# Concept of β-Breaker Dipeptides and its Application in Alzheimer's Amyloid Disruption

**A Thesis Submitted in Partial Fulfillment of the**

**Requirements for the Degree of**

***DOCTOR OF PHILOSOPHY***

**Submitted by**

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**Dedicated to**

# Lord Krishna & Parents


# Indian Institute of Technology Guwahati

## Department of Chemistry

## STATEMENT

I do hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the Department of Chemistry, Indian Institute of Technology Guwahati, India under the guidance of Dr. Bhubaneswar Mandal.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

2nd April 2013 N. K. Chaitanya

Indian Insitute of Technology Guwahati

**Certificate**


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## CERTIFICATE

This is to certify that N K Chaitanya has been working under my supervision since July 2008 as a regular registered Ph. D. student. I am forwarding his thesis entitled "**Concept of β-Breaker Dipeptides and its Application in Alzheimer's Amyloid Disruption**" for being submitted for the Ph. D. (Science) degree of this institute. I certify that he has fulfilled all the requirements according to the rules of this institute regarding the investigations embodied in his thesis and this work has not been submitted elsewhere for a degree.

2nd April 2013

**Dr. Bhubaneswar Mandal**

**Supervisor**

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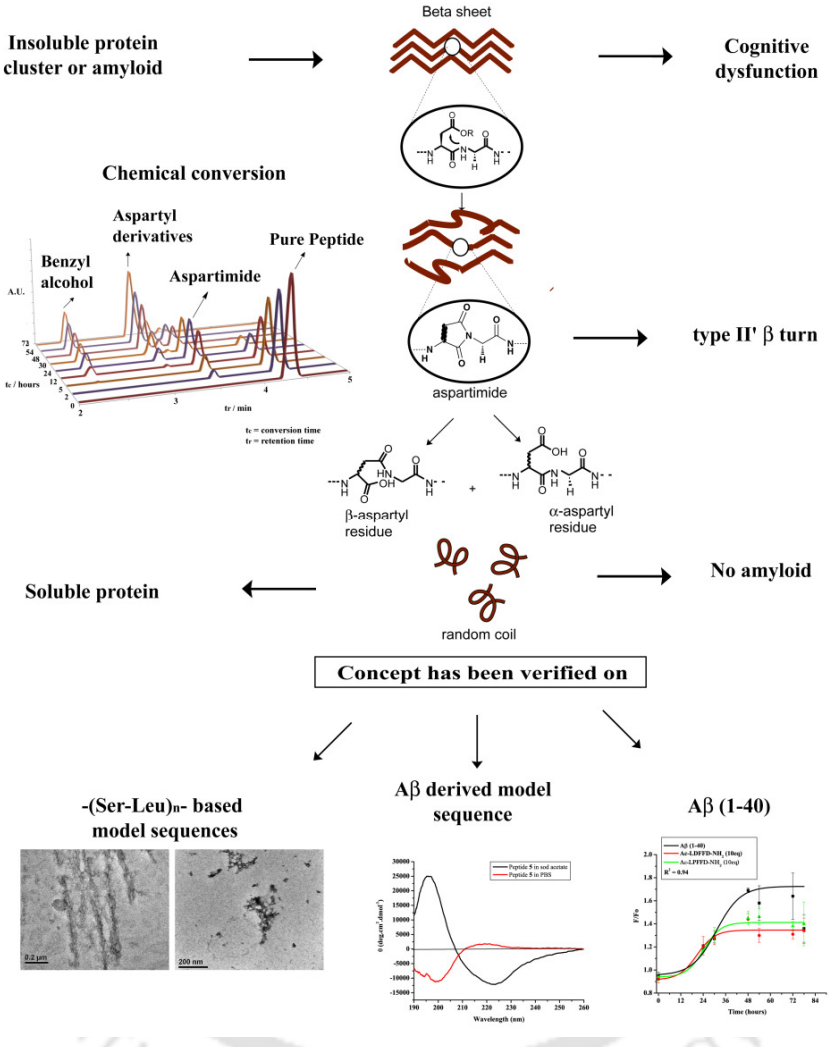
## Synopsis

Misfolding and aggregation of amyloid β peptide into fibrillar aggregates is believed to be the main cause of Alzheimer's disease (AD). This transformation of amyloid β peptide from its native state to β sheet rich ordered aggregates occurs via a nucleation dependent pathway. One of the possible approaches to inhibit or disrupt the aggregation of Aβ peptide is the use of β breaker peptides introduced by Prof. Soto and is a landmark in the domain of peptide based drug design against AD. Later other strategies, including the use of N-methylated amino acids and Aib (amino isobutyric acid) based analogues have shown promising results. However, these strategies suffer from a conceptual drawback as the preinstalled disrupting element, e.g. kink (for proline and Aib) or N-methylated amino acids may lessen the chances of proper alignment and recognition to the aggregating Aβ peptide. And if the recognition of the β-breaker peptide with the aggregating peptide is not good enough β-breaking effect cannot be expected. To solve this problem we have developed a novel concept, termed as '**the concept of β-breaker dipeptides (BBDP)**". The proof of the principle of this novel concept and its application in amyloid disruption is the main topic of this thesis.

The prime idea of the concept is based on the chemistry of aspartimide formation. Aspartimide induces type II' β turn when incorporated in a peptide and ring opening of the aspartimide residue generates α and β aspartyl residues (1:3 ratio) at physiological pH. We wanted to incorporate an Asp residue in a designed β-breaker peptide, where the side chain of the Asp is protected with a benzyl group. Most attractive feature of this novel β-breaker peptide is, as it retains the normal peptide backbone, i.e. no preinstalled kink or N-methylation is present, the β-

breaker peptide will align with another aggregating sequence properly. Therefore, no recognition problem will be there.




**Thesis overview**

Later at physiological pH, aspartimide formation will take place, which will generate a kink and disturb the planer β-sheet topology. Subsequent ring opening of the aspartimide derivative generates α and β aspartyl analogues, which brings flexibility in the system so that hydrogen bonding between β breaker peptide and other aggregating peptide breaks down. Racemization of

the Asp residue also will play a role for disruption of the β-sheet architecture. Thus, complete breakdown of the amyloid can be achieved using such a technique as shown in the above figure. Chemical conversion of β breaker dipeptide unit into aspartimide and aspartyl residues can be monitored using LC-MS.

In the first few chapters, we described the design and synthesis of a novel class of β-breaker peptides, which are BBDP containing β-breaker peptides and used them to inhibit and disrupt the aggregation of other aggregating peptides. We obtained the proof of the concept from model -$(Ser\text{-}Leu)_n$- based sequences. First it was shown that a -$(Ser\text{-}Leu)_n$- based BBDP containing β-breaker peptide inhibit self aggregation and that can be used to inhibit the aggregation of other aggregating peptide with homologous sequence. From the thioflavin T experiment we have demonstrated that amount of fibril increased first, wich was disrupted to a considerable extent with further incubation at near physiological pH *in vitro*.

Later, we have shown that Aβ derived breaker peptide could inhibit self aggregation. Also, Aβ derived shorter aggregating sequence which is likely to behave similar to the full length Aβ (1-40) was synthesized and it was demonstrated that aggregation of that sequence was also inhibited considerably when mixed with an Aβ derived β-sheet breaker peptide at near physiological condition *in vitro*. Most significant result was that the β-sheet breaker peptide which was designed using the BBDP concept was able to inhibit the aggregation of the full length Aβ (1-40). Based on all the findings, we can conclude that the BBDP concept is a valid concept for β-sheet disruption and can be applied for the development of peptide based therapeutics against Alzheimer's disease. Further this concept can be extended to the drug design against other amyloid diseases as well.

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Nadimpally Krishna Chaitanya

**Abbreviations**

| | |
|---|---|
| Aβ | Amyloid beta |
| $Ac_2O$ | Acetic anhydride |
| AD | Alzheimer's disease |
| APP | Amyloid precursor protein |
| BBDP | β-breaker dipeptide |
| Boc | *tert*-butyloxycarbonyl |
| BOP | Benzotriazol-1-yloxy tris(dimethylamino) phosphonium hexafluorophosphate |
| Bzl | Benzyl |
| Cbz | Benzyloxycarbonyl |
| CD | Circular dichroism |
| CR | Congo red |
| DCM | Dichloromethane |
| DIPEA | Diisopropylethyl amine |
| DMF | N, N dimethyl formamide |
| ESI MS | Electrospray ionization mass spectrometry |
| $Et_2O$ | Diethyl ether |
| Fmoc | 9-Fluorenylmethoxycarbonyl |
| FT-IR | Fourier transformation infra red spectroscopy |
| HFIP | 1,1,1,3,3,3-hexafluoro-2-propanol |
| HOBt | 1-hydroxy benzotriazole |
| HPLC | High pressure liquid chromatography |
| LC-MS | Liquid chromatography mass spectrometry |
| mM | milli mol |

| | |
|---|---|
| nm | Nanometre |
| µM | micro mol |
| MW | Molecular weight |
| NMI | *N*-methyl imidazole |
| NMP | *N*-methyl pyrolidine |
| NMR | Nuclear magnetic resonance |
| OtBu | *tert*-butyl ester |
| PBS | Phosphate buffer solution |
| PyBOP | Benzotriazole-1-yl-oxy-tris-pyrolidine-phosphonium hexafluorophosphate |
| RP | Reverse phase |
| SPPS | Solid phase peptide synthesis |
| tBu | *tert*-butyl |
| TEM | Transmission electron microscopy |
| TFA | Trifluoroacetic acid |
| TFE | Trifluoroethanol |
| ThT | Thioflavin T |
| Trt | Trityl |
| UPLC | Ultra pressure liquid chromatography |
| UV | Ultraviolet |

**Amino Acids**:

| | | |
|---|---|---|
| Ala | A | Alanine |
| Arg | R | Arganine |
| Asn | N | Asparagine |
| Asp | D | Aspartic acid |
| Cys | C | Cysteine |
| Gln | Q | Glutamine |
| Glu | E | Glutamic acid |
| Gly | G | Glycine |
| His | H | Histidine |
| Ile | I | Isoleucine |
| Leu | L | Leucine |
| Lys | K | Lysine |
| Met | M | Methionine |
| Phe | F | Phenylalanine |
| Pro | P | Proline |
| Ser | S | Serine |
| Thr | T | Threonine |
| Trp | W | Tryptophan |
| Tyr | Y | Tyrosine |
| Val | V | Valine |

# Chapter 1: Introduction

## 1 Introduction

The proof of the principle of a novel concept for the disruption of Alzheimer's amyloid β, which is termed as the concept of β breaker dipeptides (BBDP), is demonstrated in this thesis. Before going into the concept of β sheet disruption, it is necessary to discuss some fundamental topics associated with it. Insight on the problem is given and the way that we have chosen to solve it is described.

### Peptides and proteins

Peptides and proteins are important class of bio macromolecules which play a crucial role in the normal functioning of living systems. Proteins are essentially polypeptide chains with usually more than 50 amino acids and the size may vary from few hundred to several thousand kilo Daltons. Proteins in the form of enzyme play a significant role (catalysts) in many metabolic processes. They also serve as hormones, neurotransmitters, cytokines and growth factors.

Proteins and peptides consist of a series of amino acids attached in a linear fashion via covalent bonds. Such covalent bonds are called peptide bonds. Amino acids are the building blocks of the proteins in which a central carbon atom called α carbon linked to an amino group, a carboxylic acid group, a hydrogen atom and a distinctive R group. R group is referred to as side chain (figure 1).

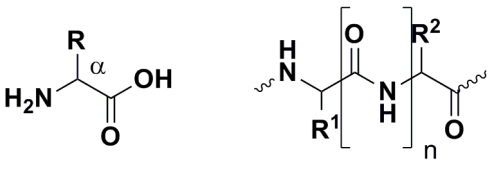



**Figure 1: General structure of amino acid (left) and general formula of a peptide bond.**

There are twenty different side chains varying in size, charge, hydrogen bonding capacity, hydrophobic character and chemical reactivity which are known to constitute proteins. These R groups decide the chemical reactivity of a particular amino acid. Amino acids like Serine, Threonine, Cysteine, Tyrosine, Asparagine and Glutamine residues comes under a category of polar residues. Charged amino acids are Aspartic acid, Glutamic acid, Lysine, Arginine and Histidine. Glycine, Alanine, Valine, Leucine, Isoleucine, Proline, Phenyl alanine, Tryptophan and Methionine are hydrophobic and non polar amino acids.

## Peptide bond

A peptide bond (amide bond) is formed by the condensation reaction between an amino group and a carboxylic acid group with loss of a water molecule. This repeating unit is called the main chain or the backbone with side chains lying outside. Peptide backbone is capable of hydrogen bonding because carbonyl oxygen is good hydrogen bond acceptor and NH is good hydrogen bond donor (except proline). In general, hydrogen bonding between the polypeptide

backbones stabilizes the protein. Peptide bond is planar and that is due to delocalization of electrons between the carbonyl group and the amino group as shown in figure 2.

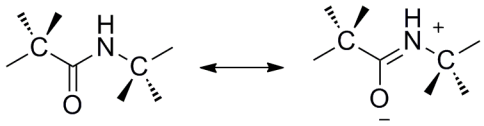


**Figure 2: Peptide bond resonance structure.**

Planarity of the peptide bond enables the geometry around the bond in two different forms: trans and cis as shown in figure 3.

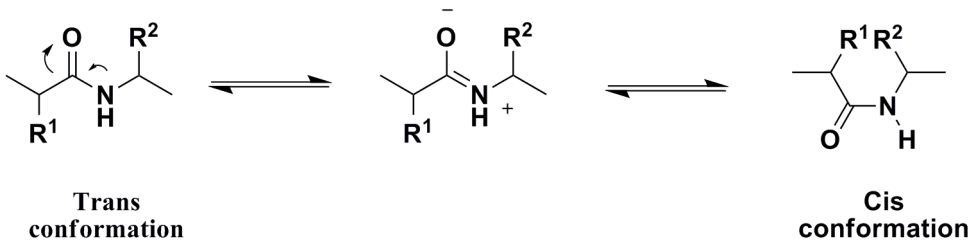



**Figure 3: Cis-trans isomerization of the peptide bond.**

## Torsion angles and Ramachandran plot

Although peptide bond is planar, rotation is possible between N-$C_\alpha$ and C-$C_\alpha$ which gives flexibility to the peptide chain. Phi ($\phi$) is the angle of rotation about the bond connecting N and $C_\alpha$ atom, whereas Psi ($\psi$) is the angle of rotation about the bond connecting C and $C_\alpha$ atom.

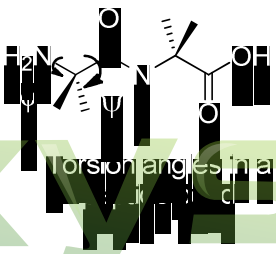


**Figure 4: Torsion angles in a peptide bond.**

Ramachandran plot is a four quadrant plot of Psi vs Phi and tells about the allowed values of those torsion angles (figure 5). Based on these values the stable conformations of the peptides are determined. Such plot was first reported by Ramachandran (named after him) and Sasisekaran who used solid spheres to analyze the angles.[1]

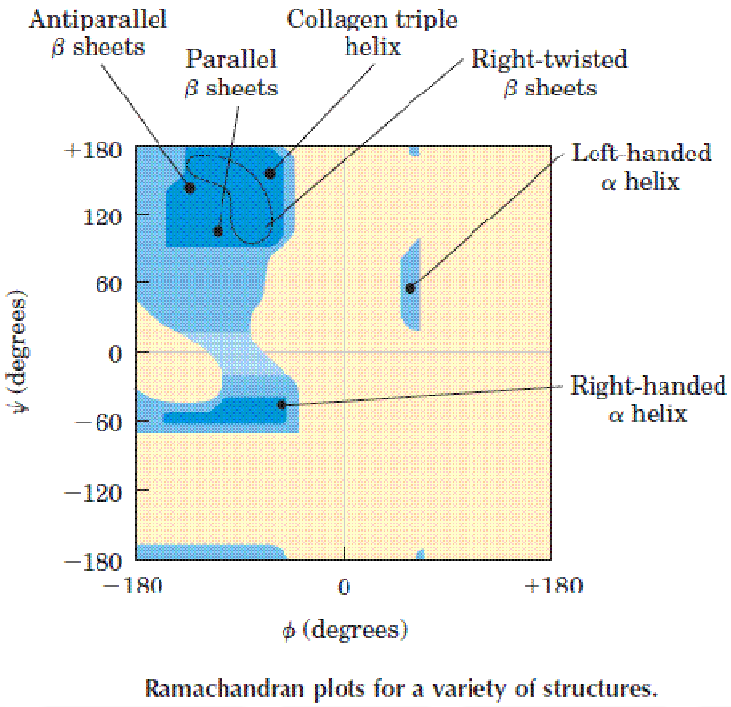



**Figure 5: Ramachandran plot (image taken from Nelson, D. L. and Cox, M. M. Lehninger principles of biochemistry, fourth edition).**

## Secondary structures of proteins

Primary structure consists of the sequence of amino acids attached in a linear fashion to form a poly peptide chain. The position of amino acid decides the stable secondary structure of a protein. Various levels of structural descriptions of proteins are known, e.g. primary, secondary, tertiary and quaternary structure. Among those, secondary structure consists of three main kinds of conformations, e. g. $\alpha$-helix, $\beta$-sheet and $\beta$-turn.

### *α*-helix

Linus Pauling and Robert Corey proposed α-helix structure of protein.[2] According to them; there is an imaginary axis and polypeptide backbone runs longitudinally like a coil, where R groups (side chain of the amino acids) are lying outside the helical backbone. A representative picture is shown in figure 6.

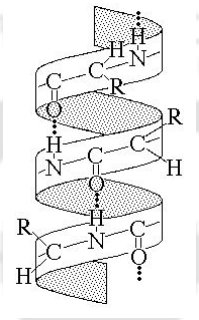


**Figure 6: Structure of α helix.**

### *β*-sheet

Polypeptide chains sometimes exist in zigzag fashion and one chain is connected to another chain through *'hydrogen bonding'* between the carboxy group of one unit and amino group of another unit. This produces a series of polypeptide chains connected to each other which is known as *beta pleated sheet*. Pleat is due to Van der Waals repulsions between the R groups (H in case of glycine) of different amino acids other than proline.

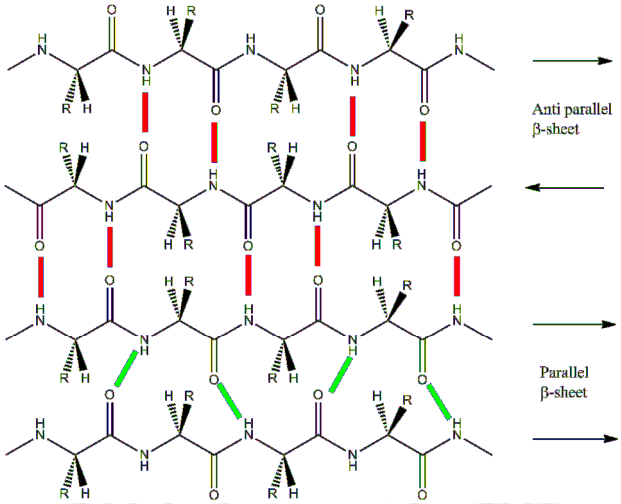



**Figure 7: Structure of parallel and anti parallel β sheet.**

Two strands are separated by a distance of 3.5 Å. Adjacent β strands can run either in the same direction (parallel β sheet) or in anti parallel direction (anti parallel β sheet). Anti parallel hydrogen bonding follows a simple pattern where NH group is bonded with CO of the next strand and vice versa. However, in parallel β sheet NH group is bonded with CO on adjacent strand while CO is bonded to NH of the amino acid residue lying two residues farther on adjacent chain as shown in figure **7.**

***β turn or β bend***

180° fold or turn involving four amino acids and there exists hydrogen bonding between the carbonyl oxygen of the first residue and amine of the fourth residue (from *i* to *i*+3). Remaining two residues present at the middle do not participate in any kind of interaction (figure 8).[3]

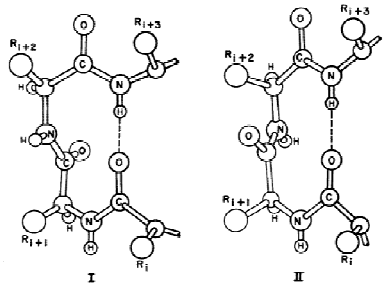


**Figure 8: Beta turn (Courtesy: Image is taken from Lewis, P. N.; Momany, F. A.; Scheraga, H. A. *Proc. Nat. Acad. Sci. USA*, 1971 68, 2293-2297).**

## Protein folding and misfolding

Protein once synthesized in ribosome attains a thermodynamically stable conformation, which is essential for a proper functioning of a cell. However, the complete mechanism of protein folding is unclear till date. *Protein folding* is thought to be a highly cooperative process. In case a small portion of the folded structure becomes disrupted, the interactions between it and the remaining structure are lost. As a result, entire protein gets destabilized. Covalent interactions like disulphide bonds and non covalent interactions like Van der Waals interactions, hydrogen bonds, dipolar interactions and hydrophobic interactions decides the stable conformation of a protein.

Protein folding, unfolding and misfolding are commonly occurring processes in biological systems. A phenomenon in which protein undergoes a dynamic change which encompasses hydrophobic side chains move away from water and polar groups like CO and NH develop hydrogen bonding between each other is called as protein folding. Protein folding depends on the type of amino acid residues present. α-Helix is stabilized by hydrogen bonding between CO and NH groups within the same strand whereas in β strand hydrogen bonding is between next neighboring strand. Hydrogen bonding is the stabilizing force between the strands. Higher order structures like β sheets and β hairpins are based on these secondary structures.

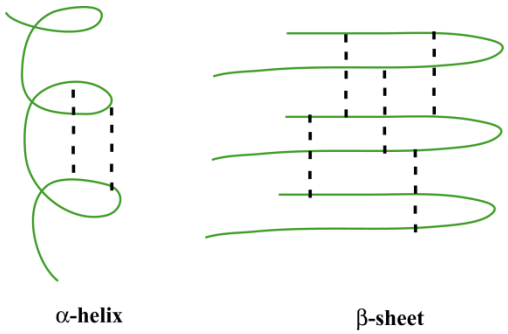



**Figure 9: Secondary structures of proteins. Green line indicates peptide strand and black line (dotted) indicates hydrogen bonding.**

When a specific protein/peptide transforms from one of its functional conformation into another structure that differs from its native state is referred to *protein misfolding*. This state of change in conformation leads to various diseases commonly known as protein misfolding/protein conformational diseases. It is a pathological state in which the availability of given protein for its normal functionalization falls down due to misfolding. In many cases, peptide/protein converts from its soluble form (random coil) into highly ordered fibrillar aggregates (β sheet) and these insoluble extracellular fibrillar species known as amyloid fibrils or plaques which are responsible for a pathological condition, 'Amyloidosis'.

Sometimes protein misfolding involves transition from normal secondary structure into ordered β sheets. This transition depends on various parameters like pH, ionic strength, genetics and mutations. Number of diseases comes under the category of 'Amyloidosis' in which peptide/protein aggregates into insoluble amyloid plaques. Few examples are given in table 1[4]

| Disease | Aggregating peptide/ protein | Number of residues | Native structure |
|---|---|---|---|
| **Alzheimer's disease** | **Amyloid β** | **40 or 42** | **Natively unfolded** |
| Parkinson's disease | α-synuclein | 140 | Natively unfolded |
| Huntington's disease | Huntingtin with poly Q expansion | 3144 | Largerly natively unfolded |
| Type II diabetes (neuropathic localized) | Amylin, Islet amyloid precursor protein | 37 | Natively unfolded |

**Table 1: List of some protein misfolding or protein conformational diseases.**

## Alzheimer's disease

"*Considering everything, it seems we are dealing with a special illness*".

Alois Alzheimer

Psychiatrist, Germany

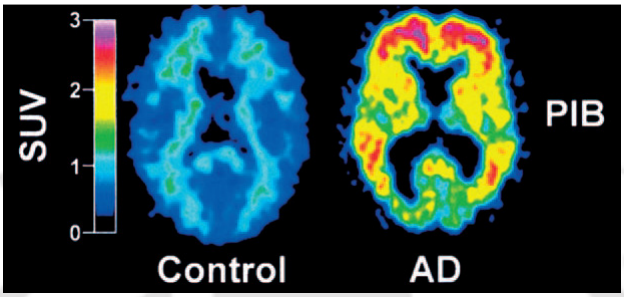



**Figure 10: SUV (PIB) image of normal and AD patient.** [8]

Alzheimer's disease is the most common form of dementia and is prevalent in the adults of the age group of 60 years and above.[5, 6] In 2000, Alzheimer's disease (AD) affected more than 4.5 million people in the U.S. alone and presently 24 million are suffering worldwide. It is estimated that by 2050 number of cases will increase by almost 3-fold.[7, 8] In 1906, Alois Alzheimer (disease named after him), a psychiatrist at a hospital in Munich examined the brain tissue of a patient who died because of some kind of mental abnormalities that accumulated with time.[9] He found discrete interneuronal fibrillar deposits called neuro fibrillary tangles (NFT) inside the patient's brain and also dense deposits outside and around the nerve cells called senile plaques (SP) as displayed in figure 11. Senile plaques composed of amyloid β (Aβ) peptide whereas neurofibrillary tangles consist of Tau proteins. He mentioned this abnormality as a special illness. Common symptoms include short term memory loss, failure in understanding, auditory hallucinations.

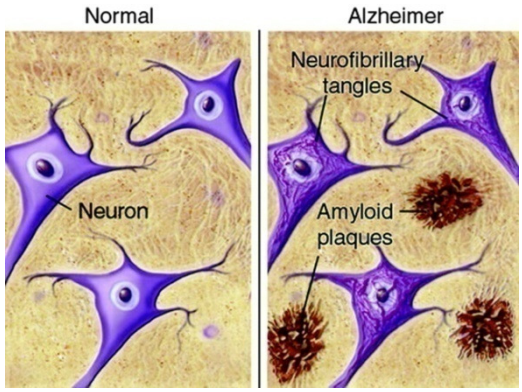



**Figure 11: Comparison between a healthy neuron and diseased neuron comprising amyloid plaques and neurofibrillary tangles. (image taken from http://pakmed.net/academic/age/alz/alz030.htm)**

Amyloid deposits consists of a peptide called Amyloid β peptide, expressed from type I membrane spanning glycoprotein called amyloid precursor protein (APP) of nearly 770 amino acids. Function of APP is still not clear. APP gene is coded at chromosome number 21 and people with an extra copy of this chromosome i.e. people with Down syndrome are likely to suffer from Alzheimer's disease by the age of 40 years.[10] In a healthy individual Aβ peptide concentration in human brain is about 2 μg/g whereas, in AD patients it is around 31 μg/g.

AD can either be early onset AD (EOAD) below 60 years of age or may develop at the ending stages of life that is late onset (LOAD). Majority of people are suffering due to LOAD and it is estimated that by 2020, 42 million people will suffer with LOAD worldwide. Memory is due to electrical impulses along the neuronal connections in the brain by opening and closing of synapses to form a circuit to encode specific information. Learning involves new synaptic connections and synthesis of proteins at synapses. In AD these synaptic connections gets completely destroyed and leads to episodic memory impairment.

## Amyloid β peptide

Aβ peptide is produced at the plasma membrane and comes out in extra cellular space and gets deposited as senile plaques (SPs) with β fold. This peptide is produced by enzymatic cleavage of APP by a class of secretase enzymes. It is normally cleaved by α secretase between Lys 16 and Leu 17. However, abnormal cleavage is due to other two 'secretases' namely β secretase which cuts at N-terminus between Met and Asp of APP and γ secretase cuts at C-terminus and exact position may not be the same and it varies between 39 to 43 as depicted in figure 12.[10] Existence of Aβ 40 is 10 times prominent than Aβ 42 which is more neurotoxic.

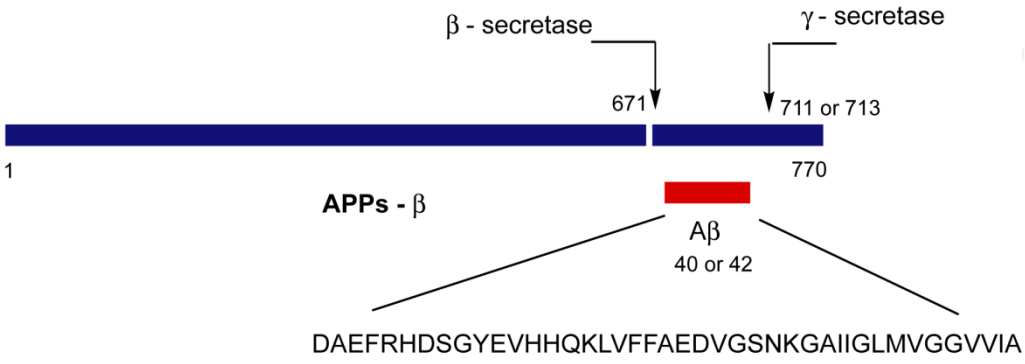



**Figure 12: Structure showing enzymatic cleavage of APP to produce Aβ peptide.**

First 16 amino acids are hydrophilic and the remaining hydrophobic residues are involved in β sheet structure stabilized by either hydrophobic or π-π interactions. Aggregation is mainly due to the nucleation of monomer unit to produce oligomer(s) and finally to produce amyloid fibril. Evidences suggest that oligomers are more neurotoxic than other counterparts.[11, 12, 13]

Kinetic studies suggested that fibril formation thought to proceed via a nucleation dependent pathway.[14] It is a two step process comprising of initial lag phase during which nuclei is

formed followed by an exponential phase in which protofibrils grow and assemble into mature amyloid fibril which are in equilibrium with monomer units (figure 13).

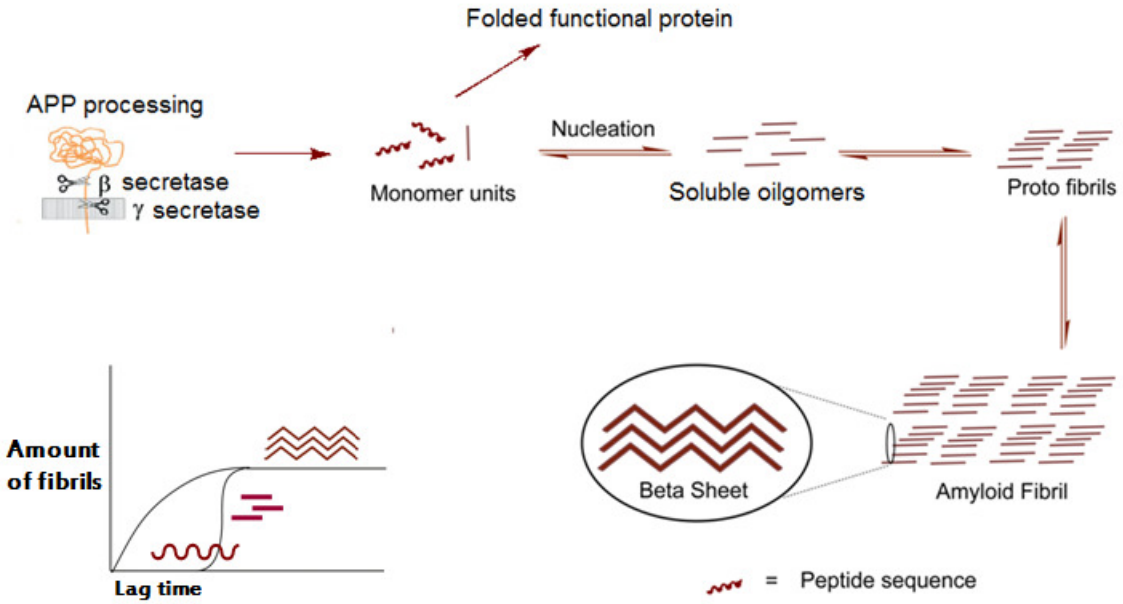



**Figure 13: Amyoid fibril formation via a nucleation polymerization mechanism.**

Excessive accumulation of Aβ peptide particularly Aβ 42 causes neurodegeneration and oxidative stress.[15, 16, 17] Oxidative stress accounts for drastic reduction in the levels of anti oxidants (vitamin E, C and glutathione), increase in amount of 8-hydroxy-2'-deoxyguanosine (product of oxidative damage of DNA) and lipid peroxidation. Aβ on reaction with metal ions and oxygen produces reactive oxygen species like hydrogen peroxide which causes lipid and protein oxidation. Metal ions such as copper binds to three Histidine residues and one Tyrosine residue with three nitrogens and one oxygen donor ligand N3O1 coordination and this is confirmed from computational modeling.[18, 19] Met[35] residue of Aβ is also responsible for oxidative stress and neurotoxicity but the exact mechanism of oxidative stress and neurotoxicity is still lacking. Replacement of Met 35 with other non polar amino acid like Ile enabled reduced levels of neurotoxicity.

## 1.8 Current treatment strategies

Despite the rigorous efforts and enormous progress made in both diagnosis and treatment of this disease, there is no cure available till date. The available drugs on the market are good at improving the patient's condition of anxiety, insomnia or depression only.

1. To restrain β and/or γ secretases for the production of amyloid β from APP is a strategy for drug design against AD. Numerous potent molecules have been discovered which inhibit the protease activity.[20, 21] Drug called 'Flurizan™' from Myriad genetics has proven to reduce the levels of Aβ42 by modulating the activity of γ secretase.

2. Use of small molecules as potent chemotherapeutic agents is another promising strategy against AD. A molecule, which binds to Aβ, crosses blood-brain-barrier and significantly reduces Aβ levels in the brain is an ideal drug candidate. A company, Neurochem produced a small molecule called Tramiposate Alzhemed™, has entered clinical trials.[22] Cellular inflammation is noticed in cerebral cortex of patients suffering from AD. Anti inflamamatory drugs have direct influence on cyclooxygenase and other inflammatory mediators. Approaches based on antioxidant, neuroprotective and neurotrophic are also in use but none of the strategies could stop cognitive dysfunction.[23]

3. Based on HRMS and cyclic voltametry experiments it was proved that Cu (II) binds to Aβ 42 and forms 1:1 complex. In the prescence of redox active molecules like ascorbic acid, pyruvic acid, lactic acid, Vitamin $B_{12}$, glutathione and electron sources like $NAD^+$ this Cu (II):Aβ complex reduces oxygen and form hydrogen peroxide responsible for oxidative stress.[19] If somehow a ligand which can competitively bind to these metal ions can inhibit Aβ deposition. Clioquinol™, known $Cu^{2+}/Zn^{2+}$ chelator has shown a promising result in transgenic mice. Clioquinol and its derivatives are in clinical trial stage.

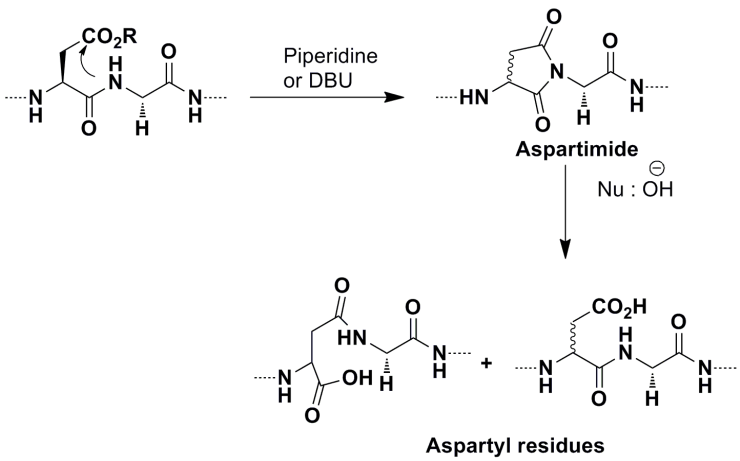
4. To inhibit Aβ fibrillization or dissolution of preformed fibrils using peptide based molecules is most reasonable approach in the direction of therapautics. Tjernberg first introduced a short peptide fragment (KLVFF) from the Aβ sequence itself which was shown to inhibit fibrillogenesis in vitro.[24] Later, Soto's peptide showed a promising result on inhibition of fibrillization and dissolution of preformed fibrils in rat brain.[25] However, the peptide molecule was needed in twenty equivalents to exhibit the desired result. The added advantage of this strategy is that there is always a lower risk of unanticipated side effects.

5. Use of antibodies as potential therapeutic agents is another treatment strategy against the AD. It was first demonstrated that antibodies against Aβ peptide could block the formation of amyloid fibrils in vivo.[26] In 1999, it was demonstrated that vaccinating transgenic mice against the Aβ peptide could dramatically reduce deposition of fibrillar amyloid. More importantly, the stoichiometry of antibody to Aβ is in the range of 10 to 100 times for depolymerizing the fibrils.[27]

## 1.9 Aspartimide formation

Aspartimide ring closure is well known side reaction in the Fmoc based synthesis of peptides containing aspartic acid. This side reaction occurs due to presence of base like piperidine or DBU during Fmoc cleavage step as depicted in scheme 1.[28, 29, 30] Aspartimide formation takes place by attack of backbone NH of *i* + 1 residue on side chain ester.

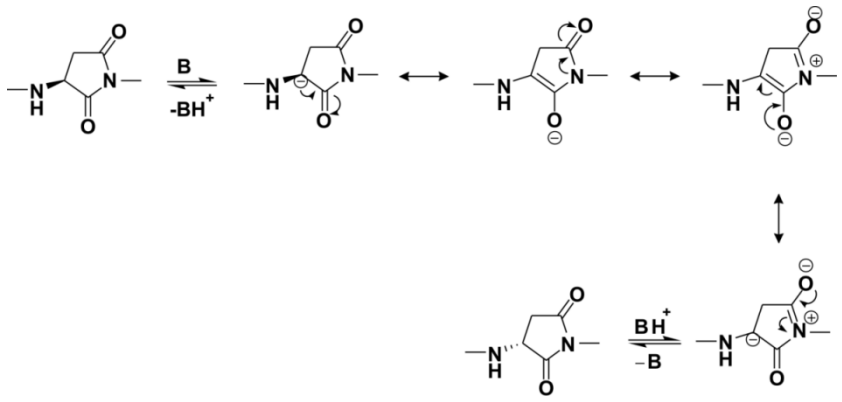
Acid catalysed apartimide formation is also noticed in some cases in Boc based SPPS.[31] Tendency of aspartimide formation depends on a) neighbouring amino acid residue[30, 32] and b) side chain ester. Asp (OBzl) is known to generate aspartimide easily than other counterparts.[31, 32]




**Scheme 1: Aspartimide formation.**

Aspartimide ring closure is a side reaction occurs in the presence of bases like piperidine or DBU during Fmoc cleavage step in SPPS. That means in basic medium aspartimide formation occurs. Similarly, in basic buffer such as phosphate buffer, backbone NH of *i* + 1 residue (neighbouring amino acid) becomes more nucleophilic which results in O to N acyl migration.

Aspartimide results from nucleophilic attack of nitrogen on the gamma carbonyl (side chain carbonyl) with loss of alcohol (ROH). In many cases, side chain acid is protected by esters like tBu, Bzl and this facilitates loss of alcohol in basic condition. In basic condition, acyl migration takes place from O to N results in the formation of five member cyclic imide. Ring opening takes place due to attack of either $H_2O$ or piperidine to form respective adducts. Attack of water produces a mixture of α and β aspartyl residues where β aspartyl dominates α aspartyl by three times.[33] Exact reason for this predominance is not clear though, it is due to some electronic, steric and intramolecular catalytic effects.[34] Rate of acyl migration can be regulated by temperature and pH of the solution. Catalytic amount of piperidine may accelerate kinetics of acyl migration. In the presence of base the acidic α proton of the aspartimide is extracted out and the resulting conjugate base is stabilized by resonance as shown in scheme 2.[35, 36]



**Scheme 2: Resonance stabilization of aspartimide.**

## 1.10 Utility of aspartimide formation

Though aspartimide formation is undesirable side reaction in Fmoc based SPPS, this succinimide can be used to induce type II' β turn in secondary structures of proteins.[37, 38, 39] Succinimide residue is similar to a proline which is known to generate turn in secondary structures of proteins (figure 14). Relative frequency of proline to induce turn in secondary conformations is 1.91, helix is 0.52 and β-sheet is 0.64.

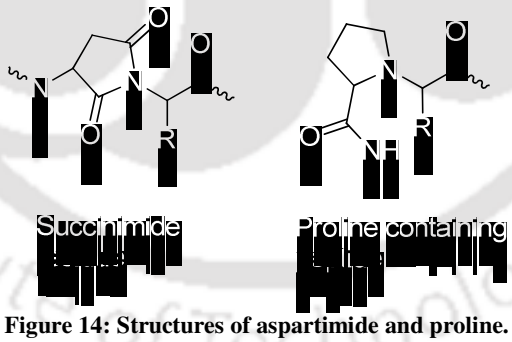



**Figure 14: Structures of aspartimide and proline.**

Induction of turn is confirmed from molecular simulations, NMR and crystallographic data obtained from short peptides containing succinimide residue. It was reported that rate of acyl migration depends on neighboring amino acid residue at the carboxyl side.[37] Bulky and unreactive side chain (like leucine) makes migration slower and conversely, presence of any

small group (glycine, alanine) and if additional hydrogen bonding potential groups are present (serine) reaction progress faster.

| Aspartyl dipeptides | Small (Gly, Ala, Ser) | Large (His, Tyr, Trp, Arg) |
|---|---|---|
| Intracellular | 28 | 6 |
| Extracellular | 18 | 10 |

**Table 2: Distribution of aspartyl residues in proteins.** [37]

Proteins in general containing aspartimide has a propensity to induce type II' β turn. This was comprehensible from the Psi (ψ) and Phi (ϕ) values calculated for a particular type I or type II β turn. Psi value for type II' β hairpin turn is also known to be around -120° and Psi value (angle between α carbon and carbonyl carbon) for succinimide residue is also -120°.[37] Therefore, succinimide residue has a propensity to induce turn on main frame of peptide chain similar to that of proline.

## 1.11 Concept of β-breaker dipeptides in β-sheet disruption

Use of β breaker peptides, first introduced by Soto *et al*., was a landmark in the direction of amyloid disruption.[25] He prepared peptide based molecules containing proline as β breaker element incorporated in a recognition sequence. Proline is known to generate turn due to its unique structure. The kink generated by proline helps to disrupt the aggregation between the neighbouring β strands. Another promising approach was N-methylation of peptide inhibitors which interfere with the hydrogen bonding of peptide backbone.[40] Aib (α-Aminoisobutyric acid) substituted short peptides were also used to disrupt the β sheet structures.[41] Aib with two methyl residues generates helical conformation. Aib as a β breaker element has been used to disrupt the aggregation of hIAPP, a 37 amino acid peptide responsible for type II diabetes.

A common problem with all the reported strategies for the design of β-breaker peptides is that, the element responsible for β-sheet disruption is already installed in the sequence and that may prevent proper recognition of the added β-breaker peptide and existing β-sheet forming aggregating peptide. If recognition is not good breaking activity cannot be expected to be good.

To address the problem of recognition, we prepared for the first time a novel class of 'β breaker dipeptide' (BBDP) containing β-breaker peptides for amyloid disruption. A dipeptide of general formula [Asp(OBzl)-Axx] can act as a β-breaker unit (BBDP). Asp(OBzl) stands for an aspartic acid residue with side chain protected as benzyl ester and Axx can be any amino acid. According to the concept of β-breaker dipeptides (BBDP concept), a β-breaker dipeptide unit (BBDP) containing β-sheet breaker peptide properly aligns with Aβ peptide or any other aggregating peptide at the initial stage, that undergoes a chemical change in

biological condition (pH 7.4, 37 °C) and results in a chemically modified peptide that disrupts β-sheet and inhibits fibrillogenesis (figure 15) *in situ*.

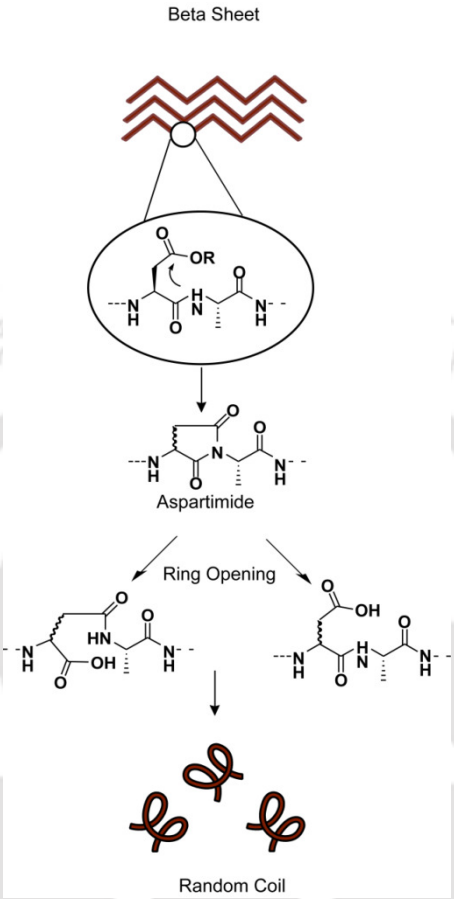



**Figure 15: β sheet disruption using chemistry of aspartimide formation.**

Better recognition at initial stage is expected as the BBDP containing β-breaker peptide retains its native peptide backbone and β-breaker element is not installed. The β-breaker element will be formed *in situ* by a chemical change. Mentioned chemical change may be the formation of the aspartimide from BBDP at near physiological pH. And the *in situ* chemically modified peptide can be either the aspartimide containing version of the peptide or the β-aspartyl version of the peptide. Succinimide ring induces type II' β turn in secondary structures of proteins.[37] This is confirmed from molecular simulations, NMR and crystallographic data obtained from short peptides containing succinimide residue. Therefore,

*in situ* conversion of the native peptide to aspartimide version is supposed to generate a kink and destabilize β-sheet network. Ring opening of aspartimide generates α and β aspartyl residues in 1:3 ratio.

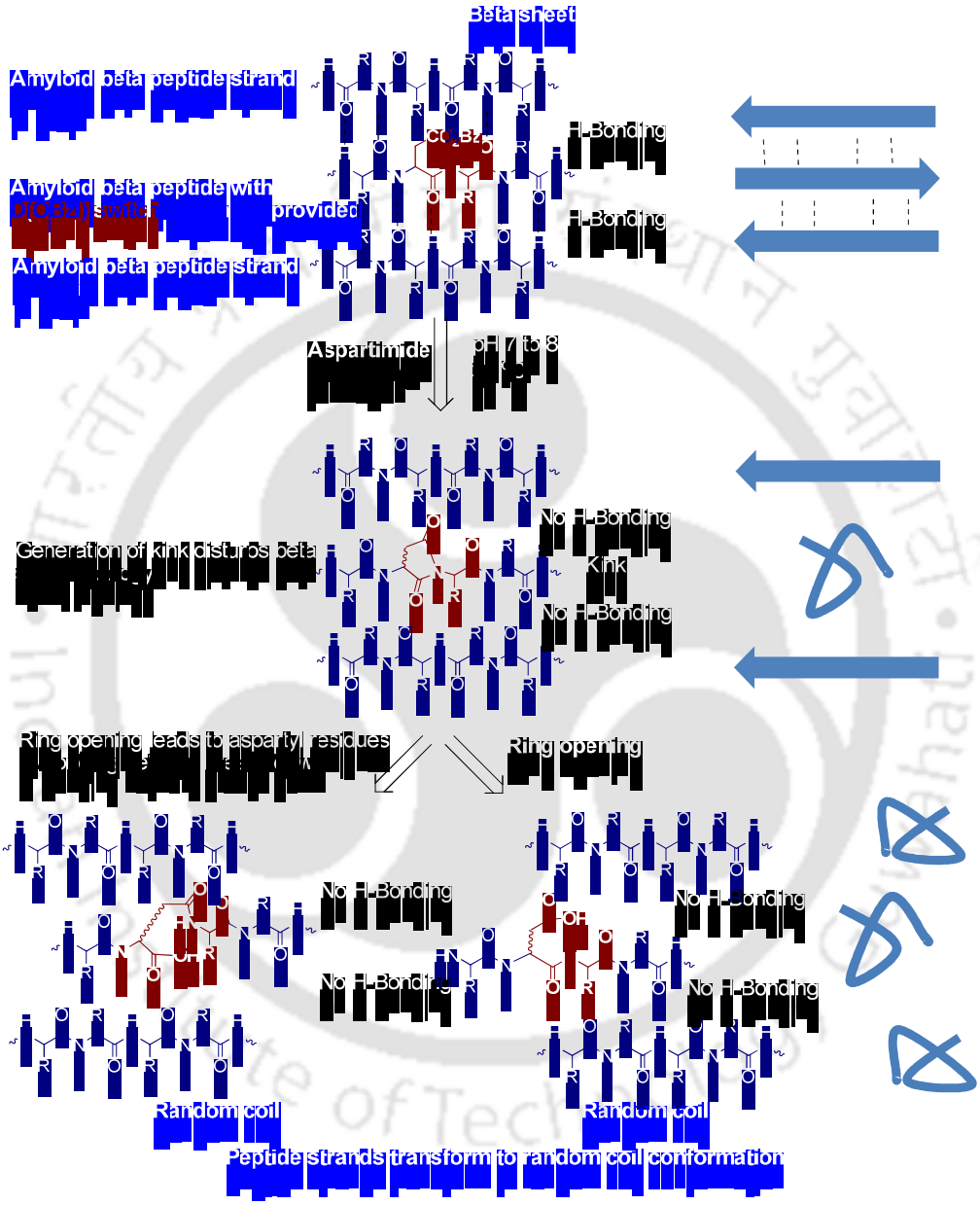



**Figure 16: Disruption of hydrogen bonding in a β strands via chemistry of aspartimie formation.**

Conversion of the native α-peptide to the β-aspartyl peptide brings flexibility in the peptide backbone and the H-bonding network between the two neighbouring peptides gets disturbed.

Finally, 1/3 portion of the aspartimide version of the peptide gets converted back to the α-aspartyl form of the peptide, 50 % of which gets converted to the D-aspartyl form. Those peptides with inverted configuration Asp also destabilizes the β-sheet network formed by the L amino acids.

Therefore, such a BBDP containing peptide is supposed to get aligned perfectly with and recognized by the β-sheet forming peptides at the initial stage and may get incorporated in the β-sheet network of the disease forming aggregating peptides. Such incorporated BBDP containing β-breaker peptide is supposed to get converted first to the aspartimide version of the peptide which again converts to the β-aspartyl version and α-aspartyl version of D-configuration with time at physiological pH. Generated aspartimide, β-aspartyl and D-amino acid containing versions of the added β-breaker peptide should destabilize the H-bonding network of the β-sheet architecture (figure 16). Thus total disruption of aggregation could be achieved.

## 1.12 Biophysical methods to monitor β-sheet and fibril formation

### a) Circular dichroism

Circular dichroism is most commonly used technique to examine the secondary structure of protein/peptide in a solution. The main principle involved in the CD is differential absorption of two circularly polarized components and the resulting radiation possesses elliptical polarization and is reported in terms of ellipticity (θ) in degrees.

$$\Delta A = A_L - A_R$$

Secondary structures of a protein comprises of helix, sheet and turns and in general peptide bond absorbs at 220 nm and 190 nm due to n to $\pi^*$ and $\pi$ to $\pi^*$ transitions. Also, side chains of aromatic amino acids may contribute in some cases. Different types of secondary structures give characteristic CD spectra in far UV region. The result can be compared with that of XRD or NMR to quantitate the secondary structure. In order to obtain the reliable data, exact protein concentration must be known and CD instrument should be properly calibrated.[42]

**b) Fourier Transformation Infra Red spectroscopy (FT-IR)**

FT-IR is one kind of qualitative technique used to determine the presence of secondary structure of a protein or a peptide. This is one kind of quantitative technique is primarily based on the asymmetric stretching frequencies of amide I band of the peptide. Amide I band which is used to assign secondary structures, occurs in the region between 1600 and 1700 $cm^{-1}$. Typically, sample is examined before and after fibril formation. FT-IR has also been used to suggest the presence of either parallel or anti parallel β sheet in a particular peptide or protein.[43]

**c) Thioflavin T**

Thioflavin T is one kind of benzothiazole fluorescent dye which exhibit characteristic spectral shift and/or intensity change upon binding with peptide fibrils. ThT assay is most commonly used technique to monitor the formation of amyloid. This dye is weakly fluorescent in water with excitation and emission maxima at 350 and 440 nm respectively. This dye upon binding to amyloid fibrils excitation and emission maxima shifts to higher wavelength at 435 to 490 nm respectively.[44] ThT is one quantitative technique to monitor formation of amyloid with time and intensity of peak is proportional to amount of fibrils formed in a given set of conditions. On the other hand, decrement in peak intensity indicates

inhibition of fibrillar assembly and the kinetics of amyloid formation and its inhibition on addition of inhibitor can be monitored easily by ThT assay.[45, 46] Although this technique is widely accepted technique to monitor fibril growth, there is an ambiguity associated, that is, ThT may bind to amorphous aggregates and may give false result. At the same time inhibitor peptides may displace mature fibrils and bind to ThT. Therefore this technique cannot be considered as direct evidence of fibril growth other techniques such as EM (electron microscopy) studies, FT-IR, birefringence assay are performed in parallel.

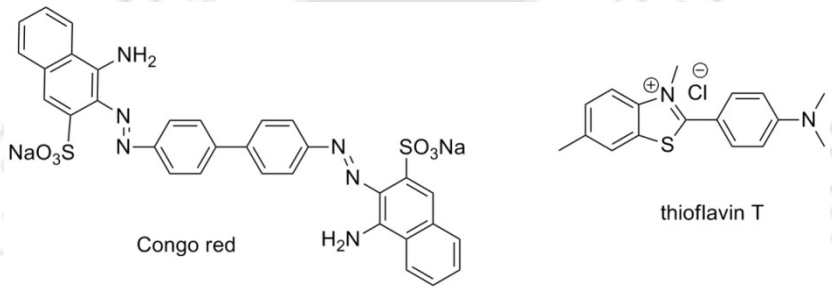



**Figure 17: Structure of Congo red and thioflavin T.**

Mechanism of ThT action is not understood clearly till date though some reports claim ThT similar to congo red form micelles at low concentrations (sub micromolar range) which was confirmed from conductivity measurements. Critical micelle concentration was also determined to be around 4 ± 0.5 μM in water. Therefore, it is very likely that ThT binds to cross β srands of amyloid fibrils by both ionic and hydrophobic interactions.[47]

**b) Transmission electron microcopy (TEM)**

TEM is widely used qualitative technique to visualize fibrils formed from any class of aggregating peptides. This technique is based on the principle of electron diffraction similar to projector presentation of transparencies where a ray of light interacts with the subject and

projects it in enlarged and high resolution image. Similarly, in TEM a ray of electron interacts with sample and gives an image of it which is recorded by in-built camera.

Tungsten filament is common source of electrons which are passed through the anode. Condenser lens focus this electron beam over sample grid. Objective lens and the aperture define the aperture angle. Once image is formed electrons pass through diffraction lens for magnification. To visualize the peptide samples are stained with a negative staining agent like uranyl acetate or phosphotungstic acid. This staining helps the clarity and resolution of image when viewed under low voltage conditions at 80 kV.[48, 49]

**c) Congo red birefringence**

Dye binding is a specific characteristic property of amyloid fibrils. Upon staining with congo red these fibrils show green-gold birefringence when visualized under optical polarisable microscope.[50, 51] It is a simple qualitative technique to monitor the formation of amyloid. Only mature fibrils and amyloid is known to show birefringence and remaining other intermediates like oligomers or ADDLs do not give such birefringence.

## 1.13 Aim of the work

Works described in this thesis is focused on the generation and exploration of a novel concept, which we termed as 'the concept of β-breaker dipeptides (BBDP)' and its application in β-sheet disruption. We wanted to introduce this novel concept for development of the peptide based lead molecules which can be used against various protein conformational diseases. However, in this thesis we focused our attention on the application of the strategy on Aβ (1-40) and its related sequences. Since Aβ peptide is known to play a key role in the pathogenesis of Alzheimer's disease our primary target was to disrupt the amyloid formation in the presence of suitable β-breaker peptides.

Handling Aβ (1-40) is difficult and troublesome that too during initial stages of optimization of the concept. Therefore, we started from simple model -$(Ser\text{-}Leu)_n$- based aggregating peptides and Aβ derived sequences which are likely to behave similar to Aβ (1-40). Once the proof of concept was established we prepared a suitable β-breaker peptide similar to Soto's peptide and compared its β-breaking potential with that of a designed BBDP containing β-breaker peptide of Aβ(1-40).

To start with, we wanted to explore the concept of β-sheet disruption on self aggregation of model BBDP containing peptides. Model BBDP containing peptides were designed in such a way that these peptides aggregate in a specific condition, at the same time BBDP unit inhibits the aggregation. It was essential to examine the disruption potential of self aggregation, because then and only then such BBDP containing peptides can be used to inhibit or disrupt the fibril formation of other aggregating peptide when mixed in a given experimental condition.

Next study was to examine the β breaking potential of BBDP containing peptide on model aggregating sequences. -(Ser-Leu)$_n$- based model aggregating peptide which was demonstrated to form amyloid like fibrils in vitro was used as a tool to study conformational changes when mixed with BBDP containing breaker peptide. Similar to that model aggregating peptide, Aβ$_{14-23}$ fragment embedded between -(Ser-Leu)$_n$- residues namely Aβ derived aggregating peptide was shown to form amyloid like fibrils. This Aβ derived sequence was opted as a suitable mimic for Aβ (1-40) to evaluate the β breaking potential of suitable BBDP containing peptide.

Having obtained the proof of the proposed concept of β sheet disruption on self aggregation and model aggregating sequences, we wanted to extend and investigate the final objective of amyloid disruption of Aβ (1-40). In order to achieve that, we wanted to prepare a suitable β breaker peptide which breaks the fibril assembly of Aβ(1-40) peptide. To demonstrate the robustness of the concept, the β-breaking efficiency of the BBDP containing β breaker peptide designed with the intention of disruption of aggregation of the Aβ(1-40), its potential was compared with that of the Soto's peptide.

We also wanted to prepare -D(OBzl)-Axx- version of the Aβ(1-40) peptide, which when mixed with Aβ (1-40), first recognizes and later disrupts self aggregation and aggregation of neighbouring peptide as well. In order to find out the suitable positions we wanted to prepare a proline mutant version of Aβ, which is likely to behave similar to -D(OBzl)-Axx- containing analogue, and its aggregation behaviour with that of Aβ will be compared.

The conformational behavior of the peptides was thought to be analyzed using CD and FT-IR followed by monitoring fibril formation by thioflavin T and TEM and finally amyloid formation by Congo red birefringence assay.

# Chapter 2: β-breaker dipeptide containing model peptides and inhibition of self aggregation

## 2.1.1 Design of the β-breaker dipeptide containing model peptides

As a starting point, we wanted to design a model aggregating peptide which is intended to aggregate to form amyloid like fibrils at specific condition. We designed a sequence in which hydrophilic and hydrophobic amino acids were placed at alternate positions, so that all hydrophilic side chains align on one side and the hydrophobic side chains aligns on the opposite side. Such peptides are known to form β-sheets, which eventually forms fibrils and amyloid like aggregates.[52] -(Ser-Leu)$_n$- can constitute such a peptide. The value of $n_c$, β (critical chain length of for β-sheet formation for such sequence) was decided to be 7 as it was known that synthesis of such peptides becomes difficult when n is greater than 4. Therefore, n is equal to 4. Moreover, in cases where $n_c$, β is less than 6, β sheet formation does not occurs easily. That means, critical chain length for β-sheet formation for such a sequence is 7.[53] We wanted the insertion of the β breaker dipeptide (BBDP) unit in the middle of the designed sequence. That BBDP unit was decided to be **-D(OBzl)S-** sequence.

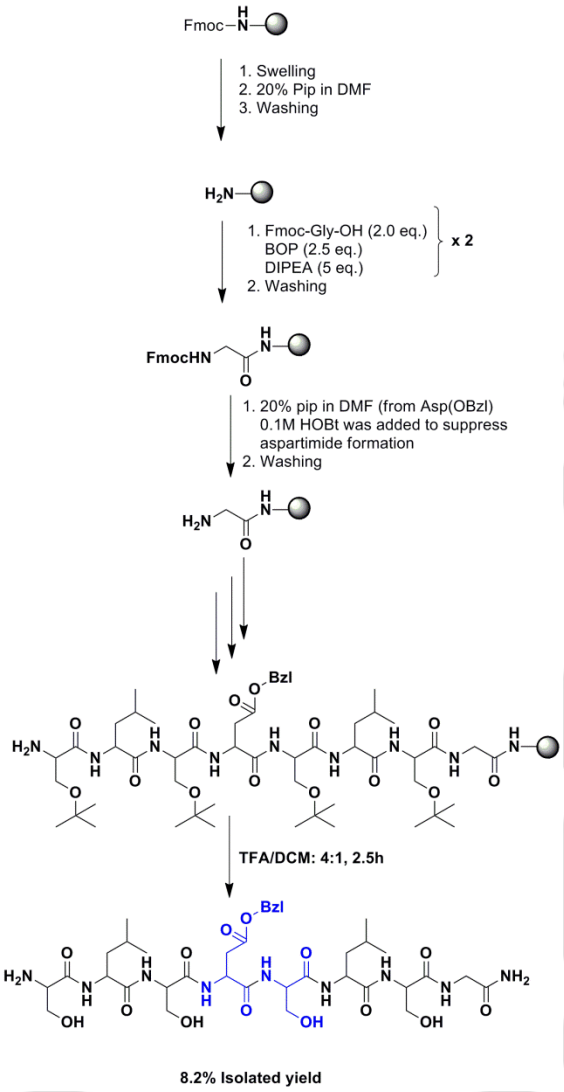
As it was mentioned in chapter one, for the insertion of the BBDP unit, the BBDP containing peptide will stay as β-sheet at the initial stage, which is intended to disrupt that β-sheet architecture by aspartimide formation and subsequent ring opening at near physiological pH. One glycine residue was kept at the C-terminus as a spacer. Therefore, our first β sheet forming β breaker dipeptide (BBDP) containing sequence becomes the following.

**Model BBDP containing peptide: $H_2N$-$^{1}$SLSD(OBzl)SL$^{7}$S-$GNH_2$ (peptide 1)**

## 2.1.2 Synthesis and characterization of model β breaker dipeptide containing peptide 1

Model BBDP containing peptide was synthesized according to standard Fmoc/tBu protection strategy on Rink amide resin following a reported protocol.[54, 55, 56, 57] Coupling was carried out using 2 equivalents of Fmoc amino acid, BOP (2.5 equivalents) in the presence of DIPEA (5 equivalents). Coupling was monitored at each step using Kaiser's test and micro cleavage test. In case of incomplete acylation coupling cycle was repeated and capping was done using acetic anhydride and *N*-methyl imidazole (2 equivalents) for 30 minutes in two cycles. $N^{\alpha}$-Fmoc removal was carried out using 20% piperidine in DMF for 21 minutes (7 min x 3 times). From Asp(OBzl) amino acid 0.1M of HOBt was added in piperidine/DMF to prevent aspartimide formation.[58] After N-terminal Fmoc removal peptide was washed with DMF and DCM (scheme 1). Peptide was cleaved from the resin using TFA in DCM (4:1) for nearly 3 hours. Resin was washed three times with TFA. Peptide was precipitated using cold diethyl

ether to obtain white solid mass. Based on 0.33 mmol scale 23 mg (8 %) of purified peptide was obtained in the form of white fluffy powder.




**Scheme 1: Synthetic scheme of model β breaker dipeptide containing peptide 1 using Fmoc/tBu based SPPS.**

Peptide **1** was purified using C18 μ Bondapak semi preparative HPLC column using solvent A ($H_2O$ with 0.09% TFA) and solvent B ($CH_3CN$ with 0.09% TFA). Gradient of 20 % acetonitrile for 5 min and 60 % acetonitrile for 20 minutes with total run time of 30 minutes was used. Dual wavelength was selected at 214 and 254 nm. Peptide purity was confirmed

from LC-MS using C18 column using solvent A (water with 0.1% FA) and solvent B (acetonitrile with 0.1% FA). Mass was confirmed from MS (ESI +ve mode) a representative figure of the LC-MS profile of the pure peptide **1** is shown below (Figure 1). The LC profile of the pure peptide is depicted as panel (a) in figure 1, whereas the peak corresponding to the mass of the peptide is shown in panel (b).

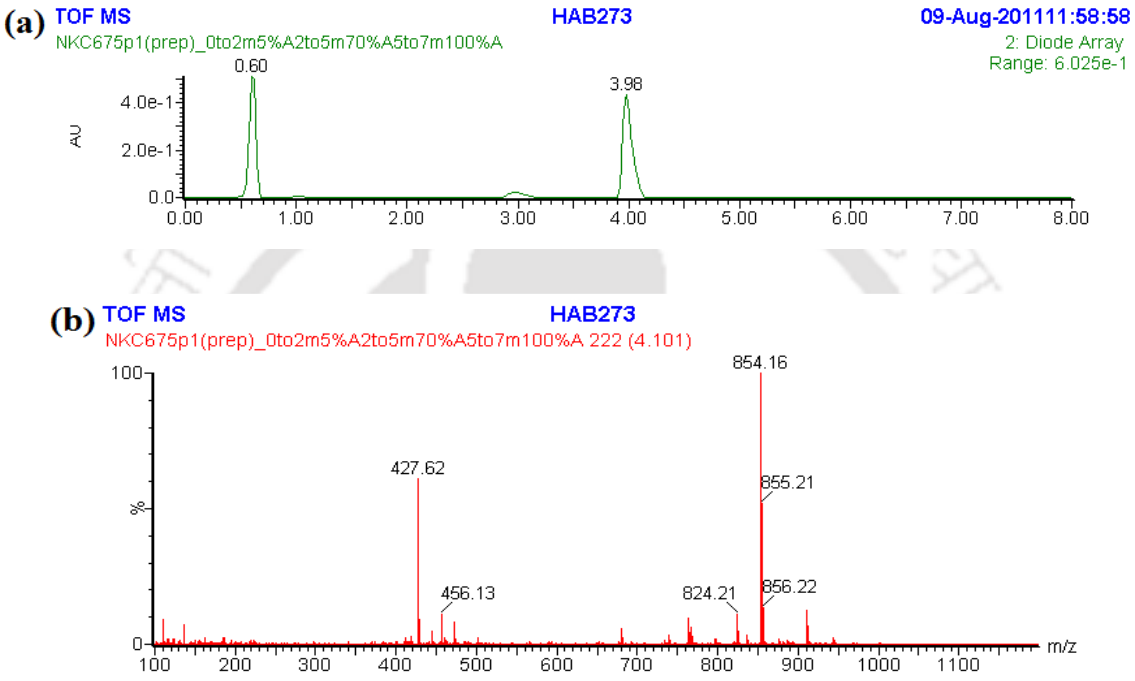



**Figure 1: LC-MS picture of model BBDP containing peptide 1. Calculated mass for $C_{37}H_{60}N_9O_{14}$ was 854. 42 $[M+H]^+$ and 854.16 $[M+H]^+$ and 427.62 $[M+2H]^{2+}$ were observed.**

## 2.1.3 Monitoring O to N acyl migration of peptide 1 by LC-MS

Kinetics for the conversion of BBDP unit into corresponding aspartimide and aspartyl residues was monitored by LC-MS. As polarity of the aspartimide and aspartyl residues was close, proper gradient (5% $CH_3CN$ for 0-2 min and 5-70 % $CH_3CN$ for 2-5 min in a total run time of 8 minutes) was selected through which distinct peaks were observed corresponding to different forms of the peptide.

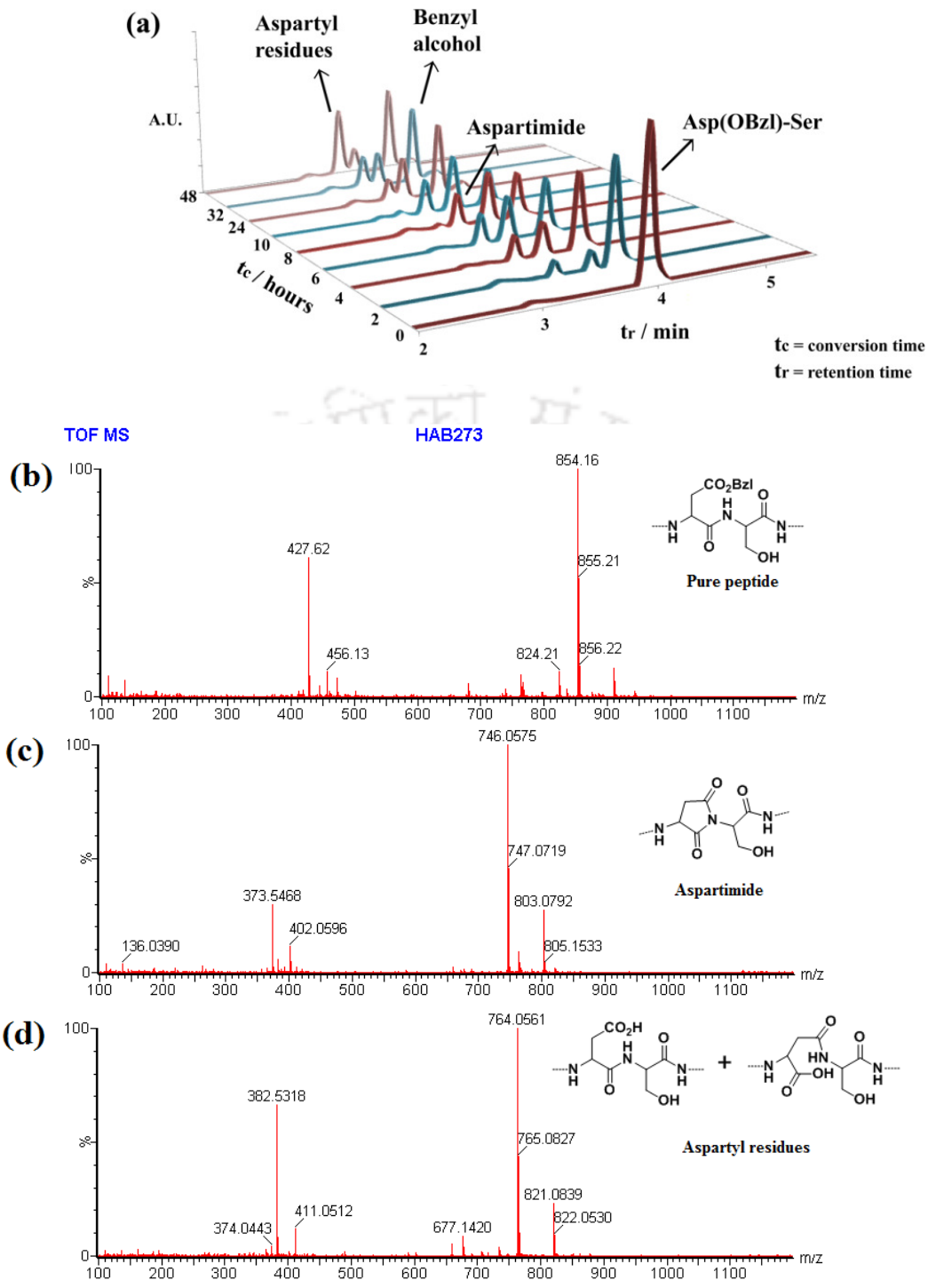



**Figure 2: Kinetics of O to N acyl migration of model β breaker dipeptide containing peptide (peptide 1) using LC-MS.**

20 µL of the sample from the stock solution (1.1 mM) in PBS (0.1 M, pH 7.0) was taken and injected in LC-MS at different time intervals. The stock solution was sonicated and vortexed

for 1 min each before taking the sample for injection. This was continued till the peaks corresponding to the aspartimide and the aspartyl residues were emerged. At the end, all the LC profiles were plotted using MS-Excel software (figure 2a). Corresponding ESI-MS data are displayed in panel b to d in figure 2.

The peak eluting prior to the pure peptide intensified with time and we contemplated it to be the by-product, benzyl alcohol. In order to confirm that we injected pure benzyl alcohol in the LC-MS and the retention time matched. The peak eluting prior to the peak assigned for benzyl alcohol was due to the formation of the aspartimide derivative of the peptide **1**, which was confirmed from the corresponding ESI-MS (panel c, figure 2). After around 8 h, we noticed the peak corresponding to the aspartimide derivative to disappear slowly and emergence of two new peaks in place of that. Those two new peaks were assigned to the α-aspartyl and β-aspartyl residues, which were confirmed from the ESI-MS (panel d). The ratio of the α-aspartyl and β-aspartyl residues was found to be 1:3 approximately, which is in agreement with the reported value.[59] In this way we could monitor the desired chemical conversion of the BBDP containing aggregating peptide **1** from its native form to aspartimide state which eventually converted to the aspartyl derivatives.

### 2.1.4 Monitoring stability of peptide 1 in acidic pH by LC-MS

Stability of the BBDP containing peptide **1** was studied in acidic environment in which no aspartimide formation is expected. BBDP containing Peptide **1** was dissolved in a solution of water (0.1% TFA, pH 1.0) to obtain a final concentration of 1.1 mM and incubated at 37 °C. Stock solution was sonicated and vortexed for 1 min each periodically to prevent fibril deposition. 20 µL of peptide sample was taken from the stock solution and injected in LC-MS for next seven days (Figure 3).

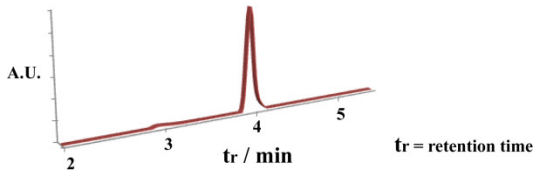



**Figure 3: LC chromatogram of model BBDP containing peptide 1 in acidic environment. No acyl migration was observed for 7 days.**

No change in the LC profile was noticed for seven days. This indicates that the BBDP unit is quite stable in the acidic pH as no peak corresponding to either benzyl alcohol or aspartimide was noticed.

## 2.1.5 Monitoring conformation of BBDP containing peptide 1 by circular dichroism (CD) studies

After getting evidence for the desired chemical conversion, we were interested to study the conformational behaviour of the model BBDP containing peptide **1** in basic pH, since in basic pH we expect chemistry of aspartimide formation comes into play and peptide no longer stays in β-sheet conformation. 0.5 mg of peptide **1** was dissolved in 1.1 ml of (PBS, 50 mM, pH 7.4) to obtain a final concentration of 500 μM and incubated at 37 °C for 3 days and its conformation was studied.

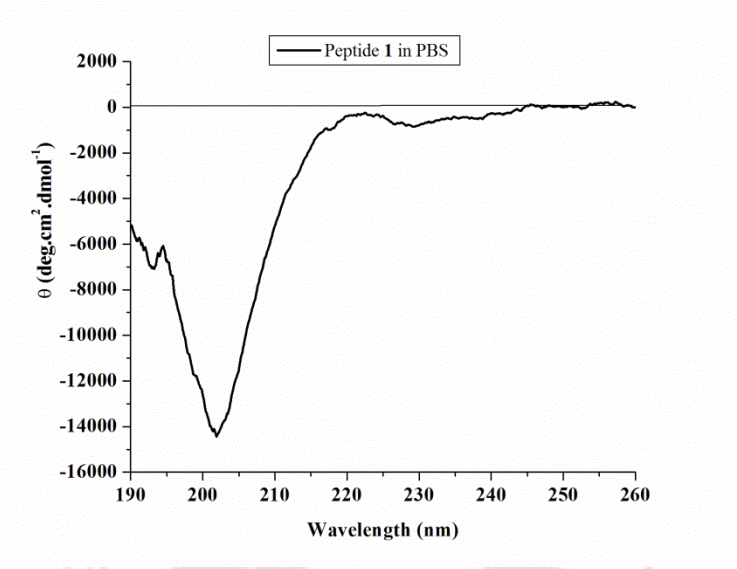



**Figure 4: CD spectrum of peptide 1 in PBS after 3 days of incubation. Y-axis indicates mean residue ellipticity.**

Ellipticity units of millidegrees was converted to mean residue molar ellipticity using the following equation (where *n* is the number of peptide bonds in protein and ellipticity is the raw data from the instrument).

$$\theta\,(deg.cm^2.dmol^{-1}) = Ellipticity\ (mdeg).\ 10^6\ /\ Pathlength\ (mm).\ [Protein]\ (\mu M).n$$

CD spectrum produced a characteristic minimum of 200-205 nm which was assigned to unordered conformation of the peptide as displayed in figure 4. From the deconvolution analysis we observed 61 % of the random coil conformation and 21 % of β content (table 1). This confirms that the BBDP containing peptide adapts random coil conformation in PBS, which is probably because of the generation of an aspartimide and aspartyl residues from the BBDP unit. This is likely to disturb the fibril formation.

| Peptide **1** in PBS | |
|---|---|
| | **Ratio** |
| Helix | 3.3 |
| Beta | 21.6 |
| Turn | 13.7 |
| Random | 61.4 |
| Total | 100 |

**Table 1: Detailed information of the ratio of different secondary structures.**

## 2.1.6 Monitoring conformational conversion of BBDP containing peptide 1 by fourier transformation infra-red spectroscopy (FT-IR) studies.

FT-IR is another technique from which we obtained an idea of the secondary structure of peptides. 20 µL of the peptide sample after three days of incubation as prepared before (section 2.1.4 and 2.1.5) was mixed with KBr and pellet was prepared. 32 scans accumulated for a sample followed by subtraction of the background to obtain the final spectra.

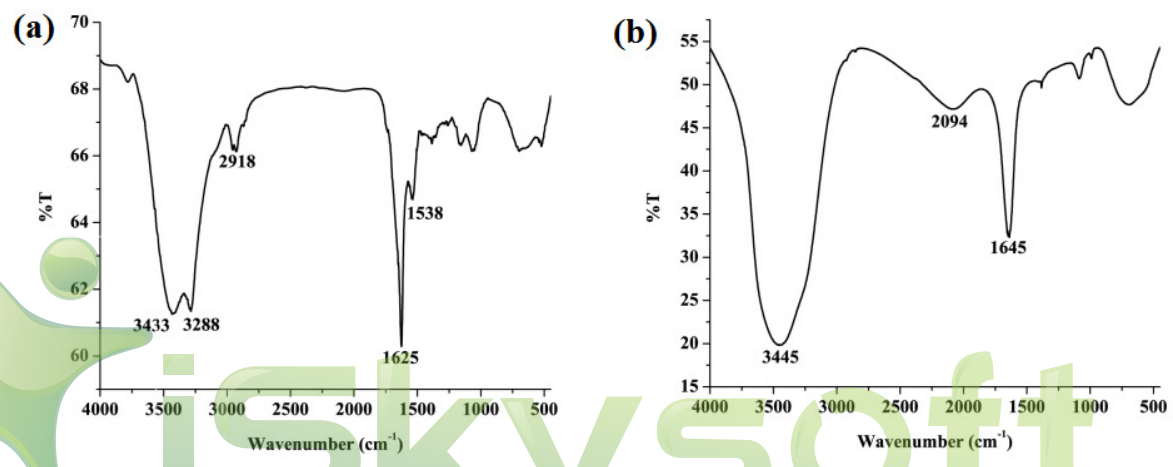



**Figure 5: FT-IR spectra of peptide 1 in (a) $H_2O$ (0.1 % TFA). (b) PBS. Analyzed after three days.**

We noticed presence of strong band at 1625 $cm^{-1}$ for the peptide **1** (from 0. 1% TFA water mixture) indicating amide I of β sheet (figure 5a).[60, 61] At the same time peptide sample from PBS was analysed and a band at 1645 $cm^{-1}$ was observed which indicates random coil conformation (figure 5b). Thus, it is confirmed that BBDP containing peptide **1** stays in β-sheet conformation in acidic pH whereas it stay as random coil in basic pH.

### 2.1.7 Monitoring fibril formation of peptide 1 using thioflavin T fluorescence assay

Having obtained the idea about the desired chemical and conformational conversion using different techniques, described above, we wanted to investigate whether peptide **1** can disrupt fibril at near physiological pH. It is discussed in the chapter 1 (section 1.12) that monitoring fluorescence emission of a peptide solution in presence of thioflavin T provides quantitative idea on fibril formation.

0.5 mg of lyophilized peptide **1** was dissolved in 3.5 ml of PBS (50 mM, pH 7.4) to obtain a final concentration of 160 μM and incubated at 37 °C over water bath. At different time intervals, 20 μL of peptide sample was mixed with 200 μL of thioflavin T solution (50 μM), total volume was made up to 400 μL with PBS (50 mM, pH 7.4) and fluorescence was measured ($\lambda_{ex}$ = 435 nm and $\lambda_{em}$ = 485 nm).

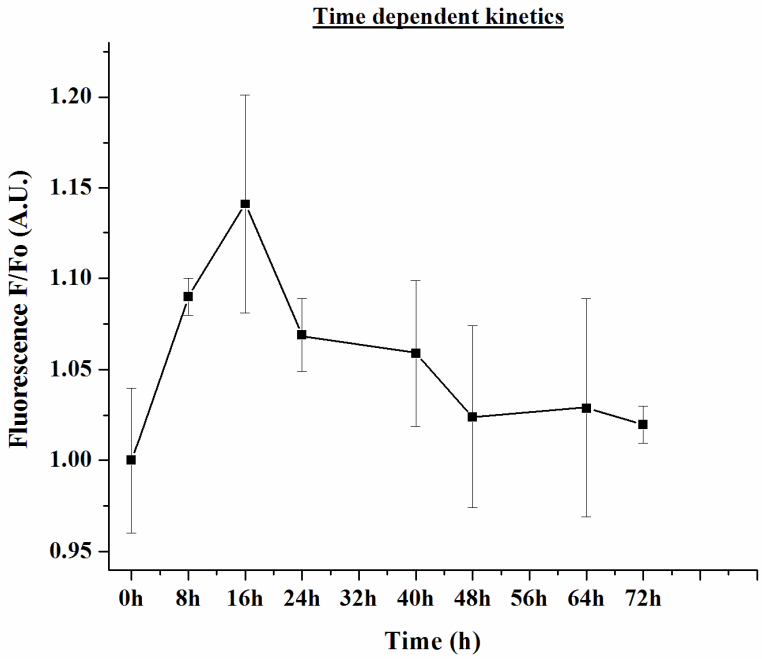



**Figure 6: Time dependent kinetics of fibril formation of BBDP containing peptide 1.**

We noticed an increase in the fluorescence signal for first 16 h and after that a decrement was noted (figure 6). This is probably due to the fact that the model BBDP containing peptide **1** first forms β-sheet which aggregates to fibrils. Those fibrils get disrupted with time, probably because the chemistry of aspartimide formation and subsequent hydrolysis takes place and peptide loses its usual peptide backbone. This is in agreement with our hypothesis.

## 2.1.8 Monitoring fibril formation of peptide 1 using transmission electron microscopy (TEM)

Previous experiments suggested that our designed BBDP containing aggregating peptide **1** undergoes desired chemical conversion and as a result of that it can disrupt β-sheet and fibril formed by itself at a specific condition. Next we wanted to verify inhibition of fibril

formation using TEM also. Electron microscopy provides the direct evidence for the presence and absence of fibrils.

The samples taken from the stock solutions which were incubated for seven days in acidic pH and in basic pH in parallel were viewed under electron microscope. 10 µL of the peptide sample from the stock solution was added over a carbon coated grid, followed by addition of 10 µL of 2 % uranyl acetate over it. The droplet was allowed to stand for few minutes and excess solution was removed carefully by blotting paper. Grid was allowed to dry in open air and examined at 80 kV under TEM.

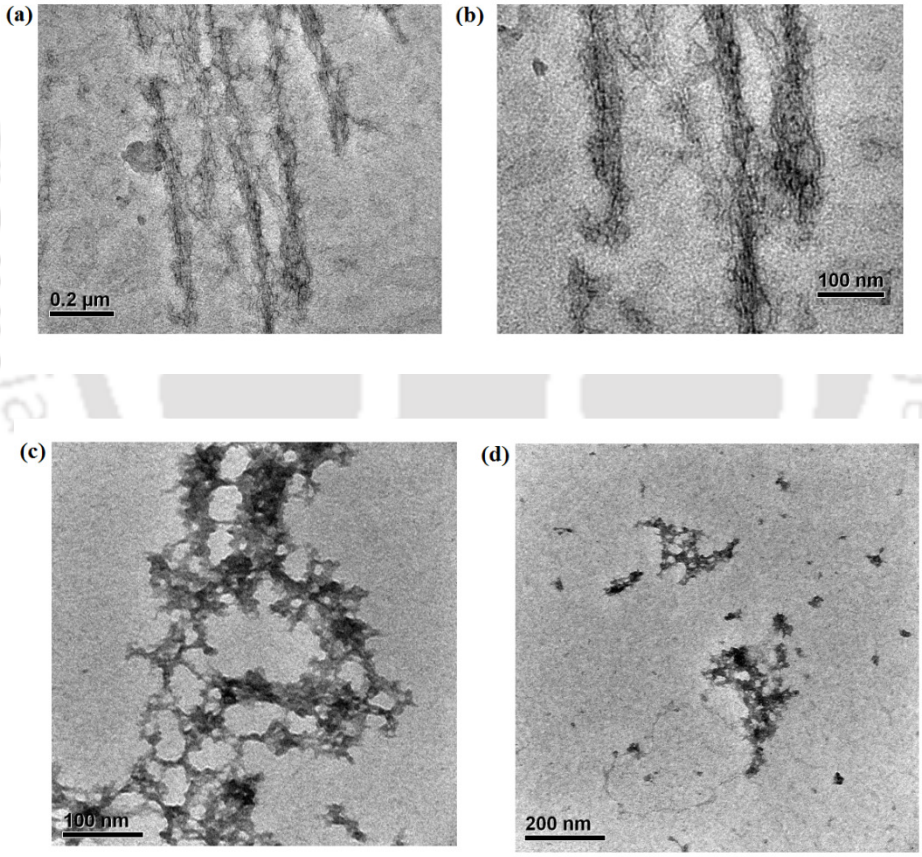



**Figure 7: TEM images of peptide 1 (a) and (b) in $H_2O$ ( 0.1 % TFA) and (c) and (d) in PBS after seven days.**

We noticed presence of thread like fibrils of nearly 10-15 nm in diameter (figure 7a and 7b) from the sample which was incubated in $H_2O$ (0.1 % TFA, pH 1). On the other hand, we

noticed some amorphous and disordered clusters from the peptide sample when taken from the PBS set (figure 7c and 7d) and no fibril like structure was observed. From this experiment it can be inferred that model BBDP containing aggregating peptide forms β-sheet and fibrils in acidic pH and inhibits self aggregation at physiological pH. This can be accounted for the fact that in acidic pH no aspartimide formation and subsequent ring opening takes place. Therefore, the peptide backbone remains intact. On the other hand, at basic pH disruption of the peptide backbone takes place via chemistry of aspartimide formation. As peptide backbone is broken, formation of β-sheet and fibril is not expected.

### 2.1.9 Monitoring amyloid formation of peptide 1 using Congo-red birefringence assay

Green gold birefringence upon staining with Congo red is another characteristic property of amyloid(s) when viewed under polarisable microscope. However, under normal light it appears red.

20 μL from the stock solution which was used in LC-MS and thioflavin T was taken and placed over a glass slide and air dried. 20 μL of the saturated Congo red solution (method of sample preparation is mentioned in experimental section) was added over it and allowed to dry. Dried spot was viewed under optical polarizable microscope.

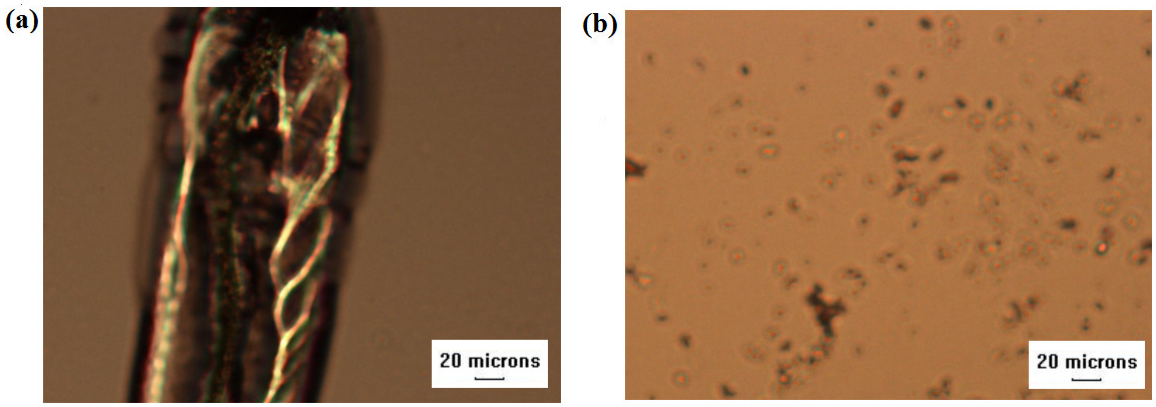



**Figure 8: (a) Microscopic image of mature fibril on staining with Congo red and viewed under polarized microscope. (b) Microscope image of the peptide in basic condition under polarised light. Scale bars indicate 20 microns.**

We noticed a characteristic green gold birefringence for BBDP containing peptide sequence **1** when the stock solution was incubated in water (0.1% TFA) mixture (figure 8a). On the other hand, we did not notice any birefringence when peptide was incubated in PBS (figure 8b). It indicates that BBDP containing aggregating peptide **1** forms amyloid in acidic pH, whereas, amyloid formation is inhibited in basic pH.

Based on the experimental results of the model BBDP containing peptide **1** it was clear that the concept of β sheet disruption based on the chemistry of aspartimide formation was proved efficient. As model BBDP containing peptide produced aggregates in acidic pH and inhibited its own aggregation in a neutral to basic pH ranges. Based on the result of thioflavin T fluorescence measurement we can conclude that BBDP containing peptides not only inhibits self aggregation but also disrupts fibrils. In order to authenticate the concept of β sheet disruption we wanted to test the hypothesis on another peptide with similar aggregation potential to that of peptide **1**.

## 2.2.1 Design of model BBDP containing aggregating peptide 2

We designed another peptide which is likely to behave in a similar way to peptide **1** in a given experimental condition. -(Ser-Leu)$_n$- comprising sequence was designed such that alternate hydrophilic and hydrophobic amino acids governs the secondary conformation. However, this time peptide with total six residues was selected for the study. Chemical synthesis and purification of such shorter peptide sequence was expected to be easier compared to the peptide **1**. Glycine was kept as a spacer at the C-terminus and N-terminus was kept free to aid the solubility. BBDP unit -**D(OBzl)S**- was incorporated in the middle of the sequence.

**Model BBDP containing peptide: $H_2N$-$^1$LS<u>D(OBzl)S</u>L$^6$S-$GNH_2$ (peptide 2)**

## 2.2.2 Synthesis and characterization of model BBDP containing peptide 2

Model BBDP containing aggregating peptide **2** was synthesized in a similar way as peptide sequence **1** on Rink amide resin using Fmoc/tBu protection strategy (similar to procedure in section 2.1.2). Based on 0.33 mmol scale 22 mg of pure peptide was obtained as white powder. Peptide purity was confirmed from LC-MS.

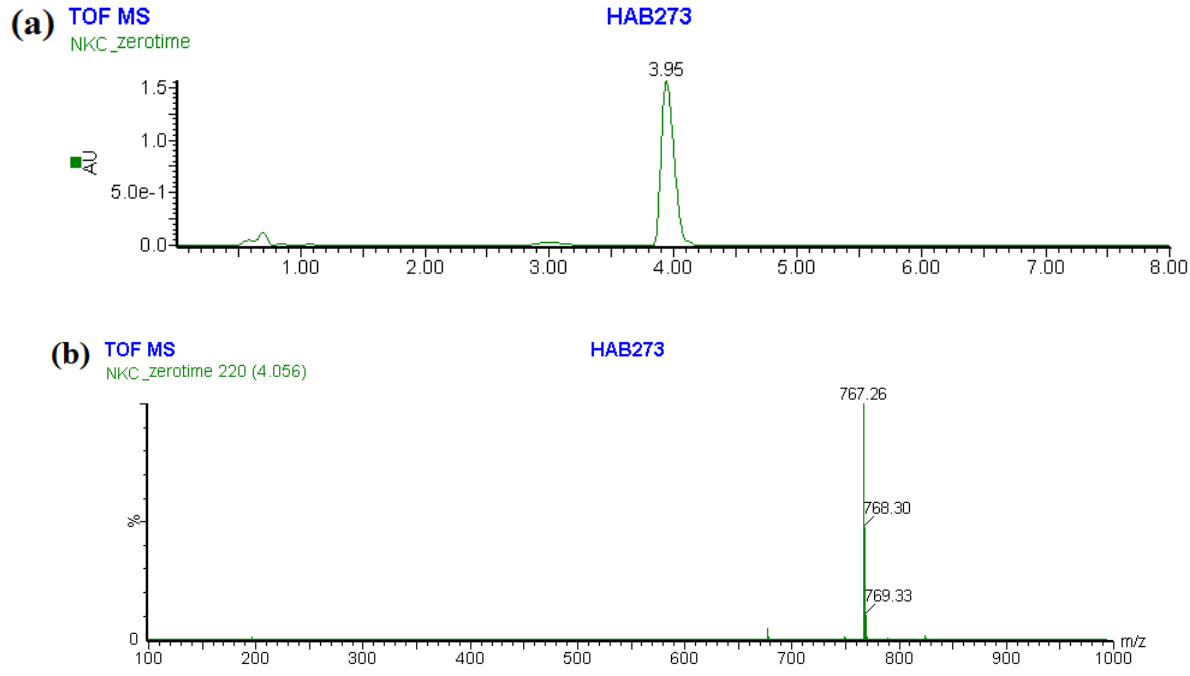



**Figure 9: LC-MS picture of the purified model BBDP containing peptide 2. Calculated mass for $C_{34}H_{55}N_8O_{12}$ is 767.39 and observed mass 767.26 $[M+H]^+$.**

Peptide **2** was purified using semi preparative HPLC and purity was confirmed from LC-MS. $[M+H]^+$ was observed in MS (ESI-MS positive mode). Representative figure of LC profile is shown in figure 9a and mass is shown in figure in 9b.

## 2.2.3 Monitoring of O to N acyl migration of model BBDP containing peptide 2

We investigated the time required for the chemical conversion of BBDP unit into aspartimide and further into aspartyl residues. Gradient of 5% $CH_3CN$ for 2 minutes and 5-70 % $CH_3CN$ for 2-5 minutes (linear gradient) with total run time of 8 minutes was used to resolve the peaks corresponding to different forms of the peptide.

Lyophilised sample was dissolved in PBS (0.1M, pH 7.0) to obtain a final concentration of 1.1 mM and incubated at 37 °C over water bath. Stock solution was sonicated and vortexed for 1 min each and 20 µL of the peptide sample was taken and injected in LC-MS.

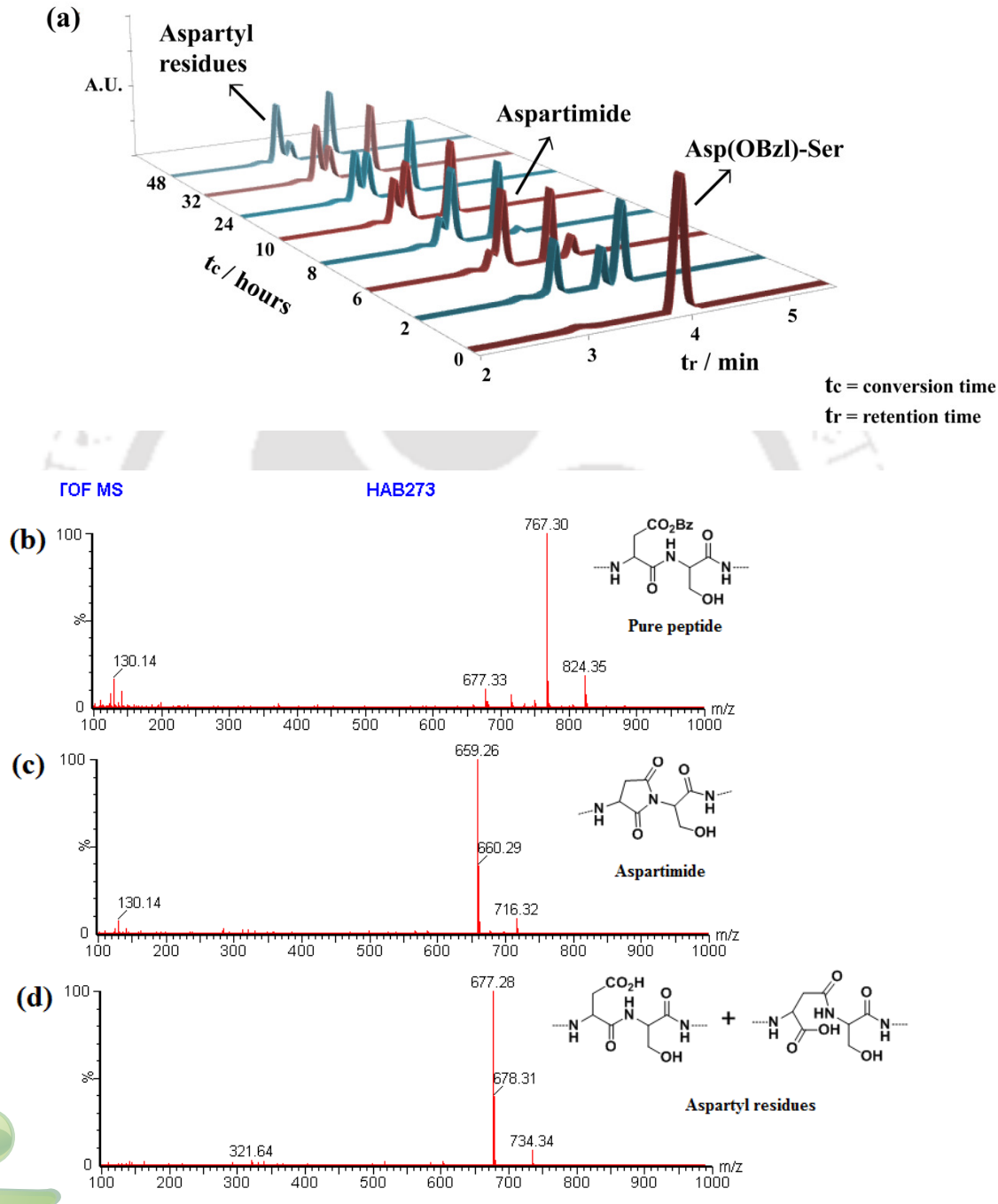



**Figure 10: Study on kinetics of O to N acyl migration of model β breaker dipeptide containing peptide 2 using LC-MS.**

Since BBDP unit **-D(OBzl)S-** was similar to that of peptide **1**, it was found that time required for aspartimide formation and ring opening to produce aspartyl residues was also similar to that of peptide **1**. After 8 h, we noticed aspartimide peak disappeared and two new peaks for α and β aspartyl residues emerged. This continued for 48 h and no change was noticed after that as displayed in figure 10a. Mass of various forms of peptide that is aspartimide and aspartyl residues were observed and matched to the expected value (figure 10b-d). We could monitor the kinetics of chemical conversion using LC-MS.

## 2.2.4 Monitoring stability of peptide 2 in acidic pH by LC-MS

Stability of BBDP unit -D(OBzl)S- was monitored using LC-MS. In acidic pH, aspartimide formation was not expected and peptide retained its native peptidic backbone. To test that, lyophilized peptide sample was dissolved in water (0.1% TFA) mixture and incubated at 37 °C. Stock solution was sonicated periodically and 20 µL of the peptide sample was taken and injected in LC-MS for seven days. No change in the LC profile was observed for seven days (figure 11). This indicates that the BBDP unit did not undergo any chemical change in acidic pH.

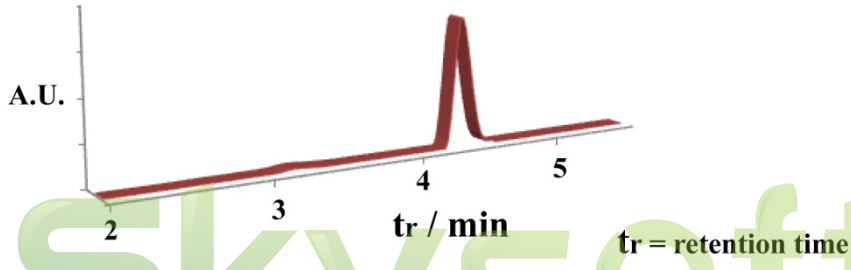



**Figure 11: UV chromatogram of model BBDP containing peptide 2 in acidic pH after seven days.**

## 2.2.5 Monitoring conformation of BBDP containing peptide 2 by circular dichroism (CD) studies

CD spectroscopy was used to check the conformation of the peptide **2** in basic pH. 0.5 mg of peptide **2** was dissolved in 1.3 ml of PBS to obtain a final concentration of 500 μM, incubated at 37 °C for three days. 100 μL of the peptide sample was taken from the stock solution and diluted with PBS to obtain final concentration of 160 μM and analyzed by CD.

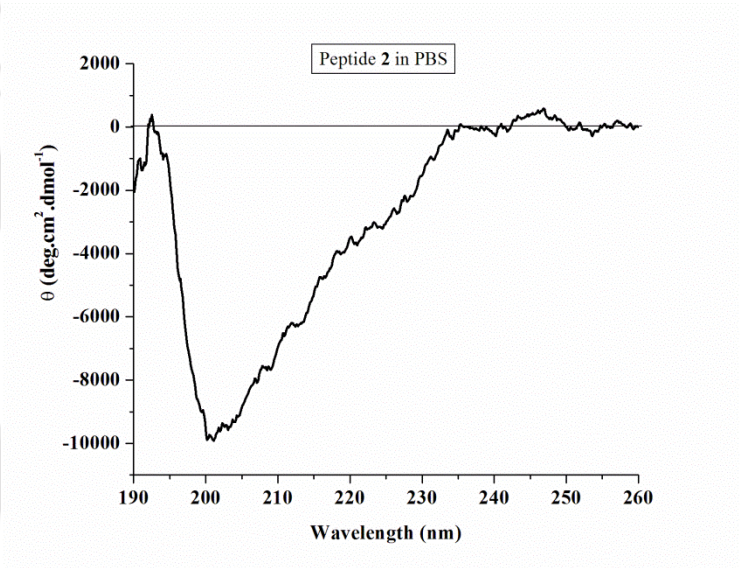



**Figure 12: CD spectrum of the peptide 2 in PBS after three days of incubation.**

We observed a spectrum with a minimum at 200 nm which is assigned to random coil conformation (figure 12). Peptide **2** in basic pH undergoes chemical conversion, where BBDP unit converts into aspartimide and subsequent ring opening produces aspartyl residues. Due to this conversion peptide no longer stays in β sheet conformation and is confirmed from CD study.

| Peptide **2** in PBS | |
|---|---|
| | **Ratio** |
| Helix | 2.2 |
| Beta | 39.5 |
| Turn | 4.2 |
| Random | 54.1 |
| Total | 100 |

**Table 2: Detailed information of ratio of different secondary structures.**

From table 2, 54 % of the random coil conformation and 39 % of β-sheet conformation of peptide 2 was obtained. It is suggested that at that particular time of analysis conversion of β sheet to random coil was in progress.

### 2.2.6 Monitoring conformational conversion of BBDP containing peptide 2 by fourier transformation infra-Red spectroscopy (FT-IR) studies

Secondary structure of the peptide **2** was also studied using FT-IR. 20 μL of the peptide sample was taken from the stock solution used in LC-MS and ThT analysis, mixed with KBr and pellet was prepared. 32 scans were taken for the sample followed by the subtraction of the background to obtain final spectra.

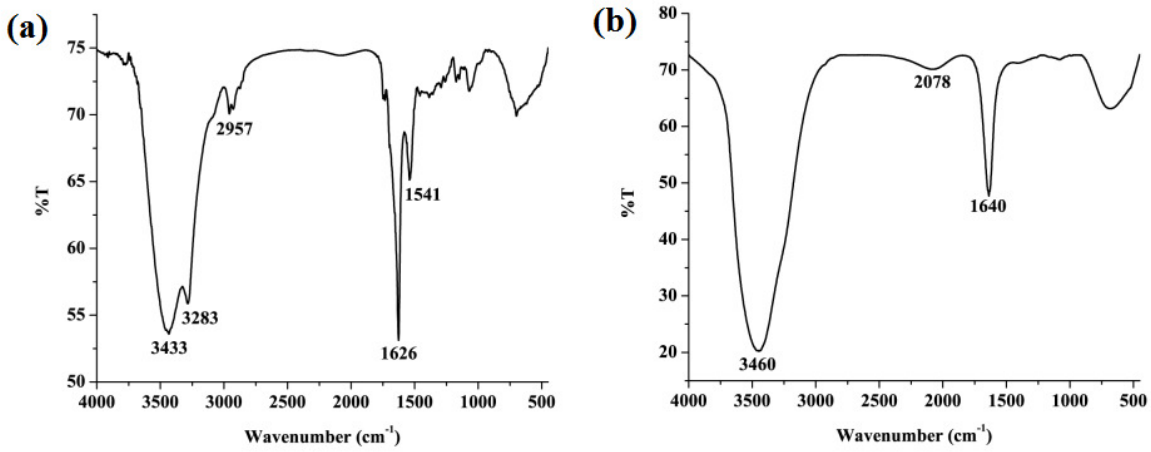



**Figure 13: FT-IR spectra of peptide 2 in (a) $H_2O$ (0.1 % TFA) and (b) PBS and analyzed after three days.**

Peptide **2** produced a strong band at 1626 $cm^{-1}$ which is assigned to amide I of β sheet conformation when sample was taken from water (0.1% TFA) mixture (figure 13a). On the other hand, peptide sample from PBS produced a band at 1640 $cm^{-1}$ corresponding to random coil (figure 13b). Disappearance of 1626 $cm^{-1}$ band from the basic environment revealed that peptide no longer stays in β sheet conformation and this is probably due to conversion of peptide from its native form into modified peptide which lacks native backbone.

## 2.2.7 Monitoring fibril formation of peptide 2 using thioflavin T fluorescence assay.

0.5 mg of the lyophilized peptide **2** was dissolved in 4 ml of PBS (50 mM, pH 7.4) to obtain a final concentration of 160 μM and incubated at 37 °C over water bath. At different time intervals, 40 μL of peptide sample was mixed with 200 μL of thioflavin T solution (50 μM), total volume was made up to 400 μL with PBS (50 mM, pH 7.4) and fluorescence was measured ($\lambda_{ex}$ = 435 nm and $\lambda_{em}$ = 485 nm).

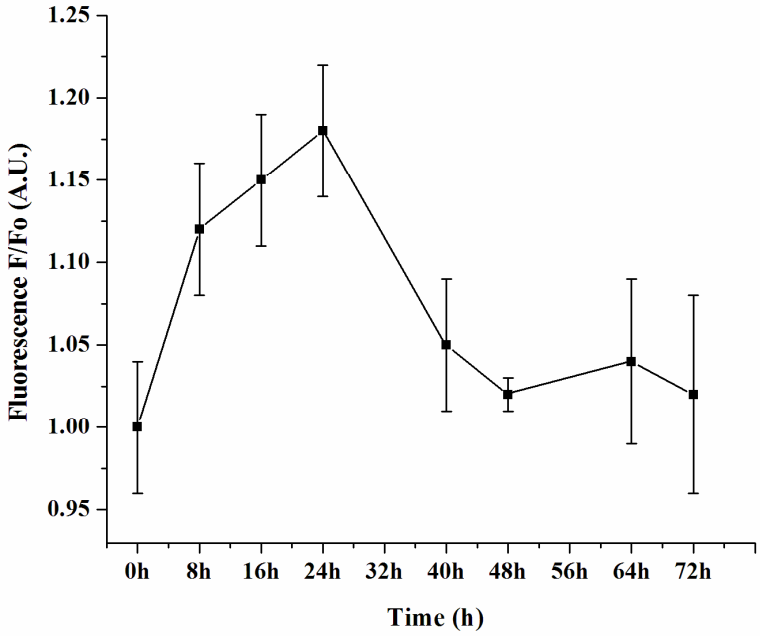



**Figure 14: Time dependent kinetics of fibril formation of BBDP containing peptide 2**

We observed an increase in the fluorescence signal for first 24 h and thereafter a decrement was noticed as displayed in figure 14. The reason attributed is that the BBDP containing peptide **2** first aligns to form β sheet rich fibrils and in due course of time fibril formation is disrupted due to chemistry of aspartimide formation which is in agreement with our hypothesis.

## 2.2.8 Monitoring fibril formation of peptide 2 using transmission electron microscopy (TEM)

Conformation of the peptide **2** was studied from circular dichroism and FT-IR characteristic tools. Electron microscopy gave information on the morphological characteristics of peptide fibrils. Sample was prepared in a similar way as described in section 2.1.8.

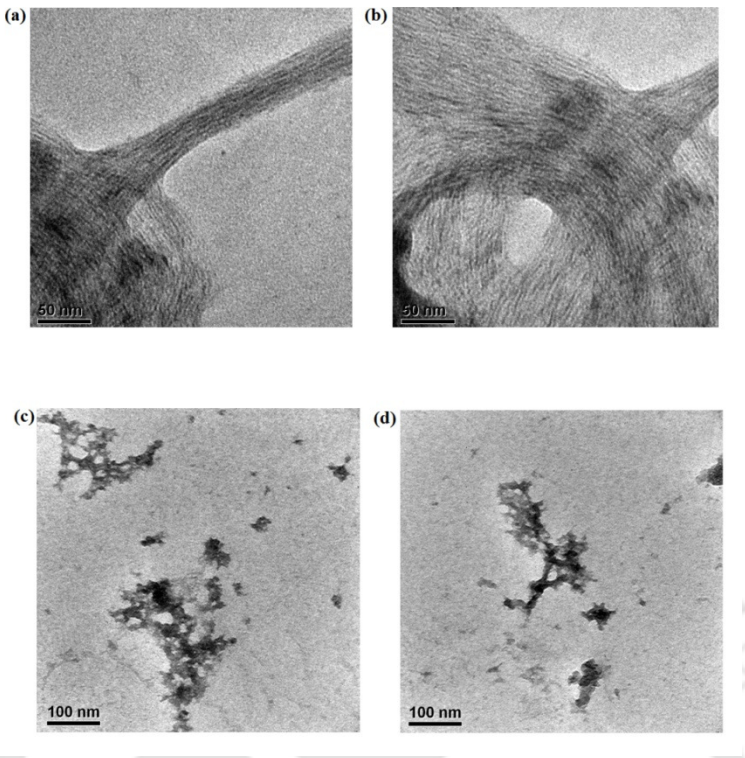



**Figure 15: TEM images of peptide sequence 2 in acidic and basic environment.**

We noticed presence of thick bunch of fibrils of nearly 10 nm in diameter for peptide 2 which was incubated in water (0.1 % TFA) mixture as displayed in figure 15a and 15b. Peptide **2** from the PBS exhibited amorphous aggregates. No fibrils were noticed as shown in figure 15c and 15d. This again confirms that BBDP unit undergoes chemical modification in basic environment to produce aspartimide and aspartyl residues which destroys native backbone and as a result fibril formation is inhibited.

### 2.2.9 Monitoring amyloid formation of peptide 2 using Congo-red birefringence assay

Green gold birefringence under optical polarizable microscope is a unique property of amyloid. To investigate the same peptide sample was prepared in a similar way to that of the peptide **1** (discussed in experimental section) and viewed under optical polarizable microscope.

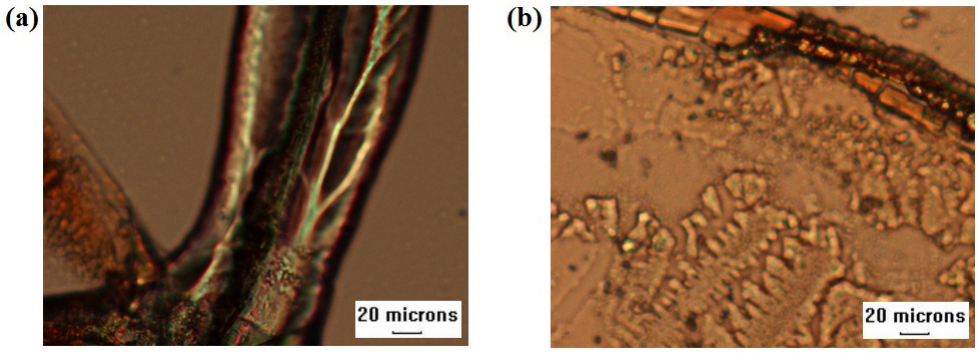



**Figure 16: (a) Microscopic image of mature fibril on staining with Congo red in acidic pH (b) Microscopic image of peptide 2 sample in basic condition under polarized light. Scale bars indicate 20 microns.**

We noticed green gold birefringence for peptide **2** when incubated in water (0.1 % TFA) mixture as displayed in figure 16a and no birefringence was noticed from PBS mixture figure 16b. This indicates that peptide **2** forms amyloid similar to peptide **1** in acidic environment and in a basic condition BBDP unit inhibits β sheet aggregation and amyloid formation.

## 2.3 Conclusion

On the basis of various experimental findings we can conclude that BBDP containing peptides (sequence **1** and sequence **2**) formed amyloid like fibrils in acidic pH as no aspartimide formation and subsequent ring opening occurred and native peptide backbone was retained. Once pH was adjusted from neutral to basic range, BBDP containing peptide produced aspartimide and aspartyl residues, which finally inhibited β sheet, fibril and subsequent amyloid formation. Results obtained from thioflavin T fluorescence measurement indicated that such peptides can disrupt preformed fibril as well. This concept was tested on two model aggregating sequences. In another word, model BBDP containing aggregating peptides have a potential to aggregate in one particular condition (pH < 7.0) and to inhibit self assembly in another specific condition (pH ≥7.0, 37 °C). Next we would like to test whether such BBDP containing peptides can be used to disrupt β sheet aggregation and to stop amyloid formation of other aggregating peptides when mixed.

# Chapter 3: Inhibition of aggregation of model amyloid forming peptide using BBDP containing model peptide 1

## 3.1 Introduction

In the previous chapter we discussed aggregation as well as inhibition of self assembly of model BBDP containing peptides at different pH. We observed that BBDP containing peptides (peptide **1** and **2**) aggregates to form amyloid like fibrils in a specific condition (pH < 7.0). Later, when pH was maintained from neutral to basic (≥ 7.0), complete disappearance of fibrillar aggregates was observed which was confirmed using various biophysical tools. Hence, our next target was to investigate the potential of BBDP containing peptide to inhibit or disrupt aggregation caused by other model aggregating peptides when mixed. This is to explore therapeutic potential of such peptides.

### 3.1.1 Design of model aggregating peptide

We wanted to obtain a molecular mimic of Aβ peptide which aggregates in vitro in a similar fashion to that of the Aβ peptide, at the same time we wanted to have the control on the rate of its aggregation. That peptide can act as a model aggregating peptide. In this context, we wanted to use our BBDP containing model peptide **1** as a β-breaker peptide. It is necessary to have sequence homology between the aggregating peptide and the β-breaker peptide for proper recognition to take place.[52] If proper recognition is not there between aggregating peptide and β-breaker peptide, we cannot expect β-breaking efficiency of the breaker peptide. Mutter *et al.* reported -(Ser-Leu)$_n$- based peptide that forms amyloid like fibrils rich in β sheet architecture in a specific experimental condition (basic pH). They inserted a depsi bond in the middle of the sequence using the side chain of a specific serine residue. Class of such peptides was named as "Switch peptides",[62] where depsi bond connected serine acted as a switch unit (figure 1). Absence of the peptidic backbone forbids attaining β sheet conformation at the initial stage of the peptide as the chain length of the peptides connected to the serine switch from both sides was less than the critical chain length of β-sheet formation ($n_c$, β =7). Once the peptide attains normal peptide backbone at basic pH via O to N acyl migration, its chain length becomes greater than the $n_c$, β. Then it can form β-sheet which finally converts to fibril and amyloid.

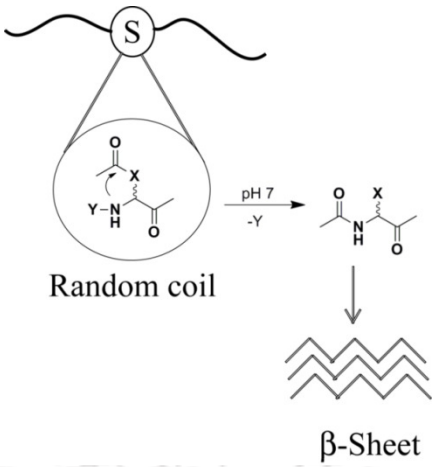


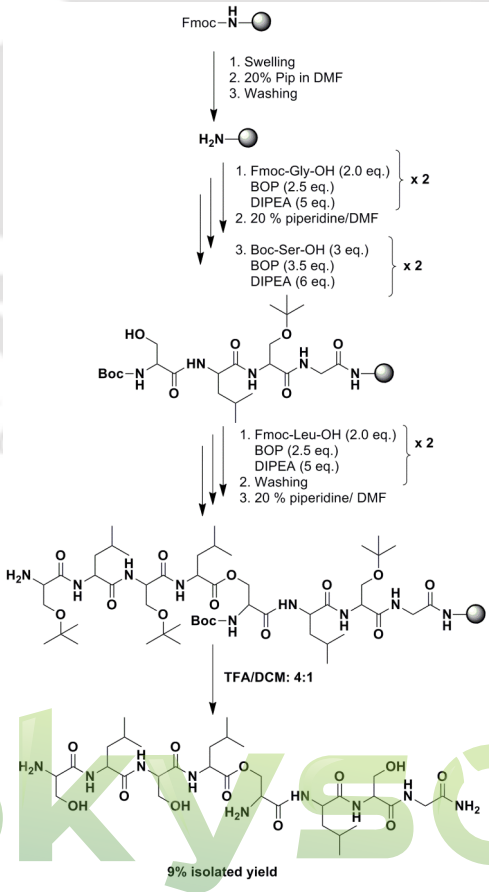

**Figure 1: Switch peptide with one serine switch element. Acyl migration takes place to convert peptide from depsi state to native state.**

We have designed our model aggregating peptide based on the concept of switch peptides, described above. We took a -(Ser-Leu)$_n$- based model aggregating peptide to keep sequence homology with our breaker peptide. Chain length of the final peptide was decided to be seven amino acids. The switch forming depsi bond was incorporated at the side chain of the serine residue at position 5, so that chain length of the fragment peptides connected to the switch element becomes less than $n_c$, β. Therefore, it is expected that the designed aggregating peptide will stay in unstructured form initially, which at basic pH would undergo O to N acyl migration and adopt native peptide backbone. At that final stage the chain length of the peptide exceeds $n_c$, β, therefore, it forms β-sheet conformation and forms fibril similar to that of the Aβ peptide. N-terminus was kept free to aid the solubility of the peptide. Glycine at the C-terminus was kept as a spacer.

**Model aggregating peptide: $H_2N$-$^{1}$SLSL($H^+$)SL$^{7}$S-$GNH_2$ (peptide 3)**

## 3.1.2 Synthesis and characterization of peptide 3

Model amyloid forming peptide was synthesized according to standard procedures of solid phase peptide synthesis (SPPS) similar to peptide **1** described in the section 2.1.2.[53, 54, 55,56, 63] Coupling of serine residue corresponding to switch element was performed using $N^{\alpha}$-Boc protected and hydroxyl side chain free derivative of amino acid (Boc-Ser-OH). This allowed coupling of $N^{\alpha}$-Fmoc protected leucine to side chain hydroxyl group via ester bond. Boc-Ser-OH (3 eq.) in 1 mL of DMF was taken in a 5 mL plastic syringe first, followed by PyBop (3.5 eq.) and DIPEA (7 eq.) in 2 mL of DMF and kept for rotation for 2 hours and this procedure was repeated for 3 times until Kaiser test shows negative. Next, esterification using Fmoc-Leu-OH (3 eq.) was performed using PyBOP (3.5 eq.) and DIPEA (7 eq.) for 2 h. See experimental section, page no. 140 for detailed description. The synthetic scheme is shown below (scheme 1).




**Scheme 1: SPPS scheme of model amyloid forming peptide 3.**

Peptide was purified using semi preparative HPLC and purity was confirmed by LC-MS.A representative figure of LC-profile is shown in panel 2a and mass profile is shown in panel 2b.

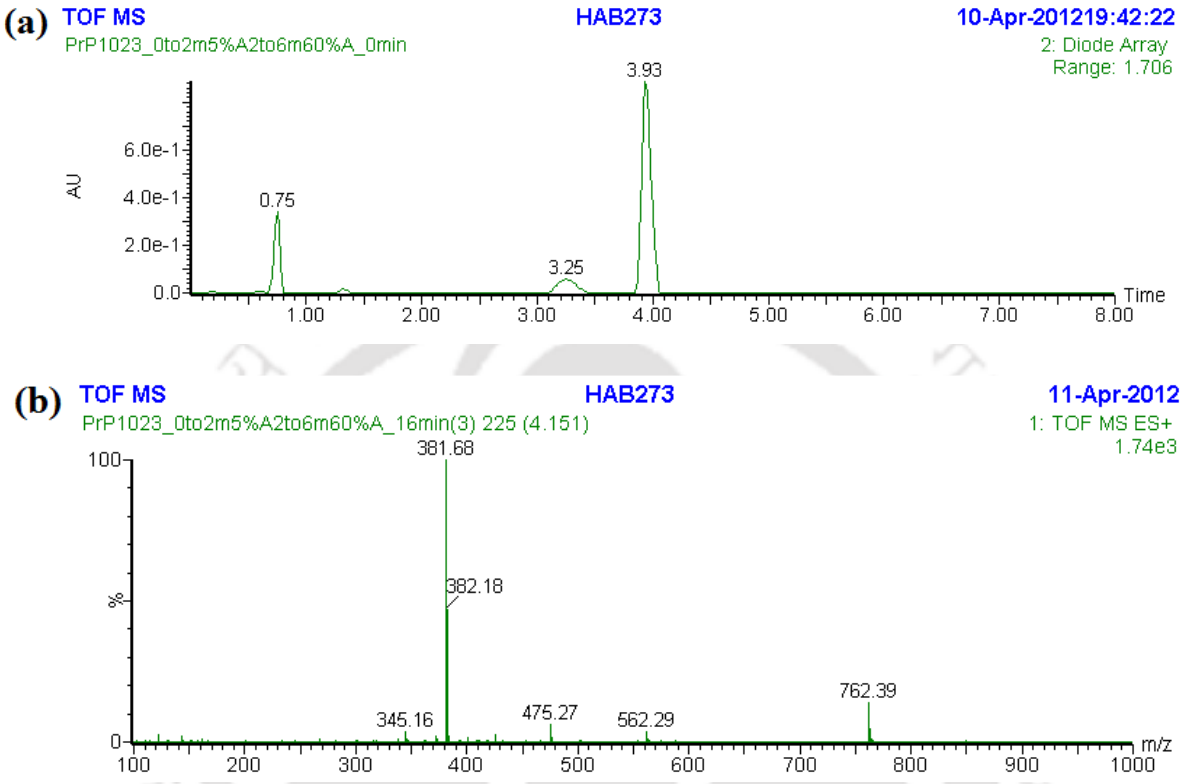



**Figure 2: LC-MS diagram of model amyloid forming peptide. 762.35 $[M+H]^+$ was observed from the peak at 3.93 minutes.**

### 3.1.3 Kinetics of O to N acyl migration of peptide 3

Rate of O to N acyl transfer was studied using LC-MS. This rate of acyl shift can be controlled by pH of the system. Polarity of the peptide in depsi form and in native form was close. In order to resolve the peaks a gradient of 5% acetonitrile for 2 minutes and 5-60 % acetonitrile for 6 minutes (linear gradient) in a total run time of 8 minutes was used.

1 mg of the lyophilized peptide sample was dissolved in 1.2 ml of PBS (0.1M, pH 7.2) to reach a final concentration of 1 mM. The stock solution was incubated at 37 °C on water bath. At different time intervals sample (50 µL) from stock solution was taken in LC-MS

vials, quenched using 1N HCl (adjusted the pH of the peptide solution to pH 1.0) and injected in LC-MS. At the end all the text files were collected and plotted using MS-excel (figure 3).

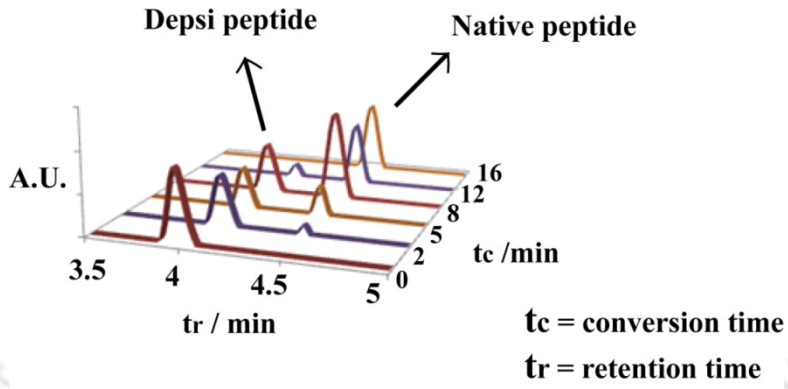



**Figure 3: UV chromatograms monitoring O to N acyl migration of model aggregating peptide. (gradient used 5 to 60% acetonitrile for 2 to 6 minutes).**

At zero time, we observed only one major peak at $t_R$ 4.1 min, corresponding to the pure peptide **3**. Slowly after a couple of minutes, a new peak emerged at $t_R$ 4.5 min. bearing the same mass value as displayed in figure 4a and 4b.

In LC-MS we noticed, peptide 3 in depsi form is more polar ($t_R$ 4.1 min) than peptide in native form ($t_R$ 4.5 min). This confirms peptide in native form is more hydrophobic than peptide in depsi form. Since, presence of ester linkage prevents the inter strand interaction. On the other hand, native backbone enables inter strand interaction which generates β sheet. This was confirmed from other biophysical techniques as well. The first peak was assigned to the peptide in depsi form which slowly transformed to the native form in pH 7.0. Presence of two peaks continued for near about 16 min and later, that peak at $t_R$ 4.1 min. disappeared completely.

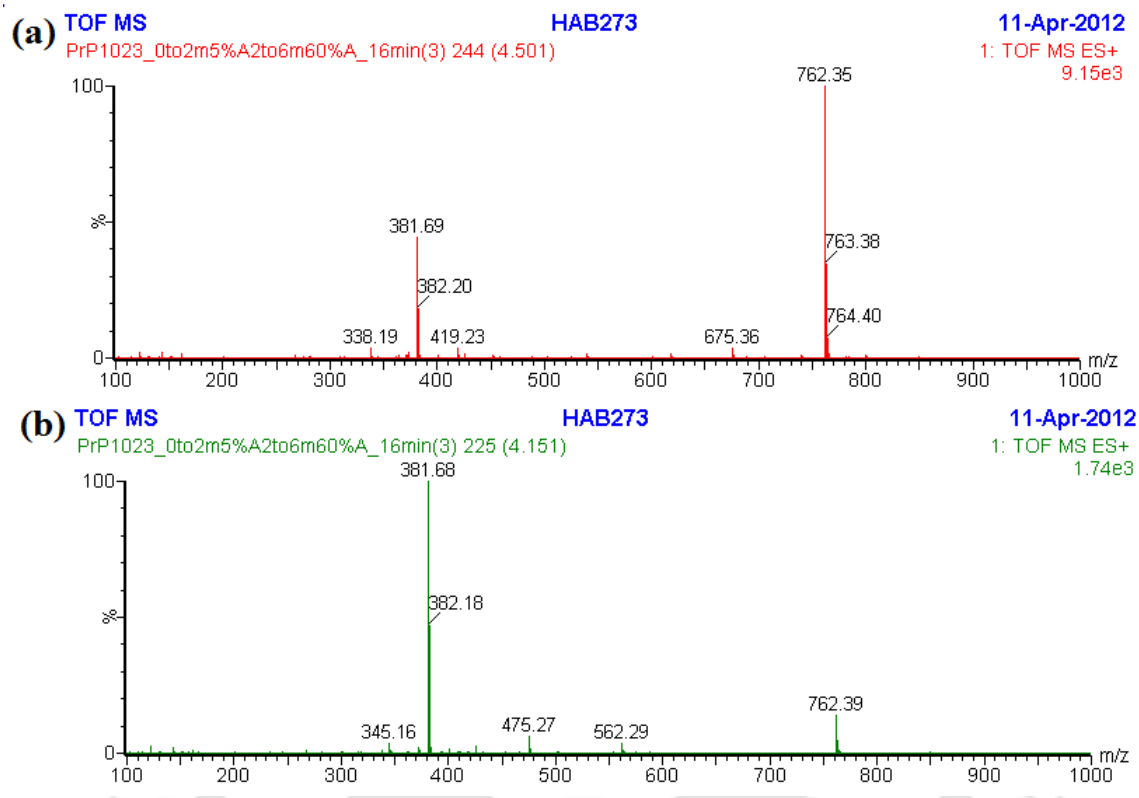
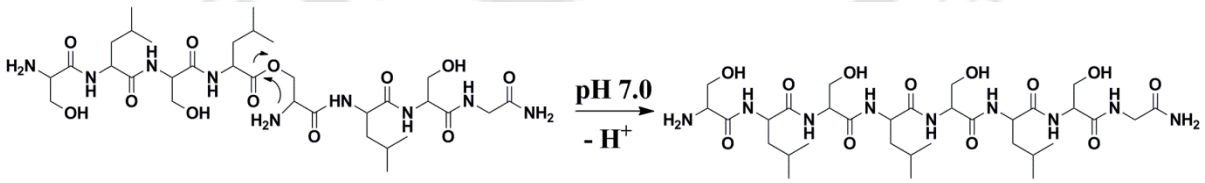



**Figure 4: MS spectra of the model aggregating peptide 3 in depsi form and native form.**

When pH was adjusted to 7.0 protonated form gets converted to free amine due to which O to N acyl migration starts and peptide attains native backbone with required chain length for β sheet formation. ($n_c$, β).[53]




**Depsi peptide** **Native peptide**

**Random coil** **β-sheet**

**Scheme 2: pH induced O to N acyl shift of peptide 3.**

### 3.1.4 Monitoring conformational transition of peptide 3 by circular dichroism (CD) studies

We designed the model aggregating peptide **3** such that it aggregates in specific condition and form amyloid like fibrils. Therefore, it was essential to check the β sheet forming potential, before using it for mixing experiment.

Lyophilized peptide **3** was dissolved in sodium acetate buffer (50 mM, pH 4.0) and PBS (0.1 M, pH 7.0) independently to reach a final peptide concentration of 0.5 mM and incubated at 37 °C on water bath. Conformational transitions were studied using two different biophysical techniques, i.e. CD and FT-IR.

For CD analysis, peptide from the stock solution was diluted from respective buffer to obtain a final cuvette concentration of 150 μM. From the CD experiment, we noticed a maximum centred at 190-195 nm and a minimum at 210-215 nm for peptide 3 taken from PBS which probably indicates β sheet. On the other hand, peptide **3** from sodium acetate buffer produced a spectrum with a minimum at 190-195 nm which is usually assigned to random coil conformation.

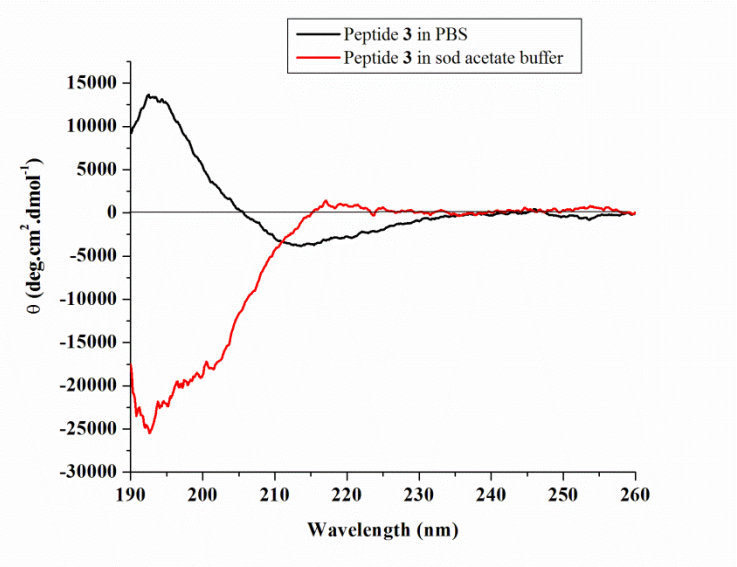



**Figure 5: CD spectra of peptide 3 in sodium acetate buffer (red line) and PBS (black line).**

From the CD experiment it can be inferred that the peptide **3** at pH 4.0 stays in random coil conformation due to shortage of critical chain length for β sheet formation ($n_c$ β). However, when pH was maintained in basic range O to N acyl migration takes place and peptide attains native peptidic linkage with required number of residues for β sheet formation.

### 3.1.5 Monitoring conformational conversion of peptide 3 by Fourier Transformation Infra-Red spectroscopy (FT-IR) studies

Conformations of the peptide **3** in different conditions were also studied by FT-IR spectroscopy. An aliquot from the stock solution (mentioned in section 3.1.4) was taken and mixed with KBr, pellet was prepared and checked under FT-IR.

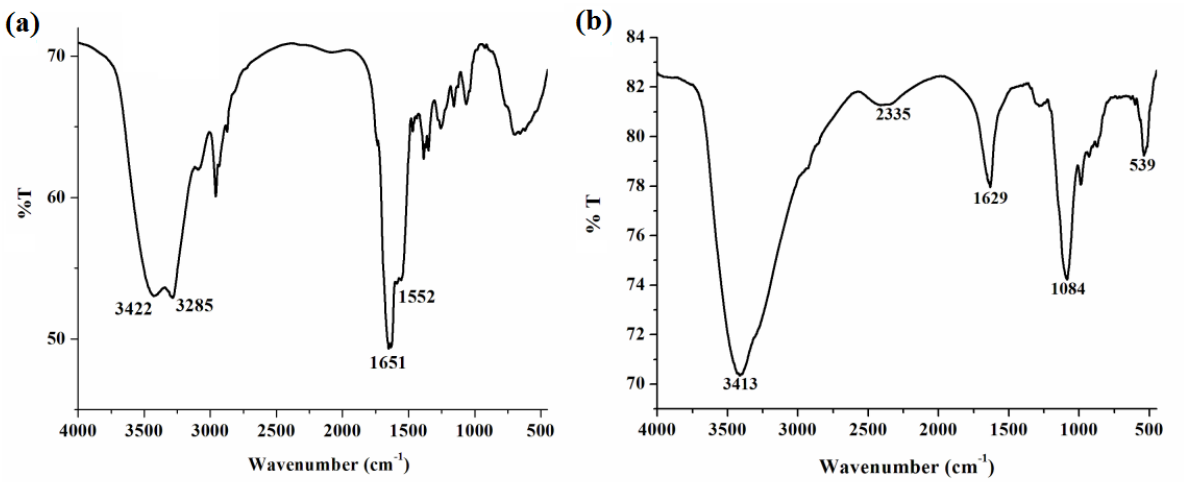



**Figure 6: FT-IR of the peptide 3 in (a) sodium acetate buffer and (b) PBS**

A band at 1651 $cm^{-1}$ was observed from the peptide sample incubated in sodium acetate buffer solution (pH 4.0, figure 6a), which was assigned to amide I band for random coil conformation of peptide **3**. In parallel, we examined the peptide sample from PBS (pH 7.0) also and observed a band at 1629 $cm^{-1}$ which was assigned to the β sheet conformation of peptide **3** (figure 6b). This indicates that peptide **3** forms β sheet in basic pH and does not form β-sheet in acidic pH.

### 3.1.6 Monitoring fibril formation of peptide 3 using thioflavin T fluorescence assay.

Thioflavin T analysis provides the quantitative information on fibril formation. Once conformational changes of the peptide **3** were studied and confirmed using CD and FT-IR, we wanted to investigate the rate of fibril formation using thioflavin T fluorescence assay.

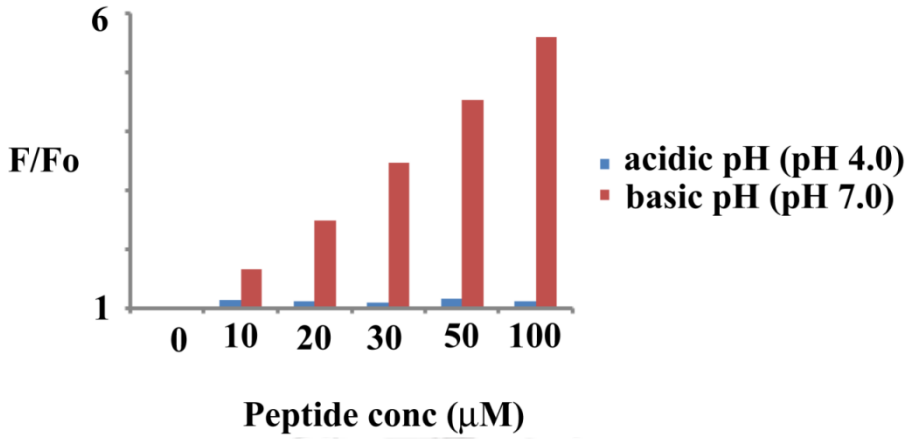



**Figure 7: Concentration dependent fluorescence study of model aggregating peptide 3.**

We noticed a considerable increment for the peptide sample taken from the stock solution in PBS (mentioned in section 3.1.4) and no change was noticed for the peptide sample which was incubated in sodium acetate buffer (figure 7). This confirms that model aggregating peptide aggregates to form fibrils in basic pH, whereas, it does not form such fibrils in acidic pH.

### 3.1.7 Monitoring fibril formation of peptide 3 using TEM

Presence of fibrils formed from model aggregating peptide **3** was analyzed using transmission electron microscopy. Stock solutions of peptide **3**, prepared as described in section 3.1.4, was used for electron microscopy analysis after incubation of three days.

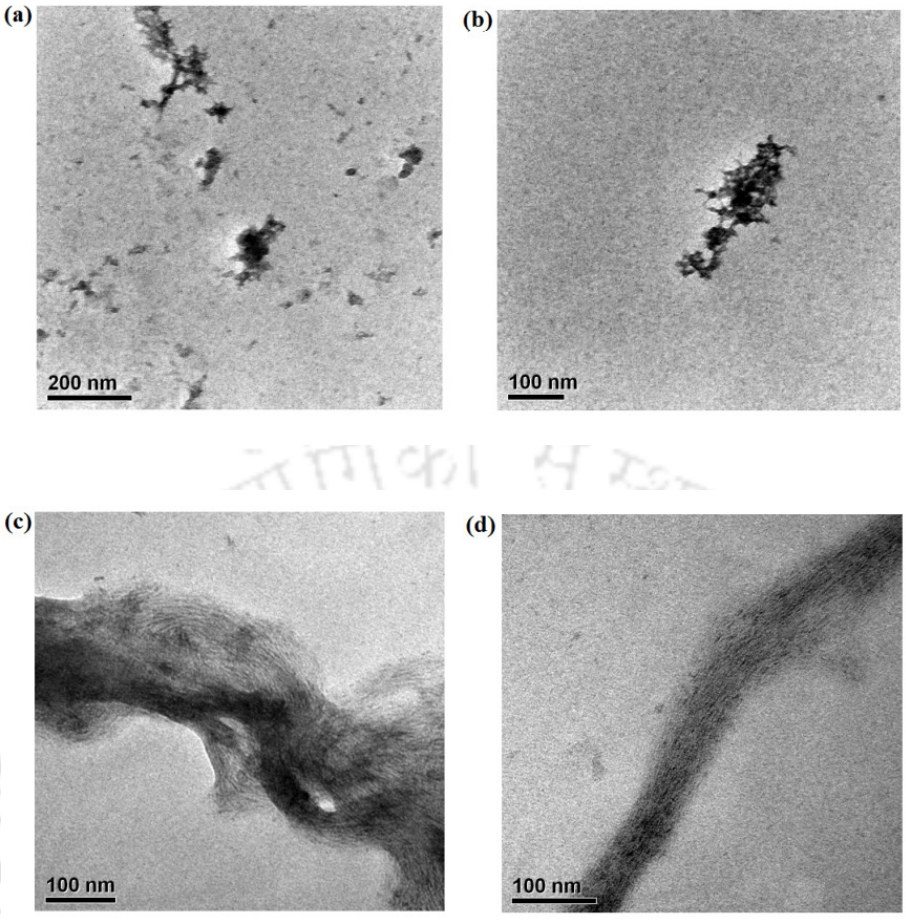



**Figure 8: TEM images of the peptide sample taken from (a and b) sodium acetate buffer, (c and d) PBS after three days of incubation.**

We could not find any ordered aggregates for the peptide sample incubated in sodium acetate buffer (figure 8a and 8b) instead, some disordered structures were noticed. On the other hand, peptide **3**, incubated in PBS formed fibrillar structures of nearly 10 nm in thickness (figure 7c and 7d). This result indicates peptide **3** does not form fibrils in acidic environment due to non availability of peptide backbone. Once peptide attained native backbone through O to N acyl shift, peptide **3** formed fibrils.

### 3.1.8 Monitoring amyloid formation of peptide 3 using Congo red birefringence assay

We also studied amyloid formation by model aggregating peptide **3** under optical polarisable microscope after staining with Cong red. 20 μL from the stock solution (mentioned in section 3.1.4) was taken over the glass slide in a similar way as it was mentioned in section 2.1.9 and viewed under polarizable microscope.

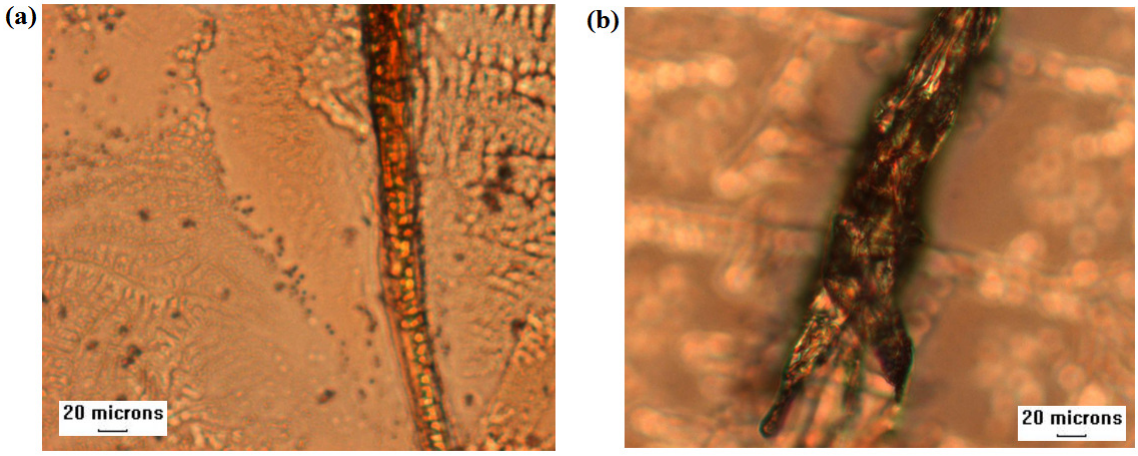



**Figure 9: Optical polarizable microscope images of the peptide sample incubated in (a) sodium acetate buffer (b) PBS.**

No green gold birefringence was observed from sodium acetate buffer (pH 4.0) solution of the model aggregating peptide **3** after seven days of incubation time as displayed in figure 9a. On the other hand, the peptide **3** exhibited green gold birefringence after incubation in PBS (pH 7.0) for same time. This indicates model aggregating peptide self assembled to produce amyloid like fibrils in basic pH.

### 3.1.9 Conclusion

We could finally design, synthesize and characterize a model aggregating sequence, which is a seven residue switch peptide. This peptide has sequence homology with the BBDP containing β-sheet breaker peptide **1**. At the same time we can control the rate of aggregation of such a peptide by controlling the pH of the system. This aggregates in a similar fashion to that of the Aβ peptide in vitro which was demonstrated using various biophysical tools. We want to use this peptide as an Aβ mimic peptide to investigate the β-breaking potential of our BBDP containing β-sheet breaker peptide **1**.

## 3.2. To investigate the β-breaking efficiency of peptide 1 against the aggregation of peptide 3

Our results confirm that model aggregating peptide **3** formed amyloid like fibrils at neutral to basic pH and that can be used as a miniature model of Aβ peptide in vitro. On the other hand, BBDP containing peptide **1** breaks self aggregation under same experimental condition. Therefore, the next question came to our mind was, can peptide **1** inhibit aggregation of peptide **3** when mixed in the same experimental condition (figure 10).?

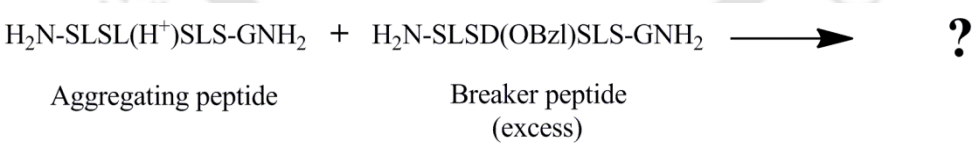



**Figure 10: Mixing experiment to investigate the β-breaking efficiency of peptide 1 against peptide 3**

To answer this question, peptide **1** was taken in three equivalents to suppress the aggregation model aggregating peptide **3** as necessary and checked at first with circular dichroism.

### 3.2.1 Monitoring conformational transition by circular dichroism (CD) studies

3.0 mg of peptide **1** (three equivalents) was taken in an eppendorf tube containing 1 mg of peptide **3** and dissolved in 2.6 ml of (PBS 50 mM, pH 7.0) to obtain a peptide solution of 0.5 mM concentration. To initiate fibrillization, samples were kept for incubation on water bath at 37 °C for three days. From the stock solution (described in section 3.2), 50 µL of the sample was taken and diluted with 450 µL of (PBS 50 mM, pH 7.0) to obtain a peptide solution of 50 µM which was taken in a 300 µL cuvette of 1 mm path length and sample was analyzed using Jasco J-815 instrument.

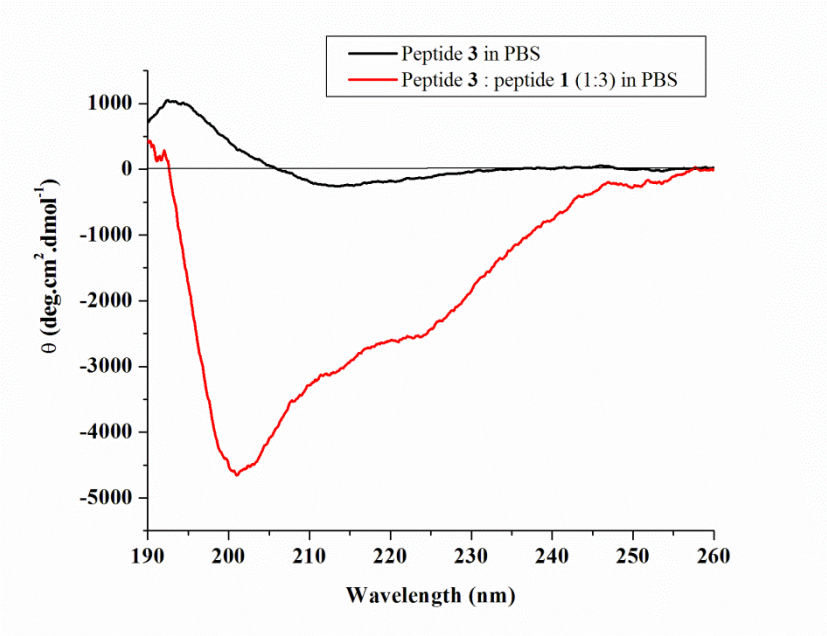



**Figure 11: CD spectra of peptide 3 alone (black line) and in presence of 3 equivalents of peptide 1 (red line).**

CD spectrum of peptide **3** in presence of three equivalents of peptide **1** produced a minimum centred at 200-205 nm as shown in figure 11. This confirms the transformation of peptide **3** from β sheet to random coil conformation in agreement to our concept. Therefore, peptide **1** helps to disrupt the fibril formation by peptide **3** when mixed in three equivalents.

### 3.2.2 Monitoring conformational transition by FT-IR studies

20 µL of the stock solution was taken, mixed with KBr and pellet was prepared. 32 scans were taken followed by background subtraction to obtain final spectra.

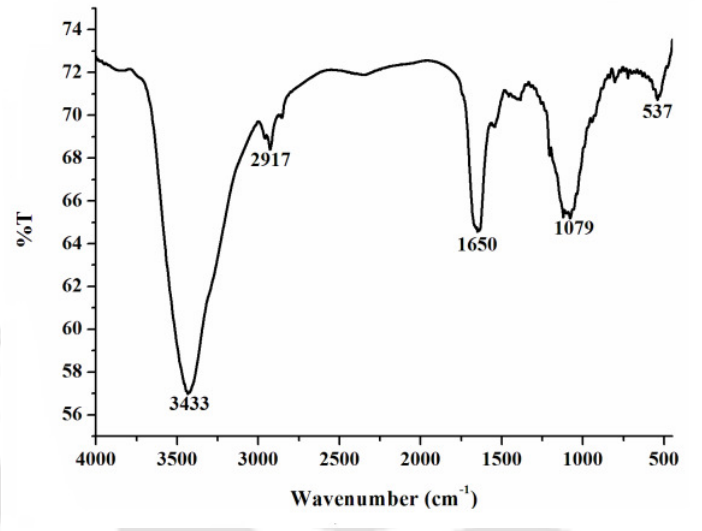



**Figure 12: FT-IR spectra of peptide 3 in the presence of three equivalents of peptide 1.**

From FT-IR spectroscopic studies we observed a broad band at 1650 $cm^{-1}$ for the aggregating peptide **3** when mixed with three equivalents of peptide **1** (figure 12). Previously, for model aggregating peptide a band at 1629 $cm^{-1}$ was noticed for β sheet conformation (figure 6b). This indicates BBDP containing peptide (peptide **1**) could stop aggregation of model aggregating peptide when mixed in excess.

### 3.2.3 Monitoring fibril formation using thioflavin T fluorescence assay

Kinetics of fibril formation was studied using thioflavin T fluorescence experiment. Unlike other experiments, thioflavin T fluorescence was carried out in PBS (50 mM) of pH 7.4. Triplicate of 0.8 mg of model aggregating peptide was taken in eppendorf tube and dissolved in 2 ml of PBS to obtain a final peptide concentration of 0.52 mM. For initiating fibril

formation samples were incubated at 37 °C on water bath. In parallel 3 fold molar excess of peptide **1** (2.6 mg) was dissolved in similar eppendorf tubes (another triplicate set) containing aggregating peptide **3** and kept for incubation. Kinetics of fibril formation in the presence and absence of breaker peptide was analyzed independently using thioflavin T assay.

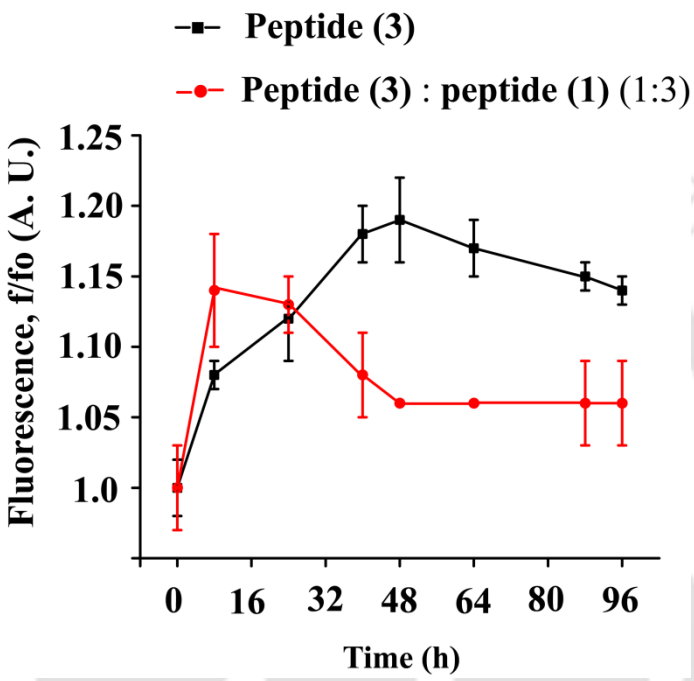



**Figure 13: Time dependent thioflavin T assay of peptide 3 (black line) and peptide 3 mixed with three equivalents of peptide 1 (red line).**

From the thioflavin T fluorescence study displayed above in figure 13, we have noticed that the model aggregating peptide **3** alone aggregates with time in a linear fashion upto 48 h. On the other hand, when three equivalent of breaker peptide **1** was mixed with the aggregating peptide **3**, we noticed increment of fibril formation up to 6 h which then slowly decreased. That increment of the fibril at the first phase can be accounted for co-aggregation of the breaker peptide 1 and the aggregating peptide **3**, probably due to proper recognition. The decrement of the fibril at the later stage is probably due to the aspartimide formation and subsequent hydrolysis of the aspartimide to form aspartyl residues. When such chemical

conversion occurs the breaker peptide **1** is forced to change its conformation from β-sheet to random coil which forces β-sheet and fibril disruption of peptide **1**. As the breaker peptide **1** changes its β-sheet conformation, which probably was integrated inside the β-sheet formed by peptide **3** already due to co-aggregation, disrupts the β-sheet and fibrillar architecture of peptide **3** as well.

### 3.2.4 Monitoring fibril formation using transmission electron microscopy (TEM)

First direct evidence of amyloid disruption was observed from electron microscopic studies. An aliquot (10 μL) of the seven days old peptide sample was added over TEM grid and over it 10 μL of 2% uranyl acetate was added allowed to stay for a minute. Excess solution was removed by blotting paper and visualized under electron microscope at 80 kV.

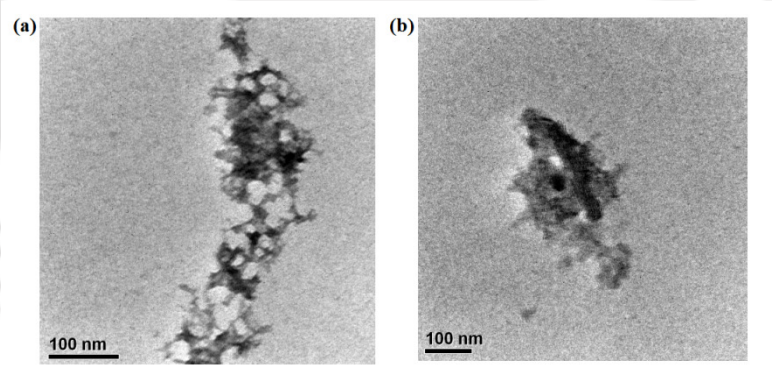



**Figure 14: TEM images of the peptide sample when mixed with three equivalents of breaker peptide 1.**

Peptide **3** when incubated in the presence of peptide **1**, no ordered fibrils were noticed instead some disordered clusters were seen as displayed in figure 14. This explicitly revealed that BBDP containing peptide **1** is capable of disruption of aggregates formed by any other model

aggregating peptide when mixed. As a positive control, the representative picture of fibril when peptide **3** alone was incubated for three days can be recalled (figure 8c and 8d).

### 3.2.5 Monitoring amyloid formation using Congo red birefringence assay

Congo red birefringence assay provided information on amyloid disruption of model aggregating peptide (peptide **3**) in presence of BBDP containing peptide **1**.

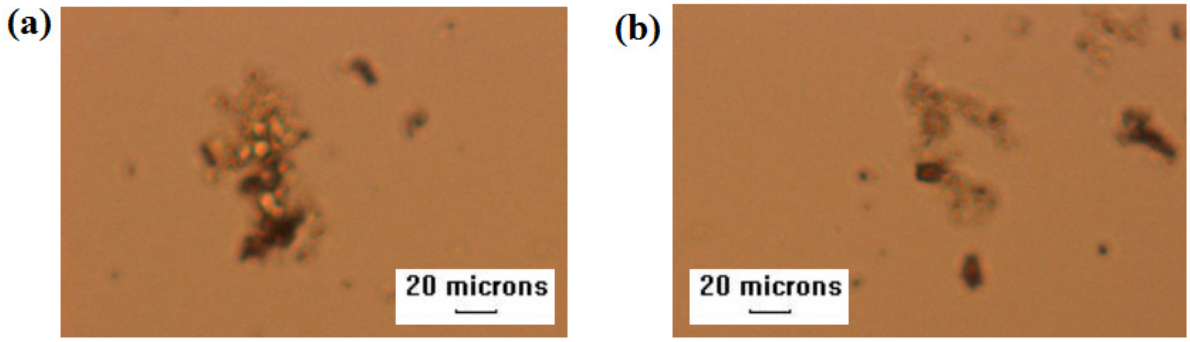



**Figure 15: polarizable microscope images (a) and (b) peptide mixed with three equivalents of breaker peptide.**

Above pictures (figure 15 a and b) revealed that gold birefringence was noticed at few places only over the slide. This indicates that peptide **1** disrupted amyloid formation by peptide **3** quite substantially when co-incubated in three equivalents. For a comparison please see the figure 9b, which was obtained with peptide **3** alone.

### 3.2.6 Conclusion

The results of the section 3.1 established that the peptide **3** formed amyloid like fibrils, which we could use as a model aggregating peptide. Next, we investigated the β-breaking potential of our BBDP containing β-breaker peptide **1** against the peptide **3**. Based on the outcome of thioflavin T fluorescence it was noticed that peptide **1** could disrupt the aggregation of peptide **3** considerably. Nevertheless, electron microscopy studies showed complete disappearance of fibrils. It can be concluded that BBDP containing peptide can be used to disrupt the aggregation of any other aggregating peptide with homologous sequence.

# Chapter 4: Inhibition of aggregation of Aβ derived model peptide by BBDP containing homologous breaker peptide

In the previous chapters, we discussed that BBDP containing -(Ser-Leu)$_n$- based model peptide **1** inhibits self aggregation and aggregation of model homologous aggregating peptide **3** as well to a considerable extent when mixed in three equivalents. Having obtained the proof of the concept from model sequences our target was to check the hypothesis of β sheet disruption on full length Aβ peptide. However, synthesis and handling full length Aβ peptide is difficult. It requires special synthetic and purification skills. Therefore, we thought if we have a system that is readily accessible and behave similar to the full length Aβ peptide it would be easy to test the concept further. Nevertheless, the peptide fragment Aβ$_{14-23}$ with -(Leu-Ser)$_n$- residues flanked on both sides was used as a substitute of Aβ peptide. Such peptides were named as 'host guest switch peptide' and fibrillization of host guest switch peptide was reported to be similar to the full length Aβ peptide.[64] Therefore, we have decided

to prepare an aggregating peptide and homologous BBDP containing breaker peptide similar to the 'host guest switch peptide' to test our concept on such peptides.

### 4.1.1 Design of Aβ derived model aggregating peptide

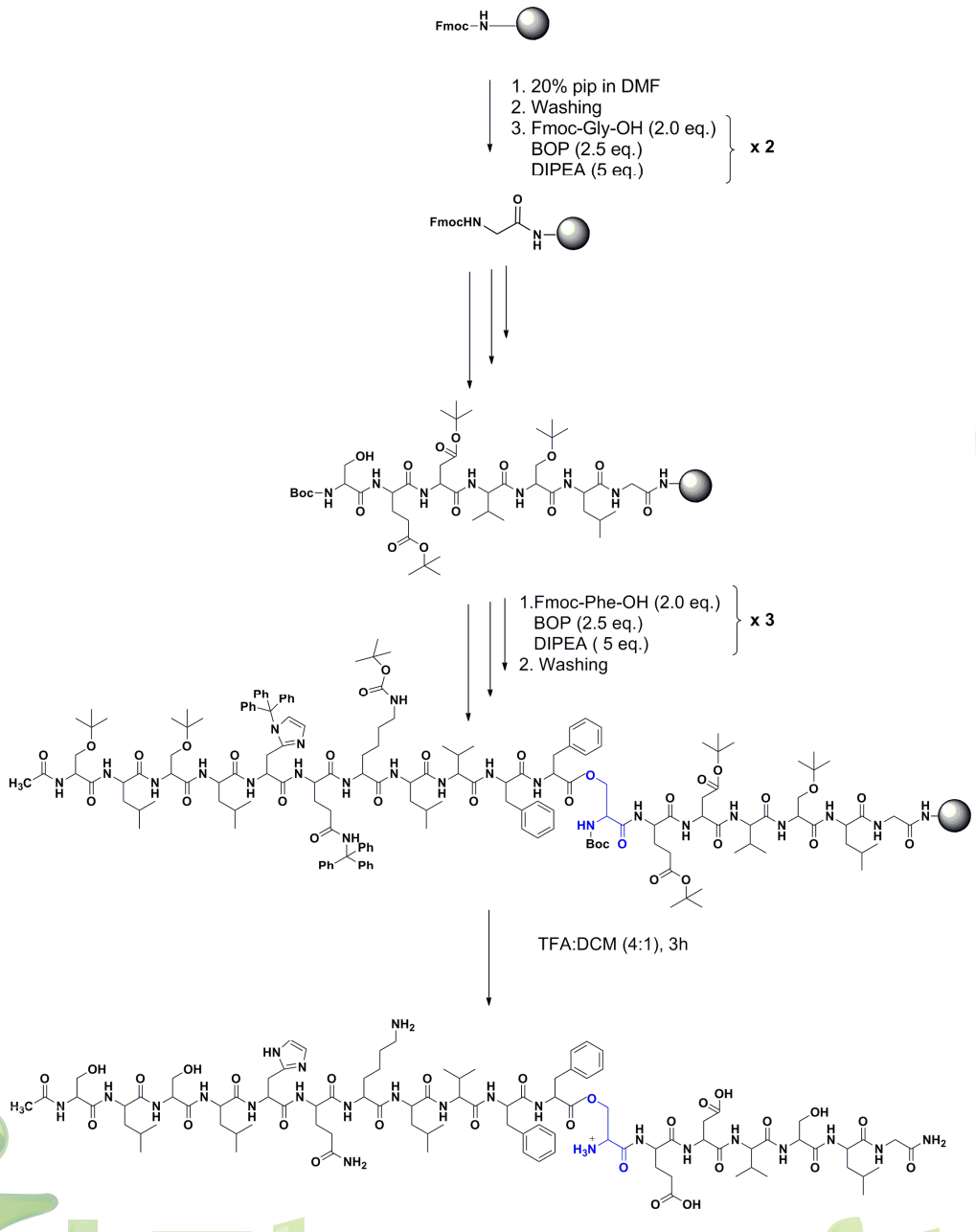
Mutter *et al*. reported a 'host guest switch peptide' with a sequence in which $A\beta_{14-23}$ fragment was embedded in between -$(Leu\text{-}Ser)_n$- residues which can be used as an alternative to the Aβ peptide. $A\beta_{14-23}$ fragment itself does not form fibrils on its own.-$(Leu\text{-}Ser)_n$- residues probably help to promote the aggregation and solubility of the peptide sequence, similar to model aggregating peptide. More importantly, one serine switch element was incorporated which controls the self assembly and that can be regulated by pH of the system. Glycine was used as a spacer at C-terminus as its helps in attachment to the resin. N-terminus was acetylated to increase the aggregation potential. We decided to use this peptide as aggregating Aβ derived model peptide. The efficiency of our relevant β-breaker peptide wished to be tested on the aggregation of this peptide. The sequence of the peptide is shown below.

**Aβ derived model aggregating peptide: Ac-$^{1}$SLSLHQKLVFF(H$^{+}$)SEDVS$^{17}$ L-$GNH_2$ (peptide 4)**

### 4.1.2 Synthesis and characterization of peptide 4

Aβ derived aggregating peptide **4** was synthesized according to Fmoc/tBu orthogonal protection strategy as described in experimental section.[54, 55, 56, 57] $N_{\alpha}$-Boc serine was used at switch position (scheme 1). Peptide was cleaved from the resin using TFA/DCM (4:1) for

three hours. Based on 0.33 mmol scale we obtained 28 mg (4 %) of the pure peptide in the form of white fluffy powder.




**Scheme 1: SPPS synthetic scheme of peptide 4 using Fmoc/tBu protection strategy.**

Purity of the peptide **4** was confirmed using LC-MS. Linear gradient of 60% $CH_3CN$ for 6 min in a total run time of 8 minutes was used. A representative picture of LC profile is shown in figure 1a. $[M+H]^+$ and $[M+2H]^{2+}$ was observed in the ESI MS (+ve mode) as shown in figure 1b.

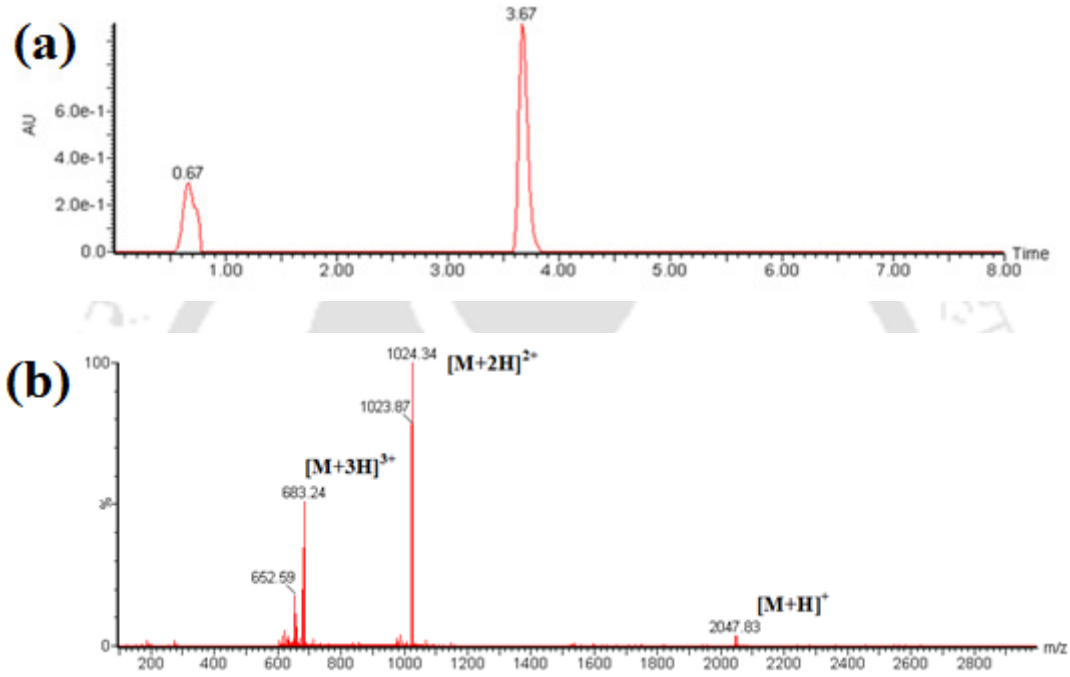



**Figure 1: (a) LC picture of the peptide 4. (b) MS data of the peptide.**

## 4.1.3 Monitoring O to N acyl migration of peptide 4

0.5 mg of the lyophilized sample was dissolved in 1 ml of PBS (50 mM, pH 7.4) to obtain a final peptide concentration of 240 µM. Peptide sample was incubated on 37 °C water bath. Sample was sonicated and vortexed frequently to prevent the fibril deposition. At different time intervals with 10 min duration gap sample from the stock solution was taken, quenched by adding 0.2N HCl and injected in LC-MS.

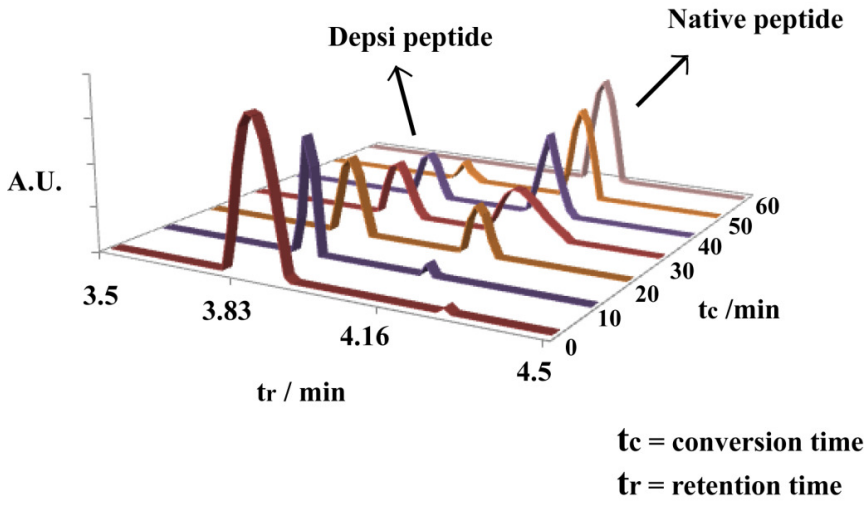
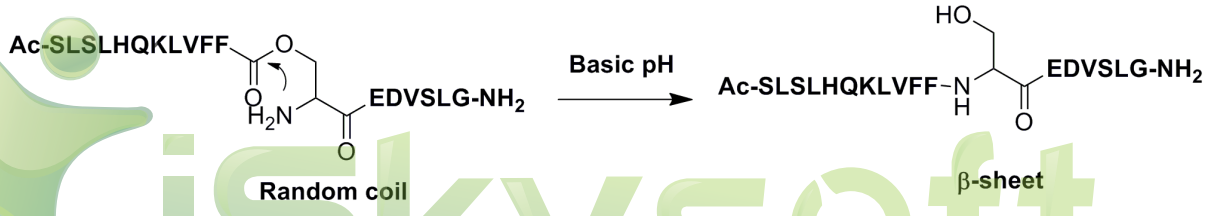



**Figure 2: pH induced kinetics of acyl migration.**

At zero time we noticed one major peak $t_r$ 3.85 min corresponding to the peptide in the depsi form. Slowly, a new peak emerged at $t_r$ 4.18 min with similar mass value (figure 2). This study was continued till complete disappearance of the first peak (nearly 60 minutes) occurred.

From this study it could be inferred that at biological pH, O to N acyl migration for Aβ derived aggregating peptide **4** is slower as compared to -(Ser-Leu)$_n$- model aggregating peptide **3**. This may be attributed to the fact that presence of bulky phenyl alanine residue next to serine makes conversion slower.




**Scheme 2: O to N acyl migration of Aβ derived aggregation peptide at pH 7.4.**

Our next target was to investigate the β sheet forming potential of Aβ derived aggregating peptide **4** in a specific condition using various biophysical tools. We compared the aggregation behaviour in acidic and basic pH conditions in parallel. We expect that in acidic pH no acyl shift takes place as a result, peptide exists in random coil conformation and vice versa.

| Condition | Conformation |
|---|---|
| pH < 7.0 | Random coil |
| pH ≥ 7.0 | β-sheet |

**Table 1: Conformation of peptide 4 in different pH.**

### 4.1.4 Monitoring conformational conversion of peptide 4 by circular dichroism (CD) studies

1 mg of lyophilised sample was dissolved in 3 ml each of sodium acetate buffer (50mM, pH 4.0) and PBS (50 mM, pH 7.4) separately to obtain a final peptide concentration of 160 μM and incubated at 37 °C on water bath. Conformational changes and fibril formation were studied.

Circular dichroism was used to determine secondary structure in different pH. Sample from the stock solution was used directly for CD studies after three days of incubation. Peptide **4** showed a minimum centred at 205-210 nm indicates an unordered structure. On the other hand, in PBS showed a minimum at 225 nm and maximum at 195-200 nm which corresponded to β sheet signal (figure 3).

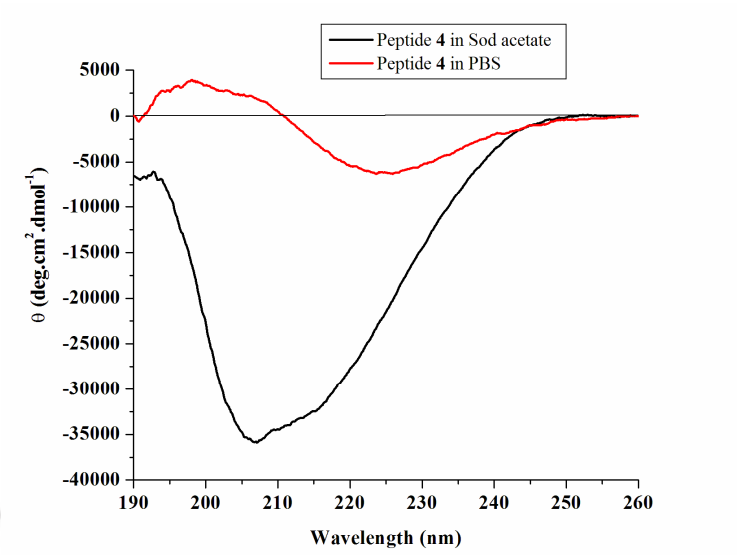



**Figure 3: Conformational changes in CD spectra of Peptide 4. Red line indicates peptide 4 in PBS and black line in sodium acetate buffer.**

| Peptide **4** in sodium acetate buffer (pH 4.0) | | Peptide **4** in PBS (pH 7.4) | |
|---|---|---|---|
| | Ratio | | Ratio |
| Helix | 0.0 | Helix | 8.6 |
| Beta | 49.7 | Beta | 91.4 |
| Turn | 0.0 | Turn | 0.0 |
| Random | 50.3 | Random | 0.0 |
| Total | 100.0 | Total | 100.0 |

**Table 2: Detailed information of the different secondary structures of peptide 4 in sodium acetate buffer and PBS**

This CD experiment confirms that at physiological pH, O to N acyl migration begins and peptide **4** produces fibrils. We observed an equal ratio of random coil to β sheet for peptide **4** from sodium acetate buffer and this may be due to high concentration of peptide which forced to aggregate even in acidic pH.

### 4.1.5 Monitoring conformational conversion of peptide 4 by FT-IR studies

FT-IR spectra were recorded to determine the internal conformation of the peptide. Aβ derived aggregating peptide **4** in it's depsi form revealed a band at 1640 $cm^{-1}$ corresponding to non β conformation of the peptide (figure 4a). On the other hand, peptide sample from PBS produced a peak at 1631 $cm^{-1}$ which is assigned to β sheet conformation (figure 4b).

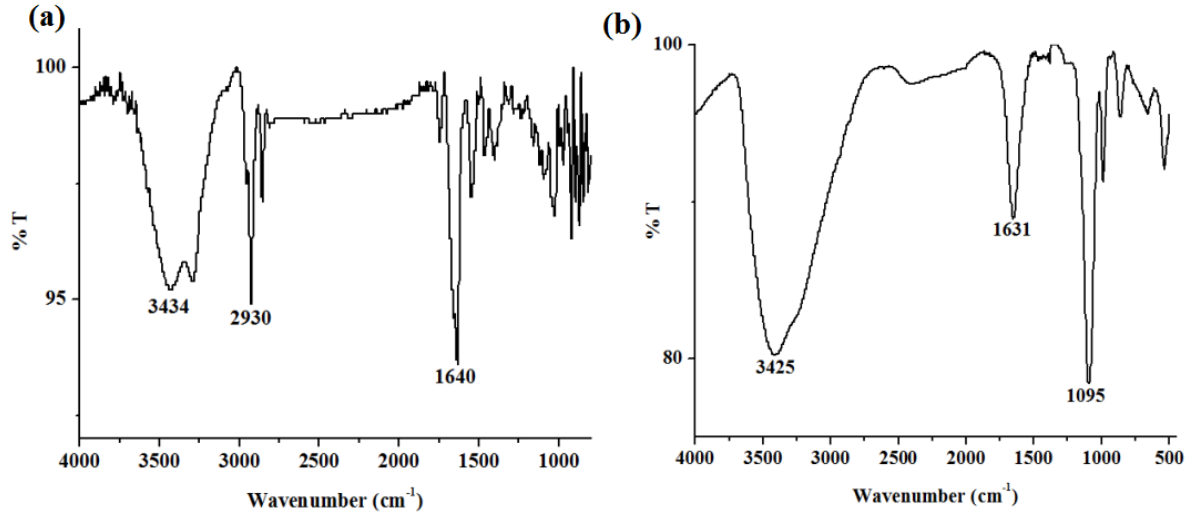



**Figure 4: FT-IR spectrum of peptide 4. (a) Peptide sample was incubated in sodium acetate buffer (pH 4.0). (b) Peptide sample from PBS, pH 7.4. Sample was analysed after three days.**

From the results of FT-IR experiments it could be inferred that once pH was triggered to basic range, due to O to N acyl shift peptide **4** attained native backbone which resulted in β sheet and fibril formation.

### 4.1.6 Monitoring fibril formation of peptide 4 using thioflavin T fluorescence assay

Having obtained the idea about the desired chemical and conformational conversion using different techniques our next target was to scrutinize the amount of fibril formed by thioflavin T fluorescence assay.[37]

For thioflavin T fluorescence studies a stock solution of 0.4 mM was prepared in sodium acetate buffer and PBS separately and incubated as required. Concentration dependent fluorescence was measured after three days of incubation. An aliquots were taken from the stock solution and mixed with 200 µL of thioflavin T (50 µM) and total volume was made up to 400 µL by addition of respective buffer solutions. Fluorescence was measured at emission wavelength of 485 nm.

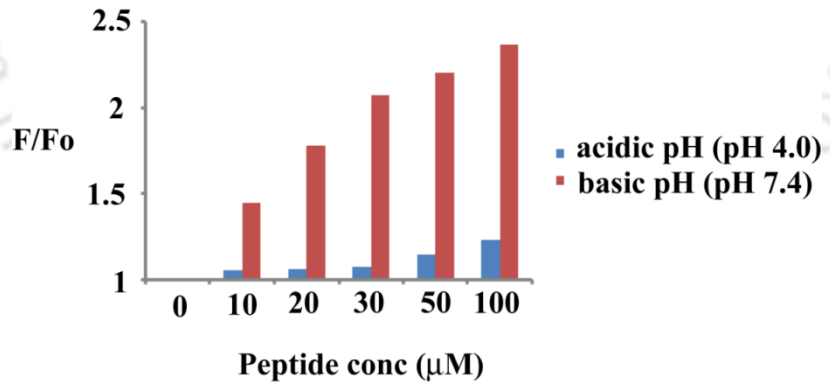



**Figure 5: Concentration dependent thioflavin T fluorescence after three days of incubation. Blue bar indicates sample from sodium acetate buffer and red bar indicates sample from PBS.**

We noticed negligible increment in the fluorescence when sample was taken from sodium acetate buffer (blue bar, figure 5). However, a considerable increment was noticed from the sample incubated in PBS (red bar, figure 5). Moreover, at higher concentration, change of the

fluorescence intensity was substantial. So this confirms that in basic pH, peptide **4** aggregates to form fibrils which bind to thioflavin T. And at acidic pH such fibril formation is not significant. That is probably because in basic condition O to N acyl migration takes place, the peptide regains its native backbone, it forms β-sheet and aggregates to form fibril.

### 4.1.7 Monitoring fibril formation of peptide 4 using TEM

To evaluate the morphology of Aβ derived aggregating peptide **4**, electron microscopy was used. Seven days old stock solution was used to prepare the sample on carbon coated copper grid. Parallel examination of samples from acidic and basic solutions was carried out.

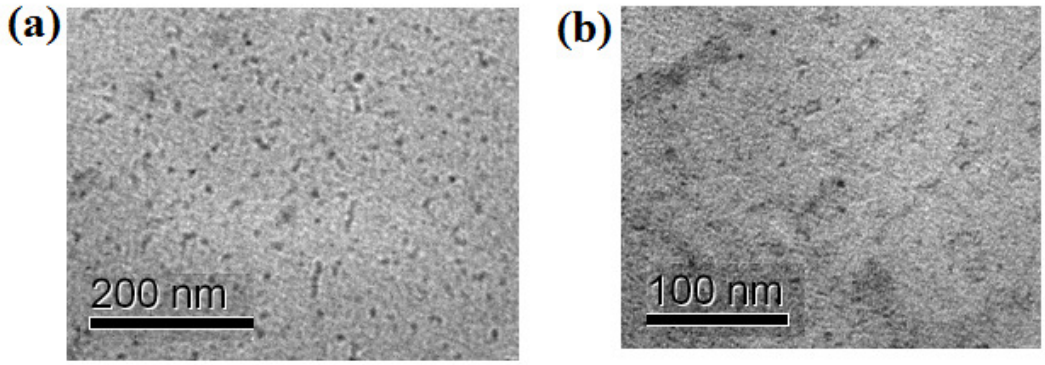



**Figure 6: TEM images of peptide 4 in sodium acetate buffer.**

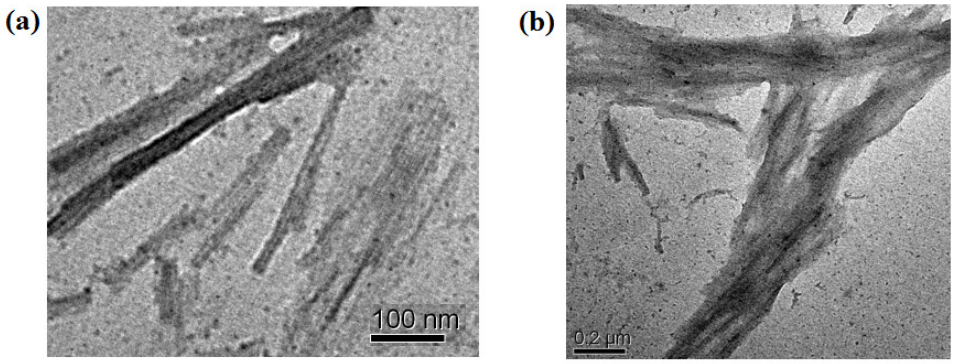



**Figure 7: TEM images of the peptide 4 from PBS set.**

No aggregates were noticed from the sample which was incubated in sodium acetate buffer, pH 4.0 (figure 6a-b). On the other hand, we noticed long needle like fibrils of nearly 10 nm in diameter for the peptide **4** which was incubated in PBS. At some places enormous aggregates were also noted (figure 7a-b). This TEM examination confirms that peptide **4** form fibrils in basic pH and can be used as a substitute of Aβ peptide.

### 4.1.8 Monitoring amyloid formation of peptide 4 using Congo-red birefringence assay

Upon staining with Congo red, amyloid exhibits green gold birefringence when viewed under optical polarisable microscope. No such green gold birefringence was observed when peptide sample from sodium acetate buffer was analysed (figure 8a). However, gold birefringence was observed from the sample which was taken from the PBS incubated batch as exhibited in figure 8b. Parallel examination was done after 10 days.

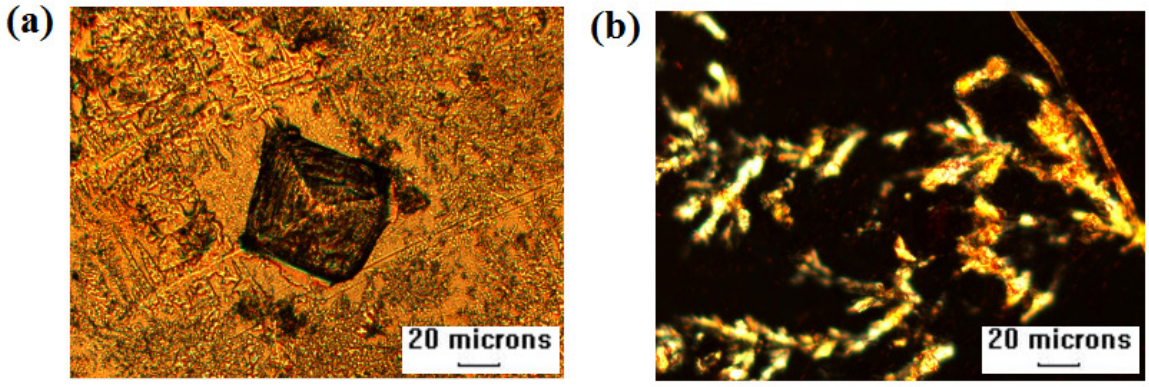



**Figure 8: TEM images of the peptide 4 (a) from the sodium acetate buffer (b) from PBS set after ten days.**

From the above experimental findings, it was clear that Aβ derived aggregating peptide **4** aggregates in a controlled manner and also form amyloid like fibrils which can be used as mimic of full length Aβ for testing our concept of β sheet disruption. Having a mimic in hand, our next target was to design and synthesize suitable breaker peptide.

### 4.2.1 Design of Aβ derived BBDP containing homologous β breaker peptide

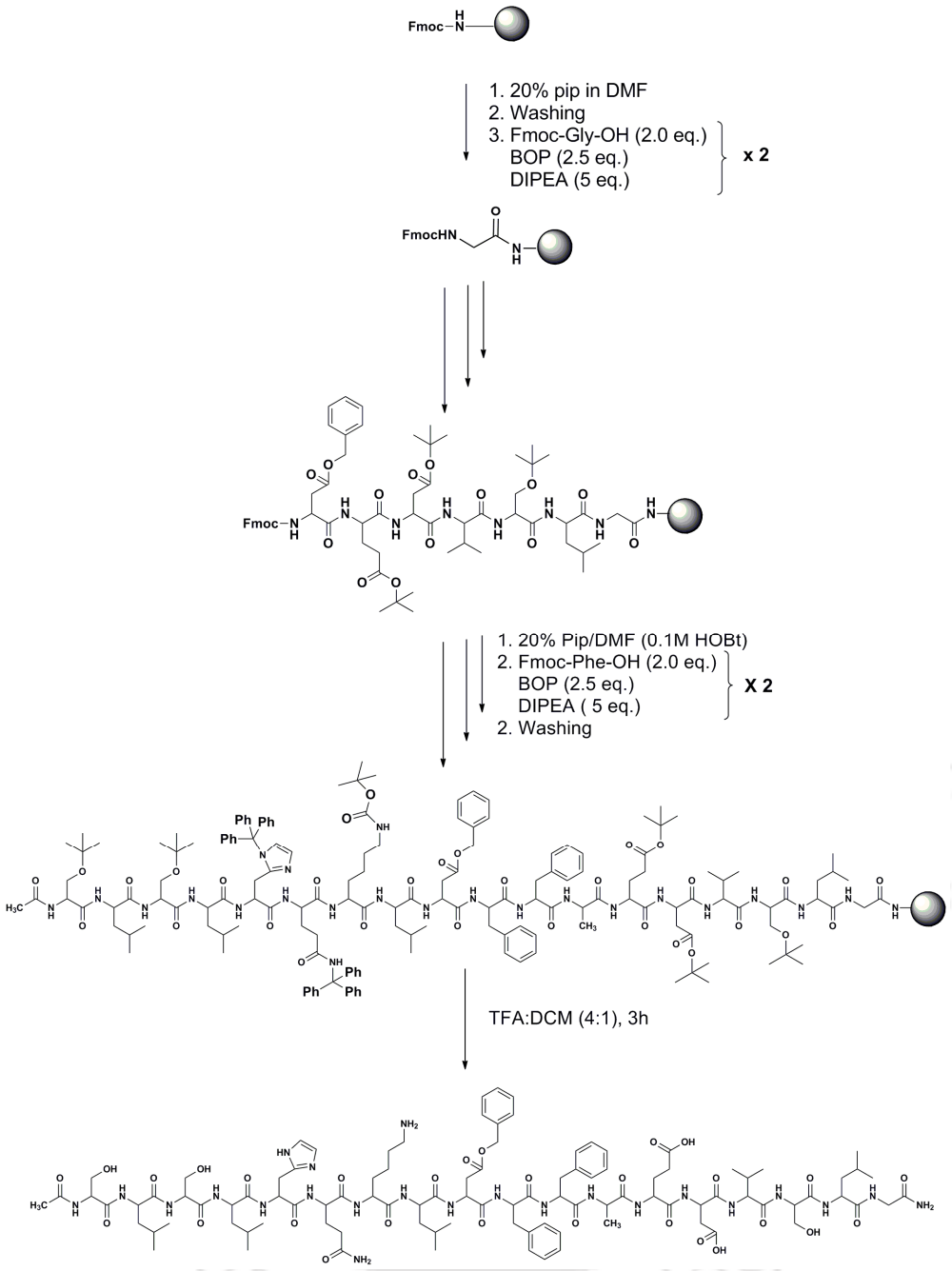
We wanted to design a β-breaker peptide which can first assemble with Aβ derived aggregating peptide and slowly, at physiological pH, undergoes a chemical modification i.e. aspartimide formation and subsequent ring opening it breaks its self assembly and aggregation of neighbouring peptide as well. Since Aβ derived aggregating peptide **4** contained $A\beta_{14\text{-}23}$ fragment embedded in between -$(Ser\text{-}Leu)_n$- residues, therefore, for keeping sequence homology for proper recognition we decided to keep similar sequence in the Aβ derived BBDP containing β breaker peptide also. $Val^{18}$ of the $A\beta_{14\text{-}23}$ fragment was replaced with Asp(OBzl) and the rest of the sequence was similar to peptide **4**. **Asp(OBzl)F**

was a BBDP unit in this case which is likely to generate aspartimide and aspartyl residues on ring opening. The sequence of the Aβ derived BBDP containing β breaker peptide is as shown below.

**Aβ derived model breaker peptide: Ac-$^{1}$SLSLHQKL<u>D(OBzl)F</u>FAEDVS$^{17}$L-GNH$_2$ (5)**

## 4.2.2 Synthesis and characterization of peptide 5

Peptide **5** was synthesized in a similar way to that of peptide **4** using Fmoc/tBu protection strategy. In this case however, we used 0.1 M of HOBt in piperidine/DMF mixture after incorporation of D(OBzl) in the sequence.[48] Based on 0.33 mmol scale we obtained 15 mg (2 %) of the pure peptide. Synthetic scheme is briefly depicted in scheme 3.




**Scheme 3: Synthetic scheme of peptide 5 using Fmoc/tBu protection strategy.**

Purity was confirmed from LC-MS. We observed a peak at $R_t$ 4.35 min which was our peak of interest (figure 9a). In ESI MS (+ve) mode we noticed $[M+H]^+$, $[M+2H]^{2+}$ and $[M+3H]^{3+}$ as displayed in figure 9b.

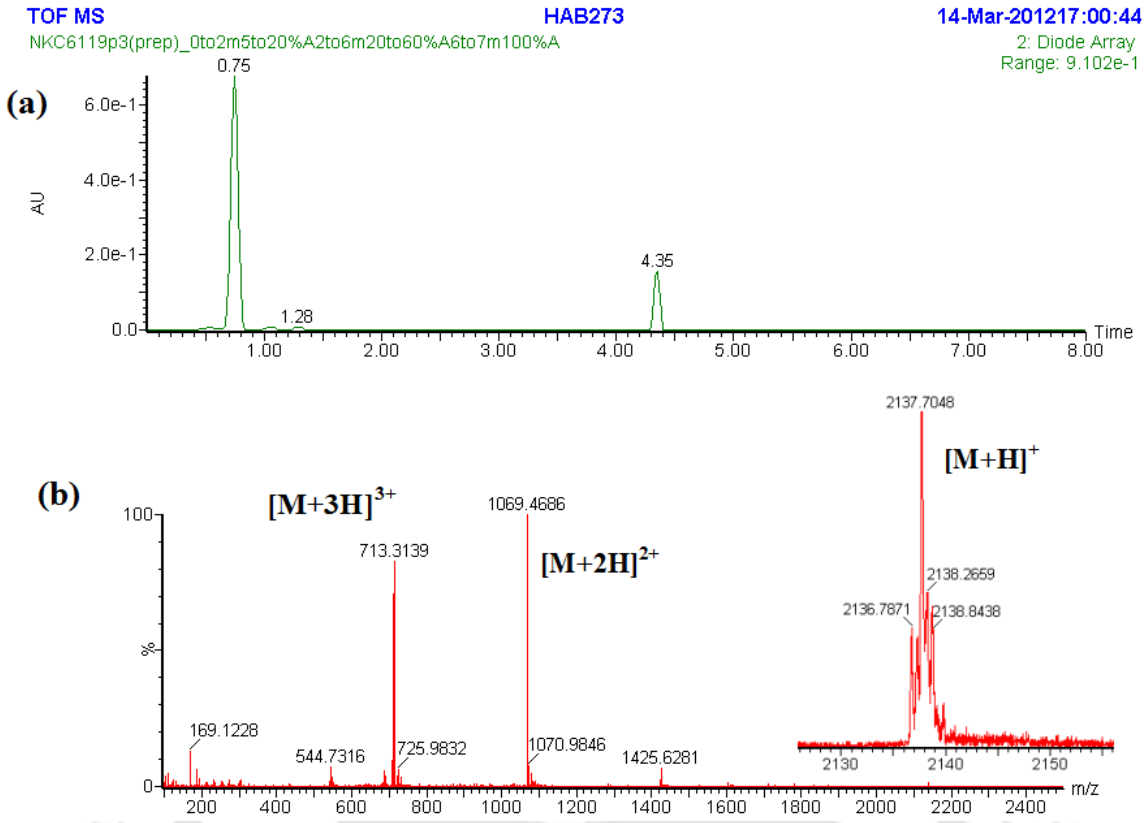
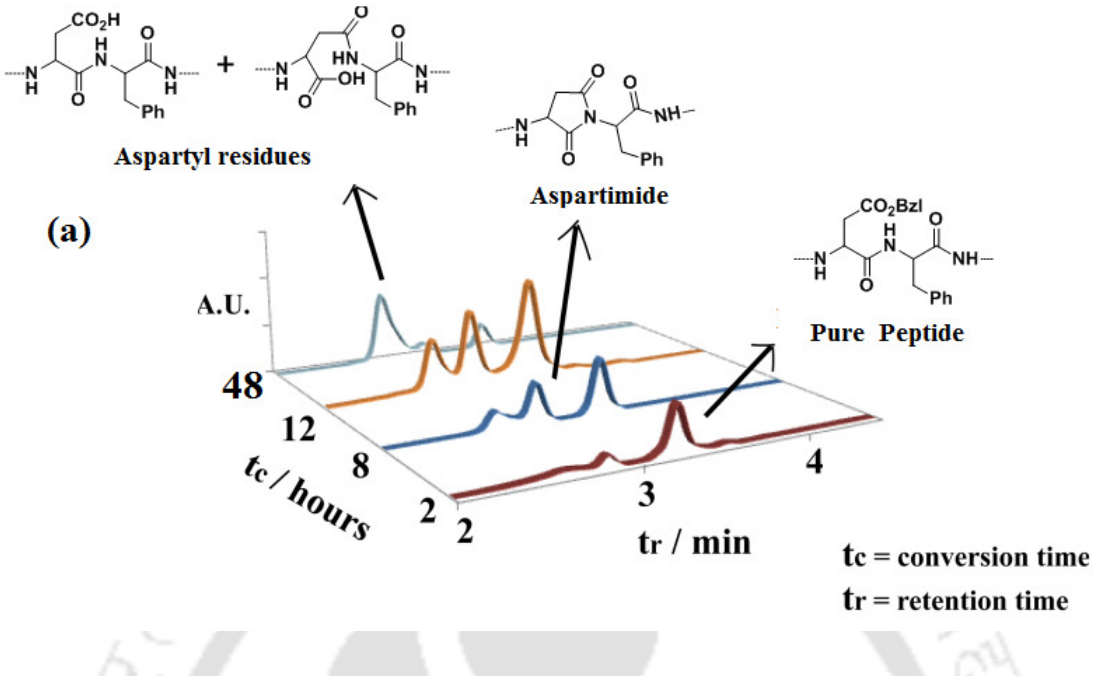



**Figure 9: LC-MS profile pictureof the peptide 5.**

## 4.2.3 Monitoring aspartimide formation of peptide 5 by LC-MS

Chemical conversion of BBDP unit into corresponding aspartimide and aspartyl residues was monitored using LC-MS. 0.5mg of lyophilized sample was dissolved in 1ml of PBS (50 mM, pH 7.4) to obtain a final concentration of 230 µM and incubated at 37 °C. At different time intervals stock solution was sonicated and vortexed to prevent fibril deposition.

We observed that in two hours peak for aspartimide derivative emerged. It continued for 12 h and at nearly 48 h we have seen complete conversion of aspartimide into aspartyl residues (figure 10a). Corresponding mass of all the intermediates of peptide **5** was observed and shown in figure 10b-d.



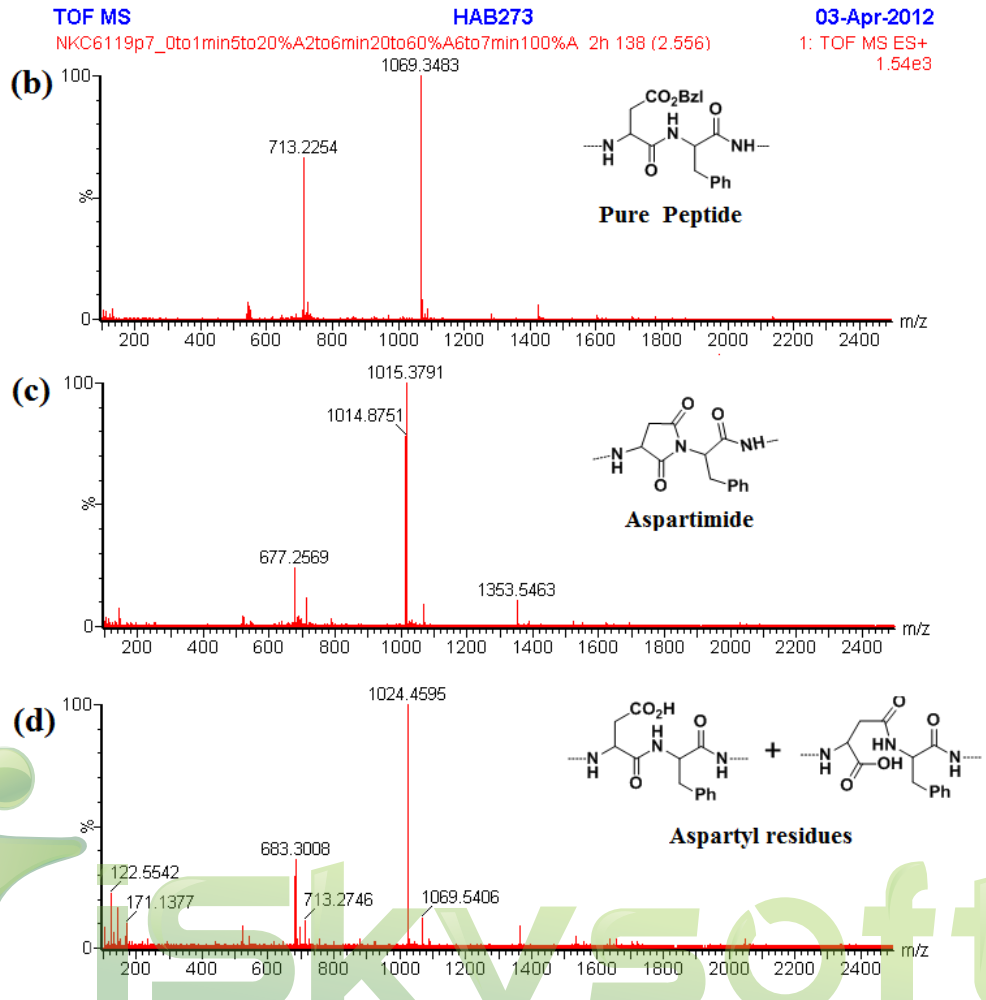




**Figure 10: Monitoring chemical conversion of peptide 5.**

After monitoring chemical conversion of BBDP unit of peptide **5** into aspartimide derivative and further into aspartyl residues using LC-MS, our next objective was to investigate the β sheet forming potential and inhibition of self assembly of peptide **5** at a specific pH.

Peptide **5** was designed in a way such that it first aggregates in acidic pH to form fibrils and once pH was adjusted to basic range it should break self assembly via the chemistry of aspartimide formation.

### 4.2.4 Monitoring conformational conversion of peptide 5 by circular dichroism (CD) studies

We prepared two sets of peptide samples, 1 mg of lyophilised sample was dissolved in 3 ml each of sodium acetate buffer (50mM, pH 4.0) and PBS (50 mM, pH 7.4) separately to obtain a final peptide concentration of 150 μM and incubated at 37 °C on water bath. Two parallel studies were conducted to examine the behavior of the peptide **5**. Conformational changes were studied using CD after three days of incubation.

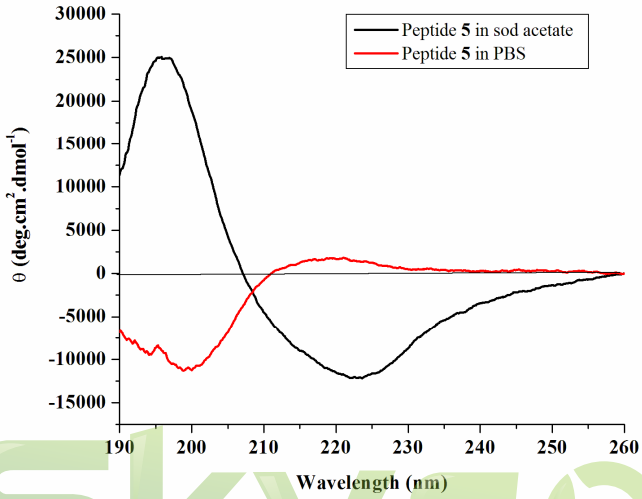



**Figure 11: CD spectra of peptide 5. Black line indicates peptide 5 in sodium acetate buffer and red line in PBS.**

| Peptide **5** in sodium acetate buffer (pH 4.0) | | Peptide **5** in PBS (pH 7.4) | |
|---|---|---|---|
| | Ratio | | Ratio |
| Helix | 8.3 | Helix | 0.9 |
| Beta | 91.7 | Beta | 0.0 |
| Turn | 0.0 | Turn | 18.1 |
| Random | 0.0 | Random | 81 |
| Total | 100.0 | Total | 100 |

**Table 3: Detailed information of the ratio of different secondary structures of peptide 5 in sodium acetate buffer and PBS**

CD spectrum of peptide **5** from sodium acetate buffer showed a characteristic minimum at 220-225 nm and maximum at 195-200 nm, a typical β sheet signal. Whereas, peptide **5** from PBS revealed a characteristic minimum at 195-200 nm indicates random coil structure (figure 11). From the experiment, it was noticed that the peptide **5** aggregates in sodium acetate buffer to form fibrils rich in β sheet rich architecture. Once pH was adjusted to basic (pH 7.4) we noticed a signal corresponding to random coil. This is due to chemistry of aspartimide formation that occurred in basic pH and peptide **5** stayed in random coil conformation after three days.

### 4.2.5 Monitoring conformational conversion of peptide 5 by FT-IR

FT-IR spectra were recorded after three days to understand the conformation of the peptide **5** in acidic and basic pH. We noticed a sharp band at 1627 $cm^{-1}$ for peptide **5** which is assigned to β sheet conformation (figure 12a). On the other hand, peptide sample when incubated in

PBS new peak appeared at 1651 $cm^{-1}$ (figure 12b). This indicates an inhibition of β sheet formation by peptide **5** in basic pH after three days.

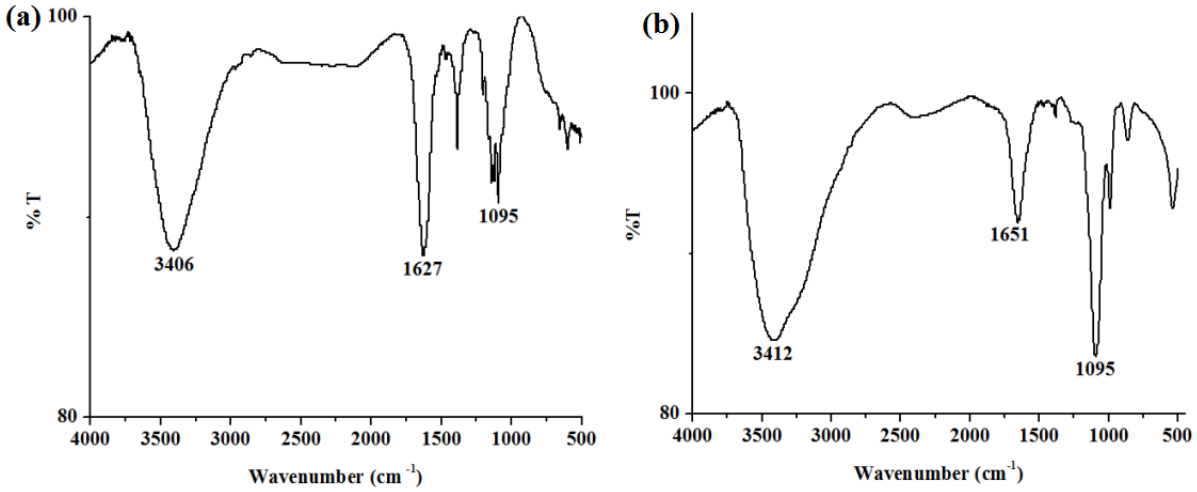



**Figure 12: FT-IR spectra of peptide 5 (a) sample incubated in sodium acetate buffer (b) sample incubated in PBS.**

## 4.2.6 Monitoring fibril formation of peptide 5 using thioflavin T fluorescence assay

Kinetics of fibril formation was monitored using thioflavin T assay. 0.5 mg of lyophilised sample was dissolved in 1.5 ml PBS (50 mM, pH 7.4) to obtain a final concentration of 150 μM. Peptide sample was incubated at 37 °C on water bath.

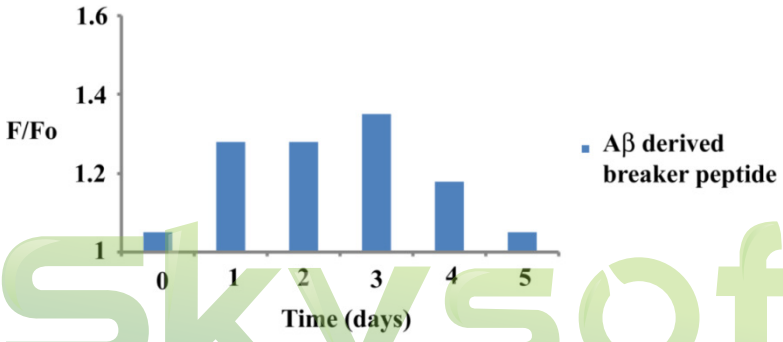



**Figure 13: Time dependent thioflavin T fluorescence of peptide 5.**

At different time interval, 20 µL of the sample was mixed with 200 µL thioflavin T (50 µM) and total volume was made upto 400 µL by addition of buffer and fluorescence was measured at $\lambda_{ex}$ = 435 nm and $\lambda_{em}$ = 485nm.

We observed a slow increase in the fluorescence at initial stages which was continued for three days as showed in figure 13. After three days we noticed a sudden decrement in the intensity. This may be due to the fact that after three days fibril formation was inhibited due to the chemistry of aspartimide formation and subsequent ring opening.

### 4.2.7 Monitoring fibril formation of peptide 5 by TEM

From thioflavin T fluorescence study we noticed that fibril formation of peptide **5** was inhibited after three days. We examined the fibril formation of the peptide **5** using TEM which also provides a direct confirmation. 10 µL of the peptide sample from seven days old stock solution was taken and spotted over the grid. Later, 10 µL of the 2 % uranyl acetate was added over the grid and allowed to stay for a min. Excess solution was removed by blotting paper.

We observed clear long fibrils of nearly 10 to 15 nm in diameter for peptide **5** when incubated in sodium acetate buffer as shown in figure 14 a-b. On the contrary, we noticed no such fibrillar aggregates on incubation in PBS for seven days (figure 14 c-d).

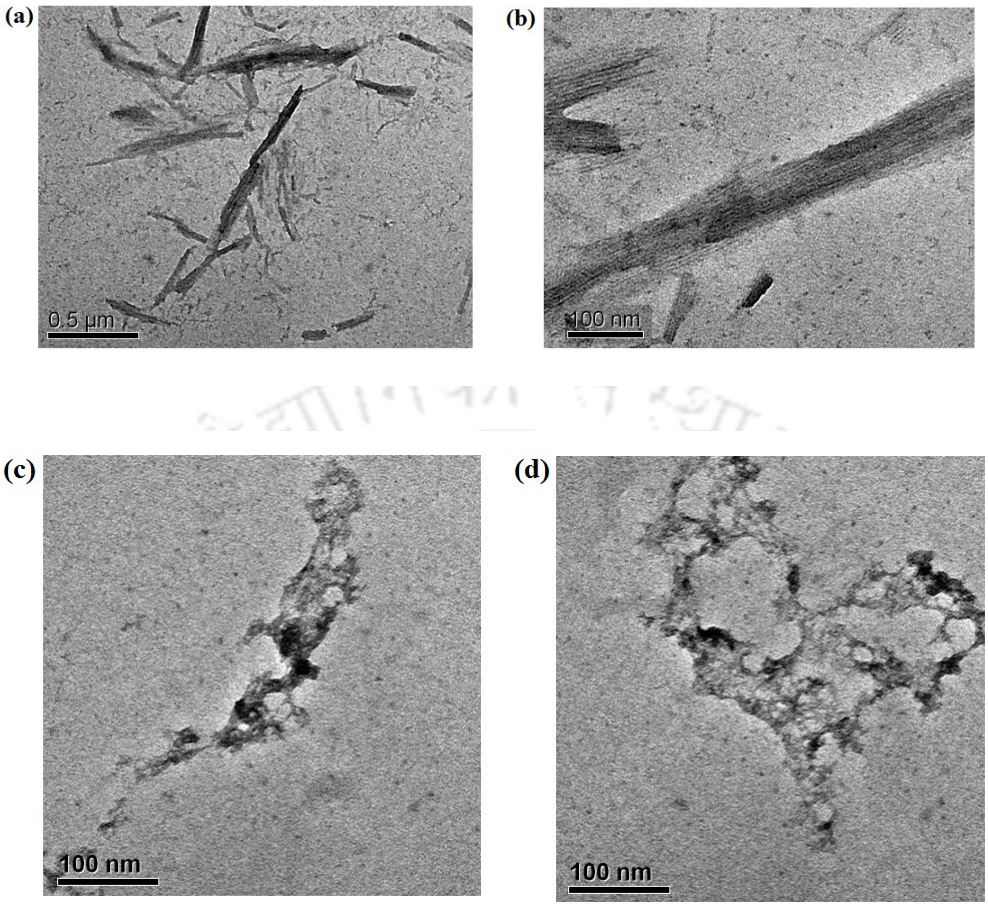



**Figure 14: TEM images of peptide 5 in (a) acidic buffer (b) PBS after seven days.**

Experiment of TEM confirmed the fibril formation by peptide **5** in a acidic condition, in which native backbone is retained. Once pH was triggered to basic condition due to aspartimide formation backbone is disrupted and due to which fibril formation is inhibited.

### 4.2.8 Monitoring amyloid formation of peptide 5 using Congo-red birefringence assay

20 µL of the peptide **5** from different buffer sets was taken over a glass slide as a droplet. Over the same droplet, 20 µL of saturated Congo red solution was added and kept for drying.

After proper drying spot was examined under optical polarisable microscope after nearly fourteen days. Peptide **5** revealed gold birefringence (figure 15a) at certain places which was not noticed when taken from PBS set (figure 15b).

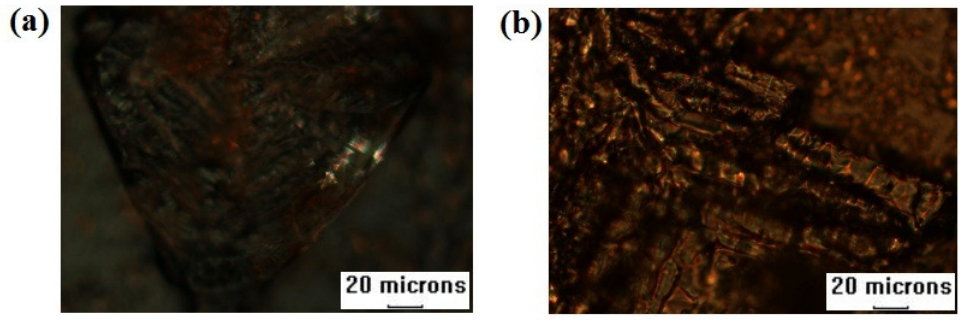



**Figure 15: Microscope images of the peptide 5 (a) from sodium acetate buffer (b) from PBS after ten days of incubation**

Peptide **5** in acidic pH form fibrils which was confirmed from thioflavin T and TEM experiment. Further, Congo red birefringence experiment confirms amyloid formation. On the other hand, in basic pH fibril and amyloid formation was not noticed in TEM and birefringence experiment respectively. Therefore, it was inferred that amyloid formation is inhibited in basic pH. Furthermore, from the results of thioflavin T assay, it can be concluded that fibril disruption also took place for peptide **5** at physiological pH.

## 4.3 To investigate the β-breaking efficiency of peptide 5 against the aggregation of peptide 4

Different biophysical experiments provided the proof that peptide **5** is capable of inhibition of self assembly. Therefore, our next objective was to test the capability of the Aβ derived β breaker peptide **5** to inhibit or disrupt fibril formed by aggregation of the Aβ derived

aggregating peptide **4** on mixing. Similar to chapter 3, we started our study using three equivalents of peptide **5** with respect to peptide **4**.

### 4.3.1 Monitoring conformational transition by CD studies

1 mg of pure peptide **4** was dissolved in 3.2 ml of PBS (50 mM, pH 7.4) to obtain a final concentration of 150 μM. Stock solution was incubated as required. In parallel, another set of peptide **4** was prepared at same concentration and to it 3 mg (three equivalents) of peptide **5** was added and incubated. All the experiments were performed taking sample from the stock solution at the given time intervals.

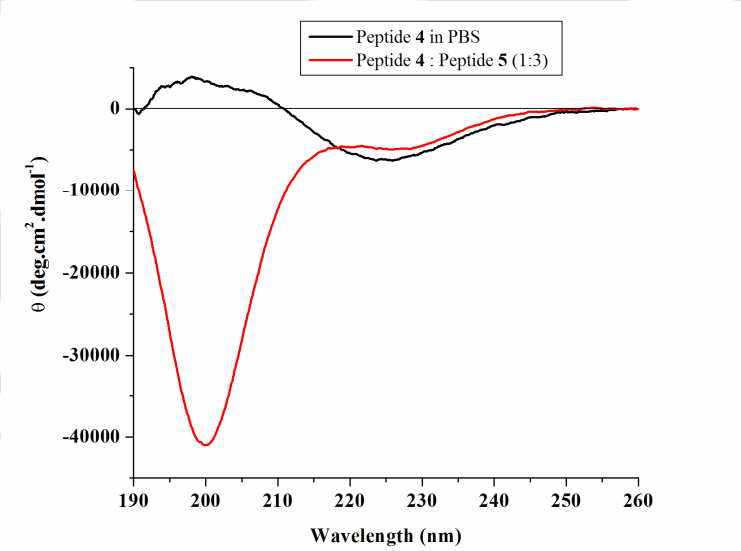



**Figure 16: CD spectra of peptide 4 alone (black line) and in presence of three equivalents of peptide 5 (red line).**

We have seen earlier that peptide **4** forms fibrils in basic pH (section 4.1.4) which was confirmed from the CD analysis. Nevertheless, when peptide **4** was mixed with three equivalents of peptide **5** we observed a CD Spectrum characterized by a minimum centred at

200 nm indicated unordered structure of peptide **4** in PBS after three days of incubation as shown in figure 16 (red line).

| Peptide **4** alone PBS (pH 7.4) | | Peptide **4** + Peptide **5** (1:3) in PBS (pH 7.4) | |
|---|---|---|---|
| | Ratio | | Ratio |
| Helix | 8.6 | Helix | 17.4 |
| Beta | 91.4 | Beta | 0.0 |
| Turn | 0.0 | Turn | 0.0 |
| Random | 0.0 | Random | 82.6 |
| Total | 100.0 | Total | 100.0 |

This indicates that three equivalents of peptide **5** could inhibit β sheet formation of peptide **4** at pH 7.4. This is probably due to conversion of BBDP unit into aspartimide and aspartyl residue which disrupted the hydrogen bonding and as a result no fibril formation was noticed for peptide 4 when mixed.

### 4.3.2 Monitoring conformational transition using FT-IR

Three days old stock solution was used to analyse the conformation of peptide **4** when mixed with peptide **5** in three equivalents.

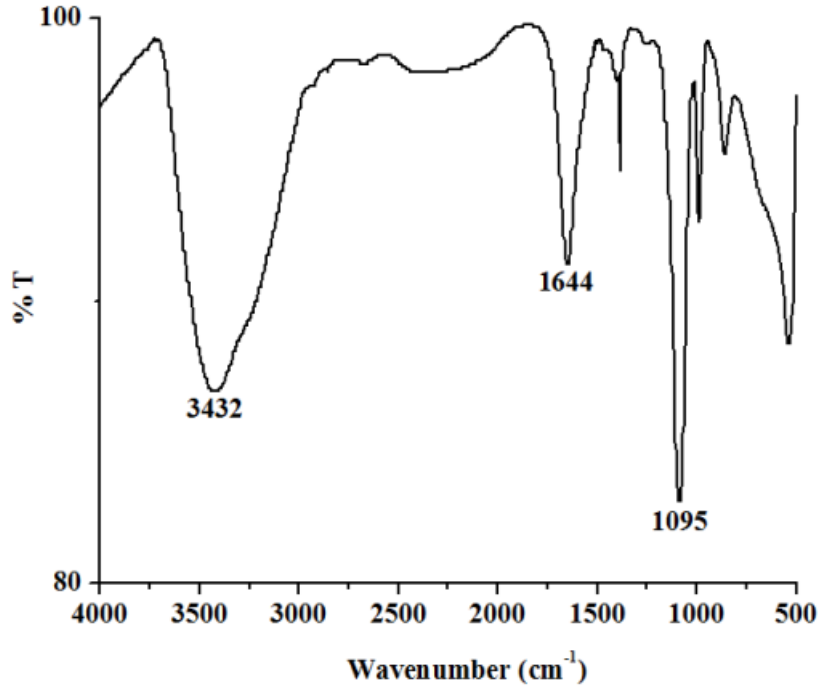



**Figure 17: FT-IR spectrum of the peptide 4 when mixed with three equivalents of peptide 5 after three days.**

We noticed a band at 1644 $cm^{-1}$ from the mixture incubated in PBS at 37 °C after three days as shown in figure 17. Earlier, peptide **4** revealed a band at 1631 $cm^{-1}$ which was assigned to β sheet conformation (figure 4b). However, on mixing with three equivalents of peptide **5** former peak at 1631 $cm^{-1}$ disappeared. This indicates that peptide **5** was capable to inhibit the aggregation of peptide **4**.

### 4.3.3 Monitoring fibril formation using thioflavin T fluorescence assay

An aliquots (20 μL) of the peptide **4** alone and another one mixed with breaker peptide **5** from the stock solution (experimental section) was mixed with 200 μL of thioflavin T (50 mM) and total volume was made up to 400 μL. Fluorescence was measured at $\lambda_{ex}$ = 435 nm and $\lambda_{em}$ = 485 nm. Experiments were carried out in triplicates.

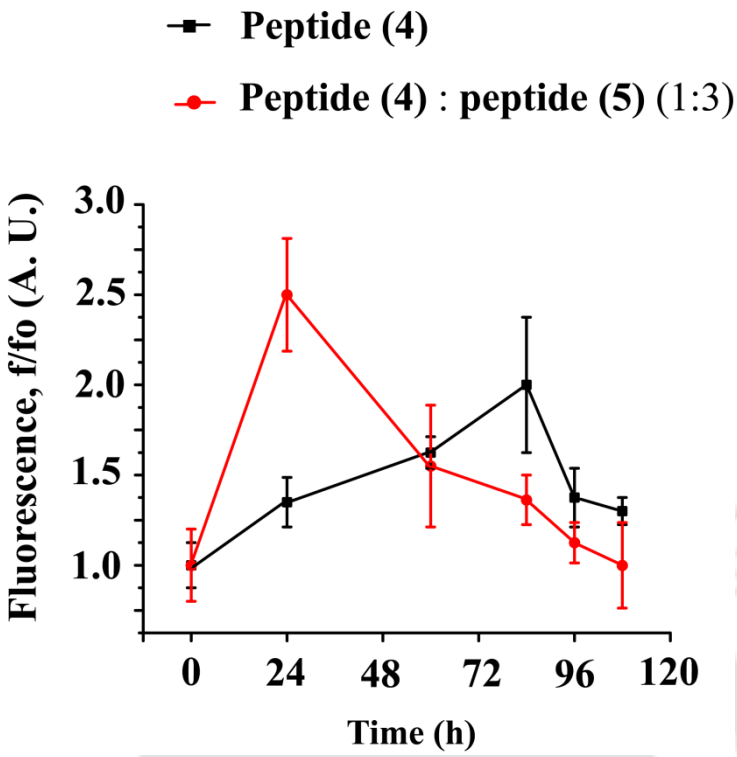



**Figure 18: Time dependent fluorescence of peptide 4 alone (black line) peptide 4 plus three equivalents of peptide 5 (red line).**

We observed a substantial decrement in the fluorescence signal of peptide **4** after 56 h when mixed with peptide **5** in three equivalents as displayed in figure 18. Therefore, it can be inferred that peptide **5** could inhibit the fibril formation of peptide **4** significantly and this may be due to chemistry of aspartimide formation which occurs in basic pH.

### 4.3.4 Monitoring fibril formation using transmission electron microscopy (TEM)

Seven days old sample from the stock solution of mixing experiment set was analysed under electron microscope and we noticed complete disappearance of fibrils over the grid (figure 19). TEM experiment in agreement with thioflavin T evidenced that fibril disruption was achieved.

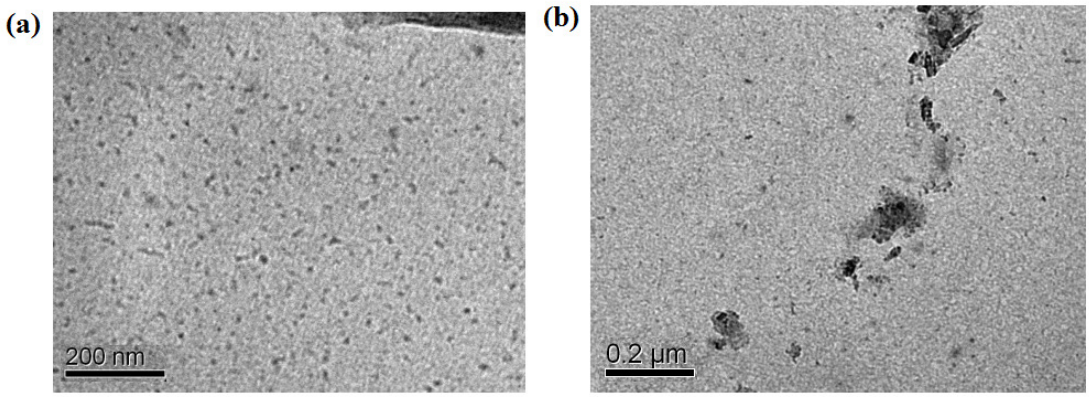



**Figure 19: TEM image of the peptide 4 when mixed along with three equivalents of peptide 5 after seven days of incubation**

This confirms that peptide **5** could inhibit the self assembly and aggregation of peptide **4** as well, when mixed in three equivalents at physiological condition. At basic pH, BBDP unit generates aspartimide and ring opening produces aspartyl residues which disrupts the fibril assembly in agreement to the concept.

### 4.3.5 Monitoring fibril formation using Congo red birefringence assay

Peptide sample **4** from the mixing experiment set was visualised under optical polarisable microscope. We did not notice any green gold birefringence from the sample. Instead, some ambiguous images were observed (figure 20). Experiment confirms that peptide **5** disrupts the amyloid formation by peptide **4** when mixed in three equivalents.

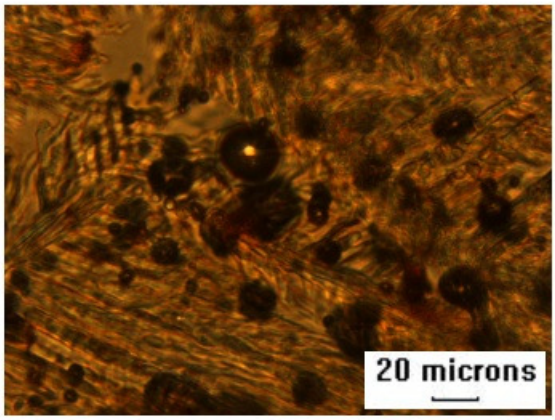



**Figure 20: Microscope image of the peptide 4 in presence of three equivalents of peptide 5.**

### 4.3.6 Conclusion

β-Sheet forming potential of peptide **4** was studied using various biophysical tools and it was recognized that peptide **4** aggregates in a specific condition to form amyloid like fibrils which was used as an Aβ mimic, in agreement to previous report on it. At the same time, we examined the aggregation and inhibition of self aggregation of our BBDP containing β breaker peptide **5**. It was confirmed that peptide **5** inhibits its self assembly and disrupts amyloid formation from TEM and Congo red birefringence assay respectively. More importantly, in case of mixing studies we noticed that aggregation of peptide **4** was brought down to a significant level, if not completely on mixing with peptide **5** in three equivalents.

# Chapter 5: Inhibition of aggregation of Aβ (1-40) by β-breaker dipeptide containing peptide

Having obtained the proof of concept of β sheet disruption on -(Ser-Leu)$_n$- based model aggregating peptide (peptide **3**) and Aβ related model peptide (peptide **4**). We decided to examine the concept further on the aggregation of amyloid β (1-40) peptide. Aβ (1-40) peptide is known to be responsible for Alzheimer's disease in human brain along with its 39 and 42 membered congeners.

**$^{1}$DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGV$^{40}$V (Aβ 1-40)**

Tjernberg, in 1996, first reported a peptide based molecule 'KLVFF', which is part of the Aβ peptide and also known as the core sequence responsible for initiation of self aggregation, as a potent inhibitor of Aβ (1-40) aggregation in vitro.[24] It was shown that Lys$^{16}$, Leu$^{17}$ and Phe$^{20}$ are crucial for binding to Aβ peptide and inhibit fibril formation. Later, Soto and co workers introduced another peptide based molecule 'LPFFD' quite popular as 'β sheet

breaker peptide' was shown to inhibit Aβ peptide aggregation and dissolution of pre formed fibrils.[25] However, twenty fold molar excess of the molecule was needed to suppress the aggregation. Presence of proline residue in 'LPFFD' makes it distorted and therefore it suffered from a structural drawback of recognition and proper alignment with full length Aβ peptide. Lack of recognition decreases efficiency.

## 5.1 Design of BBDP containing β breaker peptide

We wanted to design a peptide which would inhibit the aggregation of the full length Aβ peptide when co-incubated. More importantly, we wanted to solve the problem of recognition and alignment. For that, a β-breaker peptide was designed which first aligns with Aβ peptide easily and in a better manner as there is no kink present already in the sequence, which later undergoes a chemical modification to produce aspartimide at physiological condition to generate a kink *in situ*. Moreover, aspartimide undergoes racemization and ring opening to aspartyl residues which completely disrupts the peptide backbone and hence would disrupt aggregation. Similar to Soto's peptide we designed a pentapeptide with one BBDP element incorporated in it. **-D(OBzl)F-** is serving as BBDP unit in this case. N-terminus was acetylated, which makes it resistant towards enzymatic degradation.

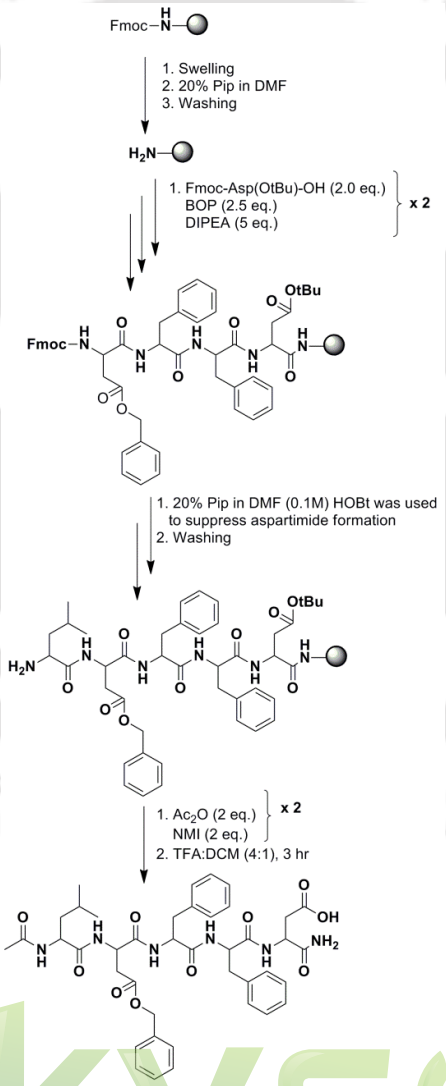
(Our deigned β breaker peptide): **Ac-LD(OBzl)FFD-$NH_2$ (peptide 6)**

We also prepared the Soto's peptide which acted as a control in our experiments. N-terminus was acetylated to keep the uniformity with peptide **6**.

(Soto's b-breaker peptide): **Ac-LPFFD-$NH_2$ (peptide 7)**

## 5.2 Synthesis and characterization of β-breaker peptide 6 and 7

Peptide **6** and **7** were synthesized using standard Fmoc/tBu protection strategy as described in section 2.1.2.[54, 55, 56, 57] Brief synthetic scheme for the peptide **6** and **7** is shown in scheme 1a and 1b respectively. In case of peptide **6**, 0.1M HOBt was added after D(OBzl) to suppress the aspartimide formation while synthesis.[58] Purity of the peptides **6** and **7** was confirmed from HPLC and ESI MS as depicted in the figure 1 and 2 respectively.




**Scheme 1: SPPS scheme for the synthesis of Ac-LD(OBzl)FFD-$NH_2$.**

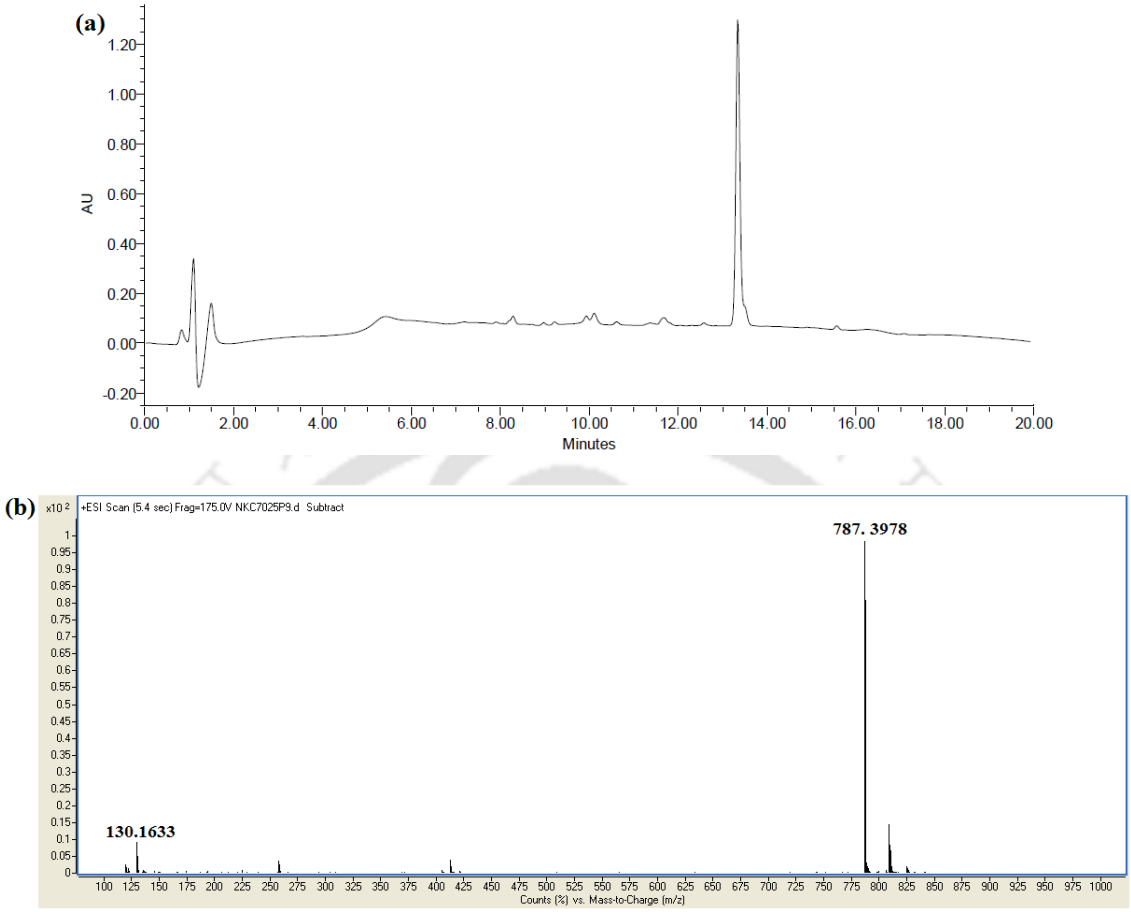



**Figure 1: (a) HPLC picture of the purified peptide sample Ac-LD(OBzl)FFD-$NH_2$. (b) ESI-MS data of Ac-LD(OBzl)FFD-$NH_2$ calculated mass for $C_{41}H_{51}N_6O_{10}$ is 787.3667 observed mass 787.3978.**

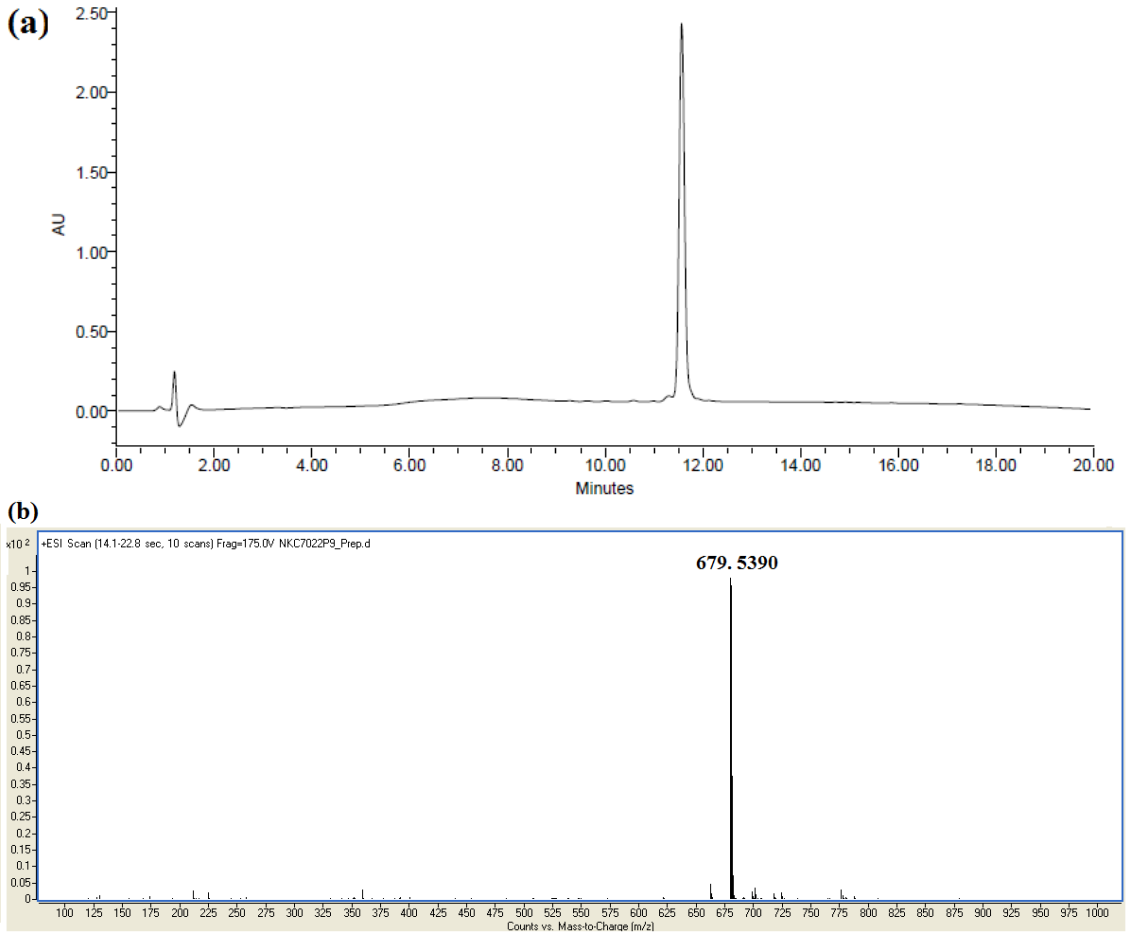



**Figure 2: (a) HPLC profile picture of purified peptide 7 (b) ESI-MS data of the peptide 7. Calculated mass for $C_{35}H_{47}N_6O_8$ is 679.3455 and observed mass is 679.5390.**

## 5.2 Kinetics of aspartimide formation

Rate of conversion of BBDP unit into aspartimide and aspartyl residues was monitored using LC-MS. Linear gradient of 0 to 95% $CH_3CN$ for 18 min in a total run time of 20 min was selected.

1 mg of the peptide **6** was dissolved in 1.2 ml of PBS (50 mM, pH 7.4) to obtain a final concentration of 1 mM. The stock solution was sonicated and vortexed for 1 min each before

taking the sample for injection. This was continued till the peaks corresponding to the aspartimide and the aspartyl residues emerged.

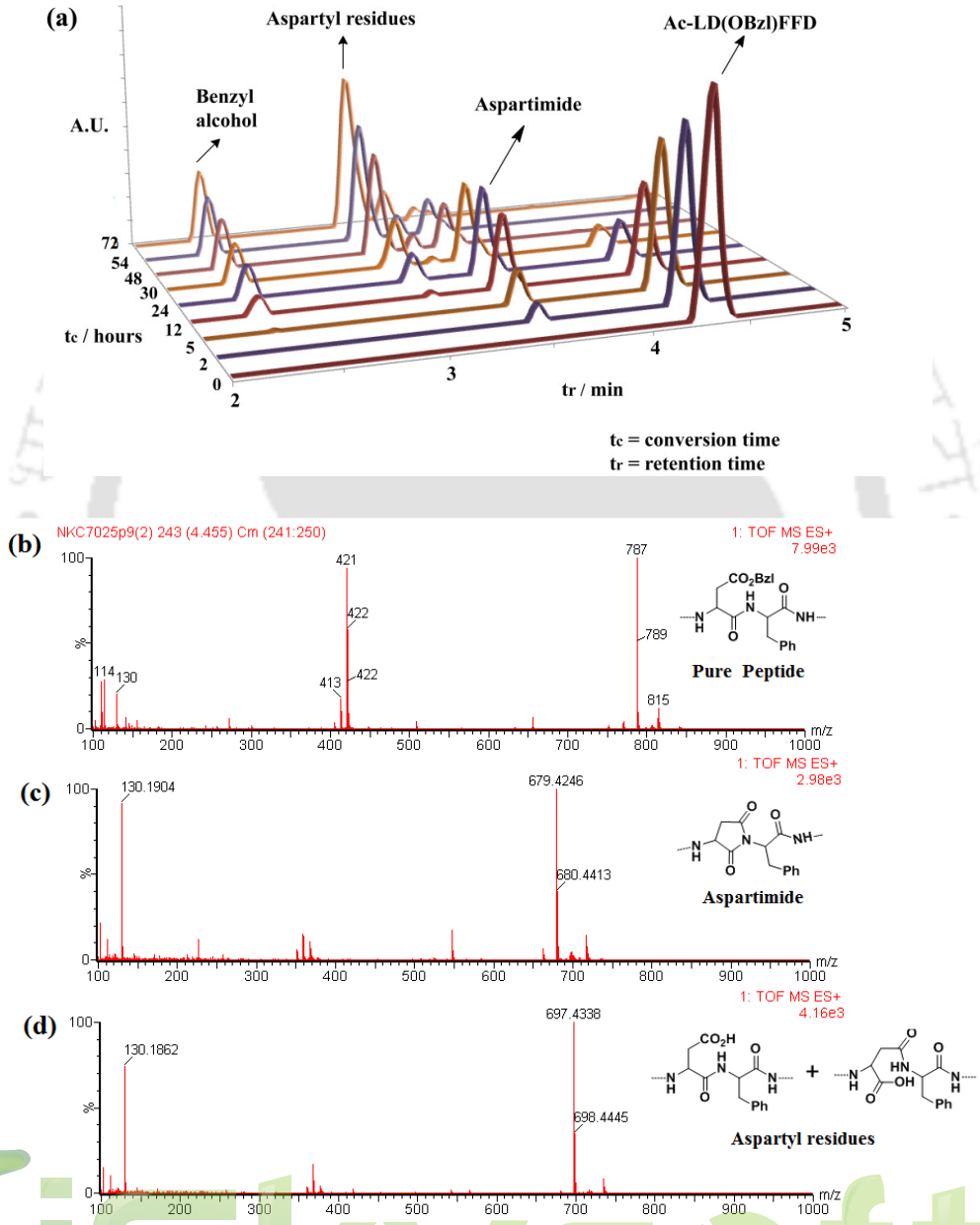



**Figure 3: Study on kinetics of O to N acyl migration peptide 6 using LC-MS. (a) LC profile picture (b) mass data of different forms of peptide 6.**

After nearly 30 h we found that pure peptide completely exhausted and peak for α and β aspartyl residues emerged (figure 3a). We noticed clear and distinct peaks for all different forms of the peptide and benzyl alcohol. All the intermediates were confirmed from the ESI MS data as shown in the panel b-d of figure 3.

## 5.3. Monitoring conformational changes by CD

0.5 mg of Aβ (1-40) was taken in a 2.0 ml Eppendorf tube and dissolved in TFA to obtain homogeneous solution free of aggregates. TFA was evaporated using $N_2$. To remove TFA completely, HFIP was added and evaporated using $N_2$ to obtain a film like material.[65] To the Eppendorf tube 2.0 ml of PBS (50mM, pH 7.4) was added followed by sonication and vortex to obtain a final concentration of 57 μM. In case of breaker peptides **5** fold molar excess (0.1 mg) of each of peptide **6** and **7** were mixed with 0.5 ml of Aβ (1-40) and kept for incubation in water bath at 37 °C. Spectra were collected after three days.

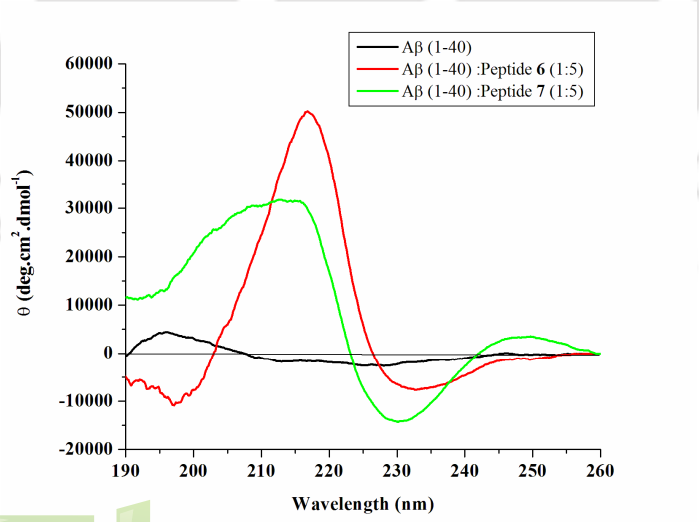



**Figure 4: CD spectra of Aβ(1-40) alone (black line), in presence of 5 equivalents of peptide 6 (red line) and in presence of 5 equivalents peptide 7 (green line).**

From the CD experiment, we observed weak signal for Aβ (1-40), spectrum with a maximum at 195-200 nm indicated a signature of β sheet (61%). However, when Aβ (1-40) was mixed with breaker peptide **6** we noticed a maximum centered at 215-220 nm and a minimum at 230-235 nm. And, when Aβ (1-40) when mixed with breaker peptide **7** we noticed a spectrum characterized by a minimum centered at 230 nm and maximum 210-215 nm (figure 4).

Use of 5 equivalents of peptide **6** produced a spectrum with 41% of turn and 59% of random coil conformation as displayed in table 1. This is due to peptide **6** at physiological pH generates aspartimide which induces type II' β turn and further ring opening to produce aspartyl residues due to which fibril formation gets disrupted and Aβ no longer stays in β sheet conformation.

| **Aβ (1-40)** | | **Aβ (1-40): Peptide 6 (1:5)** | | **Aβ (1-40): Peptide 7 (1:5)** | |
|---|---|---|---|---|---|
| | Ratio | | Ratio | | Ratio |
| Helix | 0.0 | Helix | 0.0 | Helix | 0.0 |
| Beta | 61.5 | Beta | 0.0 | Beta | 100 |
| Turn | 0.0 | Turn | 41.6 | Turn | 0.0 |
| Random | 38.5 | Random | 58.4 | Random | 0.0 |
| Total | 100 | Total | 100 | Total | 100 |

**Table 1: Detailed information of the secondary structure of Aβ (1-40) in the presence of breaker peptides.**

## 5.4 Monitoring conformational changes by FT-IR

FT-IR measurements also confirmed the presence of β sheet signature from the Aβ (1-40) sample incubated in PBS (50 mM, pH 7.4) at 37 °C for 3 days. The intense characteristic band for β-sheet was noticed at 1631 $cm^{-1}$ as shown in figure 5a.

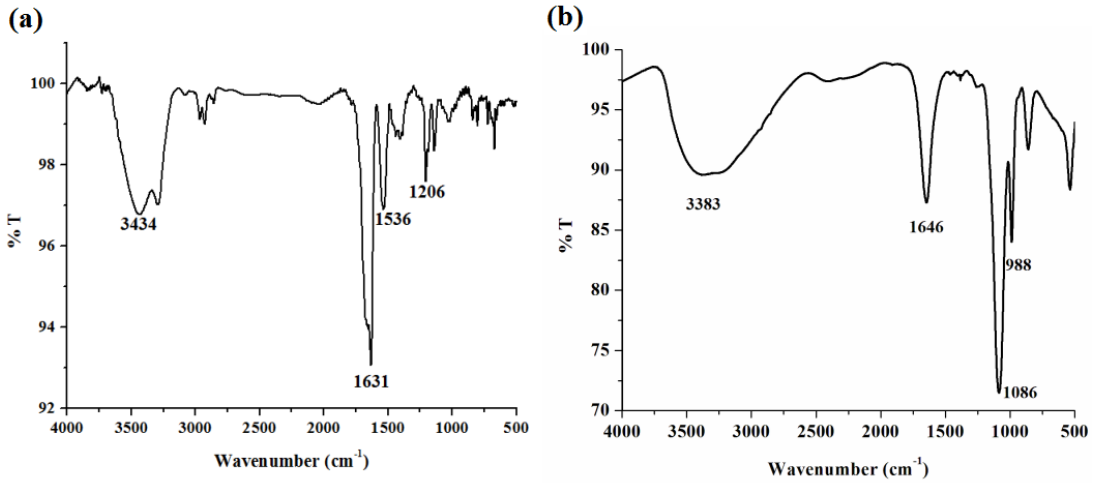



**Figure 5: FT-IR spectrum of the peptide sample (a) Aβ (1-40) and (b) Aβ plus Ac-LD(OBzl)FFD-$NH_2$ after mixing in ten equivalents.**

Co-incubation of Aβ (1-40) with ten equivalents of peptide **6** exhibited the band at 1646 $cm^{-1}$, which is a characteristic band for random coil (figure 5b). This indicates peptide **6** can inhibit/disrupt the aggregation of Aβ (1-40).

## 5.5 Monitoring fibril formation using thioflavin T fluorescence

Fibril formation was monitored using thioflavin T binding assay. 0.5 mg of Aβ (1-40) was taken in a 2.0 ml Eppendorf tube and dissolved in TFA to obtain homogeneous solution free of aggregates.[65] TFA was evaporated using $N_2$. To remove TFA completely, HFIP was added

and evaporated using $N_2$ to obtain a film like material. This process was repeated twice. To the Eppendorf tube 2.0 ml of PBS (50mM, pH 7.4) was added followed by sonication and vortex to obtain a final concentration of 50 μM. Three different sets were prepared independently. To initiate fibrillization, Eppendorf tubes were kept in water bath at 37 °C. In case of breaker peptides 10 fold molar excess (0.2 mg) of each of peptide **6** and **7** were mixed with 0.5 ml of Aβ (1-40) and kept for incubation in water bath at 37 °C.

From the stock solution an aliquot (40 μL) of the peptide sample was mixed with 200 μL of Thioflavin T solution (50 μM) and total volume was made up to 400 μL. Fluorescence was measured at excitation wavelength of 435 nm and emission wavelength at 485 nm (final concentration of peptide 2.5 μM and ThT is 25 μM).

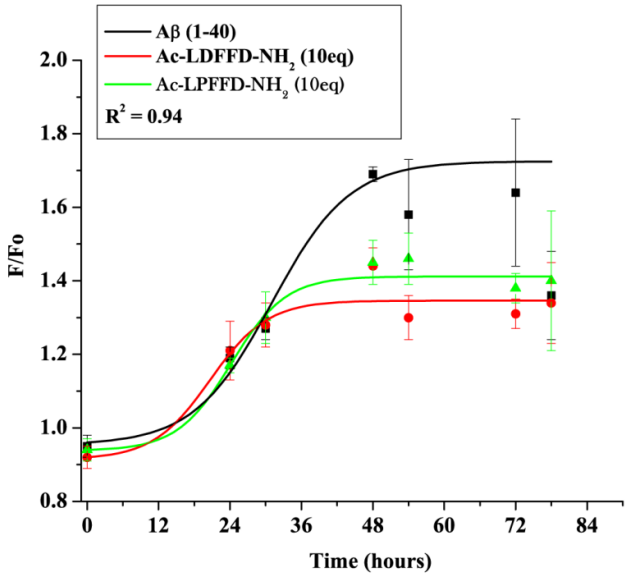



**Figure 6: Kinetics of fibril formation using ThT fluorescence assay. Black line indicates fluorescence of Aβ (1-40), green line indicates Ac-LPFFD-$NH_2$ (10 equivalents), Red line indicates Ac-LD(OBzl)FFD-$NH_2$ (10 equivalents). Results are average of triplicates, error bars indicate standard deviation.**

When monomeric Aβ (1-40) was incubated in presence of 10 equivalents of peptide **7** fluorescence is reduced to nearly 60 % in agreement to the report from Soto and co-workers.[21] Comparable decrement in the fluorescence was also noticed in case of peptide **6** as shown in figure 6. This indicates that five member aspartimide ring formed which has similar kind of geometry to that of proline and therefore the β-breaking efficiency of peptide **6** is comparable to that of peptide **7**.

## 5.6 Monitoring fibril formation by electron microscopy (TEM)

Next, we used electron microscopy technique to monitor aggregation of Aβ (1-40) alone and when mixed with ten equivalents of Ac-LD(OBzl)FFD-$NH_2$. As shown in figure 7 we noticed Aβ (1-40) display fibrillar aggregates of nearly 20 nm in diameter after seven days of incubation.

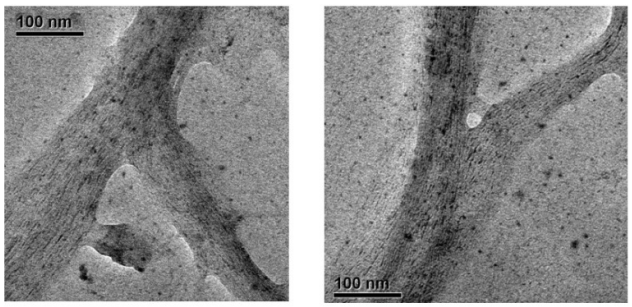



**Figure 7: TEM images of Aβ (1-40) after seven days of incubation in PBS.**

Aβ (1-40) when co-incubated for the same time with peptide **6,** fibrils were not observed instead some disordered materials were seen (figure 8). Our observation of disappearance of fibrils from the sample when mixed with peptide **6** indicates that it can inhibit/disrupt fibril formation of Aβ (1-40).

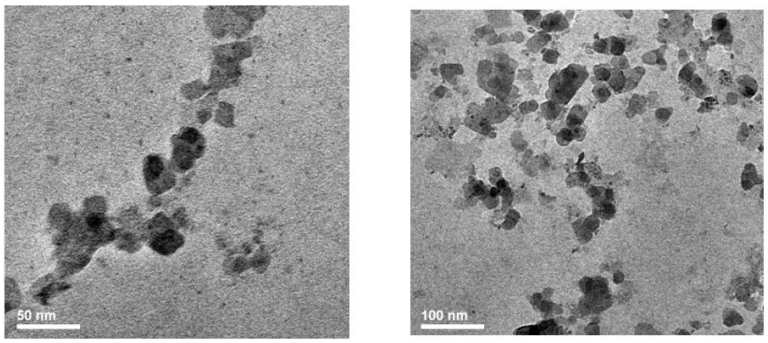



**Figure 8: TEM images of the peptide sample Aβ (1-40) mixed with 10 equivalents of Ac-LDFFD-$NH_2$.**

## 5.7 Monitoring amyloid formation using Congo red birefringence assay

Congo red staining experiments provided further evidence of amyloid formation and disruption. Samples of Aβ (1-40) and that co-incubated with peptide **6** were analyzed under optical polarizable microscope.

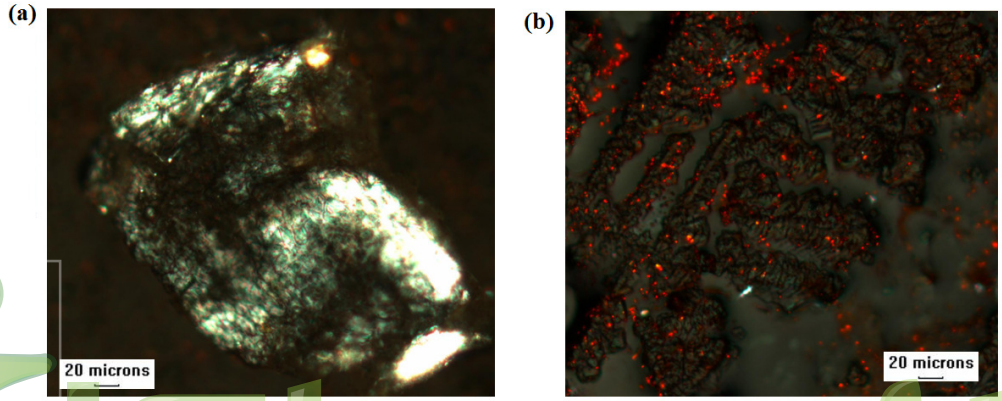



**Figure 9: Congo red photographs of the peptide sample (a) Optical polarisable microscope image of the Aβ (1-40). (b) Aβ (1-40) when mixed with ten equivalents of Ac-LD(OBzl)FFD-$NH_2$.**

We have noticed a characteristic green gold birefringence (figure 9a) under polarized light when the slide prepared from a ten days old Aβ (1-40) sample. On the other hand, from the sample of Aβ (1-40) co incubated with peptide **6** we noticed complete disappearance of intense birefringence instead at few places some shiny materials were seen as shown in figure 9b. This confirms the disruption of amyloid formation of Aβ (1-40) on mixing with ten equivalents of peptide **6**.

## 5.8 Conclusion

From the findings of the various experiments we can conclude that aggregation of Aβ (1-40) peptide can be stopped when mixed with peptide **6**. Peptide **6** containing one BBDP unit undergoes aspartimide formation and subsequent ring opening to aspartyl residues which was explicitly demonstrated using LC-MS. The experimental result of thioflavin T provided the proof of concept, since we could demonstrate that BBDP containing β-breaker peptide inhibits aggregation. Electron microscopy and Congo red birefringence studies demonstrated disruption of fibrils and amyloid formation respectively. So the concept of β sheet disruption based on the chemistry of aspartimide formation can be used to prepare lead molecules which can be used against Alzheimer's disease.

# Chapter 6: Modulation of aggregation propensity of Aβ38 by site specific multiple proline substitution

Amyloid β peptide which is generated from the amyloid precursor protein (APP) by enzymatic cleavage of β- and γ-secretase, is the principal component of the senile plaques in the neuronal cells and the main culprit of Alzheimer's disease. Sequence of the peptide upto 1-38 is shown below.

**$^{1}$DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVG$^{38}$G (Aβ38)**

Non crystalline nature and poor solubility in aqueous media makes it difficult to probe the underlying mechanism of molecular organization of Aβ peptide. Efforts are in progress to prepare the soluble analogues of Aβ peptide which enables the structural determination by NMR in solution state. High resolution molecular structures of Aβ peptide can be useful to enlighten the chemical biology of Alzheimer's disease in a profound manner. We wanted to prepare a soluble analogue of Aβ38 by substitution of proline at few positions and to investigate the aggregation potential which can be compared with the real Aβ peptide. In the

present context, such proline mutation provides us an idea about the positions where we can insert BBDPs to design a BBDP containing analogue of the full length Aβ peptide.

## 6.1 Design of soluble version of Aβ38

Unique structural feature of proline renders peptides with non β conformation.[66] Proline was never found in the interior of β sheets and this is attributed to certain constraints associated with peptidyl-prolyl bond angles. Moreover, NH of proline is not available for H-bonding to the next β-strand. Based on the structural merits of proline residue, we prepared an analogue of Aβ38 by incorporation of proline at 18 and 31 which are responsible for β-sheet formation. An extra proline was kept at position 12 to detach the unstructured flanking part from the hydrophobic regions. We named this analogue as **P3-Aβ38** for the rest of the discussion.

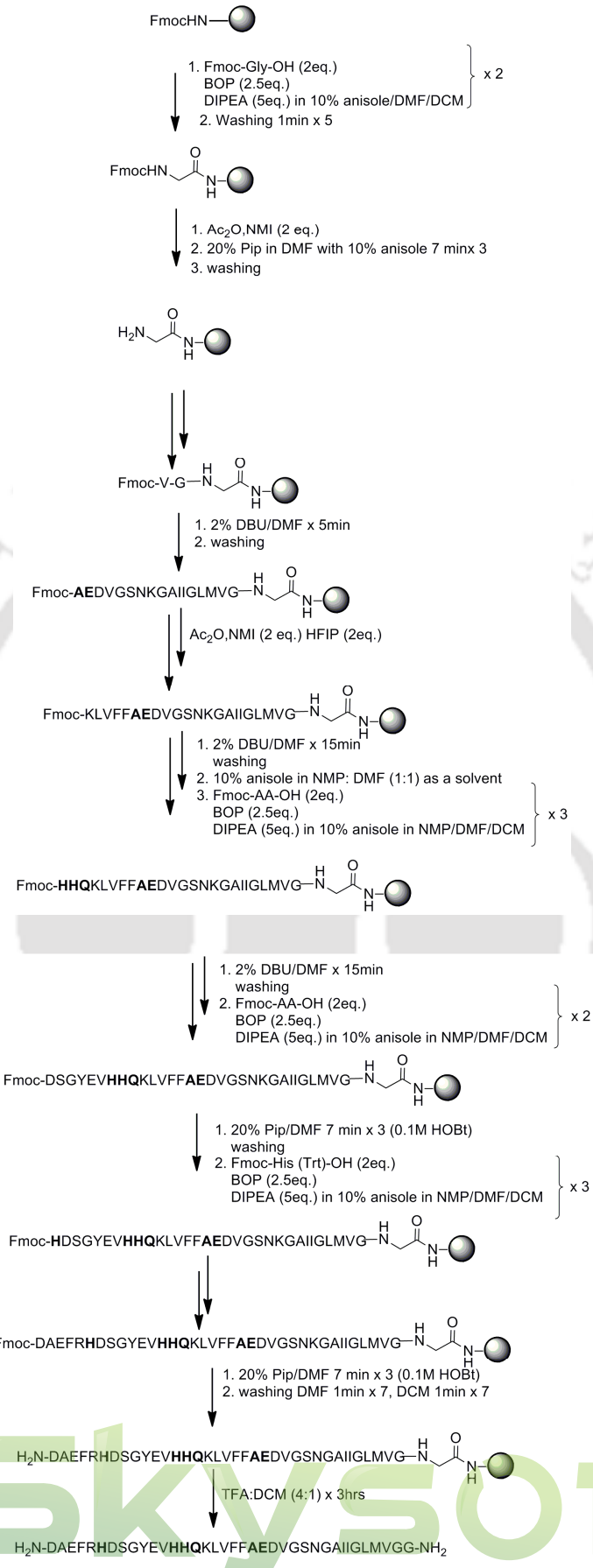
**$^{1}$DAEFRHDSGYE$^{12}$PHHQKL$^{18}$PFFAEDVGSNKGA$^{31}$PIGLMVG$^{38}$G (P3-Aβ38)**

## 6.2 Synthesis and Characterization of Aβ38

Aβ38 was synthesized by compilation of the methods based on two independent protocols that is the use of 10% anisole in DMF/DCM mixture as an additive and 2 % (v/v) DBU in DMF as a deprotecting agent until residue Ser[8].[67, 68] In case of P3-Aβ38 synthesis, 10% anisole was not necessary at coupling stage and for $N^{\alpha}$-Fmoc removal 20% (v/v) piperidine in DMF was good enough. Incorporation of proline made the synthesis easy without requirement of any additives. 150 mg of Rink amide resin was taken into a plastic syringe (5 ml) with a frit column plate. For resin swelling 3 to 4 ml of DCM was taken and kept for gentle rotation for 1 h followed by DMF 3 to 4 ml for 0.5 h. During coupling step, 2 equivalents of Fmoc amino acid dissolved in DMF: DCM (4:1) mixture containing 10%

anisole was taken into the syringe. Later, 2.5 equivalents of BOP and 5 equivalents of DIPEA were dissolved in DMF: DCM (4:1) mixture containing 10% anisole, stayed for 5 min and taken into syringe. After a coupling time of 1 h solvent was taken out and coupling step was repeated. Completion of coupling was monitored by Kaiser test (and micro cleavage test at some points). In some cases, where incomplete acylation were noticed, capping was performed to ensure no free amino groups left on resin. Capping was carried out using 2 equivalents of acetic anhydride and NMI (*N*-methylimidazole) for 1 h. For $N^{\alpha}$-Fmoc removal 2% (v/v) DBU in DMF (containing 10% anisole) for 5 min was used from $Val^{36}$ to $Ser^{8}$. For remaining residues 20% piperidine in DMF (containing 10% anisole) for 21 min (7 min x 3) was used. After final $N^{\alpha}$-Fmoc removal, peptide was cleaved from resin using a mixture of TFA: DCM (4:1) at room temperature (Scheme 1).

Presence of anisole during synthesis enhanced solvation effect between side chain protecting groups and aromatic ring of anisole. This helped in disruption of aggregation between growing peptide chains. However, use of 10% anisole as an additive could not completely eliminate aggregation. Our first danger was observed as incomplete acylation after $Ala^{21}$ and for which additional coupling step was required. This is obvious because KLVFFA is known to be highly aggregation prone sequence. Again, slow progress in acylation step was noticed after $Lys^{16}$. The reason for slow acylation can be attributed to bulky side chain protecting group in case of Fmoc-His(Trt)-OH. From $Gln^{15}$ to $Glu^{11}$ we noticed negative Kaiser test after 5 minutes of deprotection cycle and therefore it was necessary to increase the time for deprotection to 12 minutes. Later, from $His^{6}$ to $Glu^{3}$ also subtle uncertainty was noticed in Kaiser test and therefore it was essential to repeat the coupling cycle and capping. Based on 0.165 mmol scale 12 mg of purified Aβ38 peptide (2 % yield) was isolated as a white fluffy powder after lyophilisation.




**Scheme 1: SPPS scheme for the synthesis of Aβ38.**

Purity of peptide **6** was confirmed from HPLC and ESI MS. For HPLC solvent A $H_2O$ (0.09 % TFA) and solvent B $CH_3CN$ (0.09% TFA) was used. Purification was carried on $C_{18}$ μ-Bondapak column and dual wavelength of 214 nm and 254 nm was selected. We observed peak in HPLC corresponding to peptide 6 at $R_t$ nearly 11.0 min as displayed in figure 1a.

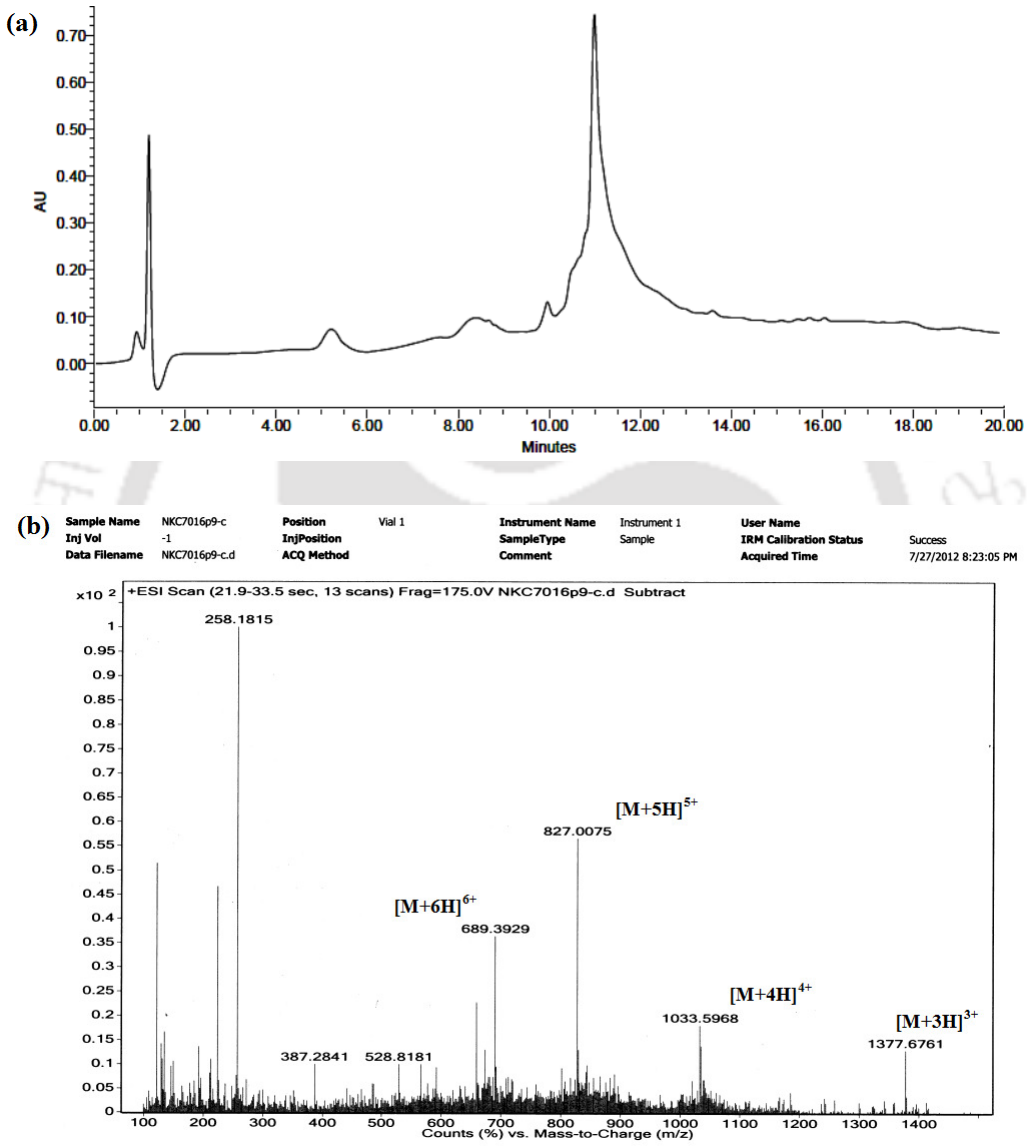
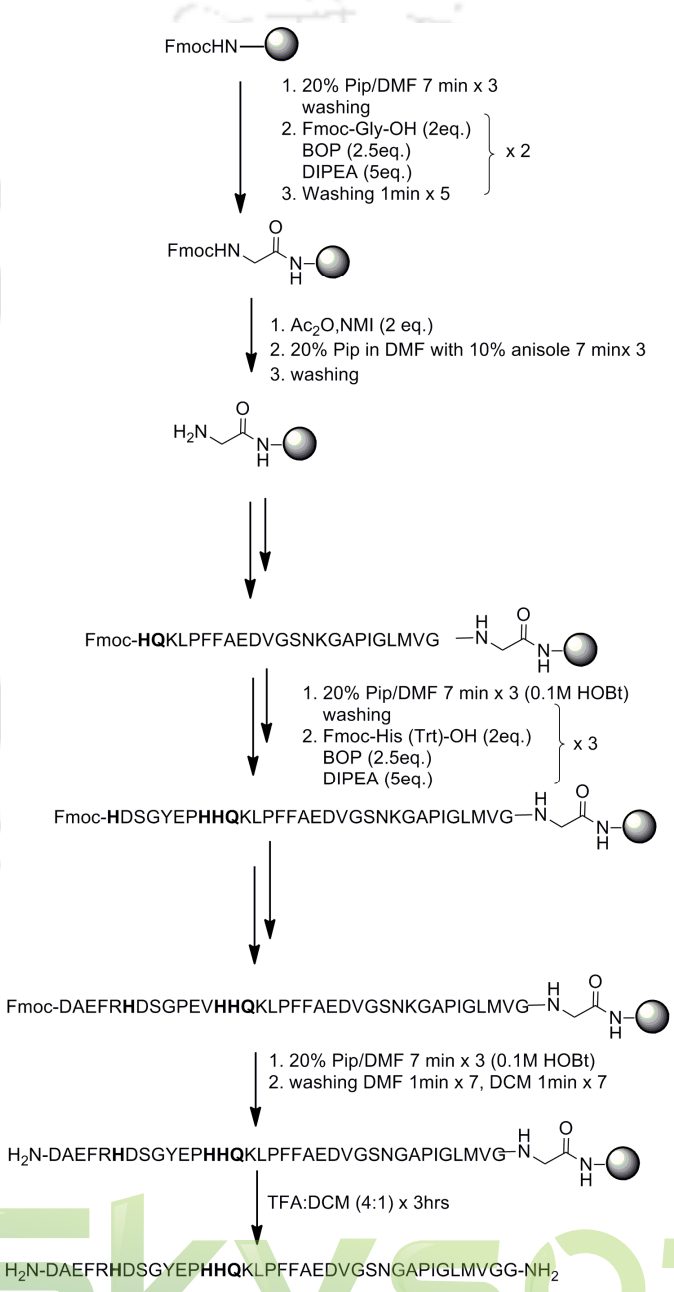



**Figure 1: (a) HPLC profile picture of peptide 9 (b) ESI MS data of peptide 9. Calculated mass for $[M+3H]^{3+}$ 1377.66 and observed mass 1377.67, $[M+4H]^{4+}$ is 1033.5 and observed mass 1033.59, $[M+5H]^{5+}$ is 827.0 observed mass 827.0, $[M+6H]^{6+}$ is 689.33 observed mass is 689.39.**

From the ESI MS (+ve mode) we observed presence of $[M+3H]^{3+}$,$[M+4H]^{4+}$,$[M+5H]^{5+}$ and $[M+6H]^{6+}$ peaks (figure 1b).

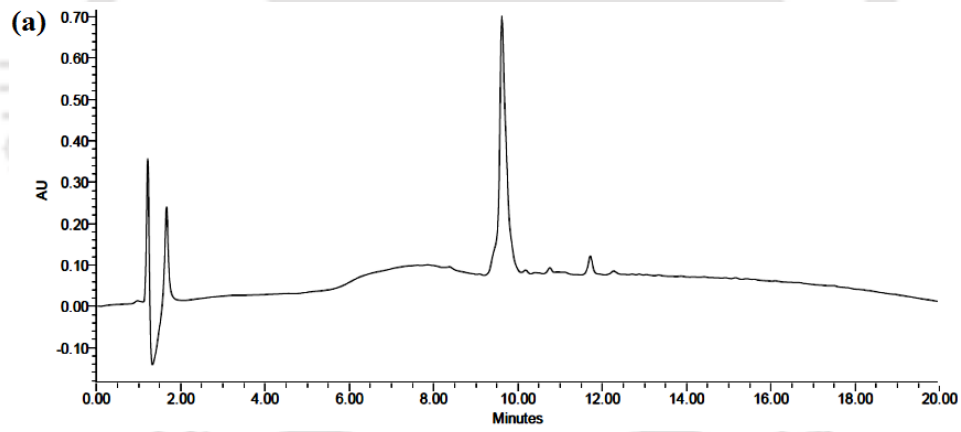
## 6.3 Synthesis and Characterization of P3-Aβ38

In case of the triple mutant version of the Aβ peptide, the synthetic procedure was similar, without need of 10% anisole and DBU for Fmoc removal. Instead, 20 % (v/v) piperidine in DMF was effective for Fmoc deprotection.




**Scheme 2: SPPS scheme for the synthesis of P3-Aβ38.**

Synthesis of P3-Aβ38 went smoothly except certain positions, e.g. $Lys^{16}$ to $His^{13}$ where coupling was required for three times. Finally, capping using acetic anhydride and *N*-methyl imidazole was performed. It was apparent from the above findings that the use of a proline helps to reduce on resin aggregation and facilitates smoother peptide synthesis. P3-Aβ38 was purified on semi preparative HPLC and purity was confirmed by analytical injection and ESI-MS analysis as shown in figure 2a. In case of P3-Aβ38 yield was improved to 24 mg (4 %) based on 0.15 mmol scale obtained as white powder. Similarly, peptide **7** was purified on semi preparative HPLC and mass was analysed in ESI MS (+ve mode). In HPLC peptide peak was observed at $R_t$ 9.8 min as shown in figure 4a and in ESI MS we observed presence of $[M+3H]^{3+}$, $[M+4H]^{4+}$, $[M+5H]^{5+}$, $[M+6H]^{6+}$ and $[M+H]^{7+}$ peaks (figure 2b).



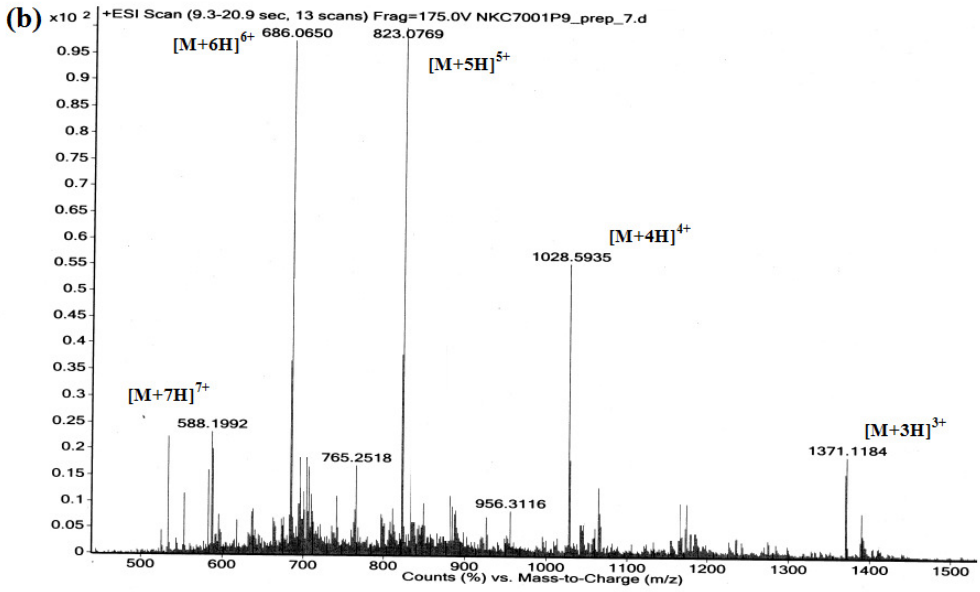



**Figure 2: (a) HPLC profile picture of peptide 8 (b) ESI MS data of peptide 8. Calculated mass for $[M+3H]^{3+}$ 1371.0 and observed mass 1371.11, $[M+4H]^{4+}$ is 1028.5 and observed mass 1028.59, $[M+5H]^{5+}$ is 823.0 observed mass 823.07, $[M+6H]^{6+}$ is 686.0 observed mass 686.06, $[M+7H]^{7+}$ is 588.14 and observed mass 588.19.**

## 6.4 Monitoring the secondary structure of Aβ38 and P3-Aβ38 by circular dichroism (CD) studies

To determine secondary structure of Aβ38 and P3-Aβ38 CD spectroscopy was used. Aβ38 and P3-Aβ38 samples of 60 µM concentration was prepared as described in the experimental section. From the seven days old stock solution 400 µL of the sample was taken in the cuvette of 1 mm path length and spectra were recorded. Data were collected as an average of four scans. Reference was subtracted from the sample data and mean residue molar ellipticity was calculated.

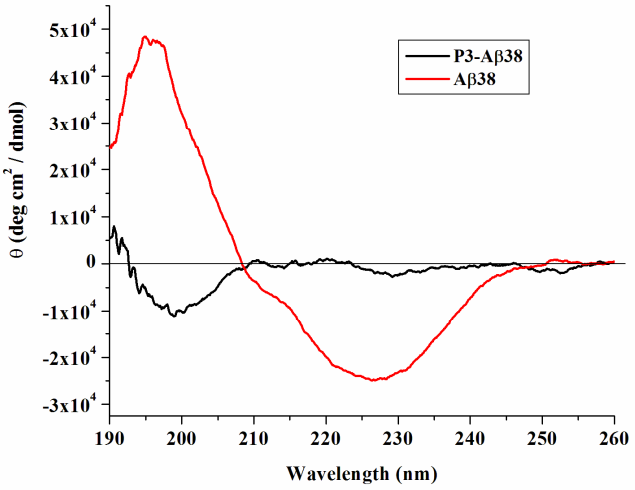



**Figure 3: CD spectra showing Aβ38 in red line and P3-Aβ38 in black line after seven days of incubation.**

Spectrum characterized by a minimum centered at 225-228 nm and maximum at 195-197 nm indicates β sheet rich structure of Aβ38 (red curve, figure 3). On the other hand, the secondary structure of the P3-Aβ38 was characterized by a minimum centred at 198 nm (black curve, figure 3).

| **Aβ38** | | **P3-Aβ38** | |
|---|---|---|---|
| | Ratio | | Ratio |
| Helix | 16.5 | Helix | 13.7 |
| Beta | 83.5 | Beta | 0 |
| Turn | 0 | Turn | 17.6 |
| Random | 0 | Random | 68.7 |
| Total | 100 | Total | 100 |

**Table 1: Ratio of different conformations of Aβ38 and P3-Aβ38.**

From the table 1, we noticed 83.5 percent of beta content from the Aβ38 sample, which indicates that Aβ38 stays mainly in the β-sheet conformation. On the contrary, P3-Aβ38 contains 68.7 percent of random coil along with 17.6 percent of turn, which again confirms that presence of proline helps P3-Aβ to stay in non β conformation even after seven days. A fraction of helix content 16.5 and 13.7 for Aβ38 and P3-Aβ38 was also noticed respectively. Hence, it is confirmed from the CD study that proline substitution can alter the conformation of a peptide which regulates the aggregation.

## 6.5 Monitoring the conformation of Aβ38 and P3-Aβ38 by FT-IR

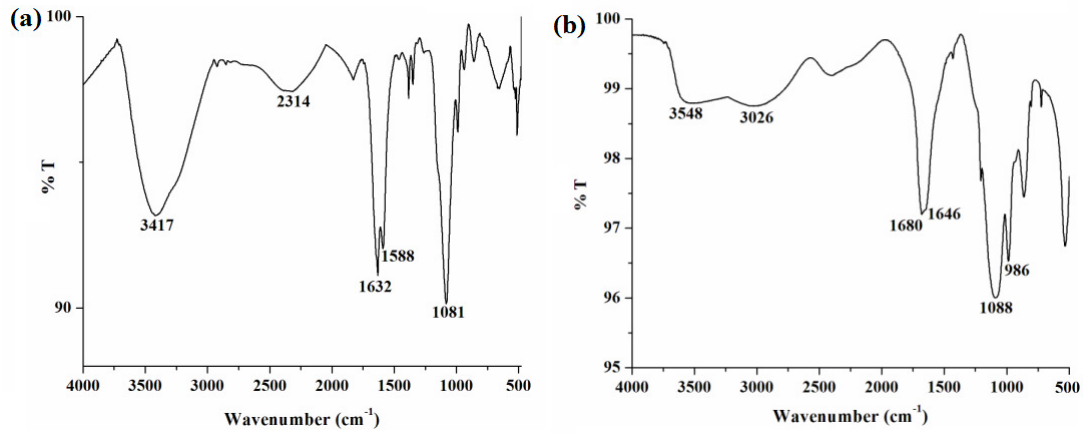



**Figure 4: FT-IR spectra of (a) Aβ38 and (b) P3-Aβ38 after seven days.**

FT-IR is another complementary technique to investigate the existence of various secondary conformations of protein/peptide. We noticed a sharp band at 1632 $cm^{-1}$ for Aβ38 which corresponds to β-sheet secondary structure (figure 4a) after seven days of incubation in PBS. On the other hand, a sharp band at 1680 and 1646 was noticed in case of P3-Aβ38 after same incubation time, which was probably for the presence of β-turn and random coil conformation as shown in figure 4b.

## 6.6 Monitoring fibril formation of Aβ38 and P3-Aβ38 by thioflavin T fluorescence

The kinetics of the fibril formation was monitored using ThT fluorescence assay at different time intervals. Peptides at 60 μM concentration were incubated in 50 mM PBS (pH 7.4) at 37 °C. An aliquot from stock solution was mixed with ThT and fluorescence was measured.

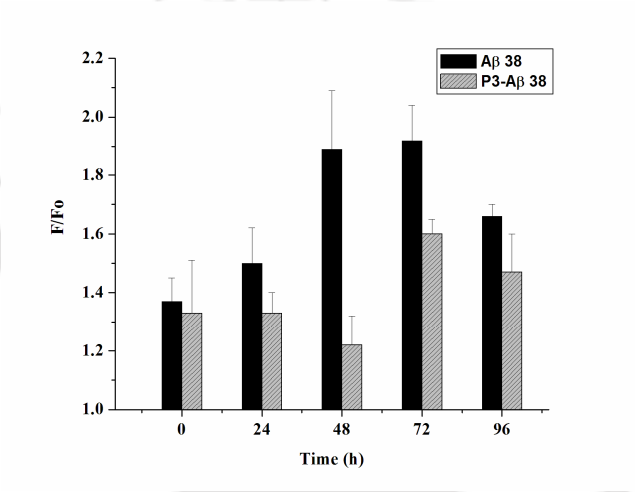



**Figure 5: Time dependent thioflavin T fluorescence assay of Aβ38 (black bar) and P3-Aβ38 (grey colour).**

In case of Aβ38, fluorescence increment was observed after 24 h which was not noticed in case of P3-Aβ38 (figure 5), which indicates that the aggregation propensity of Aβ38 is relatively higher than that of P3-Aβ38. However, slight increment of the fluorescence intensity was observed at 72 h for P3-Aβ38. This indicates enlargement of the lag time due to multiple proline insertion. A decrement of total amount of fibril formation due to multiple proline substitution was also evident from the figure.

## 6.6 Monitoring fibril formation of Aβ38 and P3-Aβ38 by TEM

Direct evidence of fibril formation of Aβ38 and P3-Aβ38 was confirmed from electron microscopy. Sample of Aβ38 and P3-Aβ38 were prepared at the same time and were examined using electron microscopy. TEM analysis was carried out after seven days of incubation.

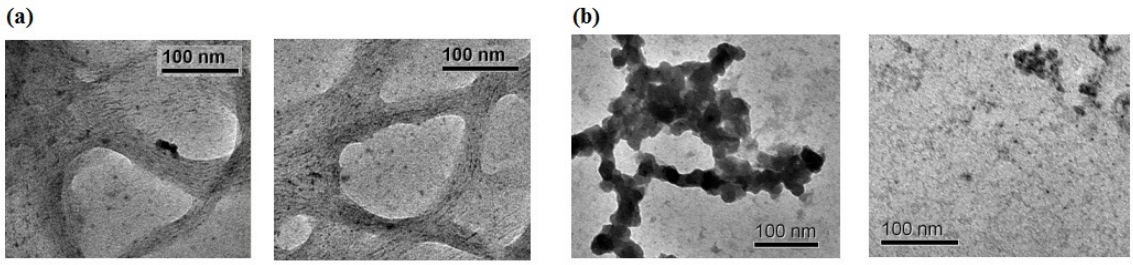



**Figure 6: TEM images (a) Aβ38 (b) P3-Aβ38 after seven days.**

Aβ38 revealed fibrillar morphology as displayed in figure 6a whereas, nonfibrillar and disordered structures were seen in case of P3-Aβ38 figure 6b. In agreement to CD and thioflavin T assay it could be inferred that presence of proline inhibited fibril formation.

## 6.7 Monitoring amyloid formation by Aβ38 and P3-Aβ38 using Congo red birefringence study

Aβ38 exhibited characteristic green gold birefringence under polarized light which was not observed in case of P3-Aβ38. This again confirms the incapability of amyloid architecture formation of P3-Aβ38. The result with Aβ38 was a positive control (figure 7). Images were taken after ten days of incubation in PBS.

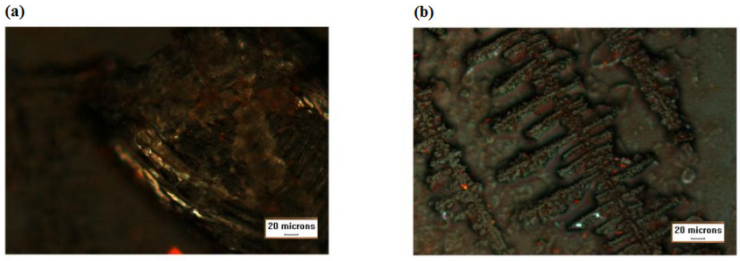



**Figure 7: Birefringent images of the peptide samples (a) Aβ38 and (b) P3-Aβ38 after ten days.**

## 6.8 Conclusion

Based on the above findings, it is suggested that P3-Aβ38 aggregation is slower compared to Aβ38 under similar experimental conditions. That indicates substitution of proline at core hydrophobic region of Aβ38 renders a less aggregation prone variant. The aggregation propensity of the amyloid β peptide can be adjusted using such multi proline replacement strategy. Proline substitution afforded good aqueous solubility which may help in solution NMR characterization with adequate time for resolution. Proline insertion at positions which can inhibit aggregation can be used for anti-alzheimer drug design. Most importantly, we can prepare BBDP version of the Aβ38 and investigate its therapeutic potential. Also, it renders synthesis of such aggregating peptides on solid support easier.

# Chapter 7: Experimental section

## 7.1 Instrumentation and general methods

### Reagents and solvents

Rink amide resin was obtained from Fluka (Loading 1.1 mmol/g). BOP, (Benzotriazole-1-yloxy) tris (dimethylamino) phosphonium hexafluorophosphate, PyBOP (Benzotriazole-1-yl-oxy-tris-pyrolidine-phosphonium hexafluorophosphate, HBTU (1- [Bis (dimethylamino) methylene]-1H benzotriazolium hexafluorophosphate 3-oxide) was purchased from Sigma. Dimethylformamide (extrapure grade), dichloromethane (extrapure grade) and acetonitrile of HPLC grade were obtained from Merck (India). Anisole and trifluoroacetic acid (TFA) of extrapure grade were purchased from SRL (India). Milli-Q water at 18.2 Ω was used. Fmoc amino acids were purchased from GL Biochem (Shanghai) with following protecting groups:

$N^G$-2, 2, 4, 6, 7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf) for Arg; *tert*-butyl for Asp, Glu, Ser, Thr and Tyr; $N^{im}$-trityl (Trt) for His, Asn, Gln and Boc for Lys.

**Chromatography**

Crude peptides were purified on Waters 600E semi preparative RP-HPLC using C18-μ Bondapak column at a flow rate of 5ml/min. Solvent system composed of solvent A (0.09% TFA in $H_2O$) and solvent B (0.09% TFA in $CH_3CN$). Waters 2489 UV detector was used with an option of dual detection at 214 and 254 nm. Gradient used for purification was 5-20% $CH_3CN$ for 5 min, 20-60% $CH_3CN$ over 5 to 25 min.

Purity of the peptides was confirmed from Waters UPLC-MS system (ESI) was used. Solvents used were solvent A (0.09% Formic acid in $H_2O$) and solvent B (0.09% FA in $CH_3CN$) on C18 column with a flow rate of 0.25 ml/min. Dual wavelength were selected at 214 nm and 254 nm. Linear gradient of 5 to 95 % $CH_3CN$ was used in a total run time of 8 min.

Purity was also confirmed using Waters 600E Analytical HPLC system, Waters C8 analytical column at a flow rate of 1 ml/min, linear gradient of 5-100% $CH_3CN$ over 18 minutes in a total run time of 20 min. Dual wavelength was selected at 214 nm and 254 nm.

**Mass spectrometry**

Mass of the peptide samples were analyzed on Waters UPLC-MS (ESI +ve mode) Micromass Q-TOF equipped with Masslynx software. In some cases, Agilent-Q-TOF 6500 instrument ESI positive mode was used equipped with Mass hunter work station software.

**Circular dichroism**

It is one kind of absorption spectroscopy which helps to identify the secondary conformations of a protein/peptide.[42] Lyophilized peptide sample was dissolved in PBS (50 mM, pH 7.4)

and sodium acetate buffer (50 mM, pH 4.0) as required to obtain a final concentration of 150 μM (60 μM in case of Aβ38 and P3Aβ38). Stock solution (was used directly for Aβ38 and P3Aβ38) was diluted from respective buffer solutions to obtain final concentration of 50 μM. 400 μL of the sample was taken in a cuvette of 10 mm path length using a bandwidth of 1 nm. Two measurements were accumulated. Spectra were recorded from 190 nm to 260 nm on JASCO J-815 instrument.

**Transmission electron microscopy**

Direct evidence of presence or absence of fibrils in a sample can be obtained using electron microscopy. Peptide solution (10 μL) was added and allowed to float for 1min on the carbon coated grid. 2% Uranyl acetate solution (10 μL) was added to the same grid and was allowed to float for 1 min. Excess solution was removed using blotting paper. Excess solution was removed. After drying TEM analysis was done on JOEL instrument at 80 kV.

**Thioflavin T assay**

Fibril formation was monitored by a thioflavin T fluorescence assay. This assay gives a direct quantification of fibrils formed. Thioflavin T (Sigma Aldrich) stock solution was prepared at a concentration of 3.14 mM, stored at 4 °C with proper protection from light to prevent quenching.[45]

Lyophilized peptide samples were dissolved in PBS (50 mM, pH 7.4) and sodium acetate buffer (50 mM, pH 4.0) as required to prepare a stock solution from 60 μM to 150 μM and incubated at 37 °C over water bath. At different time intervals, 20 μL to 40 μL of peptide sample was mixed with 200 μL of thioflavin T solution and total volume was made up to 400 μL (final cuvette cocncentration: 2 to 5 μM and thioflavin T: 50 μM). Fluorescence emission

was measured at 485 nm and excitation of 435 nm, slit of 5 nm on Fluoromax-4, Horiba instrument.

**Fourier Transformation Infra Red Spectroscopy (FT-IR)**

Peptide stock solution which was prepared for thioflavin T experiment was used for FT-IR analysis. 20 µL of the peptide sample after required time of incubation was mixed with KBr and a pellet was prepared. At first, background was obtained followed by sample with total 32 scans using Thermo Scientific instrument. To obtain final spectra background was subtracted and text files were plotted using Origin 61 software.

**Congo-red birefringence**

Upon staining with Congo red amyloid exhibits characteristic green gold birefringence under plane polarized light.[51] A peptide stock solution which was prepared for thioflavin T experiment was used for birefringence studies also.

20 µL of the peptide sample was added on a glass slide and over it 40 µL of the saturated Congo red solution was added and allowed to dry. Excess Congo red, was removed by blotting paper and sample was allowed to dry at room temperature. On the glass slide red spot was observed under Leica DM 2500P polarisable microscope.

Preparation of saturated Congo red solution: A solution of 80% ethanol was prepared and saturated amount of sodium chloride was added. The solution was stirred for a few minutes and filtered away excess sodium chloride. A saturating amount of Congo red was added, stirred and filtered to obtain final working solution.[43]

## 7.2 Solid Phase Peptide Synthesis

The syntheses [57] were carried out manually in a 5 ml plastic syringe fitted with a frit column which was attached to Stuart rotator (20 rpm). Rink amide MBHA resin was swollen in DCM for 1.5 h and DMF for 0.5 h. Distilled DCM and $N_2$ sparged DMF were used in the syntheses.

*Coupling reaction*

For a standard coupling, 2 equivalents of Fmoc amino acid was dissolved in DMF and added to the resin. 2.5 equivalents of BOP or PyBOP and 5 equivalents of DIPEA were addedto it later. Coupling time varied from 45 min to 6 h. At some critical points HBTU was used. Resin was washed with DMF and DCM for 5 min each (1 min x 5) after each coupling. All couplings were performed at room temperature.

*Fmoc deprotection*

For $N_\alpha$-Fmoc deprotection 20% piperidine in DMF was used for 21 min (7min x 3). In case of Aβ38 synthesis, 2% DBU in DMF was used to ensure complete removal. Resin was washed with DMF and DCM for 5 min each (1min x 5) after deprotection.

*Kaiser test* [69]

Kaiser A: 0.5 g ninhydrin in 10 ml ethanol

Kaiser B: 0.4 mL of 0.001M $KCN_{aq}$ in 20 mL of pyridine.

A few resin beads were placed in a small fusion tube and 100 µL of Kaiser A and Kaiser B were added. Tube was heated at 80 °C for 5 min over a sand bath. A positive test is indicated by the presence of blue colour.

*Acetylaton/ capping*

3 equivalents of $Ac_2O$ and 3 equivalents of *N*-methyl imidazole were mixed in DCM and added to the resin and the reaction was carried out for 1h (30 min x 2).

*Cleavage of peptide from the resin*

The Rink amide MBHA resin was treated with a solution of TFA/DCM (4:1) for 3h. On an average 1 mL of the cocktail was used for 100 mg of the resin. Crude peptide was precipitated using cold diethyl ether, centrifuged and purified by semi preparative HPLC.

## Synthesis

Model BBDP containing peptides were synthesized on Rink amide resin (loading 1.1 mmol/g). Each coupling was performed with 2 equivalents of Fmoc amino acid, 2.5 equivalents of BOP and 5 equivalents of DIPEA (for first coupling three equivalents were used). After each coupling resin was washed with DMF and DCM for 5 min each (1 min x 5) and Kaiser test was performed. Fmoc group was removed using 20 % piperidine in DMF mixture for 21 min (7 min x 3). After incorporation of Asp(OBzl) 0.1 M HOBt was used in piperidine/DMF mixture.

- **Synthesis of BBDP containing model β-breaker peptide, $H_2N$-Ser-Leu-Ser-Asp(OBzl)-Ser-Leu-Ser-Gly-$NH_2$ (1)**
-

Rink amide resin: (loading 1.1 mmol/g); 300 mg; 0.33 mmol

| Amino acid/ amount (mg) / eq. | | Coupling time | Kaiser test | Capping | Notes |
|---|---|---|---|---|---|
| Fmoc-Gly-OH/ | 294/3 | 1.5 h | -ve | yes | - |
| Fmoc-Ser(OtBu)-OH/ | 252/2 | 45 min | -ve | - | - |
| Fmoc-Leu-OH/ | 232/2 | 1 h | -ve | - | - |
| Fmoc-Ser (OtBu)-OH/ | 252/2 | 45 min | -ve | - | - |
| Fmoc-Asp(OBzl)-OH/ | 440/3 | 1 h x 2 | -ve | - | - |
| Fmoc-Ser(OtBu)-OH/ | 252/2 | 45 min | -ve | - | - |
| Fmoc-Leu-OH/ | 232/2 | 1 h | -ve | - | - |
| Fmoc-Ser(OtBu)-OH/ | 252/2 | 45 min | -ve | - | - |

*Cleavage of protecting groups and peptide from resin*

After final Fmoc removal, peptide was cleaved from the resin using a mixture of TFA/DCM (4:1) 3 mL for 3 h. Crude peptide was precipitated using cold diethyl ether, centrifuged and washed 3 times with ether. Peptide was purified on semi preparative HPLC ($C_{18}$ μ-Bondapak, 20% $CH_3CN$ for 5 min and 20 to 60 % $CH_3CN$ for 5 to 25 min in a total run time of 30 min) and lyophilized to obtain peptide **1**.

Texture: white powder

Yield: 24 mg; 8.2%, with respect to the resin loading

ESI-MS: (m/z) 854 $[M+H]^+$, 427 $[M+2H]^{2+}$

LC-MS: $R_t$ 3.9 min ($C_{18}$ 5 % $CH_3CN$ for 2 min, 5 to 70 % $CH_3CN$ for 2 to 5 min in a total run time of 8 min linear gradient).

- **Synthesis of BBDP containing model β-breaker peptide, $H_2N$-Leu-Ser-Asp(OBzl)-Ser-Leu-Gly-$NH_2$ (2)**

Rink amide resin: loading 1.1 mmol/g; 300 mg; 0.33 mmol

| Amino acid/ amount (mg) / eq. | | Coupling time | Kaiser test | Capping | Notes |
|---|---|---|---|---|---|
| Fmoc-Gly-OH/ | 294/3 | 1.5 h | -ve | yes | - |
| Fmoc-Ser(OtBu)-OH/ | 252/2 | 45 min | -ve | - | - |
| Fmoc-Leu-OH/ | 232/2 | 1 h | -ve | - | - |
| Fmoc-Ser (OtBu)-OH/ | 252/2 | 45 min | -ve | - | - |
| Fmoc-Asp(OBzl)-OH/ | 440/3 | 1 h x 2 | -ve | - | - |
| Fmoc-Ser(OtBu)-OH/ | 252/2 | 45 min | -ve | - | - |
| Fmoc-Leu-OH/ | 232/2 | 1 h | -ve | - | - |

*Cleavage of protecting groups and peptide from resin*

After final Fmoc removal peptide was cleaved from the resin using a mixture of TFA/DCM (4:1) 3 mL for 3 h. Crude peptide was precipitated using cold diethyl ether, centrifuged and washed 3 times with ether. Peptide was purified on semi preparative HPLC ($C_{18}$ μ Bondapak, 20% $CH_3CN$ for 5 min and 20 to 60 % $CH_3CN$ for 5 to 25 min in a total run time of 30 min) and lyophilized to obtain peptide **2**.

Texture: white powder

Yield: 22 mg, 9 %, with respect to the resin loading

ESI-MS: (m/z) 767 $[M+H]^+$

LC-MS: $R_t$ 3.9 min ($C_{18}$ 5 % $CH_3CN$ for 2 min; 5 to 70 % $CH_3CN$ for 2 to 5 min in a total run time of 8 min linear gradient).

- **Synthesis of the model aggregating peptide $H_2N$-Ser-Leu-Ser-Leu-($H^+$)Ser-Leu-Ser-Gly-$NH_2$ (3)**

Model aggregating peptide was synthesized on Rink amide resin (loading 1.1 mmol/g). Each coupling was performed using 2 equivalents of Fmoc amino acids, 2.5 equivalents of PyBOP and 5 equivalents of DIPEA. After each coupling resin was washed with DMF and DCM for 5 min each (1 min x 5) and acylation was monitored using Kaiser test. At serine switch position Boc-Ser-OH (3 eq.) in 1 mL of DMF followed by PyBop (3.5 eq.) and DIPEA (7 eq.) in 2 mL of DMF was taken and kept for rotation for three cycles. Next, Fmoc-Leu-OH was also used in three equivalents and continued to ensure complete coupling.

Rink amide resin: loading 1.1 mmol/g; 300 mg; 0.33 mmol

| Amino acid/ amount (mg) / eq. | | Coupling time | Kaiser test | Capping | Notes |
|---|---|---|---|---|---|
| Fmoc-Gly-OH/ | 294/3 | 1.5 h | -ve | yes | - |
| Fmoc-Ser(OtBu)-OH/ | 252/2 | 45 min | -ve | - | - |
| Fmoc-Leu-OH/ | 232/2 | 1 h | -ve | - | - |
| Boc-Ser-OH/ | 203/3 | 1 h x 2 (3 times) | -ve. | - | Esterification |
| Fmoc-Leu-OH/ | 349/3 | 1 h x 2 (3 times) | N.A. | - | - |
| Fmoc-Ser(OtBu)-OH/ | 252/2 | 45 min | -ve | - | - |
| Fmoc-Leu-OH/ | 232/2 | 1 h | -ve | - | - |
| Fmoc-Ser(OtBu)-OH/ | 252/2 | 45 min | -ve | - | - |

*Cleavage of protecting groups and peptide from resin*

After final Fmoc removal peptide was cleaved from the resin using TFA/DCM (4:1) 3 mL for 2.5 h. Crude peptide was precipitated on cold diethyl ether, centrifuged and washed with ether for three times. Peptide was later purified on semi preparative HPLC ($C_{18}$ µ Bondapak,

5 to 60 % $CH_3CN$ for 25 min linear gradient, total run time of 30 min) and lyophilized to obtain peptide **3**.

Texture: white fluffy powder

Yield: (22 mg, 9 %)

ESI-MS: (m/z) 762 $[M+H]^+$, 381 $[M+2H]^{2+}$

LC-MS: $R_t$ 4.0 min ($C_{18}$ 5 % $CH_3CN$ for 2 min; 5 to 60 % $CH_3CN$ for 2 to 6 min, in a total run time of 8 min linear gradient).

- **Synthesis of Aβ derived model aggregating peptide Ac-Ser-Leu-Ser-Leu-His-Gln-Lys-Leu-Val-Phe-Phe-($H^+$)Ser-Glu-Asp-Val-Ser-Leu-Glu-$NH_2$ (4)**

Peptide was prepared by stepwise solid phase peptide synthesis using Fmoc/tBu protection strategy on Rink amide resin (loading 1.1. mmol /g). Resin was swollen in DCM for 1.5 h and DMF for 0.5 h. 2 equivalents of Fmoc amino acids, 2. 5 equivalents of PyBOP and 5 equivalents of DIPEA were used. Coupling time varied from 45 min to 6 h. Each coupling was monitored by Kaiser test and in case of incomplete acylations coupling cycle was repeated followed by capping. Fmoc removal was achieved using 20 % piperidine in DMF mixture for 21 min (7 min x3).

Rink amide resin: loading (1.1 mmol/g); 300 mg; 0.33 mmol

| Amino acid/ amount (mg) / eq. | | Coupling time | Kaiser test | Capping | Notes |
|---|---|---|---|---|---|
| Fmoc-Gly-OH/ | 294/3 | 1.5 h | -ve | yes | - |
| Fmoc-Leu-OH/ | 232/2 | 1 h | -ve | - | - |
| Fmoc-Ser(OtBu)-OH/ | 252/2 | 45 min | -ve | - | - |

| | | | | | |
|---|---|---|---|---|---|
| Fmoc-Val-OH/ | 223/3 | 1 h | -ve | - | - |
| Fmoc-Asp(OBut)-OH/ | 271/2 | 1 h x 2 | -ve | - | - |
| Fmoc-Glu(OBut)-OH/ | 376/2 | 1 h x 2 | -ve | - | - |
| Boc-Ser-OH/ | 203/3 | 1 h x 2 | N.A. | - | Esterification |
| Fmoc-Phe-OH/ | 383/3 | 2 h x 2 | -ve | - | - |
| Fmoc-Phe-OH/ | 255/2 | 1 h x 2 | -ve | - | - |
| Fmoc-Val-OH/ | 272/2 | 1 h x 2 | -ve | - | - |
| Fmoc-Leu-OH/ | 232/2 | 1 h x 2 | -ve | - | - |
| Fmoc-Lys(Boc)-OH/ | 308/2 | 1 h x 2 | -ve | - | - |
| Fmoc-Gln(Trt)-OH/ | 603/3 | 2h x 3 | +ve | yes | - |
| Fmoc-His(Trt)-OH/ | 612/3 | 2h x 3 | +ve | yes | - |
| Fmoc-Leu-OH/ | 232/2 | 1 h x 2 | -ve | - | - |
| Fmoc-Ser(OtBu)-OH/ | 252/2 | 45 min | -ve | - | - |
| Fmoc-Leu-OH/ | 232/2 | 1 h | -ve | - | - |
| Fmoc-Ser(OtBu)-OH/ | 252/2 | 45 min | -ve | yes | - |
| | | | | Final | |
| | | | | Capping | |

*Cleavage of protecting groups and peptide from resin*

After final Fmoc removal resin was washed with DMF and DCM for 5 times each (1 min x 5). Peptide was acetylated using $Ac_2O$ (3 equivalents) and *N*-methyl imidazole (3 equivalents) in DCM solvent for 1 h (30 min x 2). Finally peptide was cleaved from the resin using TFA/DCM (4:1) 3 mL for 3 h was used. Crude peptide was precipitated using cold diethyl ether resin was washed with TFA for three times. Crude peptide was centrifuged, washed with ether for 3 times and purified using semi preparative HPLC ($C_{18}$ µ Bondapak,

linear gradient of 0 to 60 % $CH_3CN$ for 0 to 20 min was used in a total run time of 25 min) and lyophilized to obtain peptide **4**.

Texture: white fluffy powder

Yield: 28 mg, 4 %, with respect to the resin loading.

ESI-MS: (m/z) 2047 $[M+H]^+$, 1024 $[M+2H]^{2+}$, 683 $[M+3H]^{3+}$

UPLC: $R_t$ 3.6 min ($C_{18}$ linear gradient of 60 % $CH_3CN$ 6 min, in a total run time of 8 min).

- **Synthesis of the Aβ derived homologous BBDP containing peptide Ac-Ser-Leu-Ser-Leu-His-Gln-Lys-Leu-Asp(OBzl)-Phe-Phe-Ala-Glu-Asp-Val-Ser-Leu-Gly-$NH_2$ (5)**

Peptide was synthesized on Rink amide resin (loading 1.1 mmol /g). Resin was swollen in DCM for 1.5 h and DMF for 0.5 h. Each coupling was performed with 2 equivalents of Fmoc amino acid, 2.5 equivalents of PyBOP and 5 equivalents of DIPEA. After each coupling resin was washed with DMF and DCM 5 min each (1 min x 5). Coupling was monitored by Kaiser test. For Fmoc deprotection 20 % piperidine in DMF was used for 21 min (7 min x 3). From Asp(OBzl) 0.1 M HOBt was added in piperidine/DMF mixture.

Rink amide resin loading 1.1 mol/g; 300mg; 0.33 mmol

| Amino acid/ amount (mg) / eq. | | Coupling time | Kaiser test | Capping | Notes |
|---|---|---|---|---|---|
| Fmoc-Gly-OH/ | 294/3 | 1.5 h | -ve | yes | - |
| Fmoc-Leu-OH/ | 232/2 | 1 h | -ve | - | - |
| Fmoc-Ser(OtBu)-OH/ | 252/2 | 45 min | -ve | - | - |
| Fmoc-Val-OH/ | 223/3 | 1 h | -ve | - | - |
| Fmoc-Asp(OBut)-OH/ | 271/2 | 1 h x 2 | -ve | - | - |

| | | | | | |
|---|---|---|---|---|---|
| Fmoc-Glu(OBut)-OH/ | 376/2 | 1 h x 2 | yes | - | - |
| Fmoc-Ala-OH/ | 205/2 | 1 h | -ve | - | - |
| Fmoc-Phe-OH/ | 255/2 | 1 h x 2 | -ve | - | - |
| Fmoc-Phe-OH/ | 255/2 | 1 h x 2 | -ve | - | - |
| Fmoc-Asp(OBzl)-OH/ | 293/2 | 1 h x 2 | -ve | - | - |
| Fmoc-Leu-OH/ | 232/2 | 1 h x 2 | -ve | - | - |
| Fmoc-Lys(Boc)-OH/ | 308/2 | 1 h x 2 | -ve | - | - |
| Fmoc-Gln(Trt)-OH/ | 603/3 | 2h x 3 | +ve | yes | - |
| Fmoc-His(Trt)-OH/ | 612/3 | 2h x 3 | +ve | yes | - |
| Fmoc-Leu-OH/ | 232/2 | 1 h x 2 | -ve | - | - |
| Fmoc-Ser(OtBu)-OH/ | 252/2 | 45 min | -ve | - | - |
| Fmoc-Leu-OH/ | 232/2 | 1 h | -ve | - | - |
| Fmoc-Ser(OtBu)-OH/ | 252/2 | 45 min | -ve | yes | - |

*Cleavage of protecting groups and peptide from resin*

After final Fmoc removal resin was washed with DMF and DCM for 5 times each (1 min x 5). Peptide was acetylated using $Ac_2O$ (3 equivalents) and *N*-methyl imidazole (3 equivalents) in DCM solvent for 1 h (30 min x 2). Finally peptide was cleaved from the resin using TFA/DCM (4:1) 3 mL for 3 h was used. Crude peptide was precipitated using cold diethyl ether resin was washed with TFA for three times. Crude peptide was centrifuged, washed with ether for 3 times and purified using semi preparative HPLC ($C_{18}$ µ Bondapak, linear gradient of 0 to 60 % $CH_3CN$ for 0 to 20 min was used in a total run time of 25 min) and lyophilized to obtain peptide **5**.

Texture: white powder

Yield: 17 mg, 2.4 %, with respect to the resin loading.

ESI-MS: (m/z) 2137 $[M+H]^+$, 1069 $[M+2H]^{2+}$, 713 $[M+3H]^{3+}$

UPLC: $R_t$ 4.3 min ($C_{18}$ linear gradient 5 to 20 % $CH_3CN$ for 2 min; 20 to 60 % $CH_3CN$ for 2 to 6 min, in a total run time of 8 min linear gradient).

**Synthesis of β breaker peptides**

Both the β breaker peptides were synthesized on Rink amide resin (loading 1.1 mmol/g). Each coupling was performed with 2 equivalent of Fmoc amino acid, 2.5 equivalent of PyBOP, 5 equivalent of DIPEA. After each coupling resin was washed with DMF and DCM for 5 min each (1 min x 5). Coupling was monitored by Kaiser test. Fmoc deprotection was achieved using a solution of 20 % piperidine in DMF for 21 min (7 min x 3). 0.1 M of HOBt was used in case of peptide 6 after Asp(OBzl).

- **Synthesis of BBDP containing β-breaker peptide Ac-Leu-Asp(OBzl)-Phe-Phe-Asp-$NH_2$ (6)**

Rink amide resin: loading 1.1 mmol /g, 300 mg, 0.33 mmol

| Amino acid/ amount (mg) / eq. | | Coupling time | Kaiser test | Capping | Notes |
|---|---|---|---|---|---|
| Fmoc-Asp(OBut)-OH/ | 406/3 | 1 h x 2 | -ve | yes | - |
| Fmoc-Phe-OH/ | 255/2 | 1 h x 2 | -ve | - | - |
| Fmoc-Phe-OH/ | 255/2 | 1 h x 2 | -ve | - | - |
| Fmoc-Asp(OBzl)-OH/ | 440/3 | 1 h x 2 | -ve | - | - |
| Fmoc-Leu-OH/ | 349/3 | 1 h x 3 | -ve | - | - |
| | | | | Final acetylation | |

*Cleavage of protecting groups and peptide from resin*

After final Fmoc removal peptide was acetylated using $Ac_2O$ (3 equivalents) and *N*-methyl imidazole (3 equivalents). Peptide was cleaved from the resin using TFA/DCM (4:1) for 3 h. Crude peptide was precipitated using cold diethyl ether and purified using semi preparative HPLC ($C_{18}$ μ Bondapak, linear gradient of 5 to 95% $CH_3CN$ for 25 min in a total run time of 25 min) and lyophilized to obtain peptide **6**.

Texture: white powder

Yield: 20 mg, 7.9 %, with respect to the resin loading.

ESI-MS: (m/z) 787 $[M+H]^+$

HPLC: $R_t$ 13.5 min ($C_8$ linear gradient 0 to 100% $CH_3CN$ for 18 min, in a total run time of 20 min).

➢ **Synthesis of the control β-breaker peptide Ac-Leu-Pro-Phe-Phe-Asp-$NH_2$ (7)**

Rink amide resin: loading 1.1 mmol /g, 300 mg, 0.33 mmol

| Amino acid/ amount (mg) / eq. | | Coupling time | Kaiser test | Capping | Notes |
|---|---|---|---|---|---|
| Fmoc-Asp(OBut)-OH/ | 406/3 | 1 h x 2 | -ve | yes | - |
| Fmoc-Phe-OH/ | 255/2 | 1 h x 2 | -ve | - | - |
| Fmoc-Phe-OH/ | 255/2 | 1 h x 2 | -ve | - | - |
| Fmoc-Pro-OH/ | 333/3 | 1 h x 2 | -ve | - | - |
| Fmoc-Leu-OH/ | 349/3 | 1 h x 3 | -ve | - Final acetylation | - |

*Cleavage of protecting groups and peptide from resin*

After final Fmoc removal peptide was acetylated using $Ac_2O$ (3 equivalents) and *N*-methyl imidazole (3 equivalents). Peptide was cleaved from the resin using TFA/DCM (4:1) for 3 h. Crude peptide was precipitated using cold diethyl ether and purified using semi preparative HPLC ($C_{18}$ μ Bondapak, linear gradient of 5 to 95% $CH_3CN$ for 25 min in a total run time of 25 min) and lyophilized to obtain peptide **7**.

Texture: white powder

Yield: 36 mg, 16 %, with respect to the resin loading.

ESI-MS: (m/z) 679 $[M+H]^+$

HPLC: $R_t$ 11.5 min ($C_8$ linear gradient 0 to 100% $CH_3CN$ for 18 min, in a total run time of 20 min).

- **Synthesis of the triple mutant analogue of the Aβ peptide, $H_2N$-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Pro-His-His-Gln-Lys-Leu-Pro-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Pro-Ile-Gly-Leu-Met-Val-Gly-Gly-$NH_2$ (P3Aβ38, peptide 8)**

Triple mutant P3Aβ38 was assembled on 150 mg of Rink amide MBHA resin (loading 1.1 mmol/ g). Each coupling was performed with 2 equivalents of Fmoc amino acids, 2.5 equivalents of PyBOP and 5 equivalents of DIPEA. At Pro and other bulky residues number of equivalents was increased to 3 and coupling step was repeated. After each coupling resin was washed with DMF and DCM for 5 min each (1min x 5). Coupling was monitored by

Kaiser test and micro cleavage test at some points. Fmoc deprotection was achieved using 20 % piperidine in DMF.

Rink amide resin: loading (1.1 mmol/g); 150 mg; 0.165 mmol

| **Amino acid/ amount (mg) / eq.** | | **Coupling time** | **Kaiser test** | **Capping** | **Notes** |
|---|---|---|---|---|---|
| Fmoc-Gly-OH/ | 147/3 | 1.5 h | -ve | - | - |
| Fmoc-Gly-OH/ | 98/2 | 45 min + 1h | -ve | - | - |
| Fmoc-Val-OH/ | 113/2 | 1 h | -ve | - | - |
| Fmoc-Met-OH/ | 122/2 | 1 h | -ve | - | - |
| Fmoc-Leu-OH/ | 116/2 | 1 h | -ve | - | - |
| Fmoc-Gly-OH/ | 98/2 | 1 h | -ve | - | - |
| Fmoc-Ile-OH/ | 116/2 | 1 h | -ve | - | - |
| Fmoc-Pro-OH/ | 111/2 | 1 h x 3 | -ve | - | - |
| Fmoc-Ala-OH/ | 102/2 | 1 h x 3 | -ve | - | 2 eq extra |
| Fmoc-Gly-OH/ | 98/2 | 1 h x 2 | -ve | - | - |
| Fmoc-Lys(Boc)-OH/ | 154/2 | 1 h x 3 | -ve | - | - |
| Fmoc-Asn-OH/ | 196/2 | 1h x 2 | -ve | - | - |
| Fmoc-Ser(tBu)-OH/ | 127/2 | 1 h x 2 | -ve | - | 1258 [M+H]<br>629 $[M+2H]^{2+}$ |
| Fmoc-Gly-OH/ | 98/2 | 1h x 2 | -ve | - | - |
| Fmoc-Val-OH/ | 112/2 | 2 h | -ve | - | - |
| Fmoc-Asp(tBu)-OH/ | 135/2 | 2 h | -ve | - | - |
| Fmoc-Glu(OtBu)-OH/ | 140/2 | 2 h x 2 | +ve | yes | - |
| Fmoc-Ala-OH/ | 102/2 | 1h x 2 | -ve | - | - |
| Fmoc-Phe-OH/ | 127/2 | 1h x 3 | -ve | - | - |
| Fmoc-Phe-OH/ | 191/3 | 1h x 3 | -ve | - | - |

| | | | | | |
|---|---|---|---|---|---|
| Fmoc-Pro-OH/ | 167/3 | 1h x 3 | -ve | - | 2043 $[M+H]^{H+}$, 1022$[M+2H]^{2H+}$ |
| Fmoc-Leu-OH/ | 171/3 | 1 h x 3 | -ve | - | - |
| Fmoc-Lys(Boc)-OH/ | 121/2 | 1 h x 3 | +ve | - | - |
| Fmoc-Glu(tBu)-OH/ | 302/3 | 1 h x 3 | -ve | yes | - |
| Fmoc-His(Trt)-OH/ | 306/3 | 1 h x 3 | +ve | yes | - |
| Fmoc-His(Trt)-OH/ | 306/3 | 1 h x 3 | -ve | yes | - |
| Fmoc-Pro-OH/ | 167/3 | 1 h x 3 | -ve | - | - |
| Fmoc-Glu(tBu)-OH/ | 210/3 | 1 h x 3 | -ve | Yes | - |
| Fmoc-Tyr(But)-OH/ | 227/3 | 1 h x 2 | -ve | - | - |
| Fmoc-Gly-OH/ | 147/3 | 1 h | -ve | - | - |
| Fmoc-ser(tBu)-OH/ | 189/3 | 1 h x 2 | -ve | - | 1732 $[M+H]^{+}$ 1156 $[M+2H]^{2+}$ 867 $[M+3H]^{3+}$ (Fmoc protected) |
| Fmoc-Asp(tBu)-OH/ | 203/3 | 1 h x 2 | -ve | - | - |
| Fmoc-His(Trt)-OH/ | 102/3 | 1 h x 3 | -ve | - | - |
| Fmoc-Arg(Pbf)-OH/ | 106/3 | 1 h x 3 | -ve | - | - |
| Fmoc-Phe-OH/ | 127/3 | 1 h x 3 | -ve | - | - |
| Fmoc-Glu(tBu)-OH/ | 140/3 | 1 h x 3 | -ve | - | - |
| Fmoc-Ala-OH/ | 102/3 | 1 h x 3 | -ve | - | - |
| Fmoc-Asp(tBu)-OH/ | 135/3 | 1 h x 3 | -ve | - | 1371 $[M+3H]^{3+}$ 1028 $[M+4H]^{4+}$ 823 $[M+5H]^{5+}$ 686 $[M+6H]^{6+}$ |

After last Fmoc removal peptide resin was washed with DMF and DCM and protecting groups and peptide was cleaved from the resin using TFA/DCM (4:1) for 3 h. Crude peptide was precipitated using cold diethyl ether. Resin was washed 3 times with TFA. Peptide was purified on semi preparative HPLC ($C_{18}$ μ Bondapak, linear gradient of 5 to 20 % $CH_3CN$ for 5 min and 20 to 60 % $CH_3CN$ for 5 to 25 min in a total run time of 30 min) and lyophilized to obtain peptide **8** (P3Aβ38).

Texture: white powder

Yield: 24 mg; 4%, with respect to the resin loading

HPLC $R_t$ = 9.7 min ($C_8$ linear gradient 5 to 95 % $CH_3CN$ for 0 to 18 min in a total run time of 20 min).

ESI-MS: (m/z) 1371 $[M+3H]^{3+}$; 1028 $[M+4H]^{4+}$; 823 $[M+5H]^{5+}$; 686 $[M+6H]^{6+}$, 588 $[M+7H]^{7+}$.

- **Synthesis of Aβ38, $H_2N$-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-$NH_2$ (peptide 9)**

Aβ38 was assembled on 150mg of Rink amide MBHA resin (loading 1.1 mmol/g). Resin was swollen in DCM for 1.5 h and DMF for 0.5 h. 10 % anisole in DMF/ DCM mixture was used as an additive in all the steps of peptide synthesis. Coupling was carried out with 2 equivalents of Fmoc amino acid, 2.5 equivalents of PyBOP (at some points

HBTU) and 5 equivalents of DIPEA was used. After each coupling resin was washed with DMF and DCM for 5 times (1 min x 5). Coupling was monitored using Kaiser test and micro cleavage test sometimes. In case of incomplete acylation capping was performed using $Ac_2O$ (3 equivalents) and *N* Methyl imidazole (3 equivalents) for 1 h (30 min x 2) For Fmoc deprotection 2 % DBU (in DMF containing 10 % anisole) was used till $Ser^8$ and time varied from 5 min to 15 min. For remaining residues 20 % piperidine in DMF (containing 10% anisole was used).

Rink amide resin: loading: 1.1 mmol/g; 150 mg; 0.165 mmol

| Amino acid/ amount (mg) / eq. | | Coupling time | Kaiser test | Capping | Notes |
|---|---|---|---|---|---|
| Fmoc-Gly-OH | 130/2 | 1 h x 2 | -ve | Yes | - |
| Fmoc-Gly-OH | 130/2 | 1 h x 2 | -ve | - | - |
| Fmoc-Val-OH | 149/2 | 1 h x 2 | -ve | - | - |
| Fmoc-Met-OH | 244/3 | 1 h x 2 | -ve | - | 584 $[M+H]^+$ (Fmoc protected) |
| Fmoc-Leu-OH | 232/3 | 1 h x 2 | -ve | - | - |
| Fmoc-Gly-OH | 196/3 | 1 h | -ve | - | - |
| Fmoc-Ile-OH | 232/3 | 1.5 h | -ve | - | - |
| Fmoc-Ile-OH | 232/3 | 1.5 h | -ve | - | - |
| Fmoc-Ala-OH | 205/3 | 1.5 h | -ve | - | - |
| Fmoc-Gly-OH | 196/3 | 1 h | -ve | - | - |
| Fmoc-Lys(Boc)-OH | 308/3 | 1 h x 3 | -ve | - | - |
| Fmoc-Asn(Trt)-OH | 393/3 | 1h x 2 | -ve | - | - |
| Fmoc-Ser(tBu)-OH | 252/3 | 1 h x 2 | -ve | - | - |
| Fmoc-Gly-OH | 196/3 | 2.5 h | -ve | yes | - |

| | | | | | |
|---|---|---|---|---|---|
| Fmoc-Val-OH | 223/2 | 2 h | -ve | - | - |
| Fmoc-Asp(tBu)-OH | 271/3 | 2 h x 2 | -ve | yes | - |
| Fmoc-Glu(OtBu)-OH | 280/3 | 2 h x 2 | -ve | - | - |
| Fmoc-Ala-OH | 205/3 | 1h x 2 | -ve | yes | - |
| Fmoc-Phe-OH | 255/3 | 1h x 3 | -ve | yes | - |
| Fmoc-Phe-OH | 255/3 | 1h x 3 | -ve | yes | - |
| Fmoc-Val-OH | 223/3 | 1h x 2 | -ve | - | - |
| Fmoc-Leu-OH | 232/3 | 1 h x 2 | -ve | - | - |
| Fmoc-Lys(Boc)-OH | 308/3 | 1 h x 3 | -ve | yes | - |
| Fmoc-Glu(tBu)-OH | 402/3 | 1 h x 3 | -ve | yes | - |
| Fmoc-His(Trt)-OH | 408/3 | 1 h x 3 | +ve | yes | - |
| Fmoc-His(Trt)-OH | 408/3 | 1 h x 3 | +ve | yes | - |
| Fmoc-Val-OH | 223/3 | 1 h x 3 | +ve | yes | - |
| Fmoc-Glu(tBu)-OH | 280/3 | 1 h x 3 | not | yes | - |
| Fmoc-Tyr(But)-OH | 302/3 | 1 h x 2 | clear | yes | - |
| Fmoc-Gly-OH | 196/3 | 1 h | -ve | - | - |
| Fmoc-Ser(tBu)-OH | 252/3 | 1 h x 3 | -ve | - | - |
| Fmoc-Asp(tBu)-OH | 271/3 | 1 h x 2 | -ve | yes | - |
| Fmoc-His(Trt)-OH | 408/3 | 1 h x 3 | -ve | yes | - |
| Fmoc-Arg(Pbf)-OH | 427/3 | 1 h x 3 | -ve | yes | - |
| Fmoc-Phe-OH | 255/3 | 1 h x 3 | -ve | - | - |
| Fmoc-Glu(tBu)-OH | 280/3 | 1 h x 3 | -ve | - | - |
| Fmoc-Ala-OH | 205/3 | 1 h x 2 | -ve | - | - |
| Fmoc-Asp(tBu)-OH | 271/3 | 1 h x 2 | -ve | | 1377[M+3H]$^{3+}$ |
| | | | -ve | | 1033 [M+4H]$^{4+}$ |

827 $[M+5H]^{5+}$

689 $[M+6H]^{6+}$

*Cleavage of protecting groups and peptide from resin*

After final deprotection of the last Fmoc group, peptide was cleaved from the resin using TFA/DCM (4:1) for 3h and precipitated using cold diethyl ether. Crude peptide was purified by semi preparative HPLC ($C_{18}$ μ Bondapak, linar gradient of 5 to 30 % $CH_3CN$ for 5 min, 30 to 60% $CH_3CN$ for 5 to 25 min in a total run time of 30 min) and lyophilized to obtain peptide **9** (Aβ38)

Texture: white powder

Yield: 12 mg, 2%, with respect to the resin loading.

HPLC: $R_t$ = 11.3 ($C_8$ 5 to95 % $CH_3CN$ for 0 to 18 min in a total run time of 20 min, linear gradient)

ESI-MS: (m/z) 1377 $[M+3H]^{3+}$; 1033 $[M+4H]^{4+}$; 827 $[M+5H]^{5+}$; 689 $[M+6H]^{6+}$

# Conclusions and future directions

The work presented in this thesis mainly centred on the development of the concept of 'β-breaker dipeptides and it's application in Alzheimer's amyloid disruption'. A dipeptide of the sequence "Asp(OBzl)-Axx" serves as the β-breaker dipeptide (BBDP). A BBDP containing peptide aligns with neighbouring (aggregating) peptide possibly co-aggregates when sequence homology is retained. At physiological pH, such BBDP undergoes a chemical conversion to aspartimide and subsequent ring opening which generates a kink and finally disrupts the native backbone of the peptide. Such chemical and structural change of the BBDP containing peptide forcefully disrupts the aggregation. Such concept can be useful for drug design against amyloidosis.

Our result indicates that the concept of β breaker dipeptides has a useful impact on the aggregation of model -(Ser-Leu)$_n$- based aggregating peptides, Aβ related smaller model aggregating peptides and full length Aβ (1-40) peptides. In the second chapter we have demonstrated that BBDP containing peptides (peptide 1 and 2) could inhibit self aggregation in a condition when BBDP unit generated an aspartimide and aspartyl residues, in agreement with our concept. Later, we used one of the BBDP containing peptide (peptide 1) to inhibit the aggregation of a model aggregating peptide (peptide 3) and demonstrated that peptide 1 indeed could stop the fibril assembly of the peptide 3 based on thioflavin T and TEM studies. Amyloid disruption of the peptide 3 in a similar condition was also demonstrated based on the results obtained from Congo red birefringence studies. Results of these experiments delivered the proof of the concept.

Having obtained the proof of the concept on model -(Ser-Leu)$_n$- based aggregating sequences we further explored the usefulness of the concept on Aβ derived sequences which behaves similar to the full length Aβ, though shorter in length (peptide 4). Aβ derived BBDP containing β-sheet breaker peptide (peptide 5) was demonstrated to inhibit or disrupt self aggregation and was used for β sheet disruption of Aβ derived model aggregating peptide (peptide 4). In mixing experiment, a substantial decrement in fibril assembly was noted.

Finally, we could design and employ a β-breaker dipeptide containing β-breaker peptide, Ac-LD(OBzl)FFD-$NH_2$ (peptide 6), similar to Soto's peptide Ac-LPFFD-$NH_2$ (peptide 7) against the aggregation of Aβ(1-40). From the experimental results we observed that peptide 7 had a **effect** comparable to Soto's peptide in terms of β sheet disruption and amyloid disruption.

We also wanted to prepare BBDP containing version of the Aβ(1-40) peptide, which when mixed with Aβ (1-40), first recognizes and later disrupts self aggregation and aggregation of neighbouring peptide as well. This can be one possible strategy against the aggregation of Aβ peptide. Prior to that we need to know the appropriate positions of -Asp(OBzl)-Axx- (BBDP) units where it can execute its action at best. Synthesis of -Asp(OBzl)-Axx- version of Aβ (1-40) is difficult and requires special purification skills. Therefore, in order to find out the suitable positions we prepared a proline mutant version of Aβ(1-38), which is likely to behave similar to the -Asp(OBzl)-Axx- containing analogue, and compared its aggregation behaviour with that of Aβ (1-38). It can be concluded that the positions we targeted (positions 12, 18 and 31) are crucial for inhibition of aggregation, where –Asp(OBzl)-Axx- unit can be incorporated. Moreover, better positions than these are likely to exist but needs a detailed scrutiny to find these out.

Although the proof of the concept is obtained, a more comprehensive investigation is necessary for extension of the concept. For example, we need to examine the toxic nature (cytotoxicity) of BBDP containing peptides against various cell lines, number of equivalents required (dose dependent studies) and appropriate positions. This directs to the development of such BBDP containing peptides as potential lead molecules for the development of therapeutics against Alzheimer's disease. Next we would like to explore the concept on other aggregating peptides like IAPP known to cause diabetes type II, and the work is likely to be carried out by my successors. We are also interested to replace Bzl (benzyl) group with other small units, e.g. hexafluoroisopropanol, ethanol, *etc.* to reduce probably associated toxicity and to check β breaking potential of such peptides.

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**Title**: "Concept of the β breaker dipeptides and its application in Alzheimer's Amyloid disruption."

Supervisor: Dr. Bhubaneswar Mandal

## Honors and Awards

- Qualified **Graduate Aptitude Test in Engineering (GATE)** 2007 organized by Ministry of Human Resource Development, Government of India.
- Secured **second position** in M.Sc. in 2007

## Research experience

- **2010-Onwards**: **Senior research fellowship** in department of chemistry, Indian Institute of Technology Guwahati, from Indian Institute of Technology Guwahati, India.
- **2008-2010**: **Junior research fellowship** in department of chemistry, Indian Institute of Technology Guwahati, from Indian Institute of Technology Guwahati, India.

## Skills

- **Instrumentation**: NMR spectroscopy, mass spectrometry (MS/LC-MS), HPLC, Fluorescence spectroscopy, Optical polarisable microscope, FT-IR spectroscopy, UV-Visible spectroscopy, polarimeter.
- **Computer** (software familiar with): MS-Office, Adobe Illustrator, Chem Draw, Scifinder, Origin, Sigma Plot.

## Teaching experience

- Both tutorial and practical (B.Tech. and M. Sc.) teaching assistant (TA) at Indian Institute of Technology Guwahati, India.

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- Cricket, soccer, travelling

## List of Publications

1. **Nadimpally**, **K**. **C**., Paul, A. and Mandal, B. "β Breaker Dipeptides and Their Application in Alzheimer's Amyloid Disruption" (*communicated*).

2. **Nadimpally**, **K**. **C**., Paul, A., Saha, A., and Mandal, B. (2013) "Modulation of Aggregation Propensity of Aβ38 by Site Specific Multiple Proline Substitution." *Int*. *J*. *Pept*. *Res*. *Ther*. 19, 365-371.

3., Saha, A.[§], **Nadimpally**, **K**. **C**.[§], Paul, A. and Mandal, B. (2014) "Phenolic Ester Mediated Oligopeptide Synthesis Promoted by HOBt." *Protein Peptide Lett*. 21, 188-193. (§ equal contribution).

4. Kishore, T., **Nadimpally**, **K**. **C**., Chakraborty, M. P., Paul, A. and Mandal, B. (2013) "Ethyl 2-(*tert*-Butoxycarbonyloxyimino)-2-Cyanoacetate (Boc-Oxyma) as Coupling Reagent for Racemization free Esterification, Thioesterification, Amidation and Peptide Synthesis" *Adv*. *Synth*. *Catal*. 355, 448-462.

5. Palakurthy, N. B., Dev, D., Rana, S., **Nadimpally**, **K**. **C**. and Mandal, B. (2012) "Sulphonamide synthesis via Oxyma-O-Sulphonates: Compatibility to Acid Sensitive Groups and SPPS" *Eur*. *J*. *Org*. *Chem*. 2013, 2627-2633.

6. Kishore, T., **Nadimpally**, **K.**, **C**., Paul, A. and Mandal, B. (2012) "Waste Reduction in Amide Synthesis by a Continuous Method Based on Recycling of the Reaction Mixture" *RSC Adv*. 2, 6838–6845.

7. **Nadimpally**, **K**. **C**., Kishore, T., Palakurthy, N. B., Saha, A. and Mandal, B. (2011) "Catalyst and Solvent Free Amidation of Inactive Esters of *N*-protected Amino Acids" *Tetrahedron Lett*. 52, 2579-2582.

## Conferences / Symposiums

1. **Nadimpally**, **K**. **C**., Paul, A., and Mandal, B. “Towards the development of the concept of β breaker dipeptides to disrupt Alzheimer’s amyloid.” *4th Indian Peptide Symposium-2013*, 21-23 Feb, Saha Institute of Nuclear Physics, Kolkata.

2. **Nadimpally**, **K**. **C**., Paul, A., and Mandal, B. Modulation of aggregation propensity of amyloid beta (1-38) by site specific multiple proline substitution", *8th J-NOST (National Organic Symposium Trust) Conference,* 15 -17 Dec, 2012 IIT Guwahati.

3. **Nadimpally**, **K**. **C**., Thalluri, K., Paul, A., Palakurthy, N. B., and Mandal, B. "Catalyst and solvent free amidation of inactive esters of *N*-protected amino acids: A green chemistry perspective", *CRSI-2012 (national symposium)*, IISER Trivandrum & NIIST Trivandrum, 3–5 February, Trivandrum.

4. Saha, A., **Nadimpally**, **K**. **C**., and Mandal, B. "A rapid repetitive and cost effective chemoselective ligation protocol for peptide synthesis", *International Symposium on the Advances in Chemical Sciences*, IIT Guwahati, 30th January, Guwahati.

5. **Nadimpally**, **K**. **C**., Kishore, T., Mandal, S., and Mandal, B. “Facile Synthesis of Water Soluble Peptide Based Polymers.” *FICS-2010* (*national symposium*), IIT Guwahati, 3-4 December, Guwahati.