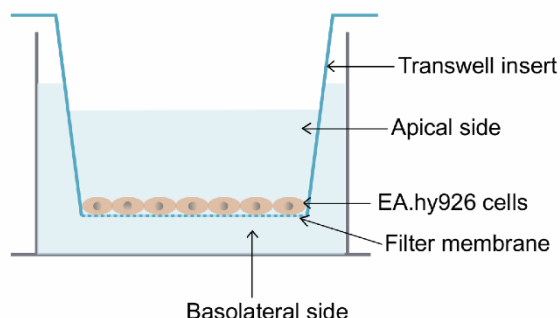
MTT assays were performed to assess the cytotoxicity of treated and untreated Ac-VQIVYK-am aggregates using human neuroblastoma SH-SY5Y and IMR-32 neuronal cell lines purchased from the repository of the National Centre for Cell Science (NCCS) Pune, India. The SH-SY5Y and IMR-32 cells were maintained T25 culture flask containing Dulbecco's modified Eagle's medium (DMEM). All the

cells were supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin at 37 °C under 5% CO<sub>2</sub> and were maintained for further assay by subsequent re-culturing. Prior to the MTT assays, each of the cells (80 µL) were cultured in 96-well plates at a density of  $5 \times 10^3$  cells/well for 24 hours in a CO<sub>2</sub> cell culture box (Forma SteriCycle CO<sub>2</sub> Incubator 371, Thermo Scientific, USA). Then, the cells were treated with 20 µL control as well as treated peptide samples (25 µM) and for 24 hours. Post incubation, 10 µL MTT solutions (5.5 mg/mL) were added into each well. The medium was replaced with 100 µL DMSO to dissolve the formazan crystals after 4h. The absorbance at 570 nm was measured by a multifunctional microplate reader (TECAN Austria GmbH, Grödig, Austria) to estimate the cell viability under respective treatments. Six replicates were performed, and the data were averaged.

#### **7.2.9 Blood Brain Barrier Transwell Permeability Assay (*in vitro*)**

An *in vitro* BBB model comprising of a monolayer immortalized human brain endothelial cells (EA.hy926) growing on the apical side of a permeable membrane placed between two compartments was used to evaluate peptide translocation. Human brain endothelial cells (EA.hy926) (ATCC, CRL-2299) were cultured in DMEM supplemented with 10% FBS and 100 U/mL penicillin and 100 U/mL streptomycin in a humidified condition having 5% CO<sub>2</sub> and 95% air at 37 °C. The cells adhered as monolayers were harvested after reaching confluence using trypsin-EDTA, and 3500 cells/well were then seeded in Transwell filter insert plates (1 µm pore size) (Corning). Cells were allowed to attain a confluent monolayer (5-6 days) until full confluency was reached and media was changed every two days. The full confluence resulted in the establishment of a stable, tight layer mimicking the natural BBB (Figure 7.2)





**Figure 7.2** Schematic representation of Blood-Brain Barrier Model.

The perturbant peptide solution was added on the apical side of the BBB model and allowed to permeate for 5 hours. 5(6)-Carboxyfluorescein labeled cell penetrating peptide (CPP), TAT (47 - 57) known to have very high unidirectional influx across the BBB, was used as a positive control for BBB permeability in our experimental model. Post incubation, the solution in the basolateral side was collected. The peptide translocation was quantified by measuring the absorption for tyrosine present in each of the perturbant peptides. The following established calculation steps were used:

$$\text{i) } A = \epsilon cl \text{ (Beer-Lambert law)}$$

where, A is absorbance 280 nm,  $\epsilon$  is molar extinction coefficient ( $\sim 1280$  for tyrosine), c is the concentration of the sample and l is the length of light path used (l=1 cm).

$$\text{ii) \% translocation} = \frac{C_f}{C_i} \times 100$$

where,  $C_f$  is the concentration of peptide recovered from the basolateral side of the BBB model, post incubation of 5 hours,  $C_i$  is the initial concentration of peptide

sample added to the apical side of BBB. For estimating the translocation of 5(6)-Carboxyfluorescein labeled TAT<sub>(47-57)</sub>, fluorescence intensity was measured, and the percentage translocation was calculated using the following equation:

$$(iii) \% \text{ translocation} = \frac{F_f}{F_i} \times 100$$

where,  $F_f$  is fluorescence intensity of TAT<sub>(47-57)</sub> recovered from the basolateral side of BBB model, post incubation of 5 hours,  $F_i$  is the initial concentration of the same added to the apical side of BBB.

#### **7.2.10 Blood Brain Barrier integrity evaluation assay**

The integrity of the BBB model post-treatment with peptide perturbants was evaluated by adding fluorescein isothiocyanate (FITC)-conjugated dextran (FD40, with a molecular weight of 40 kDa) as a probe to wells pre-treated with perturbants. A 25 mg/ml stock of FD40 was prepared in water and was later diluted in DMEM without phenol red, to a final absorbance value below 0.1. Diluted FD40 probe was added to the apical side for 2 hours. Post incubation, FITC fluorescence was estimated at 493 nm, and emission was ranging from 500 nm to 600 nm using a spectrofluorometer, to detect any damage to the BBB.

#### **7.2.11 Pre-formed aggregate study**

To investigate the effect of inhibitor peptides on the pre-formed A<sub>3</sub> (Ac-VQIVYK-NH<sub>2</sub>) aggregates, we allowed A<sub>3</sub> peptide to aggregate for 12 hours before treating with inhibitor peptides. ThT fluorescence measurements were recorded, as described above, at 24<sup>th</sup> and 36<sup>th</sup> hour to estimate the modulatory effect of peptide treatment.

## **7.3 RESULTS AND DISCUSSION**

### **7.3.1 Design, synthesis and characterization of the peptides**

To test our hypothesis, we synthesized the model peptide (end-capped) Ac-VQIVYK-am as well the designed seven peptide-based aggregation perturbants. The peptide sequences and their respective codes are shown in Figure 7.1 (c). They were synthesized manually employing the solid-phase peptide synthesis protocol, using Fmoc-chemistry and purified by high-performance liquid chromatography (HPLC) followed by characterization using MALDI-TOF mass spectrometry (Appendix Table A1).

### **7.3.2 Amyloidogenicity assessment of the designed peptide-based perturbants**

We tested the self-assembly propensity of all the designed molecular perturbants using Thioflavin T (ThT) fluorescence, Fourier-transform infrared spectroscopy (FTIR) spectroscopy, Dynamic Light Scattering (DLS), Field-emission transmission electron microscopy (FETEM) along with cytotoxicity experiments.

#### **7.3.2.1 Thioflavin T fluorescence assay**

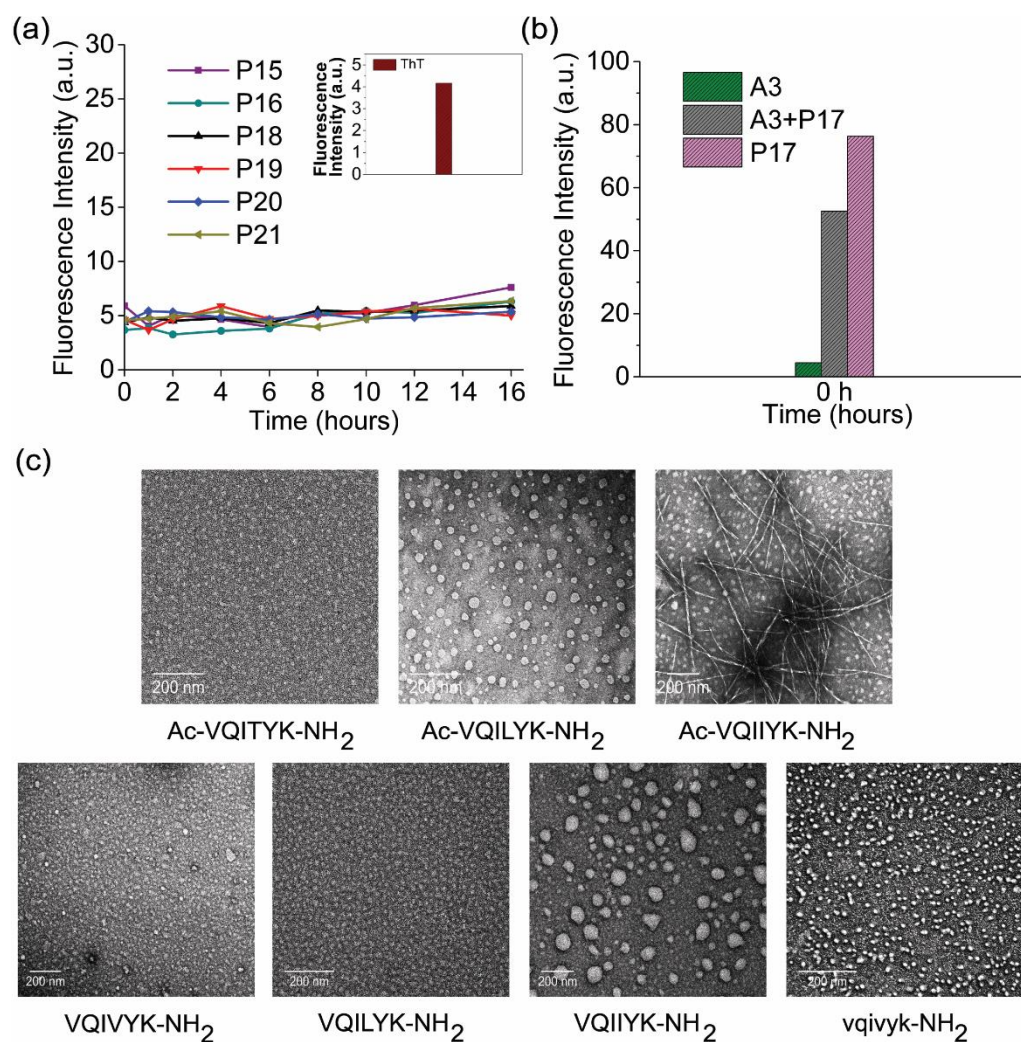
Thioflavin T is a dye that attaches to amyloid fibrils and gives an enhanced fluorescence emission upon binding, and the intensity of fluorescence is directly proportional to the extent of fibrillogenesis [24]. The perturbant peptides were dissolved in water. The aggregation propensity was estimated intermittently up to 16 hours, by adding ThT dye. The results show that all the perturbant peptides (P15-P21) except P17, have very low fluorescence signal, comparable to blank ThT dye. (Figure 7.3 (a)) suggesting the non-amyloidogenic nature of the perturbants. P17 peptide, however, showed 17 times higher fluorescence intensity compared to the model aggregating peptide (A3) at the 0th hour. Thus P17 was excluded from further analysis (Figure 7.3 (b)).

### **7.3.2.2 FETEM Analysis**

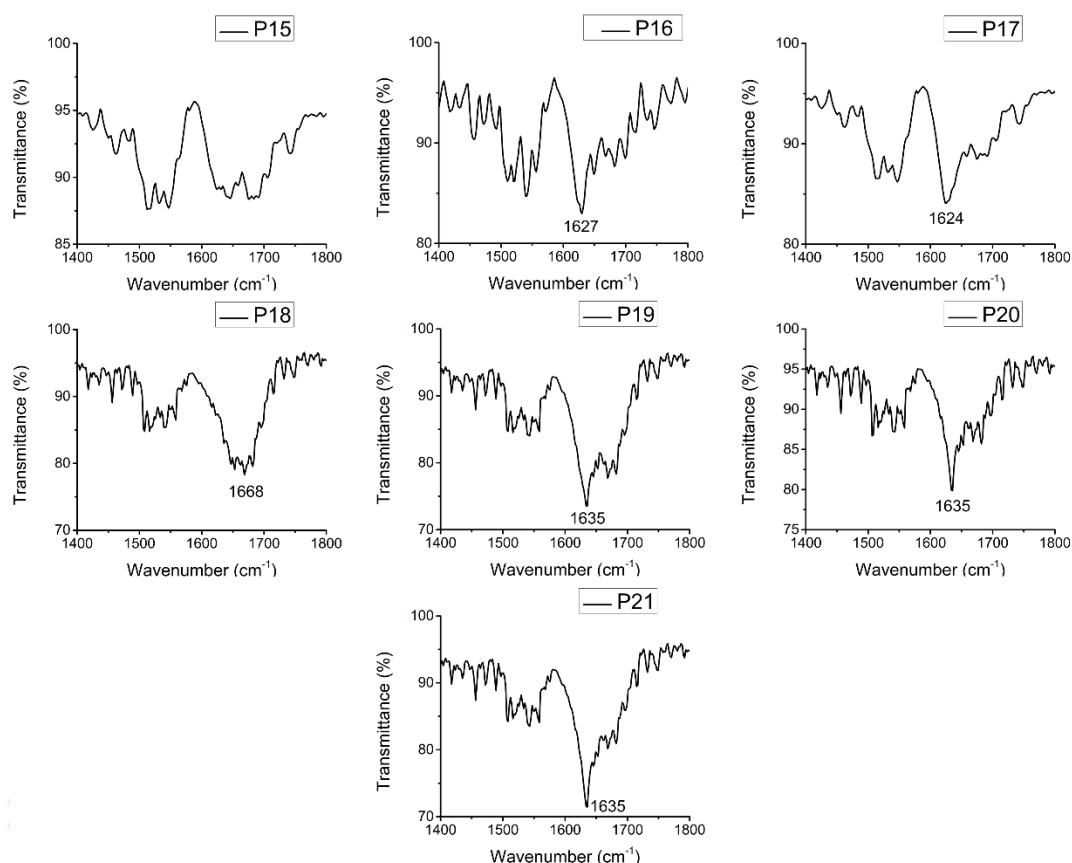
FETEM micrographs of amyloidogenic peptides can provide a qualitative impression of fibrillar assemblies. To further verify the self-assembling nature of our designed peptide, we performed FETEM imaging of the dried peptide samples. No fibrillar assemblies were observed for each of the perturbant peptides except P17, which showed dense fibrillar structures, a morphology characteristic of amyloidogenic peptides/proteins (Figure 7.3 (c)).

### **7.3.2.3 FTIR Analysis**

To probe into the secondary structure conformation of the perturbant peptides FTIR spectra of the dried films of the peptide samples were recorded (Figure 7.4). P17 showed a band around  $1624\text{ cm}^{-1}$ , a characteristic of  $\beta$ -sheet structure which correlates with the intense ThT fluorescence signal observed. While P16 displayed a signal around  $1624\text{ cm}^{-1}$  and P19, P20 and P21 showed band around  $1635\text{ cm}^{-1}$  suggesting that  $\beta$ -sheet conformation of these peptides is retained in the dried films. Peptide P18 displayed a signal around  $1668\text{ cm}^{-1}$  which is suggestive of random coil conformation [25]. Perturbant P15 did not show any characteristic band.



**Figure 7.3** (a) ThT assay profiles of perturbant peptides. The data shows time-dependent ThT fluorescence measurements for perturbant peptides allowed to aggregate under ambient conditions. The fluorescence intensity values are comparable to value for blank ThT dye (shown in inset), (b) ThT assay profiles of P17 perturbant peptide and A<sub>3</sub> treated with equimolar P17 (A<sub>3</sub>+P17) at 0<sup>th</sup> hour in comparison with A<sub>3</sub> peptide. High initial fluorescence values indicate, P17 has aggregation propensity, and (c) FETEM micrographs of perturbant peptides.



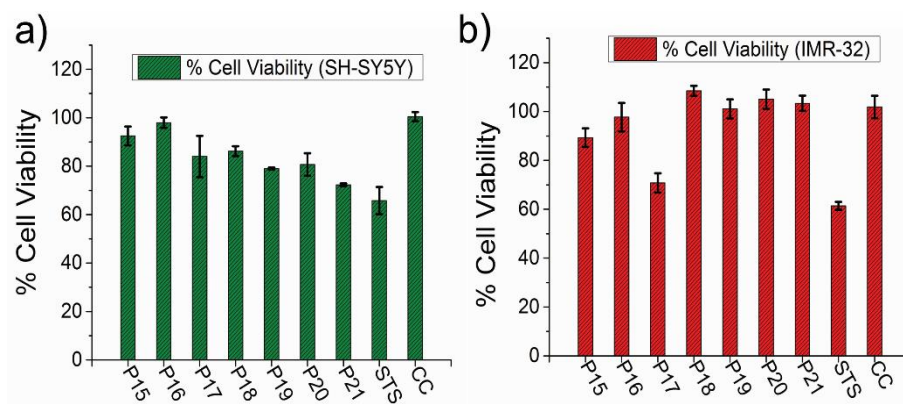
**Figure 7.4** FTIR spectra of perturbant peptides.

### 7.3.2.4 Toxicity Estimation Study

Minimal cellular toxicity is a pre-requisite for the designed perturbant peptides to be eligible as a potential therapeutic agent against tauopathies. The cytotoxicity of the perturbant peptides was tested by *in vitro* toxicity analysis on Human neuroblastoma SH-SY5Y and IMR-32 neuronal cell lines. The peptide solutions were added to the cells and incubated for 48 hours. Staurosporine (STS) added and untreated cells (CC) served as positive and negative control respectively. The toxicity was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. The SH-SY5Y cell viability estimates were, 92%, 98%, 84%, 86%, 79%, 81% and 72% for P15, P16, P17, P18, P19



P20 and P21 peptides respectively (Figure 7.5 (a) and Table 7.1). While the IMR-32 cell viability percentages were, 89%, 97%, 71% for P15, P16, P17 respectively and 100% for P18, P19, P20 and P21 peptides (Figure 7.5 (b) and Table 7.1).



**Figure 7.5** Perturbant toxicity estimation. The inherent toxicity of designed peptide perturbants was estimated by adding them to (a) SH-SY5Y, and (b) IMR-32 cells. The cytotoxicity was determined using an MTT assay. STS and CC denote positive control (Staurosporine treated) and negative control (untreated cells), respectively.

**Table 7.1** Summary of MTT assay data for IMR-32 and SH-SY5Y cell viability upon treatment with the designed peptide-based perturbants.

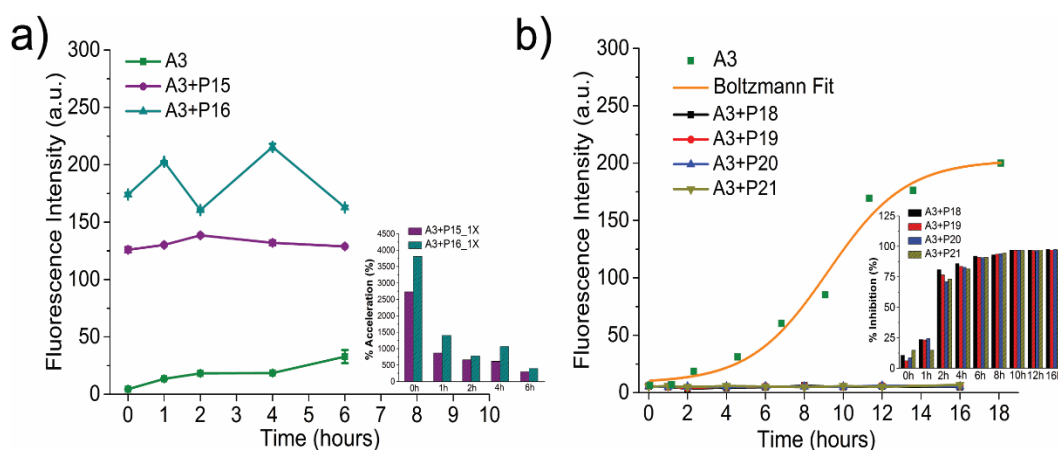
| Experimental Condition | % Cell Viability (IMR-32) | Std. Dev. | % Cell Viability (SH-SY5y) | Std. Dev. |
|------------------------|---------------------------|-----------|----------------------------|-----------|
| P15                    | 89.31232                  | 3.8268    | 92.48989                   | 3.9075    |
| P16                    | 97.70774                  | 5.8418    | 97.97805                   | 2.11789   |
| P17                    | 70.80229                  | 3.94733   | 83.99769                   | 8.50869   |
| P18                    | 108.49284                 | 2.07301   | 86.19295                   | 2.0087    |
| P19                    | 101.11748                 | 3.88795   | 78.97169                   | 0.43615   |
| P20                    | 105.02388                 | 3.99953   | 80.70479                   | 4.63206   |
| P21                    | 103.40019                 | 3.08014   | 72.27036                   | 0.60036   |
| STS                    | 61.43266                  | 1.595     | 65.74235                   | 5.61411   |
| CC                     | 101.89112                 | 4.57101   | 100.46216                  | 1.91689   |

### **7.3.3 Effect of the designed peptides on model aggregating peptide**

We tested the modulatory effect of designed molecular perturbants on self-assembly kinetics of Ac-VQIVYK-am (A<sub>3</sub>). The aggregating peptide, A<sub>3</sub> was allowed to aggregate in the presence and absence of equimolar concentration of each of the perturbant peptide. The effect of treatment was monitored using various biophysical, cytotoxicity and blood-brain barrier (BBB) experiments.

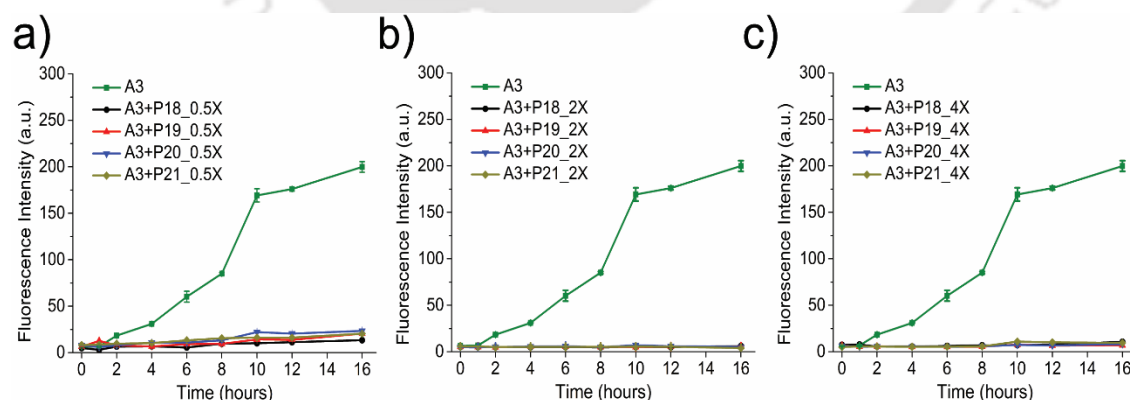
#### **7.3.3.1 Examining the kinetics of self-assembly using Thioflavin T fluorescence assay**

A time-dependent ThT fluorescence assay monitored the quantitative estimation of aggregation in treated and untreated A<sub>3</sub> peptide. The fluorescence intensity of untreated A<sub>3</sub> peptide increased with time, showing aggregation in treated and untreated A<sub>3</sub> peptide. The fluorescence intensity of untreated A<sub>3</sub> peptide increased with time, showing a characteristic sigmoidal curve. The peptide sample was allowed to aggregate in the presence of P<sub>15</sub>, P<sub>16</sub> and P<sub>17</sub> displayed 25-fold, 35-fold and 15-fold higher fluorescence intensities from the 0th hour, suggesting an accelerated aggregation upon treatment (Figure 7.6 (a)). On the contrary, the peptide sample allowed to aggregate in the presence of the P<sub>18</sub>-P<sub>21</sub> perturbant peptides, showed 40-fold lesser fluorescence intensities compared to untreated A<sub>3</sub> peptide, indicating an impeded aggregation in the case of P<sub>18</sub>-P<sub>21</sub> peptide treated samples (Figure 7.6 (b)).



**Figure 7.6** Effect of designed peptide perturbants on model amyloidogenic peptide. Fibrillation kinetics of A<sub>3</sub> was examined by ThT fluorescence emission in the presence and absence of equimolar concentration of (a) accelerator and (b) inhibitor peptides.

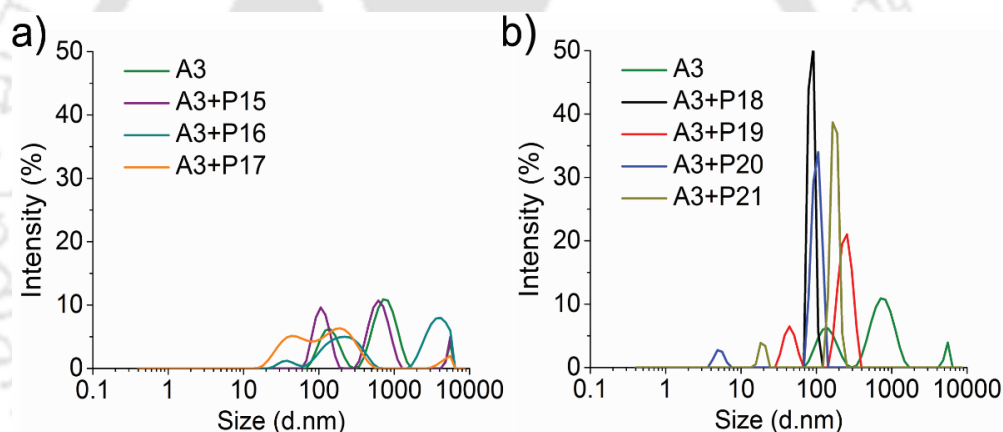
Owing to their ability to accelerate the aggregation of the core-peptide segment of tau, A<sub>3</sub>, peptides P15, P16 and P17 were designated as ‘accelerators’ while peptides ‘P18, P19, P20, and P21 were designated as ‘inhibitors.’ Similar ThT fluorescence-based aggregation profiles were obtained in the presence of 4x, 2x and 0.5x molar equivalents of inhibitor peptides (Figure 7.7).



**Figure 7.7** ThT fluorescence assay based aggregation kinetics profiles of A<sub>3</sub> peptide treated with perturbant inhibitor peptides having (a) 0.5X, (b) 2X, and (c) 4X molar concentration.

### 7.3.3.2 Monitoring the size distribution using DLS

The size of amyloid aggregate is known to increase with increased rate of aggregation [26]. Dynamic light scattering measurements were done to compare the size distribution of amyloid-assemblies formed by peptide sample treated with accelerator and inhibitor peptides compared to the untreated ones. The DLS plot of the untreated and accelerator treated A<sub>3</sub> peptide samples, show the presence of large-sized aggregates (Figure 7.8 (a)). A<sub>3</sub> peptide sample treated with inhibitor peptides showed the presence of particles with ten-fold reduced size compared to untreated, and accelerator peptide treated samples (Figure 7.8 (b)). The DLS data complements our observations from ThT fluorescence measurements.

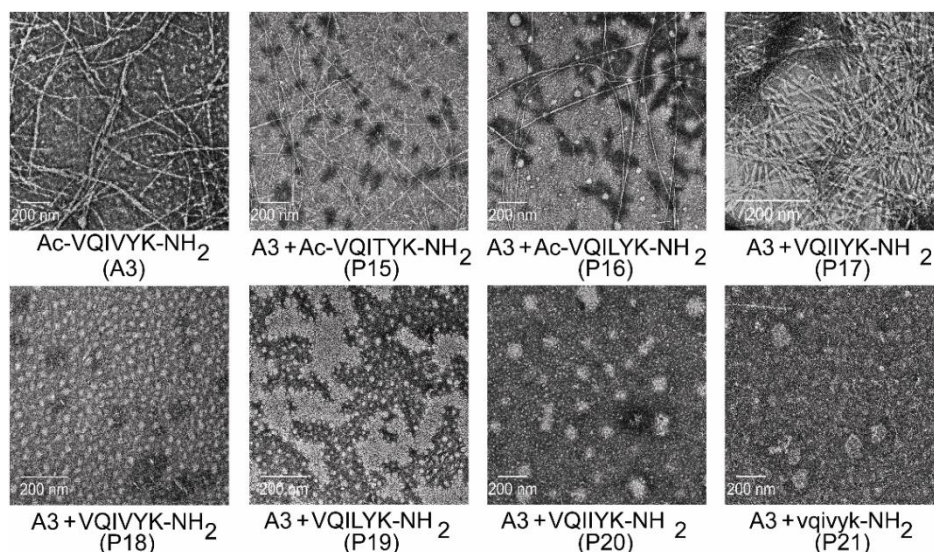


**Figure 7.8** Effect of designed peptide perturbants on model amyloidogenic peptide. Size estimation using dynamic light scattering (DLS) in the presence and absence of equimolar concentration of (a) accelerator peptides, and (b) inhibitor peptides, is shown.

### 7.3.3.3 Morphological examination using FETEM

Field-emission transmission electron microscope (FETEM) imaging was performed to examine the effects of the designed molecular perturbants on the morphology of A<sub>3</sub> aggregates. The untreated sample (A<sub>3</sub>) as well as the sample treated with accelerators (P15 and P16) show the presence of dense fibrils.

Contrastingly, samples treated with inhibitors (P18-P21) showed spherical structures, and no fibers were observed, indicating the absence of the amyloids in the mixture (Figure 7.9).



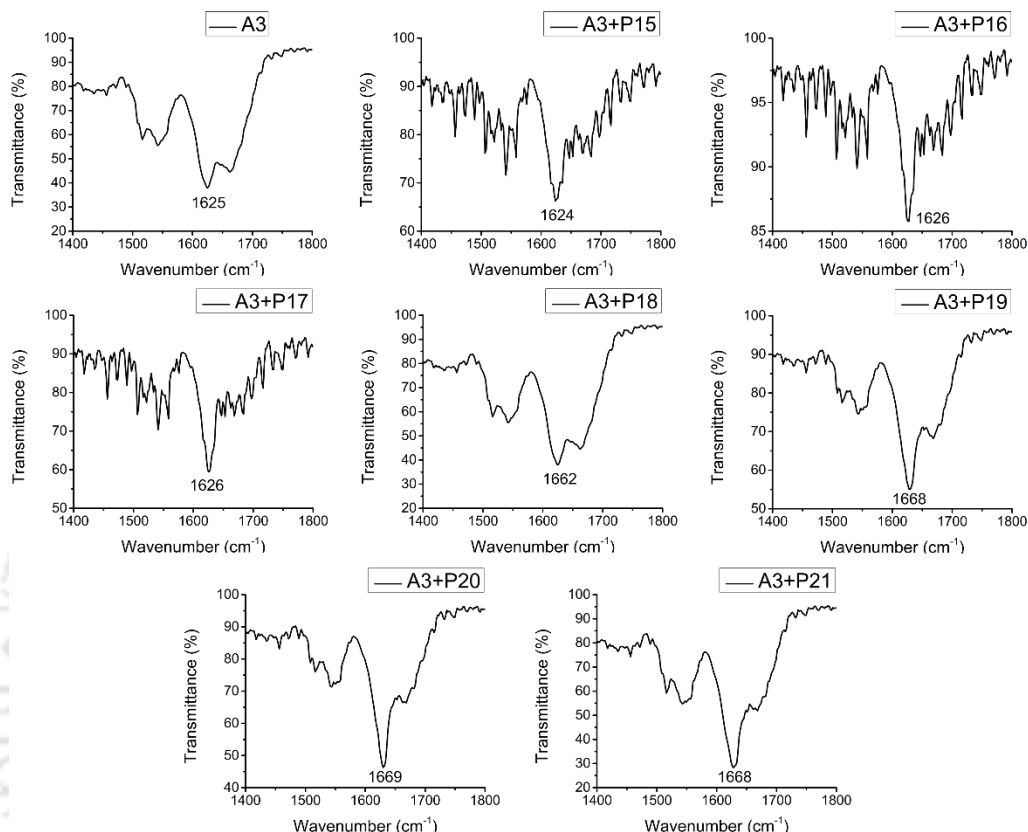
**Figure 7.9** FETEM analysis. FETEM micrographs of perturbant treated and untreated A<sub>3</sub> peptide.

#### 7.3.3.4 Secondary structure elucidation using FTIR

The secondary structure conformations of the treated and untreated A<sub>3</sub> samples was determined by FTIR analysis. While untreated A<sub>3</sub> peptide showed a characteristic amide I band of  $\beta$ -sheet, A<sub>3</sub> treated with peptides P15, P16 and P17 also displayed a peak around  $1625\text{ cm}^{-1}$  suggesting that the treated samples retain  $\beta$ -sheet conformation in dried films (Figure 7.10). A<sub>3</sub> peptide treated with perturbants P18, P19, P20, and P21 showed prominent peaks centered around  $1662\text{--}1669\text{ cm}^{-1}$  which has been assigned to random coil formation [25]. FTIR results suggest that though treated and untreated sample differs in their aggregation kinetics and morphology, they fold to the same  $\beta$ -sheet conformation in their dried form. This result indicates that the morphological difference induced by



perturbant treatment is expressed at the supramolecular assembly and not at the molecular level.



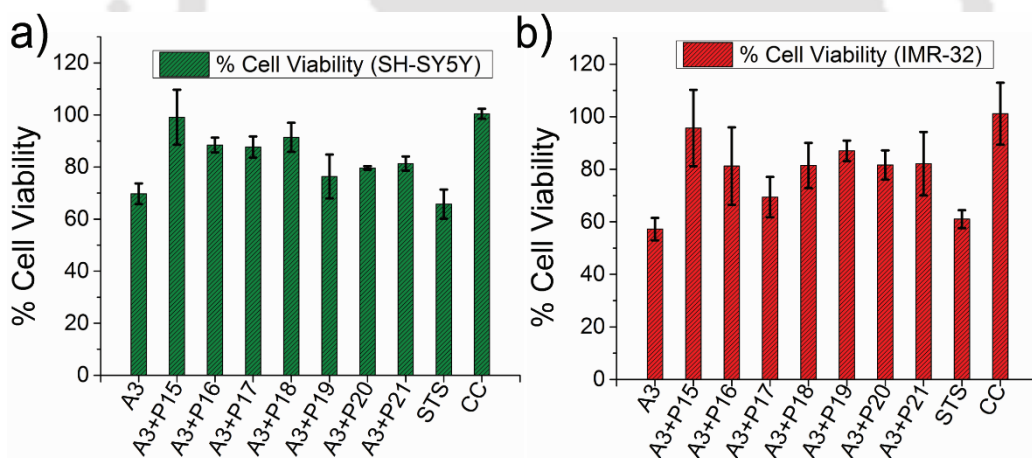
**Figure 7.10** Conformational analysis. FTIR spectra of perturbant treated and untreated A<sub>3</sub> peptide.

### 7.3.3.5 Cytotoxicity reduction analysis

The cytotoxic amyloid assemblies of tau protein aggregates are considered as the hallmark of tau linked pathogenesis in AD as well as other tauopathies. Millions of people, currently suffer from tauopathies, yet no FDA approved anti-amyloid therapy is available. The toxic tau aggregates have been ascribed to cause synaptic dysfunction and neuronal loss [27, 28]. Further, an increasing number of recent reports propose oligomeric intermediates formed during fibrillization to be more toxic than mature fibrils [29-31]. Hence, arresting the tau in its non-toxic form or accelerating it to self-assembly to mature fibrillar state may aid as a therapeutic



option. The impedance/acceleration of A<sub>3</sub> self-assembly, confirms the potential efficacy of the designed molecular perturbants for therapeutic applications in amyloidogenic diseases. Therefore, we measure the enhancement in the cell viability as a result of perturbant administration using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Human neuroblastoma SH-SY5Y and IMR-32 neuronal cell lines were treated with 25  $\mu$ M of perturbant treated and untreated A<sub>3</sub> peptide solution. A<sub>3</sub> samples were initially allowed to aggregate in the presence (treated) or absence (untreated) of an inhibitor/accelerator perturbant peptide. The test results show that the untreated amyloidogenic A<sub>3</sub> peptide solution lowered the cell viability of SH-SY5Y and IMR-32 to 57% and 69% respectively. The treatment with designed perturbant peptides, however, resulted in cell viability improvements from 6% to 30% in SH-SY5Y and 13% to 38 % in IMR-32 cell lines (Figure 7.11 and Table 7.2).



**Figure 7.11** Effect of peptide perturbants on the cellular toxicity of A<sub>3</sub> peptide. Post incubation, A<sub>3</sub> samples allowed to aggregate in the presence and absence of perturbant peptides were added to (a) SH-SY5Y, and (b) IMR-32 cells. The cytotoxicity effect was determined using an MTT assay. The molecular perturbation reduced the toxicity of A<sub>3</sub> peptide evident from the increased cell viability compared to the untreated sample. STS and CC denote positive control (Staurosporine) and negative control (untreated cells), respectively.

The cell viability experiment results endorse the neuroprotective potential of the designed perturbant peptides.

**Table 7.2** Summary of MTT assay data for IMR-32 and SH-SY5Y cell viability upon treatment with the designed peptide-based perturbant treated and untreated A<sub>3</sub> samples.

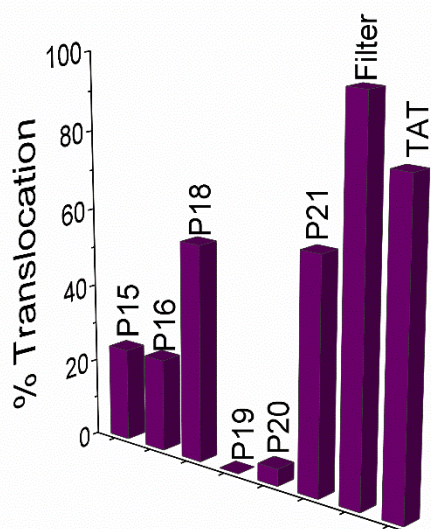
| Experimental Condition          | % Cell Viability (IMR-32) | Std. Dev. | % Cell Viability (SHSY-5y) | Std. Dev. |
|---------------------------------|---------------------------|-----------|----------------------------|-----------|
| A <sub>3</sub>                  | 57.23698                  | 4.28355   | 69.72848                   | 3.99116   |
| A <sub>3</sub> +P <sub>15</sub> | 95.71182                  | 14.52605  | 99.13345                   | 10.53207  |
| A <sub>3</sub> +P <sub>16</sub> | 81.22705                  | 14.72776  | 88.44598                   | 2.84953   |
| A <sub>3</sub> +P <sub>17</sub> | 69.42104                  | 7.68682   | 87.69497                   | 4.05338   |
| A <sub>3</sub> +P <sub>18</sub> | 81.45388                  | 8.61147   | 91.39226                   | 5.56845   |
| A <sub>3</sub> +P <sub>19</sub> | 87.00583                  | 3.8956    | 76.37204                   | 8.41998   |
| A <sub>3</sub> +P <sub>20</sub> | 81.6375                   | 5.5454    | 79.66493                   | 0.70042   |
| A <sub>3</sub> +P <sub>21</sub> | 82.12357                  | 12.06088  | 81.34027                   | 2.72011   |
| STS                             | 61.0175                   | 3.43297   | 65.74235                   | 5.61411   |
| CC                              | 101.17736                 | 11.81267  | 100.46216                  | 1.91689   |

### 7.3.3.6 BBB translocation assay

One of the prime causes behind the failure of potential antibody-based and other molecular therapies has been the failure to cross the blood-brain barrier owing to their large size [15]. Experimental studies support the capability of low molecular weight, small hydrophobic molecules to cross BBB [32, 33]. The small size and hydrophobic nature of our design scheme can aid the ability of our perturbant peptides in crossing the BBB. To test this hypothesis, we performed BBB permeability test on an *in vitro* model of BBB. We used a BBB model comprising of human brain endothelial cells, EA.hy926, cultured on the apical side of a permeable membrane placed between two sections, to assess the peptide

translocation. Perturbant peptide solution was added on the apical side of the BBB model and allowed to permeate for 5 hours. 5(6)-Carboxyfluorescein labeled cell penetrating peptide (CPP), TAT (47 - 57) known to have very high unidirectional influx across the BBB, was used as a positive control for BBB permeability in our experimental model [34]. Post incubation the solution in the basolateral side was collected. The peptide translocation was quantified employing the method discussed in section 7.2.9 of this chapter.

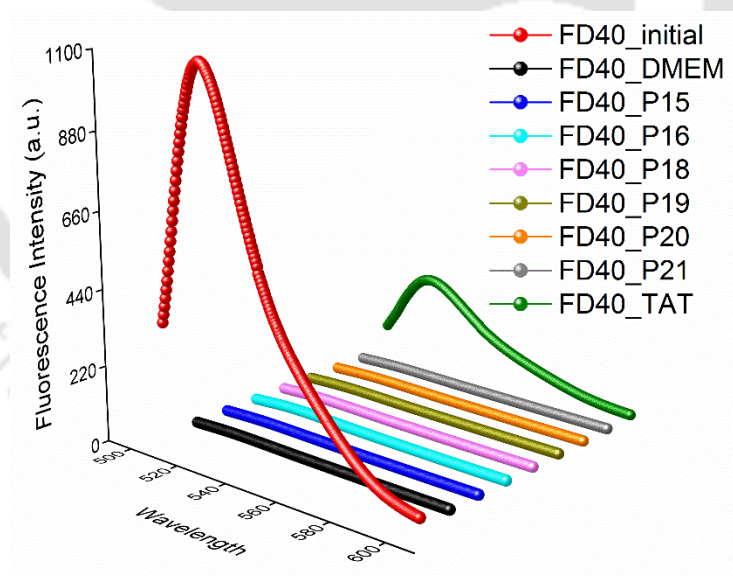
The experimental results show that after 5 hours, approximately 24% P15 and P16, 56% P18, 4.4% P20 and 61% P21 translocated across model BBB system. P19 shows 0% and positive control showed 83% translocation across model BBB system (Figure 7.12).



**Figure 7.12** BBB translocation assay. An in vitro BBB model comprising of an immortalized human brain endothelial cell line EA.hy926 grown as a monolayer on the apical side of the Transwell plate was employed. The perturbant peptides were added on the apical side of the plate and percentage translocation was estimated by comparing the initial (apical side) and final (basolateral side) peptide concentration. The measurements were compared to two controls: one an empty Transwell (“Filter”) and the other Tat<sub>(47-57)</sub> peptide added as positive control.

### 7.3.3.7 BBB integrity determination

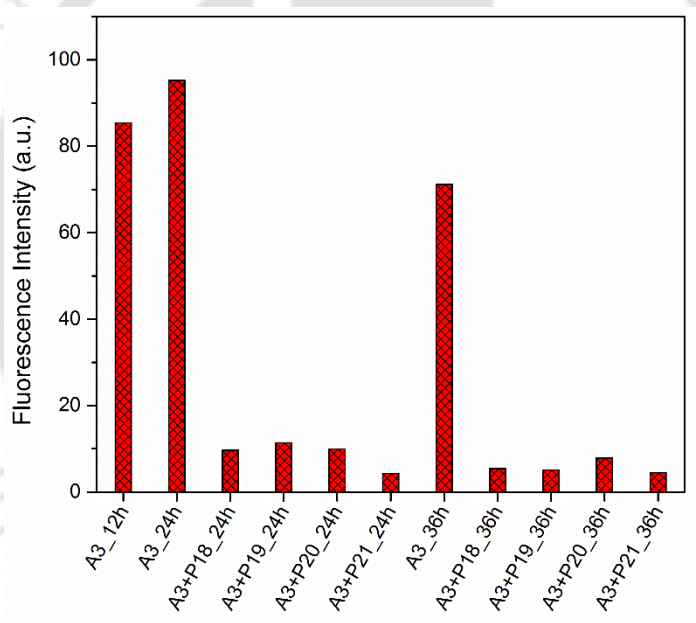
The BBB has an overall protective function, and thus the therapeutic designs should ensure from any impairment or disruption to normal functioning of BBB. Therefore, the integrity of BBB model post-treatment with peptide perturbants was evaluated by adding fluorescein isothiocyanate (FITC)-conjugated dextran (FD40, with the molecular weight of 40 kDa), to perturbant pre-treated wells for 2 hours and estimating FITC fluorescence post incubation to detect any damage to the BBB [35, 36]. The results of BBB integrity evaluation show the very low FITC fluorescence for perturbant peptides, indicating that they had negligible influence on the barrier integrity and hence no paracellular leakage (Figure 7.13). Positive control peptide TAT (47 - 57), however, shows high FITC fluorescence and thus affected the barrier permeability and may have caused a paracellular leakage.



**Figure 7.13** BBB integrity assay. Any loss of integrity of BBB model due to the effect of perturbant peptides was evaluated by adding a high molecular, FITC labelled FD40 compound and recording the FITC fluorescence to detect any damage to the membrane permeability.

### 7.3.4 Effect of inhibitor peptide on pre-aggregated A<sub>3</sub> peptide

A<sub>3</sub> peptide, allowed to aggregate for 12 hours was treated with inhibitor peptides, and ThT fluorescence measurements were recorded at 24th and 36th hour to investigate the effect of inhibitor peptide on the pre-formed A<sub>3</sub> aggregates. We observed that A<sub>3</sub> peptide treated with P18, P19, P20, and P21, showed an approximately 10-fold, 8-fold, 10-fold and 22-fold decrease in fluorescence intensities at 24th hour respectively, compared to untreated A<sub>3</sub>. After the 36th hour the reduction in treated A<sub>3</sub> fluorescence intensity with P18, P19, P20, and P21, was 13-fold, 13-fold, 9-fold, and 16-fold respectively (Figure 7.14).



**Figure 7.14** Time-dependent ThT assay profiles of pre-aggregated A<sub>3</sub> peptide treated with perturbants.

The results of this experiments indicate that not only designed peptide-based inhibitors could prevent the amyloidogenesis of A<sub>3</sub> peptide but they also can disaggregate the pre-formed A<sub>3</sub> self-assemblies, and P21 peptide was the most potent amyloid breaker of the experimental set.



## **7.4 CONCLUSIONS**

The molecular intervention of amyloidogenesis continues to be the motivation behind many drug discovery efforts against tauopathies and several molecular modulators have advanced to animal or human clinical trials. Here, we designed a series of peptides targeting the minimal amyloidogenic hexapeptide “VQIVYK” (A<sub>3</sub>). This peptide has been reported to play a critical role in the abnormal aggregation of tau. We demonstrated that our peptide-based modulators were non-amyloidogenic and showed minimal cytotoxicity on two model neuronal cell lines SH-SY5y and IMR-32. The treatment of A<sub>3</sub> with acetylated peptides accelerated the rate of its self-assembly while the non-acetylated counter-parts prevented A<sub>3</sub> from self-assembling. The effect the designed peptides on the size of aggregates, morphology, secondary structure conformation and cytotoxicity of the A<sub>3</sub> peptide was also investigated. The treatment of A<sub>3</sub> with perturbants impeded the formation of large aggregates and prevented the formation of toxic fibrillar morphologies. The treated A<sub>3</sub> peptide displayed the  $\beta$ -sheet conformation in FTIR experiments, indicating that the modulatory effect of perturbants is limited to the assembly level. The results of MTT assay-based cytotoxicity studies showed that there are up to 38% improvement in the cell viability of the peptide-treated A<sub>3</sub> samples, while preliminary BBB permeability experiments demonstrated the BBB crossing ability of the peptides. The addition of perturbant peptides to pre-aggregated A<sub>3</sub> samples, lowered the extent of aggregation. In conclusion, this class of designed peptide molecules are highly potent in modulating the aggregation of tau protein, reducing the aggregation induced cytotoxicity, having BBB permeability and ability to break pre-formed self-assemblies.



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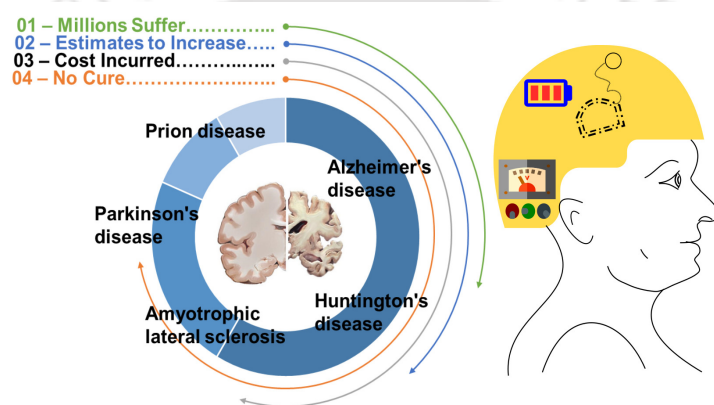
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## CHAPTER 8

# A PROTOTYPE DEVICE FOR NON-INVASIVE TREATMENT OF NEURODEGENERATIVE DISORDERS



Alzheimer's disease (AD) is the most prevalent form of dementia in the elder population, affecting 48 million people worldwide. Alzheimer's Association estimates one new case every 33 seconds. This number may almost triple by 2050 in the absence of any cure or prevention methods. Although dementia mainly affects older people, it is not a normal part of ageing. AD currently has no available cure. There is an urgent need to develop effective therapy to treat the disease, delay its onset, and also prevent it from developing. This chapter is logical extension of our experiments at molecular-scale. We present the prototype of a futuristic device that can be used as a non-invasive therapeutic option. However, a series of *in vivo* studies, which we are pursuing in mouse models, and device optimization, would be required to develop this model to a futuristic therapeutic device.

An international patent on this work is: PCT patent no. WO/2019/012556



## **8.1 INTRODUCTION**

Most of the neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis are characterized by selective loss or degeneration of specific regions of the brain, and accumulation of protein or peptide aggregates, also known as amyloids, in neurons or other cells or extracellularly in patients. Among these, Alzheimer's disease, and Parkinson's disease are age-related diseases characterized with progressive loss of neurons with increasing age.

Alzheimer's disease is the most prevalent form of dementia in the elder population and as per WHO estimates, it affects nearly 48 million people worldwide [1]. It is characterized by the presence of dense insoluble deposits of amyloid-beta ( $A\beta$ ) peptide outside and around neurons. These deposits, often referred to as "plaques", are a result of abnormal aggregation of  $A\beta$  peptide into toxic aggregates. As per the amyloid cascade hypothesis, amyloid precursor protein (APP), (a transmembrane protein found in the brain cells which helps in synaptic activities and also protects brains cells from injuries) is cleaved by enzyme  $\alpha$ -secretase, and the cleaved fragments are exported to extra-cellular space where they re-fold to form soluble oligomers. These soluble oligomers coalesce to form insoluble fibrils which aggregate further to form amyloid plaques [2].

Approximately hundred potential drugs for treatment of Alzheimer's disease have failed between 1998 and 2011 [3]. And the ones that have been approved by Food and Drug Administration have not shown much promise and have several side-effects [4-6]. There are two types of drugs for treatment of Alzheimer's disease; memantine and four acetylcholinesterase inhibitors (rivastigmine, galantamine, tacrine, and donepezil). At times they are often used in combination [7]. These drugs however have failed to cure the disease or even prevent its progression and can only provide symptomatic treatment [8].



Several small molecule therapeutic strategies have been suggested for treating Alzheimer's disease such as:

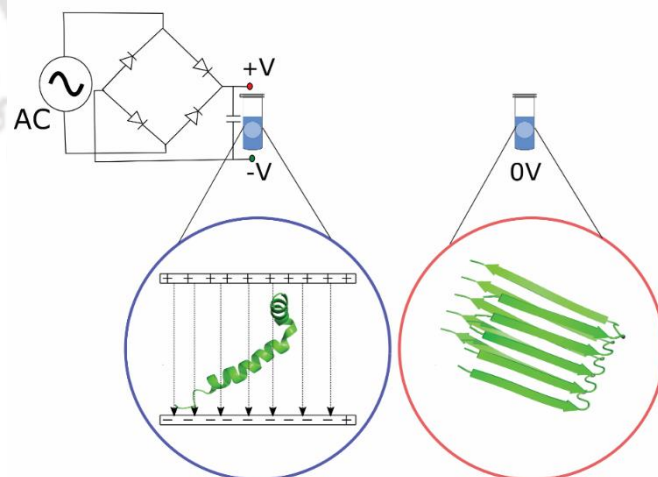
- (a) *stabilizing the native state*: unfolding and thus aggregation is inhibited by using proteins or small molecules that bind to the native folded state of protein and prevent it from unfolding [9];
- (b) *sequestering monomeric peptide*: antibodies with help of protein-engineering as well as designed peptides have been used to bind to aggregation-prone regions of monomeric peptides to prohibit it from self-assembling [10-12];
- (c)  *$\beta$ -sheet breakers that terminate fibril elongation*:  $\beta$ -Sheet formation is fundamental for amyloid aggregation; fibrils grow by adding new strands via hydrogen bonding to the sheet; in this approach the fibrils are capped by adding peptide/pseudo-peptides which prevents fibril growth [13-16];
- (d) *inhibiting gamma ( $\gamma$ )-secretase or  $\beta$ -secretase or activation of alpha secretase*: to prevent abnormal cleavage amyloid precursor protein (APP) [17, 18];
- (e) *removing existing amyloid plaques*: using vaccines or antibody based approaches [18]; and
- (f) *microglial activation using scanning ultrasound*: Microglia act like microphage and digest amyloid plaques by phagocytosis [19].

Though these strategies have shown promise, some have inhibited fibril formation while others have arrested fibrillar growth, however, most of them show poor ability to penetrate the blood-brain barrier and are often highly toxic.

The electric field studies on biomolecules (like DNA and proteins) have shown permanent or induced dipole formation along the direction of applied field, indicating that electric field interaction could modulate their dipole moments [20-23]. Against this backdrop, there is a pressing need to discover improved

therapeutic strategies for the treatment of Alzheimer's disease. In particular, an inexpensive and non-invasive approach, for treating Alzheimer's, which may also avoid the limitations of drug therapy.

In this chapter, we discuss a potentially inexpensive and non-invasive technique for treating Alzheimer's disease using electric field. Figure 8.1 shows the graphic illustration of our hypothesis on electric field mediated modulation of amyloid assembly. We propose a device that generates focused AC/DC electric field specifically targeting dense insoluble deposits of amyloid-beta ( $A\beta$ ) peptide outside and around neurons in the brain of an Alzheimer's patient. To verify the efficacy of our approach we have employed an additive step by step strategy and have tested field based perturbation first on the core recognition motif of  $\beta$ -amyloid polypeptide [24]. The shortest molecule known to self-assemble into ordered nano-structures: diphenylalanine (L-Phe-L-Phe, FF), discussed in detail in Chapter 4. We have further tested our strategy on KLVFFAE [ $A\beta$  (16-42)] which is one the shortest amyloid forming peptides, with FF as the core aggregating segment.



**Figure 8.1** Hypothesis on an electric field mediated modulation of  $\beta$ -peptide to a non- $\beta$  conformation.

The stretch LVFFA is said to form the central hydrophobic core essential for fibril formation [25-28]. And finally the field set up have also been used for assessing the effect of our strategy on the full length A $\beta$  (1-42) peptide by my colleague in the laboratory.

## **8.2 DESCRIPTION OF THE DEVICE PROTOTYPE**

The present invention describes a device that can generate focused AC/DC electric field specifically targeting dense insoluble deposits of amyloid-beta (A $\beta$ ) peptide outside and around neurons in the brain of an Alzheimer's patient. This device can serve as a potential non-invasive therapeutic tool for disruption of the inherent  $\beta$ -sheet conformation known to be the state for fibril formation.

The total circuit can generally be divided into 4 stages, namely:

- the power supply/charging circuit for battery inside,
- oscillator/transistor stage,
- the voltage booster stage.
- and two parallel aluminum electrodes separated by a distance of 17 cm, anchored on to the helmet

### **8.2.1 Constitution of the device prototype**

The present invention provides a potential device for treating neurodegenerative diseases, including, but not limiting to Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis using electric field. More specifically, the invention provides a device which generates focused AC/DC electric field specifically targeting dense insoluble deposits of amyloid-beta (A $\beta$ )

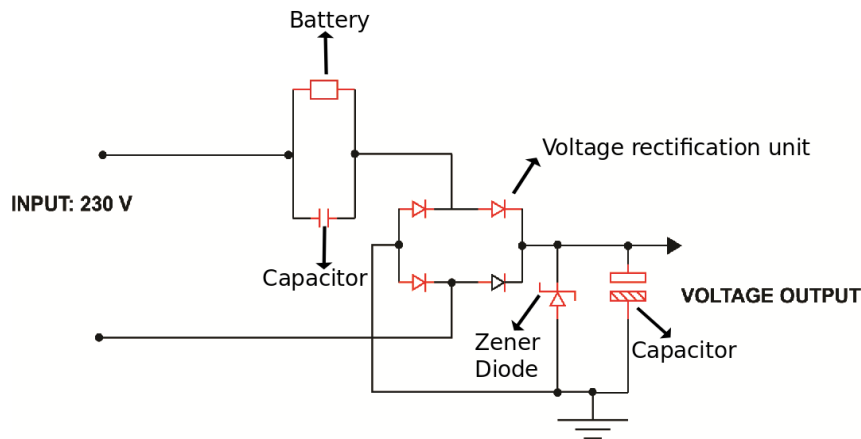
peptide outside and around neurons in the brain of a patient. This mode of action serves as a non-invasive therapeutic tool for disruption of the inherent  $\beta$ -sheet conformation known to be the reason for fibril formation.

The device typically comprises of an electrode assembly adapted to be placed on the head of a patient secured with an insulating material. Power is supplied to the electrode assembly through a battery; the battery in turn is connected to a charging circuit unit which sources low power electricity sufficient enough to charge the battery. At least one oscillator and one voltage booster are connected in line with the battery for amplifying the voltage received, and this amplified voltage is provided to the electrodes in a continuous manner. An electric field is generated across the electrodes upon receiving the voltage.

The device prototype comprises of the following major components:

#### **8.2.1.1 Power Supply/ Charging Circuit**

It's a simple capacitive power supply which can source few milli amps sufficient enough to charge the battery inside. The initial capacitor helps in limiting the current and then the voltage is rectified. This rectified voltage is brought to the desired level (battery voltage level) using a Zener Diode and a capacitor to filter the noise of the DC voltage produced. This would charge the battery inside, while plugged into the mains. This may be accompanied with a switch to enable charging (Figure 8.2).



**Figure 8.2** A schematic illustration of the power module comprising of a battery and a charging circuit unit, equipped with a capacitor which limits the current from the mains and the battery and a voltage rectifier for providing DC voltage, the Zener diode and the second capacitor to filter the noise on the DC voltage.

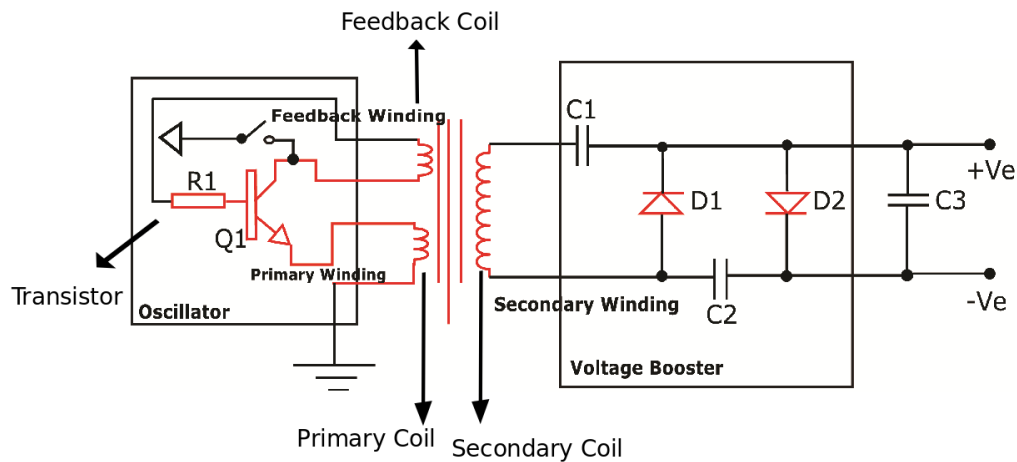
#### 8.2.1.2 Oscillator

Current is enabled through a transistor (generally npn) which allows the current to flow through a primary coil, inducing voltage in the secondary coil and secondary coil in return induces voltage in feedback coil. This counter voltage in the feedback coil causes the transistor to stop conducting and the magnetic field in the ferrite core to collapse via electrical energy from secondary coil. This process helps the transistor to conduct again, repeating the process and creating pulsed DC. The changing magnetic field induces high voltage in the secondary coil. The transformer used here is a kind of flyback transformer, unlike the normal mains transformers (Figure 8.3-8.5).

#### 8.2.1.3 Voltage Booster

This voltage is now further boosted using a combination of Diodes ( $D_1$  and  $D_2$ ) and Capacitors ( $C_2$  and  $C_3$ ), which is typically a Voltage multiplier. Typically, the

circuit uses Voltage Triple, which triples the available voltage from secondary winding of transformer (Figure 8.3-8.5).



**Figure 8.3** Schematic representation showing the circuit diagram of the oscillator and voltage amplification unit of the prototype device.

#### 8.2.1.4 Electrodes

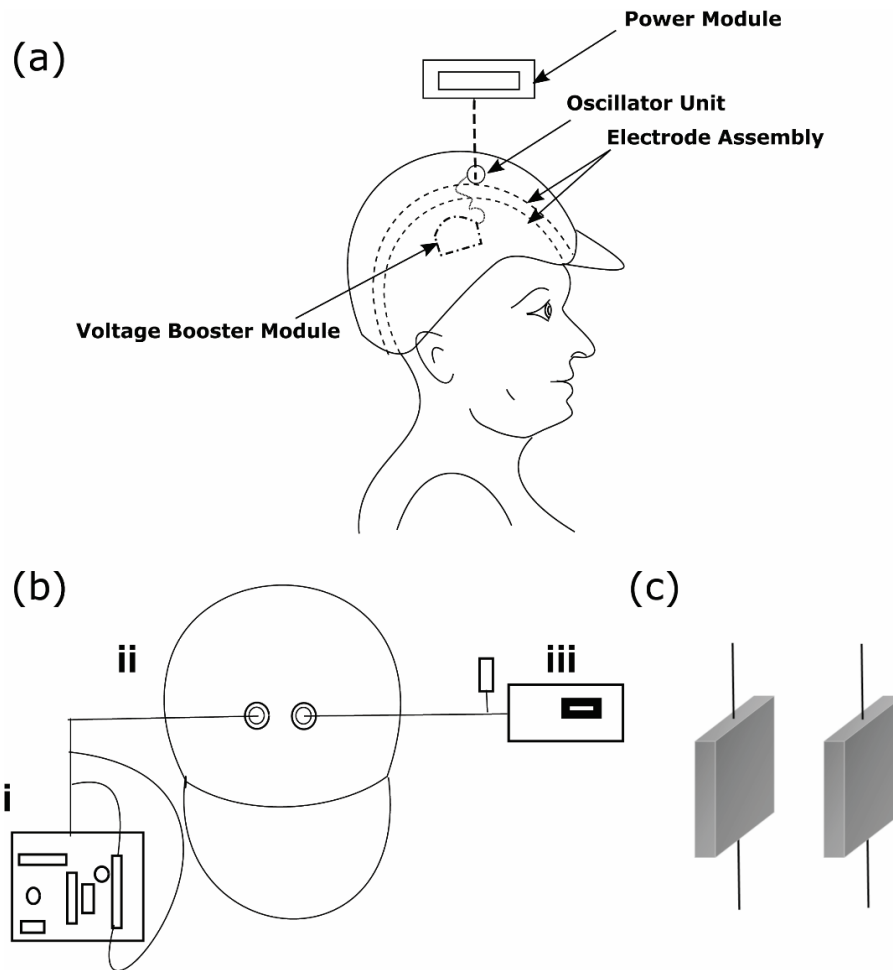
The high voltage generated by the voltage booster unit is passed onto the wires connected to two parallel aluminum electrodes separated by a distance of 17 cm, anchored on to the helmet. The electrodes have a uniform DC electric field between them. These parallel electrodes are insulated by thick plastic lamination and are adapted to be worn on the head of a patient, typically secured to a head-gear or a helmet (Figure 8.4, 8.5).

An alternate version of this device will be capable of generating an AC field across the electrodes. In this prototype, the charging circuit unit comprises of at least two capacitors, and at least one Zener diode; unlike the DC voltage setup, voltage rectifier is not used in this case.

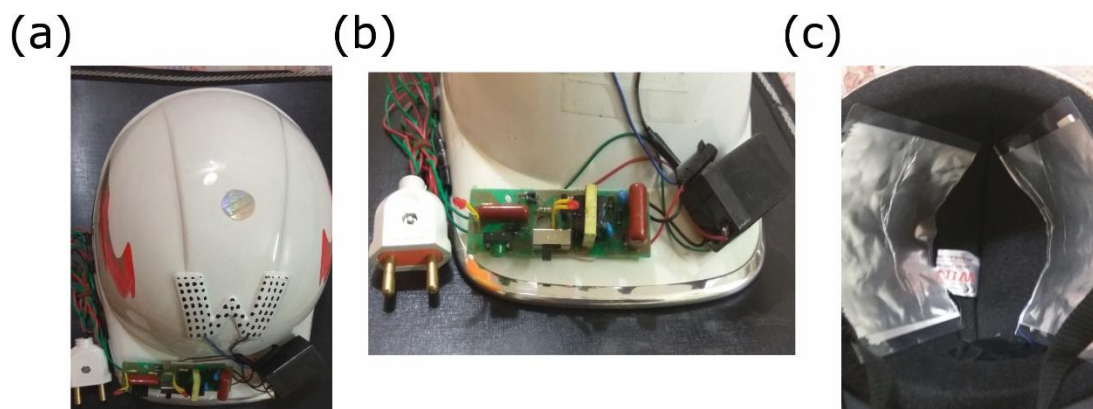


### 8.2.2 Drawings of the device prototype

The following section shows diagrammatic illustrations of the design schemes of the set-up of our porotype device.



**Figure 8.4** A schematic illustration of (a) A device for non-invasive treatment of neurodegenerative diseases using electric field. The device comprises of an electrode assembly, safely secured and adapted to be worn on head of a patient, a power module, one electronic oscillator, and a voltage booster module. (b) Cross-section view of electric field therapy device comprising of: (i) a cap in which two parallel aluminium electrodes connected to (ii) to voltage booster unit powered by (iii) DC voltage supply, and (c) cross-section view of two insulated parallel aluminium electrodes present inside electric field therapy device.



**Figure 8.5** (a) Top view of a wearable electric field therapy device of the present invention, (b) Inside view of an electric field device of the present invention, showing aluminium electrodes separated by a distance of 18 cm to produce a horizontal DC electric field and, (c) Top view of a wearable electric field therapy device of the present invention.

### 8.3 CONCLUSIONS

Through this study, we propose the design of an experimental set-up to assess the electric field based modulation of peptide aggregation and amyloid formation in disease model systems. We presented the outlines of a prospective device as a future therapeutic option. Series of *in vivo* studies and clinical trials, for its potential applications as a therapeutic option against amyloid triggered disorders, is pending.

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## CHAPTER 9

### CONCLUSIONS AND FUTURE DIRECTIONS







## **9.1 CONCLUSIONS**

Amyloid fibrils originating from abnormal protein misfolding are involved in a wide range of functional and pathological cellular processes. The amyloids are associated with several devastating human diseases collectively known as proteopathies. The understanding of the exact mechanism of amyloid formation and their toxicity is still imprecise. The absence of treatments, debilitating effects and economic burden that proteopathies imparts on society compels discovery of effective therapies at the earliest possible time. The spontaneous association of amyloids has also inspired the efforts to employ their self-assembly principles to develop peptides that can potentially self-assemble into ordered nanostructures. The development of controlled and directed nano-assemblies can deliver the raw materials for the fabrication of novel nano-materials and devices, an emerging area in the field of bionanotechnology.

The work put forth in this thesis primarily aimed to understand the mechanism of protein aggregation and investigate the effects of perturbants, both physical and molecular as modulators of amyloid self-association. The main approaches employed as modulators are electric field and peptides. The first strategy has a physical non-invasive mode of operation and involves the application of electric field mediated stress to perturb the peptide self-assembly. The idea of non-invasive methods as potential therapeutics for amyloid-induced disorders is gaining interest because; such therapies could surmount some of the shortcomings of their invasive counterparts. The second approach has an intrusive chemical means of action and comprises of de novo designed peptides as perturbants of amyloid formation.

The main body of the thesis consists of six working chapters. The introductory chapters (chapter 1 and 2) provide an uninitiated reader the kind of orientation in the basics of protein aggregation, the background to, and summary of, the previous and current research in the working chapters. Chapter 3 elucidated the potential of the electric field as perturbant of FF self-

assembly. The experimental results showed that electric-field mediated modulation of electrostatic interactions directing the self-assembly of FF could arrest the self-assembly of FF and fabricate nanostructures with distinct morphologies without any chemical additives. FF being the derived from AD-associated A $\beta$  (1-42) peptide, electric field mediated arrest of its self-assembly encouraged us to expand its application on other amyloidogenic peptides, and explore its efficacy as a potential non-invasive therapeutic for amyloid-induced disorders. Chapter 4 highlighted the differential aggregation kinetics of short steric zipper peptides of the same length and examined the geometrical basis of their varying rates of aggregation. The efforts to establish the role of side-chain geometry on the protein aggregation kinetics are still in progress.

Chapter 5 discussed the development of structure-based peptides as perturbants targeting the core hydrophobic fragment of the amyloid beta polypeptide. Our experimental results showed that treatment of amyloidogenic model peptide with the perturbants decelerated its aggregation kinetics and prevented the formation of toxic fibrillar morphologies. Chapter 6 demonstrated the application of the electric field as a modulator of tau aggregation, a key player in numerous proteopathies. The experimental findings established that the electric field exposure decelerated the aggregation kinetics of two hexapeptides, forming the aggregation core of the pathological tau. The structures formed by both the peptides under field conditions were lacking the typical fibrillar morphology, were smaller in size, and were 18 to 45 % less toxic in two neuronal and one normal cell lines. Chapter 7 presented the de novo designed novel class of highly potent potential modulators of tau self-assembly. The addition of perturbant peptides to the hexapeptide "VQIVYK" (reported to catalyze the pathological aggregation of tau) modulated its self-assembly by either arresting or accelerating it. The inhibition, as well as acceleration of aggregation, lead to a reduction in the aggregation induced cytotoxicity. The perturbant peptides

showed minimal cellular toxicity to model neuronal cell lines and exhibited the BBB permeability, and when added to pre-aggregated peptide samples, they also showed the ability to break pre-formed self-assemblies, making them potent candidates to counter pre as well post pathogenic interactions of tau. The positive results from our experimental studies employing electric field as an aggregation modulator, motivated us to propose a prototype device (Chapter 8) that can serve as a futuristic non-invasive therapeutic option for targeting amyloidogenesis in AD and other proteopathies.

The work presented in this thesis may provide new avenues to optimize both non-invasive as well as peptide-based therapies against the amyloid formation. However, how our findings in the reductionist *in vitro* peptide-based systems translate to the complex environment of a living system will be an interesting investigation.

## **9.2 FUTURE DIRECTIONS**

The culmination of the present work presents the foundation stone for the initiation of multiple future avenues pertaining to the development and assessment of molecular and physical perturbants as a therapeutic option for protein misfolding disorders and as a design tool for nanofabrication. The following opportunities could be explored as part of the future work of the thesis:

Investigate the combined effects of designed peptide-based perturbants and electric field on the aggregation kinetics of disease associated amyloidogenic peptide and proteins.

- *in vivo* studies on AD animal models to investigate the efficacy of electric field as a potential therapeutic option.
- Examining the role of side-chain geometry on the aggregation kinetics of amyloidogenic proteins/peptides.

- Design of novel peptide-sequences targeting amyloid aggregation based on our current set of designs.
- *in vivo* testing of the designed peptides to modulate the amyloid and tau self-assembly.
- Examine the effects of peptide-based aggregation modulators on full-length tau protein.
- *in vivo* studies on tau animal models to investigate the efficacy of electric field as a potential therapeutic.
- Development of a future peptide-based drug for AD.



APPENDIX







**Table A1** list of synthesized peptide sequences and their molecular mass estimated using MALDI-TOF. D-amino acids are shown in small alphabets.

| S No.                                       | Peptide Sequence | Calculated Molecular Weight (Da) | Observed Molecular Mass (m/z) |
|---------------------------------------------|------------------|----------------------------------|-------------------------------|
| <b>List of Short Aggregating Peptides</b>   |                  |                                  |                               |
| 1                                           | Ac-KLVFFAE-am    | 894.063                          | 895.508                       |
| 2                                           | MVGGVVIA-am      | 743.950                          | 767.235 [+Na] <sup>+</sup>    |
| 3                                           | Ac-VQIVYK-am     | 789.956                          | 791.197                       |
| 4                                           | SSTNVG-am        | 562.563                          | 567.037                       |
| 5                                           | NKGAIL-am        | 613.741                          | 614.431                       |
| 6                                           | NFGAIL-am        | 632.743                          | 655.384 [+Na] <sup>+</sup>    |
| 7                                           | LSFSKD-am        | 694.768                          | 696.351                       |
| 8                                           | GNNQQNY-am       | 835.814                          | 857.896 [+Na] <sup>+</sup>    |
| 9                                           | SSTSAA-am        | 521.511                          | 544.009 [+Na] <sup>+</sup>    |
| 10                                          | Ac-VQIINK-am     | 754.911                          | 756.422                       |
| <b>List of Designed Peptide Perturbants</b> |                  |                                  |                               |
| 1                                           | Ac-KLVfFAE-am    | 894.063                          | 895.576                       |
| 2                                           | Ac-KLVfFAE-am    | 894.063                          | 895.508                       |
| 3                                           | Ac-KLvFFaE-am    | 894.063                          | 894.318                       |
| 4                                           | Ac-KLVHHAE-am    | 873.993                          | 875.409                       |
| 5                                           | Ac-KLVHFfAE-am   | 884.028                          | 885.486                       |
| 6                                           | Ac-KLVFHAE-am    | 884.028                          | 885.521                       |
| 7                                           | KLVfFAE-am       | 852.026                          | 853.358                       |
| 8                                           | KLVfFAE-am       | 852.026                          | 853.252                       |
| 9                                           | KLVFHAE-am       | 841.991                          | 843.372                       |
| 10                                          | KLVHFfAE-am      | 841.991                          | 843.393                       |
| 11                                          | klvffae-am       | 852.026                          | 853.415                       |
| 12                                          | klvFfae-am       | 852.026                          | 853.396                       |
| 13                                          | Ac-klvffae-am    | 894.063                          | 895.258                       |
| 14                                          | Ac-klvFfae-am    | 894.063                          | 895.415                       |
| 15                                          | Ac-VQITYK-am     | 791.928                          | 792.533                       |
| 16                                          | Ac-VQILYK-am     | 803.983                          | 804.466                       |
| 17                                          | Ac-VQIIYK-am     | 803.983                          | 804.666                       |
| 18                                          | VQIVYK-am        | 747.918                          | 749.777                       |
| 19                                          | VQILYK-am        | 761.945                          | 761.955                       |
| 20                                          | VQIIYK-am        | 761.945                          | 762.533                       |
| 21                                          | vqivyk-am        | 747.918                          | 748.977                       |



## List of Publications and Conferences

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### Patents

#### International Patent

- (1) Patent No. WO/2019/012556. Dated 17.01.2019. Title: A device treatment of neurodegenerative diseases.

#### Indian Patents

- (1) Patent No. TEMP/E-1/36478/2019-KOL. Dated 27.08.2019. Title: Peptide-based modulators of amyloidogenic peptides.
- (2) Patent No. 243/KOL/2015. Dated 09.03.2015. Title: Generation and usage of stimulus responsive nanostructures.
- (3) Patent No. 10258/2016-KOL. Dated 31.03.2016. Title: Magnetic hydrocarbon crystals.

#### Journal Articles

- (1) Jahnu Saikia, **Gaurav Pandey**, Sajitha Sasidharan, Ferrin Antony, Harshal B. Nemade, Sachin Kumar, Nitin Chaudhary, and Vibin Ramakrishnan. Electric Field Disrupts Amyloid Aggregation; Potential Non-invasive Therapy for Alzheimer's Disease. *ACS Chemical Neuroscience*, (2019), DOI:10.1021/acscemneuro.8b00490
- (2) **Gaurav Pandey**, Sudhir Morla, Harshal B. Nemade, Sachin Kumar and Vibin Ramakrishnan. Modulation of Aggregation with Electric Field; Scientific Roadmap for a Potential Non-Invasive Therapy Against Tauopathies. *RSC Advances* (2019), DOI:10.1039/c8ra09993f.
- (3) **Gaurav Pandey**, Jahnu Saikia, Sajitha Sasidharan, Deep C. Joshi, Subhash Thota, Harshal B. Nemade, Nitin Chaudhary, and Vibin Ramakrishnan. Modulation of Peptide Based Nano-Assemblies with Electric and Magnetic Fields. *Scientific Reports*, (2017), DOI: 10.1038/s41598-017-02609-z.

## List of Publications and Conferences

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### Conference Proceedings

- (1) **Gaurav Pandey**, and Vibin Ramakrishnan. Modulation of Aggregation with Electric Field; Electric field assisted inhibition of aggregation in amyloidogenic proteins. *Proceedings of the 35th European Peptide Symposium*, (2018), DOI: 10.17952/35EPS.

### Manuscripts Under Preparation

- (1) Title: Modulation of Tau Protein Aggregation using 'Trojan' sequences
- (2) Title: Molecular Perturbation of Amyloid Self-Assembly.
- (3) Title: An Investigation on The Length Dependency of RADA Peptide Self-Assembly.
- (4) Title: Geometric Origins of Protein Aggregation Kinetics.
- (5) Title: Protein Aggregation: Invasive and Non -Invasive Therapeutics (review paper).