

Design, Synthesis, and Evaluation of Peptide Conjugates for Alzheimer's Amyloid Disruption

*A Dissertation Submitted to the
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Dedicated
to
My Parents & Almighty



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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 supervision of Prof. Bhubaneswar Mandal.

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

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CERTIFICATE

This is to certify that **Mr. Tanmay Mondal** has been working under my supervision since July 2013 as a regular registered Ph. D. student. I am forwarding his thesis entitled “**Design, Synthesis, and Evaluation of Peptide Conjugates for Alzheimer’s Amyloid Disruption**” for being submitted for the Ph. D. degree from 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.

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Synopsis

The thesis entitled, “Design, Synthesis, and Evaluation of Peptide Conjugates for Alzheimer’s Amyloid Disruption” is divided into five main chapters. The abstracts of the main chapters are described below,

Chapter 1: Introduction

Despite the enormous efforts and progress made in both the diagnosis and treatment of Alzheimer’s disease (AD), there is no cure available to date.¹ The available drugs on the market are good at improving the patient’s condition of anxiety, insomnia, or depression only. Therefore design and development of an efficient drug for the treatment of AD is highly desirable.

Several unfolded and misfolded proteins accumulate and deposit as insoluble fibrous protein aggregates known as amyloid (abundant in β -sheet structure) by changing the conformational structure. Deposition of amyloid caused various human diseases called amyloidoses, such as AD, Parkinson’s disease, and type-2 diabetes mellitus. Amyloid β (A β) peptides; including soluble monomers, oligomers, and insoluble fibrils and plaques, are considered as the foremost culprit of AD neuropathology. According to “amyloid cascade hypothesis”,² fibril formation and plaque deposition of A β peptides outside nerve cells cause neuronal dysfunction, dementia, and death in AD. Despite the existing controversy on the amyloid cascade hypothesis, it is accepted that the main determinants of A β toxicity lie in its aggregation state.³ However, aggregation of hyperphosphorylated tau protein in the form of neurofibrillary tangles inside the neuron is also believed to have a causative role.⁴

A β peptides containing 39-42 residues are generated in the amyloidogenic pathway of the amyloid precursor protein (APP) processing by sequential proteolysis by β - and γ -secretases. Therefore many strategies are being developed for the reduction of aggregation⁵ and facilitation of excretion of A β from the brain.⁶ However, works on the controlled production of A β is relatively less; that again, mostly depends on the development of β/γ secretase inhibitor.⁷

Alternatively, non-amyloidogenic APP processing pathway includes site-specific cleavage by α -secretase to release neuroprotective APP fragment soluble APP- α (sAPP α) and prevents the generation of neurotoxic and pathogenic A β peptide.⁸ Therefore, the non-amyloidogenic pathway is healthy and does not cause dementia. Increasing the α -secretase-mediated processing of APP is, therefore, a therapeutic option for the treatment of AD.⁹ Design and development of enhancer or agonist of α -secretase also can be a promising approach for drug design against AD.

Chapter 2: *De novo* designed peptidic constructs cleave Amyloid β at the α -secretase cleavage site by aggregation driven cascades of chemical reactions

Inspired by the mechanism of proteolytic maturation of the Host Cell Factor-1 (HCF1),¹⁰ we have designed and developed a peptide-based construct that is intended to recognize the homologous sequence on A β or APP and cleave them at the α -secretase cleavage site to generate non-toxic sAPP α and to reduce toxic A β . Such constructs are mentioned as artificial α -Secretases (ASs). ASs are supposed to mimic the APP processing activity of α -secretase specifically, although in a non-catalytic manner, but leave all other substrates of α -secretase unaffected, reducing side effects. Usually, α -Secretase cleaves soluble folded APP, but cannot metabolize aggregated A β due to its solvent inaccessibility. However, the ASs are designed to act by self-assembly driven proteolysis; therefore, can truncate and dissolve aggregated A β as well. Such constructs may lead to a robust strategy for drug design against not only AD but also other amyloidoses.

Plausible routes of the proteolytic activity of ASs on model A β (functional mimic of A β) and A β were drawn from MALDI-TOF mass analyses; where *in-situ* trans-amidation and imide bond formation mechanism were found to play important roles towards the self-assembly driven site-selective proteolysis between ¹⁶Lys-¹⁷Leu and ¹⁷Leu-¹⁸Val. Higher proteolytic efficacy of AS4 was found due to a synergistic effect in place compare to AS1-AS3. Again, site-specific nature (towards aggregated protein) of AS4 was confirmed after performing a negative control experiment with 28 membered mutant diphtheria toxin (DTP28) sequence by replacing ⁸Val and ²³Lys by Pro. The kinetics of amyloid accumulation in the form of fibrils was monitored *in vitro* by many biophysical tools; e.g., ThT-assay, TEM, Congo red birefringence, AFM, and DLS study.^{11,12} The conformational alterations during the aggregation process were characterized by CD,¹³

FTIR; and cell membrane disruption capability was checked by vesicle leakage study to confirm that AS4 disrupted the preformed amyloid of A β into non-toxic metabolites.^{14,15}

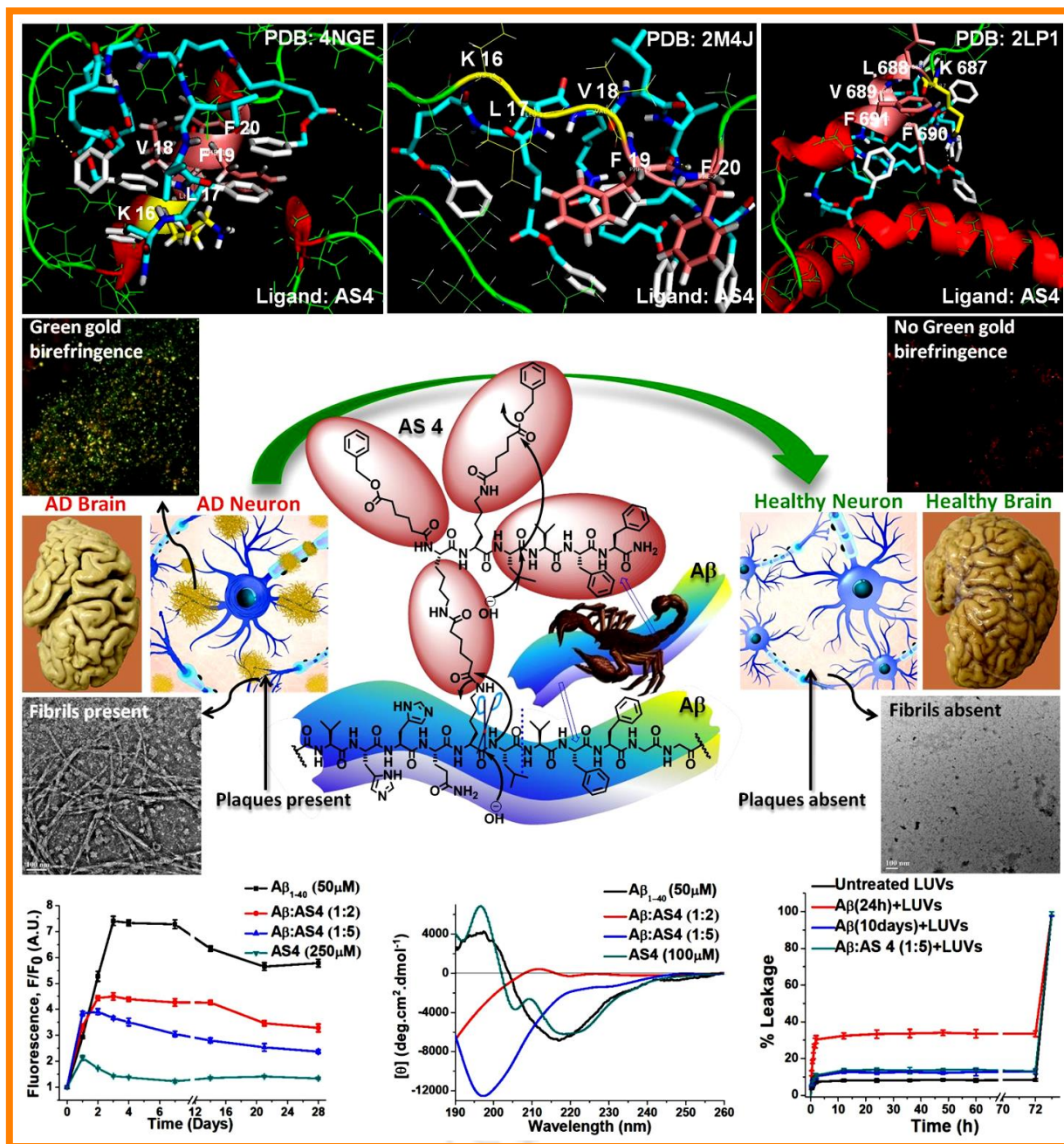


Figure 1. The Concept of function specific proteolytic activity of "Artificial α - Secretases" and the kinetics of amyloid accumulation of A β ₁₋₄₀ in absence and presence of AS4 in PBS pH 7.4 (50 mM) at 37 °C.

From ThT-assay, it was found that the fluorescence intensity of A β increased with time in the absence of AS4. However, in the presence of AS4, though a significant increase in fluorescence signal of A β was observed initially probably due to the formation of the

intermediate (peptide conjugate) and co-aggregation which were slowly suppressed thereafter probably due to the formation of various fragments resulting from site-selective cleavage. The β -sheet prevailing structure of A β was obtained when incubated alone, but AS4 prevented its β -sheet formation. The prevailing random coil structure of A β was inferred from the -ve Cotton effect around 195 nm and absence of the same around 214 nm. A β alone formed clear fibrillar structure viewed under TEM, but in the presence of AS4, no characteristic fibrillar structure was observed. On the other hand, AS4 did not appear as a fibrillar structure. After staining with Congo red, characteristic green-gold birefringence was observed for A β when viewed under cross-polarized light, but no such green-gold birefringence was observed either for the mixture of A β and AS4 or for AS4 alone. The results suggest that AS4 inhibits fibrillization and disrupts existing fibril in a dose-dependent manner.

Chapter 3: Digestion of Amyloid β by Gly-integrated miniature artificial proteases

Targeted protein degradation is a hot field of modern drug discovery as it provides a new therapeutic opportunity to eliminate disease-causing proteins by inhibiting the protein function selectively.^{16,17} Enzymatic hydrolysis of a peptide bond is initiated usually by an amino acid nucleophile polarized by a catalytic base in touch with it. In this context the conventional Ser-His-Asp triad is probably the most well-known in existing protease family and the essentially identical stereochemistry of triad is maintained in several diverse groups of peptidases whose folding patterns are exclusively different.¹⁸ The proteolytic function of Miniature Artificial Proteases (mAPs) by Gly integrated new main chain framework is described in this work, unlike the DHS catalytic triad exist in different folding scaffolds. mAPs were designed in such a way that its active site has a pocket where Asp, His, and Ser are linked via intramolecular hydrogen bonding so that Ser participates the hydrolysis of a peptide bond adjacent to it. Invented mAPs cleave a smaller A β fragment (A β ₁₂₋₂₁ or APP₆₈₃₋₆₉₂, mA β) and A β also in a site-selective manner; and it cleaves every amide bond in-between Lys₁₆-Phe₂₀ initially at the physiological condition, which was confirmed from MALDI-TOF mass analyses. The cleavage of the backbone of a peptide or protein related to disease may treat the disease to heal. Results obtained from several biophysical tools (ThT-assay, CD, FTIR, TEM, Congo red birefringence, AFM, DLS, *etc.*) suggest that mAPs are non-amyloidogenic in nature and significantly disrupted the preformed amyloid of A β into non-toxic metabolites *in vitro*. A

relatively lower dose of mAP3 was found to inhibit significantly and also disrupt the preformed amyloid aggregation of A β ₁₋₄₀ *in vitro* and may reduce the side effects. Hence our innovative paradigm based on mAPs offers a new therapeutic option against amyloidoses.

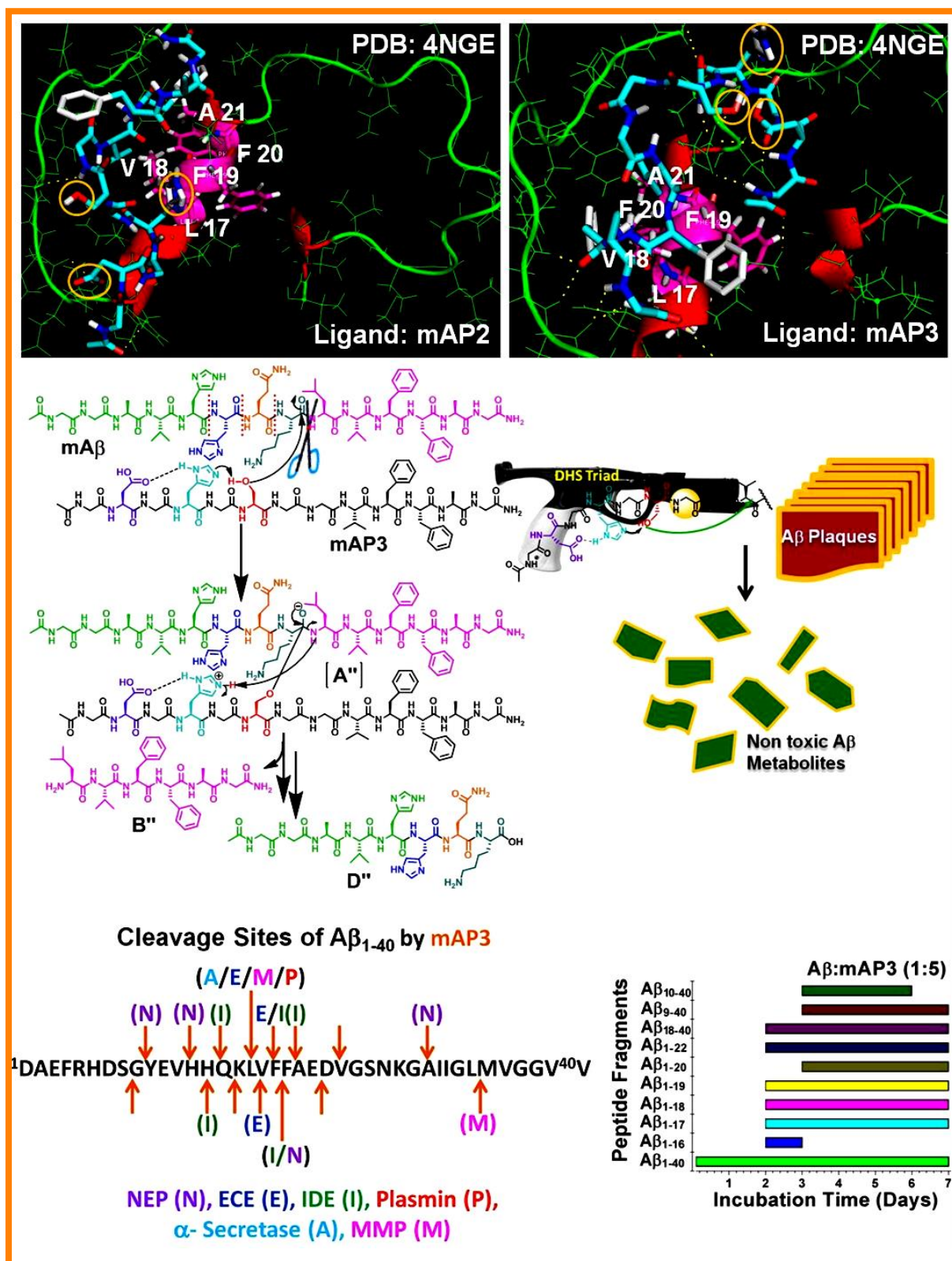


Figure 2. Concept, plausible route of the proteolysis, and multiple proteolytic enzyme activities of mAP3.

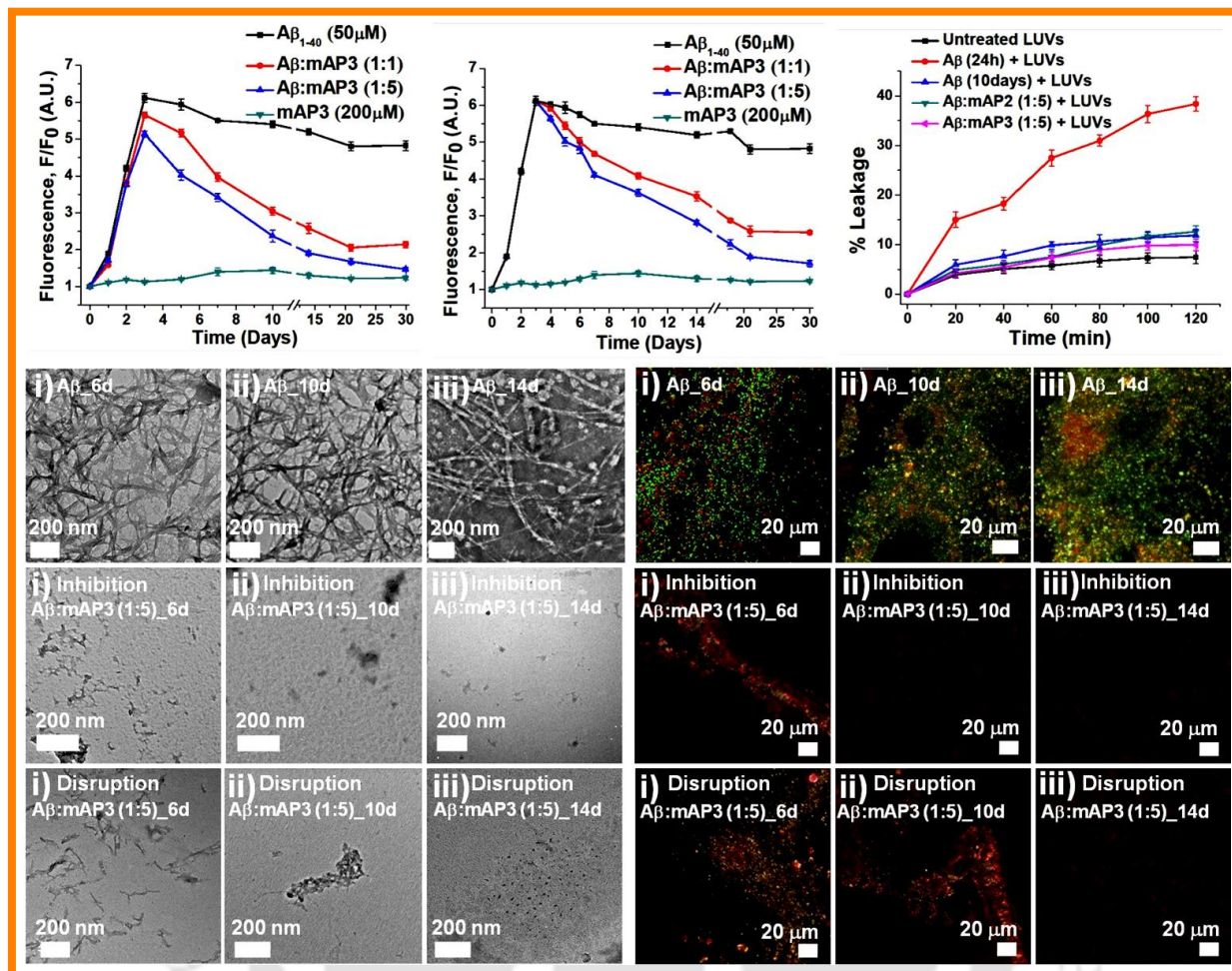


Figure 3. Various biophysical studies to monitor the kinetics of amyloid accumulation of Aβ₁₋₄₀ in the absence and presence of mAP3 in PBS pH 7.4 (50 mM) at 37 °C.

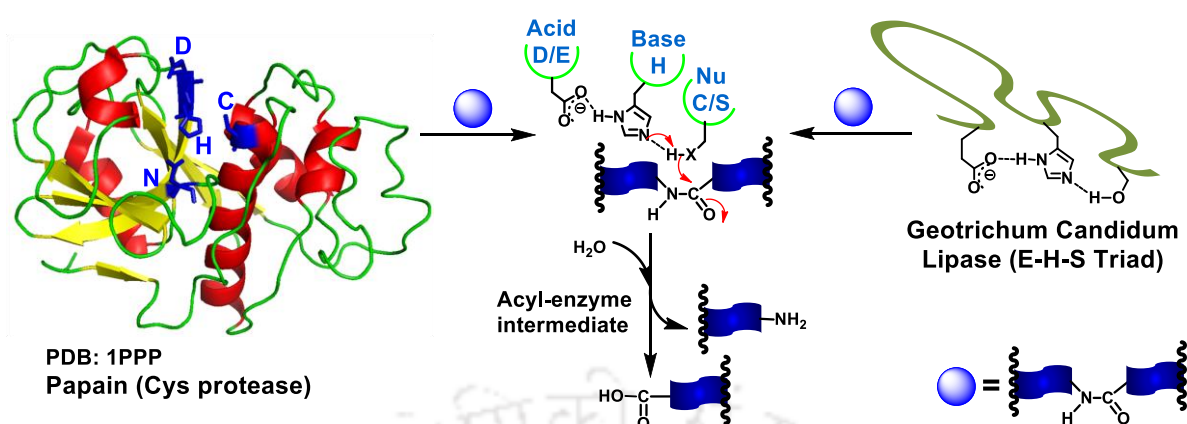
From the result of dose-dependent ThT-assay, the fluorescence intensity of Aβ₁₋₄₀ was found to be increased with time in the absence of mAP3, but decreased with time in the presence of mAP3 as the doses were increased from one-fold to five-fold molar excess due to the formation of various Aβ₁₋₄₀ fragments cleaved by mAP3, indicating the excellent inhibitory efficacy of mAP3 against amyloid formation of Aβ₁₋₄₀. From the time-dependent TEM and Congo red stained birefringence studies, it was observed that while the fibrillar structure and green-gold birefringence continued for Aβ₁₋₄₀ during the entire duration of the experiment, they gradually decreased and finally disappeared at 14 days of incubation in the presence of five-fold mAP3. After analyzing various

experiments, it was concluded that mAP3 was able to inhibit and disrupt the amyloid formation of aggregated $A\beta_{1-40}$.

Chapter 4: Native and non-native triad based protease mimics digest Amyloid β

Targeted protein degradation is imperative for both chemical biology and drug design research. Though new well-designed techniques namely, endosome targeting chimeras (ENDTAC)¹⁹ and lysosome targeting chimeras (LYTACs)²⁰ have been demonstrated to internalize and degrade extracellular proteins, no proteolysis targeting chimeras (PROTACs)²¹ or photoswitchable PROTACs (PHOTACs)²² is known yet for extracellular $A\beta$ degradation. In this chapter, a series of conventional and unconventional Triad based peptide-conjugate are designed linking a target protein recognizing unit with a small proteolytic unit, termed as “Protease Mimic (PM)”. The important feature of the design is that the proteolytic activity of a catalytic triad of Papain (cysteine protease) or *Geotrichium Candidum* Lipase (serine protease) active site could be mimicked by integrating the Triad residues by flexible Gly between each of them that acted as the small proteolytic unit. Notably, the catalytic mechanism of the cysteine-based Triad is boosted by thiol/disulfide exchange cleavage.²³ The thiolate of Cys attacks the intermolecular disulfide bond (formed by PM) in a nucleophilic substitution reaction resulting in a target protein bound intermediate (by hydrophobic interaction in-between PM and $A\beta$) with a new thiolate which can shear the adjacent protein. The designed PM cleaved a smaller fragment of $A\beta$ ($A\beta_{12-21}$, m $A\beta$) and the full-length of $A\beta_{1-40}$ at the physiological pH and temperature, which was confirmed by MALDI-TOF mass spectra analyses. Results obtained from several biophysical tools (ThT-assay, CD, FTIR, TEM, Congo red based birefringence, and DLS) suggest that PM is inherently non-amyloidogenic but significantly inhibited the aggregation of both m $A\beta$ and $A\beta_{1-40}$ and also disrupted the preformed amyloid of $A\beta_{1-40}$ into non-toxic metabolites.

a) Proteolysis by Papain and Geotrichum Candidum Lipase:



b) Proteolysis by Protease Mimic (PM):

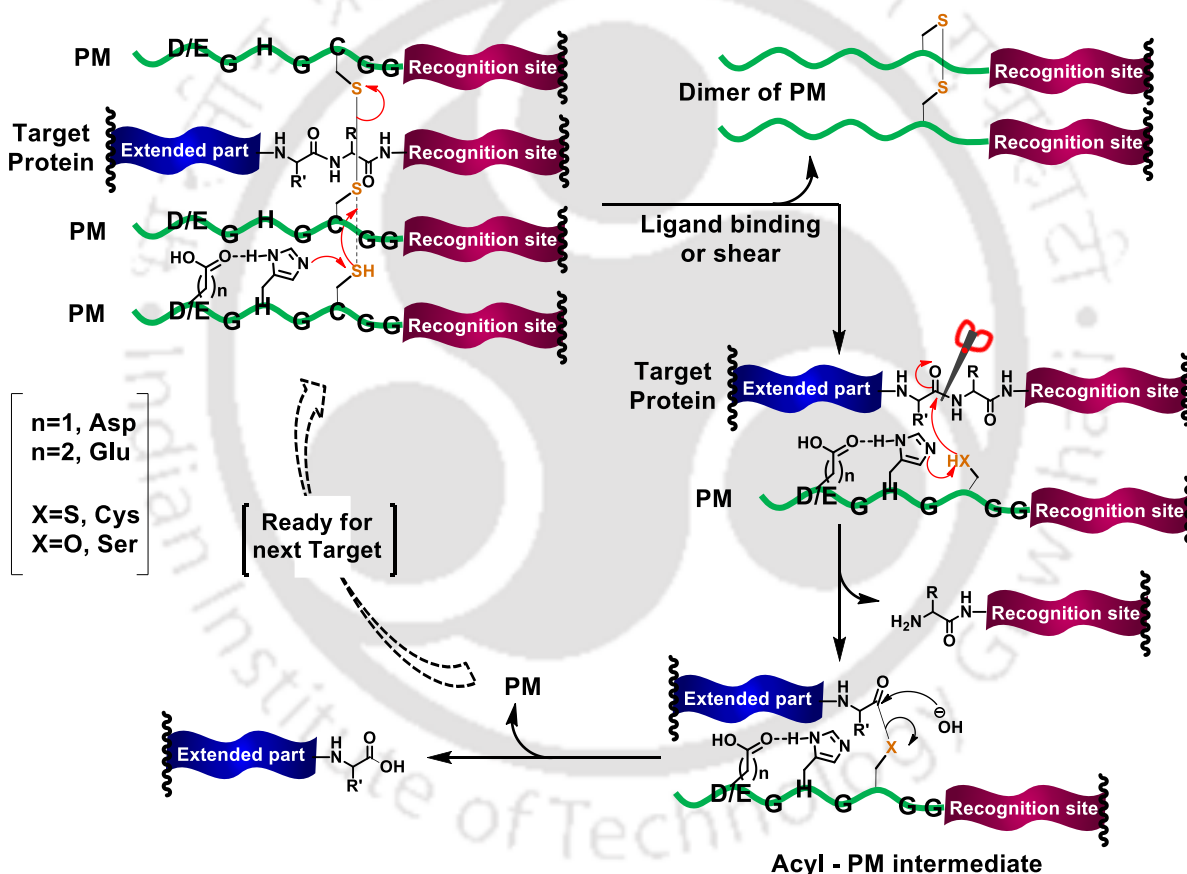


Figure 4. Design principles of PM. a) Proteolysis of a polypeptide substrate by Papain (Cys protease) and Geotrichum Candidum Lipase (Ser protease). b) Plausible mode of action of PM: target specific proteolysis by mimicking the catalytic triad of Cys or Ser protease.

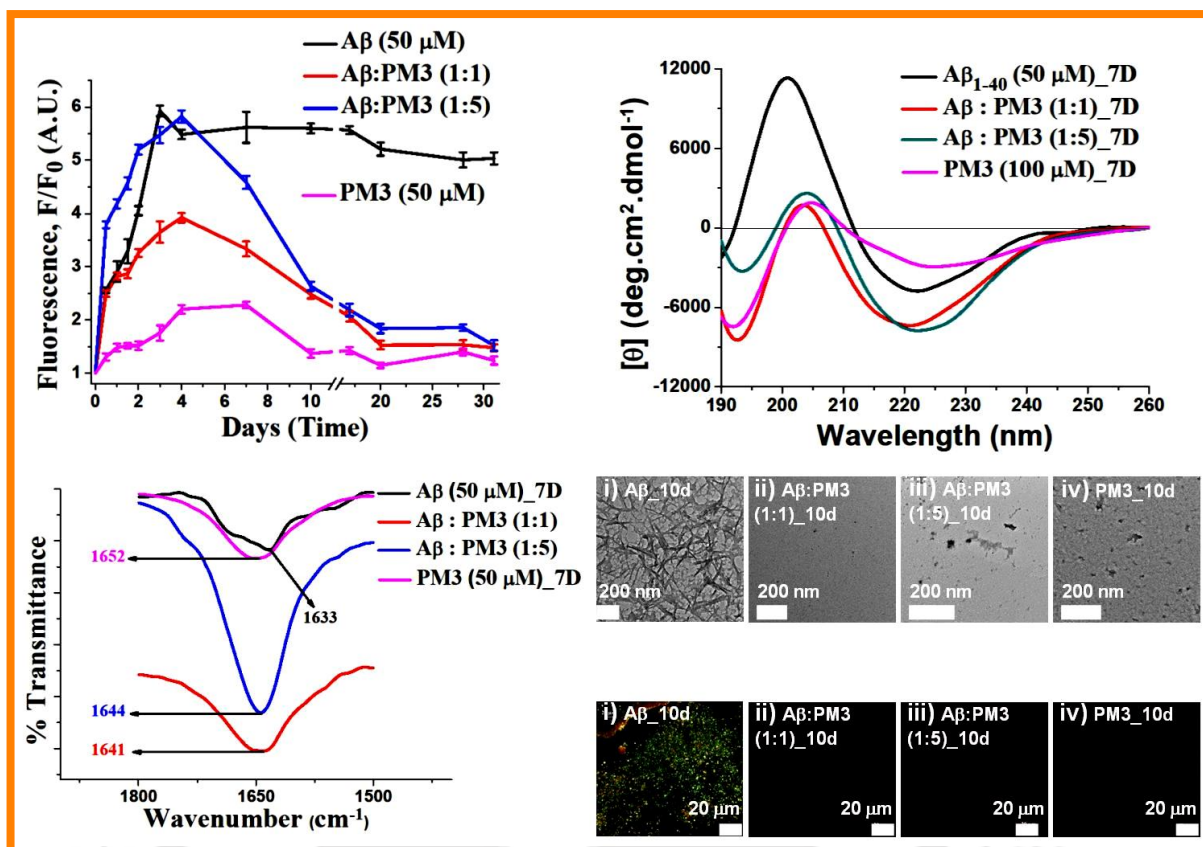


Figure 5. The kinetics of amyloid accumulation of $A\beta_{1-40}$ in the absence and presence of PM3 in PBS pH 7.4 (50 mM) at 37 °C.

It was found that the fluorescence intensity of $A\beta_{1-40}$ increased with time in the absence of PM3 and became static after 4 days after a slight lowering in intensity. But in the presence of one-fold PM3, a significant decrease in fluorescence signal of $A\beta_{1-40}$ was observed since the 2nd day and was slowly suppressed thereafter probably due to the generation of various peptide fragments resulting from several site-selective cleavages. PM3 converted the β -sheet structure of $A\beta_{1-40}$ into a non- β -sheet structure. The results of FT-IR experiments indicated that $A\beta_{1-40}$ adopted a β -sheet conformation in the absence of PM3 but adopted either random coil or helical structure in the presence of PM3. The results obtained from TEM and Congo Red birefringence studies were in agreement with those of the fluorescence experiments for the inhibition of aggregation of $A\beta_{1-40}$ in the presence of PM3 at the physiological condition.

Chapter 5: Design and development of a taurine containing peptide conjugate and its application in aggregation inhibition

Several neurodegenerative diseases are characterized by intra- and/or extracellular deposition of fibrillar protein aggregates which is believed to be the end state of protein misfolding whereas the actual culprits are non-fibrillar, oligomeric assemblies to initiate pathogenesis. In the case of AD, aggregation of A β in the presence of redox-active metal ions like Cu²⁺, Zn²⁺, Fe³⁺ are responsible for the cellular toxicity.^{24,25} Thorough experimental and computational studies have recommended that multiple binding modes of metal ions in A β peptides could be present simultaneously. Herein, taurine containing metal scavenger peptide (MSP) has been presented as the inhibitor as well as the disruptor of A β aggregation *in vitro*. We have designed the peptide conjugate which contains VFFA as recognition part and taurine unit attached with side chain of Glu, in such a way that –SO₃H groups of taurine unit are believed to lie around ¹³His and ¹⁴His of A β , because Cu²⁺, Zn²⁺ ions form salt bridge predominantly through ¹³His-Metal-¹⁴His conformation as well as bridges with ⁶His of A β .²⁶ Sequences of the peptide used are Ac-G-[E(Tau)-G]₄-VFFAG-NH₂. To check the inhibition of amyloid fibrillar aggregates of A β in the presence of metal ion, MSP was co-incubated separately with A β in PBS of pH 7.4 at 37 °C on water bath up to 7 days in parallel, and the kinetics of the amyloid accumulation was monitored using various biophysical tools.¹⁰⁻¹⁴ From different results, it was concluded that MSP does not form β -sheet, non-amyloidogenic in nature and can quench Cu²⁺, Zn²⁺, Fe³⁺ ions from A β peptide at the physiological condition. Also, the inhibitory effects of the MSP upon the amyloid formation of A β were demonstrated.

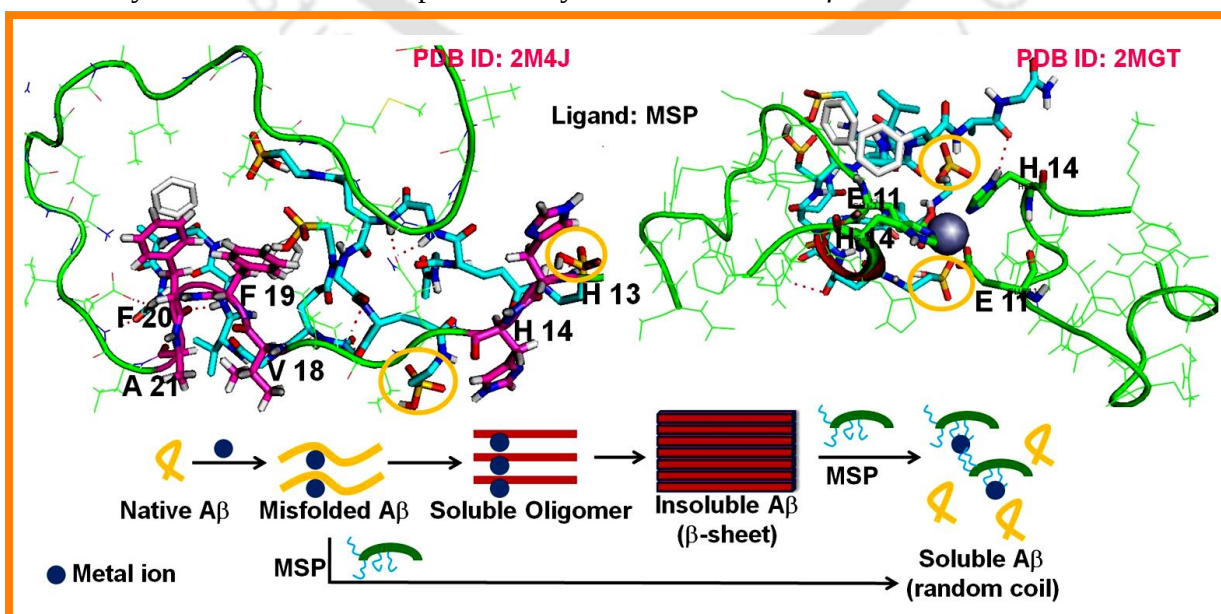
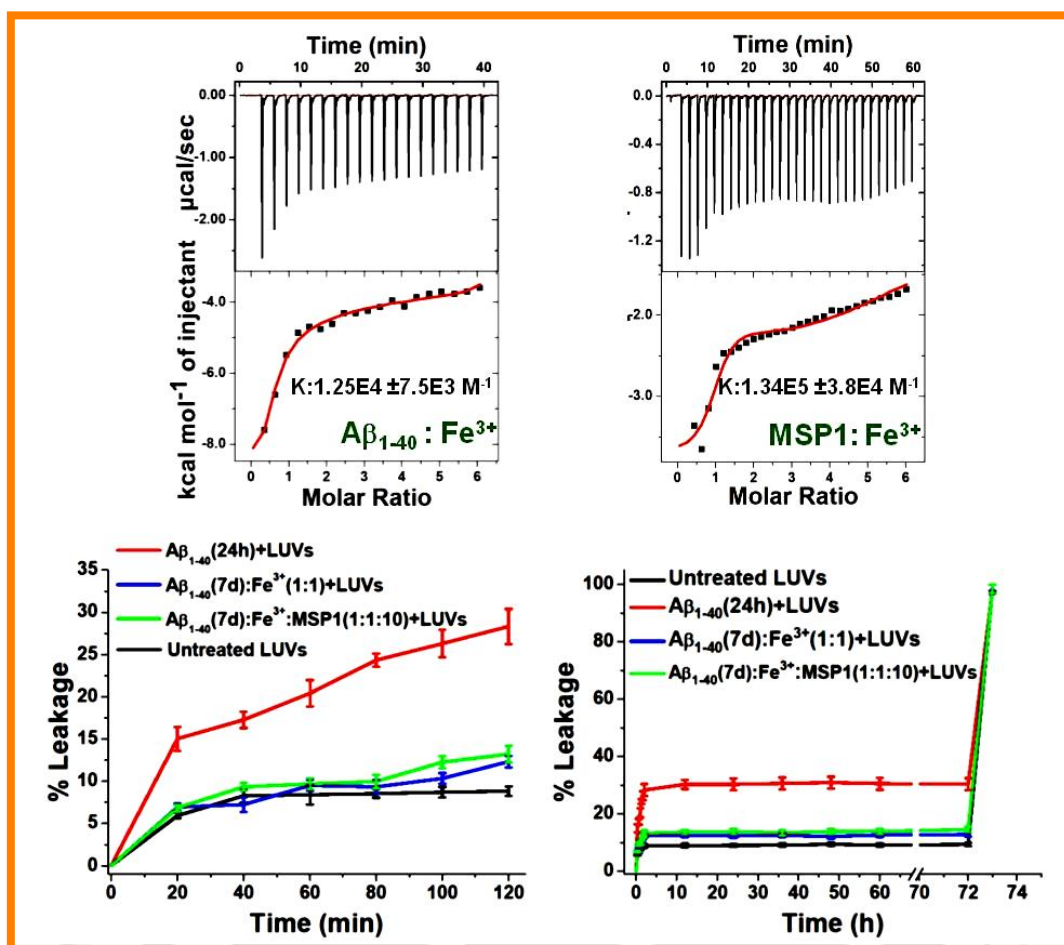


Figure 6. Hypothesis for the inhibition of amyloid formation by MSP.

Figure 7. ITC diagram (above) and vesicle leakage study (below) of Aβ₁₋₄₀ in absence and presence of MSP1.

From the ITC measurements, it was found that the binding constant of MSP to the metal ions (Fe³⁺, Cu²⁺, and Zn²⁺) was higher than that of Aβ₁₋₄₀. Thus it can be concluded that MSP can bind and extract such metal ions from the metal-Aβ₁₋₄₀ complex. From TEM and Cong Red birefringence studies, it was concluded that metal-Aβ₁₋₄₀ neither formed fibril nor formed amyloid, respectively, in the presence of MSP. These results are in agreement with the same of the ThT experiments. Cell membrane disruption capability by additives was examined by the vesicle leakage study. Entrapped dye releases by membrane rupture of LUVs in the solutions were monitored by time-dependent fluorescence assay. It was observed that oligomers caused more pore formation on LUVs and hence they were more toxic than the mature fibrils. The results confirmed that MSP disrupted the preformed amyloid of metal-Aβ₁₋₄₀ into non-toxic metabolites.

Conclusion:

In conclusion, we have developed a novel artificial α -Secretases which cleave A β site selectively by aggregation driven chemical reactions, although in a non-catalytic manner, without affecting all other substrates of α -Secretase. Inspired by the action of serine protease, we have designed and synthesized a series of conventional and unconventional Triad based protease mimic so that the side chains of these amino acids can come closer and be linked via intramolecular hydrogen bonding that may help Ser or Cys to hydrolyze an adjacent peptide following a similar mechanism of native chymotrypsin. It was found that the designed miniature Artificial Proteases cleaved a smaller A β fragment (model A β) and also the full-length A β site selectively. In the thesis, taurine containing metal scavenger peptides have been presented as the inhibitor of metal-A β aggregation *in vitro*. All the strategies can be applied to the drug design against Alzheimer's disease and amyloid-related disorders.

Throughout this thesis, three different kinds of approach for A β peptide cleavage and consequent disruption is demonstrated. However, cell-based and animal-based studies could not be performed by me for non-availability of facility and time. Such biological studies should be carried out to demonstrate the potential of such compounds for drug design against AD.

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List of Patents:

1. Indian patent application (complete specifications), filed on 23.01.2019; Title: “Design and Development of Functional Mimics of α -Secretase and their Application in Drug Design against Amyloidoses”; Application No. **201931002772**; Inventors: Dr. Bhubaneswar Mandal and **Tanmay Mondal**.
2. Indian patent application (under preparation), Title: “Robust Technique to Cleave Amyloid β by *De novo* designed Gly Integrated Triad Containing Peptides”; Inventors: Dr. Bhubaneswar Mandal and **Tanmay Mondal**.

List of Publications:

1. **Tanmay Mondal** and Bhubaneswar Mandal*. “Engineered peptidic constructs metabolize amyloid β by self-assembly-driven reactions” **ChemComm**. 2019, 55, 4933-4936.
2. **Tanmay Mondal** and Bhubaneswar Mandal*. “Targeted Amyloid Degradation by Miniature Artificial Protease”. **ChemComm**. 2020, 56, 2348-2351.
3. **Tanmay Mondal** and Bhubaneswar Mandal*. “Conventional and Unconventional Triad Based Protease Mimic Digest Amyloid” (Manuscript to be submitted)
4. **Tanmay Mondal**, Sujan Kalita, Rinku Dutta, and Bhubaneswar Mandal*. Design and development of a novel taurine containing peptide conjugate and their application in drug design against Alzheimer’s Disease and related disorders. (Manuscript to be submitted)
5. **Tanmay Mondal**, Rinku Dutta, and Bhubaneswar Mandal*. “Designed peptide conjugates metabolize model Amyloid β ”. (Manuscript under preparation)

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6. Ashim Paul, Krishna Chaitanya Nadimpally, **Tanmay Mondal**, Kishore Thalluri, and Bhubaneswar Mandal*. Inhibition of Alzheimer's Amyloid- β Peptide Aggregation and its Disruption by a Conformationally Restricted α/β Hybrid Peptide. *ChemComm.*, 2015, 51, 2245-2248.
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8. Mahesh Agarwal, **Tanmay Mondal**, and Biplab Bose*. "Peptides Derived From A Short Stretch of The Receptor-Binding Domain of Diphtheria Toxin Bind To Its Receptor". *Toxicon*, 2019, 169, 109-116.
9. Dharm Dev, Tapasi Kalita, **Tanmay Mondal**, and Bhubaneswar Mandal*. "Synthesis of Amide from Ketoxime via Beckmann Rearrangement using Catalytic amount of Ethyl 2-cyano-2-(2-nitrobenzenesulfonyloxyimino)acetate (o-NosylOXY)". (Manuscript under review)

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Abbreviations

A β	A myloid b eta
Abs	A bsorbance
ACE	A ngiotensin- c onverting e nzyme
Ac ₂ O	A cetic anhydride
AD	A lzheimer's d isease
ADAM	A d isintegrin and m etalloproteinase
Adi	A dipic acid
AFM	A tomic f orce m icroscopy
AICD	A myloid precursor protein i ntercellular d omain
APP	A myloid p recursor p rotein
AS	A rtificial α - S ecretase
AU	A rbitrary u nit
BnAdp	B enzyl a dipate
Boc	<i>tert</i> - b utyloxy c arbonyl
BOP hexafluorophosphate	B enzotriazol-1-yl o xy tris(dimethylamino) p hosponium
Bzl	B enzyl
CD	C ircular d ichroism
CF	5(6)- C arboxy f luorescein
CHCA	α - C yano-4- h ydroxy c innamic a cid
CR	C ongo r ed
DCM	D ichloro m ethane
DFT	D ensity f unctional t heory
DIPEA	D iisopropylethyl a mine
DLS	D ynamic l ight s cattering

DMF	N, N dimethyl formamide
DMPC	1,2- Dimyristoyl -sn-glycero-3- phosphocholine
DPPC	1,2- Dipalmitoyl -sn-glycero-3- phosphocholine
DTP	Diphtheria toxin protein
ECE	Endothelin-converting enzyme
ENDTAC	Endosome targeting chimeras
ESI MS	Electrospray ionization mass spectrometry
Et ₂ O	Diethyl ether
Fmoc	9- Fluorenylmethoxycarbonyl
FTIR	Fourier transformation infra red spectroscopy
GlcNAc	N-acetylglucosamine
GM1	Monosialotetrahexosylganglioside
HCF-1	Host cell factor-1
HEPES	N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid
HFIP	1,1,1,3,3,3- hexafluoro-2-propanol
HOBt	1- hydroxy benzotriazole
HPLC	High pressure liquid chromatography
IDE	Insulin-degrading enzyme
KBr	Potassium bromide
LC-MS	Liquid chromatography mass spectrometry
LUV	Large unilamellar vesicles
LYTACs	Lysosome targeting chimeras
mA β	Modified Aβ
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
mAP	Miniature artificial protease
mL	milli liter
mM	milli mol

MMPs	M atrix m etallo p roteinases
nm	N anometre
μM	m icro m ol
MSP	M etal scavenger p eptide
MW	M olecular w eight
NC	N egative c ontrol
NEP	N eprilysin
NFT	N eurofibrillary t angles
NMI	N - m ethyl i midazole
OGT	O -linked N-acetyl g lucosamine t ransferase
PDB	P rotein d ata b ank
PBS	P hosphate b uffer s olution
P	P lasmin
PM	P rotease m imic
PROTACs	P roteolysis t argeting c himeras
RP	R everse p hase
SPPS	S olid p hase p eptide s ynthesis
Tau	T aurine
tBu	t ert- b utyl
TEM	T ransmission e lectron m icroscopy
TFA	T rifluoroacetic a cid
TFE	T rifluoro e thanol
THF	T etrahydro f uran
ThT	T hioflavin T
Trt	T rityl
UV	U ltraviolet

Amino Acids:

Ala	A	Alanine
Arg	R	Arginine
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
Pyl	O	Pyrrolysine
Sec	U	Selenocysteine
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine

Chapter 1: Introduction, Objectives, and Experimental Methods

1.1. Introduction:

Dementia is caused by several diseases and conditions that damage brain cells or connections between brain cells. Symptoms of dementia include memory decline and decline in several cognitive abilities such as 1) ability to recognize objects, 2) ability to give a reasonable speech, or to understand spoken or written languages, and 3) ability to think or plan properly about complex tasks. Amyloid aggregation is the hallmark of the degenerative disease.^{1,2} The common types of dementia are 1) Alzheimer's disease (most common type, account for 60% to 80 % of cases), 2) Parkinson's disease, 3) Vascular dementia, 4) Dementia with Lewy bodies, and 5) Huntington's disease.

1.2. Alzheimer's Disease (AD):

In 1906, Dr. Alois Alzheimer first described "a peculiar disease", examining the post-mortem report of the brain of one of his patients. The patient died after years of severe memory problems. In an autopsy report of the patient's brain, Dr. Alzheimer saw dramatic shrinkage and abnormal deposits in and around nerve cells.³

“Considering everything, it seems we are dealing with a special illness”.

“Alois Alzheimer”

Psychiatrist

Munich, Germany

1906

1.2.1. Symptoms of AD:

Short term memory loss is the most common early symptom of AD. Apathy and depression are also early symptoms. As the disease advances, symptoms can include confusion with time and place, challenges in planning or solving problems, new problems with words in writing or speaking, withdrawal from work or social activities, changes in mood and personality, *etc.*⁴

1.2.2. Statistics on AD:

An estimated 44 million people were suffering from AD worldwide in 2010. By 2050, it is estimated to increase to 135 million. In America, one in eight people aged 65 and older (13 percent) have AD and almost half of the people aged 85 and older (43 percent) have this disease.⁴

1.2.3. Probable Origin of AD:

The causes of AD are not yet fully understood. However, according to Amyloid Cascade Hypothesis, it is believed that mainly aggregation of the Amyloid β ($A\beta$) peptide outside neurons and accumulation of the tau protein inside neurons result in the development of AD in the brain.^{5,6} In a healthy human brain, $A\beta$ peptide concentration is about 2 $\mu\text{g/g}$ whereas, in AD patients, it is around 31 $\mu\text{g/g}$. A healthy adult brain contains 100 billion neurons; each with long and branching extensions which assist individual neurons in forming specific connections with other neurons called synapses. Information released by one neuron flows in tiny chemical pulses through synapses and detected by the receiving neuron. The brain has 100 trillion synapses which allow signals to travel rapidly through the brain's circuits constantly.

In AD, information transfer begins to fail at synapses, the number of synapses declines, and neurons finally die. Neuron-to-neuron communication hampers due to the accumulation of $A\beta$ outside these synapses are assumed to and contribute to cell death. Inside the neuron, abnormal forms of tau detach from microtubules and accumulate in the neurofibrillary tangles that block the transport of nutrients and other necessary molecules throughout the cell, also contributes to cell death. In the brain, a higher concentration of

metal ions mainly Cu^{2+} , Zn^{2+} , Fe^{3+} , and Al^{3+} causes rapid aggregation of $\text{A}\beta$ to generate soluble oligomers which help to produce reactive oxygen species (ROS) resulting synaptic dysfunction and finally the neuronal cell death.⁷ In advanced AD patient, many regions of the brain begin to shrink dramatically from cell loss and widespread debris from dead and dying neurons (Figure 1.1).

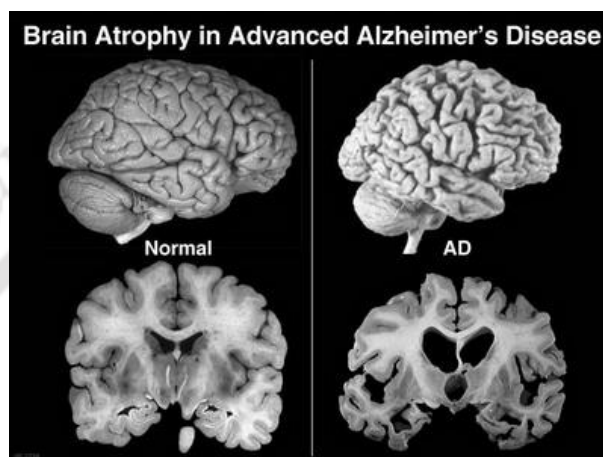


Figure 1.1. Comparison: Normal and Alzheimer's Diseased Brain.

[Courtesy: This figure is from <http://gazettereview.com/wp-content/uploads/2015/04/Alzheimers-Disease.jpg>].

1.3. Amyloid:

Amyloids are insoluble fibrous protein aggregates defined by shared specific structural traits. For some unknown reason, several unfolded and misfolded proteins accumulate and deposits as insoluble material known as amyloid by changing the conformational structure. Generally, amyloids are formed due to protein misfolding.¹

The amino acid sequence in a peptide is significant, more precisely the side chain plays a significant role in recognition followed by self-aggregation to form amyloid. In a peptide sequence, the hydrophobic side chain of amino acid tend to form β -sheet and hence can accumulate easily; where the hydrophilic side chain is responsible for random interaction with the solvent molecule as well as with another peptide sequence, preferably remains as random coil conformation. The presence of consecutive hydrophobic side chain of amino acids in a peptide sequence causes rapid aggregation followed by Amyloid

deposition.^{8,9,10} Aromatic interactions are known to be vital in globular proteins and are supposed to play a crucial role in amyloid formation.⁹

1.4. Protein folding and misfolding:

Proteins are involved in all stages of neural activity and biological activity in the folded form. Protein folding is such a process by which a protein structure acquires its functional conformation, or in other words, a polypeptide can fold itself into its characteristic and functional three-dimensional arrangement from random coil. The folded protein is known as the native state. During protein folding some part of the protein remains as unfolded or some part of the folded protein changes its native conformation and fails to get back to its native conformation. This process is called protein misfolding. A protein can only function if the specific three-dimensional structure is retained.¹

A linear polypeptide chain is freely arranged into a space-filling, compact, and well defined three-dimensional structure. In a globular protein, mainly hydrophobic amino acid residues are found to form the internal core and mostly charged, and polar side chains tend to be on the surface of the globule. The flexibility of a polypeptide backbone and the specific, regular intermolecular interactions of the side chains of amino acid determine the specific conformation of a protein. The native conformation must be energetically stable. From a thermodynamic point of view, the free energy of a protein molecule is influenced by the following major contributions: (1) Hydrophobic effect, (2) Energy of hydrogen bonds, (3) Energy of electrostatic interactions, and (4) Conformational entropy due to the restricted motion of the main chain and the side chains.¹¹

1.5. Amyloid Precursor Protein (APP) processing:

Abnormal production and deposition of A β in the form of senile plaques in the brain of AD patients are the pathological hallmark of AD. A β peptides are generated in the amyloidogenic pathway of APP processing by sequential proteolysis by β - and γ -secretases. Alternatively, in the non-amyloidogenic APP processing pathway, site-specific

cleavage by α -secretase release neuroprotective APP fragment soluble APP- α (sAPP α) and prevents the production of neurotoxic and pathogenic A β .^{5,12,13,14}

α - and β -secretase cleave APP in its extracellular domain to release a soluble fragment sAPP α or sAPP β , respectively, in the extracellular space, and produce carboxy-terminal fragments, CTF α or CTF β , which can be processed subsequently by the γ -secretase complex. The γ -secretase complex is composed of presenilin, γ -secretase activating protein (GSAP), nicastrin (NCT), pen-2, and aph-1. Finally, amyloidogenic pathway produces toxic A β . A β accumulation in the cellular compartments damages cellular functions including mitochondrial dysfunction, synaptic dysfunction, and hyperphosphorylation of tau. Such accumulation causes dementia and AD. But the non-amyloidogenic pathway generates AICD (APP intracellular domain), 3-kDa peptide p3, and sAPP α , which are non-toxic and easily clear from the brain. Therefore, non-amyloidogenic pathway is healthy and does not cause dementia. **Increasing the α -secretase-mediated processing of APP is, therefore, a therapeutic option for the treatment of Alzheimer's Disease (AD).**^{11,12,13}

1.5.1. α -Secretase Cleavage Sites:

Secretases are enzymes that cleave a longer protein in the cell membrane. Among other roles in the cell, secretases cleave the APP into three fragments as described in the earlier section. The non-pathogenic middle fragment generated by an α/γ cleavage sequence is known as 3-kDa peptide p3.^{5,12,13}

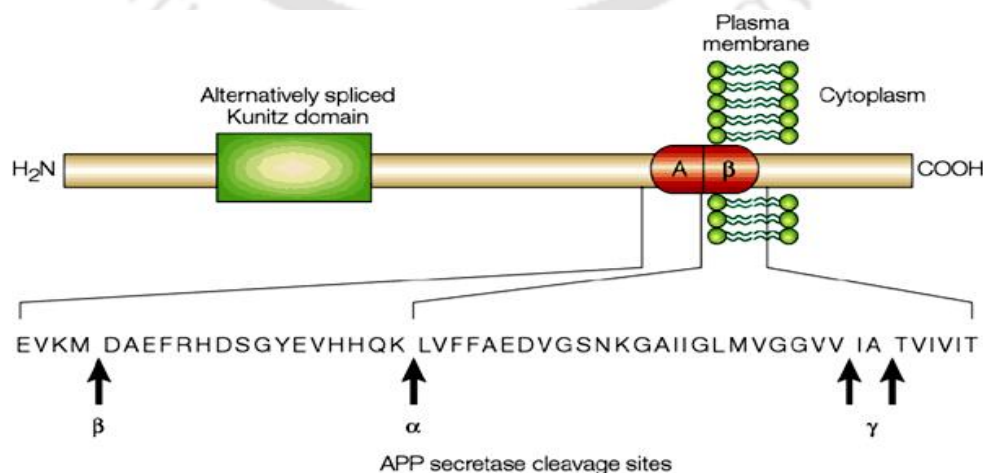


Figure 1.2. Cleaving position of α -, β - and γ -secretases in APP.

[Courtesy: This figure is from http://www.nature.com/nrn/journal/v1/n1/images/nrn1000_051a_i1.gif]

1.5.2. The function of α -secretase:

Specifically, α -Secretase cleaves between Lys16 & Leu17 of A β in the ectoplasmic region of APP. The α -secretase cleaves both plasma membrane inserted APP & secretory vesicle-associated APP. The activity of α -Secretase may be reduced by cholesterol or cholesterol esters. It has been suggested that acetylcholine receptor or muscarinic receptor stimulation may improve APP α -secretase activity.⁵

1.6. Targeted protein degradation:

Targeted protein degradation is vital for both investigating biological systems and developing novel therapeutics¹⁵. Proteolysis targeting chimeras (PROTACs)^{16,17} and conceptually similar degradation platforms, e.g., the degradation tag (dTAG)¹⁸, Trim-Away¹⁹, chaperone-mediated autophagy targeting²⁰ and SNIPERs²¹ have emerged as elegant techniques to manipulate these otherwise “undruggable” target proteins. These state-of-the-art protein degradation technologies rely on target engagement with the intracellular ubiquitin-proteasome system (UPS)²². Therefore, they are unable to act on extracellular secreted proteins, such as cytokines, chemokines, and transmembrane proteins²³. These secreted and membrane proteins represent ~40% of the protein-encoding genes²⁴ and play critical roles in many diseases, including cancer, neurodegeneration, autoimmunity, and infectious disease²⁵. New elegant techniques namely, endosome targeting chimeras (ENDTAC)²⁶ and lysosome targeting chimeras (LYTACs)²⁷ also have been demonstrated to internalize and degrade extracellular proteins by hijacking a GPCR receptor exploiting the intracellular endosomal-lysosomal degradation pathway, in contrast to PROTACs using the UPS. However, the internalization of external proteins remains challenging. Thus, practical strategies for targeted degradation of extracellular proteins remains an unmet need that can potentially impact human health. Although synthesized multifunctional PROTAC peptides effectively induced intracellular Tau protein degradation²⁸, no PROTAC is known yet for extracellular A β degradation.

1.7. Proteolysis by enzymes and the importance of artificial proteases:

Chemical modification of proteins is an important area of research, for example, chemical biology, protein engineering, proteomics, drug discovery, *etc.*, and site-specific cleavage of peptide bonds is among such modifications. The peptide bonds in proteins are highly stable under standard physiological conditions.²⁹ Only a few enzymes and synthetic reagents are there for hydrolysis of peptide bonds selectively. Enzymes are natural proteins, and their catalytic activity depends on the integrity of their structure as proteins for which they are highly specific in the choice of substrates. The active sites control the specificity and rate acceleration of enzyme catalysis.³⁰ By using macromolecular skeletons like proteins, nature constructs active sites by putting several elements in the right positions to build enzymes. In majority, the peptidases are residue-selective, for example, Trypsin (selectively cleaves at Arg and Lys), Chymotrypsin (at Phe, Trp, and Tyr), Pepsin (at Phe and Leu), Endopeptidase Glu-C (at Glu), and Endopeptidase Lys-C (at Lys).³¹ Chymotrypsin catalyzes the hydrolysis of a non-terminal peptide bond. Chymotrypsin is a serine protease where the active site of the enzyme consists of four close-lying parts (Asp, His, and Ser in one pocket; while Gly lies in another hydrophobic pocket) which works together to hydrolyse the peptide bonds specifically adjacent to an aromatic amino acid residues (Trp, Phe, Tyr) at physiological condition, and the reaction rate of peptide hydrolysis was found to be decreased dramatically for such a case where the amino acid residue as a substrate did not fit in the active site or hydrophobic pocket of the enzyme.³² Also, peptide hydrolysis can be done by Papain³³ like cysteine protease which acts like Chymotrypsin. The most efficient artificial proteases would be achieved by designing artificial active sites containing various functional groups to assist complexation with the substrate. Again, artificial proteases should be able to recognize the target protein and to cleave selective peptide bonds of the target protein.

1.8. Peptide bond hydrolysis by metalloenzymes:

The cleavage of peptide bond was done either by covalently attaching metal complex to the protein or by direct coordination of metal complex to the side chain of amino acid.^{34,35} Initially catalysts were designed for hydrolysis of activated analogs of peptides, for example, carboxyl esters, anilides, distorted amides, *etc.*, to avoid difficulties encountered

in the peptide hydrolysis. Although few characteristic features of proteases were reproduced with various synthetic agents by using activated analogs of peptides as substrates, the validity of “p-nitrophenyl ester syndrome” has been criticized.³⁶ Also, the peptide bond cleavage was done by oxidation instead of hydrolysis by various redox-active metal ions, such as Cu, Cr, Ce, Fe, Ni, and V. Metal ions in combination with oxidoreductive additives oxidized the backbones of peptides.³⁷ Analogs of artificial proteases cleave the proteins selectively by combining metal complexes with organic pendants which recognize the target proteins. Polyethylenimine (PEI) derivative that contains active sites consist of three convergent salicylate residues (only organic functional groups) acts as an artificial protease.³⁸ Carboxypeptidase A which exploits a metal ion as a Lewis-acid catalyst catalyzes the hydrolysis of C-terminal peptide bonds of proteins.⁷ In several cases, peptide hydrolysis occurs because of capturing metal ions by un-activated peptide bonds, but after hydrolysis the metal complexes are not regenerated due to strong product inhibition. Pt^{2+} and Mo^{4+} ions anchored to sulfur atoms of peptide substrates resulting in hydrolysis of adjacent peptide bonds.³⁹ Peptide hydrolysis also occurs due to binding of the imidazolyl side chain of His to Pt^{2+} .⁴⁰ The Cu^{2+} complex and Co^{3+} complex of 1,4,7,10-tetraazacyclododecane ($[\text{Cu}^{2+}(\text{cyclen})]$ and $[\text{Co}^{3+}(\text{cyclen})]$ respectively)) have been used widely as the proteolytic center of artificial proteases.⁴¹ Artificial metalloproteases were prepared on the surface of cross-linked polystyrene by using a guanidinium moiety (to recognize a carboxylate anion) positioned near a Cu^{2+} center and were found to selectively hydrolyzes carboxyl-containing amides of various proteins.⁴² The first artificial metalloprotease was prepared by using Myoglobin as the substrate to selectively hydrolyse the backbone of a target protein, and the first artificial metalloprotease was prepared by using peptide deformylase as the target protein to selectively hydrolyse the backbone of an enzyme related to a disease.⁴³ Cyclodextrin, which has an affinity for hydrophobic moieties, was used as a substrate-recognition site and was attached to a Pd^{2+} complex to hydrolyze peptide bonds adjacent to Pro residues.⁴⁴ Instead of being studied intensively for peptide hydrolysis, practical use of metal in protein analysis is still in early development. The methods developed for peptide cleavage are still far-away from natural peptidases concerning cleavage site-fidelity, and substrate specificity.

1.9. A β degrading enzymes:

Enzymes such as neprilysin (NEP), endothelin-converting enzyme (ECE), insulin-degrading enzyme (IDE), Plasmin (P), angiotensin-converting enzyme (ACE), and Matrix Metalloproteinases (MMPs) are known to cleave A β in a site-selective manner. These are nature's mechanism for facilitating excretion of A β plaques from the nervous system⁴⁵. However, solvent inaccessibility of the insoluble A β plaques prevents the functioning of such enzymes allowing for the accumulation of aggregates and thus disease progression. Promoting activities of such enzymes also may contribute to the drug design drive against AD. Notably, all of them cleave peptide linkages either by a catalytic triad-based mechanism or further assisted by a metal. For example, the glutamate at the catalytic site of ADAM (a disintegrin and metalloproteinase) 10, which is a Zn-binding metalloprotein, acts as the nucleophile. The glutamate is co-ordinated along with three histidines to the Zn-ion which polarizes the carbonyl group of the scissile peptide bond^{46,47}. Mimicking metalloenzyme action has been achieved by mimicking the catalytic site by protein engineering. For example, Tezcan et al. reported an artificial, *in vivo* active Metallo- β -lactamase with interfacial catalytic sites, constructed through the metal-directed supramolecular assembly of a monomeric protein, which is structurally and functionally unrelated to any Metallo- β -lactamase or hydrolytic enzyme⁴⁸. Stabilization of such metal-binding sites or mimicking catalytic triad based active sites⁴⁹ of serine proteases essentially requires the specific supramolecular arrangement of large protein constructs. Therefore, it is needed to design such compounds that would selectively cleave APP and A β preferably on the cleavage site of α -secretases and other A β degrading enzymes and make A β non-toxic but will not affect other substrates of those enzymes.

1.10. Transcriptional upregulation of ADAM 10:

ADAM is a family of widely expressed, transmembrane proteins. Approximately 750 amino acids long ADAM10 is accepted as one of the main α -secretases⁵⁰. Overexpression of ADAM10 reduced plaque load in mouse models of Alzheimer's disease (AD) and improved learning and memory⁵¹. Some small molecules such as melatonin⁵², retinoic acid receptors, and acitretin⁵³ demonstrated a significant effect on the increase of sAPP α by transcriptional upregulation of ADAM 10. However, at present, a wide variety of

substrates of ADAM10 have been identified, and they are linked to a number of physiological as well as pathological functions, including not only the immune and nervous system but also cancerogenesis⁵⁴. For example, ADAM 10 upregulation may be deleterious to the brain tumor patients as it is associated with the spreading of tumor cells⁵⁵. Therefore, addressing AD by ADAM 10 upregulation is critical as it may improve AD but increase the risk of cancer and other diseases.

1.11. Host cell factor-1 self-cleavage *in vivo*:

Host cell factor-1 (HCF-1), a transcriptional co-regulator of human cell-cycle progression, undergoes proteolytic maturation in which any of its six repeated sequences are cleaved by the O-linked N-acetylglucosamine (O-GlcNAc) transferase (OGT)⁵⁶. Initially, OGT driven proteolysis seemed mysterious, but recently Walker et al. demonstrated that HCF-1 cleavage occurs via unusual glycosylation of a glutamate side chain by OGT, which usually glycosylates Ser or Thr side chains otherwise, followed by the formation of an internal pyroglutamate that undergoes spontaneous peptide bond hydrolysis⁵⁷. Also, Asn mediated C-terminal extein expulsion in protein splicing was inspiring⁵⁸. However, nucleophilic attack of the backbone nitrogen to the glycosylated side-chain carboxylate of Glu is feasible for the favorable formation of intramolecular five-membered cyclic pyroglutamate *in vivo*, but mimicking such reaction pathways in intermolecular fashion is highly challenging. Moreover, to perturb the disease condition by externally added agents; consideration of the intermolecular attack was obvious. The mentioned external agent must recognize APP or A β peptide selectively; therefore peptide-based drug design approach is preferred as peptides are usually selective in nature and manipulation of their selectivity is easy.

1.12. Current treatment strategies of AD:

Despite the enormous efforts and progress made in both the diagnosis and treatment of this disease, there is no cure available to date. The available drugs on the market are good at improving the patient's condition of anxiety, insomnia, or depression only. Logically, three different approaches for drug design against Alzheimer's disease are possible. They are as follows:

- (a) by stopping Amyloid- β production,
- (b) by inhibiting Amyloid aggregation, and
- (c) by facilitating excretion of the β -amyloid peptide.

1.12.1. Strategies based on stopping Amyloid- β production:

Inhibiting β and γ secretase is a strategy for drug design against AD as this reduces the production of Amyloid β from APP.^{59,60} Drug named 'FlurizanTM' from Myriad genetics was identified as γ secretase inhibitor and reduce the levels of $A\beta_{42}$. Jing Zhao *et al.* invented a compound C1 which binds to C-terminal juxtamembrane lysines at the transmembrane domain of APP and could be able to inhibit γ -secretase production of $A\beta$.⁶¹ Several compounds, such as thiazolidinediones (rosiglitazone and pioglitazone) were reported as the β -secretase inhibitor.⁶² The drugs, such as semagacestat (LY-450139), E-2012, MK-0752, PF-3084014, BMS-708163, begacestat (GSI-953), and NIC5-15 are used as the γ -secretase inhibitor.⁶³ But due to the side effects of these drugs, fewer reports are available to use in AD.

1.12.2. Strategies based on inhibition of aggregation of the Amyloid β peptide:

Inhibition of $A\beta$ fibrillization or dissolution of preformed fibrils by peptide-based molecules is a reasonable therapeutic approach against AD. From the $A\beta$ sequence, Tjernberg first introduced a short peptide fragment (KLVFF) as an inhibitor of fibrillogenesis *in vitro*.⁶⁴ Later, Soto has developed a small peptide molecule (Ac-LPFFD-

NH₂) by using proline as a turn generating unit, and the peptide showed the promising result on inhibition of fibrillization and dissolution of preformed fibrils in rat brain.⁶⁵

Recently, we have demonstrated that the insertion of anthranilic acid in an amyloidogenic peptide sequence generates a novel conformationally restricted α/β -hybrid peptide that inhibits the amyloid formation of A β ₁₋₄₀ peptide and disrupts preformed fibrillar aggregates in vitro.⁶⁶

A novel class of dipeptides, named as β -breaker dipeptides (BBDPs), is also identified that can be incorporated into a self-recognizing sequence to generate a novel class of β -breaker peptide which forms β -sheet at the initial stage and then converts to the random coil in a controlled way at specific conditions. These β -breaker peptides are shown to incorporate first into the amyloid and then disrupt it as it happens in pro-drugs.⁶⁷

Use of small molecules as effective therapeutic agents towards drug design against AD is another promising strategy. A molecule, which binds to A β , crosses blood-brain-barrier and reduces aggregated A β levels in the brain considerably, is a perfect drug candidate. Neurochem made Tramiposate AlzhemedTM a small molecule is in clinical trials.⁶⁸ But it could not stop cognitive dysfunction. A number of small molecules, including epigallocatechin-3-gallate (EGCG), curcumin, methylene blue, rifampicin, resveratrol, etc., are well-known as anti-Alzheimer's agent for their antioxidant property and aggregation inhibition ability.^{69,70}

Due to weaker substrates for proteolysis (compare to L-peptides), D-peptides are also used as an inhibitor. For example, D-(PGKLVYA) and D-(KKLVFFARRRRA) was found to inhibit A β aggregation.⁷¹ Retro-inverso (RI) peptides e.g., RI-OR2-TAT reduces A β aggregation and plaque levels and also reduces oxidative damage.⁷² CLR01, a molecular tweezer, binds to Lys16 in A β to inhibit the aggregation of A β .⁷³ N-methyl peptides could be able to inhibit cytotoxic A β .⁷⁴ A series of SEN compounds SEN304, SEN1269, SEN1576, etc. were also found to inhibit A β aggregation and toxicity.^{75,76} A high molar ratio of NQTrp, a Quinone derivative, reduces the aggregation of A β and also reduces the toxicity in a transgenic AD Drosophila model.⁷⁷

Cu(II) binds to A β ₄₂ and forms 1:1 complex, and it was confirmed from HRMS and cyclic voltammetry experiments. Redox-active molecules, for example, ascorbic acid, lactic acid, pyruvic acid, glutathione, Vitamin B12, etc., and electron sources like NAD⁺ help

Cu(II):A β complex to reduce oxygen and to form hydrogen peroxide which is responsible for oxidative stress.⁷⁸ If, a ligand can competitively bind to the metal ions (Cu²⁺, Zn²⁺, and Fe³⁺) may inhibit A β deposition. ClioquinolTM, known as Cu²⁺/Zn²⁺ chelator, showed a promising result in transgenic mice. Clioquinol and its derivatives are in the clinical trial stage.

1.12.3. Strategies based on facilitating excretion of the amyloid- β peptide or aggregated amyloid- β peptide:

Antibody-based approach as a potential therapeutic agent is one more treatment strategy against AD. It was first demonstrated that antibodies against A β could block the formation of amyloid fibrils *in vivo*.⁷⁹ In 1999, vaccinating transgenic mice against A β was found to reduce the deposition of fibrillar amyloid noticeably. Significantly, for depolymerizing the fibrils, the stoichiometry of antibody to A β is in the range of 10 to 100 times.⁸⁰ Even though the mechanism of antibody-based therapeutic approach is not understandable, but up to date evidence suggest that a monoclonal antibody recognizing the central region of A β sequestered A β in the senile plaques.⁸¹

Recently, Prof Vijayalakshmi Ravindranath has demonstrated that extracts from snakeroot plant, rich in withanosides and withanolides, enhances excretion of a soluble and insoluble form of A β peptides from both brain and blood.⁸²

1.13. The objective of the thesis work:

From the above discussions, it is evident that many strategies are being developed for the reduction of aggregation of A β peptide. However, works on the degradation of the A β peptide is relatively less. Design and development of enhancer or agonist of α -secretase also can be a promising approach for drug design against AD as cleavage of A β by α -secretase produces non-toxic A β fragments.^{11,12,13}

Thus, we have planned to design and develop a functional mimic of the α -secretase enzyme (**Artificial α -Secretase**) that will cleave A β in a site-specific manner that would produce a non-toxic A β fragment. Optimistically, that may also cleave APP and produce non-toxic metabolites. This is a challenging project.

The design of such peptide conjugates ideally should consist of a “recognition or targeting part” and an activity element. Some of such recognition sequences are already known in the literature.¹⁶ On the other hand, the “activity element” should be a chemical entity that will stay unchanged in blood until it reaches its targets in the brain. Then, when the whole peptide-conjugate will be attached to the target molecule, A β or APP, in this case, the activity element should be able to cleave a peptide bond site specifically. Few chemical entities are reported as “artificial proteases” that can cleave a peptide bond *in vitro*.⁸³

1.13.1. Specific objectives:

1. At first we wanted to develop some peptide-based small molecules that will recognize Amyloid β peptide and cleave its peptide-backbone by a cascade of chemical reactions. As a result it will finally metabolize amyloid β peptide. If these designed peptides cleave APP, then they may be called as the functional mimic of α -secretases. However, in this strategy, achieving the catalytic activity was not targeted, as that would require further sophistication in the design. (Results in this direction is described in the 2nd chapter of this thesis.)

2. Next we wanted to achieve another type of functional mimic of A β -degrading proteases, keeping their catalytic activity intact by peptide-based molecule design. For that we mimicked the active site of chymotrypsin and attached that with the A β recognizing peptide sequences. (The works in this direction are described in the 3rd chapter).

3. Next we wanted to design a new catalytic site based on the active site of some of the native peptidases. The rest of the design moto was similar to the above-mentioned objectives. (The works in this direction are summarized in the 4th chapter).

4. Some transition-metal ions, mainly Cu²⁺, Zn²⁺, and Fe³⁺ are able to bind to A β and influence aggregation process. Such metal-bound A β complexes cause oxidative stress and directly involve in the physiological toxicity to the adjacent neurons. In our next goal, we wanted to design a metal ion chelator for inhibition of aggregation. (Our efforts in this direction are summarized in the 5th chapter of this thesis).

1.14. Experimental Methods:

1.14.1. Reagents and solvents:

Rink amide MBHA resin was purchased from GL Biochem (Shanghai) (Loading 0.7 mmol/g). N, N-Diisopropylethylamine (DIPEA) was purchased from Spectrochem Pvt. Ltd. Dimethylformamide (DMF, extra pure grade), dichloromethane (extra pure grade), and acetonitrile of HPLC grade were obtained from Merck (India). Acetic anhydride (synthesis grade), N-methyl imidazole (extra pure), Trifluoroacetic acid (TFA) of extra pure grade were purchased from SRL (India). Milli-Q water at 18.2 Ω was used. All Fmoc (N- terminus protected) amino acids, BOP [(Benzotriazole-1-yloxy) tris (dimethylamino) phosphonium hexafluorophosphate] and human Alzheimer's β -amyloid ($A\beta_{1-40}$) peptide were purchased from GL Biochem (Shanghai).

1.14.2. The procedure of peptide synthesis:⁸⁴

All the described peptides were synthesized by standard Fmoc/tBu solid-phase peptide synthesis (SPPS) method. 200 mg of Rink amide MBHA resin (loading 0.7 mmol) was taken in a 5 ml frit-fitted plastic syringe and was allowed to swell with DCM solvent first and then with DMF solvent. After that, the Fmoc group that was attached to the resin was cleaved with 20% piperidine in DMF. The resin was then washed with DMF. Then, 2.5 equivalent of Fmoc Amino acid, 3.0 equivalent of BOP (coupling reagent) and 5.5 equivalent of DIPEA (base) was dissolved in DMF and added to the syringe. The syringe was allowed to rotate by a Stuart blood tube rotator for coupling. After that, the reaction mixture in the syringe was washed with DMF first followed by with DCM. It was then acetylated (capping) with two equivalent of acetic anhydride, two equivalent of N-methyl imidazole in DCM. The cycle of the cleavage of the Fmoc group of the terminal amino acid and coupling of the desired amino acids was continued as mentioned above. Finally, cocktail cleavage was carried out with 2ml of TFA:DCM (8.5:1.5) and one drop of H₂O for 3h (or 5h where necessary) to cleave the C-terminus of the peptide from the Rink amide resin. After completion of the synthesis of the peptide sequence, the mixture was precipitated by cold diethyl ether to get the crude peptide that was purified and characterized as specified later.

In the case of artificial α -secretases (ASs), side-chain modification of lysine unit was carried out via coupling reaction of the crude peptide with mono-benzyl adipate in the solution phase, using 1.2 equivalent of BOP (coupling reagent) and 2.5 equivalent of DIPEA (base) in DCM. Mono-benzyl adipate was synthesized also from a coupling reaction between 5 equivalent of adipic acid and one equivalent of benzyl alcohol, using 2.5 equivalent of EDC.HCl, 14 equivalent of DIPEA, and 0.3 equivalent of HOBt in DCM for 12h followed by evaporation and performed silica gel column chromatography to get pure product.

1.14.3. Purification and characterization of the peptides:

Crude peptides were dissolved in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ mixture and purified by RP-HPLC (Waters 600E) using a C18- μ Bondapak column at a flow rate of 4 mL/ min. Binary solvent system [solvent A (0.1 % TFA in H_2O) and solvent B (0.1 % TFA in CH_3CN)] were used. Waters 2489 UV detector was used with dual detection at 214 and 254 nm. A total run time of 20 min. was used for purification, and the gradient was set as 5-100 % CH_3CN for 18 min, followed by 100% CH_3CN till 20 min.

The purity of the peptides was confirmed using a Waters 600E analytical HPLC system. An Ascentis C18 analytical column, a flow rate of 1 ml/min, a linear gradient of 5-100% CH_3CN over 18 minutes in a total run time of 20 min were used. Dual-wavelength was selected at 214 nm and 254 nm.

ESI-Mass of the peptide samples was analyzed on Agilent-Q-TOF 6500 instrument, in ESI positive mode, equipped with Mass hunter work station software.

1.14.4. Density Functional Theory (DFT) Calculation:

DFT yields total energies, forces, and electronic structure. Hence from DFT, it is possible to compute structural relaxation, phase stability, energy differences, etc. It provides a reasonable balance between computational efficiency and accuracy. The most stable conformation of the intermediates was obtained after performing DFT based methods

using B3LYP as energy functional and 6-31G as a basis set through Gaussian 5.0.9 program.

1.14.5. Molecular docking studies:⁸⁵

Molecular docking helps to investigate the binding affinity of a ligand with a protein. It also suggests whether a ligand is fitted in the binding pocket. AutoDock Vina version 1.1.2 software was used for molecular docking study. At first, a segment of helical and fibril A β ₁₋₄₀ was extracted from RCSB protein data bank (PDB ID: 4NGE and 2M4J respectively). AutoDock 4.2 MGL Tools version 1.5.6 software package was used for protein preparation. Water molecules were removed to avoid error; polar hydrogen atoms were added to the protein; Gasteiger charges of the macromolecule were added and saved as PDBQT (for protein) format. OpenBabel version 2.4.1 software was used to convert ligand in PDB format after minimizing energy [obtained from DFT calculation; Calculation Method: B3LYP, Basis Set: 6-31G] and the PDB file was modified by addition of Gasteiger charges. PDBQT format of the ligand was generated to coordinate files, which includes atomic partial charges and atom types. Torsion angles were calculated to assign the fixable and non-bonded rotation of molecules. Grid file was customized by using Auto Grid version 4.2 to calculate the grid parameters. Grid file is an imaginary box where within the range of volume, the given ligand can find the best possible binding site with lowest binding energy and higher binding affinity; short grid was generated to recognize the binding region of the protein molecule (receptor). We performed blind docking experiments of ligands with grid volume of 40x58x40 points (60x60x60 for AS4), grid spacing of 0.803 Å (1.000 Å for AS4) and centered (3.130, -3.283, -17.750) on the receptor helical A β ₁₋₄₀ peptide (PDB ID: 4NGE). Also we performed blind docking experiments of ligands with fibril A β ₁₋₄₀ peptide (PDB ID: 2M4J). In that case, grid volumes were (66x96x56 for 1 unit, 122x124x68 for 3 units, 112x112x78 for 4 units, 110x108x74 for 6 units), grid spaces were (0.814 Å for 1 unit, 0.900 Å for 3 units, 0.903 Å for 4 units, 1.000 Å for 6 units), centres were (3.655, -20.332, 101.713 for 1 unit; -8.891, -6.213, 101.870 for 3 units; -5.675, -9.676, 103.120 for 4 units; -8.735, -6.099, 104.448 for 6 units). After completion of docking, the output file was generated as ligand_out.pdbqt format which contains binding affinity (in kcal/mol) and RMSD lower bound, RMSD upper bound. PyMOL version 1.7.4.5 software was used

for molecular visualization and viewing the docking results. The active site regions were selected and labeled them as needed.

1.14.6. Biophysical methods used for the studies:

1.14.6.1. A β sample preparation. A β_{1-40} was purchased from GL Biochem Shanghai, China and directly used without purification. The required amount of A β_{1-40} was dissolved in 20 μ L of TFA to get disaggregated A β_{1-40} . TFA was evaporated using nitrogen gas. To remove TFA completely, HFIP was added and evaporated using nitrogen gas to get disaggregated A β_{1-40} peptide. This process was repeated twice. 2.0 ml of PBS (50 mM, pH 7.4) was added into the disaggregated material, followed by sonication and vortex to obtain a transparent solution and the total solution was divided equally (as required) followed by addition of 800 μ L of PBS to each portion to obtain a final concentration of 50 μ M.

1.14.6.2. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS): The isolate gives a peak of the corresponding mass when analyzed by MALDI-TOF mass spectrometry. Aliquots of various incubated samples can be tested by a MALDI-TOF mass spectrometer in a time-resolved manner to find out various fragments and intermediates from the corresponding mass values. Plausible routes of proteolytic cleavage of mA β by designed peptides can be drawn based on the mass values.

Sample preparation for MALDI-TOF mass analysis: Purified and lyophilized peptide samples were dissolved in PBS (50 mM, pH 7.4) to obtain stock solutions of 200 μ M. While mA β was pretreated with TFA and HFIP, 6% Ethanol was added to PBS (50 mM, pH 7.4) solutions of ASs to get clear stock solutions. They were co-incubated appropriately at 37 °C on a water bath and tested in a time-dependent manner.

MALDI-TOF mass of the peptide samples was analyzed using the CHCA matrix on BRUKER auto flex speed instrument which consists of a MALDI ionization source for samples in the solid-state and a TOF/TOF mass analyzer, equipped with Bruker daltonics

flex analysis software. To prepare the CHCA matrix, 1.0 mg of CHCA was dissolved in 100 μL of $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (1:1) containing 0.1% TFA. Parameters used for MALDI-TOF mass analyses were as follows: Processing method: SC_Peptide_Cent, Method: RP_700-3500_Da.par, Reflector: 3x 2076v, Sample Carrier: Random walk - complete sample mode (off mode where necessary).

MALDI-TOF MS analysis:

In general, when a particle was added multiple ions during the ionization, it will bear relative multiple positive charges, and the mass-charge ratio would be divided by the number of charges; but for MALDI-TOF mass, sometimes different results were obtained specially for proteins in the presence of salts. In this direction, Distler and Allison mentioned in their article, “The presence of salts has a much more dramatic influence on the MALDI spectra of oligonucleotides. With an ionic phosphodiester backbone, oligonucleotides can contain a large number of anionic sites that must combine with an assortment of cationic species such as H^+ , Na^+ , and K^+ to desorb in a singly charged form. Combinations of cations to provide a charge balance can lead to a substantial number of peaks representing the intact molecule.”⁸⁶ Also, Henzel, in his PNAS article, mentioned: “A tolerance of 4 Da (the difference between the mass found and the calculated mass) was used for the analysis of proteins by MALDI-TOF MS.”⁸⁷ We analysed our data following these articles.

R. M. Caprioli et al. identified proteins and peptides from the MALDI-Mass spectra as sodium and potassium ion adducts and multiply-charged ions. For example, in Table 1 of their article, calculated m/z of diacetylated α -MSH fragment (1-11) $[\text{M}+\text{H}]^+$ is 1512.6, observed 1515.5 (% difference +0.19, which correspond to $[\text{M}+4\text{H}]^+$). In Table 2, calculated m/z of hemoglobin α - chain (major) $[\text{M}+\text{H}]^+$ is 15197.0, observed 15283.4 (% difference +0.57, which correspond to $[\text{M}+2\text{Na}+\text{K}+\text{H}]^+$).⁸⁸

We have calibrated MALDI Mass and HRMS instruments regularly. In ESI-MS, $[\text{M}+\text{H}]^+$, $[\text{M}+2\text{H}]^{2+}$, $[\text{M}+3\text{H}]^{3+}$ were found for mAb (characterization data) and it follows the mass-charge ratio rules. Again, we have taken ESI-Mass data of the collected sample after HPLC kinetics of the incubated sample, and they follow the mass-charge ratio rules (Figure 2.55-2.60). But when mAb was co-incubated in PBS, $[\text{M}+\text{H}]^+$, $[\text{M}+2\text{H}]^+$,

$[M+3H]^+$, $[M+K]^+$, $[M+K+H]^+$ in a singly charged form were obtained in MALDI for mAb at different days (for example Figure 2.41-43, and 2.77-79).

1.14.6.3. High-Performance Liquid Chromatography (HPLC) and ESI-MS: HPLC is one of the most used analytical methods to isolate the components of a mixture in a liquid mobile phase. So, time-dependent HPLC analysis is a useful technique to investigate the proteolytic efficacy of designed peptides on mAb. From the intensity of gradually disappeared peaks or newly appeared peaks at some specific retention times, it is possible to identify various peptide fragments quantitatively after collecting the content of those peaks followed by ESI-MS analysis.

Kinetics study was done using a Waters 600E analytical HPLC system also. An Ascentis C18 analytical column, a flow rate of 0.8 ml/min, a linear gradient of 5-100% CH₃CN over 0-18 minutes in a total run time of 20 min were used. Dual-wavelength was selected at 214 nm and 254 nm. The collected sample (from 10d_mix) was run further in Agilent-Q-TOF 6500 LCMS instrument, at ESI-positive mode. Agilent eclipse plus C18 analytical column, a flow rate of 0.3 ml/min, a linear gradient of 5-100% CH₃CN over 6 minutes in a total run time of 7 min were used. Wavelength was selected at 214 nm.

1.14.6.4. Ultra Violet-Visible (UV-Vis) Spectroscopy:⁸⁹

UV absorbance is a useful technique to perform the kinetics of hydrolysis of p-Nitrophenyl acetate. Proteins absorb at 240-310 nm due to the presence of an aromatic side chain of Trp, Tyr, and Phe. The peptide bond generally absorbs below 230 nm due to $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ electronic transitions, while aromatic residues absorb in the near-UV region between 250 and 290 nm due to the $\pi \rightarrow \pi^*$ electronic transitions.

Purified solid peptide sample and p-nitrophenyl acetate were dissolved in PBS (50 mM, pH 7.0) with 2 vol % of ethanol to obtain a stock solution of 1 mM. To perform the UV-VIS study, the stock solution of the peptide was diluted as required and mixed with p-nitrophenyl acetate and incubated at 25 °C on a water bath. The kinetics was performed at 275 nm and 400 nm respectively. 2 mL of the sample was taken in a cuvette with a path

length of 1 cm. For UV-Vis absorption study Spectra were recorded from 200 nm to 700 nm on Perkin Elmer (lambda 35). The text files were plotted using OriginPro 8 software.

1.14.6.5. Thioflavin T (ThT) fluorescence assay:⁹⁰ ThT is a benzothiazole fluorescent dye which is weakly fluorescent in water with excitation and emission maxima at 350 and 440 nm respectively, but upon binding to amyloid fibrils excitation and emission maxima shifts to the higher wavelength at 435 and 485 nm respectively. ThT assay is the most commonly used quantitative technique to monitor the formation of amyloid fibrils with time. The fluorescence intensity of a peptide upon binding with ThT is directly proportional to the amount of fibril present in a given set of conditions. On the other hand, the decrement fluorescence intensity indicates inhibition of fibrillar assembly. So, the kinetics of amyloid accumulation in the absence and presence of additives can be monitored by ThT assay.

Thioflavin T (ThT) was purchased from Sigma Aldrich, and a concentration of 50 μM in PBS (50 mM, pH 7.4) was prepared as a stock solution and stored at 4 °C with dark cover to prevent degradation from light. Purified solid peptide samples (mA β and A β_{1-40} after TFA, HFIP treatment; and, designed peptides and negative control directly) were dissolved in PBS (50 mM, pH 7.4) to obtain a stock solution of variable concentrations (50 μM of A β_{1-40} , 200 μM of mA β and different molar ratios of designed peptides) and incubated at 37 °C on a water bath. In the case of ASs, 6% Ethanol was used along with PBS (50 mM, pH 7.4) to get a clear stock solution. To perform the fluorescence study, 40 μL of peptide sample was taken out from the stock solution and was mixed with 200 μL of ThT solution (50 μM); the final volume of 400 μL was made up with PBS (50 mM, pH 7.4). For the desired stock solution, three different replicate solutions were prepared. For ThT fluorescence assay, emission was measured at 485 nm and excitation at 440 nm, using a slit of 5 nm on a Fluoromax-4, Horiba instrument. The text file was taken from the instrument and OriginPro 8 software was used to plot the graph. Three different sets of replicate solutions were scanned separately for each data point, and the average was taken with observed standard deviation.

1.14.6.6. Circular Dichroism (CD) Spectroscopy:^{91,92,93} Fibrillization is usually associated with conformational alteration of the peptide. CD is the differential absorption of right-handed and left-handed circularly polarized light as a function of wavelength exhibited by optically active molecules. As secondary structural elements such as helix, sheet, turn, or random coil display characteristic wavelength-dependent dichroism, the CD is one of the useful techniques to determine the secondary structure of proteins. The secondary structure of peptides and proteins is characterized by CD spectroscopy in the “far-UV” region (180-250 nm) because, in general, the peptide bond absorbs at 220 nm and 190 nm due to $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ electronic transitions respectively. The β -sheet structure shows the negative band at 215 nm and a positive one at 195 nm. The negative Cotton effect around 195 nm confirms the random coil structure. The α -helix structure shows two negative bands around 205 nm and 220 nm, and a positive band around 195 nm.

Peptide stock solutions were prepared in the same way as described earlier for sample preparation of the ThT fluorescence assay. To perform the CD study, the stock solution was diluted with respective buffer solutions to obtain a final concentration of 100 μ M (50 μ M for A β_{1-40} peptide). 400 μ L of the sample was taken in a cuvette (Model SPC-001) having a bandwidth of 1 mm. Three measurements were collected. Spectra were recorded from 190 nm to 260 nm on a JASCO (Model J-1500) instrument. Observed ellipticity (mDeg) [obtained from Spectra Manager] was converted to mean residue molar ellipticity using the following equation:

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

1.14.6.7. Fourier transformation infrared (FTIR) spectroscopy:⁷ FTIR enables to obtain an idea of the secondary structure of the peptides. The presence of a strong band at around 1625-1640 cm^{-1} indicates the β -sheet structure and presence of a strong band at around 1645-1660 cm^{-1} indicates a random coil or helical conformation of peptides or proteins.

Peptide stock solutions were prepared in the same way as described earlier for sample preparation of the ThT fluorescence assay. 20 μ L aliquot was taken from the stock, mixed with KBr and dried completely at 50 $^{\circ}\text{C}$, and a pellet was prepared. For each sample, the

spectrum was recorded after the required time intervals. The background scan was subtracted from the sample scans and OriginPro 8 software was used to plot the graph from text files.

1.14.6.8. Transmission Electron Microscopy (TEM):⁷ TEM is used for morphological analysis. The presence of fibrillar structure under TEM is another characteristic property of amyloid formed by a peptide. In this technique, a cathode-ray source emits and accelerates a high-voltage electron beam which then passes through a thin, electron-transparent sample, and produces the inner structure of the sample as it reaches the TEM imaging system. To enhance the contrast of images, and reduce image noise, negative staining is used.

10 μ L aliquot from the stock peptide solution after required time intervals were added over the carbon-coated copper grid and allowed to float for 1 min. Then 2% uranyl acetate solution (10 μ L) was added on the same grid for negative staining and allowed to float for a further 1 min. Blotting paper was used to remove excess solution. The sample was air-dried at room temperature and kept in desiccators. TEM analyses were performed on a JEOL instrument (Model: JEM 2100) at 200 kV.

1.14.6.9. Atomic Force Microscopy (AFM):⁹⁴ AFM captures high-resolution topography of a sample adsorbed on mica or atomically flat surface. In AFM imaging, when the cantilever tip contacts a spot on the adsorbed sample, a repulsive ionic force would bend the AFM cantilever up. The extent of bending is translated by a photodetector to force units. AFM is very useful to investigate non-fibrillar A β species.

To perform the AFM analysis, the stock solution was diluted with water to obtain a final concentration of 5 μ M (1 μ M for A β ₁₋₄₀ peptide). 20 μ L aliquot from the diluted peptide solution was added over the coverslips, washed with Milli-Q water and dried at room temperature and analysed on Agilent STM 5500 instrument.

1.14.6.10. Congo red-stained birefringence study:⁷ Another characteristic property of amyloid is the expression of green-gold birefringence under cross-polarized light after staining with Congo red. Noticeably only mature fibrils and amyloid form of A β show green-gold birefringence, but oligomers or proto-fibrils do not show such birefringence.

Commercially available Congo red was purchased from Sigma Aldrich and dissolved in 80 % aqueous ethanol to prepare a saturated solution. Then sodium chloride solution (saturated) was added into the saturated Congo red solution and filtered to get needed Congo red solution for analysis.

After required time intervals, 20 μ L aliquot of the peptide solution was taken from the stock and placed over a glass slide followed by 40 μ L of the saturated Congo red solution. Blotting paper was used to remove excess solution. The sample was dried at room temperature and kept in desiccators. Birefringence analyses were performed on a Leica ICC50 HD polarizable microscope.

1.14.6.11. Dynamic Light Scattering (DLS) spectroscopy:^{95,96} As the intensity of the scattered light is proportional to the square of the particle mass, DLS is fundamentally biased for large aggregates. Hence, DLS is appropriate to measure minute amounts of protein aggregates. So, the size distributions of A β ₁₋₄₀ aggregates in the absence and presence of designed peptides can be determined by the DLS technique.

The size distributions of A β ₁₋₄₀ aggregates and co-incubated peptide samples were analysed using Zetasizer Nano-ZS90 (Malvern Instruments). To perform the DLS analysis, stock solutions were diluted with Milli-Q water to obtain a final concentration of 100 μ M (50 μ M for A β ₁₋₄₀ peptide). All the DLS results were reported as the average of three measurements.

1.14.6.12. Large unilamellar vesicles (LUVs) leakage study:^{97,98} Large unilamellar vesicles (LUVs) are considered as a cell membrane mimic. Dye-entrapped vesicle leakage study is extensively used to check the cell membrane disruption capability by additives. Smaller soluble oligomers of A β are found to be more toxic than insoluble fibril of A β , as soluble oligomers can form pores in the cell membrane resulting in membrane disruption.

Therefore, dye leakage studies were performed using carboxyfluorescein dye entrapped LUVs to check the toxicity of the peptide fragments generated from A β ₁₋₄₀ by proteolytic cleavage by designed peptides.

Preparation of LUVs and carboxyfluorescein entrapment: Large unilamellar vesicles (LUVs) were prepared by mixing three different lipids; DPPC, Cholesterol, and GM1 with 68:30:2 molar ratios. At first, lipids were solubilized in chloroform and methanol (2:1) to make 2 mM stock solution in a glass vial and solvents were evaporated completely using nitrogen gas followed by vacuum to make lipid films. The lipid films were hydrated with carboxyfluorescein (200 μ M, 500 μ L) in 50 mM HEPES buffer of pH 7.4. After that, the solution was vortexed vigorously for 30 min for emulsification. Then, the glass vial was dipped into liquid nitrogen for instant cooling, and after 5 min the frozen solution was dipped into a water bath at 50-60 °C, and the procedure is known as thawing process which was repeated for five times. Excess dye was removed by ultracentrifugation at 20000 rpm for 30 min at 10°C. The supernatant dye solution was discarded and the lipid pellet was re-hydrated with 50 mM HEPES buffer of pH 7.4. This step was repeated twice, and the final lipid pellet was collected. Next, 500 μ L of HEPES buffer of pH 7.4 was added to the lipid pellet and vortexed to obtain a homogenous suspension, which was filtered through 0.45 μ m polycarbonate membrane to obtain dye loaded LUVs (2 mM).

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Chapter 2: *De novo* designed peptidic constructs cleave Amyloid β at the α -secretase cleavage site by aggregation driven cascades of chemical reactions

2.1. Introduction:

Despite enormous efforts and considerable progress in different directions of research for the treatment of Alzheimer's disease (AD), there is no cure available yet.¹ Therefore the development of new strategies for drug design to obtain efficient drugs for the treatment of AD is the need of the hour. According to the "amyloid cascade hypothesis", conformational conversion, and self-assembly leading to fibril formation and plaque deposition of the 39-42 residues long Amyloid β peptides ($A\beta$) outside nerve cells causes neuronal dysfunction, dementia and cell death in AD.² In spite of the prevailing controversy on the amyloid cascade hypothesis,³ it is generally accepted that the main determinants of the $A\beta$ toxicity lie in its aggregating nature.⁴ However, aggregation of hyperphosphorylated tau protein in the form of neurofibrillary tangles (NFT) inside the neuron is also believed to have a causative role.⁵

$A\beta$ is generated by the amyloidogenic pathway of the amyloid precursor protein (APP) processing by sequential proteolysis by β - and γ -secretases. Therefore, many strategies have been and are being developed for the reduction of aggregation^{6,7,8} and the facilitation of excretion of $A\beta$ from the brain.⁹ However, works on the controlled production of $A\beta$ are relatively less and mostly depend on the development of β - and γ - secretase inhibitors.^{10,11} Alternatively, non-amyloidogenic APP processing pathway includes site-specific cleavage by α -secretase to release neuroprotective APP fragment and soluble APP- α (sAPP α), thus, prevents the generation of neurotoxic and pathogenic $A\beta$ peptide.¹² Therefore, the non-amyloidogenic pathway is healthy and does not cause dementia.

Promoting the α -secretase-mediated processing of APP is, therefore, another promising therapeutic option for the treatment of AD.¹³

Furthermore, we wanted to achieve site-selective proteolysis by mimicking Glu based cleavage mechanism as occurs in HCF-1.^{14, 15} In our judgment, although O-GlcNAc activation is sufficient for internal pyroglutamate formation, it may not be active enough for inter-strand acyl transfer. Therefore, to get a quick proof of principle, we replaced O-GlcNAc by a relatively more reactive benzyl (O-Bzl) group. However, increasing the reactivity of the carboxylic ester too much is detrimental for our purpose as it may undergo hydrolysis in physiological pH and temperature before reaching the active site. Furthermore, we considered the side chain of Glu too small to reach the backbone of a neighboring peptide for an intermolecular attack. Therefore, we replaced it by adipic acid, a six carbon-containing linear dicarboxylic acid, one carboxylic end of which was attached to the side chain of Lys, so that it is long enough to reach to the peptide backbone of the target APP or A β . Finally, we attached such constructs at the *N*-terminus of a homologous self-assembling partial sequence of A β , KLVFF, which is known to bind A β .¹⁶ As it is also a partial sequence of APP and bears self-assembling nature, it is expected to bind APP by similar supramolecular association and cleave it. Thus, we planned to design and develop some peptide-based constructs that are intended to recognize the homologous sequence of A β or APP and cleave them mainly at the α -secretase cleavage site to generate non-toxic sAPP α and to reduce toxic A β (Figure 1). As these peptide conjugates are intended to cleave at or around the α -secretases cleavage site, they are mentioned as artificial α -Secretases (ASs), hereafter. ASs are supposed to mimic precisely the APP processing activity of α -secretases, although in a non-catalytic manner, and leave all other substrates of α -secretase unaffected, reducing side effects. Usually, α -Secretase cleaves soluble folded APP, but can't metabolize aggregated A β due to solvent inaccessibility. However, the ASs are designed to act by self-assembly driven proteolysis, therefore, can truncate and dissolve aggregated A β as well. Thus ASs may mimic the functions of other A β processing enzymes, such as NEP, ECE, IDE, P, ACE, and MMPs also. Proper optimization of such constructs may lead to a robust strategy for drug design against not only the AD but also other amyloidoses.

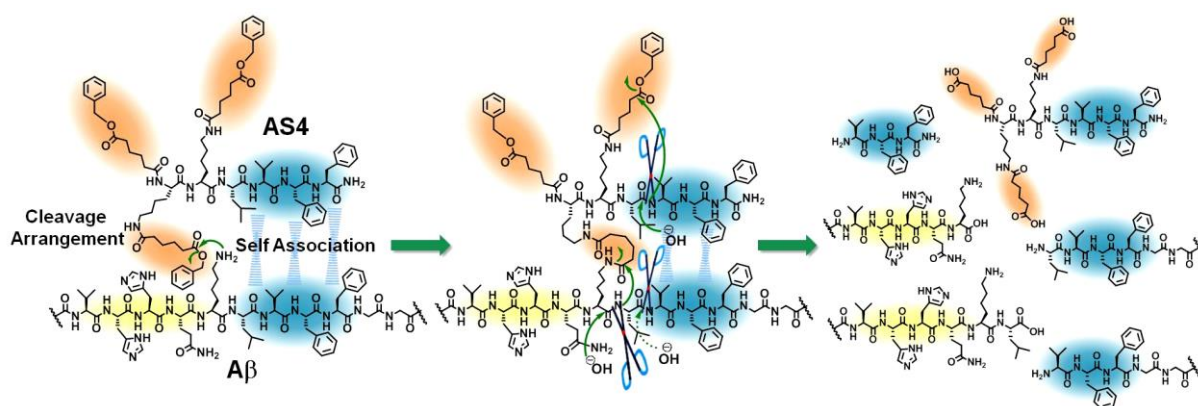


Figure 2.1. Anticipated mode of the proteolysis of $A\beta$ by AS4.

2.2. Design of the ASs and other relevant peptides:

At first, we designed four artificial α -secretases (ASs) which contain LVFF as the recognition element and benzyl adipate (BnAdp) attached at its N-terminal with a lysine unit as a scissor to cut the backbone peptide bond of nearing $A\beta$ or APP at the α -secretase cleavage site (Table 2.1, Figure 2.1). To optimize the cleavage site, the position of the BnAdp was varied. While in AS1, BnAdp was hanged to the side chain of the N-terminal Lys, in AS2 it was on the Lys that was separated by a Gly and for AS3 BnAdp was attached at the N-terminal amino group of the Ala spacers attached at the N-terminus. In AS4, a synergistic effect of all three above ASs was desired.

To test its proteolytic activity quickly, we synthesized a partial sequence of $A\beta$, which is essentially a part of the APP as well ($A\beta_{12-20}$ or $APP_{683-691}$). It contains the α -secretase cleavage site, the peptide bond connecting Lys₁₆ and Leu₁₇ of $A\beta$,¹⁷ at its middle portion. It also contains the self-assembling core hydrophobic region of $A\beta$, KLVFF. It is supposed to act as a model peptide in place of the larger $A\beta$ and APP (hereafter denoted as $mA\beta$). In $mA\beta$, Ala21 or Ala192 for $A\beta$ or APP, respectively, is replaced by Gly as it occurs in highly disease-prone Flemish mutation.¹⁸ Another Gly at the C-terminus was added as a spacer for synthetic advantage. To examine the selective nature of ASs, we also synthesized a 28 residue long unrelated negative control peptide based on the 28 membered mutant diphtheria toxin (DTP28)¹⁹ sequence by replacing Val8 and Lys23 by

Pro. Characterization data of the synthesized peptides were attached in the supporting data (Figure 2.101-113).

Table 2.1. Sequences of the peptides and their roles in the current study.

Sl. no.	Name of the peptide (short name)	Peptide sequence	Role of the peptide
1	Artificial α -Secretase 1 (AS1)	Ac-K(BnAdp)LVFF-NH ₂	Site-selective cleavage
2	Artificial α -Secretase 2 (AS2)	Ac-K(BnAdp)GLVFF-NH ₂	Site-selective cleavage
3	Artificial α -Secretase 3 (AS3)	BnAdp-AALVFF-NH ₂	Site-selective cleavage
4	Artificial α -Secretase 4 (AS4)	BnAdp-K(BnAdp)K(BnAdp)LVFF-NH ₂	Site-selective cleavage
5	Modified A β ₁₂₋₂₀ and APP ₆₈₃₋₆₉₁ (mA β)	Ac-VHHQKLVEFGG-NH ₂	The prototype of A β and APP
6	A β ₁₋₄₀ peptide	DAEFRHDSGYEVHHQKLVFFAEDV GSNKGAIIGLMVGGVV-NH ₂	Target peptide
7	Mutant DTP28	GSSDSIGPLGYGKTVDHTKVNLSPLS LFG-NH ₂	Negative control

2.3. Molecular docking studies for an initial understanding of binding affinity:

We performed molecular docking for investigating binding affinity of all the ASs with helical A β ₁₋₄₀ (PDB ID: 4NGE) in Autodock Vina 1.1.2²⁰ (Figure 2.2), and all of them interacted well in the A β ₁₋₄₀ binding pocket having the binding affinity -5.9, -5.4, -6.2 and -6.6 kcal/mol for AS 1, 2, 3 and 4, respectively. Interestingly, AS4 exhibited maximum binding affinity among the entire ASs tested (Figure 2.3. a-b). Therefore, next, we performed molecular docking studies of AS4 with fibrillar A β ₁₋₄₀ (PDB ID: 2M4J; Figure 2.3). In that case, AS4 bound well in the A β ₁₋₄₀ binding pocket independent on the number of the extracted A β ₁₋₄₀ unit used, e.g., binding affinity was -4.8, -3.9, -5.0 and -6.2 kcal/mol for one, three, four and six units, respectively. In most of the cases, the interacting amino acids in the A β ₁₋₄₀ binding pocket were ¹⁷Leu, ¹⁸Val, ¹⁹Phe, and ²⁰Phe, as expected. Finally, AS4 was docked into helical C-terminal domain of APP (C99, residues 683-728) [PDB ID: 2LP1], and it was observed that AS4 fitted well in the APP binding pocket having binding affinity -5.3 kcal/mol where the interacting amino acids in the pocket were ⁶⁸⁷KLVF⁶⁹¹F (Figure 2.4).

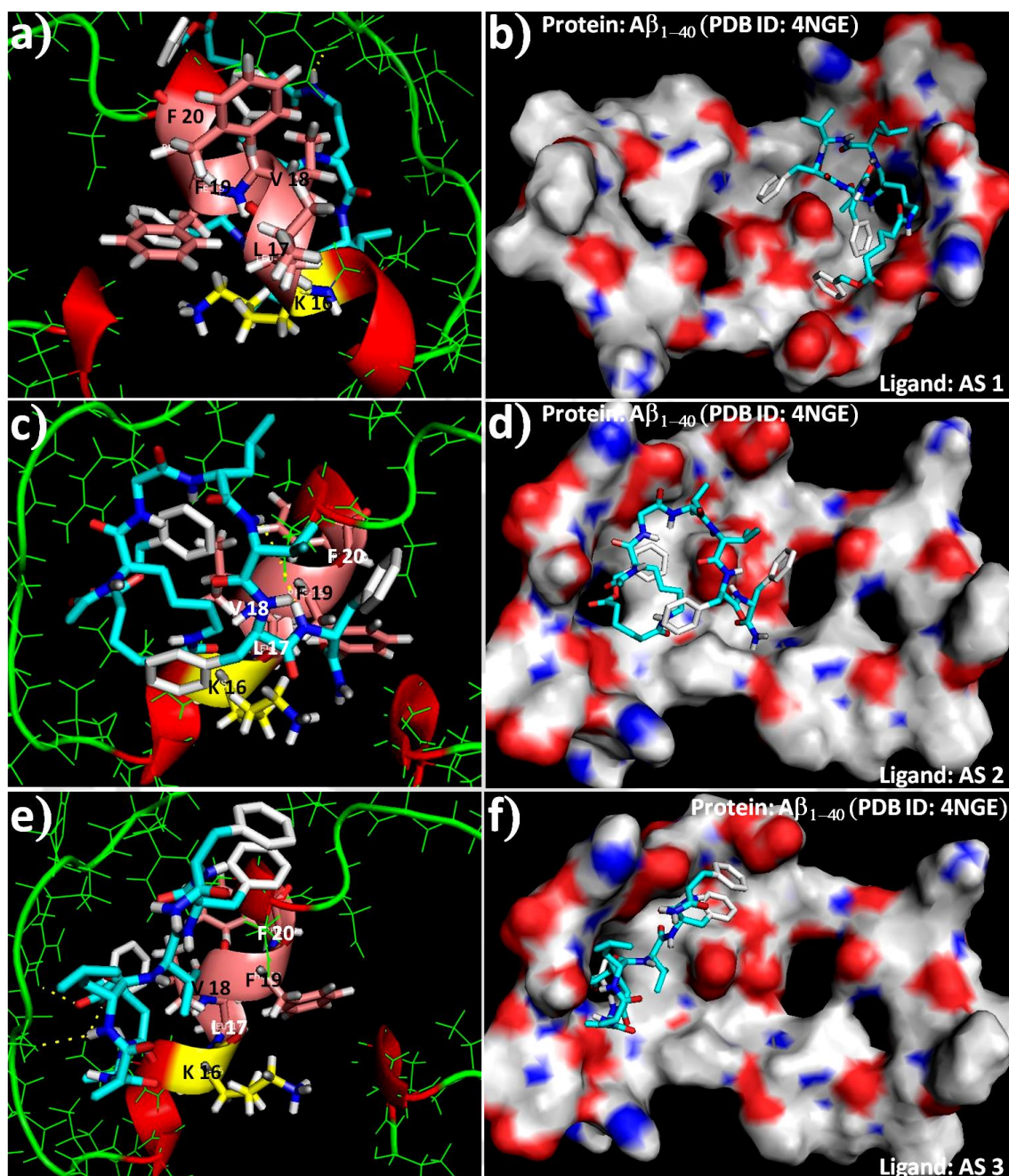


Figure 2.2. Molecular docking images of AS1 (a, b), AS2 (c, d), and AS3 (e, f) into helical A β_{1-40} (PDB ID 4NGE) reveals that ASs binds at the binding site ($^{16}\text{KLVF}^{20}\text{F}$) with binding affinity -5.9 kcal/mol, -5.4 kcal/mol, and -6.2 kcal/mol, respectively. Structures are shown as line and cartoon representation (for a, c, e), and surface representation (for b, d, f). The surface of A β_{1-40} is coloured according to the charges of the atoms where negatively and positively charged zones are represented in red and blue, respectively.

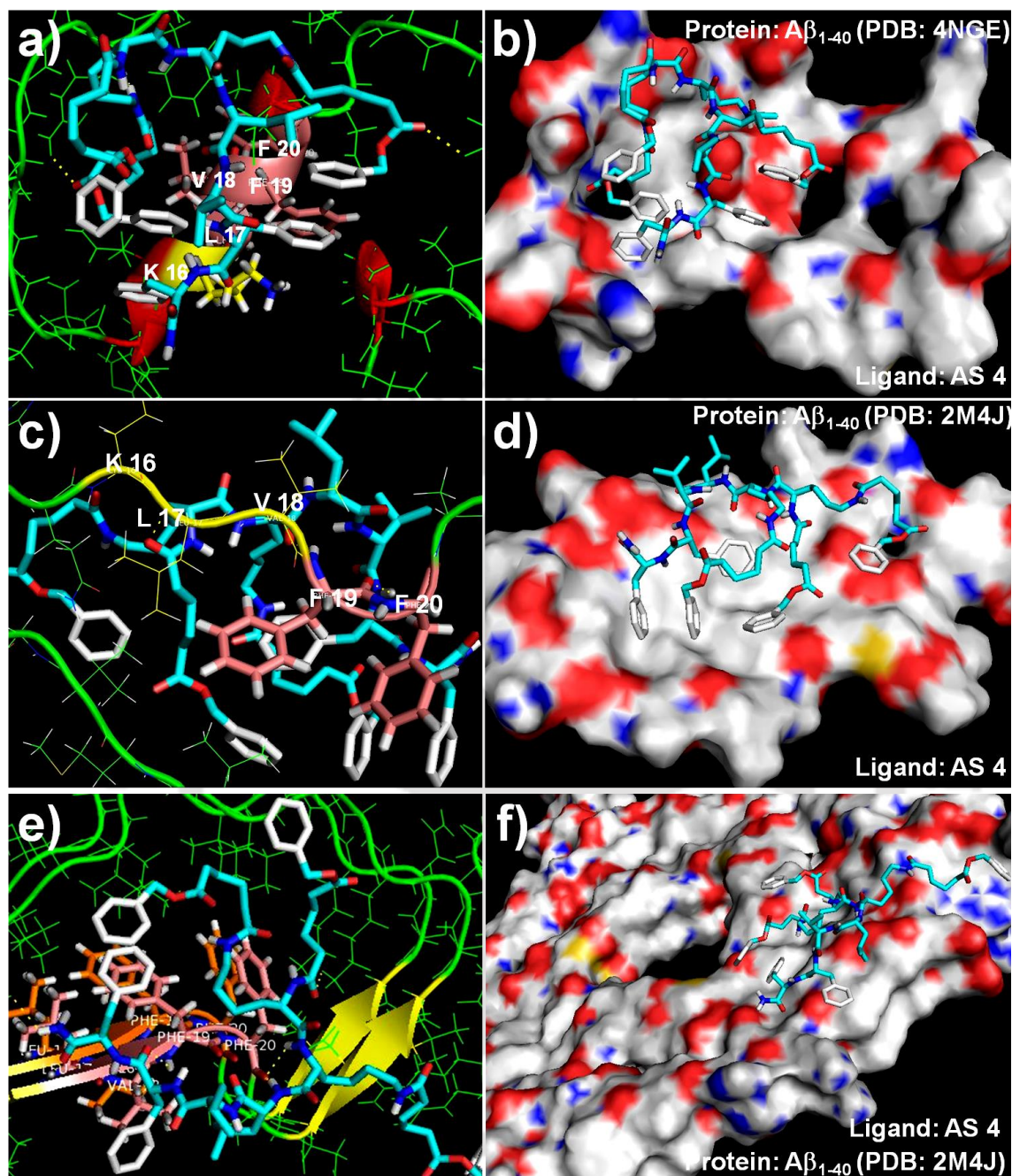


Figure 2.3. Molecular docking images of AS4 into helical (a-b), and fibril (c-f) A β ₁₋₄₀ (PDB ID 4NGE for a, b and 2M4J for c-f) reveals that AS4 binds at the binding site (¹⁶KLVF²⁰F) with binding affinity -6.6 kcal/mol, -4.8 kcal/mol, and -6.2 kcal/mol, respectively. Structures are shown as line and cartoon representation (for a, c, e), and surface representation (for b, d, f). The surface of A β ₁₋₄₀ is coloured according to the charges of the atoms where negatively and positively charged zones are represented in red and blue, respectively.

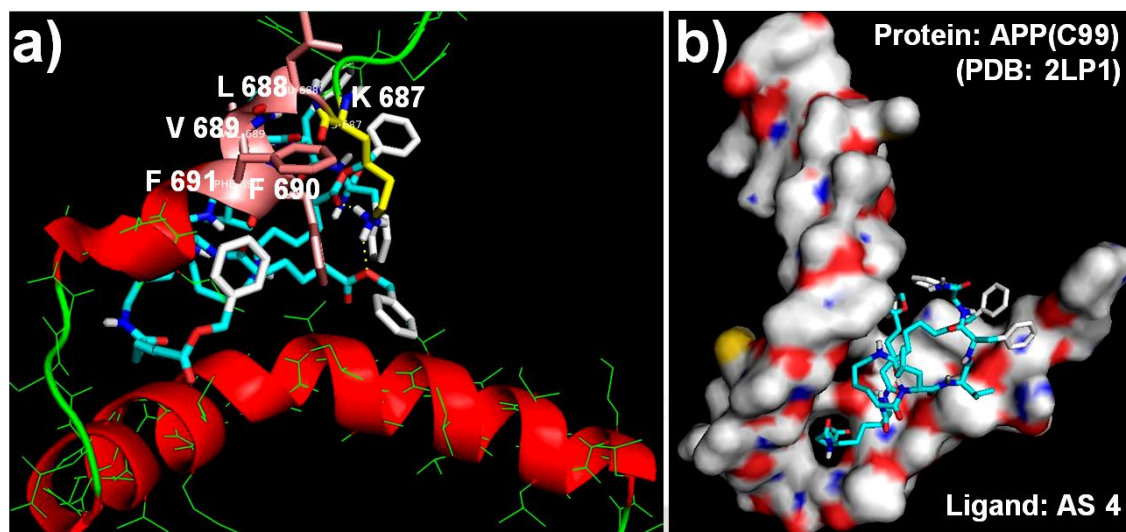


Figure 2.4. Molecular docking images of AS4 into (a-b) helical C-terminal domain of the amyloid precursor protein (C99) (PDB ID: 2LP1) reveals that AS 4 binds at the binding site (⁶⁸⁷KLVF⁶⁹¹F) with binding affinity -5.3 kcal/mol. Structures are shown as line and cartoon representation (for a), and surface representation (for b). The surface of the APP is coloured according to the charges of the atoms where negatively and positively charged zones are represented in red and blue, respectively.

2.4. Examination of the proteolytic activity of the ASs on mA β :

Before going to the A β peptide directly, which is costly and difficult to handle, we analyzed the proteolytic activity of the ASs on the mA β to get an initial idea on the proteolytic efficacy of the ASs. For that, suitable stock solutions were prepared as mentioned in the method section, co-incubated in 1:1 molar ratio of the mA β (200 μ M) and each of the ASs separately at 37 °C on a water bath. Aliquots were tested by a MALDI-TOF mass spectrometer in a time-resolved manner. ASs were stable even after 15 days at physiological pH and temperature; debenzylated products started appearing after one day, however. After 13 days of incubation, peaks corresponding to the mass of various fragments and intermediates could be observed.

2.4.1. Plausible routes of the proteolytic cleavage of mA β by AS1:

Based on the MALDI-TOF mass values, likely routes of proteolytic cleavage of mA β by AS1 were drawn (Figure 2.5-15). These results indicated AS1 cleaved the mA β in a site-selective manner between Leu-Val via route 1^{mA β -AS1} (Figure 2.5). The fragments D' and

I' may get generated by the attack of Lys side-chain nitrogen of mA β to the BnAdp carbonyl of AS1 as depicted in route 1^{mA β -AS1}. Repetition of the imidation-hydrolysis-transamidation cycle generates various intermediates and cleavage products.

Route 1^{mA β -AS1}

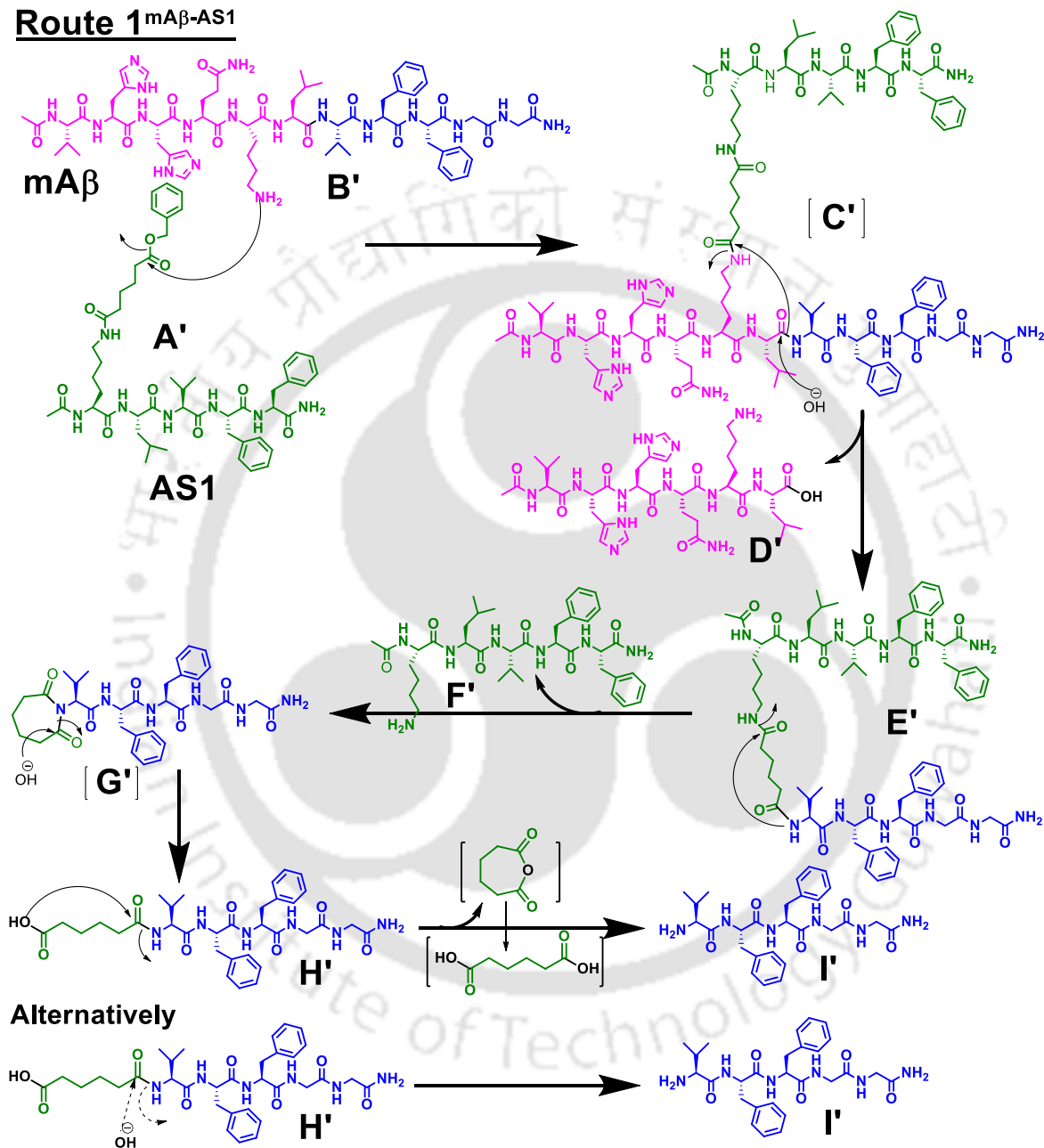


Figure 2.5. Plausible route for proteolysis of mA β by AS1 into various fragments (Route 1^{mA β -AS1}).

MALDI-TOF mass results of proteolytic cleavage of mA β by AS1 indicated that AS1 cleaved the mA β in a site-selective manner between Lys-Leu via route 2^{mA β -AS1} (Figure

2.6). In this case also various intermediates and cleavage products may get generated by the repetition of the imidation-hydrolysis-transamidation cycle from the intermediate C'.

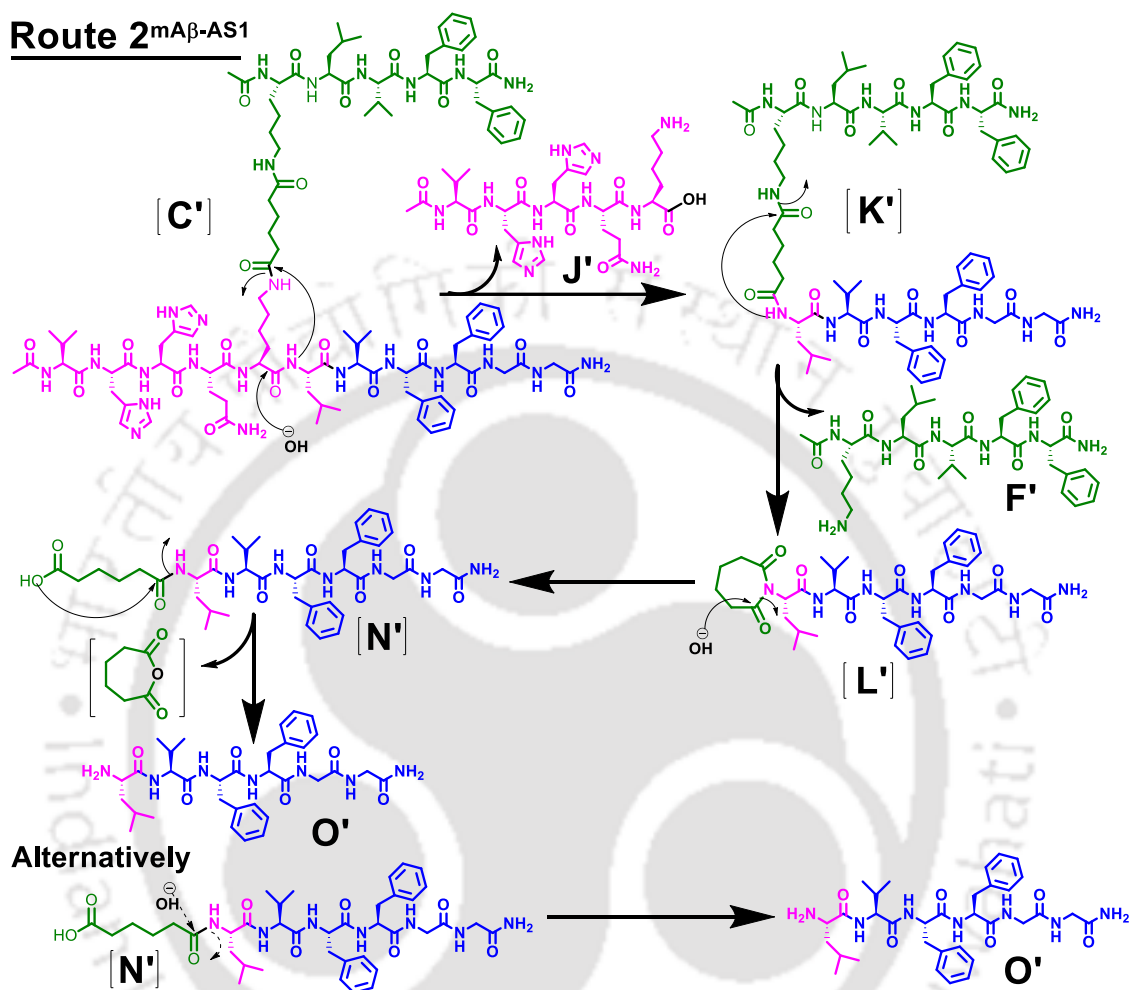


Figure 2.6. Plausible routes of cleavage of mA β into various fragments by AS1 (Route 2^{mA β -AS1}).

Alternatively, the fragments J (e.g., J', J'' and J''') and O (e.g., O', O'' and O''') also may get generated by the attack of Asn side-chain amide nitrogen of mA β to the BnAdp carbonyl of AS1, as depicted in the Route 3^{mA β -AS1} (Figure 2.7), as a representative example. However, route 3^{mA β -AS1} should be less probable than the route 2^{mA β -AS1} owing to the less availability of the electron cloud over the resonance stabilized amide nitrogen in comparison to the amine group of the side chain of Lys of mA β . The mentioned mechanism of route 3^{mA β -AS1} was not shown for other ASs as it is relatively less probable.

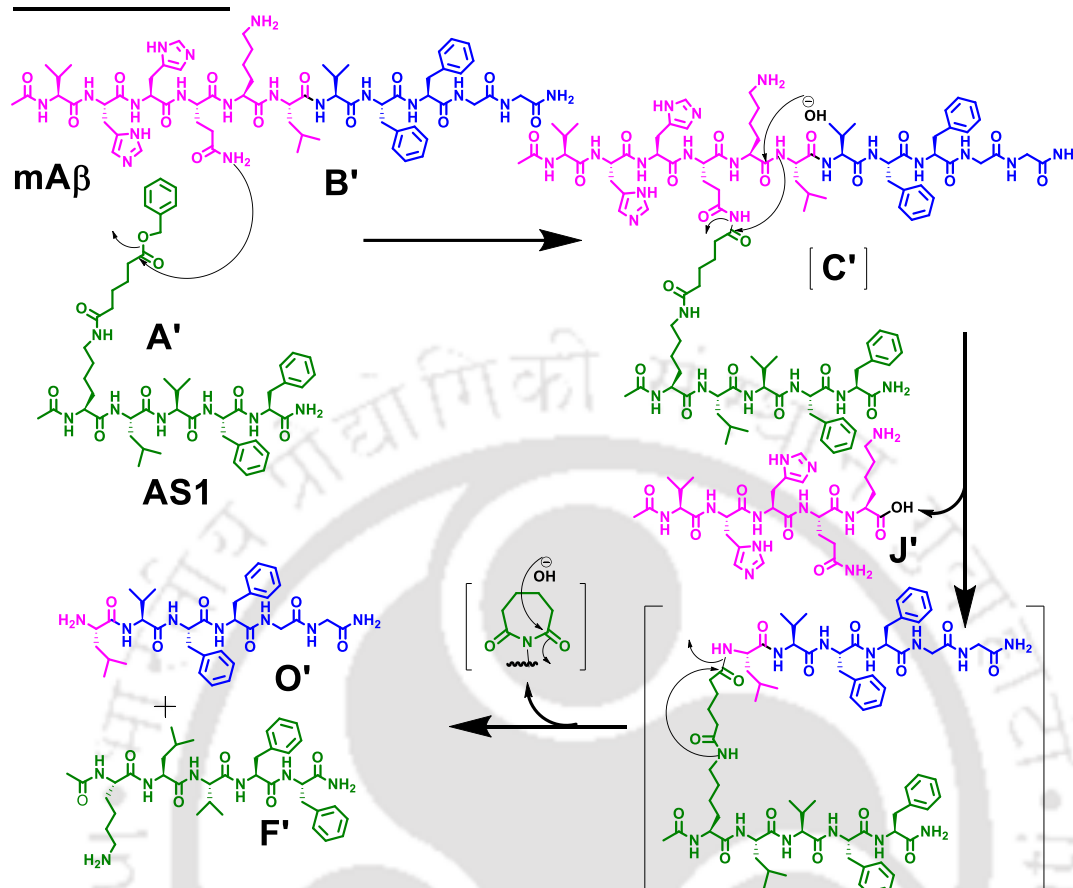
Route 3^{mA β -AS1}

Figure 2.7. Plausible routes of cleavage of mAbeta into various fragments by AS1 (Route 3^{mA β -AS1}).

Besides proteolysis products of mAbeta, AS1 generated self-degradation product Q', following probable route 1^{SD-AS1} (Figure 2.8, "SD" stands for self-degradation). To confirm that these are generated by self-degradation, we conducted time-dependent MALDI-TOF mass spectrometric studies of the AS1 incubated in the absence of mAbeta (Figure 2.15).

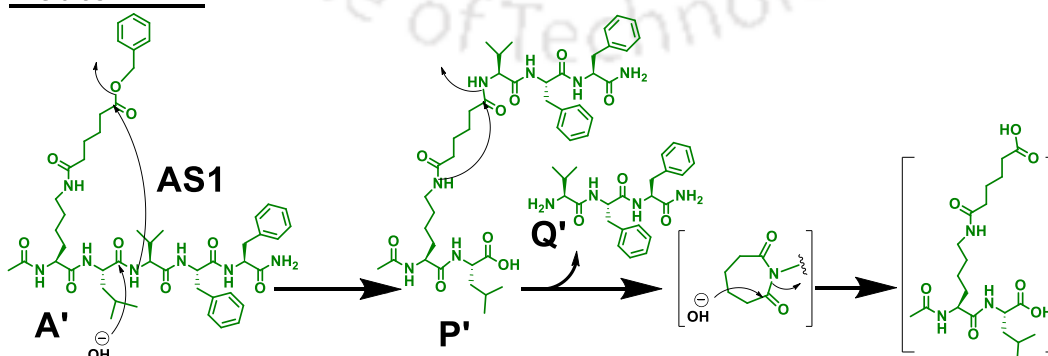
Route 1^{SD-AS1}

Figure 2.8. Plausible route of self-degradation of AS1 in the absence of mAbeta (Route 1^{SD-AS1}).

AS1 also generated self-degradation product R' (de-benzylated product), following the route 2^{SD-AS1}, (Figure 2.9).

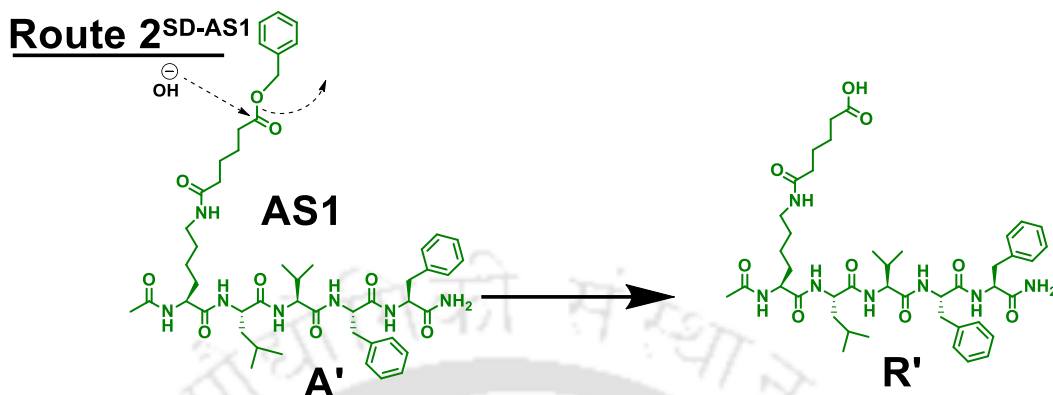


Figure 2.9. Plausible route of self-degradation of AS1 in the absence of mA β (Route 2^{SD-AS1}).

Most of the logically drawn intermediates and final fragments could be identified by time-dependent MALDI-TOF mass spectrometric analyses of the AS1 incubated in the presence of mA β , except those in parentheses (Figure 2.10-14).

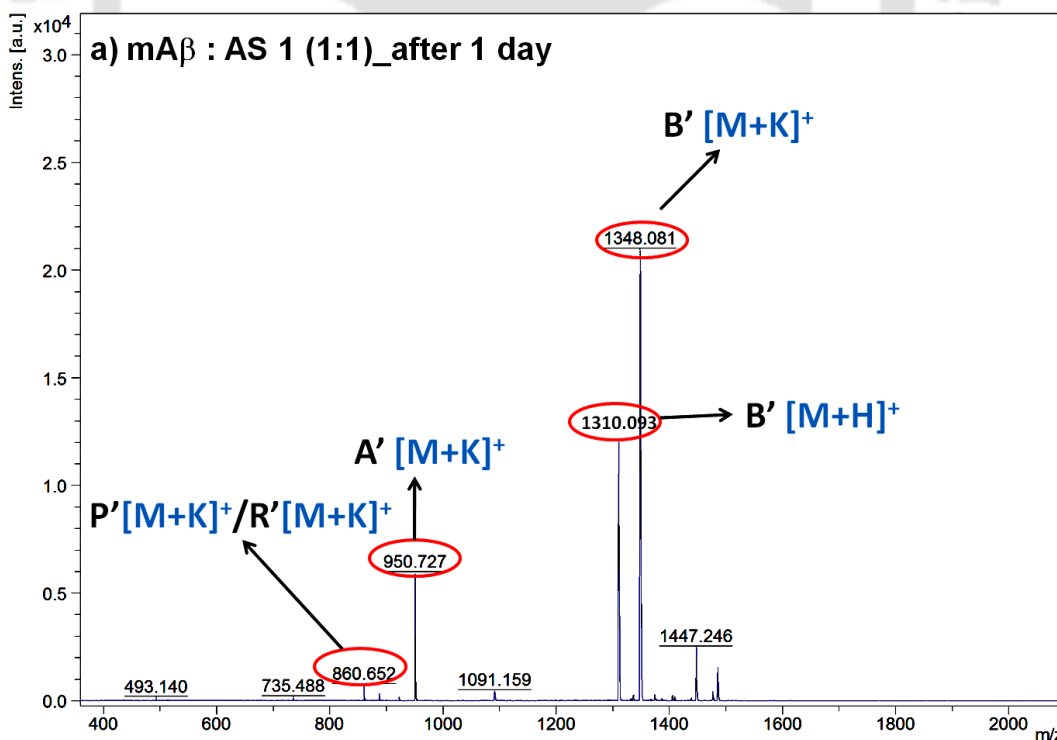


Figure 2.10. MALDI-TOF mass spectrum of mA β in the presence of AS1 (1:1) after one day of incubation in PBS pH 7.4 at 37 °C. As P' and R' are positional isomers, they exhibit same m/z value.

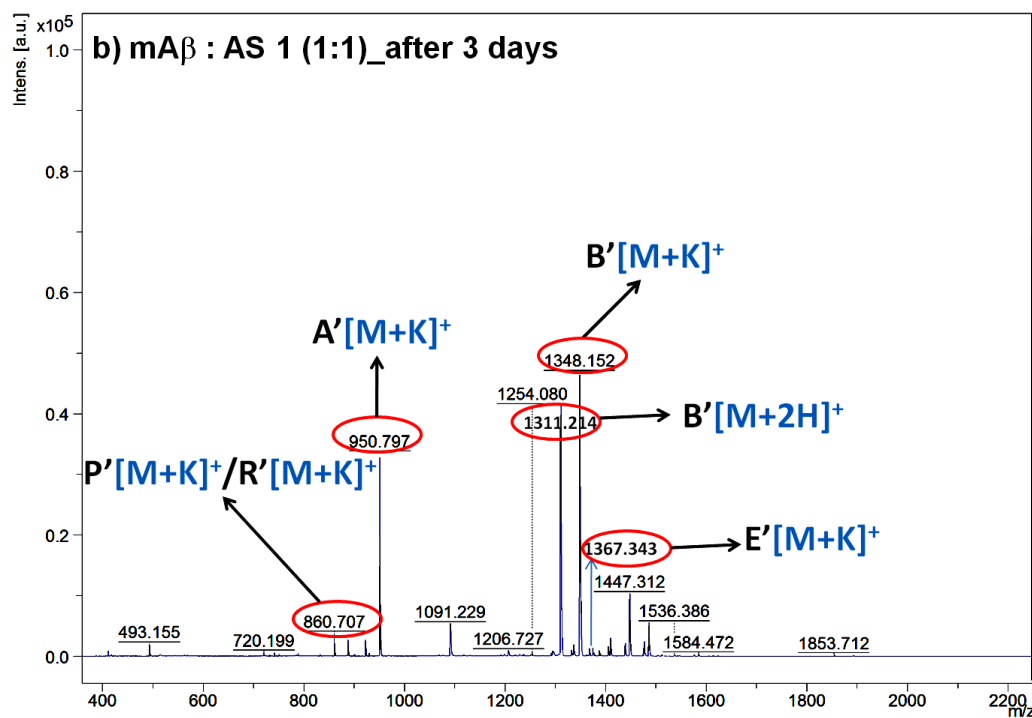


Figure 2.11. MALDI-TOF mass spectrum of mA β in the presence of AS1 (1:1) after three days of incubation in PBS.

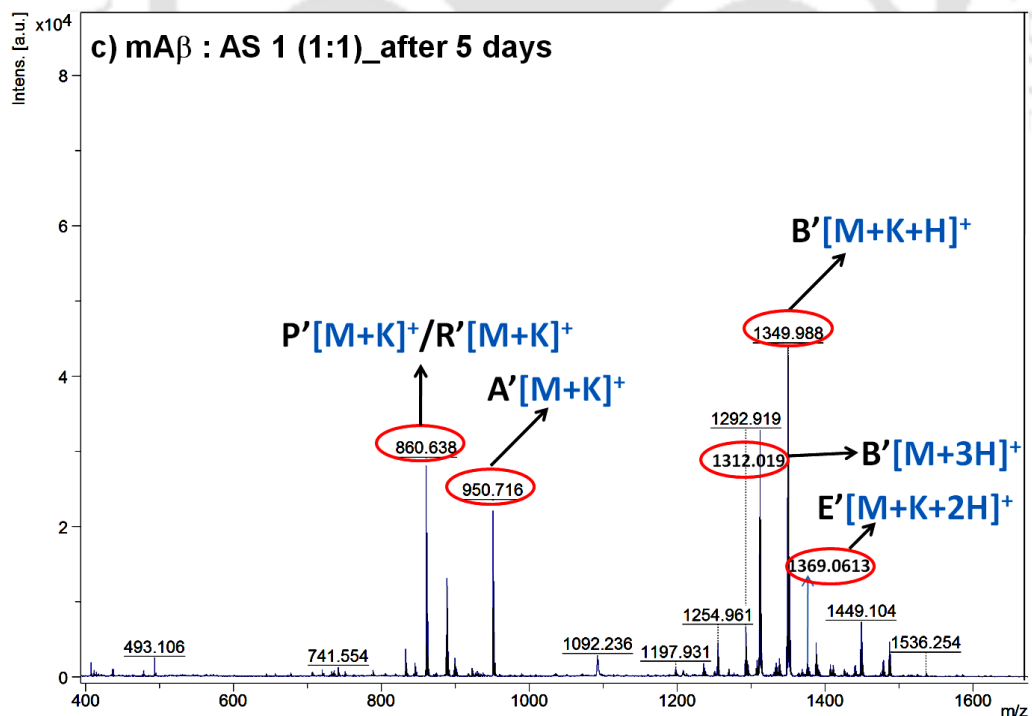


Figure 2.12. MALDI-TOF mass spectrum of mA β in the presence of AS1 (1:1) after five days of incubation in PBS.

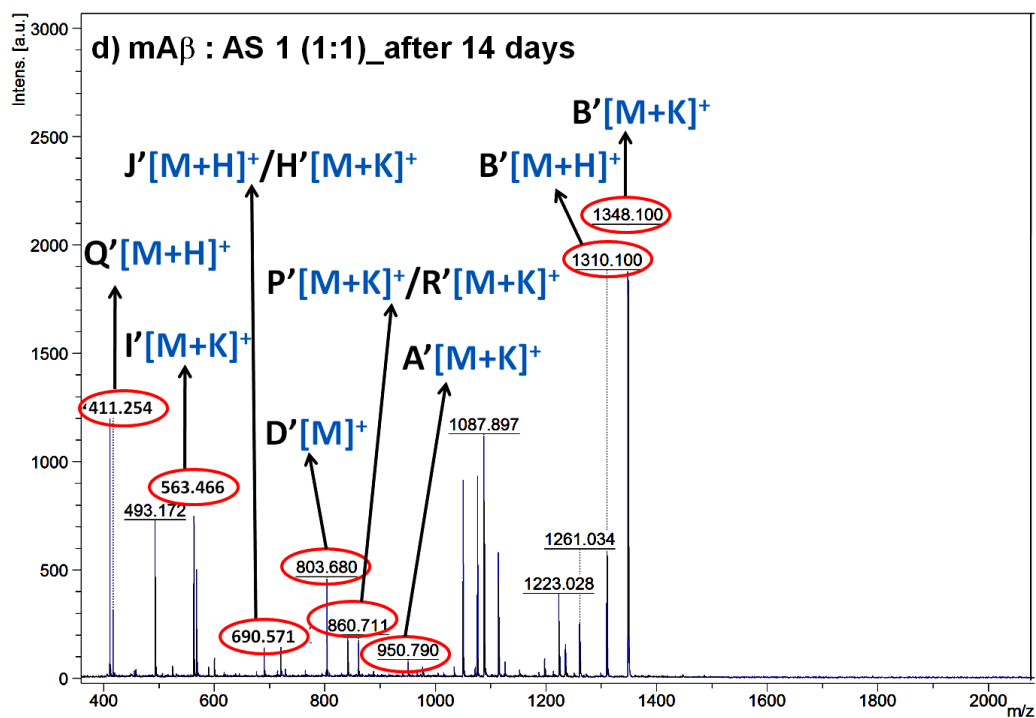


Figure 2.13. MALDI-TOF mass spectrum of mA β in the presence of AS1 (1:1) after 14 days of incubation.

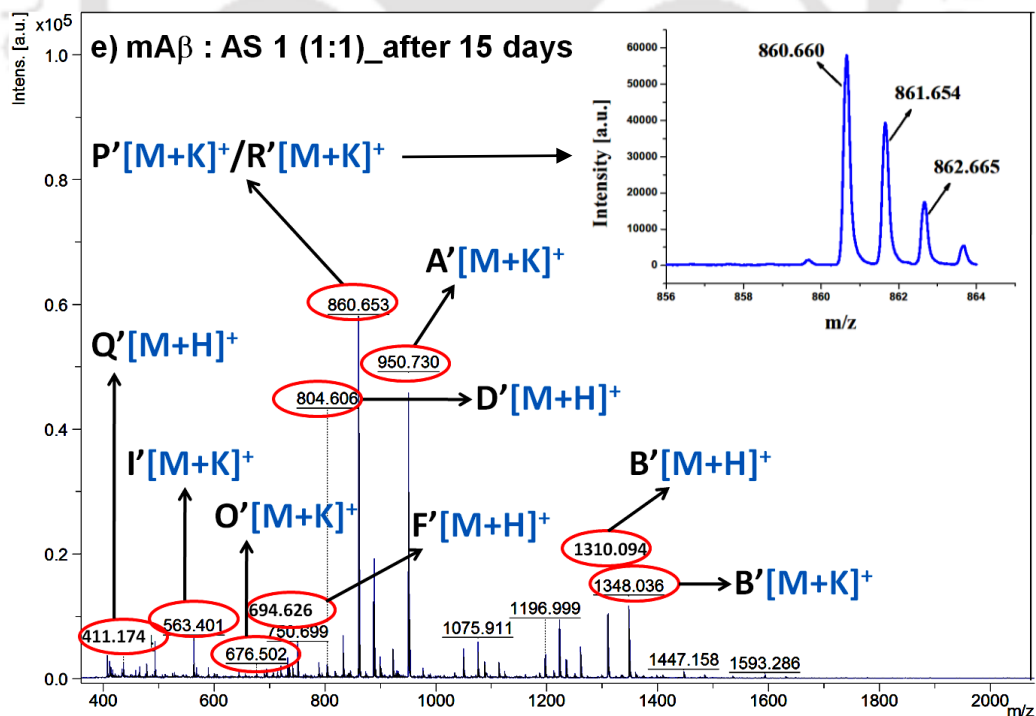


Figure 2.14. MALDI-TOF mass spectrum of mA β in the presence of AS1 (1:1) after 15 days of incubation.

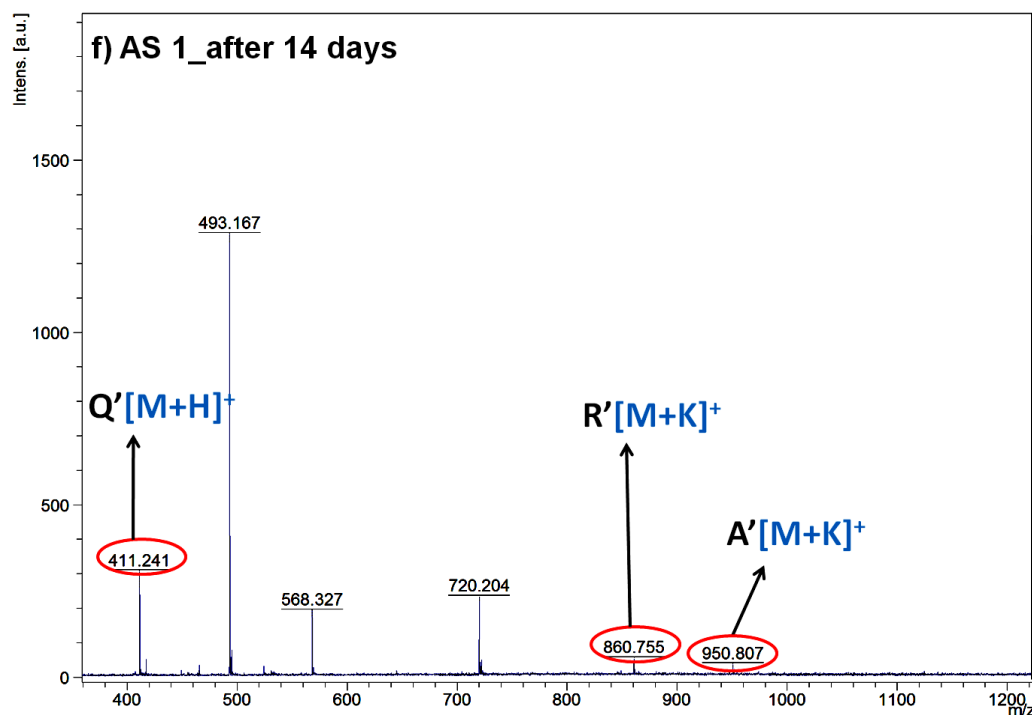


Figure 2.15. MALDI-TOF mass spectrum of AS1 in the absence of mA β after 14 days of incubation in PBS.

2.4.2. Plausible routes of the proteolytic activity of AS2 on mA β :

AS2 also cleaved mA β at the same sites in similar fashion (Figure 2.16-24). It seems, at first the amine group of the side chain of Lys of mA β got attached to the nucleophilic carbonyl of the pendant adipic acid of AS2 by the expulsion of the good leaving group, benzyl alcohol, generating the intermediate C'' via intermolecular O to N acyl migration. The intermediate C'' then either follows Route 1^{mA β -AS2} to cleave itself between Leu and Val of mA β to generate segment D'' and intermediate E'' which further gets sliced to form I''.

Besides proteolysis products of mA β , AS2 also generated self-degradation products Q'' and R'', following probable route 1^{SD-AS2} and route 2^{SD-AS2}, respectively (Figure 2.18-20, "SD" stands for self-degradation). Such products appeared for all the ASs (Figure 2.8-9, and 2.18-20). To confirm that these are generated by self-degradation, we conducted time-dependent MALDI-TOF mass spectrometric studies of the AS2 incubated in the absence of mA β (Figure 2.25). Most of the logically drawn intermediates and final

fragments could be identified by time-dependent MALDI-TOF mass spectrometric analyses, except those in parentheses (2.21-24 for AS2).

Route 1^{mA β -AS2}

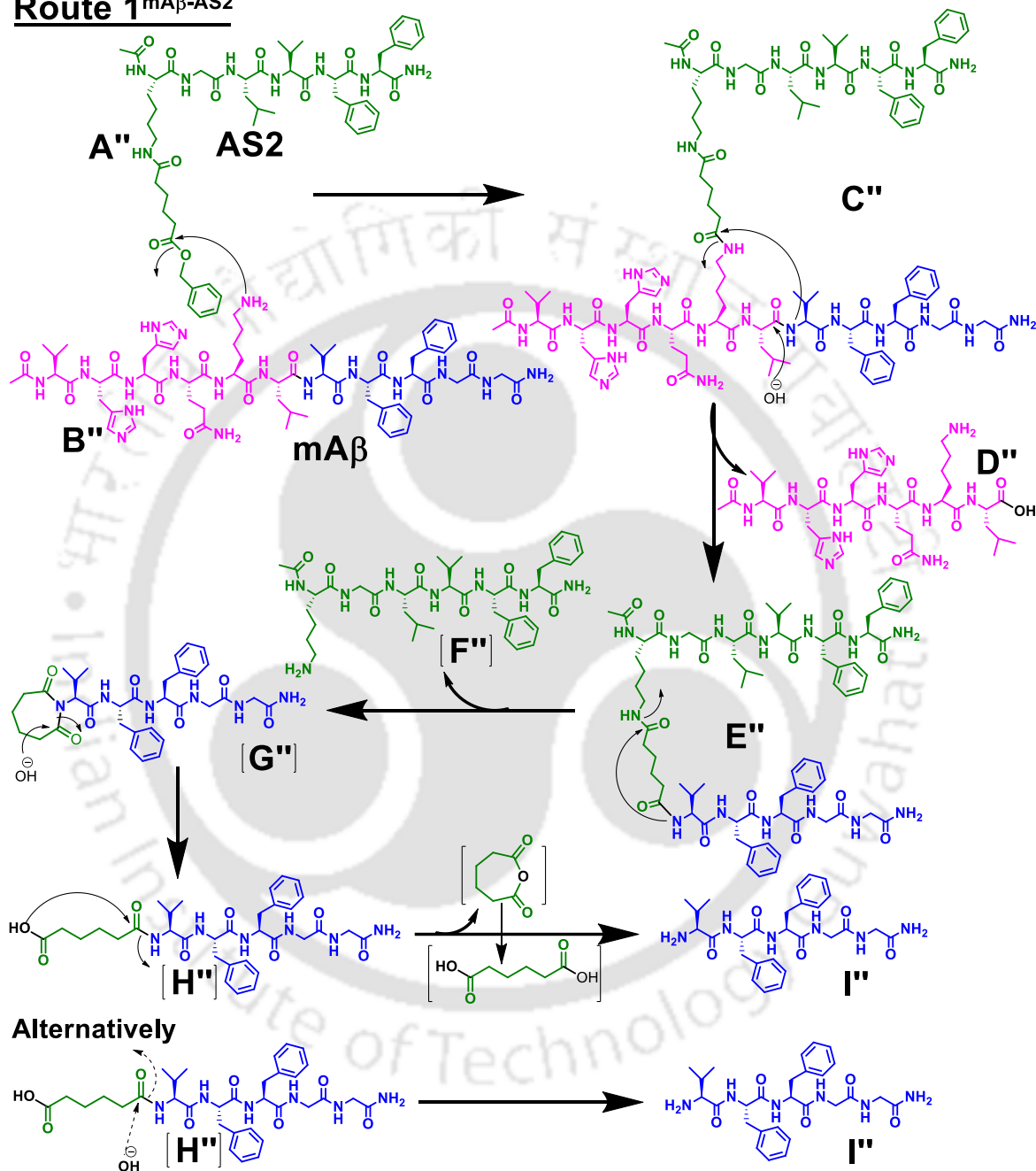


Figure 2.16. Plausible route of proteolysis of mA β into various fragments by AS2 (Route 1^{mA β -AS2}).

Alternatively the intermediate C'' may follow Route 2^{mA β -AS2} to cut itself between Lys and Leu of mA β to generate J'' and another intermediate K''. K'' may further pieces itself to form O'' by the repetition of the imidation-hydrolysis-transamidation cycle.

Route 2^{mA β -AS2}

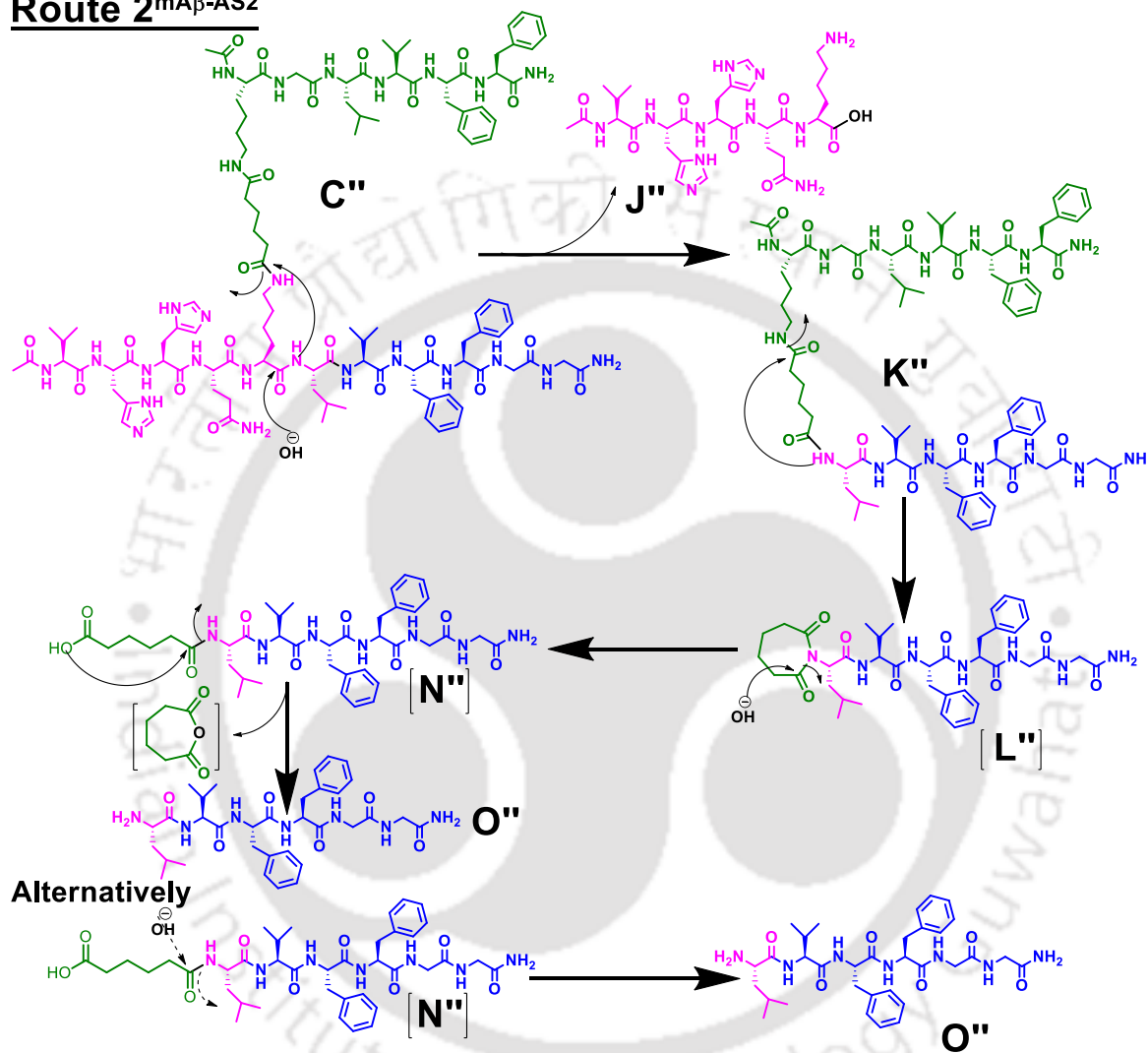


Figure 2.17. Plausible route of proteolysis of mA β into various fragments by AS2 (Route 2^{mA β -AS2}).

Besides proteolysis products of mA β , AS2 generated self-degradation product Q'', following probable route 1^{SD-AS2} (Figure 2.18, "SD" stands for self-degradation). To confirm that these are generated by self-degradation, we conducted time-dependent MALDI-TOF mass spectrometric studies of the AS2 incubated in the absence of mA β (Figure 2.25).

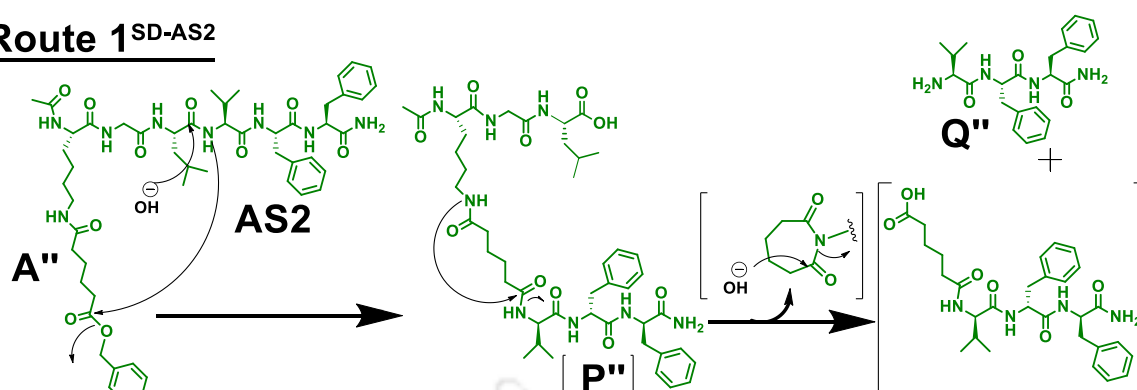
Route 1^{SD-AS2}

Figure 2.18. Plausible route of self degradation of AS2 (Route 1^{SD-AS2}).

AS2 also generated self-degradation product R'' (de-benzylated product), following the route 2^{SD-AS2}, (Figure 2.19).

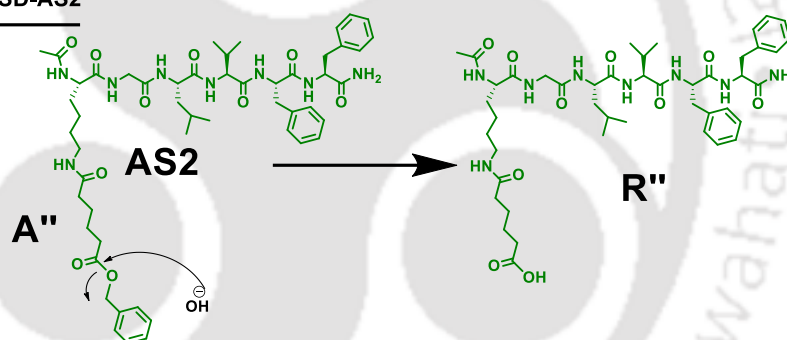
Route 2^{SD-AS2}

Figure 2.19. Plausible route of self-degradation of AS2 (Route 2^{SD-AS2}).

Alternatively the intermediate R'' may follow Route 3^{SD-AS2} to generate E-AS2''. Amine group of Lys side-chain of R'' become free after hydrolysis to form S''. E-AS2'' may get produced by the attack of Lys side-chain nitrogen of S'' to the BnAdp carbonyl of another AS2 molecule as depicted in route 3^{SD-AS2}.

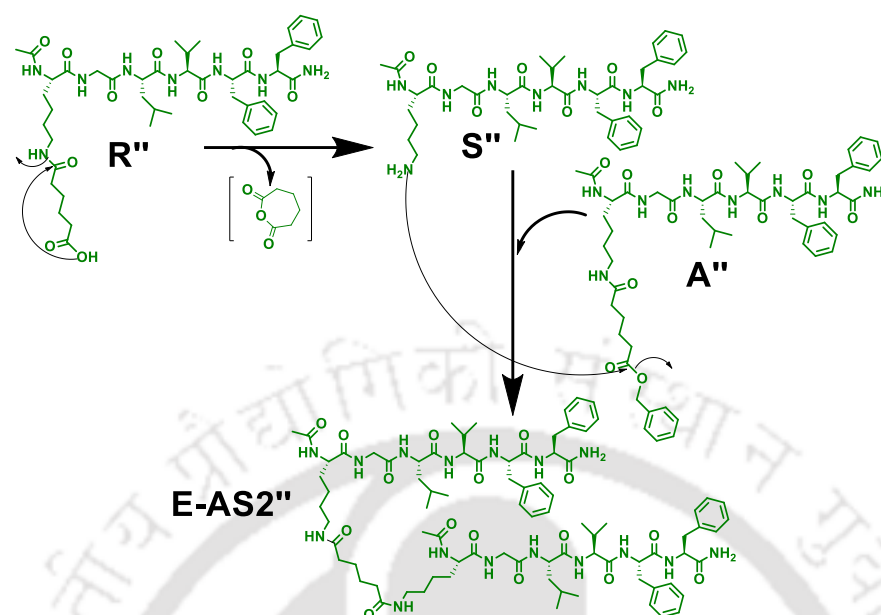
Route 3^{SD-AS2}

Figure 2.20. Plausible route of self-degradation of AS2 (Route 3^{SD-AS2}).

Most of the logically drawn intermediates and final fragments could be identified by time-dependent MALDI-TOF mass spectrometric analyses of the AS2 incubated in the presence of mA β , except those in parentheses (Figure 2.21-24).

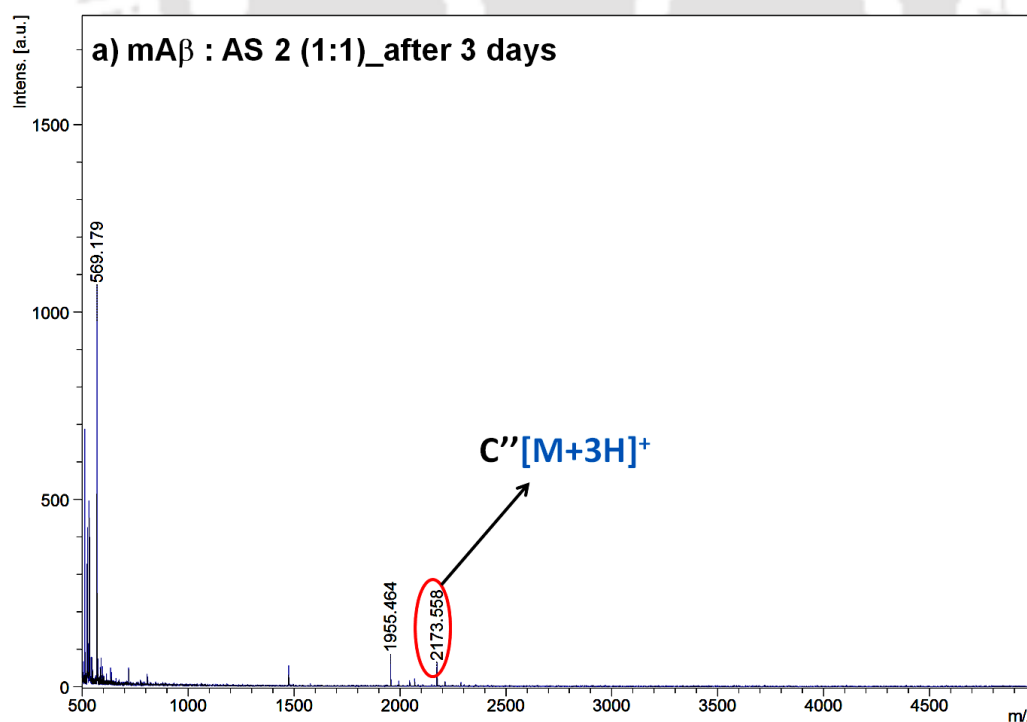


Figure 2.21. MALDI-TOF mass spectrum of mA β in the presence of AS2 (1:1) after three days.

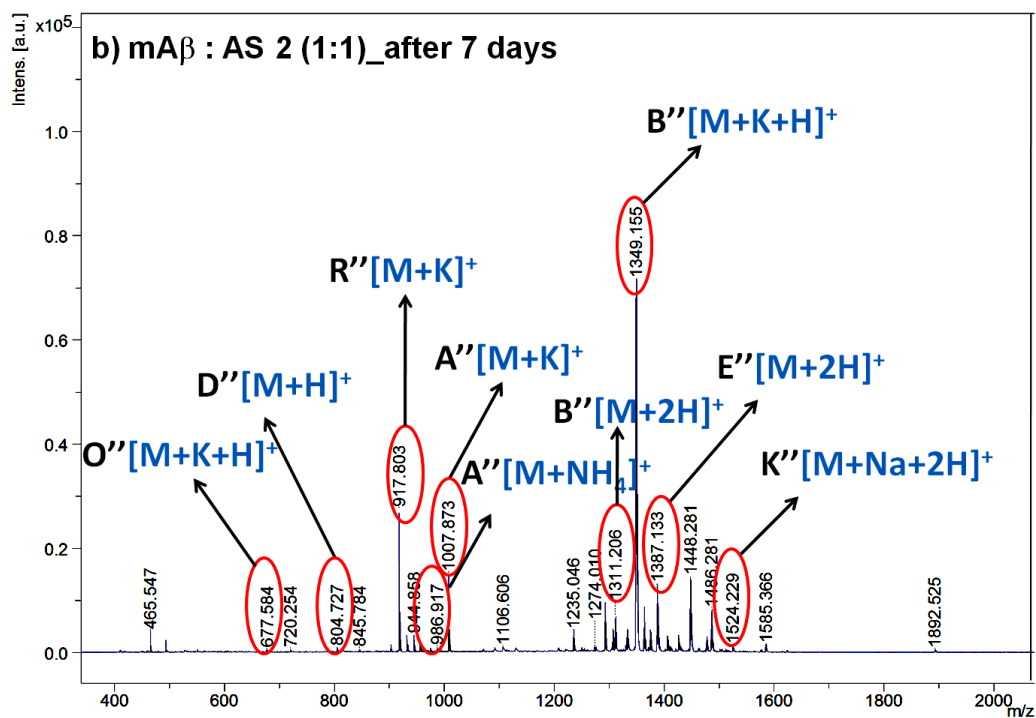


Figure 2.22. MALDI-TOF mass spectrum of mAb β in the presence of AS2 (1:1) after seven days.

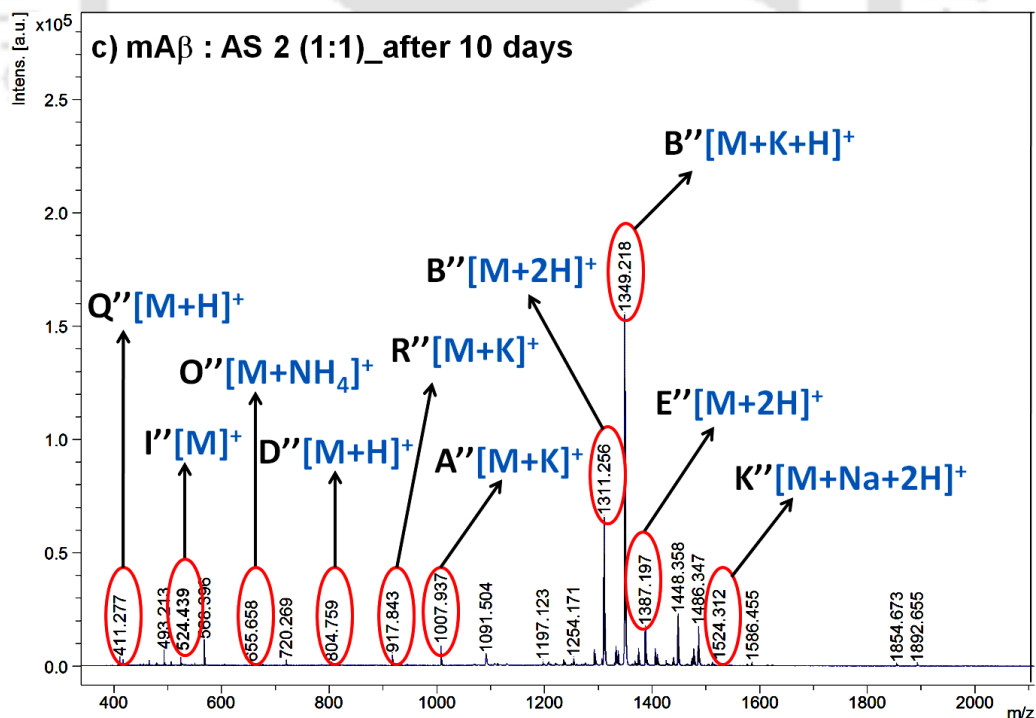


Figure 2.23. MALDI-TOF mass spectrum of mAb β in the presence of AS2 (1:1) after ten days of incubation.

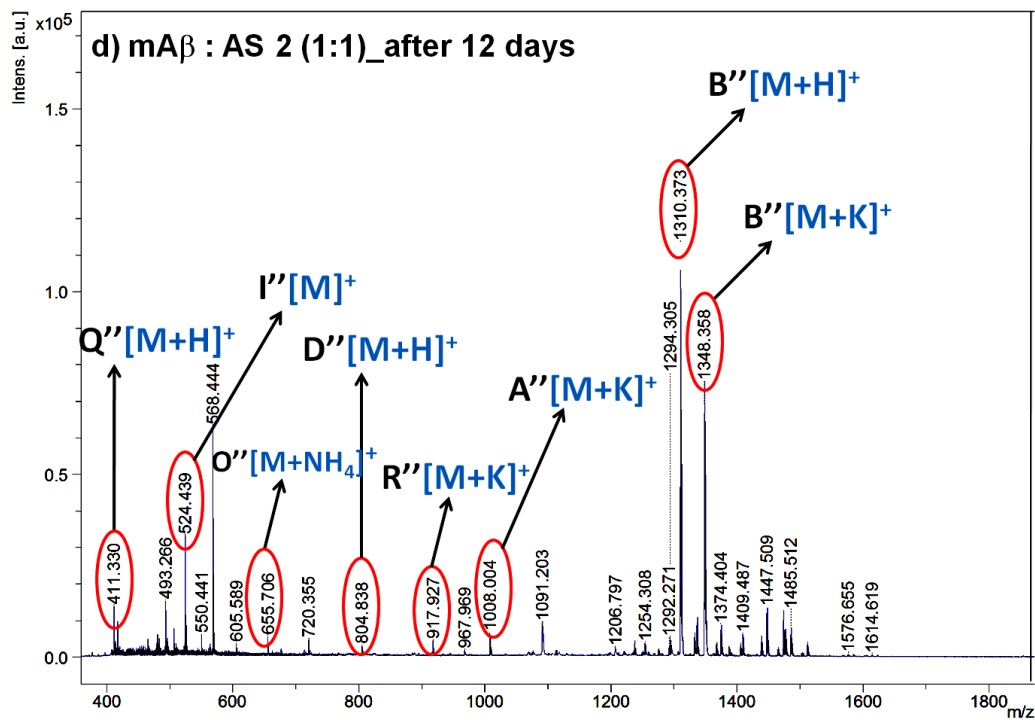


Figure 2.24. MALDI-TOF mass spectrum of mAb in the presence of AS2 (1:1) after 12 days.

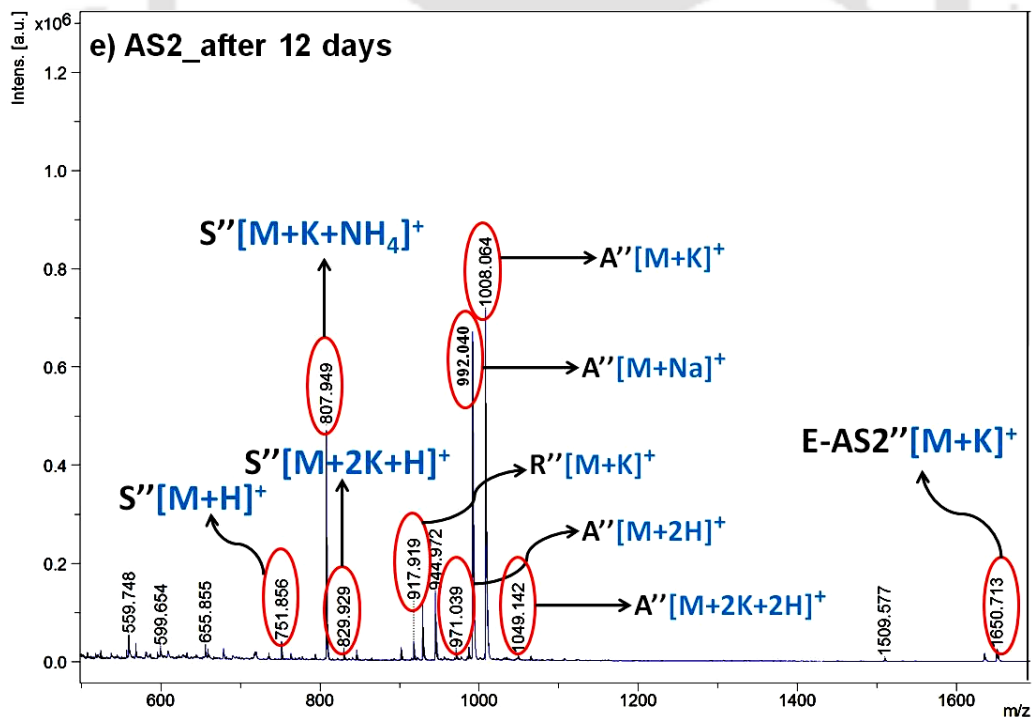


Figure 2.25. MALDI-TOF mass spectrum of AS2 alone after 12 days of incubation in PBS.

2.4.3. Plausible routes of the proteolytic activity of AS3 on mA β :

AS3 also cleaved mA β at the same sites in similar fashion (Figure 2.26-32). Interestingly, AS1, AS2, and AS3 followed similar routes (Figure 2.5-7, 2.16-17, and 2.26-28) for proteolytic degradation of mA β . The fragments D''' and I''' may get generated by the attack of Lys side-chain nitrogen of mA β to the BnAdp carbonyl of AS3 as depicted in route 1^{mA β -AS3}. Repetition of the imidation-hydrolysis-transamidation cycle generates various intermediates and cleavage products.

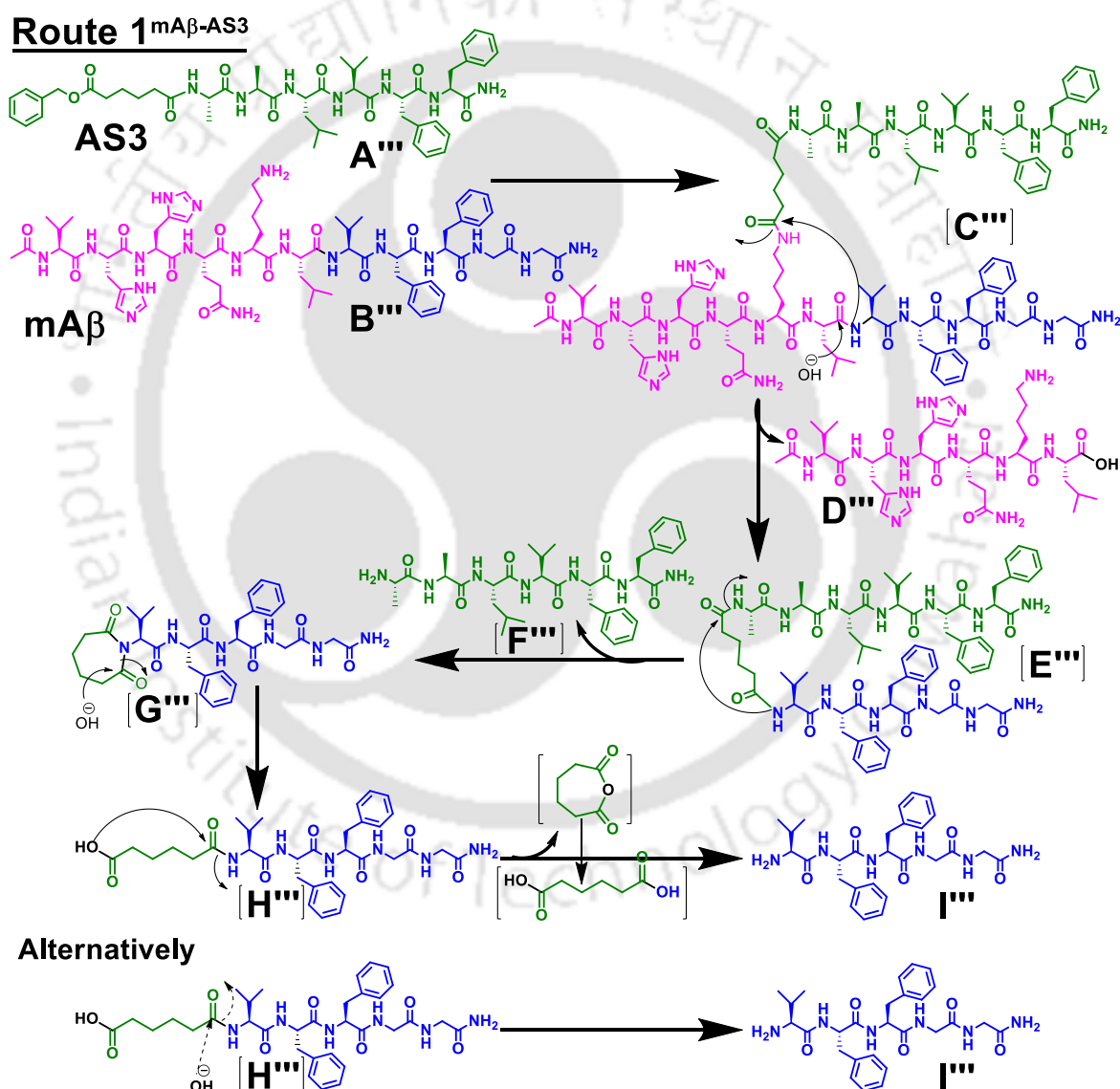


Figure 2.26. Plausible route of proteolysis of mA β into various fragments by AS3 (Route 1^{mA β -AS3}).

MALDI-TOF mass results of proteolytic cleavage of mA β by AS3 indicated that AS3 cleaved the mA β in a site-selective manner between Lys-Leu via route 2^{mA β -AS3} (Figure 2.27). In this case also various intermediates and cleavage products may get generated by the repetition of the imidation-hydrolysis-transamidation cycle from C'''.

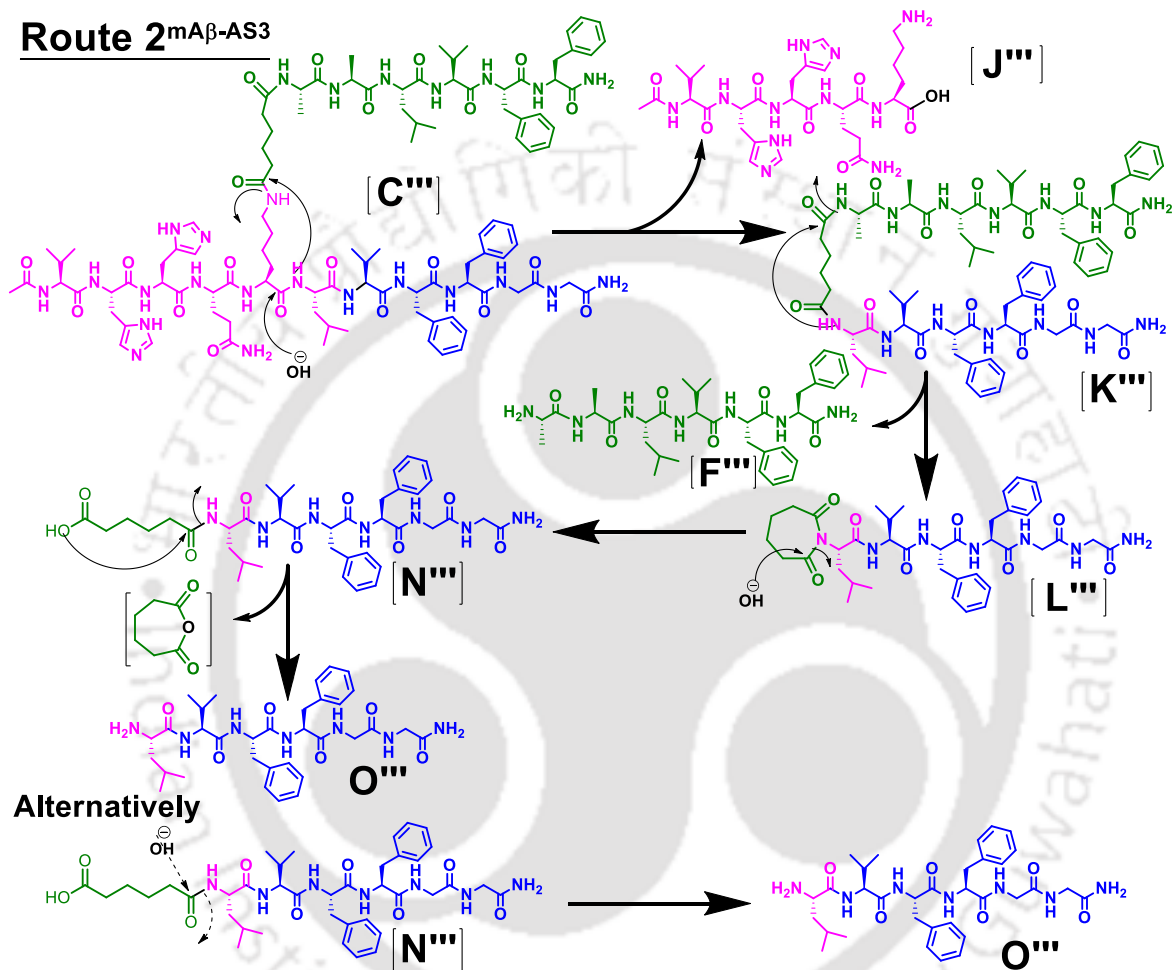


Figure 2.27. Plausible route of proteolysis of mA β into various fragments by AS3 (Route 2^{mA β -AS3}).

AS3 also generated de-benzylated product R''' following the route 2^{SD-AS3}, (Figure 2.28).

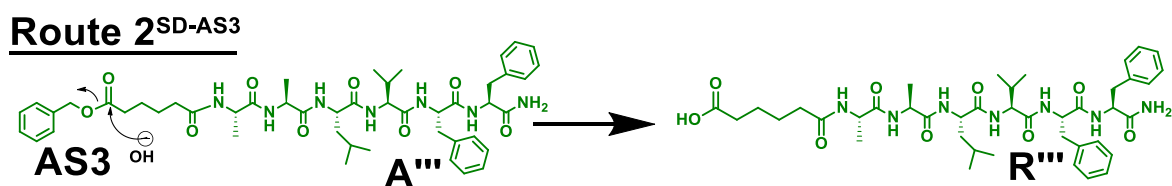


Figure 2.28. Plausible route of self-degradation of AS3 (Route 2^{SD-AS3}).

Most of the logically drawn intermediates and final fragments could be identified by time-dependent MALDI-TOF mass spectrometric analyses of the AS3 incubated in the presence of mA β , except those in parentheses (Figure 2.29-32).

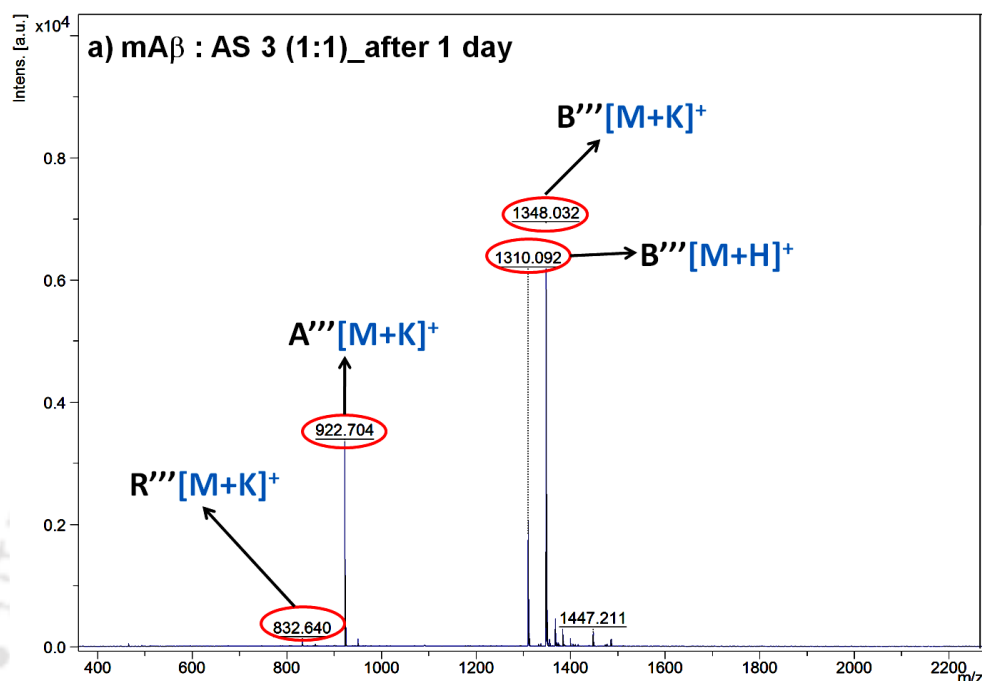


Figure 2.29. MALDI-TOF mass spectrum of mA β in the presence of AS3 (1:1) after one day.

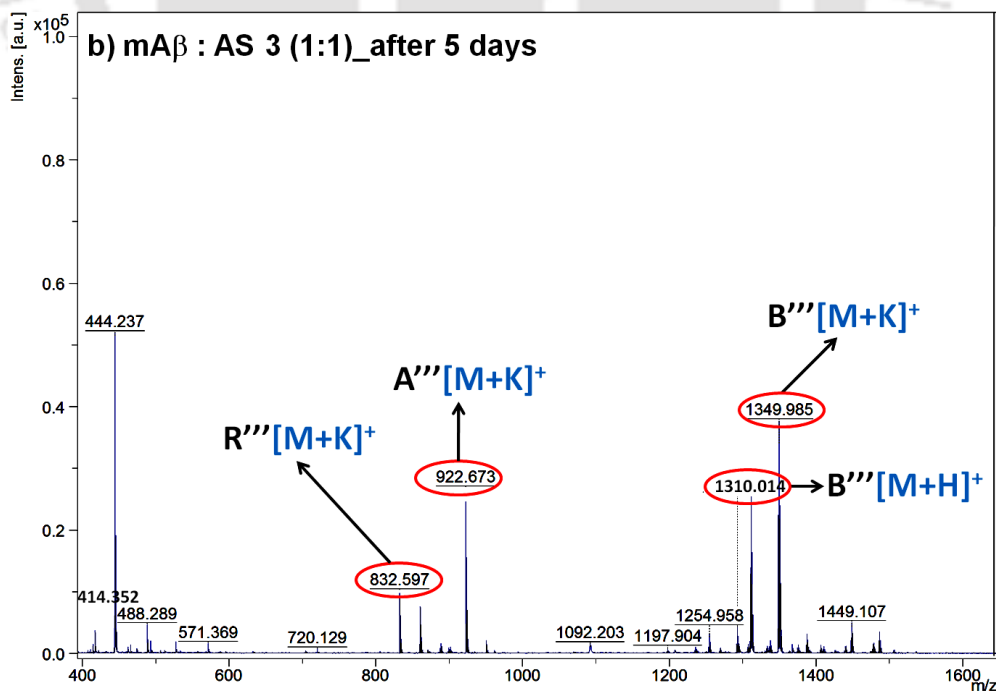


Figure 2.30. MALDI-TOF mass spectrum of mA β in the presence of AS3 (1:1) after five days.

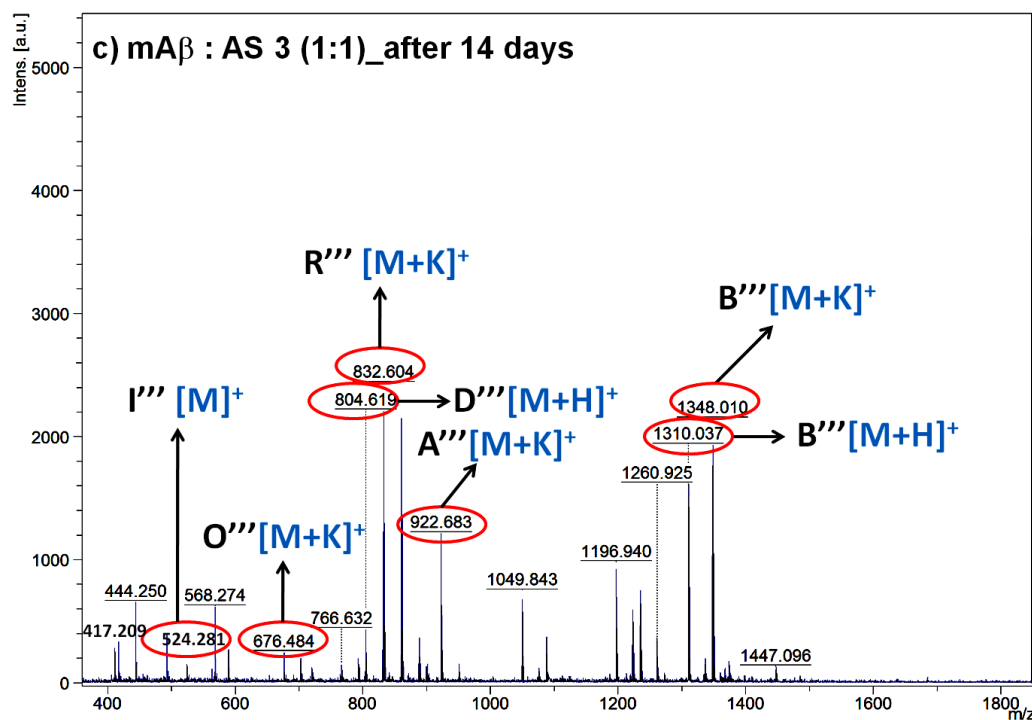


Figure 2.31. MALDI-TOF mass spectrum of mA β in the presence of AS3 (1:1) after 14 days of incubation.

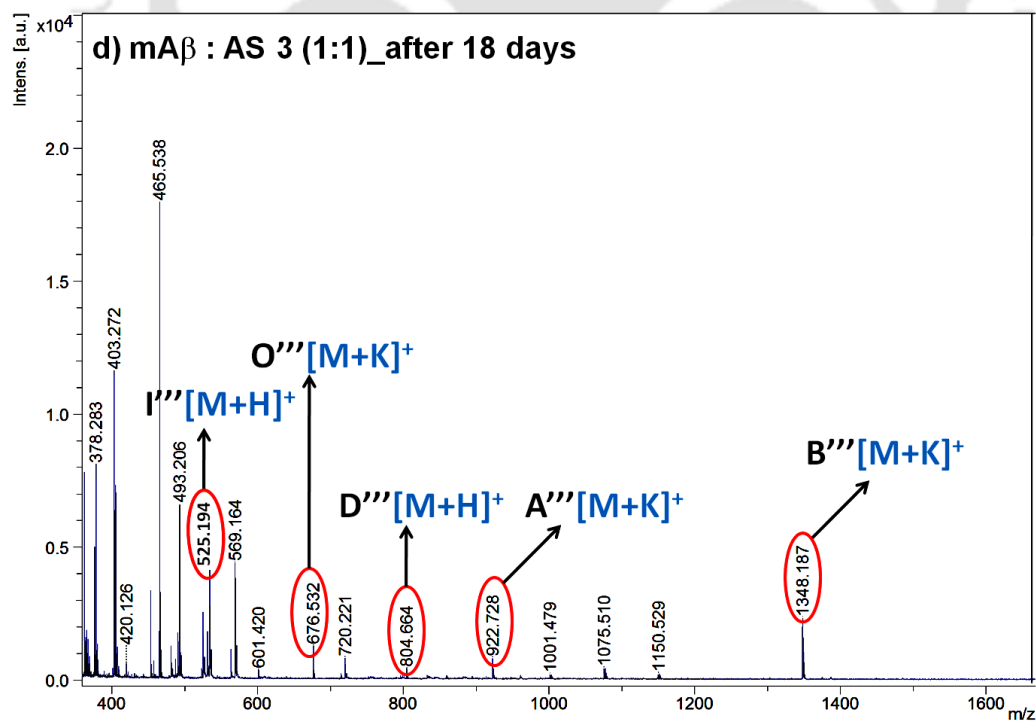


Figure 2.32. MALDI-TOF mass spectrum of mA β in the presence of AS3 (1:1) after 18 days of incubation.

2.4.4. DFT calculation of the intermediate C'':

To get an idea on why the proteolysis occurs selectively between ^{16}Lys - ^{17}Leu and ^{17}Leu - ^{18}Val , we performed DFT calculation to find the most stable conformation of the C'' (Figure 2.33). We found the bond distances between the carbonyl oxygen (of the amide bond formed by nucleophilic attack at the carbonyl group of BnAdp by side-chain amine of Lys) and the nitrogens of the backbone amide bonds of Leu and Val are 8.4 Å and 10.9 Å, respectively. This bond distance may be small enough to facilitate the nucleophilic attack by the mentioned nitrogen on the carbonyl group (Figure 2.33). Moreover, it is clear that the aromatic interaction between the Phe groups of the mAb β and the ASs forces C'' to adopt such a cycle-like structure facilitating the mentioned nucleophilic attack.

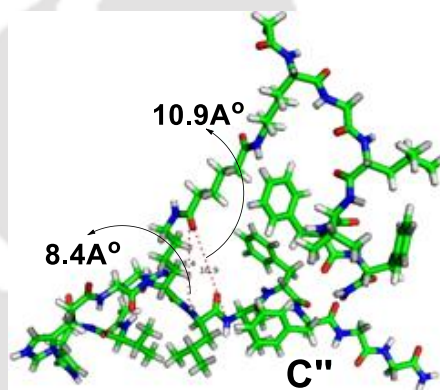


Figure 2.33. The most stable conformation of the intermediate C'' (image generated by PyMOL) obtained from DFT calculation [Calculation Method: B3LYP, and Basis Set: 6-31G].

2.4.5. Kinetics Study by HPLC and ESI-MS of mAb β in the presence of AS2:

Also, we performed a time-dependent HPLC analysis to investigate the proteolytic activity of AS2 on mAb β (Figure 2.34-35). The intensity of the peak (rt 19 min) corresponding to AS2 diminished with time but did not disappear until ten days. The peak corresponding to mAb β did not shift but changed its shape. Collecting the content of this peak and further analysis with ESI-MS revealed the existence of D'', I'', J'', O'' as well as mAb β (B'') in the same position (Figure 2.36-41). A new peak appeared at retention time 17 min. Collecting its content and further ESI-MS analysis reveals two new M/z values

that seem to correspond to two new compounds Z1'' and Z2''. Formation of such species is possible but unlikely (Figure 2.40). Also, the possibility of formation of other species with the same M/z value cannot be obviated.

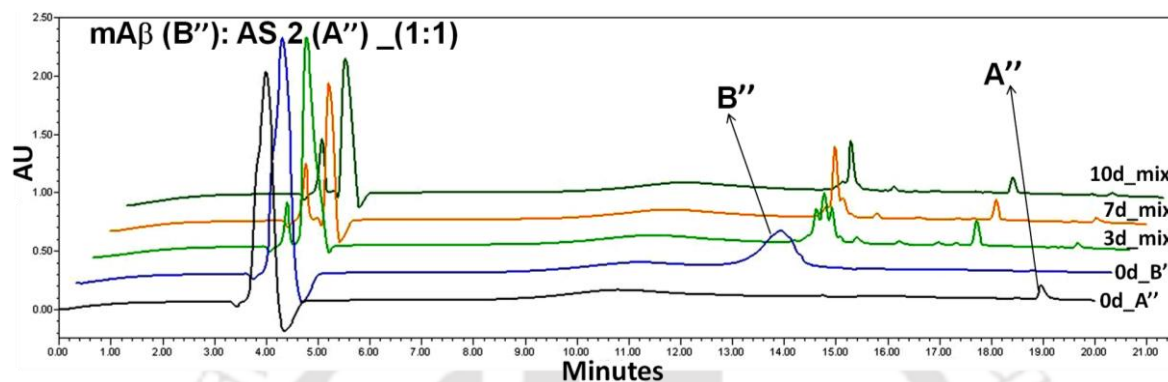


Figure 2.34. Time-dependent kinetics study of mAb β in the presence of AS2 (1:1), up to ten days of incubation in PBS pH 7.4 at 37 °C by RP-HPLC (Kinetics study was done using Waters 600E analytical HPLC system. An Ascentis C18 analytical column, the flow rate of 0.8 ml/min, a linear gradient of 5-50-100% CH₃CN over 0-10-18 minutes in a total run time of 20 min were used. Dual-wavelength was selected at 214 nm and 254 nm).

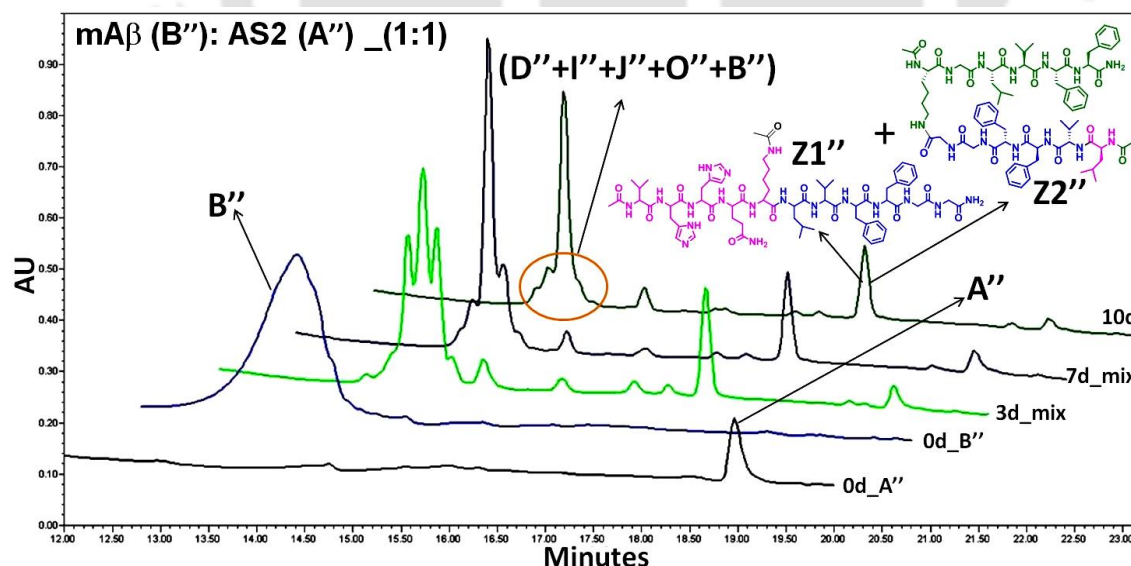


Figure 2.35. Expanded portion of the kinetics study of mAb β in the presence of AS2 (1:1), up to ten days of incubation in PBS pH 7.4 at 37 °C.

cleave Amyloid β at the α -secretase cleavage site by aggregation driven cascades of chemical reactions

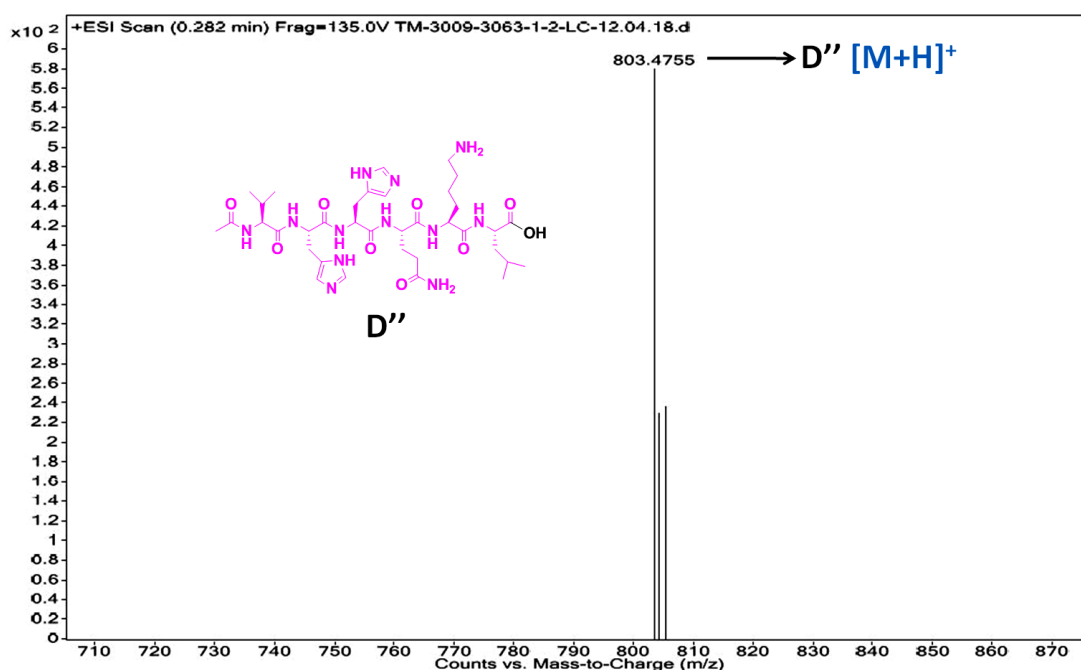


Figure 2.36. ESI-MS profile picture of the mAb fragment D''. Calculated m/z for C₃₆H₅₈N₁₂O₉ [M+H]⁺ is 803.4450, observed 803.4755.

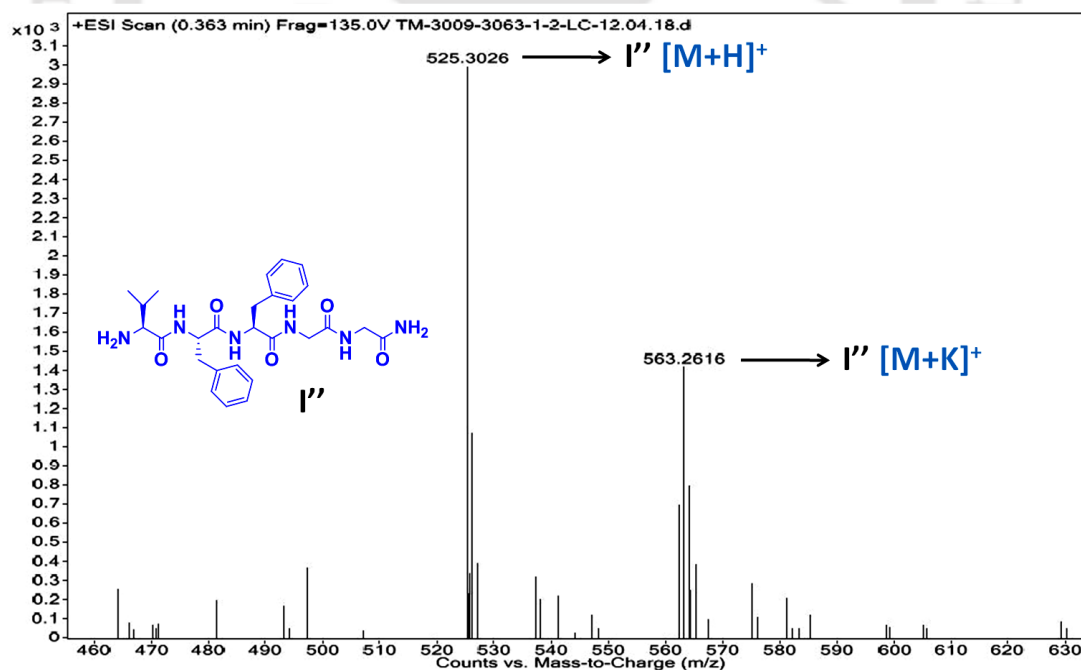


Figure 2.37. ESI-MS profile picture of the mAb fragment I''. Calculated m/z for C₂₇H₃₆N₆O₅ [M+H]⁺ is 525.2747, observed 525.3026; calculated m/z for C₂₇H₃₆N₆O₅ [M+K]⁺ is 563.2747, observed 563.2616.

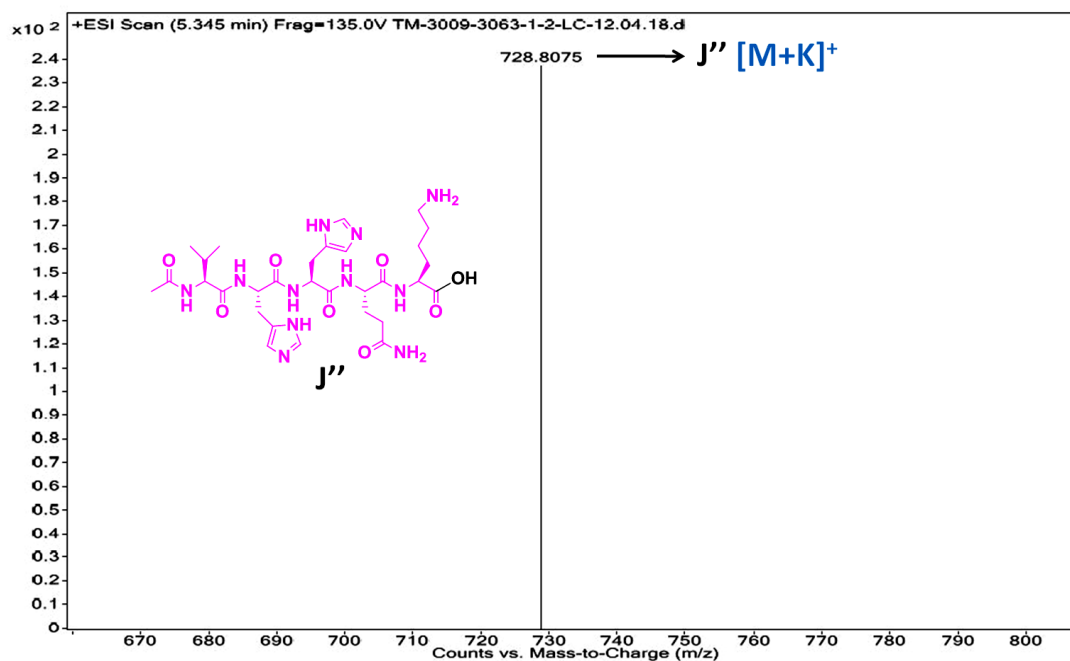


Figure 2.38. ESI-MS profile picture of the mAb fragment J'' . Calculated m/z for $C_{30}H_{47}N_{11}O_8 [M+K]^+$ is 728.7631, observed 728.8075.

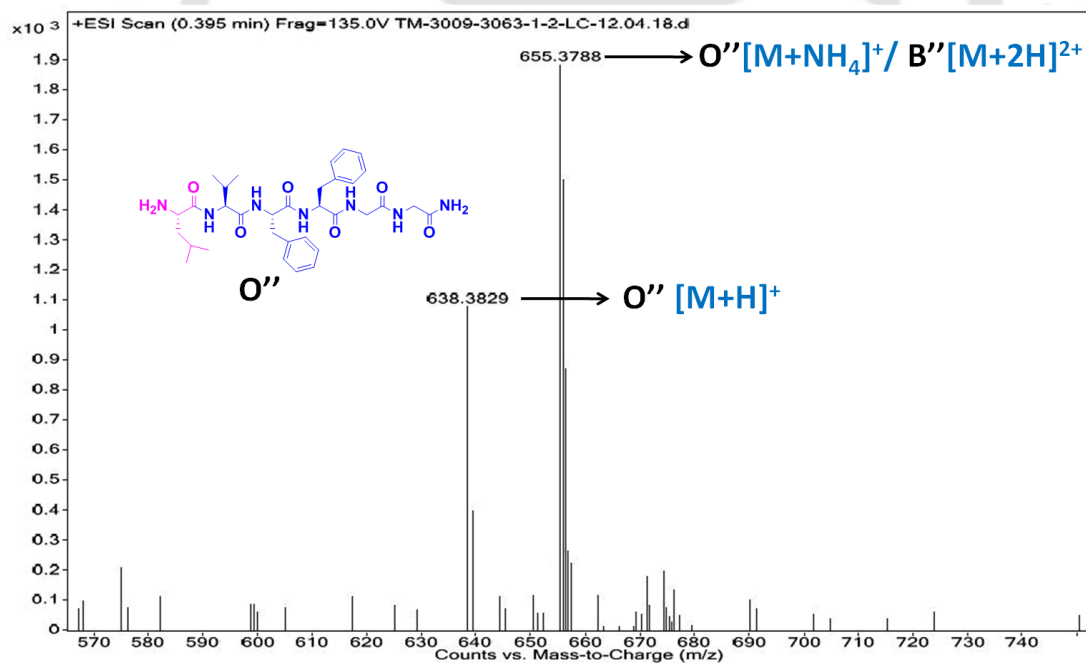


Figure 2.39. ESI-MS profile picture of the mAb fragment O'' . Calculated m/z for $C_{33}H_{47}N_7O_6 [M+H]^+$ is 638.3588, observed 638.3829; calculated m/z for $C_{33}H_{47}N_7O_6 [M+NH_4]^+$ is 655.3588, observed 655.3788. Calculated m/z for $C_{63}H_{92}N_{18}O_{13} [M+2H]^{2+}$ is 655.3548, observed 655.3788.

Plausible mechanism of formation of Z1'' and Z2''

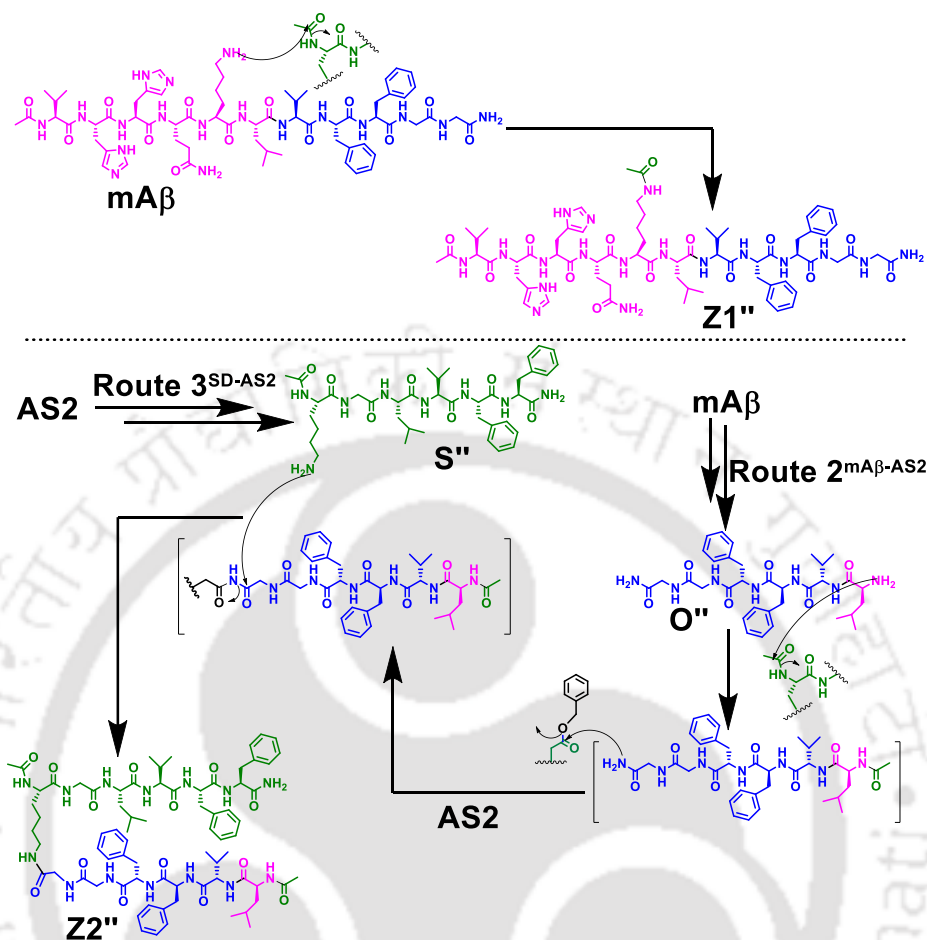


Figure 2.40. A plausible mechanism of formation of Z1'' and Z2''.

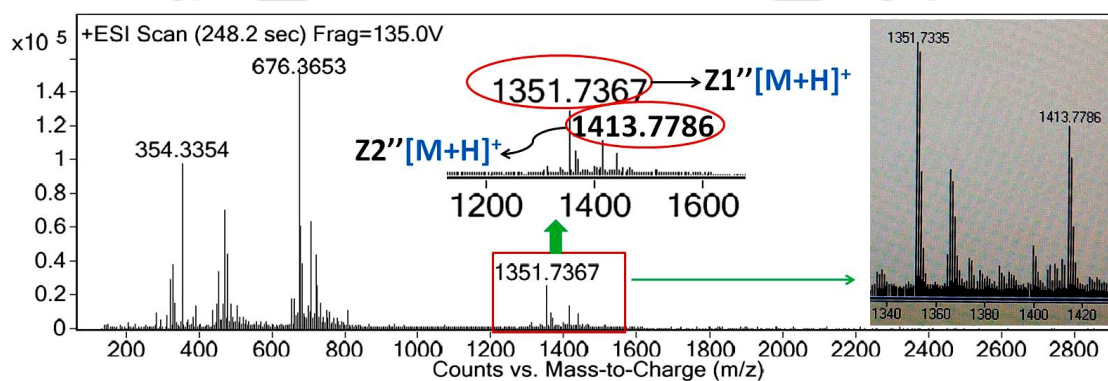


Figure 2.41. ESI-MS of Z1'' and Z2''. Calculated m/z for C₆₅H₉₄N₁₈O₁₄ [M+H]⁺ is 1351.7197, observed 1351.7367 and calculated m/z for C₇₄H₁₀₄N₁₄O₁₄ [M+H]⁺ is 1413.7856, observed 1413.7786.

2.4.6. A time-dependent persistence map:

To get a holistic view of the fragmentations, we prepared a time-dependent persistence map for each of the intermediates and fragments for AS1, AS2, and AS3 (Figure 2.42). It was clear from the map that although AS1 and AS2 started to generate proteolysis products as soon as three days of incubation AS3 took 13 days. On the other hand, while AS2 generated most of the fragments in seven days, AS1 took about 13 days. From this comparative analysis, AS2 seems to be more active than AS1 and AS3. However, in all the cases, mA β lasted even after 30 days. Therefore, a more reactive AS variant was desired.

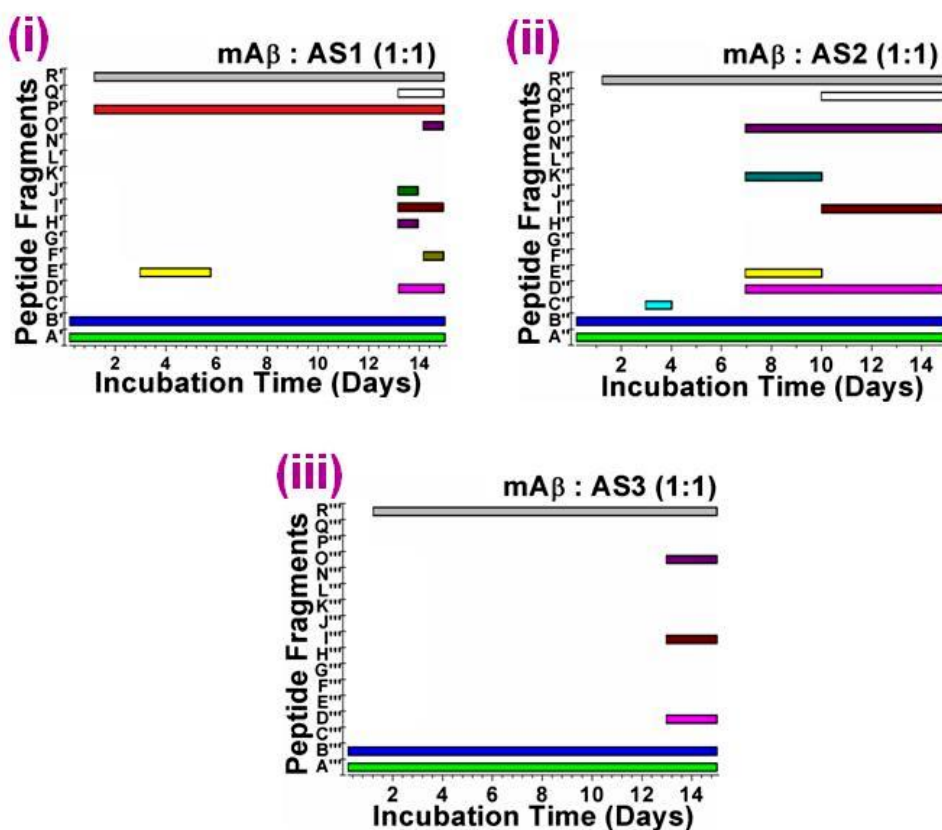


Figure 2.42. Time-dependent existence of various fragments of mA β in the presence of ASs.

2.4.7. Plausible routes of the proteolytic activity of AS4 on mA β :

To improve the reactivity, we designed AS4, which consists of three BnAdp containing Lys residues (Figure 2.43), with a motivation to observe a synergistic effect. Moreover, we performed the following proteolysis assays of mA β with the two-fold molar excess of

AS4 at the same condition (PBS pH 7.4 at 37 °C) as earlier. Probably, therefore, it was observed that AS4 cleaved the mA β completely (Figure 2.43-47) by ten days. AS4 also cleaved in a site-selective manner (between Lys & Leu, and between Leu & Val). Plausible routes of the proteolytic process of mA β by AS4 (Figure 2.43-45) was drawn based on MALDI mass (Figure 2.46-47) analyses. Intermediate C'''' followed route 1^{mA β -AS4} to cleave itself in-between Leu and Val of mA β to generate peptide fragments I'''' and D'''' via intermediate U'''' (Figure 2.43).

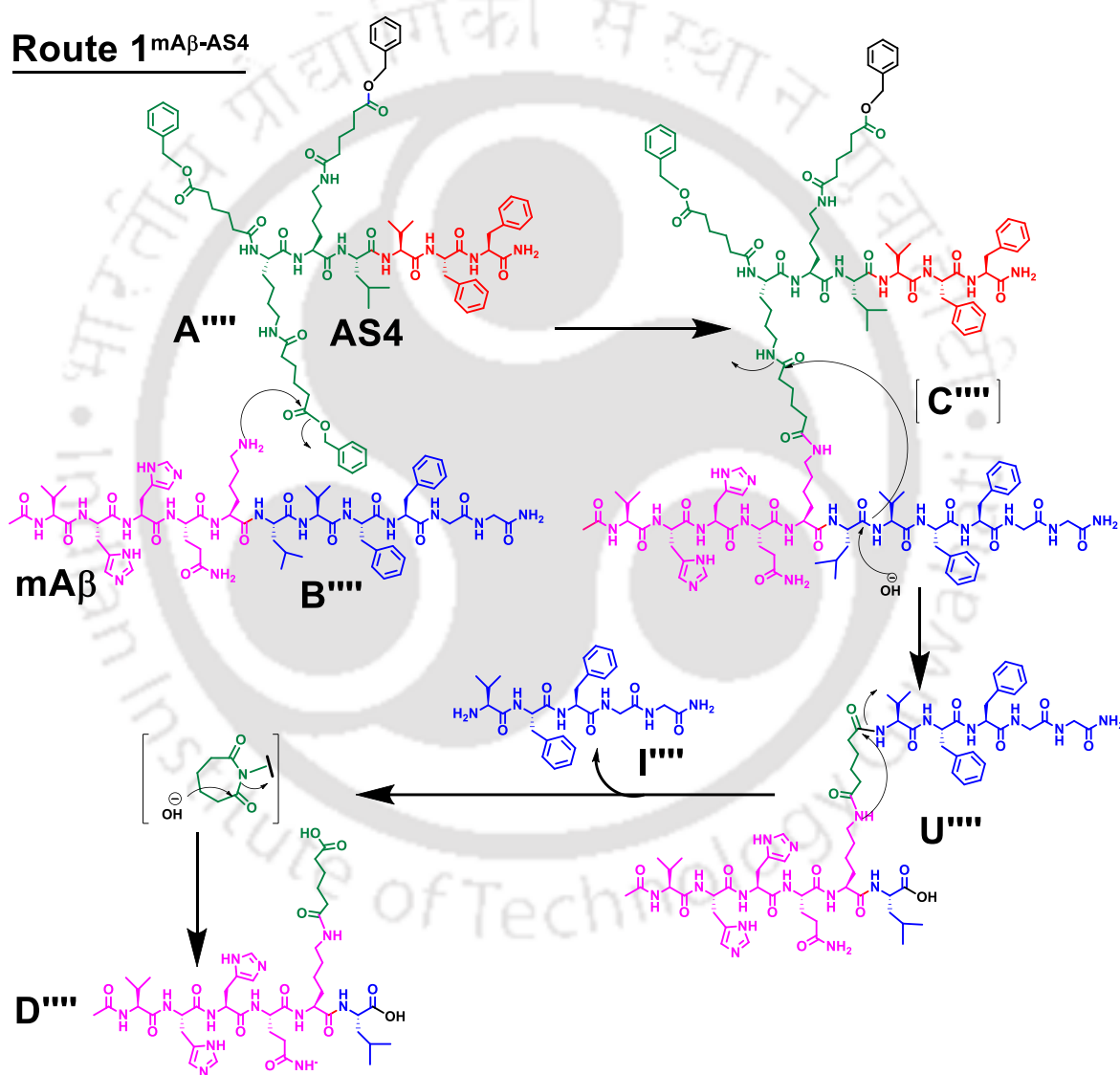


Figure 2.43. Plausible routes of proteolysis of mA β into various fragments by AS4 (Route 1^{mA β -AS4}).

Intermediate C'''' also followed route 2^{mA β -AS4} to cut itself in-between Lys and Leu of mA β segment to generate J'''' and another intermediate E'''''. The intermediate E'''' then

either followed route 4^{mA β -AS4} to generate Q'''' and O'''' via intermediates K'''' and V'''' (Figure 2.44).

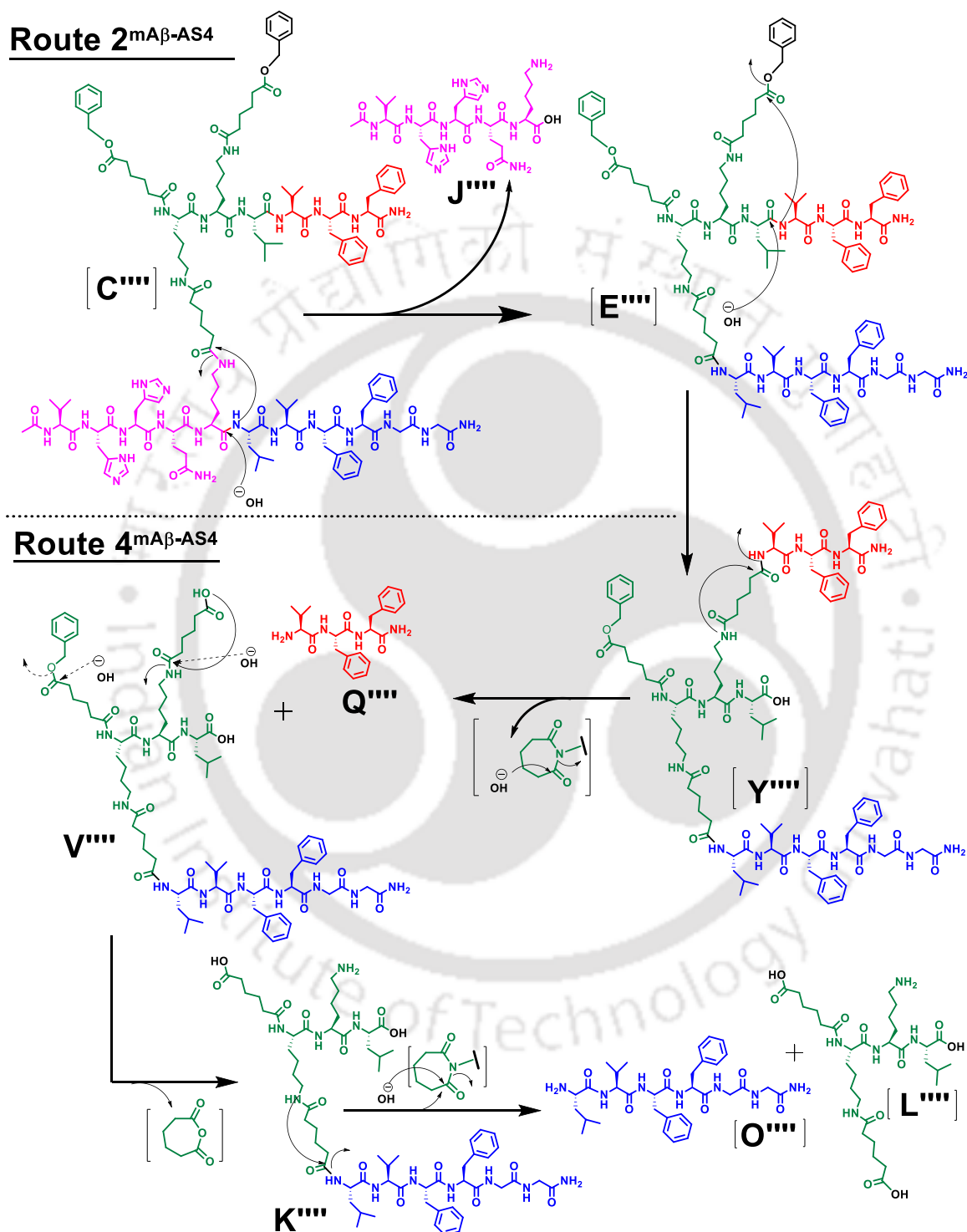


Figure 2.44. Plausible routes of proteolysis of mA β into various fragments by AS4 (Route 2^{mA β -AS4} and Route 4^{mA β -AS4}).

The intermediate E'''' also followed route 5^{mA β -AS4} to generate fragments T'''' and S''''.

Route 5^{mA β -AS4}

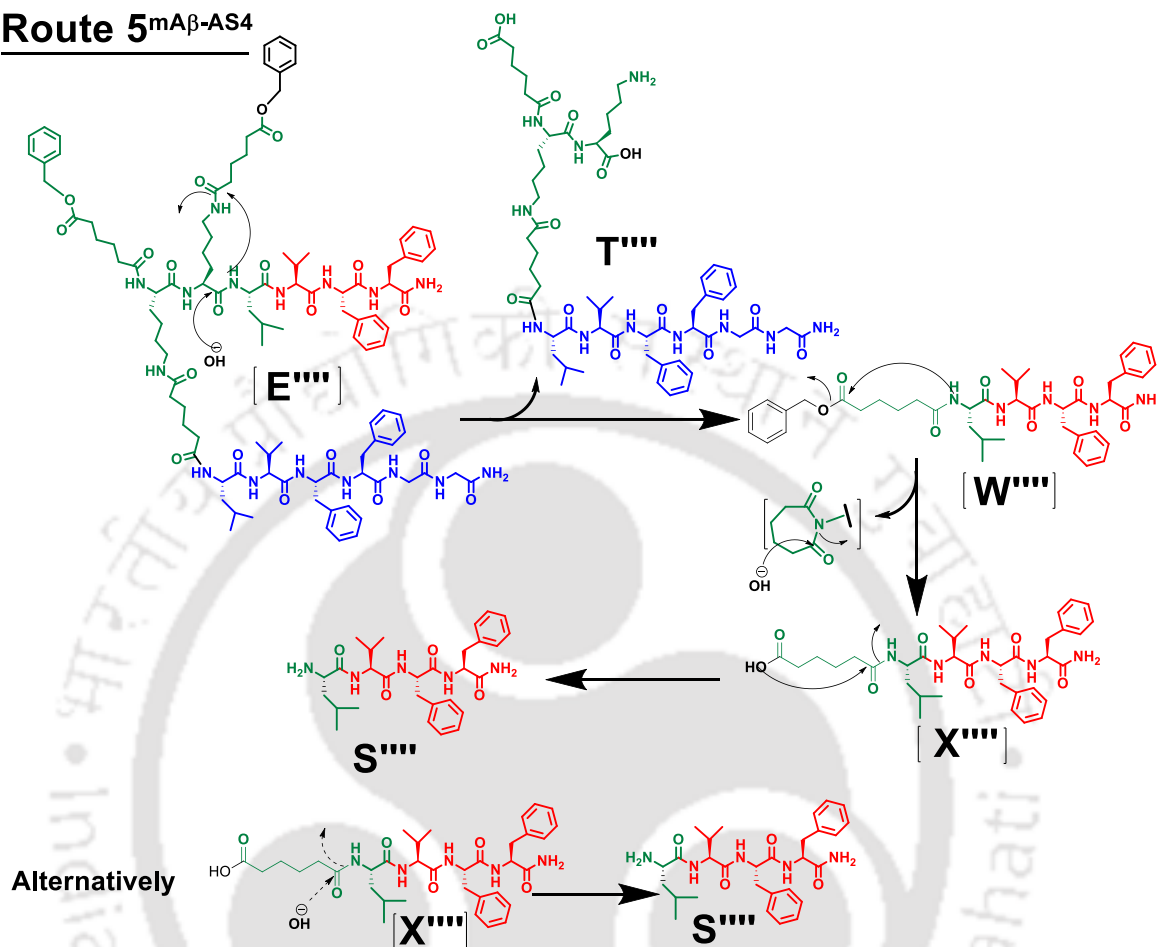


Figure 2.45. Plausible routes of proteolysis of mA β into various fragments by AS4 (Route 5^{mA β -AS4}).

Most of the logically drawn intermediates and final fragments could be identified by time-dependent MALDI-TOF mass spectrometric analyses of the AS4 incubated in the presence of mA β , except those in parentheses (Figure 2.46-47). Interestingly, it was noted that AS4 followed similar mechanistic pathways to that of AS1-AS3, but produced intermediates and fragments, having a range of lifetimes, at a faster rate than other analogs.

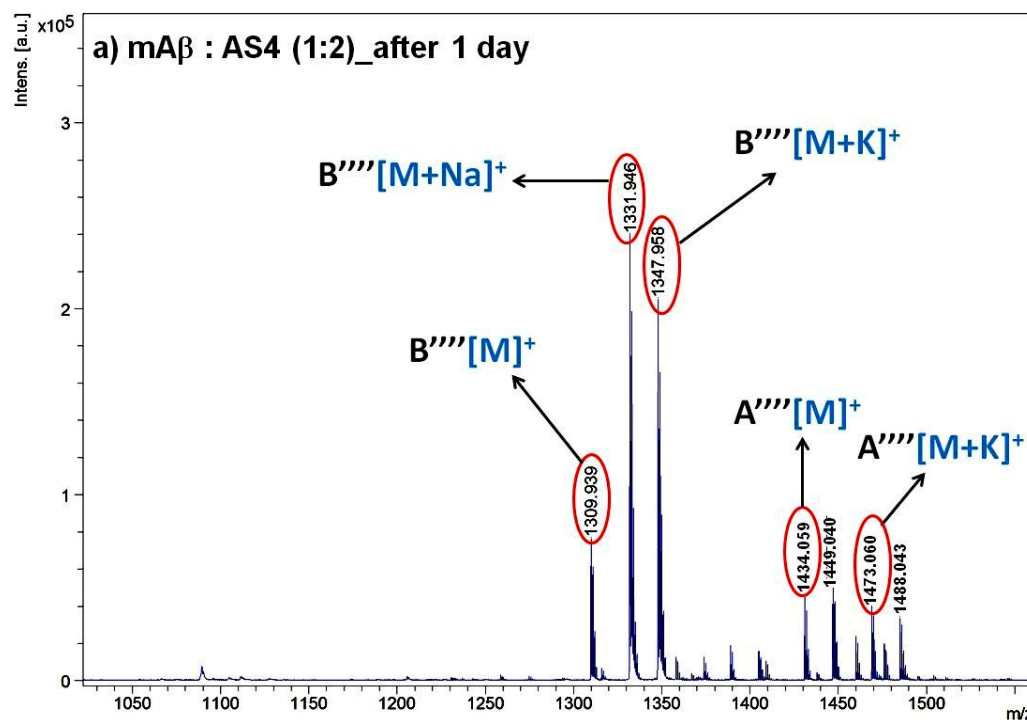


Figure 2.46. MALDI-TOF mass spectrum of mAb β in the presence of AS4 (1:2) after one day of incubation in PBS pH 7.4 at 37 °C.

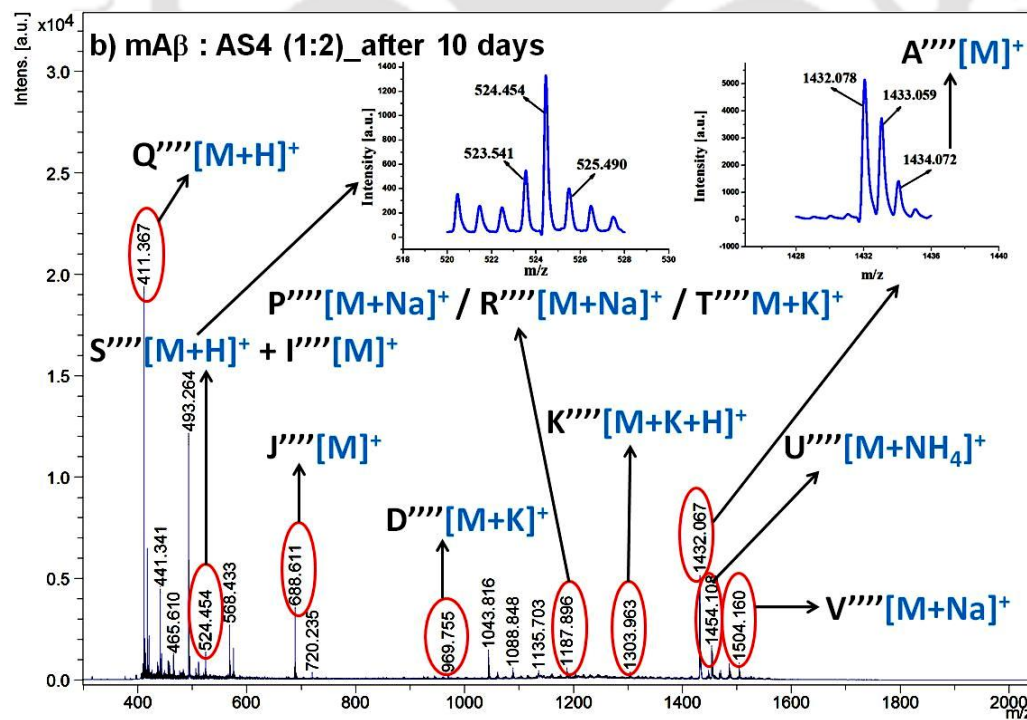


Figure 2.47. MALDI-TOF mass spectrum of mAb β in the presence of AS4 (1:2) after ten days of incubation.

2.4.8. DFT calculation of the intermediate C''':

We also performed DFT calculation of C''' (Figure 2.48) to find the most stable conformation. The bond distances between the carbonyl oxygen (of the amide bond formed by the nucleophilic attack at the carbonyl group of BnAdp by the side chain amine of Lys) and the backbone nitrogens of Leu and Val are 8.0 Å and 10.5 Å, respectively, in the most stable conformation of C'''. Additionally, flexible chains of Lys and adipic acid may favor the mentioned attacks to relieve the steric strain generated in C'''.

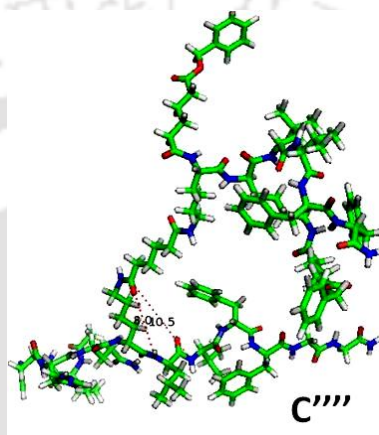


Figure 2.48. The most stable conformation of the intermediate C''' (image generated by PyMOL) obtained from DFT calculation (Calculation Method: B3LYP, and Basis Set: 6-31G).

2.4.9. Comparative map of the existence of mA β :

It was interesting to note that while all the mA β (200 μ M) was destroyed within a week in the presence of the two-fold molar excess of AS4 (400 μ M), some of it survived more than a month in the presence of the equimolar amount of other ASs (Figure 2.49).

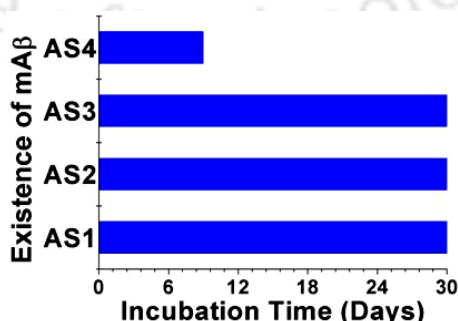


Figure 2.49. Existence of mA β up to 30 days co-incubated with ASs.

2.4.10. Plausible routes of self-degradation of AS4 (SD-AS4):

We investigated the self-degradation profile of AS4 (Figure 2.50-56) to investigate the stability of AS4 and to identify its self-degradation products. This result established the role of AS4 for proteolysis of mA β . Besides proteolysis products of mA β , AS4 generated self-degradation product Q''''', following probable route 1^{SD-AS4} (Figure 2.50, "SD" stands for self-degradation). To confirm that these are generated by self-degradation, we conducted time-dependent MALDI-TOF mass spectrometric studies of the AS2 incubated in the absence of mA β (Figure 2.53-56).

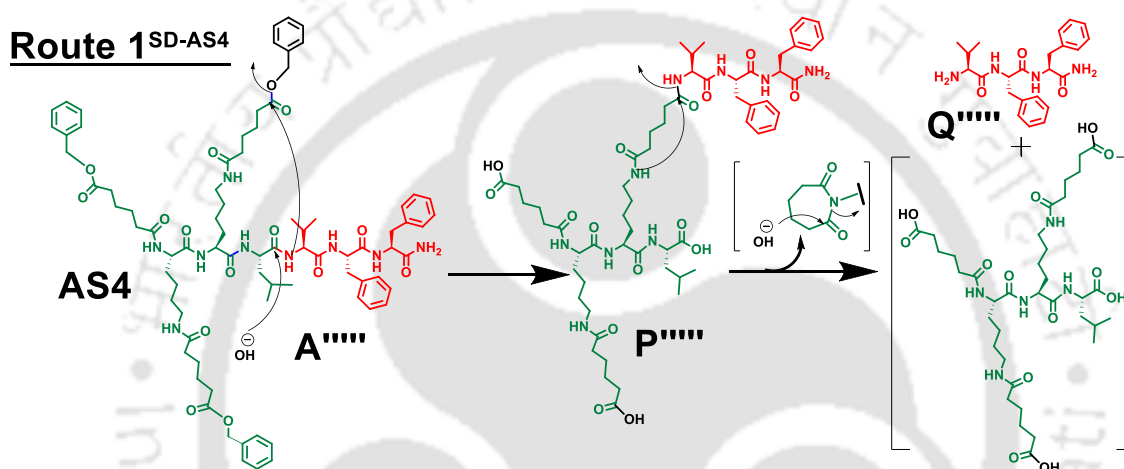


Figure 2.50. Plausible route of self-degradation of AS4 into various fragments in the absence of mA β (Route 1^{SD-AS4}).

AS4 also generated de-benzylated product R''''', following the route 2^{SD-AS4}, (Figure 2.51).

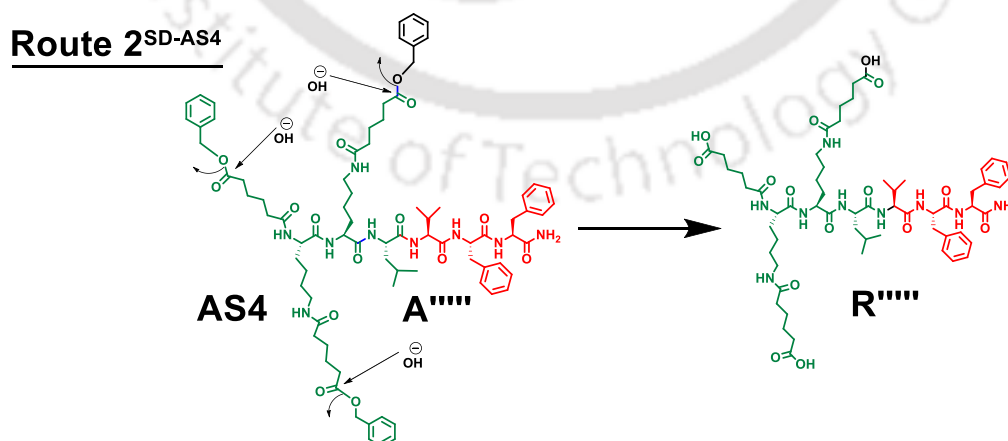


Figure 2.51. Plausible route of self-degradation of AS4 into various fragments in the absence of mA β (Route 2^{SD-AS4}).

MALDI-TOF mass results of self-degradation of AS4 indicated that AS4 cleaved itself in a site-selective manner between Lys-Leu via route 4^{SD-AS4} (Figure 2.52) to produce S'''' and M'''''. In this case also various intermediates and cleavage products may get generated by the repetition of the transamidation-hydrolysis cycle.

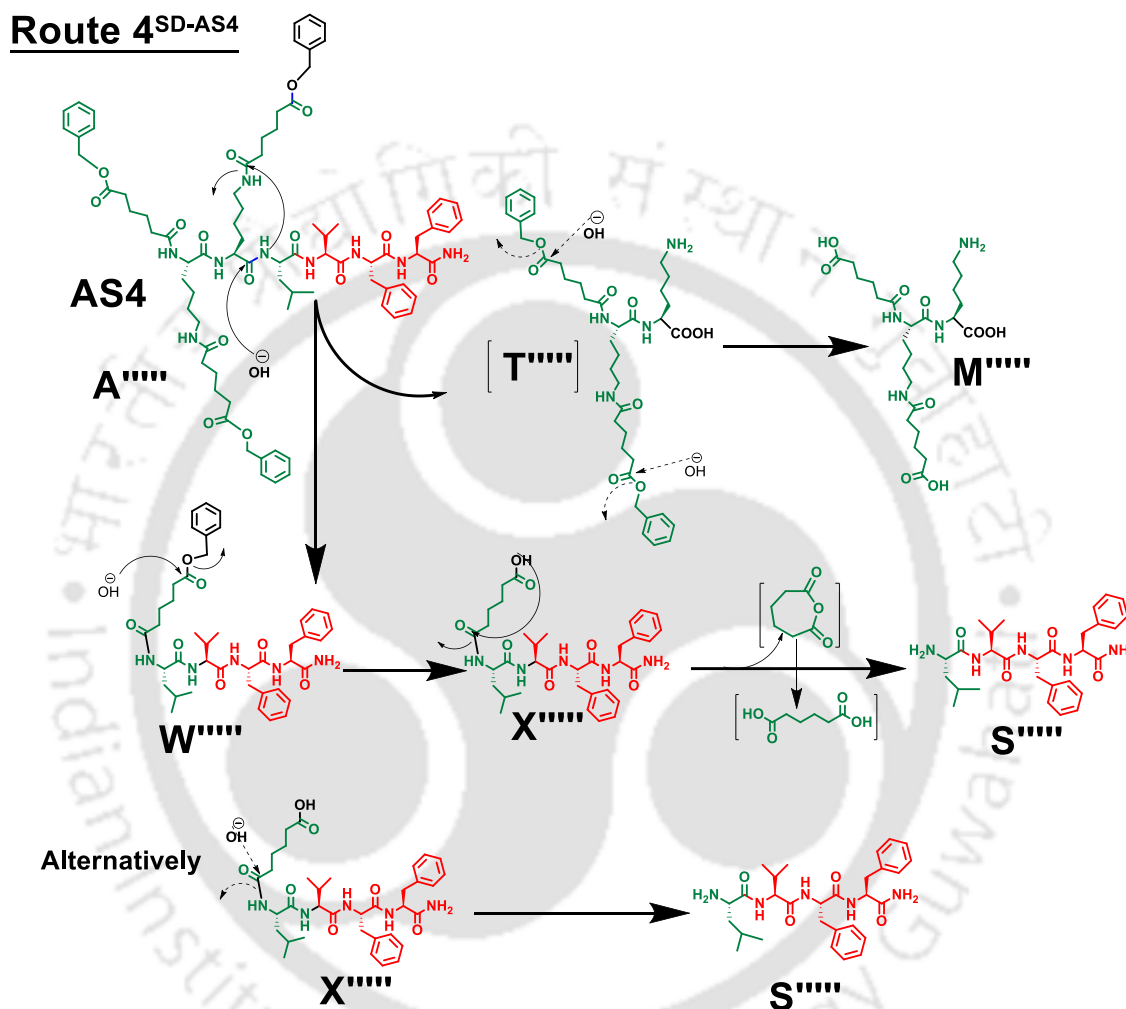


Figure 2.52. Plausible routes of self-degradation of AS4 into various fragments in the absence of mAb (Route 4^{SD-AS4}).

Most of the logically drawn intermediates and final fragments could be identified by time-dependent MALDI-TOF mass spectrometric analyses of the AS4 incubated in the absence of mAb, except those in parentheses (Figure 2.53-56).

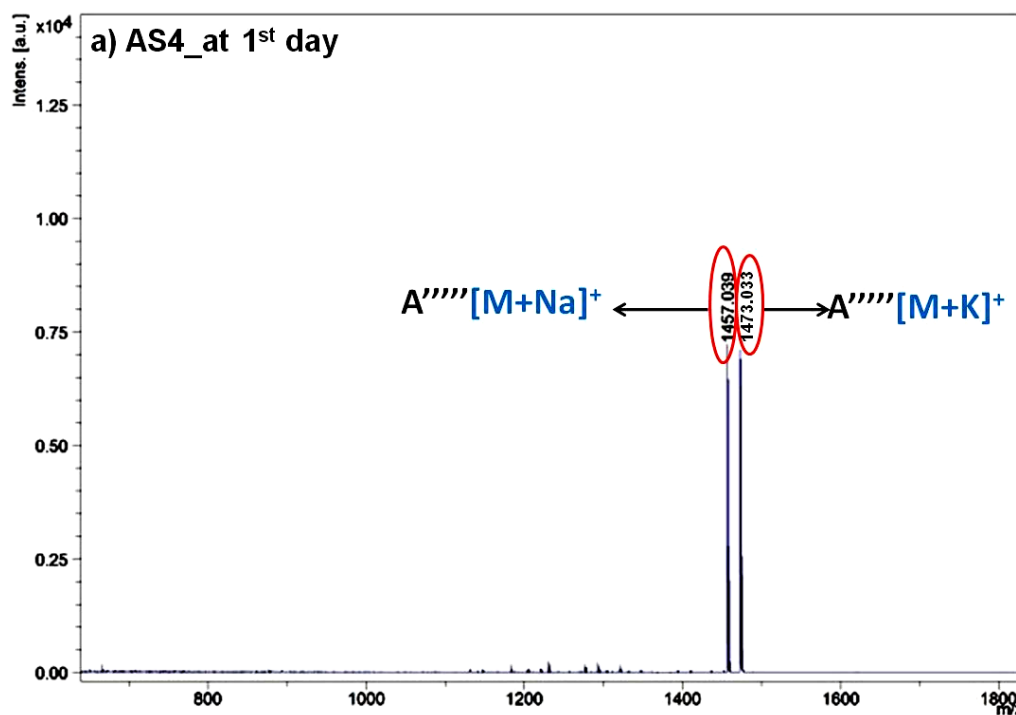


Figure 2.53. MALDI-TOF mass spectrum of AS4 at 1st day of incubation in PBS pH 7.4 at 37 °C.

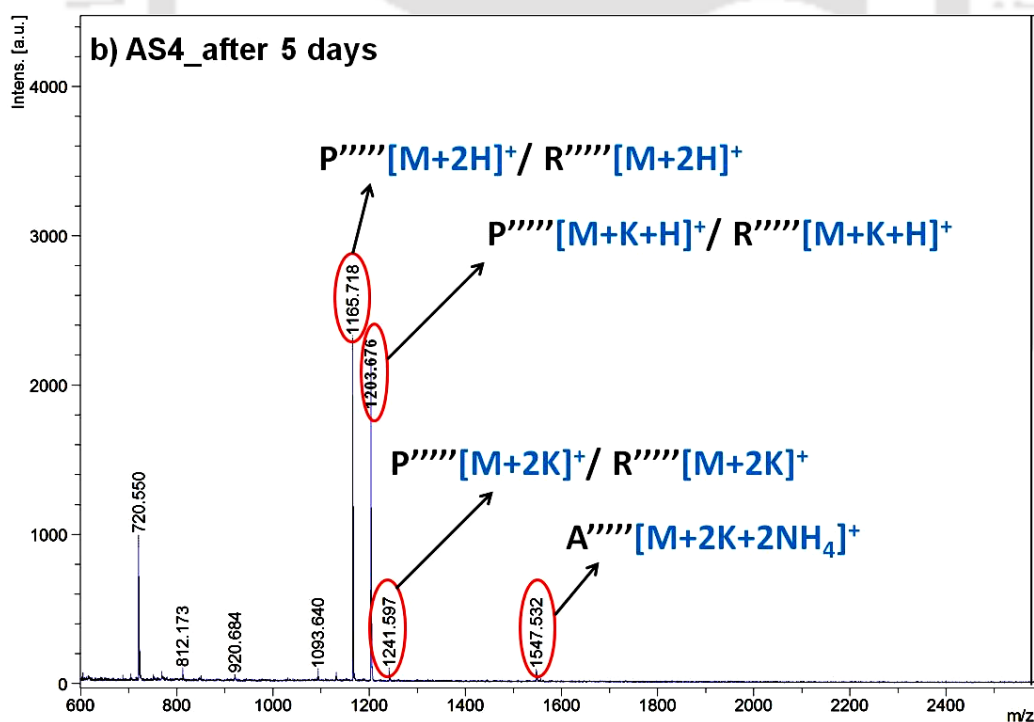


Figure 2.54. MALDI-TOF mass spectrum of AS4 after five days of incubation in PBS.

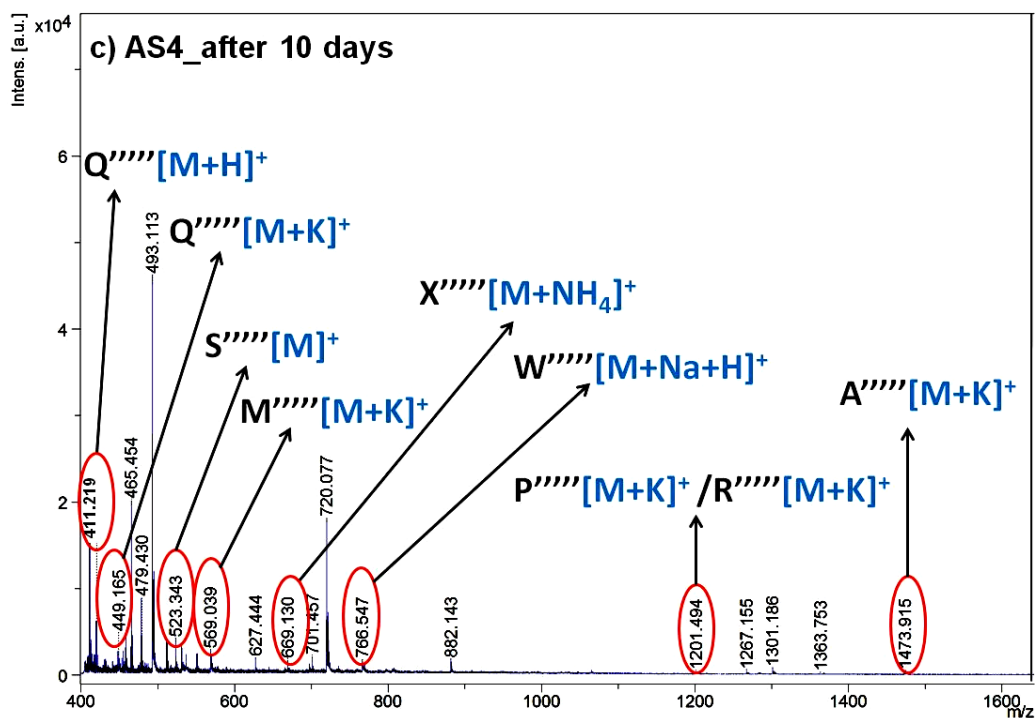


Figure 2.55. MALDI-TOF mass spectrum of AS4 after ten days of incubation in PBS.

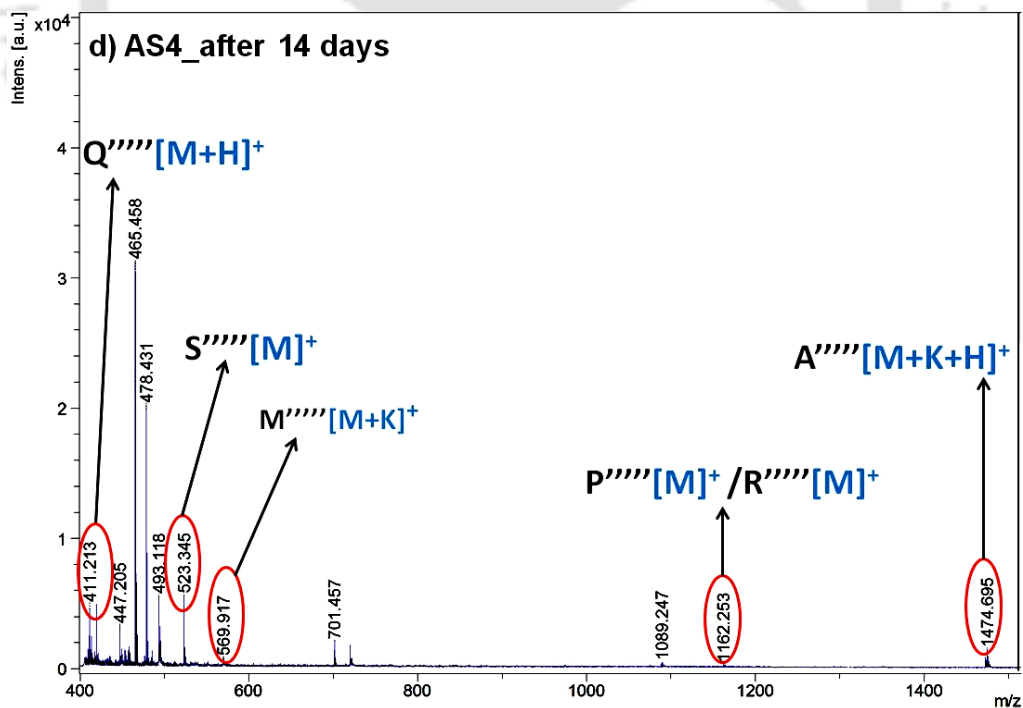


Figure 2.56. MALDI-TOF mass spectrum of AS4 after 14 days of incubation in PBS.

2.4.11. Self-degradation of mA β :

We also studied the self-degradation profile of mA β (Figure 2.57-60) and found that the self-degradation products are different from the ASs mediated proteolysis products mentioned earlier.

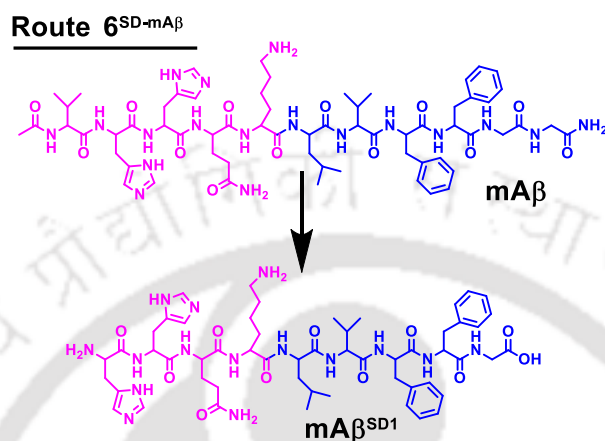


Figure 2.57. Plausible route of self-degradation of mA β into various fragments in the absence of ASs (Route 6^{SD-mA β}).

During sample preparation of mA β for MALDI-TOF mass experiments, 2 μ L of 10% TFA in water was added to break aggregation and to check self degraded fragments.

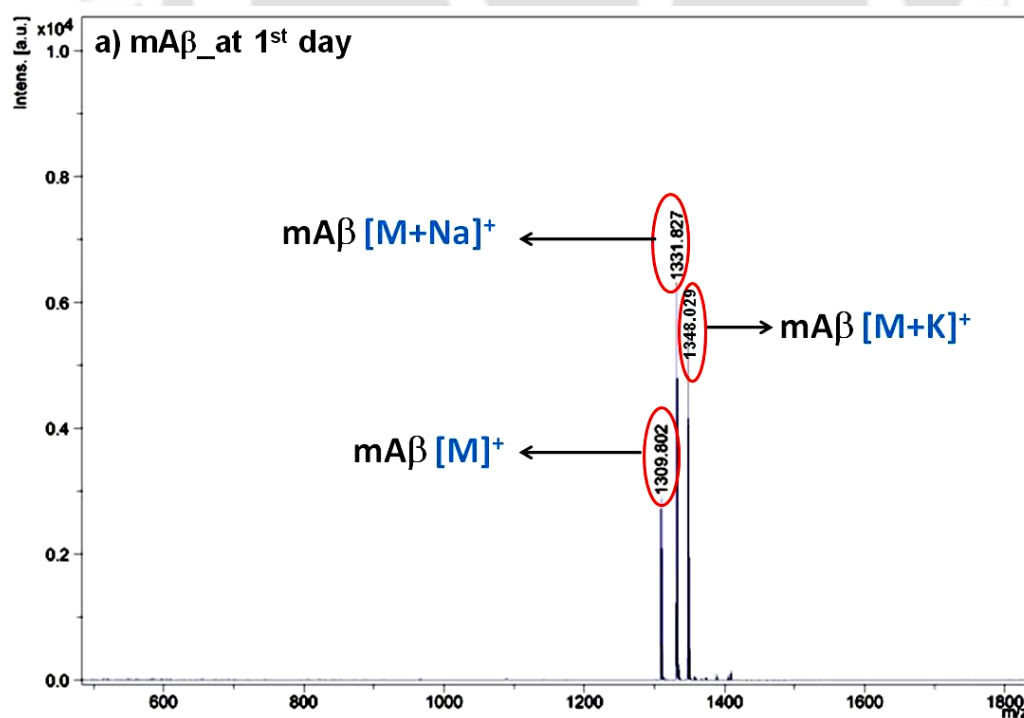


Figure 2.58. MALDI-TOF mass spectrum of mA β at 1st day of incubation in PBS pH 7.4 at 37 °C.

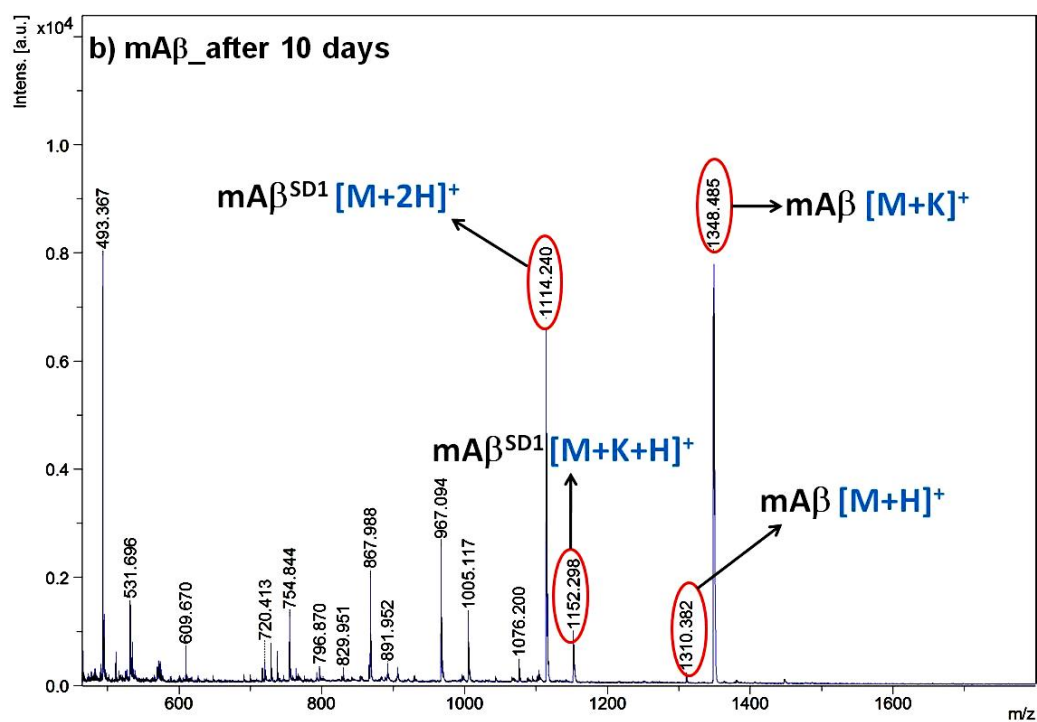


Figure 2.59. MALDI-TOF mass spectrum of mA β after ten days of incubation in PBS.

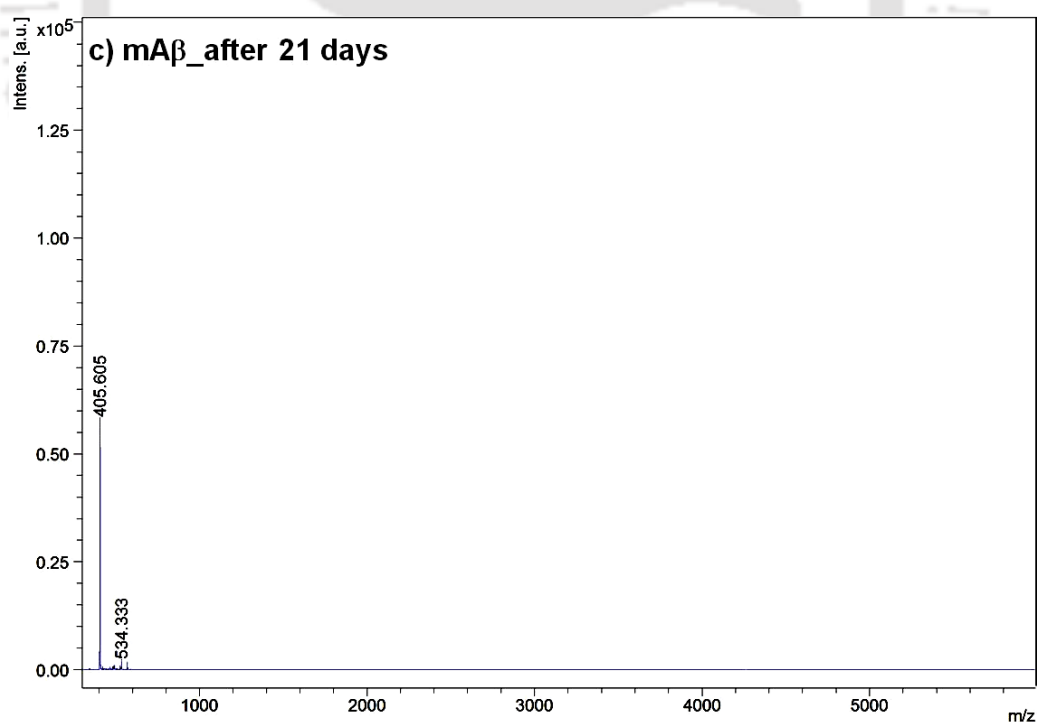


Figure 2.60. MALDI-TOF mass spectrum of mA β after 21 days of incubation in PBS.

2.5. Kinetics of amyloid accumulation of mA β in presence and absence of AS4:

The kinetics of the amyloid accumulation of mA β was monitored using various biophysical tools in absence and presence of AS4 to check the aggregation inhibition potential of AS4. At first, the purified solid peptide samples were treated with TFA and HFIP, consecutively, then dried and dissolved in PBS (50 mM, pH 7.4) appropriately to obtain the stock solutions of mA β (200 μ M) and AS4 (1 mM). In the case of AS4, 6% of ethanol was also required to get a clear stock solution. Samples were co-incubated at 37 °C on a water bath till 42 days in parallel at the desired proportions.

2.5.1. ThT fluorescence assay and CD experiments of mA β :

Fibrillization is quantitatively measured by the increment of fluorescence intensity of a peptide upon binding with ThT, and the fluorescence intensity is directly proportional to the amount of fibril present.²¹ The kinetics of fibrillization of mA β was monitored by thioflavin T (ThT) fluorescence assay (Figure 2.61-a). It was found that the fluorescence intensity of mA β (black) increased with time in the absence of AS4 and became static after 21 days after a slight lowering in intensity. But in the presence of 2 fold (red) and 5 fold (blue) molar excess of AS4, significant increase in fluorescence signal of mA β were observed until 3 days probably due to the formation of the intermediate C'''' (Figure 2.43) and co-aggregation which were slowly suppressed thereafter probably due to the formation of various fragments (Figure 2.43-45) resulting from site-selective cleavage. In case of AS4 alone (dark cyan), the fluorescence intensity was increased initially due to the presence of self-assembling LVFF segment but was slowly decreased with time probably due to self-degradation (Figure 2.50-52).

Fibrillization is usually associated with conformational alteration of the peptide.²² The conformational change of mA β was monitored by CD spectroscopy (Figure 2.61-b). The β -sheet (majorly) structure of mA β (black, Figure 2.61-b) was inferred from the negative band at 215 nm and a positive one at 195 nm, and it was observed until 42 days. The presence of the two-fold molar excess of AS4 prevented β -sheet structure (red) formation, which was more pronounced in the presence of the five-fold molar excess of AS4 (blue).

Random coil structure of mA β in the presence of the five-fold molar excess of AS4 was inferred from the -ve Cotton effect around 195 nm and absence of the same around 214 nm. The mixture of α -helix, random coil, and the β -sheet structure was observed for AS4 (dark cyan, -ve Cotton effect around 205 nm and 220 nm, and +ve Cotton effect around 195 nm) when incubated in the absence of mA β .

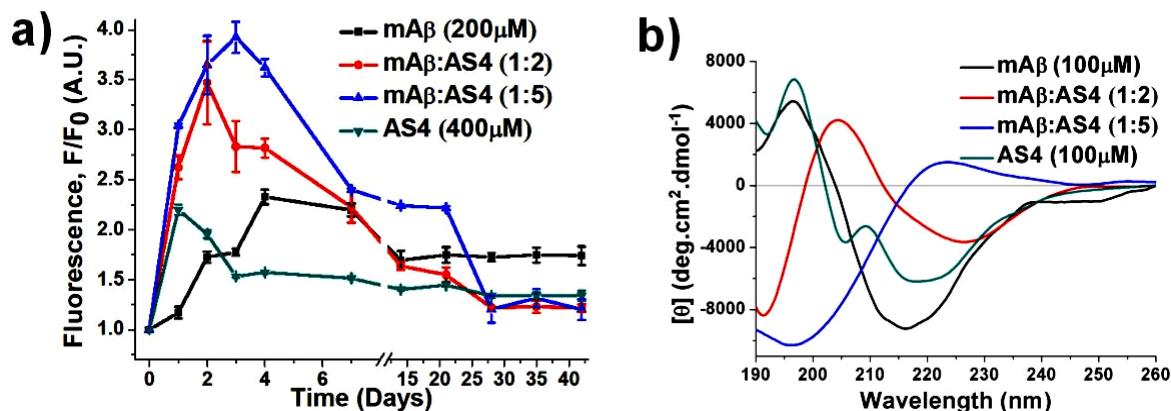


Figure 2.61. Normalized profiles of dose-dependent (a) ThT fluorescence assay, and (b) CD experiments. Spectra of mA β in the absence (black), presence of two-fold AS4 (red), five-fold AS4 (blue), and AS4 alone (dark cyan). CD Spectra were recorded after 28 days. Error bars represent standard deviations of at least three independent measurements. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

2.5.2. CD spectra of mA β alone:

A time-dependent conversion of mA β from the mainly random coil (~57%) into the mixture of β -sheet structure (~57%) and α -helix (~13%) was noted by seven days (Figure 2.62). Secondary structures of mA β were estimated by using Reed's reference.

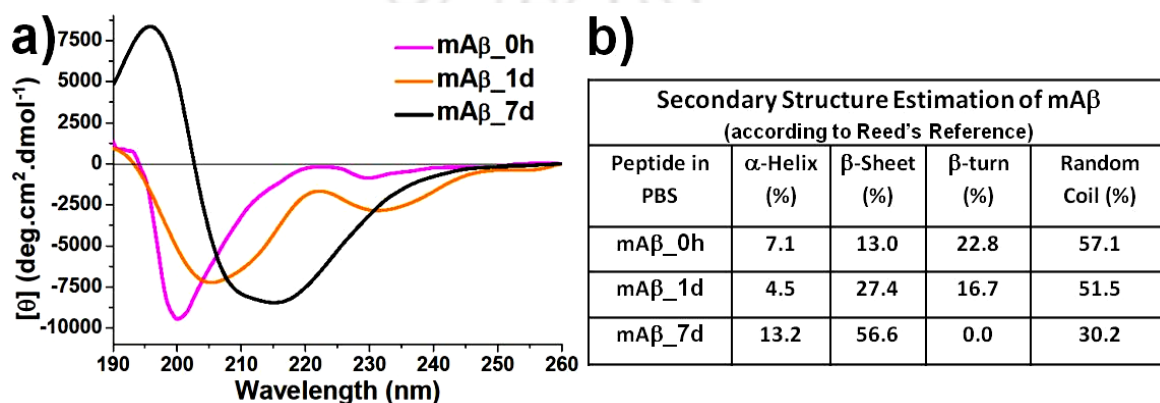


Figure 2.62. (a) CD spectra of mA β at 0 h (magenta), after one day (orange) and after seven days (black) of incubation. (b) Conversion of mA β from the random coil into the β -sheet structure. The concentration of the peptide solution in the cuvette was 100 μ M. The peptide solution was incubated in PBS pH 7.4 (50 mM) at 37 $^{\circ}$ C.

2.5.3. FTIR spectra of mA β :

FTIR is also a technique from which we obtain an idea of the secondary structure of the peptides.²¹ Presence of a strong band at around 1625-1640 cm^{-1} indicates β -sheet structure and presence of a strong band at around 1645-1660 cm^{-1} indicates random coil or helical conformation of peptides or proteins. The results (Figure 2.63) indicated that mA β adopted mainly β -sheet conformation in the absence of AS4 but adopts mainly random coil or helical structure in the presence of AS4, which were in agreement with that of the CD experiments. These results explain the inhibition of aggregation of mA β in the presence of AS4 noted in ThT based fluorescence measurements.

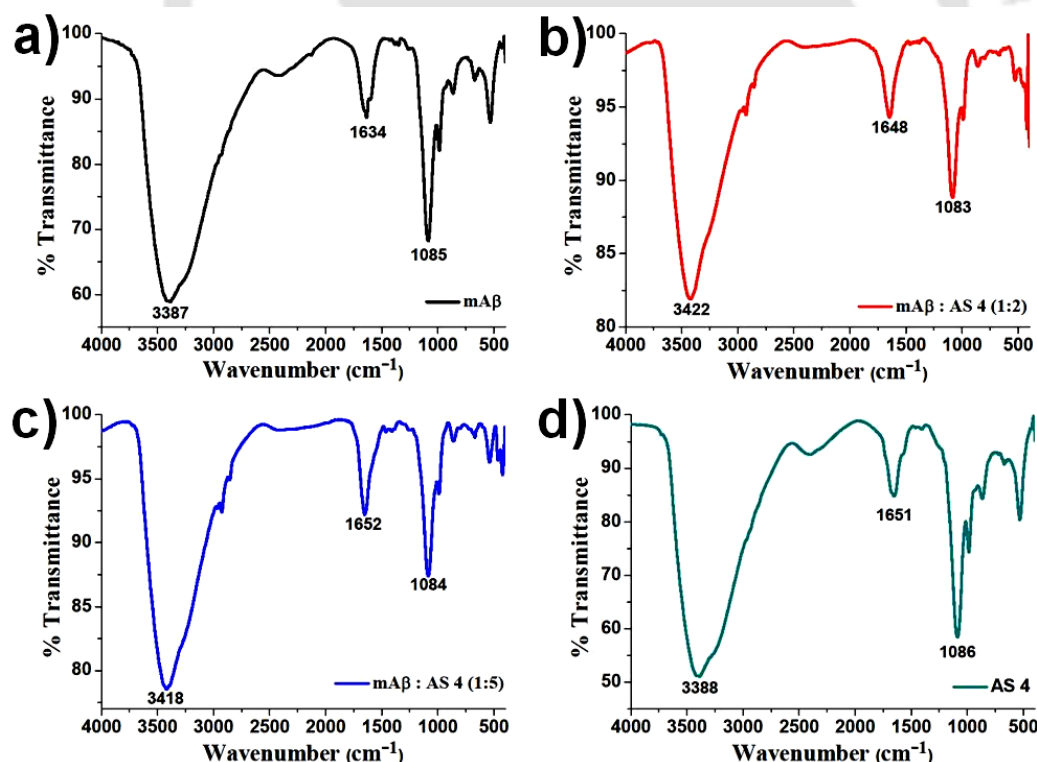


Figure 2.63. FTIR spectra of mA β (black, a) in absence, presence of (red, b) two-fold AS4, (blue, c) five-fold molar excess of AS4, and AS4 alone (dark cyan red, d). Spectra were recorded after 28 days of incubation. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 $^{\circ}$ C.

2.5.4. TEM images and Congo red stained birefringence images of mA β :

The presence of fibrillar structure under transmission electron microscopy (TEM) is another characteristic property of amyloid formed by a peptide.²¹ mA β in the absence of AS4 formed a clear fibrillar structure within a week that was stable until 28 days as observed under TEM (Figure 2.64 a-i). However, when mA β was co-incubated with AS4 (two-fold and five-fold molar excess), although tiny fibrils were noted at the 7th day, no characteristic fibrillar structure was observed later (Figure 2.64 a-ii for two-fold, and Figure 2.64 a-iii for five-fold). On the other hand, AS4 did not appear as fibrillar structure (rather off-pathway aggregates were noted, Figure 2.64 a-iv) in the absence of mA β (observed on the 28th day).

Another characteristic property of amyloid is the expression of green-gold birefringence under cross-polarized light after staining with Congo red³³. When a similar set of samples were stained with Congo red and viewed under the cross-polarized light, characteristic green-gold birefringence was observed for mA β (Figure 2.64 b-i). However, no such green-gold birefringence was observed either for the mixture of mA β and AS4 (Figure 2.64 b-ii for two-fold, and Figure 2.64 b-iii for five-fold) or for AS4 (Figure 2.64 b-iv). To get an idea about the timescale of amyloid disruption, a time-dependent Congo red birefringence study was performed, and it was noticed that while the green-gold birefringence persisted for mA β (Figure 2.64-c) during the whole span of the experiment (28 days), it gradually decreased in the presence of five-fold molar excess of AS4 (Figure 2.64-d). All the investigations were carried out under near-physiological conditions *in vitro* (PBS of pH 7.4 at 37 °C). The results of the TEM and the Cong Red staining studies indicated that mA β neither formed fibril nor formed amyloid, respectively, in the presence of AS4. These results are also in concordance with the results of the ThT experiments.

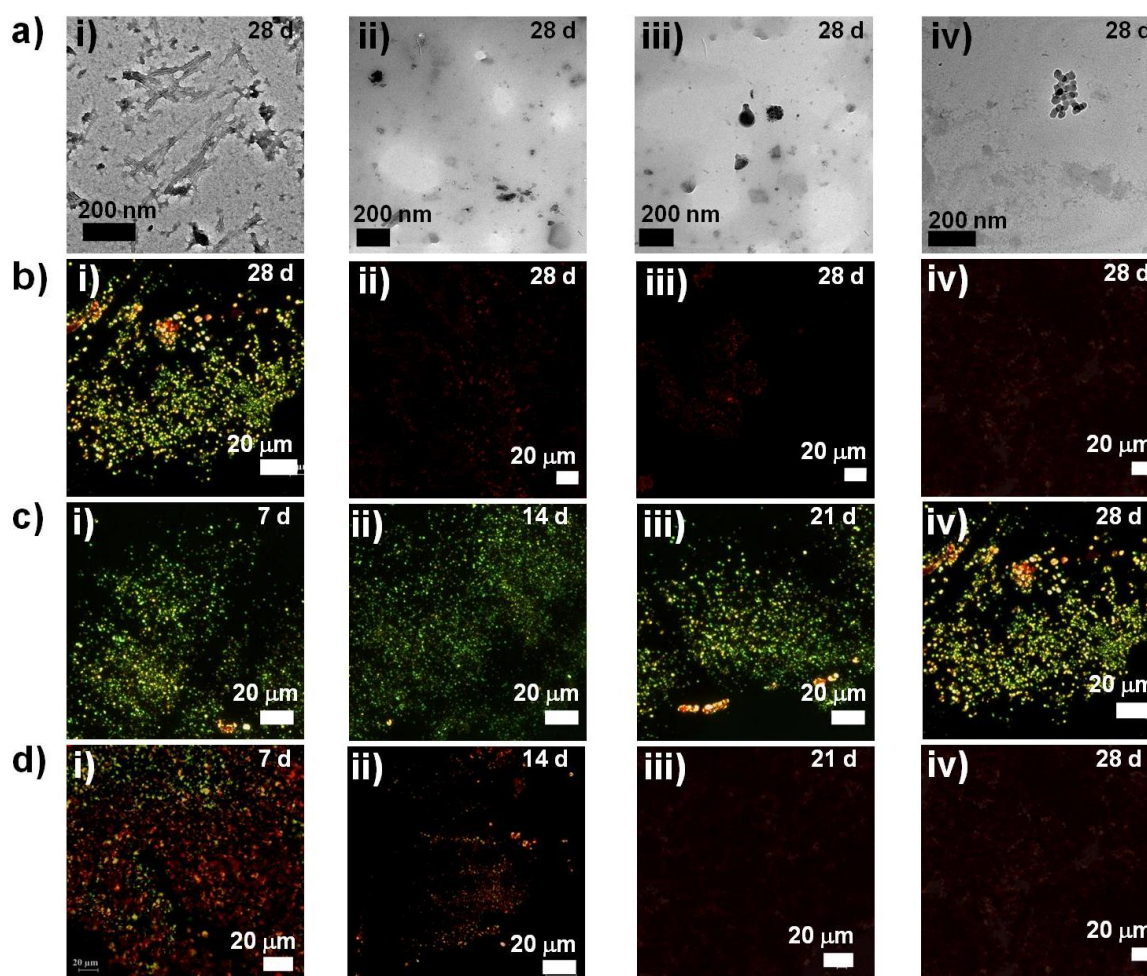


Figure 2.64. (a) TEM images, and (b) Congo red-stained birefringence images of mAb β (i) in absence, and presence of (ii) two-fold AS4, (iii) five-fold AS4, and (iv) AS4 alone. Images that were captured after 28 days are displayed. Time-dependent Congo red-stained birefringence images of mAb β fibril (c) in absence, and (d) presence of five-fold AS4. Scale bars are indicated as 200 nm for TEM images and 20 μ m for birefringence images. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 $^{\circ}$ C in parallel.

2.6. Disruption of preformed mAb β fibril by AS4:

During optimization of the ThT fluorescence assay, it seemed that AS4 first co-aggregated with mAb β then it disrupted fibril. However, it was essential to check whether AS4 can disrupt existing fibril. We also noted that the growth phase for the fibrillization of mAb β was from 24 h to 96 h. Therefore, AS4 was added to the mAb β sample at 72 h

(PBS pH 7.4 at 37 °C), i.e., on the growth phase of the fibrillation, assuming the presence of some preformed fibril and monitored further by ThT assay.

2.6.1. ThT fluorescence assay and CD experiments of mA β :

On addition of two-fold (red, in Figure 2.65-a) and five-fold (blue, in Figure 2.65-a) molar excess of AS4, the fluorescence intensity of mA β increased further, which slowly suppressed after that probably due to the same reason as mentioned before. Therefore, it can be concluded that AS4 not only inhibited fibrillization of mA β but also disrupted existing fibril. The results of a parallel CD (Figure 2.65-b) were in agreement with those of the fluorescence experiments. AS4 converted the β -sheet structure of mA β into the random coil conformation (blue, -ve Cotton effect around 1955 nm).

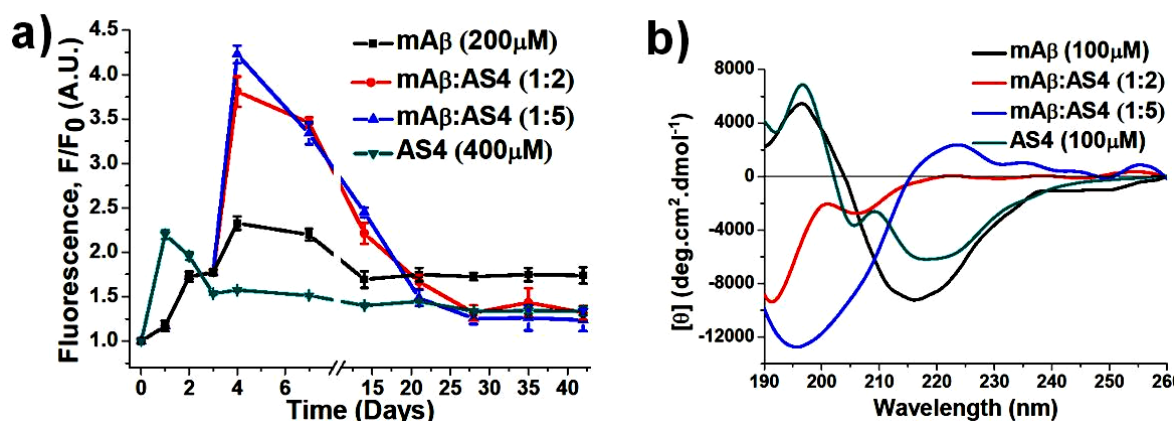


Figure 2.65. Normalized profiles of dose-dependent (a) ThT fluorescence assay, and (b) CD experiments. Spectra of mA β in absence (black), presence of two-fold AS4 (red), five-fold AS4 (blue), and AS4 alone (dark cyan). CD Spectra were recorded after 28 (3+25) days of incubation. Error bars represent standard deviations of at least three independent measurements. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

2.6.2. FTIR spectra of mA β :

The results of FTIR (Figure 2.66) study indicated that indeed, the presence of AS4 could change the β -sheet prevailing conformation of mA β to non- β -sheet structures. The peak at 1653 cm⁻¹ (red, in the presence of two-fold AS4), and 1649 cm⁻¹ (blue, in the presence of five-fold AS4) indicated the random coil structure.

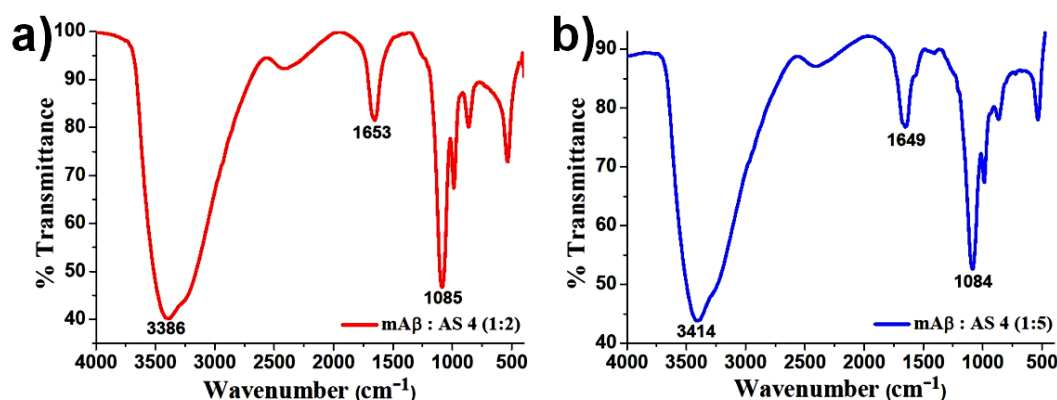


Figure 2.66. FTIR spectra of preformed mAb β fibril disruption in the presence of (red, a) two-fold AS4 and (blue, b) five-fold molar excess of AS4. AS4 added to mAb β after three days of its incubation. Spectra were recorded after 28 (3+25) days of incubation. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C.

2.6.3. TEM images and Congo red stained birefringence images of mAb β :

Also, the results of TEM (Figure 2.67-a) and Congo red birefringence studies (Figure 2.67 b-c) suggested that AS4 disrupted existing fibril and amyloid of mAb β , respectively. The results also suggest that AS4 inhibits fibrillization and disrupts existing fibril in a dose-dependent manner.

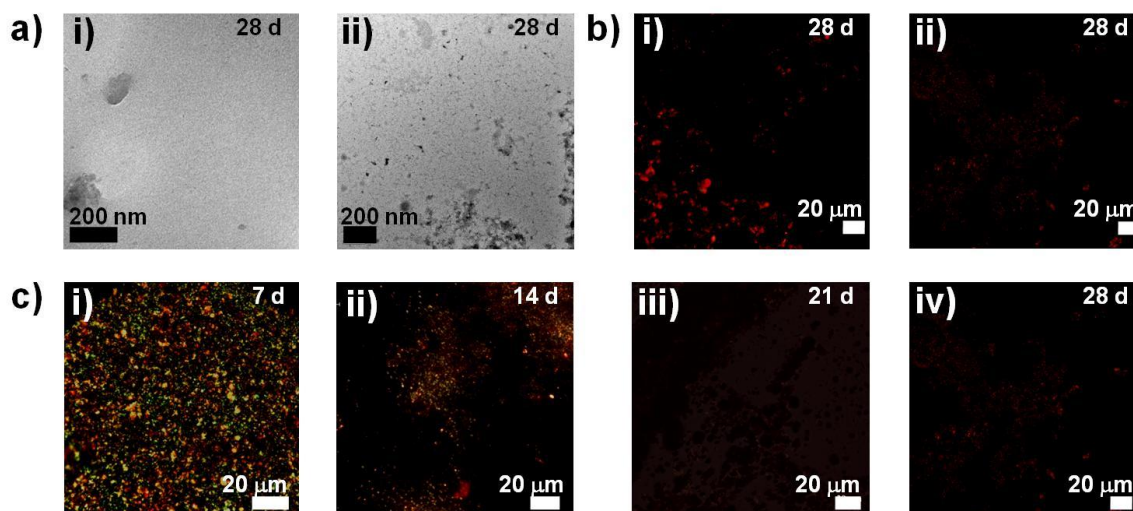


Figure 2.67. (a) TEM images, and (b) Congo red-stained birefringence images of mAb β (i) in absence, and presence of (i) two-fold AS4, (ii) five-fold AS4. Images that were captured after 28 (3+25) days are displayed. Time-dependent Congo red-stained birefringence images of mAb β fibril (c) in the presence of five-fold AS4. Scale bars are indicated as 200 nm for TEM images and 20 μ m for birefringence images.

cleave Amyloid β at the α -secretase cleavage site by aggregation driven cascades of chemical reactions

2.7. Plausible routes of the proteolytic activity of AS4 on $A\beta_{1-40}$:

Subsequently, to find out the cleavage sites of $A\beta_{1-40}$ by AS4; similar experiments were carried out using commercial $A\beta_{1-40}$ peptide. Samples were prepared similarly to that for m $A\beta$. After ten days, it was observed that AS4 cleaved $A\beta_{1-40}$ in a site-selective manner, between Lys16 and Leu17 (the α -secretase cleavage site); and Lys17 and Gln18. Various peptide fragments and intermediates of different lifetimes were also found. Plausible routes of proteolytic cleavage of $A\beta_{1-40}$ by AS4 (Figure 2.68-71) were delineated from MALDI mass (Figure 2.72-74) analyses. AS4 cleaved $A\beta_{1-40}$ between Lys16 and Leu17 to generate $A\beta_{1-16}$ segment probably via route $2^{A\beta-AS4}$ as described in route $2^{mA\beta-AS2}$.

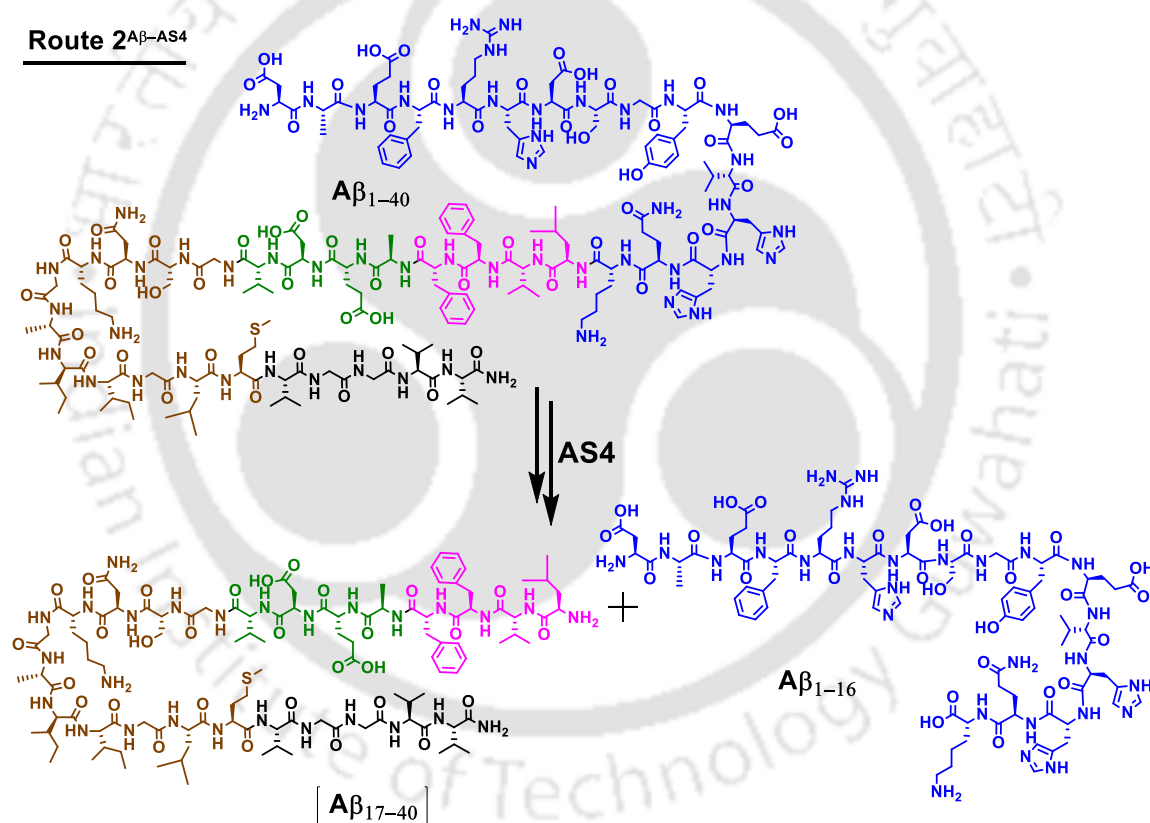


Figure 2.68. Plausible routes of proteolytic cleavage of $A\beta_{1-40}$ into various fragments by AS4 (Route $2^{A\beta-AS4}$). IM stands for Intermediate.

cleave Amyloid β at the α -secretase cleavage site by aggregation driven cascades of chemical reactions

AS4 also cleaved $A\beta$ between Lys17 and Gln18 to form $A\beta_{16-35}$ probably via route 3 $A\beta$ -AS4^{AS4}. Nucleophilic attacks of the nearest backbone nitrogens to the newly formed amide carbonyl by proximity driven interaction, spontaneous backbone amide hydrolysis and subsequent release of the amino group of the side-chain result in site-selective $A\beta$ cleavage and formation of other intermediates. Repetition of such imidation-hydrolysis-transamidation cycle generates various intermediates and cleavage products as evident from the systematic time-resolved MALDI-mass analyses on $A\beta_{1-40}$.

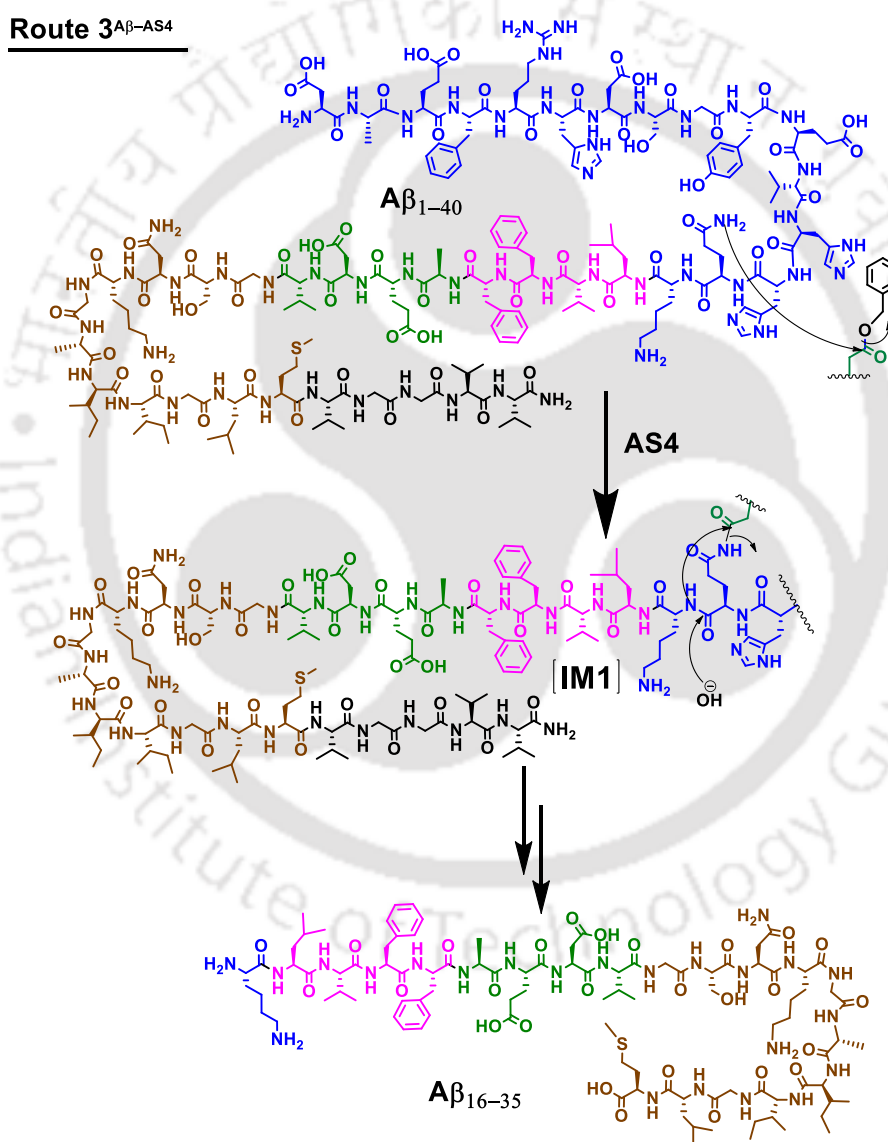


Figure 2.69. Plausible routes of proteolytic cleavage of $A\beta_{1-40}$ into various fragments by AS4 (Route 3 $A\beta$ -AS4).

cleave Amyloid β at the α -secretase cleavage site by aggregation driven cascades of chemical reactions

AS4 further sliced $A\beta_{1-16}$ into $A\beta_{12-15}$, and $A\beta_{13-15}$ probably via route 7 $A\beta$ -AS4, and 8 $A\beta$ -AS4 respectively, by prolonged incubation (21 days). In these cases also, repetition of imidation-hydrolysis-transamidation cycle generates various intermediates and cleavage products as depicted in route 7 $A\beta$ -AS4, and 8 $A\beta$ -AS4.

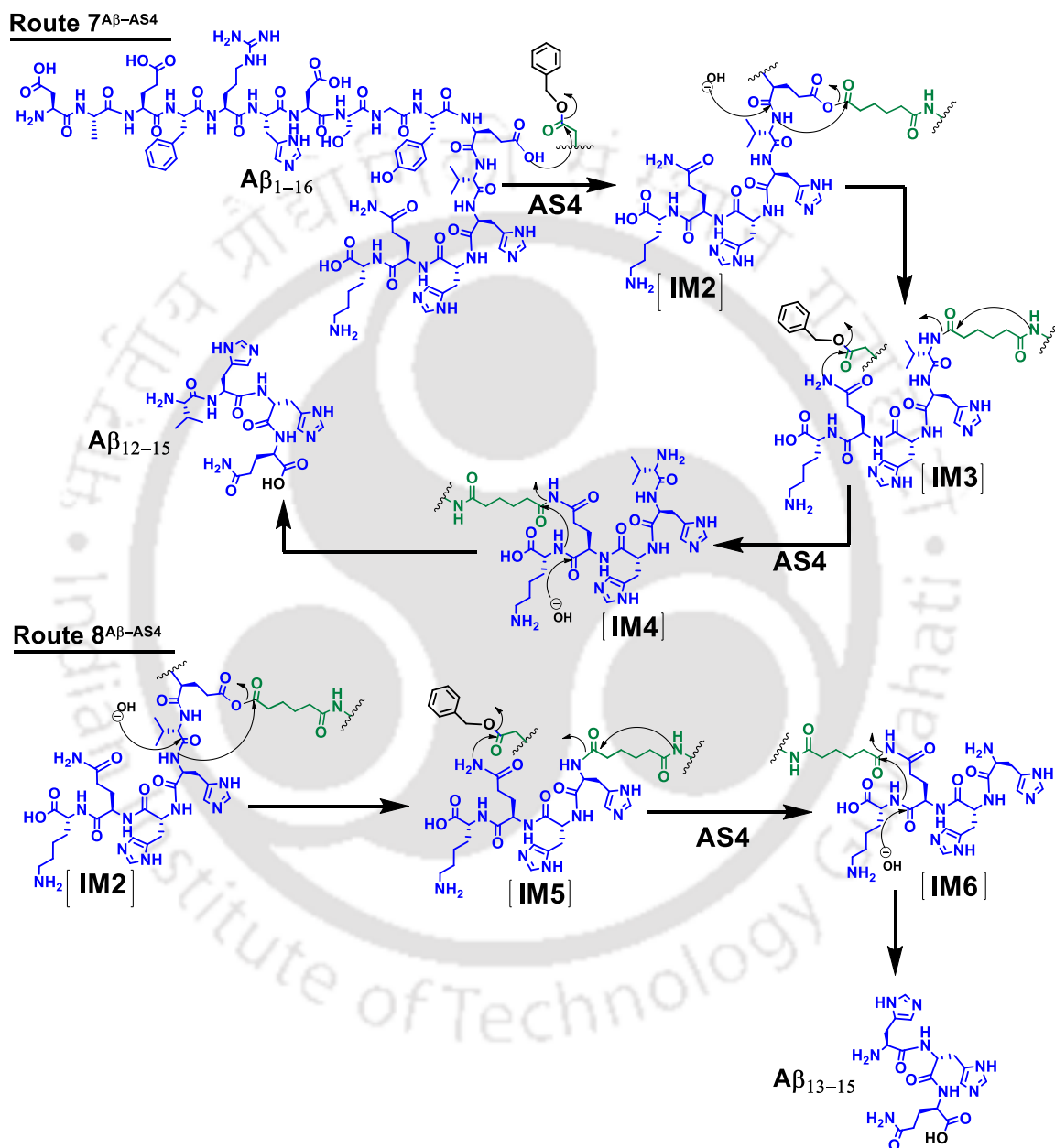


Figure 2.70. Plausible routes of proteolytic cleavage of $A\beta_{1-40}$ into various fragments by AS4 (Route 7 $A\beta$ -AS4 and Route 8 $A\beta$ -AS4).

AS4 again sliced $A\beta_{1-16}$ into $A\beta_{4-7}$ probably via 9 $A\beta$ -AS4 by prolonged incubation (21 days). The other intermediate, $A\beta_{17-40}$, also got sliced to form $A\beta_{17-24}$ probably via route

$10^{A\beta-AS4}$ with time. Nucleophilic attacks of the carboxylate anion to the carbonyl of benzyl-adipate, nucleophilic attacks of the nearest backbone nitrogens to the newly formed anhydride carbonyl by proximity driven interaction, spontaneous backbone amide hydrolysis and subsequent release of the acid functional group of the side-chain result in site-selective $A\beta$ cleavage and formation of other intermediates.

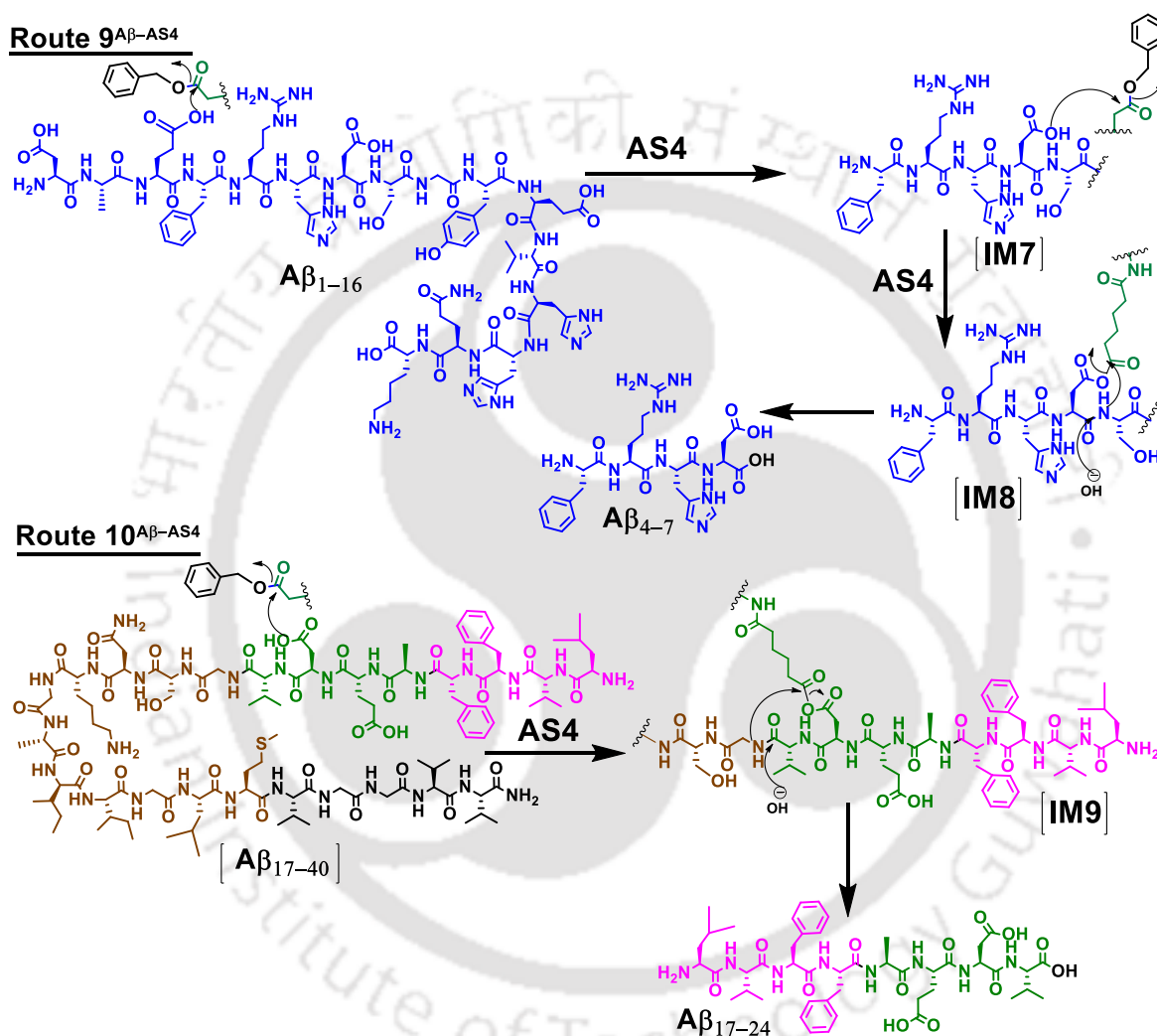


Figure 2.71. Plausible routes of proteolytic cleavage of $A\beta_{1-40}$ into various fragments by AS4 (Route 9^{Aβ-AS4} and Route 10^{Aβ-AS4}).

MALDI-TOF mass spectra of $A\beta_{1-40}$ were also analyzed in a time-dependent manner (Figure 2.72-74) in the presence of AS4. The mentioned fragments as depicted in various routes (Figure 2.68-71) were observed. Interestingly, no aggregated peak was noted in the presence of AS4; pointing to the amyloid solubilizing nature of AS4.

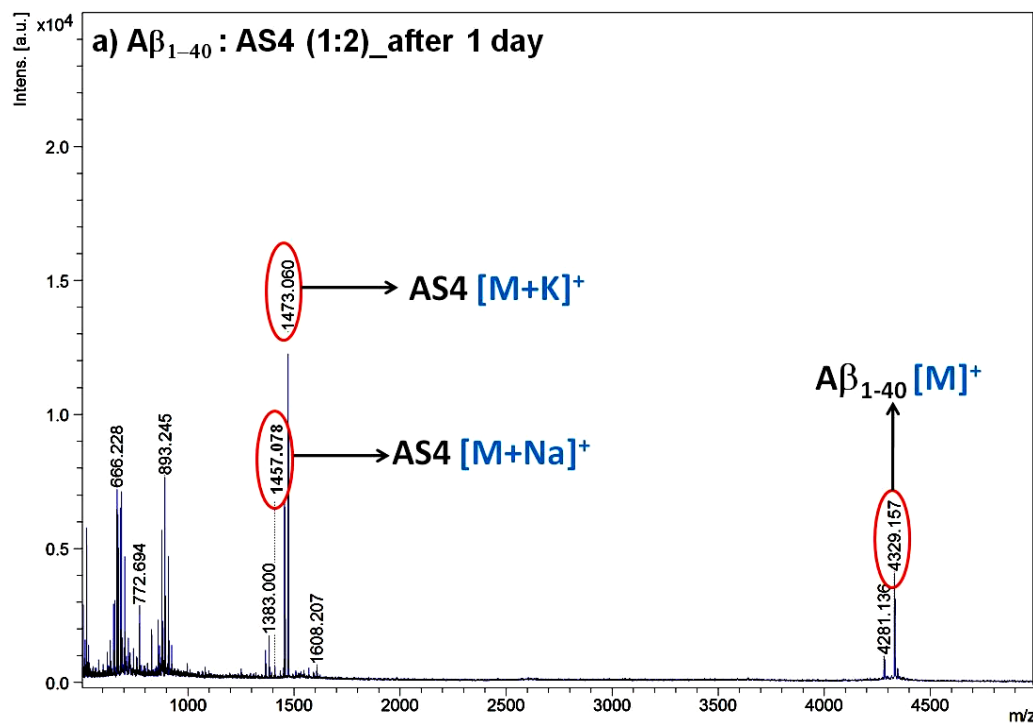


Figure 2.72. MALDI-TOF mass spectrum of $A\beta$ in the presence of AS4 (1:2) after one day of incubation in PBS pH 7.4 at 37 °C.

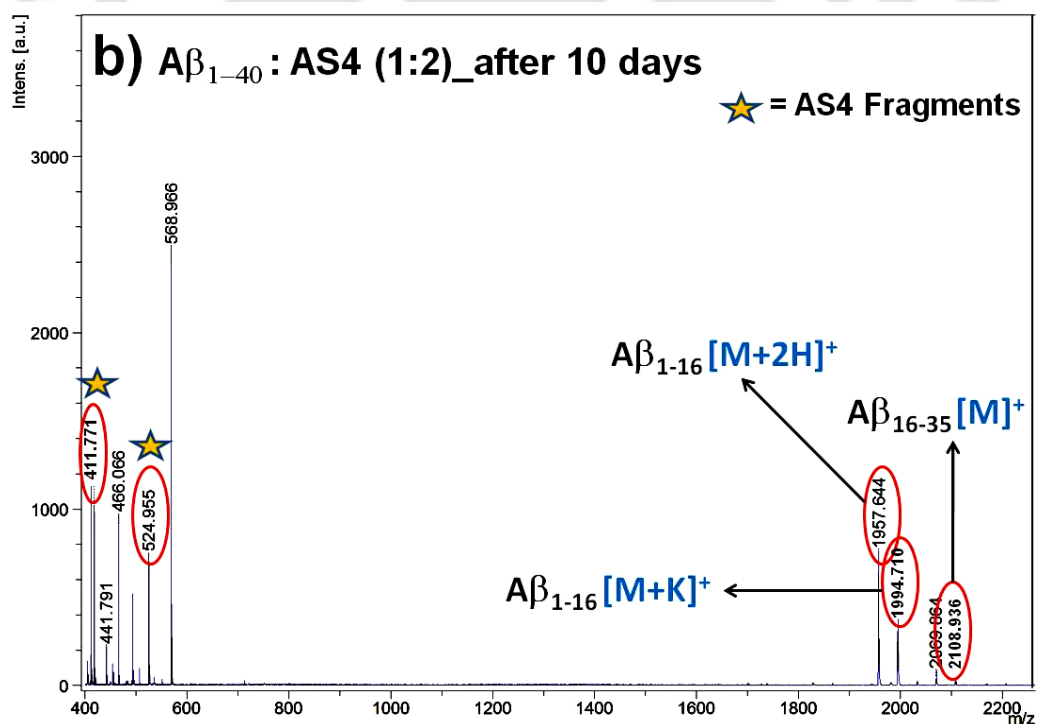


Figure 2.73. MALDI-TOF mass spectrum of $A\beta$ in the presence of AS4 (1:2) after ten days.

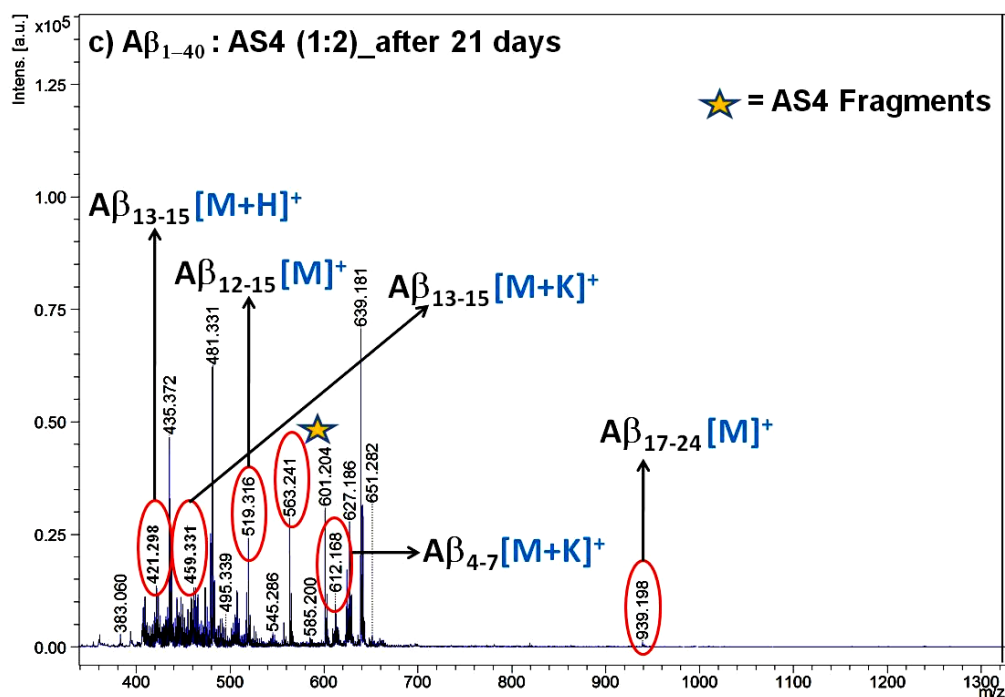


Figure 2.74. MALDI-TOF mass spectrum of $A\beta$ in the presence of AS4 (1:2) after 21 days.

A time-resolved persistence map of the $A\beta$ fragments (Figure 2.75) demonstrates the time-dependent manner of the programmed proteolysis of the smartly designed peptide-conjugate, AS4.

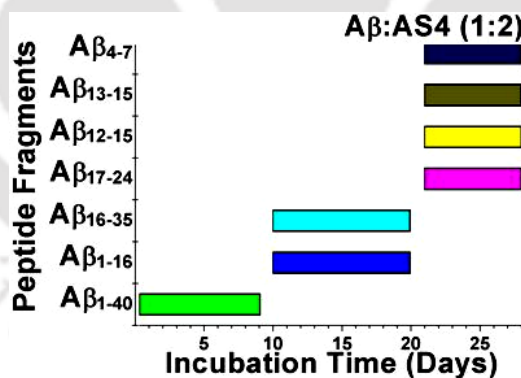


Figure 2.75. A time-resolved persistence map of the $A\beta$ fragments.

2.7.1. MALDI mass spectra of $A\beta_{1-40}$:

MALDI-TOF mass spectra of $A\beta_{1-40}$ were also analyzed in a time-dependent manner (Figure 2.76-79) in the absence of AS4. This control experiment was to check whether the mentioned peptide fragments appeared due to self-degradation of $A\beta_{1-40}$. None of the mentioned fragments were observed. Instead, the MALDI-TOF mass data indicated that $A\beta_{1-40}$ got aggregated after three days and as a result, probably it lost solvent accessibility;

thus, no degradation product was observable in MALDI-TOF mass spectra. Interestingly, the indication of such aggregation was not noted in the presence of AS4; pointing to the amyloid solubilizing nature of AS4.

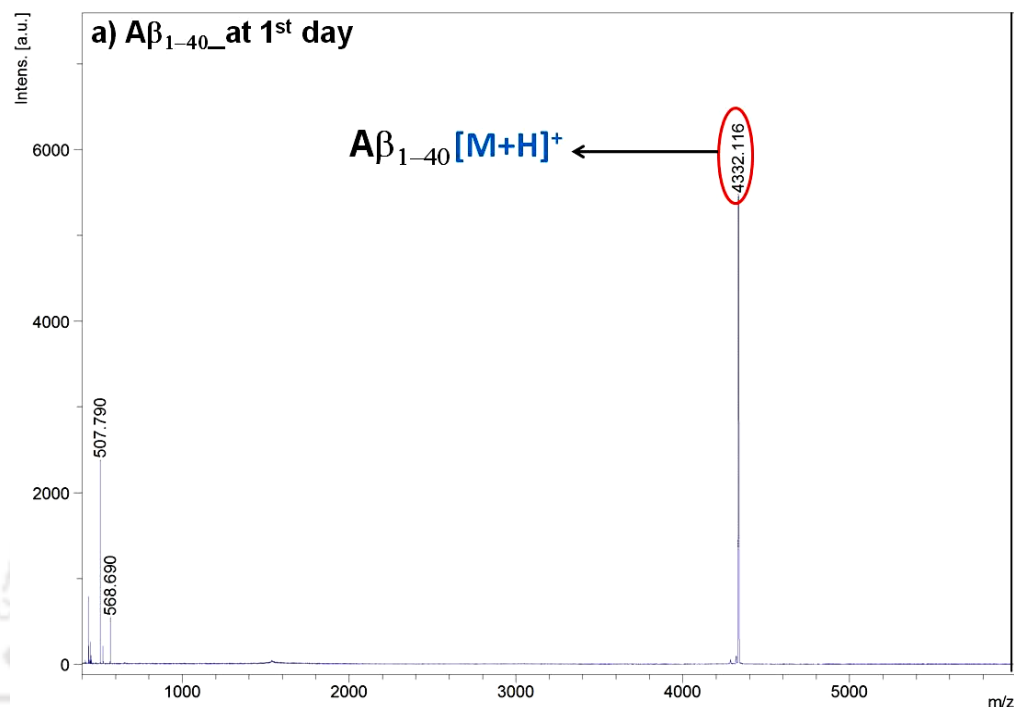


Figure 2.76. MALDI-TOF mass spectrum of $A\beta$ in the absence of AS4 at 1st day in PBS.

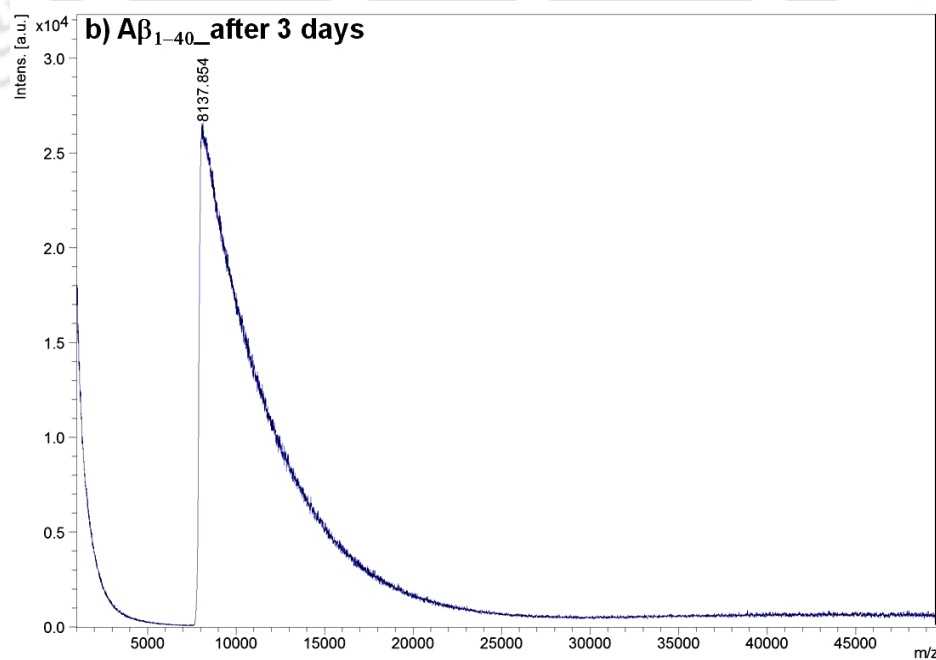


Figure 2.77. MALDI-TOF mass spectrum of $A\beta$ in the absence of AS4 after three days of incubation.

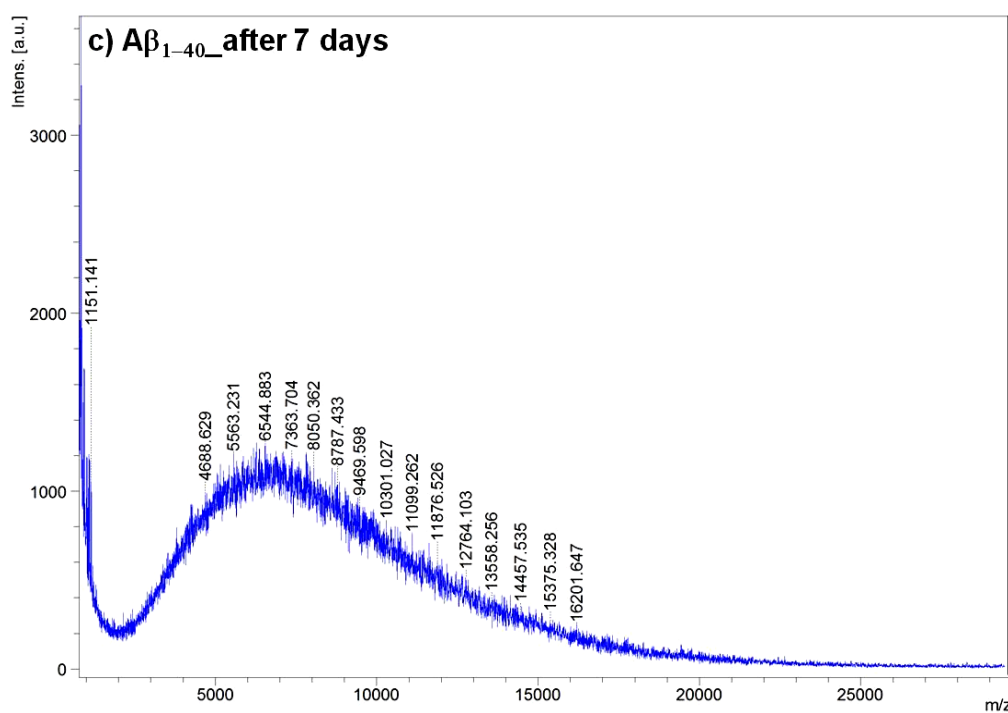


Figure 2.78. MALDI-TOF mass spectrum of A β in the absence of AS4 after seven days of incubation.

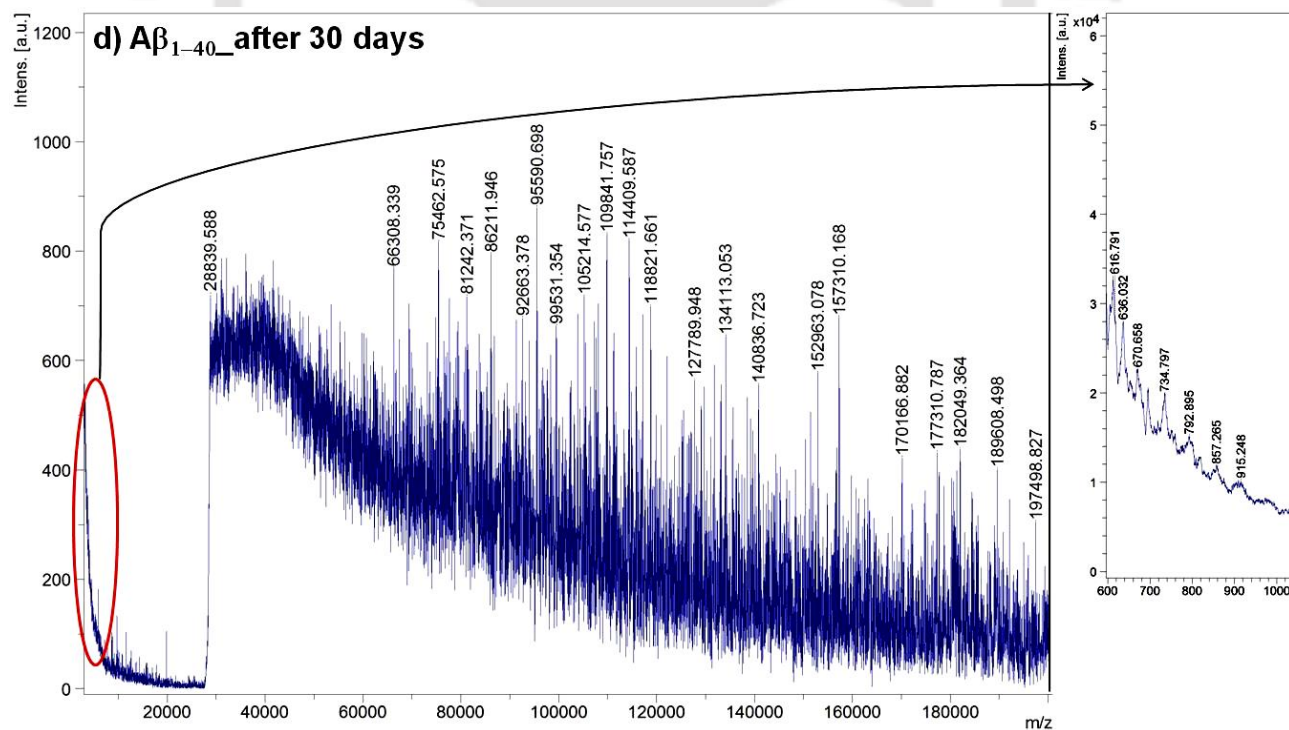


Figure 2.79. MALDI-TOF mass spectrum of A β in the absence of AS4 after 30 days of incubation in PBS.

Enzymes such as NEP, ECE, IDE, P, ACE, and MMPs also cleave A β aggregates in a site-selective manner for facilitating excretion (Figure 2.80). AS4 initially cleaved A β_{1-40} in-between Lys16 & Leu17, which is the cleavage site of not only α -secretase but also ECE, MMP2, MMP9 & P; and in-between Leu17 & Val18, the same as ECE. After prolonged incubation (>21 days), AS4 cleaved A β_{1-40} between Val12 & His13 (the cleavage site of NEP & IDE), Asp7 & Ser8 (the ACE cleavage site), and Glu3 & Phe4 (the NEP cleavage site). However, AS4 also cleaved A β_{1-40} between Glu11 & Val12 and between Val24 & Gly25, unlike any known proteolytic enzymes. Thus, AS4 itself can cleave A β_{1-40} at various sites, mimicking the proteolytic action of multiple enzymes.



2.9. Examination of the proteolytic activity of AS4 on mutant DTP28 (the negative control [NC] peptide):

Finally, to investigate the selective nature of AS4, a designed negative control (NC) peptide was co-incubated with AS4 in 1:1 molar ratio at physiological pH and temperature and followed by time-dependent MALDI mass analyses. A 28 residue long partial sequence of the fragment B of the diphtheria toxin protein (DTP) that was suitably mutated by Pro (mutant DTP28) in two positions to render a non-aggregating peptide was chosen as the negative control peptide as its sequence was entirely unrelated to both A β and the ASs but contained two Lys residues as reaction sites. The co-incubated sample did not show any of the expected fragments (Figure 2.81-86) due to cleavage by AS4, unlike mA β or A β_{1-40} (*vide supra*) even after ten days. Instead, the self-degradation products of AS4 started appearing since the first day.

Expected fragments from Negative Control (NC) Peptide

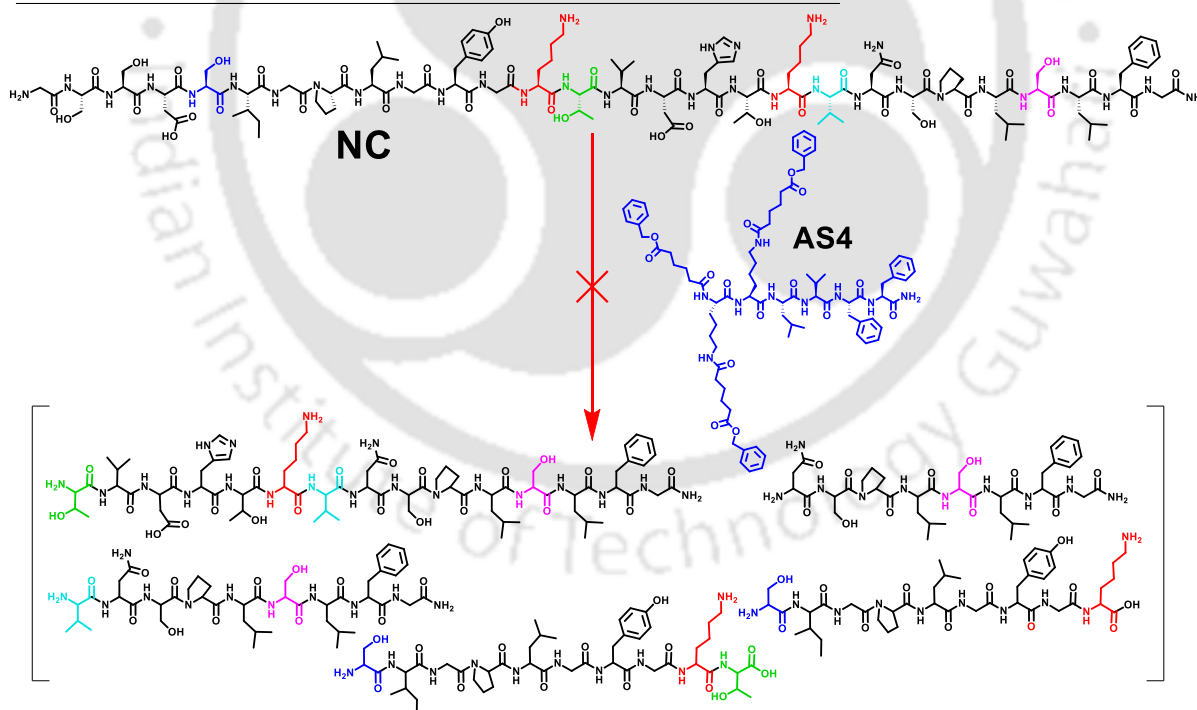


Figure 2.81. Negative control (NC) peptide didn't give expected fragments in the presence of AS4.

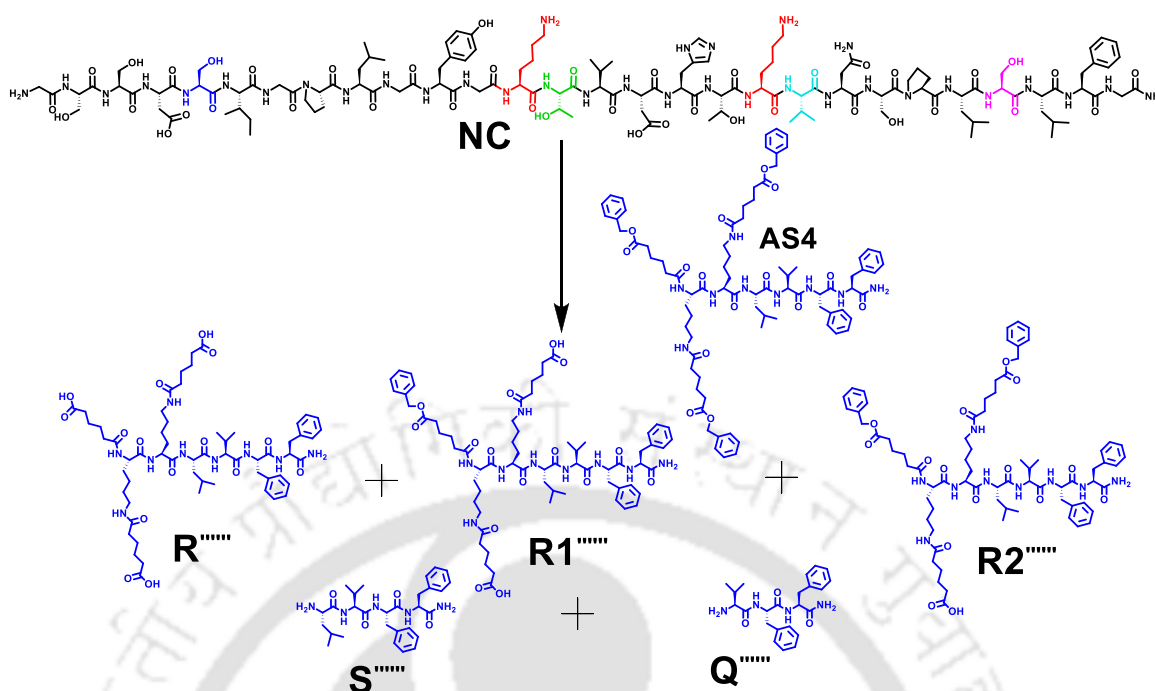


Figure 2.82. Observed self-degradation products of AS4 in the presence of NC peptide (Route 1^{SD-AS4}, Route 4^{SD-AS4} and Route 5^{SD-AS4}).

Most of the logically drawn intermediates and final fragments could be identified by time-dependent MALDI-TOF mass spectrometric analyses of the AS4 incubated in the presence of NC, except those in parentheses (Figure 2.83-86).

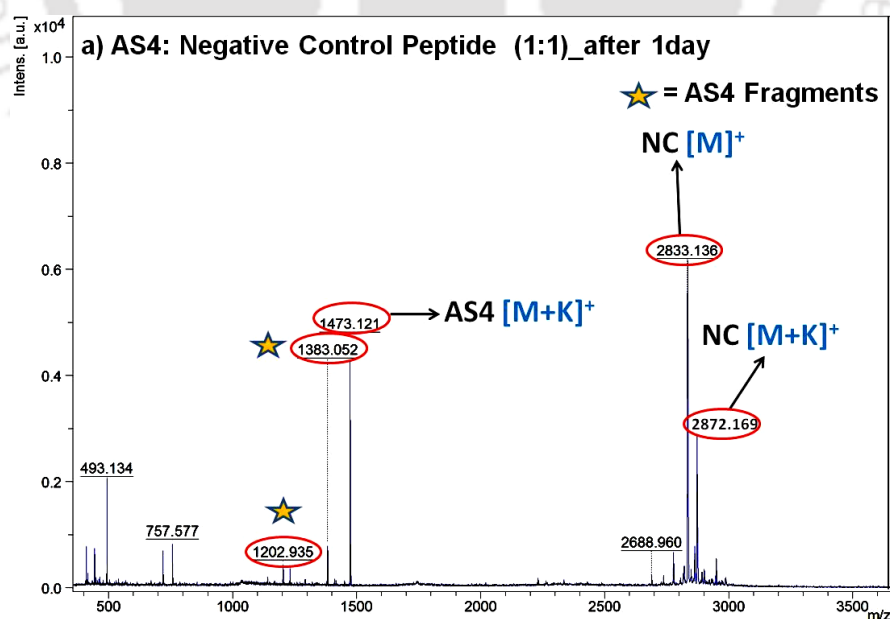


Figure 2.83. MALDI-TOF mass spectrum of NC in the presence of AS4 (1:1) after one day.

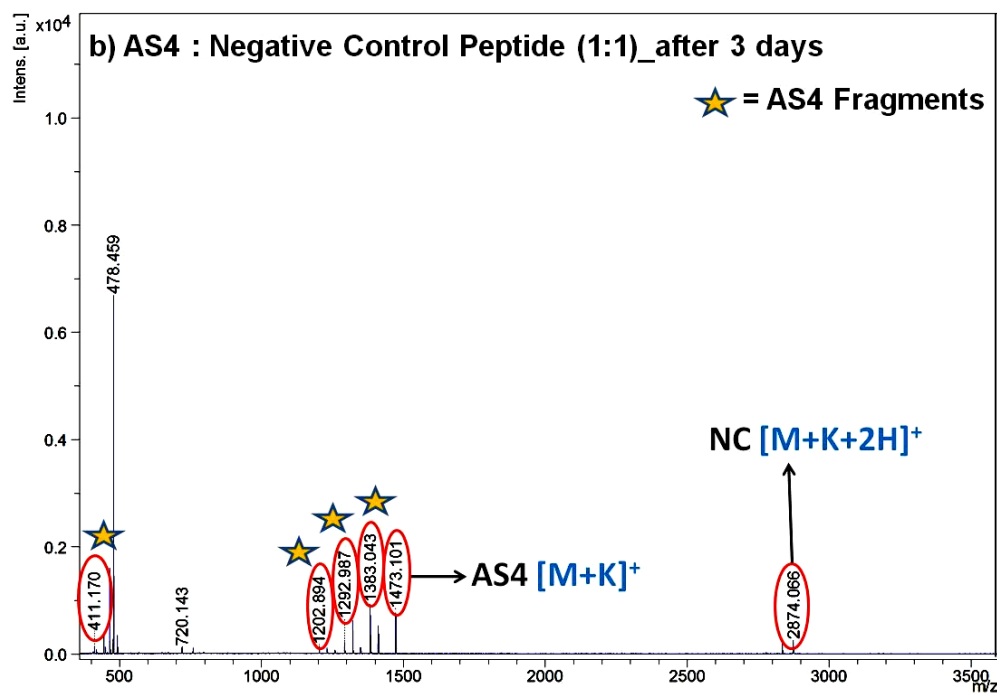


Figure 2.84. MALDI-TOF mass spectrum of negative control peptide in the presence of AS4 (1:1) after three days of incubation in PBS pH 7.4 at 37 °C.

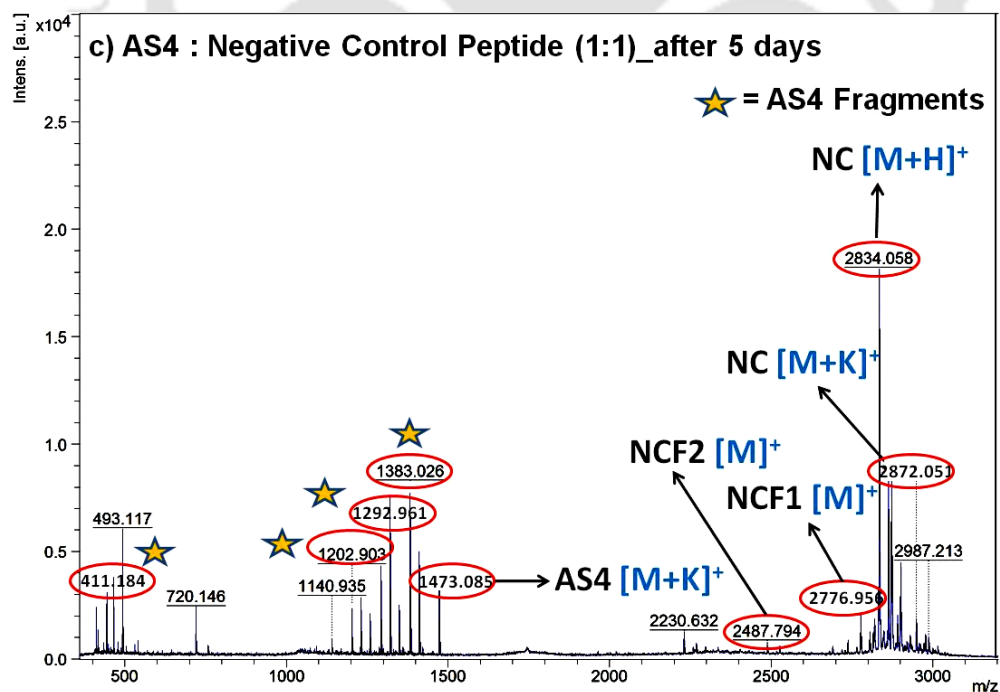


Figure 2.85. MALDI-TOF mass spectrum of negative control peptide in the presence of AS4 (1:1) after five days of incubation in PBS pH 7.4 at 37 °C.

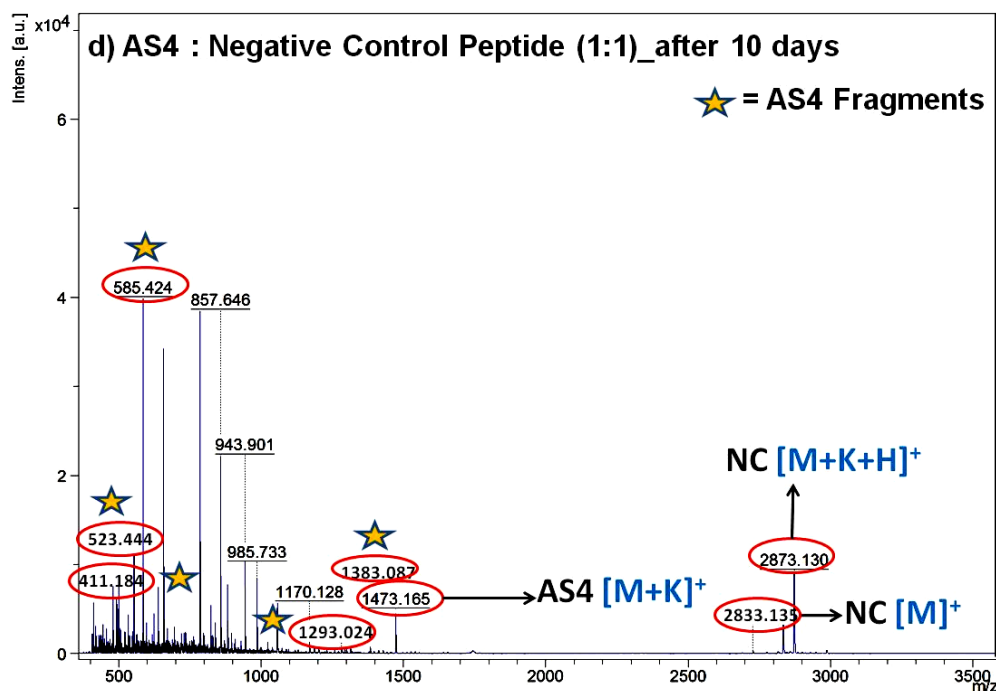


Figure 2.86. MALDI-TOF mass spectrum of NC peptide in the presence of AS4 (1:1) after ten days.

2.9.1. Self-degradation of mutant DTP 28 (NC):

After three days, NC underwent self-degradation at its Ser2 and Ser5 positions (Figure 2.87-90) subsequently, which was confirmed by a parallel experiment with NC in the absence of AS4. Such cleavage may proceed via route 11^{NC}, which is unrelated to AS4. This result not only indicates the excellent selectivity of such designed peptides but also points towards the need for self-association for the initiation of the desired proteolysis.

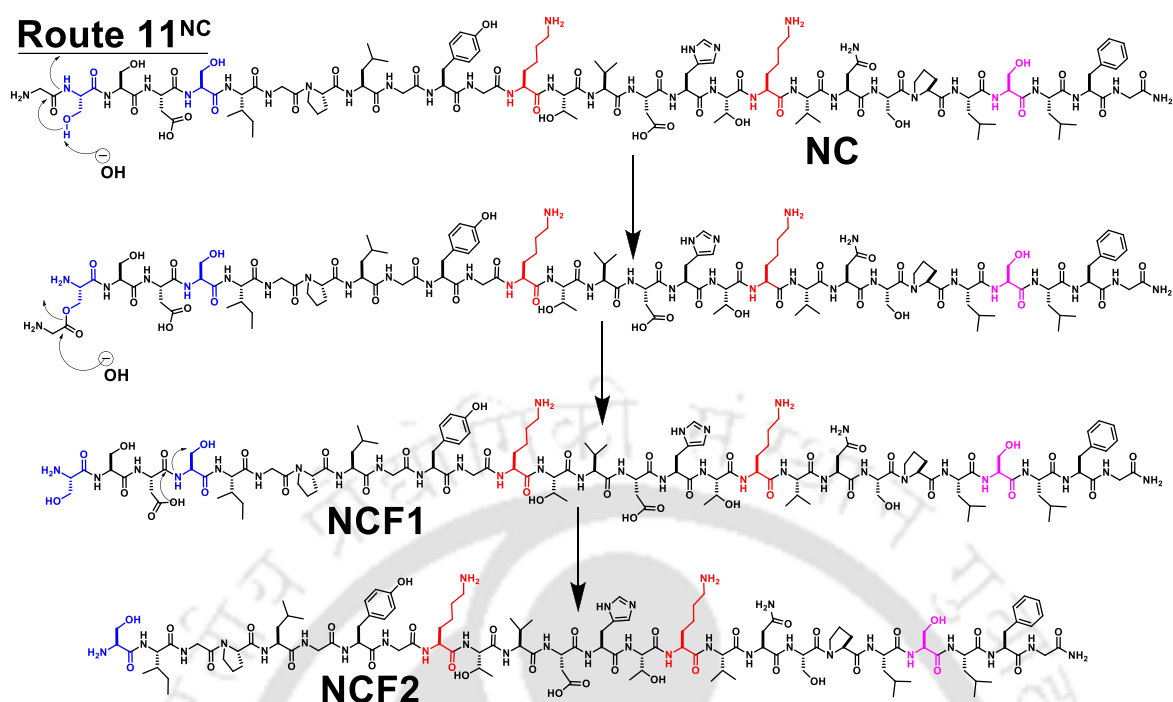


Figure 2.87. Plausible routes of cleavage of NC peptide into various fragments in the absence of AS4 (Route 11^{NC}). NCF stands for the negative control peptide fragments.

Most of the logically drawn intermediates and final fragments could be identified by time-dependent MALDI-TOF mass spectrometric analyses of the NC incubated alone (Figure 2.88-90).

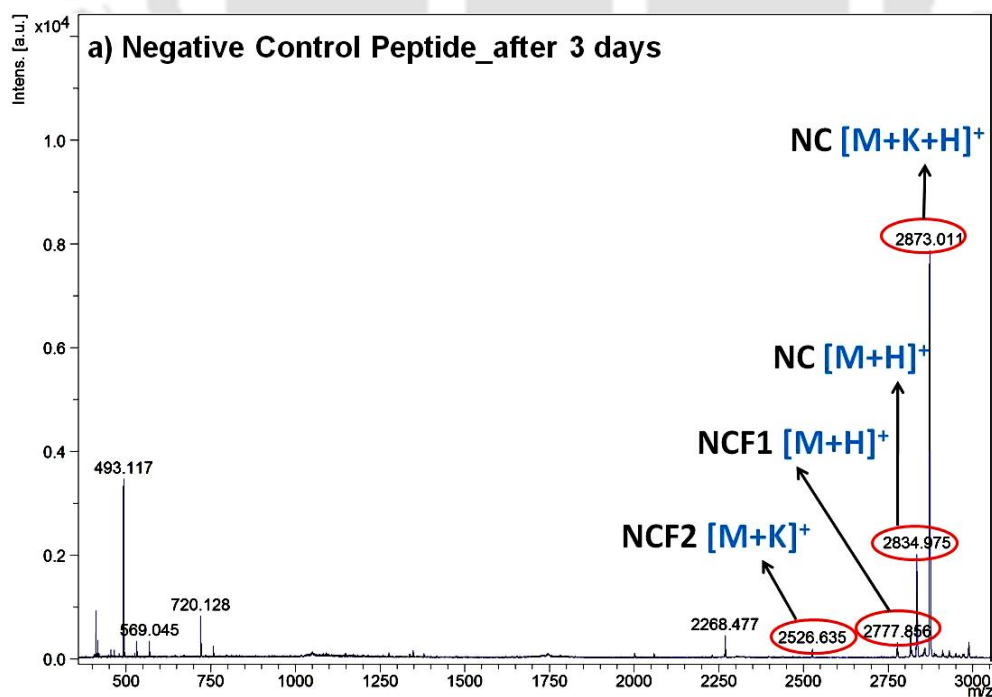


Figure 2.88. MALDI-TOF mass spectrum of NC peptide in the absence of AS4 after three days.

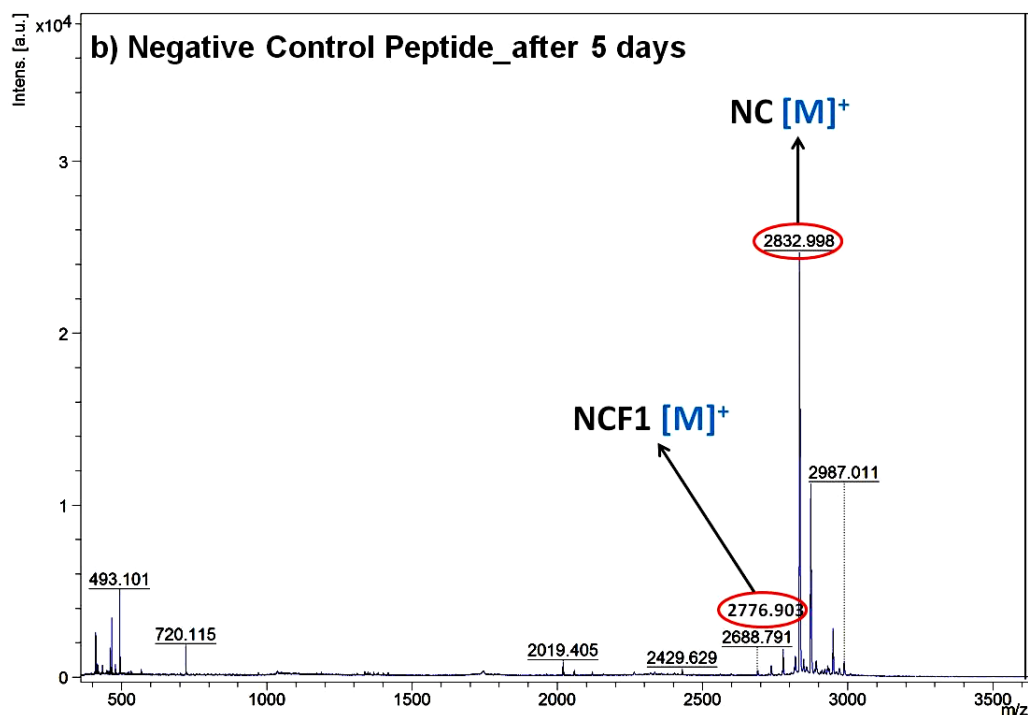


Figure 2.89. MALDI-TOF mass spectrum of NC peptide in the absence of AS4 after five days of incubation in PBS pH 7.4 at 37 °C.

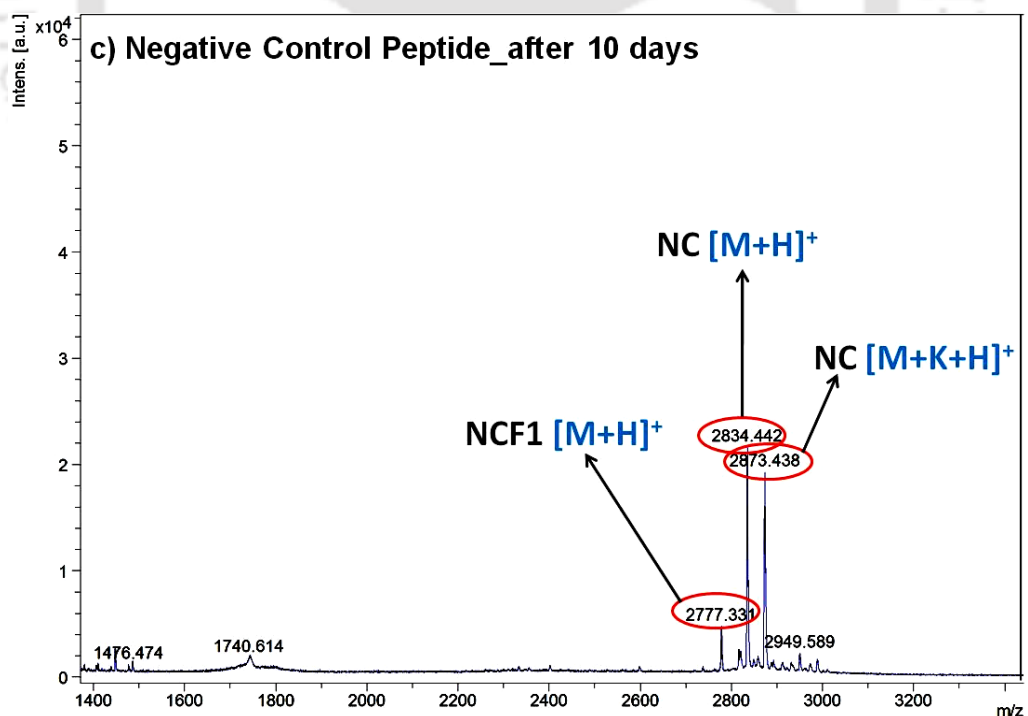


Figure 2.90. MALDI-TOF mass spectrum of NC peptide in the absence of AS4 after ten days of incubation in PBS pH 7.4 at 37 °C.

It is worth mentioning that all the routes of cascades of chemical reactions mentioned above are logically drawn probable routes, many other modes of bond formation and bond cleavage may be operating in the sample. It is also possible that we could not identify all the intermediates and fragments, present in the mixture.

2.10. Kinetics of amyloid accumulation of $A\beta_{1-40}$ in the presence and absence of AS4:

Further, to examine the efficacy of inhibition of amyloid formation of $A\beta_{1-40}$, AS4 was co-incubated in two-fold, and five-fold molar excess with 50 μ M of $A\beta_{1-40}$ in PBS of pH 7.4 at 37 °C on a water bath and the kinetics of amyloid accumulation was monitored using various biophysical tools in parallel up to 45 days.

2.10.1. ThT fluorescence assay and CD experiments of $A\beta_{1-40}$:

It was found that the fluorescence intensity of $A\beta_{1-40}$ (black, in Figure 2.91-a) increased with time in the absence of AS4 and became static after four days after slight lowering in intensity. But in the presence of two-fold (red) and five-fold (blue) molar excess of AS4; a significant decrease in fluorescence signal of $A\beta_{1-40}$ was observed since the 2nd day and were slowly suppressed thereafter probably due to the generation of various peptide fragments resulting from several site-selective cleavages (Figure 2.68-71). Increasing the dose (to five-fold) of AS4 improved the inhibition. For comparison, the ThT assay profile of AS4 alone is depicted in dark cyan.

The β -sheet based structure of $A\beta_{1-40}$ (black, Figure 2.91-b) was confirmed from the negative band at 215 nm and a positive one at 195 nm. A two-fold molar excess of AS4 altered it to a random coil structure (red). A five-fold molar excess of AS4 also converted the β -sheet structure of $A\beta_{1-40}$ into a random coil (blue) which was concluded from the -ve Cotton effect around 195 nm and absence of the same around 214 nm. AS4, when incubated alone, exhibited a mixture of α -helix, random coil, and β -sheet structure.

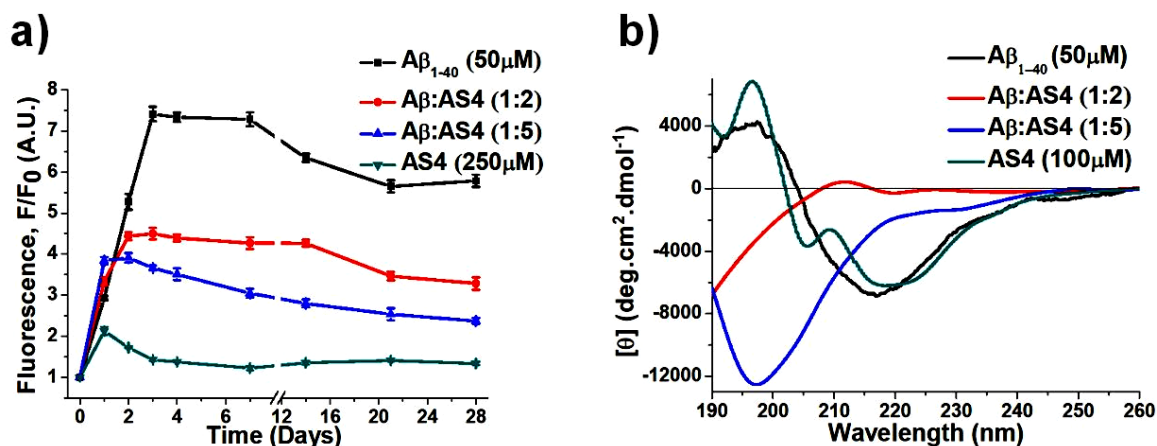


Figure 2.91. Normalized profiles of dose-dependent (a) ThT fluorescence assay, and (b) CD experiments. Spectra of A β_{1-40} in the absence (black), presence of two-fold AS4 (red), five-fold AS4 (blue), and AS4 alone (dark cyan). CD Spectra were recorded after 28 days of incubation. Error bars represent standard deviations of at least three independent measurements. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

2.10.2. FTIR spectra of A β_{1-40} :

The results of the FT-IR experiments (Figure 2.92) indicated that A β_{1-40} adopted a β -sheet conformation (1632 cm⁻¹, black) in the absence of AS4 but adopted either random coil or helical structure (1650 cm⁻¹, blue for five-fold) in the presence of AS4.

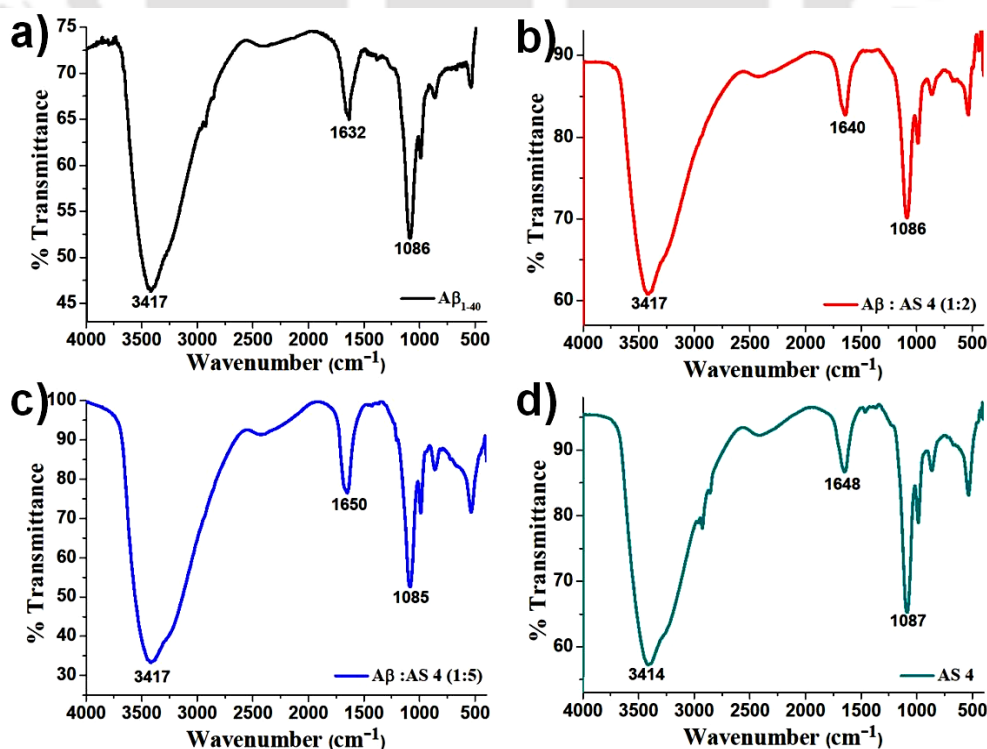


Figure 2.92. FTIR spectra of $A\beta_{1-40}$ (black, a) in absence, presence of (red, b) two-fold AS4, (blue, c) five-fold molar excess of AS4, and AS4 alone (dark cyan red, d). Spectra were recorded after 28 days of incubation. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C.

2.10.3. TEM images, Congo red-stained birefringence images, and AFM images of $A\beta_{1-40}$:

Clear fibrillar structure of $A\beta_{1-40}$ was viewed under TEM (Figure 2.93 a-i) in the absence of AS4. However, when $A\beta_{1-40}$ was co-incubated with AS4 (two-fold and five-fold molar excess), although tiny fibrils were noted for two-fold, no characteristic fibrillar structure was observed for five-fold after 45 days of co-incubation (Figure 2.93 a-ii for two-fold, and Figure 2.93 a-iii for five-fold). On the contrary, AS4 did not appear as fibrillar structure (rather off-pathway aggregates were noted, Figure 2.93 a-iv) in the absence of $A\beta_{1-40}$ (observed on the 45th day).

Characteristic green-gold birefringence of $A\beta_{1-40}$ was observed under cross-polarized light after staining with Congo Red (Figure 2.93 b-i). However, no such green-gold birefringence was observed either for the mixture of m $A\beta$ and AS4 (Figure 2.93 b-ii for two-fold, and Figure 2.93 b-iii for five-fold) or AS4 alone (Figure 2.93 b-iv). From TEM and Cong Red birefringence studies, it was concluded that $A\beta_{1-40}$ neither formed fibril nor formed amyloid, respectively, in the presence of AS4. These results are in corroboration with the same of the ThT experiments.

The presence of fibrillar structure under Atomic Force Microscopy (AFM) is another property of amyloid formed by a peptide. It was clear that the $A\beta_{1-40}$ peptide alone exhibited clear fibrillar structure (Figure 2.93 c-i) when viewed after 45 days of incubation. However, neither $A\beta_{1-40}$ along with twofold (Figure 2.93 c-ii), and five-fold (Figure 2.93 c-iii) of AS4, nor AS4 alone (Figure 2.93 c-iv) exhibited such characteristic fibrillar assembly when viewed under AFM after 45 days of incubation, respectively.

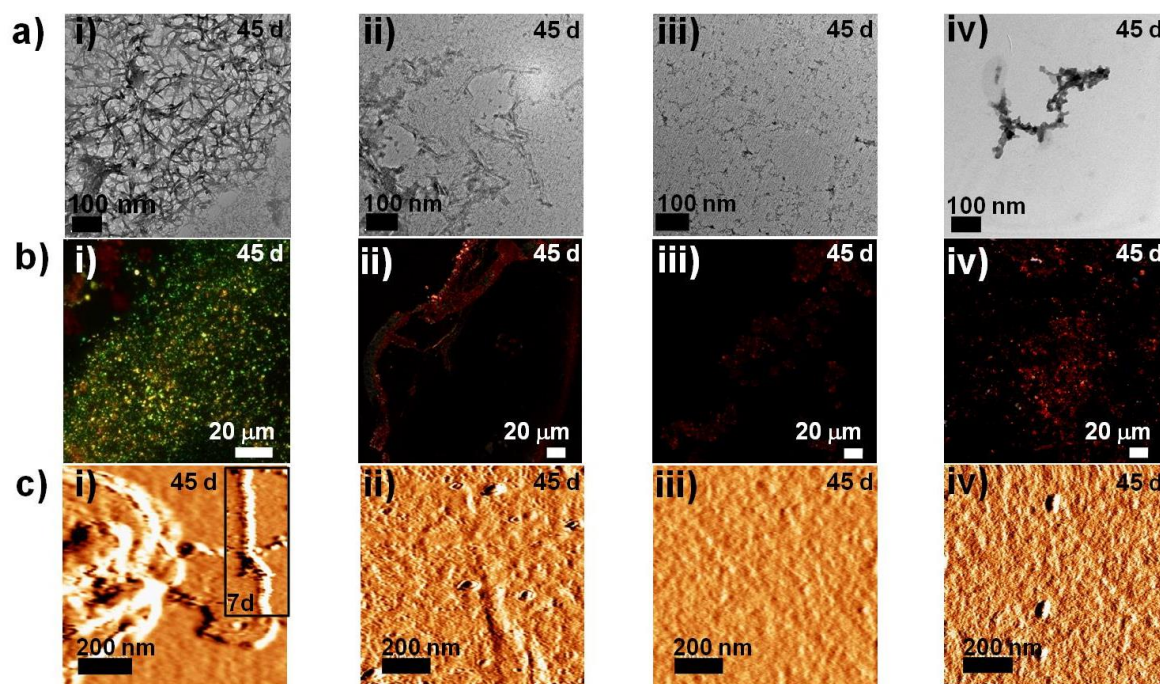


Figure 2.93. (a) TEM images, (b) Congo red-stained birefringence images, and (c) AFM images of $A\beta_{1-40}$ (i) in absence, and presence of (ii) two-fold AS4, (iii) five-fold AS4, and (iv) AS4 alone. Images that were captured after 45 days are displayed. Scale bars are indicated as 200 nm for TEM and AFM images and 20 μm for birefringence images. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 $^{\circ}\text{C}$ in parallel.

2.11. Disruption of preformed $A\beta_{1-40}$ fibril by AS4:

Further to check whether AS4 can disrupt existing fibril of $A\beta_{1-40}$, AS4 was added in the same molar ratio into the preformed fibrillar assembly of $A\beta_{1-40}$ peptide at 72 h (PBS pH 7.4 at 37 $^{\circ}\text{C}$) in parallel experiments.

2.11.1. ThT fluorescence assay and CD experiments of $A\beta_{1-40}$:

After addition of AS4, the fluorescence intensity of $A\beta_{1-40}$ increased further, which was slowly suppressed after that, probably due to the generation of different peptide fragments from site-selective cleavage by AS4 (Figure 2.94-a). The results obtained from CD spectra (Figure 2.94-b) also support the fluorescence results. A different molar excess of AS4 altered the β -sheet structure of $A\beta_{1-40}$ (black) to a random coil structure (red in the presence of two-fold, and blue for five-fold AS4; -ve Cotton effect around 190 nm).

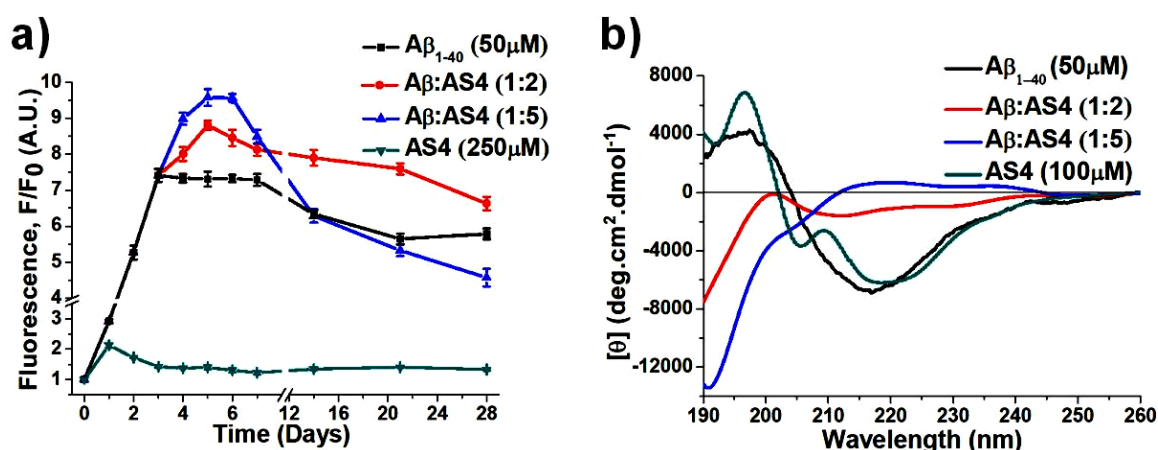


Figure 2.94. Normalized profiles of dose-dependent (a) ThT fluorescence assay, and (b) CD experiments. Spectra of A β ₁₋₄₀ in the absence (black), presence of two-fold AS4 (red), five-fold AS4 (blue), and AS4 alone (dark cyan). CD Spectra were recorded after 28 (3+25) days of incubation. Error bars represent standard deviations of at least three independent measurements. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

2.11.2. FTIR spectra of A β ₁₋₄₀:

The results of the FTIR spectra (Figure 2.95) indicated that A β ₁₋₄₀ adopted either a random coil or helical structure (1643 cm⁻¹, red for two-fold AS4; and 1654 cm⁻¹, blue for five-fold AS4), in the presence of AS4.

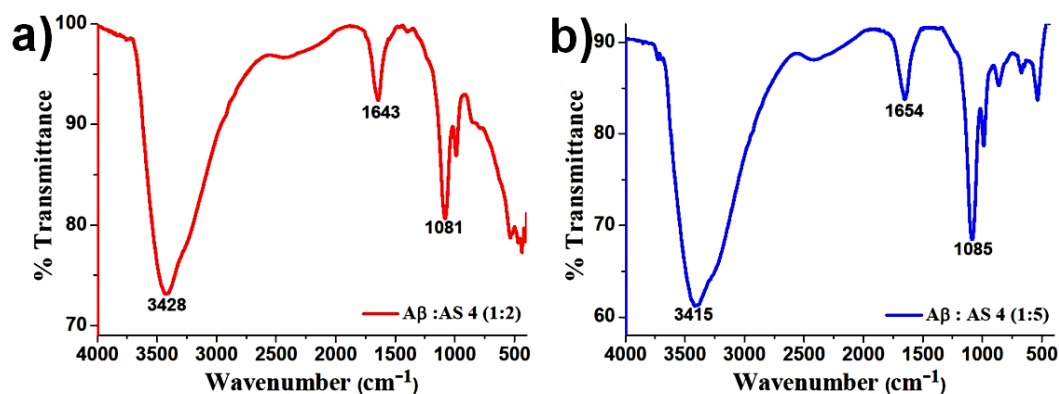


Figure 2.95. FTIR spectra of preformed A β ₁₋₄₀ fibril disruption in the presence of (red, a) two-fold AS4 and (blue, b) five-fold molar excess of AS4. AS4 added to A β ₁₋₄₀ after three days of its incubation. Spectra were recorded after 28 (3+25) days of incubation. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C.

2.11.3. TEM images & Congo red-stained birefringence images of $A\beta_{1-40}$:

The results obtained from the TEM study (Figure 2.96-a), and Congo Red birefringence study (Figure 2.96-b) were in accordance with those of the fluorescence experiments. TEM and birefringence studies suggested that $A\beta_{1-40}$ neither formed fibril nor formed amyloid, respectively, in the presence of AS4.

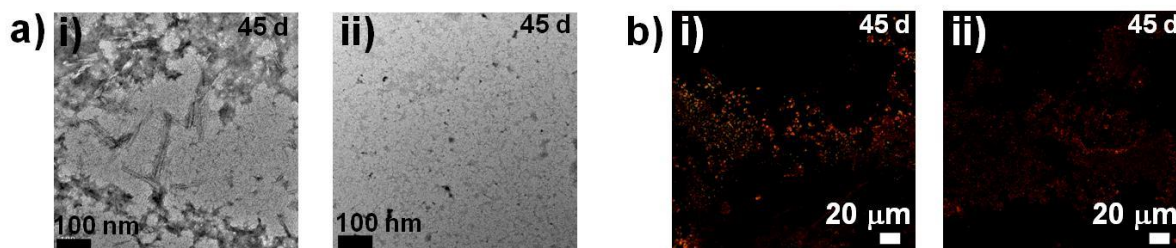


Figure 2.96. (a) TEM images, and (b) Congo red-stained birefringence images of $A\beta_{1-40}$ (i) in absence, and presence of (i) two-fold AS4, (ii) five-fold AS4. Images that were captured after 45 (3+42) days are displayed. Scale bars are indicated as 200 nm for TEM images and 20 μ m for birefringence images. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

Disruption of $A\beta_{1-40}$ aggregation was monitored by the time-dependent TEM and Congo red birefringence (Figure 2.97) studies. A five-fold molar excess of AS4 was added to the preformed fibril $A\beta_{1-40}$ after three days. As a result, amyloid aggregates of $A\beta_{1-40}$ were decreased by 14 days which were slowly disappeared later. Thus, it can be concluded AS4 could disrupt existing $A\beta_{1-40}$ amyloid as well.

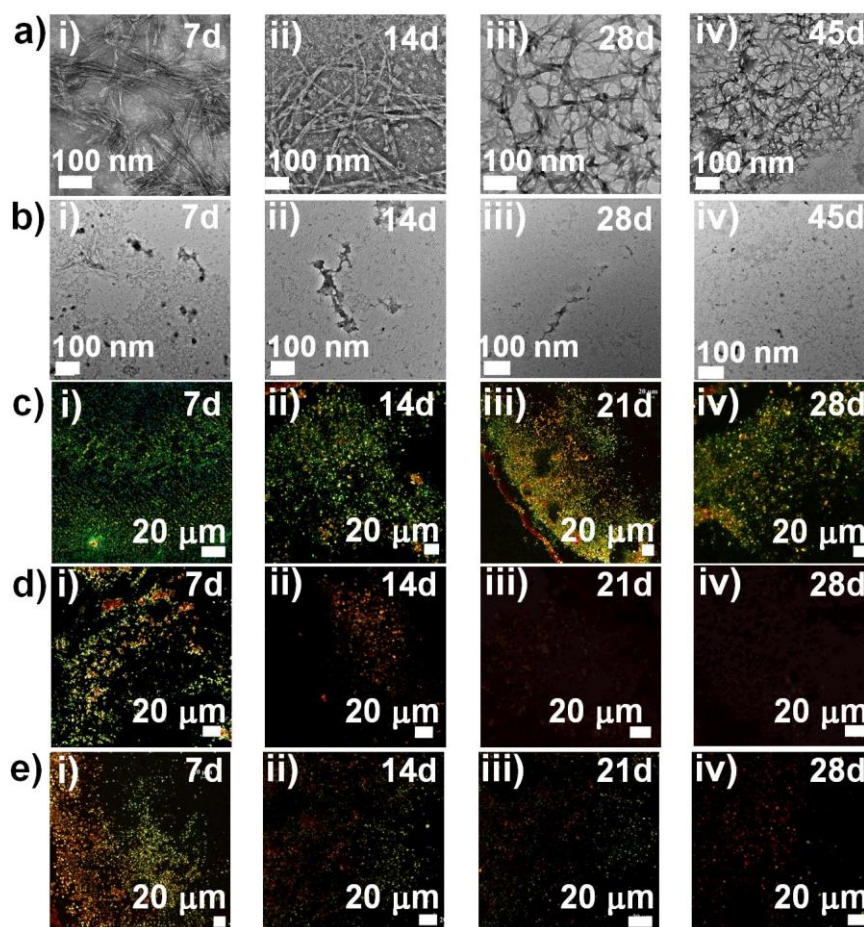


Figure 2.97. Time-dependent (a-b) TEM images, and (c-e) Congo red stained birefringence images of $A\beta_{1-40}$ (a, c) in absence, (b, d-e) in the presence of five-fold AS4. While (b, d) shows inhibition of amyloidosis, (e) disruption of pre-existing amyloid (PBS pH 7.4 at 37 °C).

2.12. DLS analysis of inhibiting $A\beta_{1-40}$ fibril formation by AS4:

From the Dynamic Light Scattering (DLS) study,²³ it was observed that the size of $A\beta_{1-40}$ aggregates increased gradually with time (Figure 2.98-a). The diameter of the dissolved particles appeared around 150 nm after 12 h of incubation. That was shifted to around 1000 nm and 5500 nm after 36 h and 72 h, respectively. However, in the presence of AS4, smaller molecular-weight aggregates with the diameter of 164 and 955 nm for two-fold (red), and 342 nm for five-fold (blue) were noticed after 72 h (Figure 2.98-b). However, there existed an extra peak around 5500 nm, but area of that peak was smaller than that of $A\beta_{1-40}$ in the absence of AS4, which indicates that the fibril network was present in all the cases after three days of incubation, but AS4 could diminish the size of $A\beta$ aggregates to

different extents in a dose-dependent manner. Fivefold molar excess AS4 showed more prominent effect than others. For a comparison, AS4 in the absence of $A\beta_{1-40}$ exhibited a peak around 72 nm (dark cyan) after 72 h. Furthermore, an intense peak around 220 nm was observed in the presence of 5 fold AS4 after seven days of incubation (Figure 2.98-c), which indicated that aggregates of $A\beta$ existed, but the size of that decreased significantly with time by AS4.

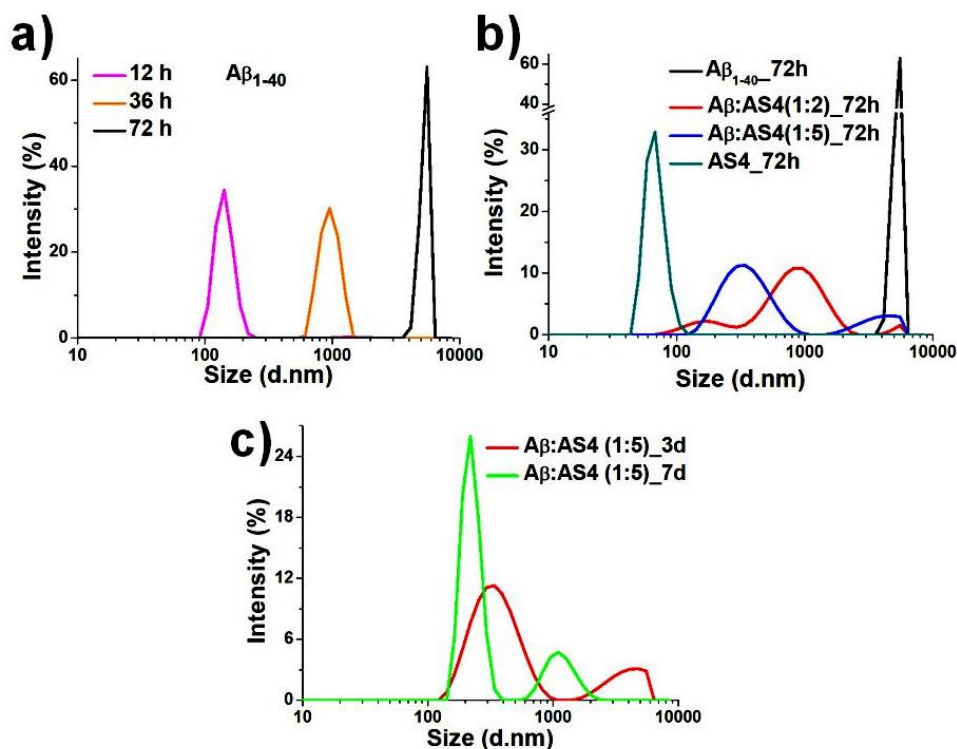


Figure 2.98. Overlay of DLS profiles of (a) $A\beta_{1-40}$ at various time points, (b) $A\beta_{1-40}$ (black), in presence of two-fold (red), five-fold (blue) molar excess of AS4, and AS4 (dark cyan) at 72h, and (c) $A\beta_{1-40}$ (black) in presence of 5 fold molar excess of AS4 after three days (red) and five days (green).

2.13. Vesicle leakage study:

It is well known that smaller soluble oligomers of $A\beta$ are more toxic than insoluble fibril of $A\beta$, mainly because soluble oligomers can form pores in the cell membrane resulting in membrane disruption.²⁴ Dye-entrapped vesicle leakage study indicates cell membrane disruption capability by additives as laboratory-made large unilamellar vesicles (LUVs) are considered as a cell membrane mimic. Therefore, we performed vesicle leakage

studies (Figure 2.100) using carboxyfluorescein dye entrapped large unilamellar vesicles to check whether AS4 disrupted mature fibrils of A β into toxic metabolites.²⁵

The formation of LUVs was characterized by transmission electron microscopy (TEM, Figure 2.99) from the negatively stained sample over carbon-coated copper grid using 2% uranyl acetate solution.

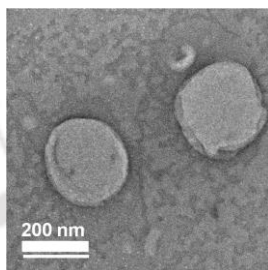


Figure 2.99. The TEM image of negatively stained LUVs. The concentration of the sample was 100 μ M. Scale bar is indicated as 200 nm.

Three sets of peptide solutions were used for the vesicle leakage studies along with untreated LUVs: (A) A β_{1-40} (incubated for 24 h), (B) A β_{1-40} (incubated for 10 days), (C) A β_{1-40} and AS 4 (1:5, AS 4 was added to the preformed fibrillar aggregates after 72 h and incubated for 10 days). Peptides and lipid were taken in 1:20 molar ratios, and the entrapped dye (carboxyfluorescein) release was monitored by time-dependent fluorescence assay (Figure 2.100). Emission was recorded for each 20 min interval up to 120 min, and then 12 h interval up to 72 h. Emission and excitation were measured at 516 nm and 485 nm respectively, using 5 nm of bandwidth on a Fluoromax-4, Horiba instrument. Finally, 10 μ L of Triton X-100 was added to obtain complete dye release from the vesicle, and the final fluorescence was measured. Untreated LUVs (natural dye leakage) were studied and used as control. The % dye release was calculated as,

$$\% \text{ Leakage} = \{(\text{Observed fluorescence} - \text{initial fluorescence}) / (\text{Total fluorescence} - \text{initial fluorescence})\} \times 100\%$$

The graph was plotted using Origin Pro 8 software from text file, and the average was taken with the observed standard deviation of three independent measurements for each data point.

cleave Amyloid β at the α -secretase cleavage site by aggregation driven cascades of chemical reactions

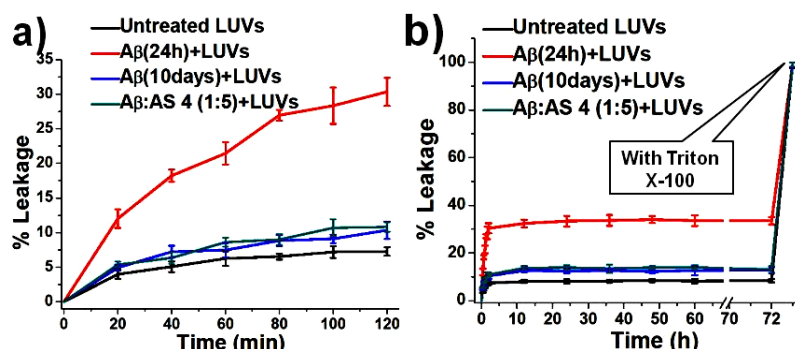


Figure 2.100. % dye (carboxyfluorescein) leakage from LUVs up to (a) 120 min and (b) up to 72 h; by untreated LUVs (black), LUVs treated with 24 h old A β protofibril (red), LUVs treated with 10 days old mature A β fibril (blue), and LUVs treated with 10 days old A β : AS4 sample (green).

It was observed that set B and C caused the lesser extent of LUV leakage in comparison to set A, which indicated that immature fibrils (protofibrils) caused more pore formation on LUVs and hence they were more toxic than the mature fibrils (B) and fibrils disrupted by AS4 (C). The results confirmed that AS4 disrupted the preformed amyloid of A β into non-toxic metabolites.

2.14. Conclusions:

While nature's mechanism of prevention of AD is based on the α -secretases mediated APP processing, it rescues us from interneuronal plaque deposition by facilitating their excretion by proteolytic degradation of A β by various enzymes. However, often solvent inaccessibility of large aggregates prevents proteolysis allowing for the accumulation of plaques. Moreover, often, these enzymes are related to many other essential physiological processes. Thus overexpression of one or more of such enzymes may help in the intervention of AD but also may lead to other severe problems. Therefore, it is necessary to design a molecular machine that can perform the proteolytic degradation of A β selectively without affecting other proteins in the system.

We noted that supramolecular interactions govern all such peptide or protein aggregation. Therefore we hypothesized that a novel way of intervention might be outlined by combining the supramolecular interactions and chemical reactions. In this work, we have

reported such an engineered peptide-based construct that selectively recognizes A β peptide and cleave it around the α -secretase cleavage site by about 4-5 days in physiological pH and temperature. Therefore we termed them as artificial α -secretases (ASs). Interestingly, results indicated that they also cleave A β on the cleavage sites of other A β degrading enzymes by prolonged incubation. Such mimicking of the proteolytic action of enzymes is governed by the self-association driven cascades of pre-programmed chemical reactions. Therefore, ASs do not work catalytically. However, most probably the hydrophobic patch on ASs recognizes the same hydrophobic patch on the APP or A β ⁸ that facilitates the nucleophilic attack of the side chain amine group of Lys of A β on the ester carbonyl of the benzyl adipate of ASs by a self-association driven interaction leading to the formation of a new amide bond by expulsion of the benzyl group. This results in a covalent attachment between A β and AS by O to N acyl migration^{26,27} generating the intermediate C. Further nucleophilic attacks of the nearest backbone nitrogens to the newly formed amide carbonyl by proximity driven interaction, spontaneous backbone amide hydrolysis²⁶ and subsequent release of the amino group of the Lys side chain result in site-selective A β cleavage and formation of other intermediates. Repetition of such imidation-hydrolysis-transamidation cycle generates various intermediates and cleavage products as evident from the systematic time-resolved MALDI-mass analyses on a smaller A β fragment (mA β) and A β ₁₋₄₀.

We have also demonstrated that AS4 could chop off A β in various other sites similar to the cleavage sites of other A β processing enzymes thus it could mimic the proteolytic action of these enzymes as well. However, it does not react with unrelated peptides, such as DTP28 indicating its excellent selectivity and operation of the self-association initiated programmed proteolysis. The energy minimized structure of intermediate C also points to the possible role of self-association.

Presence of AS4, which was designed to be and was most active among the ASs, inhibited the amyloid formation and disrupted pre-formed A β ₁₋₄₀ amyloids *in vitro* probably due to mentioned site-selective programmed proteolysis and degradation. The kinetics of amyloid accumulation and destruction was monitored by the established biophysical tools; e.g., ThT-assay, TEM, Congo red birefringence, AFM, and DLS studies. The

conformational alterations associated with aggregation were examined by CD and FTIR. The results of the LUV leakage study indicated that AS4 disrupted the preformed amyloid of A β into metabolites that were unable to rupture the vesicle membrane, which is accepted as a cell membrane mimic. This result indicates that the generated metabolites may be unable to rupture the cell membrane as well, thus non-toxic. Therefore, design and development of such constructs can be a promising approach for drug design against not only the AD but also other Amyloidoses. However, cell-based toxicity assays are required to establish their physiological relevance. Further optimization of the structure of such constructs to improve their reactivity is planned before that.

2.15. References:

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2.16. Supporting data:

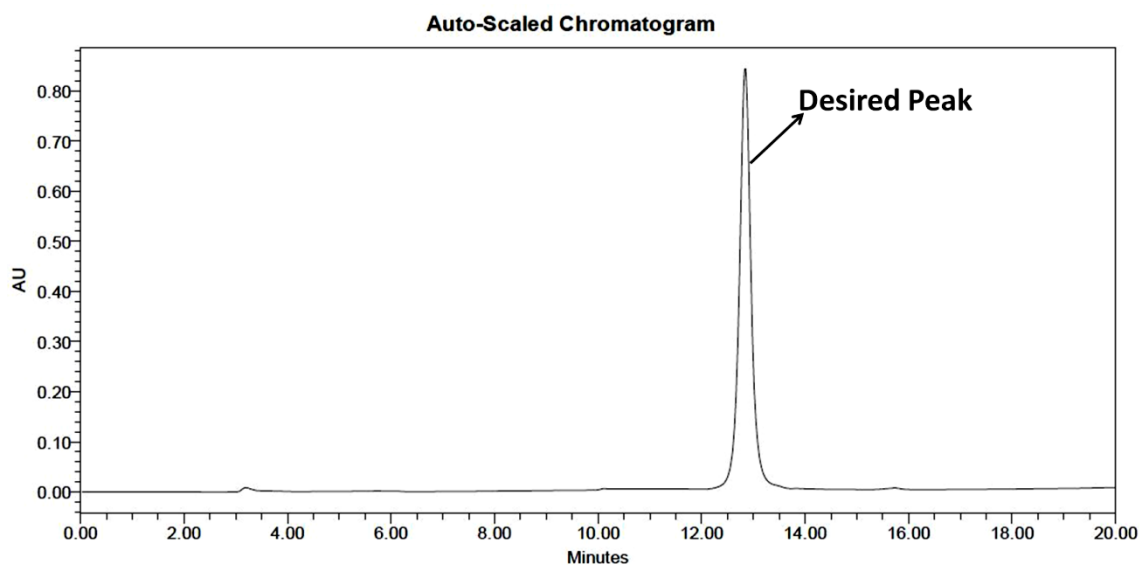


Figure 2.101. HPLC profile picture of the pure AS1.

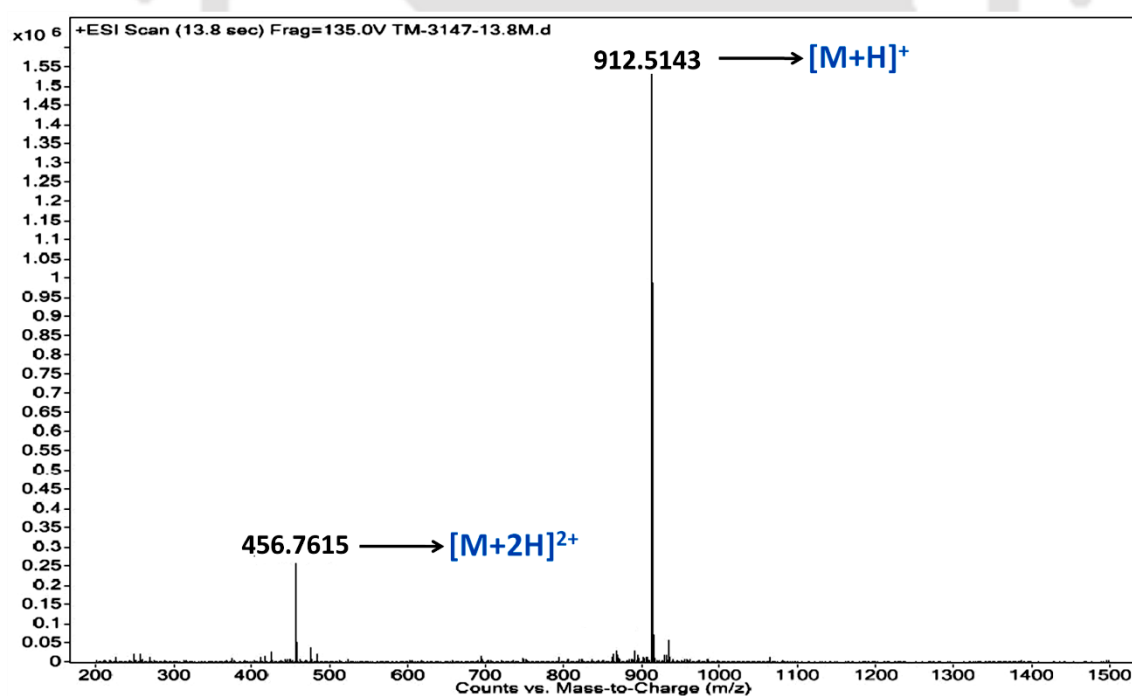


Figure 2.102. ESI-MS profile picture of the pure AS1. Calculated m/z for $C_{50}H_{69}N_7O_9$ $[M+H]^+$ is 912.5157, observed 912.5143, calculated m/z for $C_{50}H_{69}N_7O_9$ $[M+2H]^{2+}$ is 456.7578, observed 456.7615.

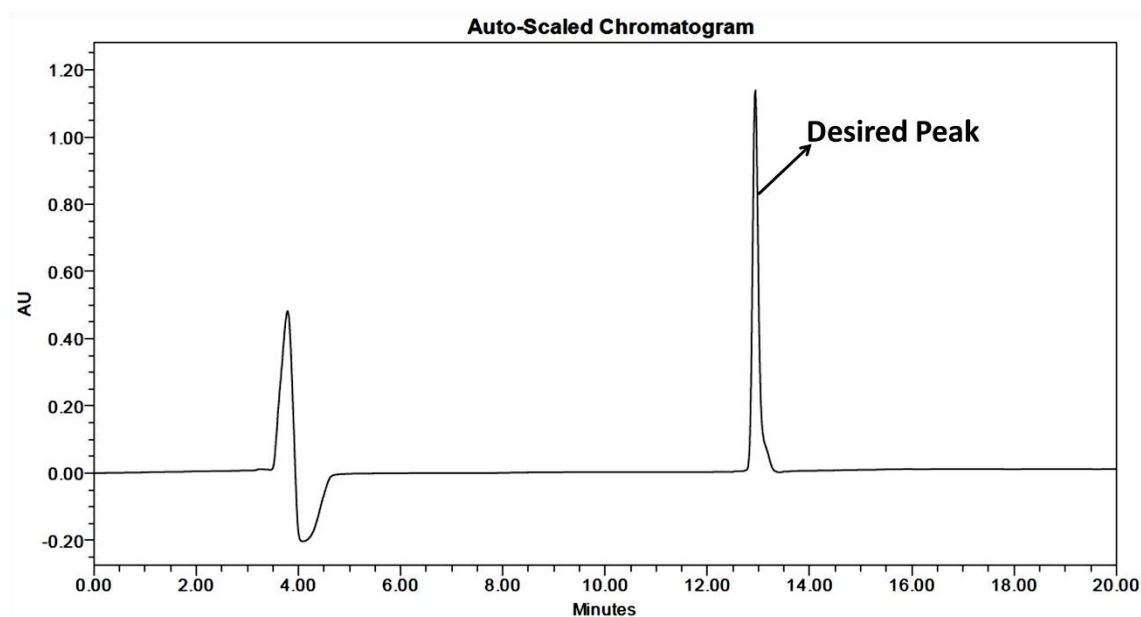


Figure 2.103. HPLC profile picture of the pure AS2.

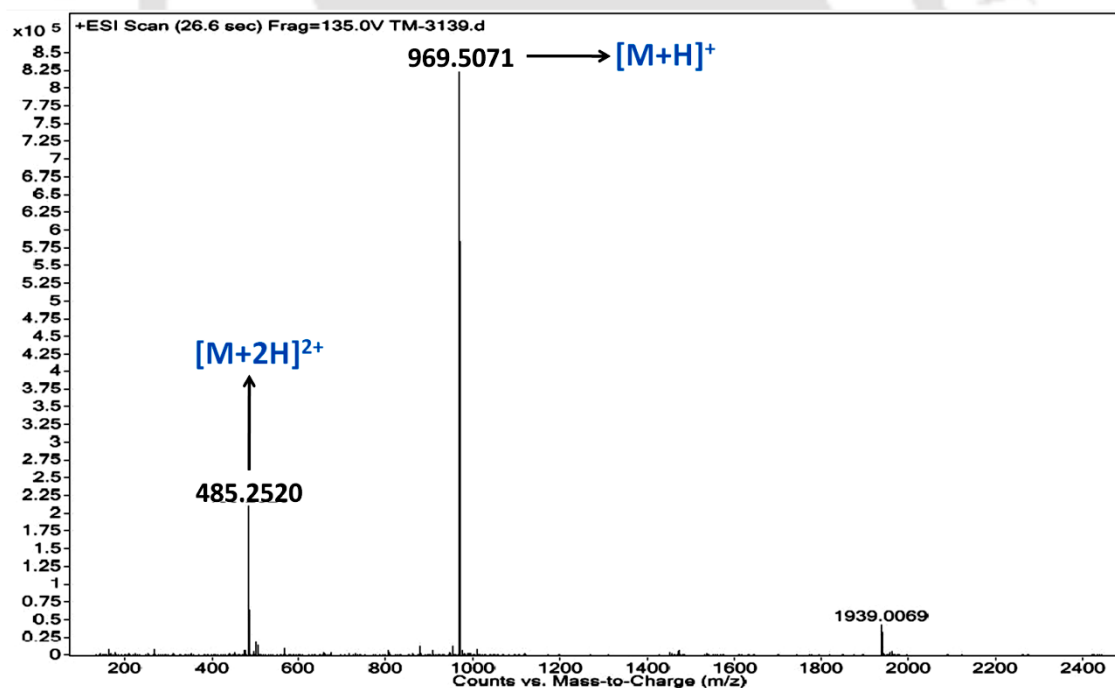


Figure 2.104. ESI-MS profile picture of the pure AS2. Calculated m/z for $C_{52}H_{72}N_8O_{10}$ $[M+H]^+$ is 969.5371, observed 969.5071, calculated m/z for $C_{52}H_{72}N_8O_{10}$ $[M+2H]^{2+}$ is 485.2685, observed 485.2520.

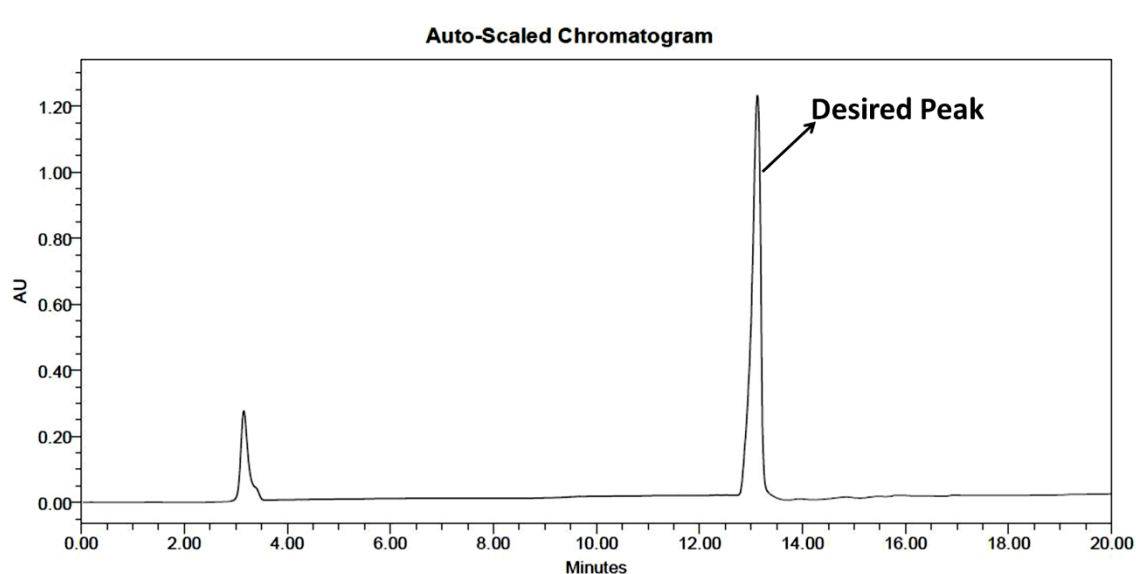


Figure 2.105. HPLC profile picture of the pure AS3.

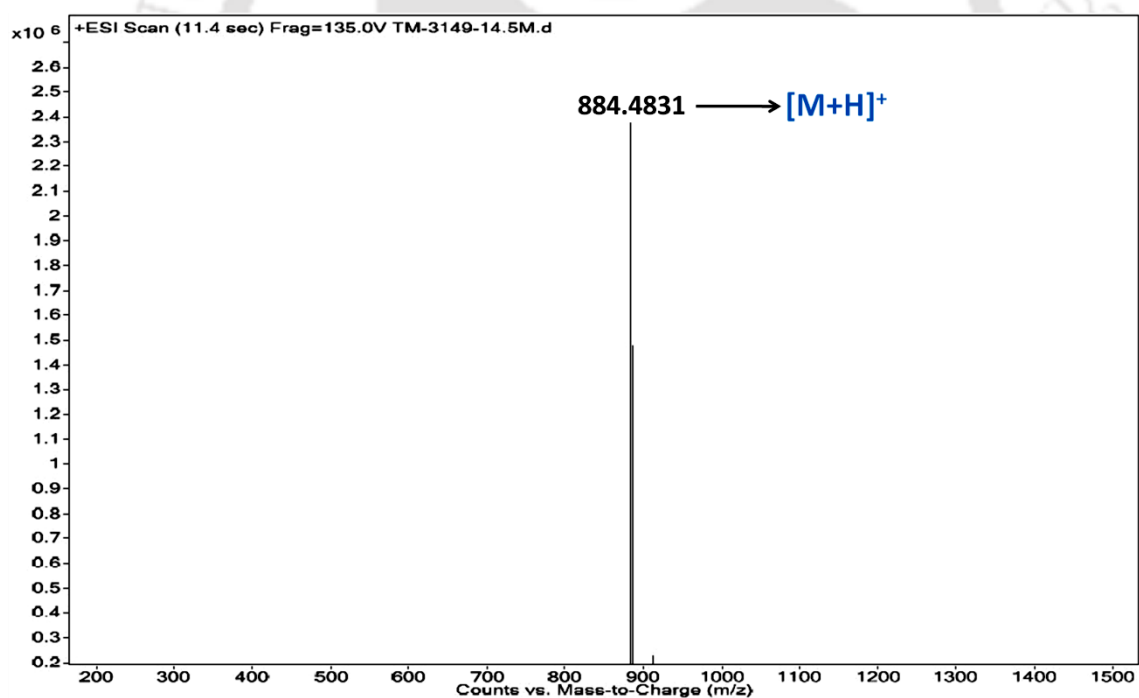


Figure 2.106. ESI-MS profile picture of the pure AS3. Calculated m/z for $C_{48}H_{65}N_7O_9$ $[M+H]^+$ is 884.4844, observed 884.4831.

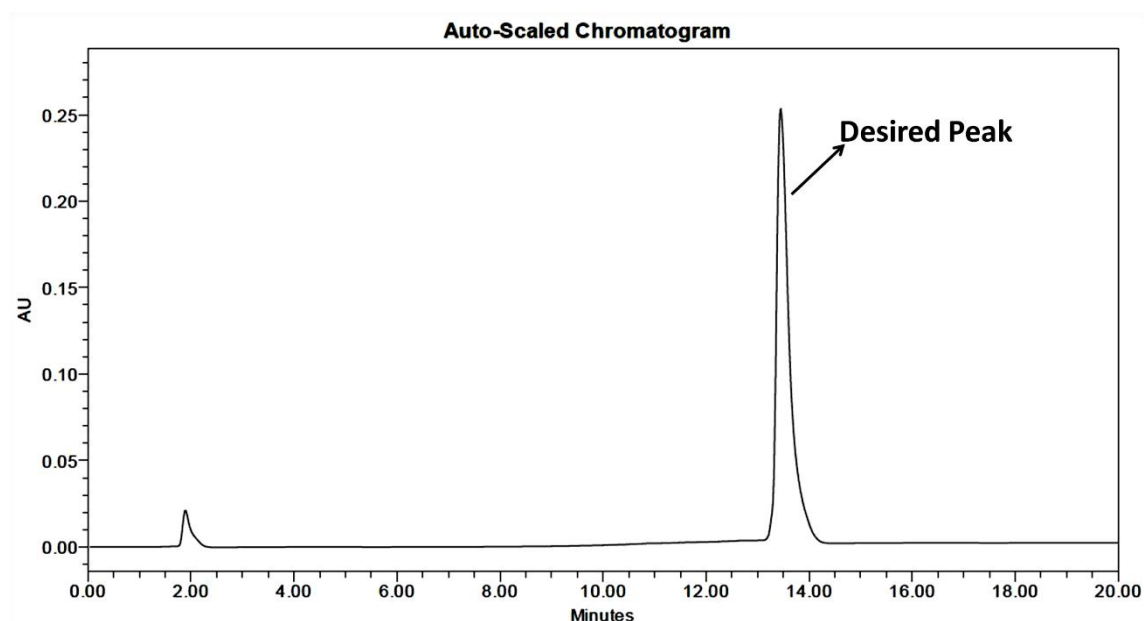


Figure 2.107. HPLC profile picture of the pure AS4.

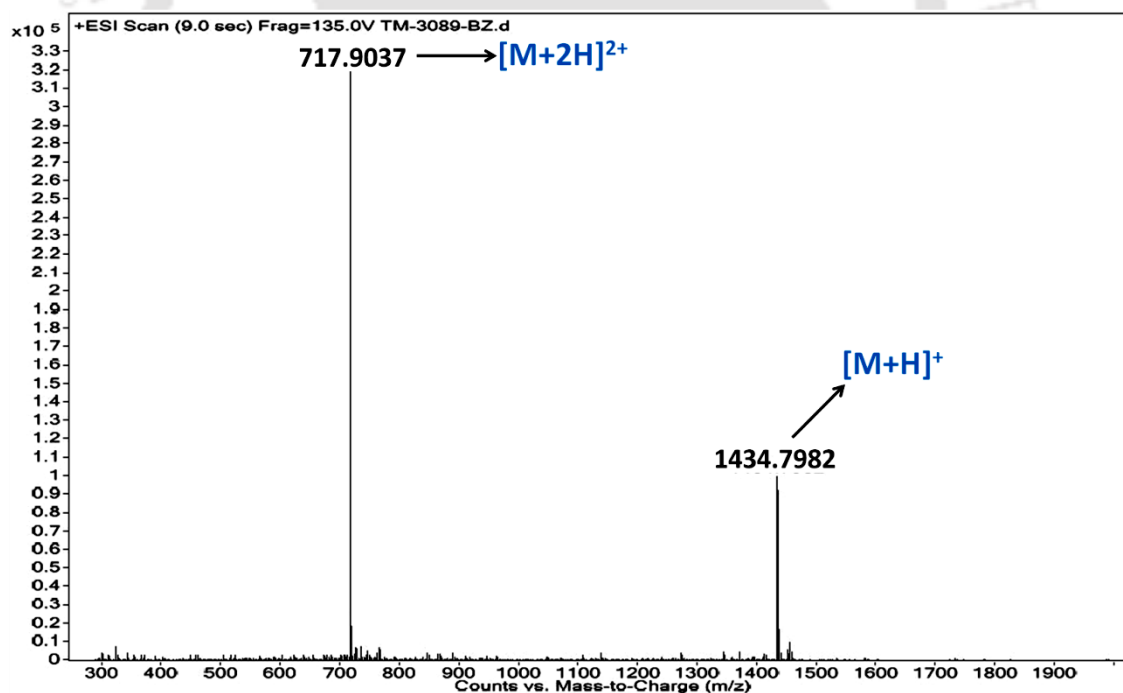


Figure 2.108. ESI-MS profile picture of the pure AS4. Calculated m/z for $C_{80}H_{107}N_9O_{15}$ $[M+H]^+$ is 1434.7900, observed 1434.7982, calculated m/z for $C_{80}H_{107}N_9O_{15}$ $[M+2H]^{2+}$ is 717.8950, observed 717.9037.

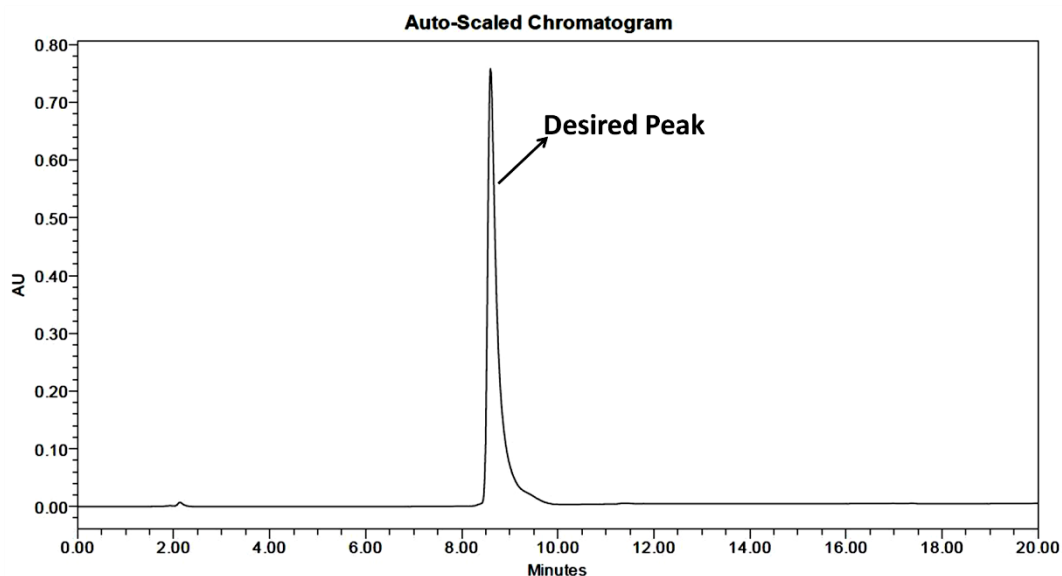


Figure 2.109. HPLC profile picture of the pure mA β peptide.

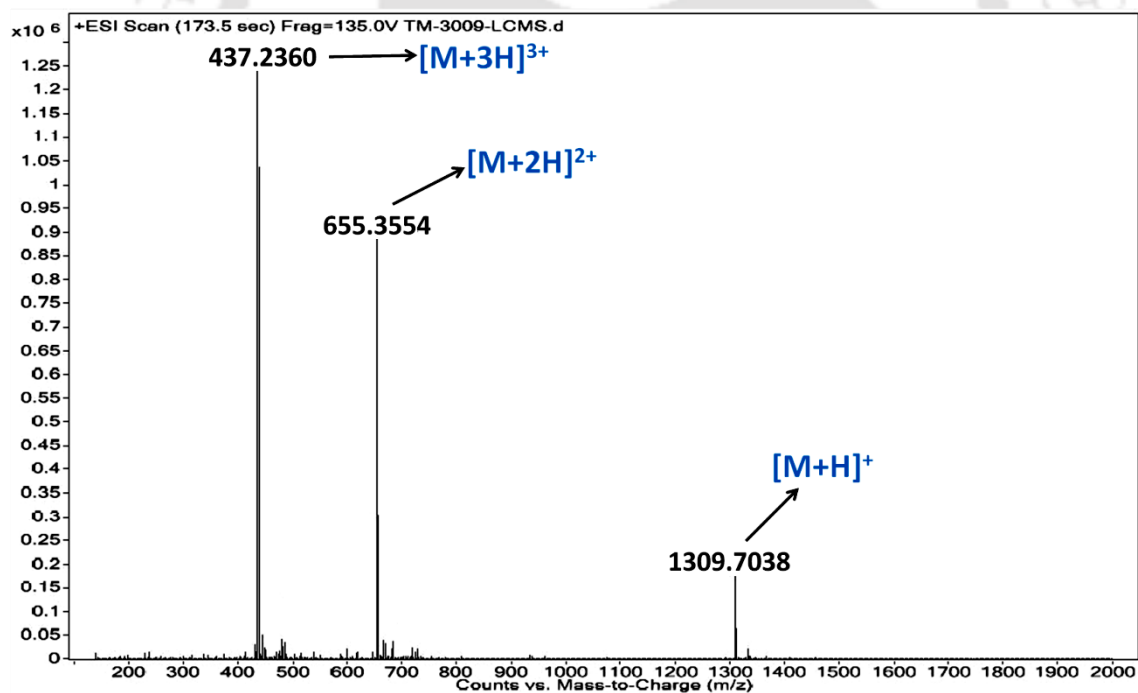


Figure 2.110. ESI-MS profile picture of the pure mA β peptide. Calculated m/z for $C_{63}H_{92}N_{18}O_{13}$ $[M+H]^+$ is 1309.7097, observed 1309.7038, calculated m/z for $C_{63}H_{92}N_{18}O_{13}$ $[M+2H]^{2+}$ is 655.3548, observed 655.3554, calculated m/z for $C_{63}H_{92}N_{18}O_{13}$ $[M+3H]^{3+}$ is 437.2365, observed 437.2360.

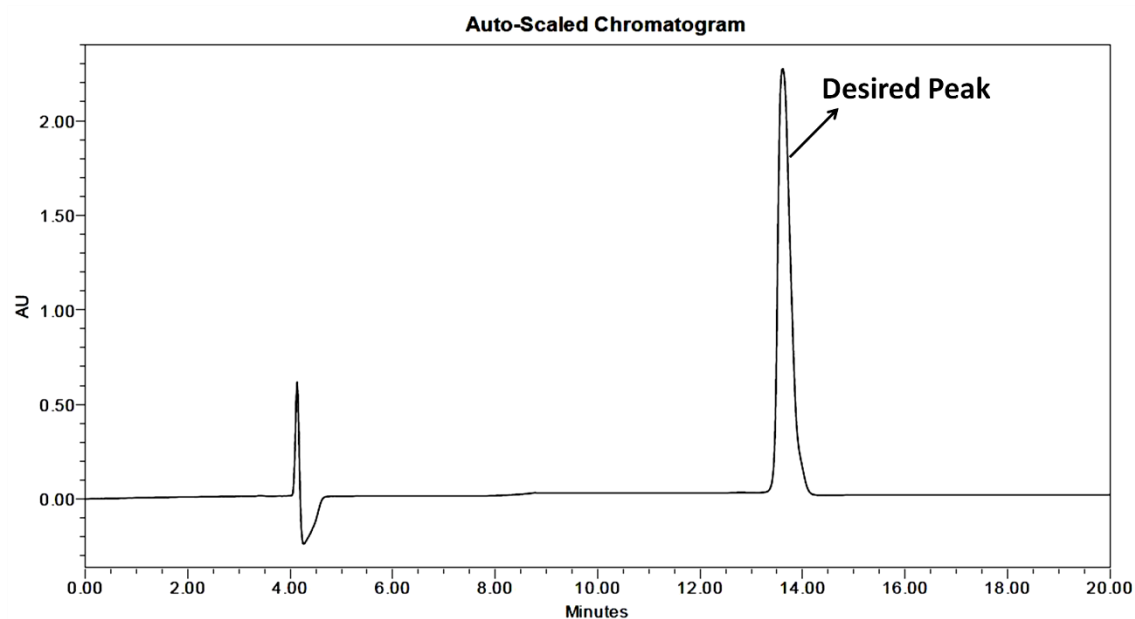


Figure 2.111. HPLC profile picture of the pure mutant DTP28.

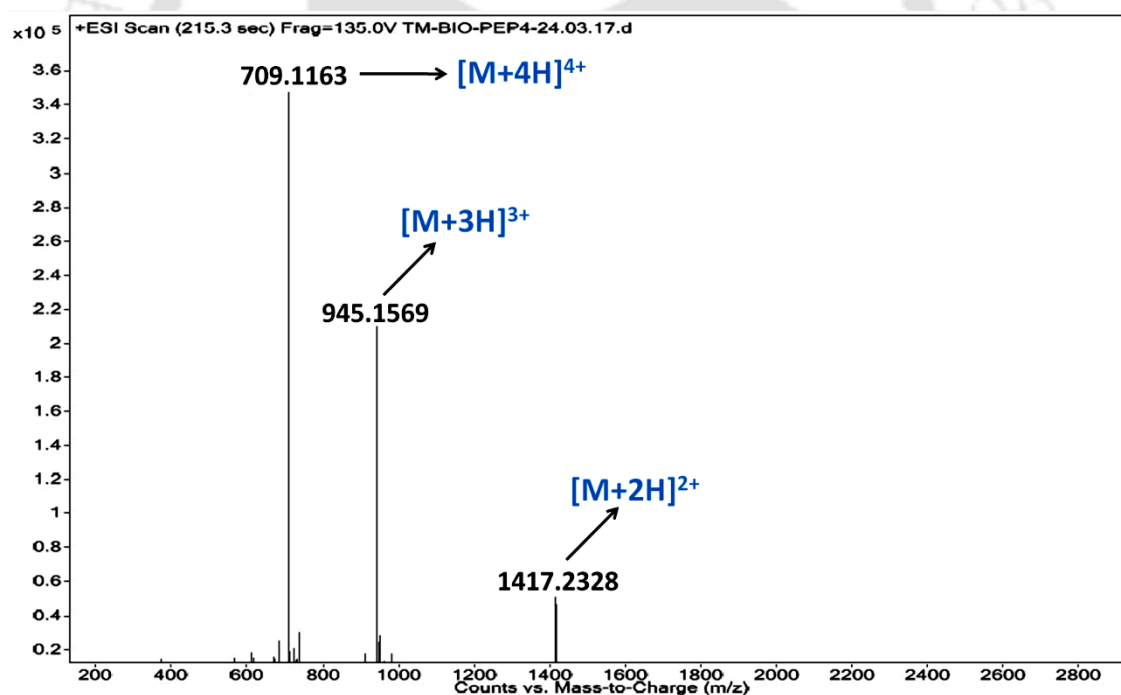


Figure 2.112. ESI-MS profile picture of the purified mutant DTP28. Calculated m/z for $C_{125}H_{198}N_{34}O_{41}$ $[M+2H]^{2+}$ is 1417.5564 and observed 1417.2328, calculated m/z for $C_{125}H_{198}N_{34}O_{41}$ $[M+3H]^{3+}$ is 945.3709 and observed 945.1569, calculated m/z for $C_{125}H_{198}N_{34}O_{41}$ $[M+4H]^{4+}$ is 709.2782 and observed 709.1163.

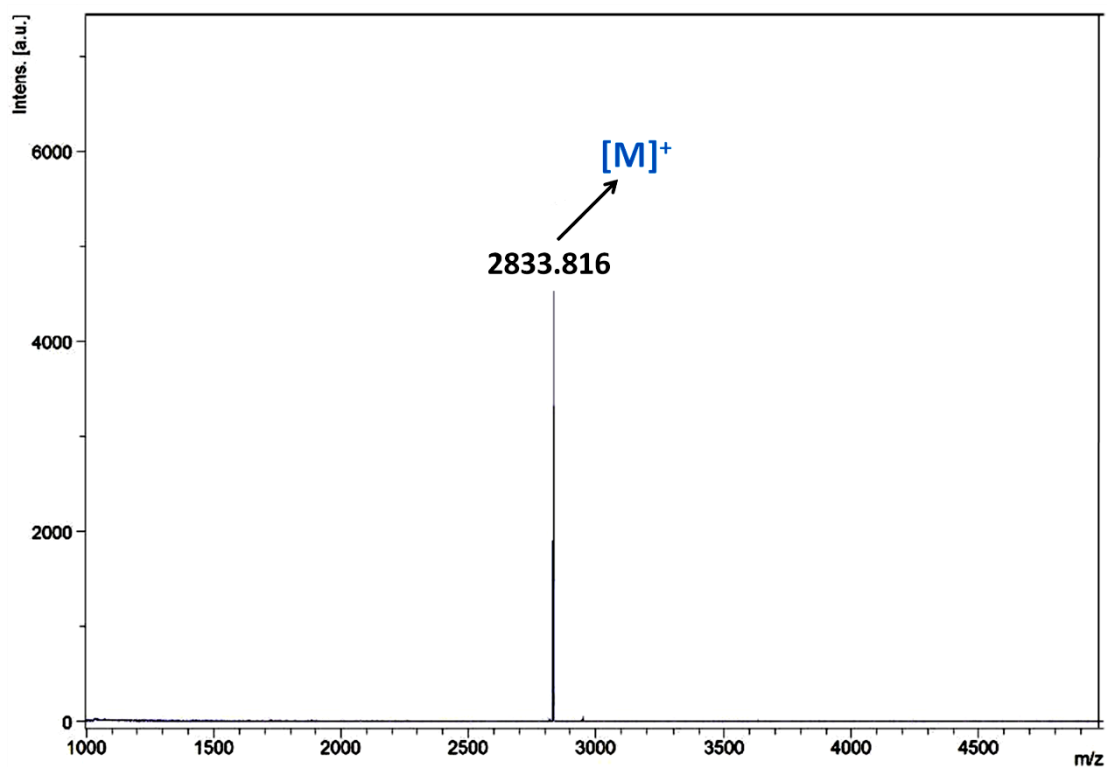


Figure 2.113. MALDI profile picture of the purified mutant DTP28. Calculated m/z for $C_{125}H_{198}N_{34}O_{41}$ $[M]^+$ is 2833.1128 and observed 2833.8160.

Chapter 3: Digestion of Amyloid β by Gly-integrated miniature artificial proteases

3.1. Introduction:

Y. Fukushima (1996) developed the alternating amphiphilic co-polypeptide poly(DLHLSL) to obtain a stable β -sheet structure having Serine protease-like hydrolytic catalytic activity¹ (due to increase of nucleophilicity by cooperative interactions) which was found to be reduced above 35 °C, because with temperature the nucleophilicity of the polypeptide and the concentration effect of the substrate reduced by decreasing β -sheet content.^{2,3} Y. M. Wong et al. (2016) introduced a peptide catalyst (Ac-FGFHFSFDF-NH₂) by mimicking the catalytic residues of a serine protease. To design the peptide, they used a repeating pattern of polar and nonpolar amino acids which favours the conversion of the peptides into amyloid-like fibrils via self-assembly to generate artificial catalysts for amidolytic activities at mild pH values.⁴

The assembly of peptides and proteins in the form of extracellular fibrillar aggregates plays a crucial role in the onset of several pathologies known as amyloidosis such as Alzheimer's disease (AD), Parkinson's disease, Prion diseases, type II diabetes, *etc.*⁵ AD is considered as the most common cause of dementia in the elderly worldwide. Deposition of amyloid aggregates associated with AD result from the abnormal folding of A β into non-native conformations.⁶ The altered conformation is responsible for forming amyloid fibrils leading to the neuronal cell death. In the non-amyloidogenic pathway, the amyloid precursor protein (APP) is cleaved by α - and γ -secretase, whereas the amyloidogenic pathway produces A β when APP is cleaved by β -secretase instead of α -secretase.^{7,8} The aggregation pathway of A β involves the following steps. Soluble monomers attach to each other to form larger complexes, ranging from dimers to heptamers, which further grow into larger soluble oligomers that again form protofibrils.

Ultimately, protofibrils grow into insoluble amyloid aggregates of mature fibrils. The process of A β aggregation depends on several factors, mostly concentration, pH, and temperature. Though many strategies are reported for the reduction of aggregation and the facilitation of excretion of A β from brain, very few works are there to destroy A β chemically, and that is the reason we want to design an artificial protease to cleave A β . Thus we designed and developed Miniature Artificial Protease (mAP) by incorporating Gly in-between the triad residues (of Chymotrypsin;¹ Asp, His, and Ser) and attached with the self-assembling part VFFA (core hydrophobic region of A β) for the site-specificity⁹ by either one or two Gly, so that mAP should be able to cleave A β .

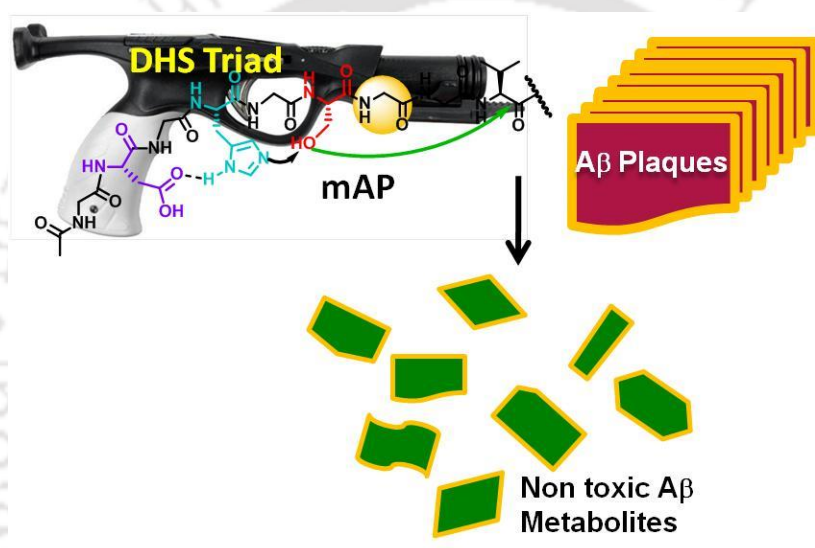


Figure 3.1. Concept of the proteolytic activity of mAP to cleave A β .

3.2. Design of the mAPs and other relevant peptides:

Keeping in view to develop mAP, first, we have screened a variety of model peptides (**mp**, Table 3.1) which contain the triad residues (Asp, His, and Ser) separated from each other either by Ala or Gly or by the combination of both. It was assumed that the side chains of the triad residues should be linked to each other by intramolecular hydrogen bonding so that they will be able to form a pocket for the hydrolysis of a peptide bond. The most stable conformation of the model peptides was achieved after calculating density functional theory (DFT) based methods using B3LYP as energy functional and 6-31G as a basis set through Gaussian 5.0.9 program. In the case of mp1, all of the triad residues (DHS) which are directly connected to each other are found in different

directions. In the case of mp2, though a bent backbone was found created by the triad residues which are separated from each other by Ala and also having Ala at the both N & C terminus, they are also placed in the different directions and there is no possibility of hydrogen bonding in-between the side chains of the triad residues. In the case of mp3, where the triad residues are separated by Ala but having Gly at the both N & C terminus, Asp and His are found to be in same directions with the possibility of intramolecular hydrogen bonding between their side chains but placed in the opposite direction of Ser. In the case of mp4, where the triad residues are separated from each other by Gly and also having Gly at the both N & C terminus, almost mp3 like structure was observed.

Table 3.1. Screening of peptide sequences.

Sl. no.	Name of the peptide (short name)	Peptide sequence
1	Model peptide 1 (mp1)	DHS-NH ₂
2	Model peptide 2 (mp2)	ADAHASA-NH ₂
3	Model peptide 3 (mp3)	GDAHASG-NH ₂
4	Model peptide 4 (mp4)	GDGHGSG-NH ₂

From the results obtained after DFT calculation of model peptides, it was clear that the incorporation of flexible Gly residues in between DHS residues are very important to design mAP. mAPs were designed (Table 3.2) by connecting the model peptides to the self-recognition motif VFFA via Ala or Gly, for the site-specificity concerning A β and also to bring all of the triad residues in a pocket with the proximal distance between their side chains. We also designed and synthesized a partial sequence of A β (A β ₁₂₋₂₁ or APP₆₈₃₋₆₉₂) which is supposed to act as a model peptide in place of the larger A β and APP (hereafter denoted as mA β) to understand the mechanism of its proteolytic activity easily. It contains both the self-assembling core hydrophobic region of A β , KLVFFA and the α -secretase cleavage site also, the peptide bond connecting Lys₁₆ and Leu₁₇ of A β . Further, we synthesized a negative control peptide, which contains 26 residues of R domain of Diphtheria toxin from S505 to F530, named as DTP28 (Gly at both N- and C-terminus as spacers). Characterization data of the synthesized peptides were attached in the supporting data (Figure 3.130-3.139).

Table 3.2. Sequences of the peptides and their roles in the current study.

Sl. No.	Name of the peptide (short name)	Peptide sequence	Role of the peptide
1	Miniature Artificial Protease 1 (mAP1)	Ac-GDAHSAVFFAG-NH ₂	Proteolytic cleavage
2	Miniature Artificial Protease 2 (mAP2)	Ac-GDGHGSGVFFAG-NH ₂	Proteolytic cleavage
3	Miniature Artificial Protease 3 (mAP3)	Ac-GDGHGSGGVFFAG-NH ₂	Proteolytic cleavage
4	Partial sequence of A β ₁₂₋₂₁ (mA β)	Ac-GGAV ₁₂ HHQKLVFFA ₂₁ G-NH ₂	Prototype of A β
5	A β ₁₋₄₀ peptide	DAEFRHDSGYEVHHQKLVFFAE DVGSNKGAIIGLMVGGVV-NH ₂	Target peptide
6	DTP28	GSSDSIGVLGYQKTV DHTKVNS KLSLFG-NH ₂	Control

3.3. The most stable conformation of mAPs:

The most stable conformation of the peptides (mAPs) was achieved (Figure 3.2-3.4 for mAP1-3 respectively) after calculating DFT based methods using B3LYP as energy functional and 6-31G as a basis set through Gaussian 5.0.9 program. In the case of mAP1, it was found that His and Ser of the triad residues (DHS) are in the opposite direction of Asp and the backbone created by DHS residues is almost linear. Though a partially bent backbone was found created by the triad residues, they are placed in the different directions in mAP2, which was not expected as mAP2 was designed from mp4. But in the case of mAP3, Asp, His, and Ser are placed in the same direction, and a prominent bent backbone was observed created by the DHS residues which can help to form the desired pocket *in vitro*. So, incorporation of flexible Gly in between DHS residues and also in-between triad and VFFA segments is very important to form intramolecular hydrogen bonding of the side chains of Asp, His and Ser.

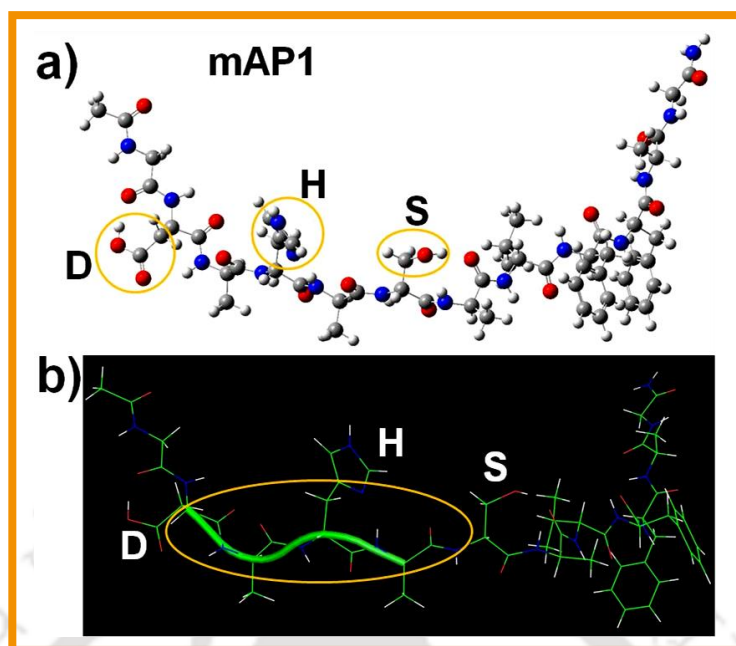


Figure 3.2. The most stable conformation of mAP1 [image generated by a) Gaussian 09W and b) PyMOL (Line & Cartoon representation)] obtained from DFT calculation [Calculation Method: B3LYP, and Basis Set: 6-31G]. Nitrogen & Oxygen atoms are represented in blue & red, respectively.

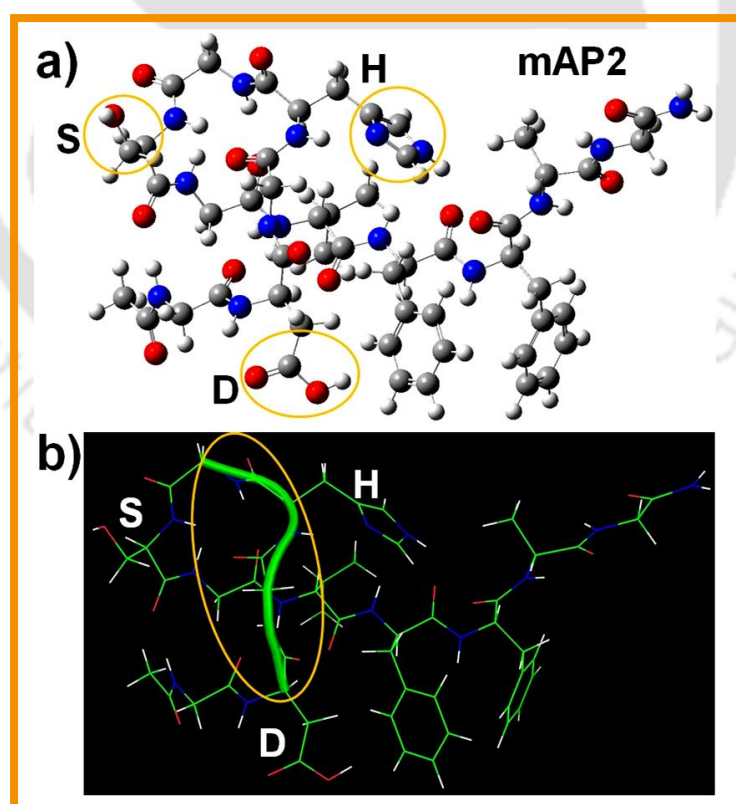


Figure 3.3. The most stable conformation of mAP2 [image generated by a) Gaussian 09W and b) PyMOL (Line & Cartoon representation)] obtained from DFT calculation [Calculation Method: B3LYP, and Basis Set: 6-31G]. Nitrogen & Oxygen atoms are represented in blue & red, respectively.

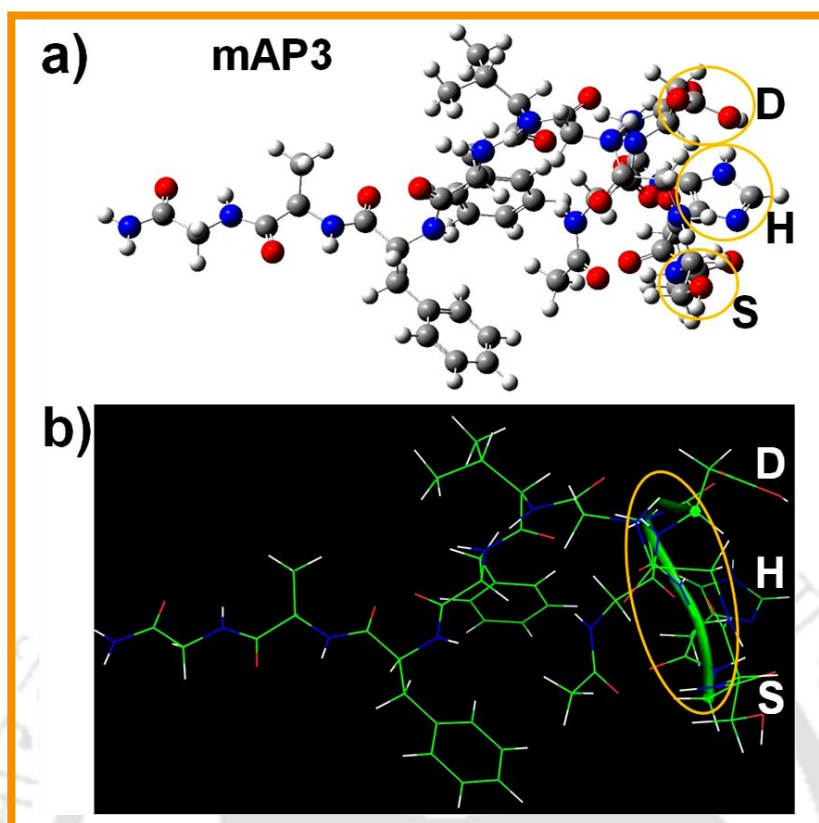


Figure 3.4. The most stable conformation of mAP3 [image generated by a) Gaussian 09W and b) PyMOL (Line & Cartoon representation)] obtained from DFT calculation [Calculation Method: B3LYP, and Basis Set: 6-31G]. Nitrogen & Oxygen atoms are represented in blue & red, respectively.

3.4. Molecular Docking:

It was detected that (Figure 3.5), mAPs interact or bind well in the helical $A\beta_{1-40}$ binding pocket having a binding affinity -5.8 kcal/mol (for mAP1), -5.4 kcal/mol (for mAP2), and -6.6 kcal/mol (for mAP3). Interestingly, mAP3 has shown maximum binding affinity among mAPs. Also, mAPs binds well (Figure 3.6) in the fibril $A\beta_{1-40}$ binding pocket having a binding affinity -6.3 kcal/mol (for mAP1), -4.7 kcal/mol (for mAP 2), and -5.7 kcal/mol (for mAP3). We observed that interacting amino acids in the $A\beta_{1-40}$ binding pocket are the hydrophobic region, *i.e.*, Leu17, Val18, Phe19, Phe20, and Ala21 in most of the cases. Finally, mAP3 was docked into the helical C-terminal domain of APP (C99, residues 683-728) [PDB ID: 2LP1], and it was found that (Figure 3.7) mAP3 fitted well in the APP binding pocket also having binding affinity -5.2 kcal/mol where the interacting amino acids in the pocket were L₆₈₈VFF₆₉₁.

From the docking results, no pocket was found created by the DHS unit in the case of mAP1. Though Asp, His, and Ser are away from one another, a pocket was found for mAP2. In the case of mAP3, a pocket with the close proximity of Asp, His, and Ser was found, created by the DHS unit.

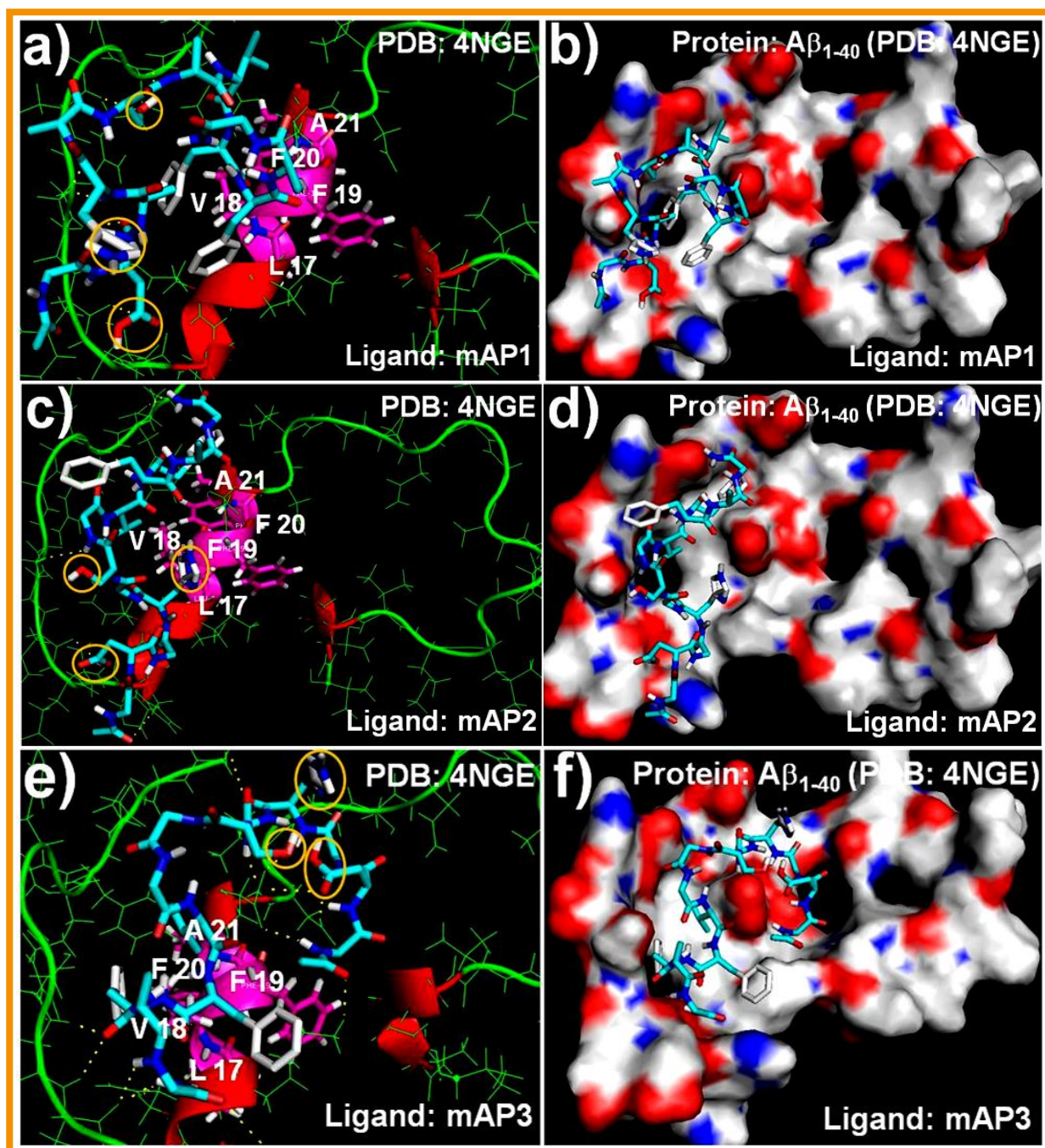


Figure 3.5. Molecular docking images of mAP1 (a, b), mAP2 (c, d), and mAP3 (e, f) into helical $A\beta_{1-40}$ (PDB ID 4NGE) reveals that mAPs bind at the binding site (V₁₈FFA₂₁) with binding affinity -5.8 kcal/mol, -5.4 kcal/mol, and -6.6 kcal/mol, respectively. Structures are shown as line and cartoon representation (for a, c, e), and surface representation (for b, d, f). The surface of $A\beta_{1-40}$ is coloured

according to the charges of the atoms where negatively and positively charged zones are represented in red and blue, respectively.

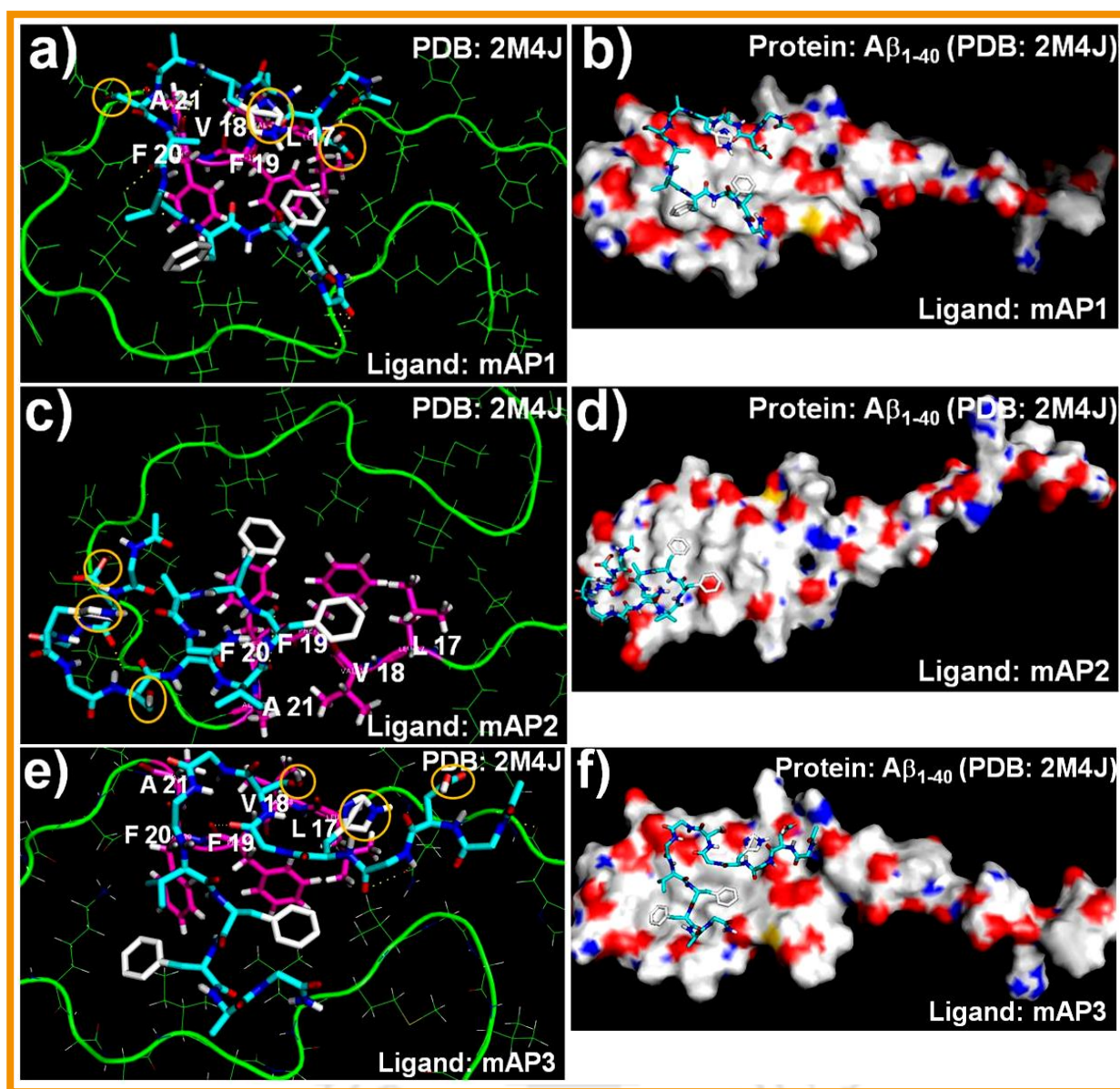


Figure 3.6. Molecular docking images of mAP1 (a, b), mAP2 (c, d), and mAP3 (e, f) into fibril $A\beta_{1-40}$ (PDB ID 2M4J) reveals that mAPs bind at the binding site (V18FFA21) with binding affinity -6.3 kcal/mol, -4.7 kcal/mol, and -5.7 kcal/mol, respectively. Structures are shown as line and cartoon representation (for a, c, e), and surface representation (for b, d, f). The surface of $A\beta_{1-40}$ is coloured according to the charges of the atoms where negatively and positively charged zones are represented in red and blue, respectively.

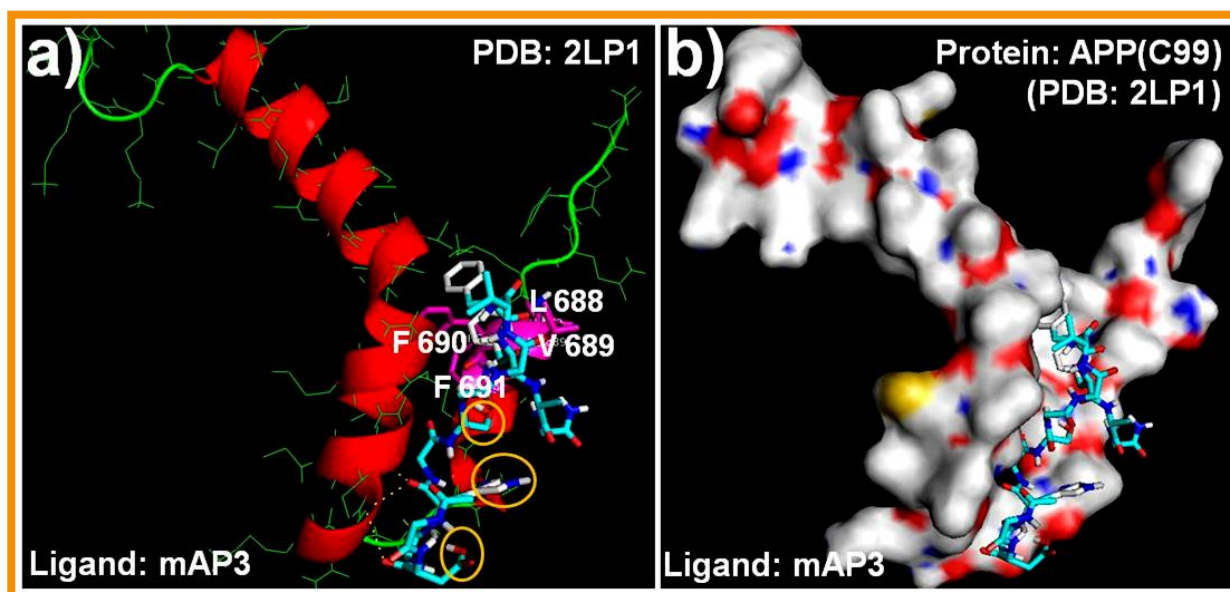


Figure 3.7. Molecular docking images of mAP3 (a, b) into helical 99 residues C-terminal domain (PDB ID: 2LP1) of APP(C99) reveals that mAP3 binds at the binding site (L₆₈₈VFF₆₉₁) with binding affinity -5.2 kcal/mol. Structures are shown as line and cartoon representation (for a), and surface representation (for b). The surface of the APP is coloured according to the charges of the atoms where negatively and positively charged zones are represented in red and blue, respectively.

3.5. Examination of the proteolytic activity of the mAPs on mA β :

To obtain a primary idea on the proteolytic efficacy of the mAPs, we examined the proteolytic activity of the mAPs on the mA β , before starting with A β peptide which is costly and not easy to handle. For that, we dissolved the purified peptides in PBS (50 mM, pH 7.4) to make suitable stock solutions and co-incubated in 1:1 molar ratio of the mA β (200 μ M) at physiological pH and temperature on a water bath. mA β alone and each of the mAPs was incubated separately at the same condition as control. Aliquots were tested by a MALDI-TOF mass spectrometer in a time-dependent way.

3.5.1. Self-degradation of mA β :

From a time-dependent MALDI-TOF mass spectrometric analyses of the mA β , dimer was found after two days of incubation, but no such degraded peak was detected after five days of incubation for its solvent in-accessibility due to the aggregation (Figure 3.8-3.12). This control experiment was performed to check self-degraded fragments, and to confirm the proteolytic activity of mAPs against mA β while performing co-incubation in the presence of mAPs.

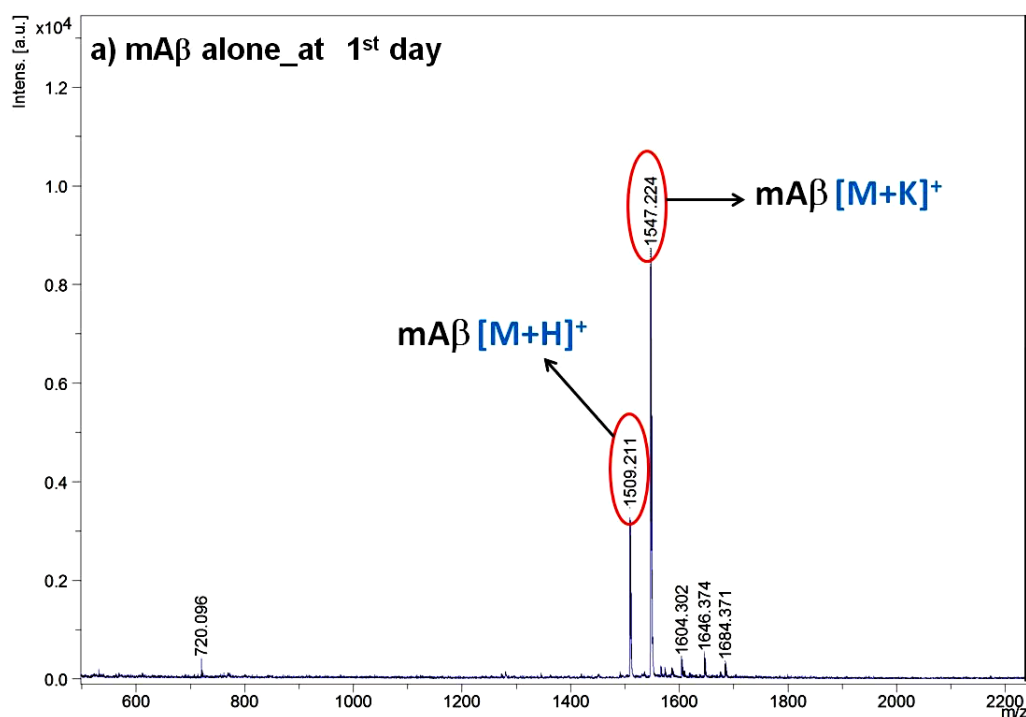


Figure 3.8. MALDI-TOF mass spectrum of mAbeta at the 1st day of incubation in PBS pH 7.4 at 37 °C.

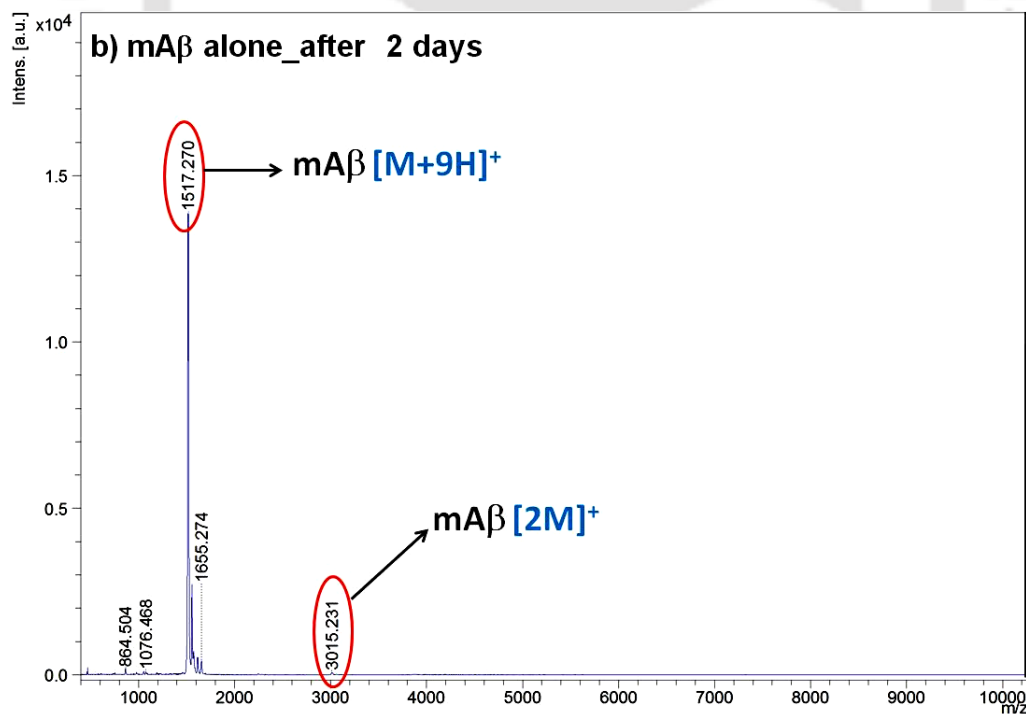


Figure 3.9. MALDI-TOF mass spectrum of mAbeta after two days of incubation in PBS.

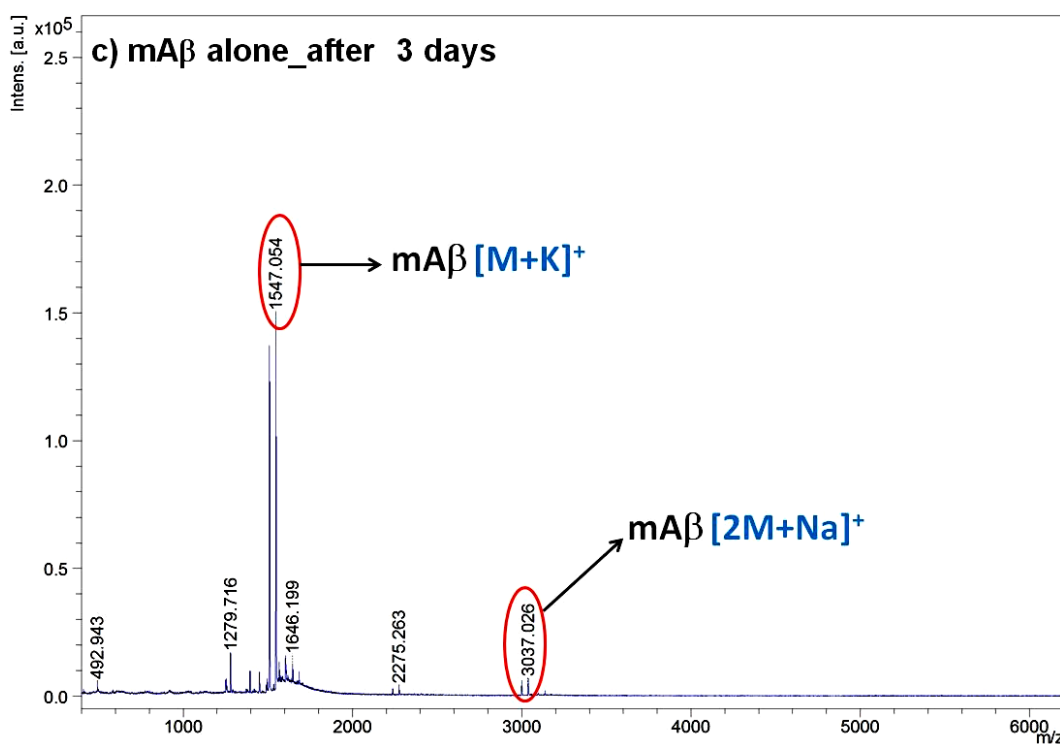


Figure 3.10. MALDI-TOF mass spectrum of mA β after three days of incubation in PBS.

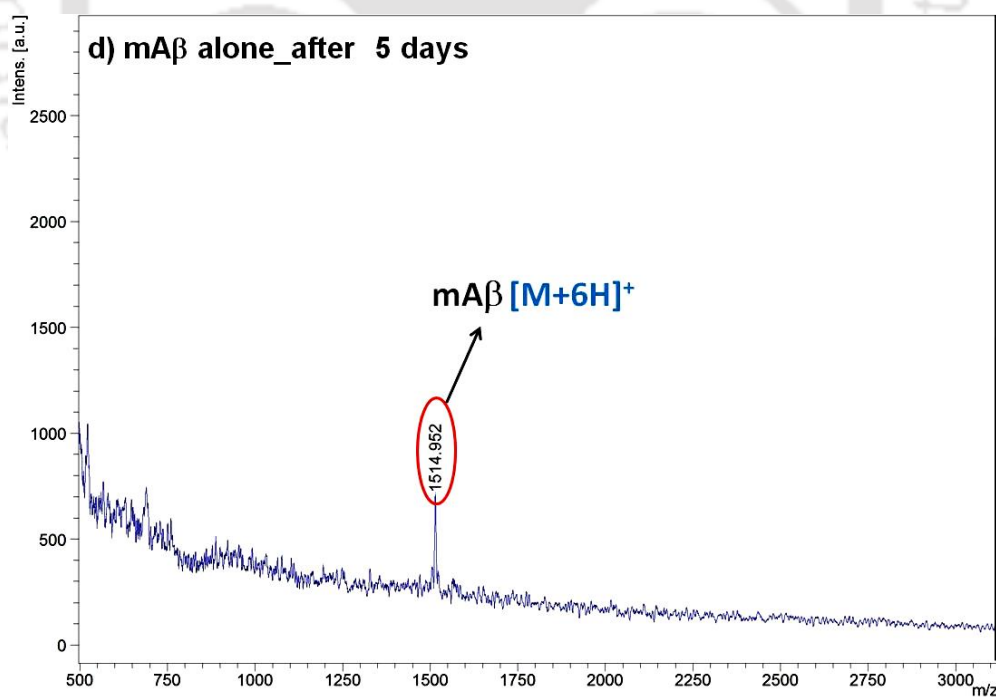


Figure 3.11. MALDI-TOF mass spectrum of mA β after five days of incubation in PBS.

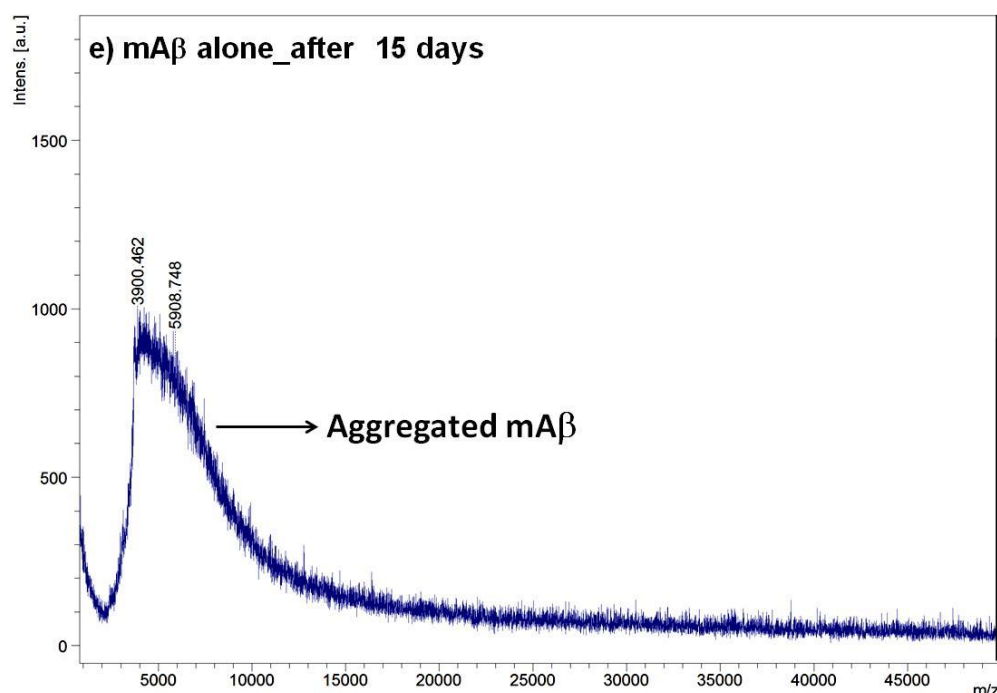


Figure 3.12. MALDI-TOF mass spectrum of mAbeta after fifteen days of incubation in PBS.

3.5.2. Self-degradation of mAP1:

Instead of the presence of some self-degraded and undetermined peptide fragments, mAP1 was found to be stable even after 21 days of incubation at the physiological condition (Figure 3.13-3.16). Though few fragments were found after seven days and 30 days of incubation, we are unable to draw the plausible mechanism, and such fragments are considered as a regular degraded product.

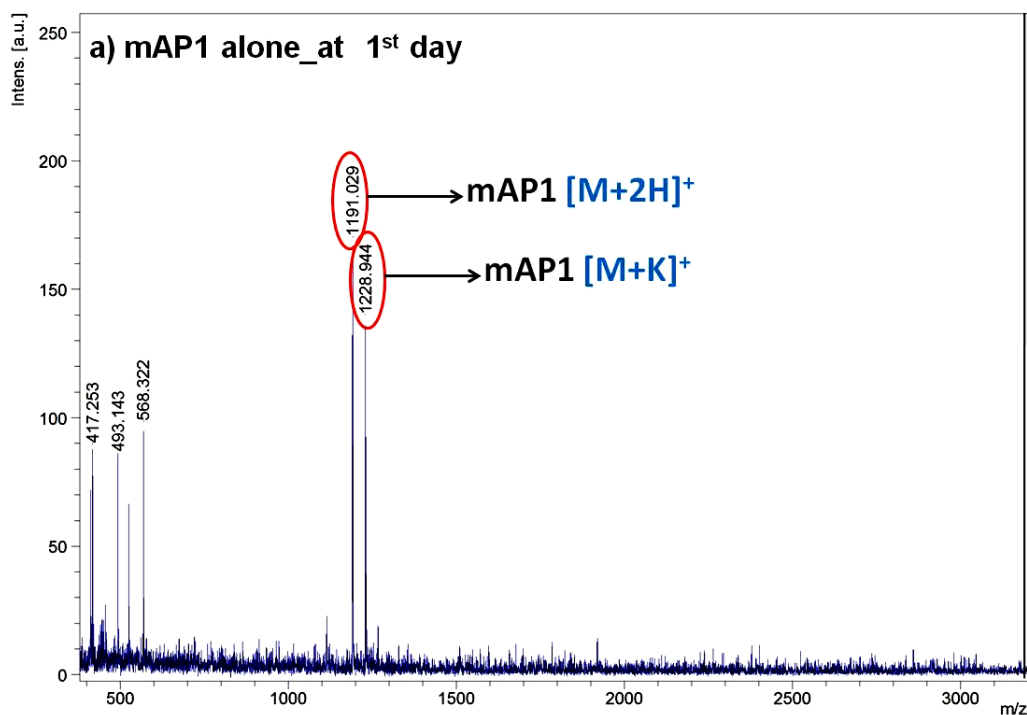


Figure 3.13. MALDI-TOF mass spectrum of mAP1 at the 1st day of incubation in PBS pH 7.4 at 37 °C.

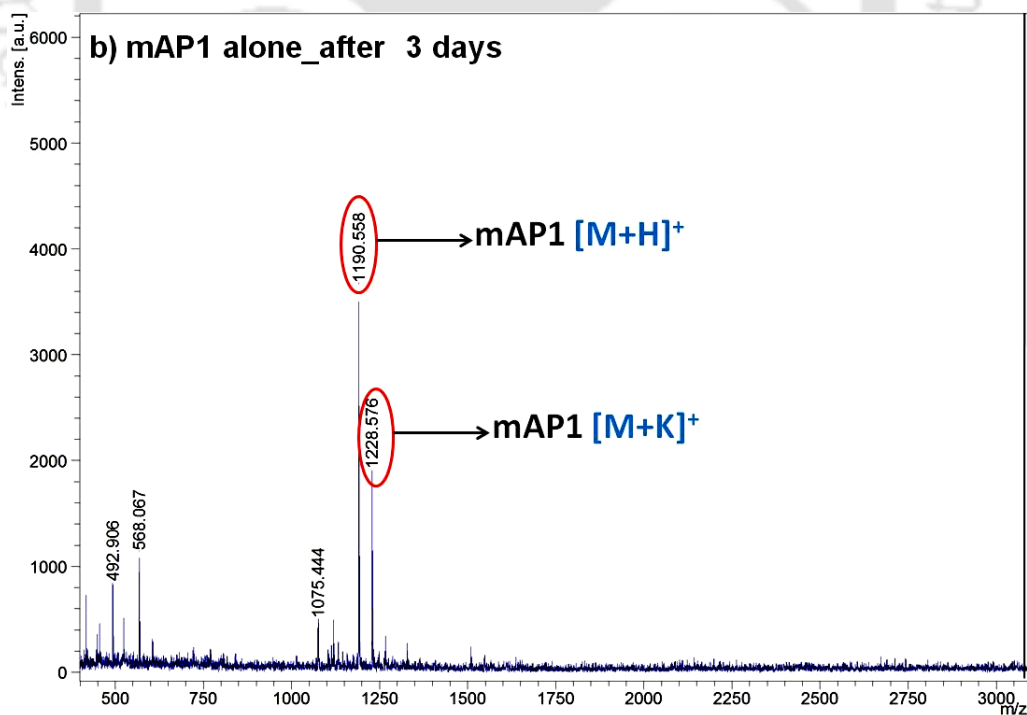


Figure 3.14. MALDI-TOF mass spectrum of mAP1 after three days of incubation in PBS.

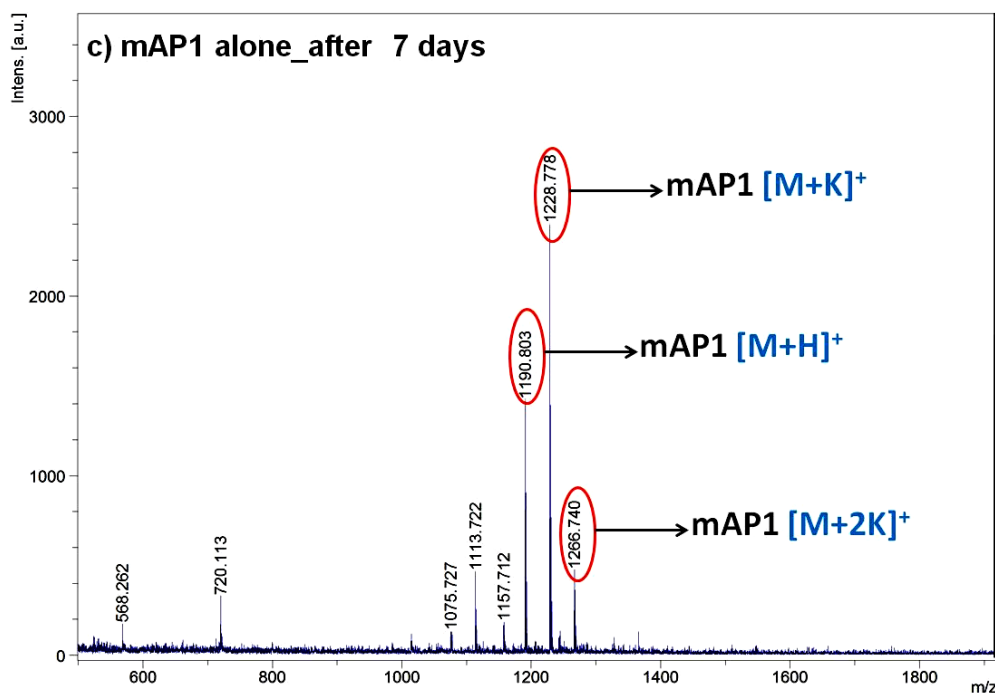


Figure 3.15. MALDI-TOF mass spectrum of mAP1 after seven days of incubation in PBS.

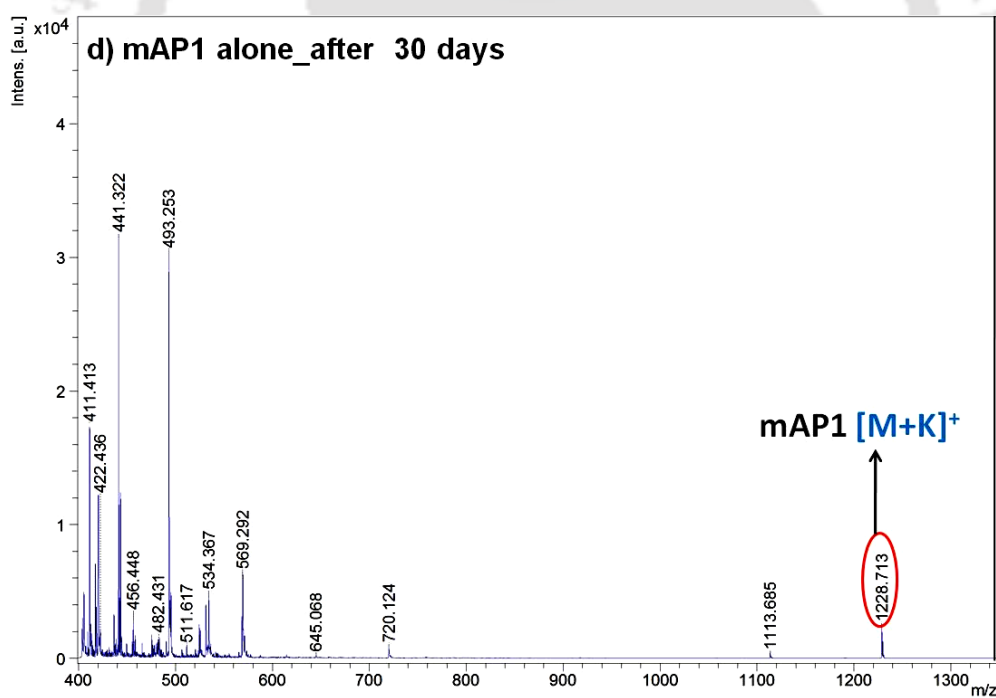


Figure 3.16. MALDI-TOF mass spectrum of mAP1 after thirty days of incubation in PBS.

3.5.3. Proteolytic activity of mAP1 on mA β :

No peak corresponding to the mass of any fragments or intermediates were noticed for mA β even after seven days or 21 days of incubation in the presence of mAP1 (Figure

3.17-3.20). But no such aggregated peak like mA β was observed, pointing to the amyloid inhibiting nature of mAP1 against mA β .

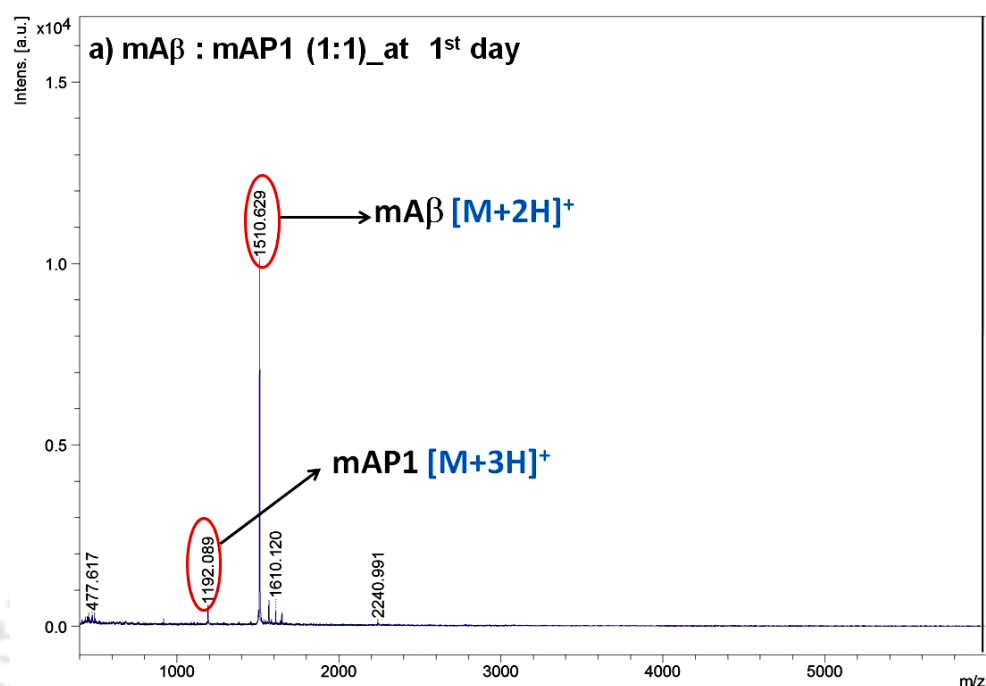


Figure 3.17. MALDI-TOF mass spectrum of mA β in the presence of mAP1 (1:1) at the 1st day.

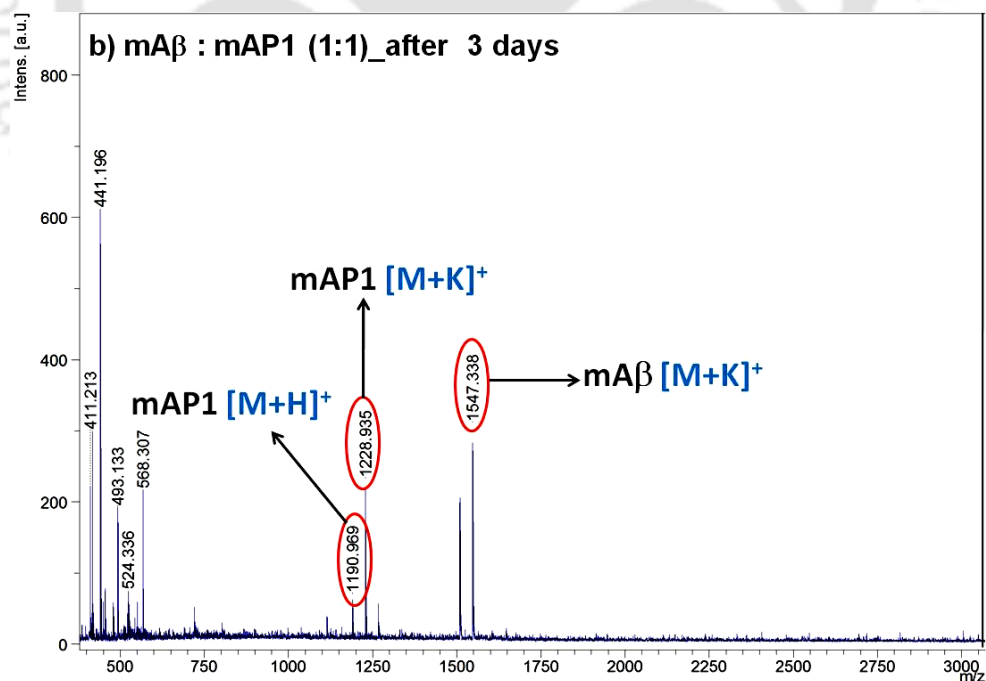


Figure 3.18. MALDI-TOF mass spectrum of mA β in the presence of mAP1 (1:1) after three days.

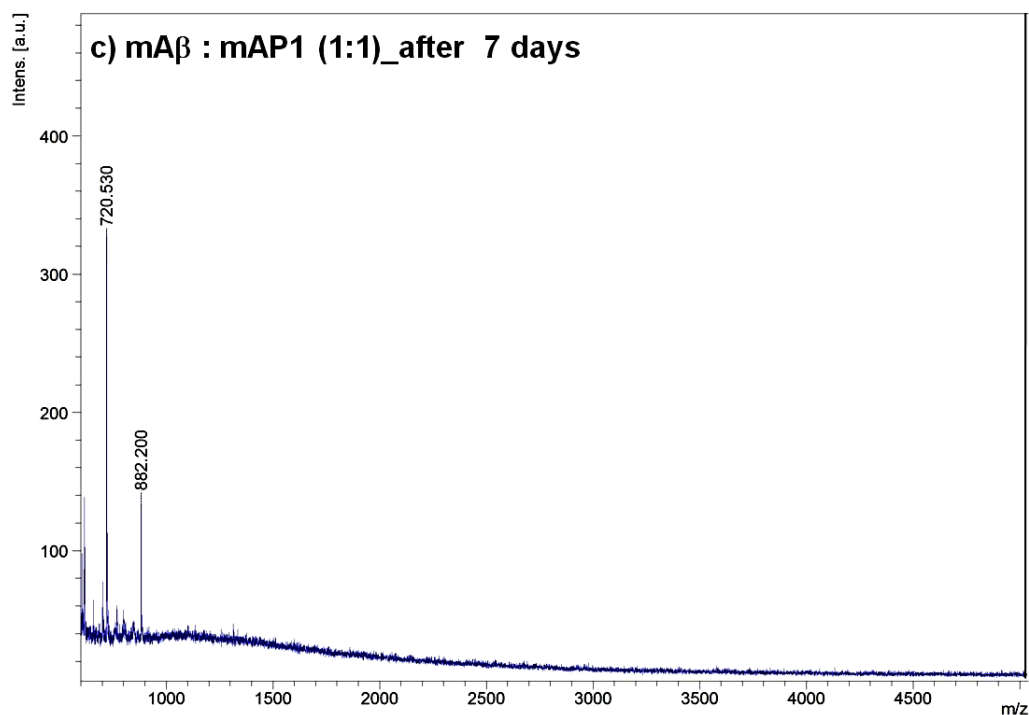


Figure 3.19. MALDI-TOF mass spectrum of mA β in the presence of mAP1 (1:1) after seven days of incubation in PBS.

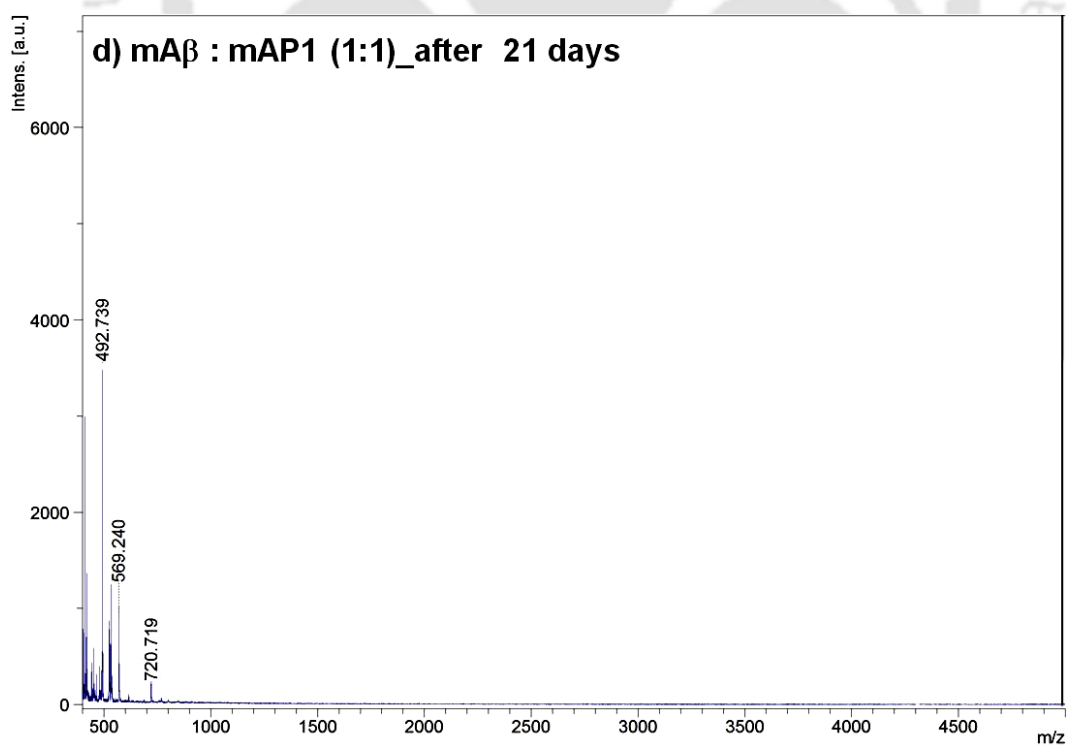


Figure 3.20. MALDI-TOF mass spectrum of mA β in the presence of mAP1 (1:1) after twenty-one days of incubation in PBS.

3.5.4. Self-degradation of mAP2:

Instead of the presence of some self-degraded and undetermined peptide fragments, mAP2 was found to be stable even after 21 days of incubation at physiological condition (Figure 3.21-3.27). The plausible mechanism of the self-degradation of mAP2 was drawn based on the MALDI-TOF mass values found after 21 days, though few fragments were found after seven days and 15 days and considered as a normal degraded product.

Route1^{SD-mAP2}

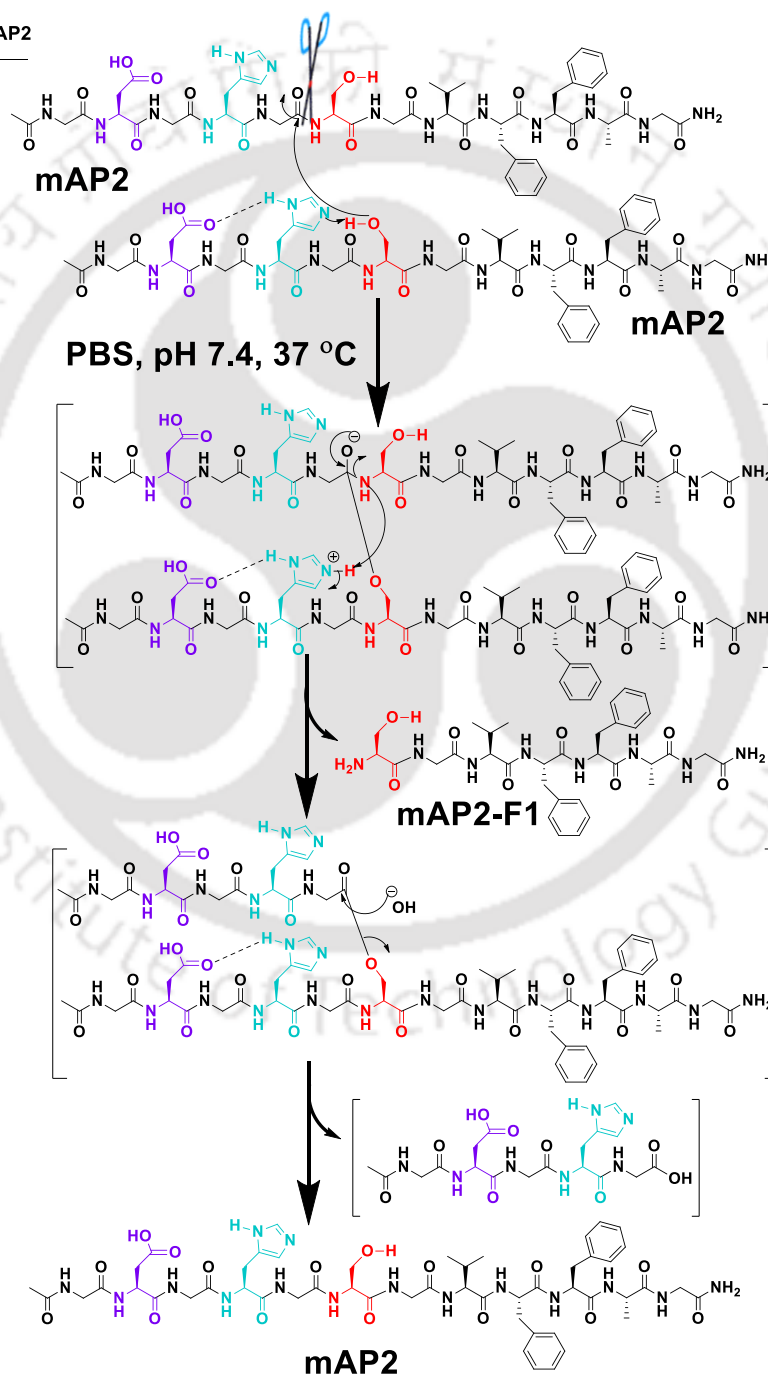


Figure 3.21. Plausible route of the self-degradation of mAP2 into various fragments (Route 1^{SD-mAP2}).

The results of MALDI-TOF mass values indicated mAP2 cleaved itself in a site-selective manner between Gly-Ser following Route 1^{SD-mAP2}. It was observed that one molecule of mAP2 became covalently attached with the second molecule by the nucleophilic attack of Ser side-chain as described in Route 1^{SD-mAP2} and cleaved the second molecule between Gly-Ser to produce mAP2-F1 peptide fragment, and finally reproduced itself pointing to the catalytic activity of mAP2.

Similarly, mAP2 cleaved itself in a site-selective manner between Gly-Asp probably following Route 2^{SD-mAP2}. Plausible routes were drawn concerning cleavage sites, but the plausible mechanism remains the same for each of the routes.

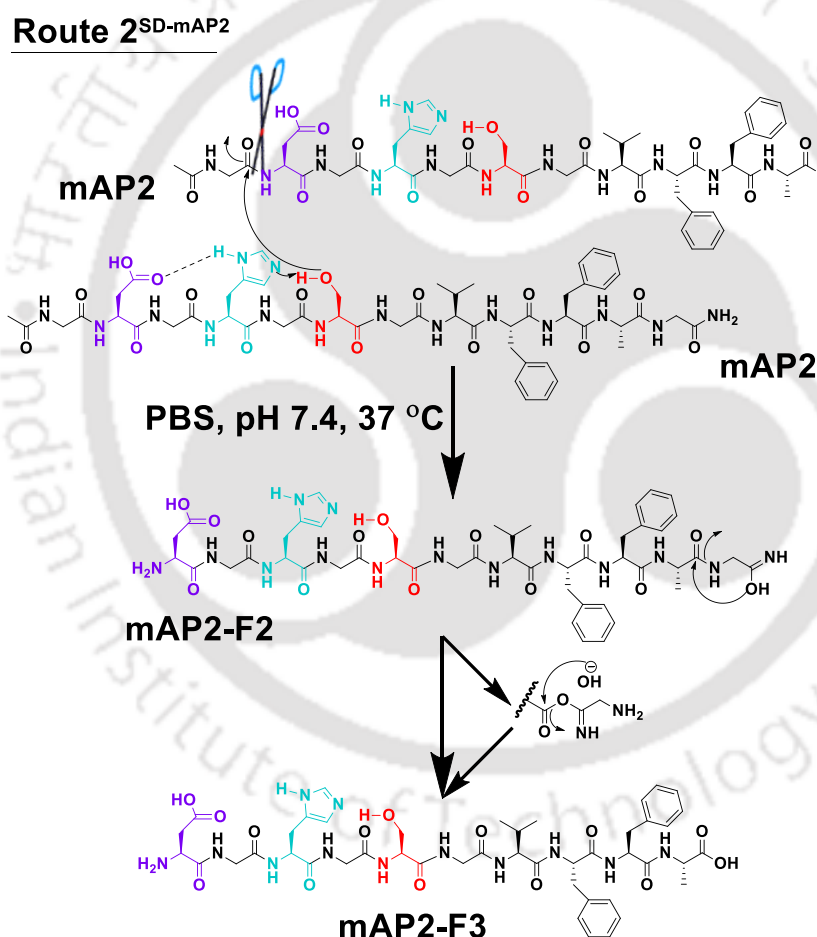


Figure 3.22. Plausible route of the self-degradation of mAP2 into various fragments (Route 2^{SD-mAP2}).

Most of the logically drawn intermediates and final peptide-fragments could be identified by time-dependent MALDI-TOF mass spectrometric analyses of the mAP2 incubated in the absence of mA β (Figure 3.23-3.27). It was observed that mAP2 was stable after 21 days of incubation at the physiological condition.

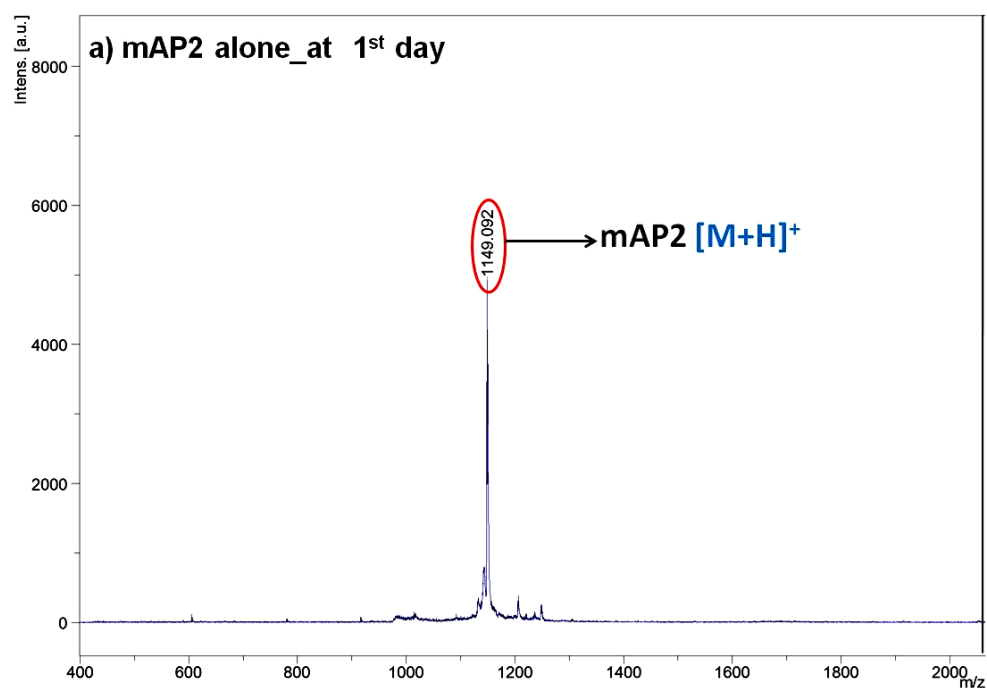


Figure 3.23. MALDI-TOF mass spectrum of mAP2 at the 1st day of incubation in PBS.

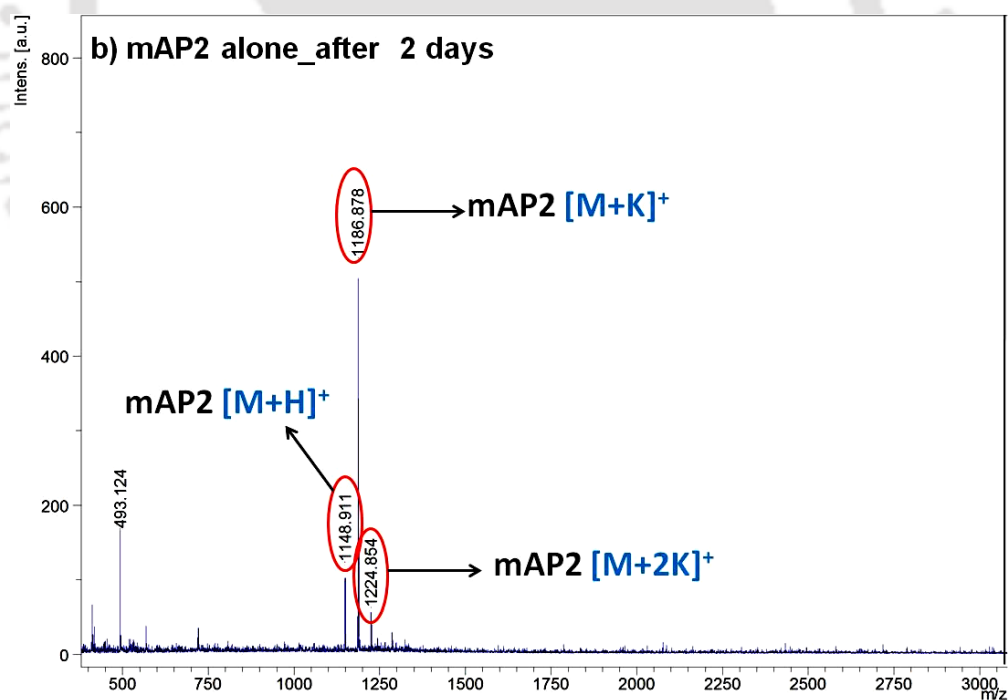


Figure 3.24. MALDI-TOF mass spectrum of mAP2 after two days of incubation in PBS.

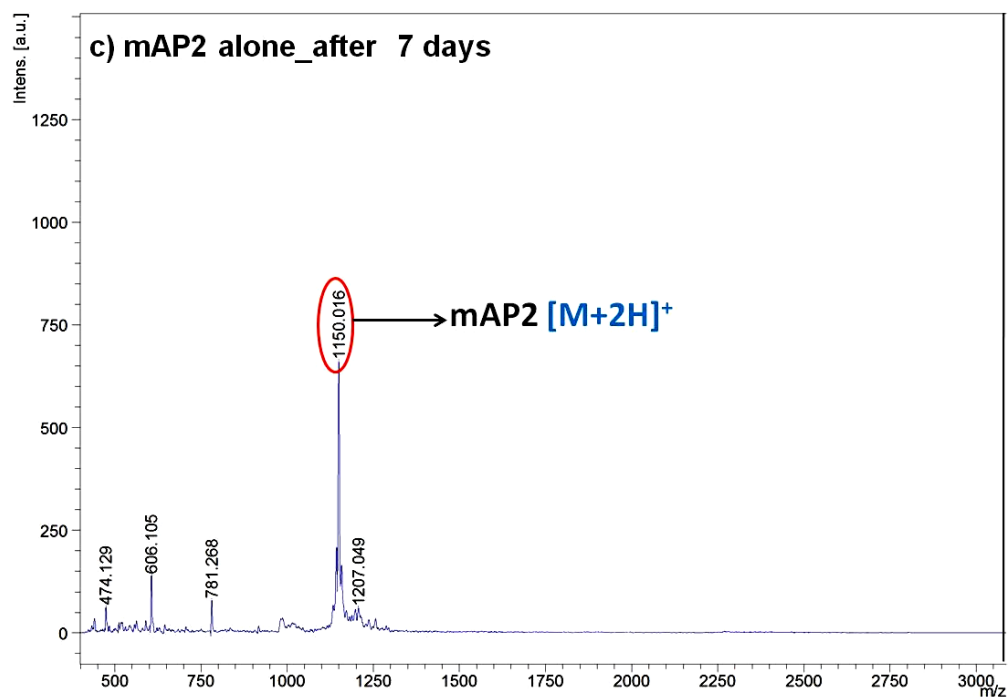


Figure 3.25. MALDI-TOF mass spectrum of mAP2 after seven days of incubation in PBS.

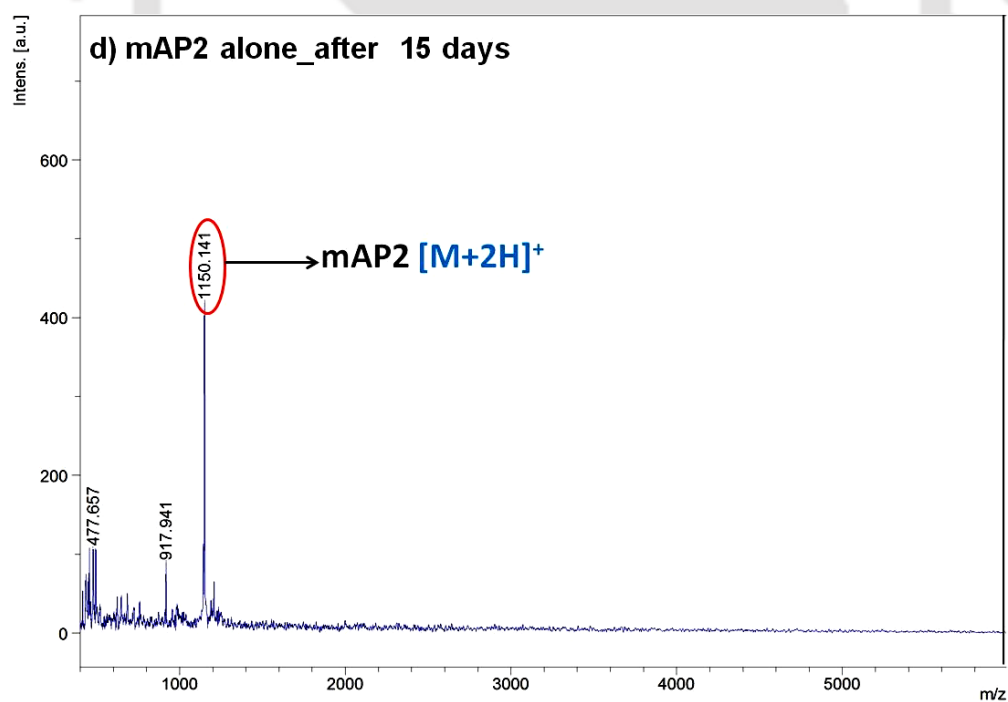


Figure 3.26. MALDI-TOF mass spectrum of mAP2 after fifteen days of incubation in PBS.

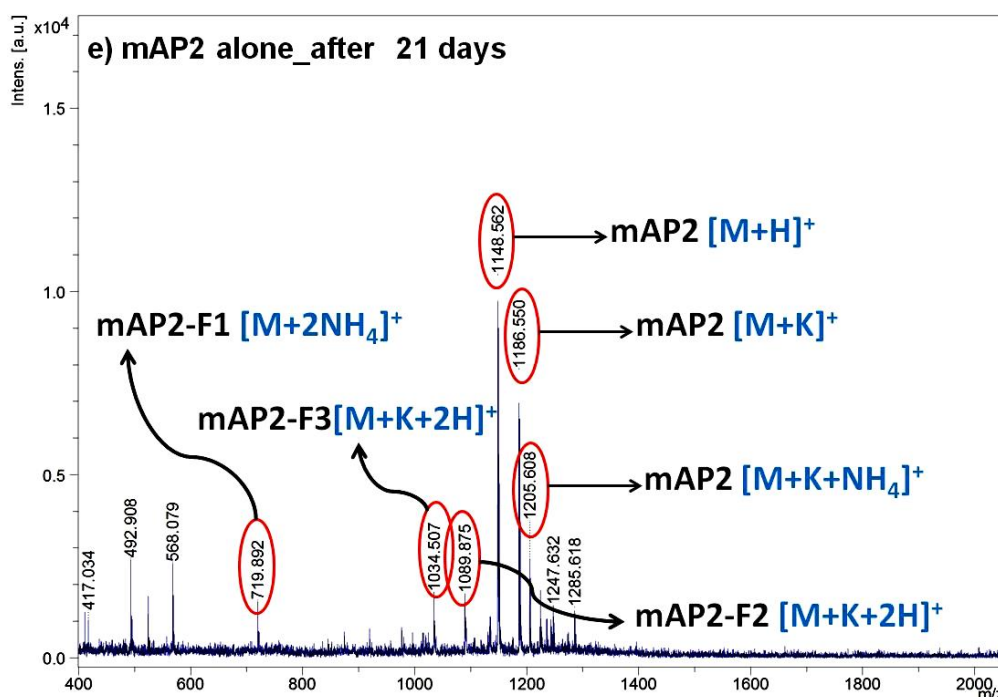


Figure 3.27. MALDI-TOF mass spectrum of mAP2 after twenty-one days of incubation in PBS.

3.5.5. Proteolytic cleavage of mA β by mAP2:

Peaks corresponding to the mass of various fragments and intermediates were detected for mA β after seven days of incubation in the presence of mAP2 (Figure 3.28-3.34). The results of MALDI-TOF mass values indicated mAP2 cleaved the mA β in a site-selective manner (between His-His, His-Gln, Gln-Lys, Lys-Leu, Leu-Val, Val-Phe, and Phe-Phe).

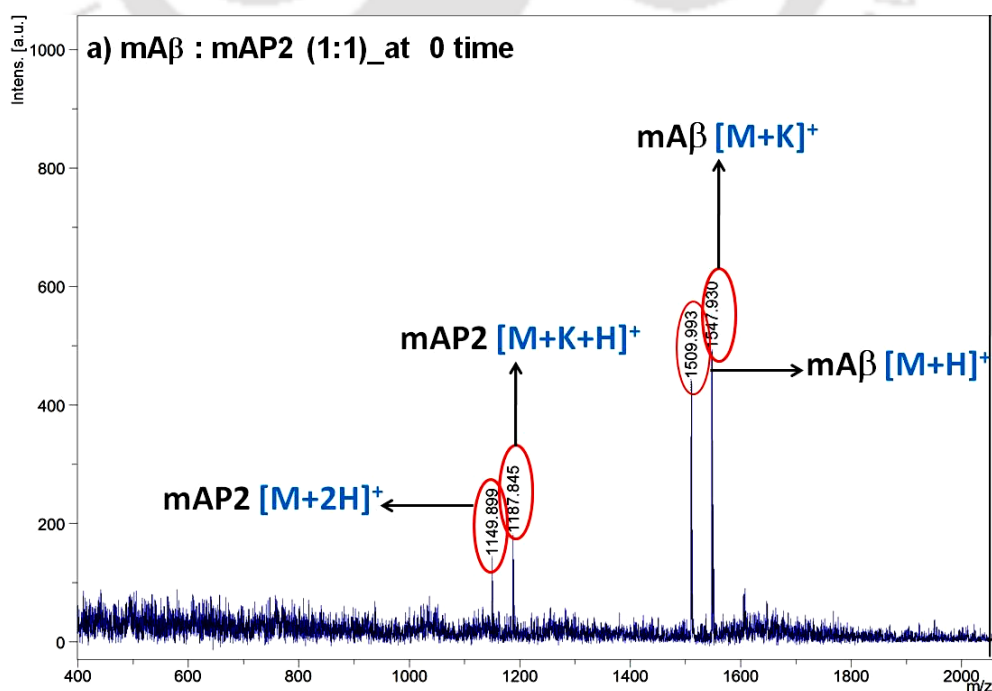
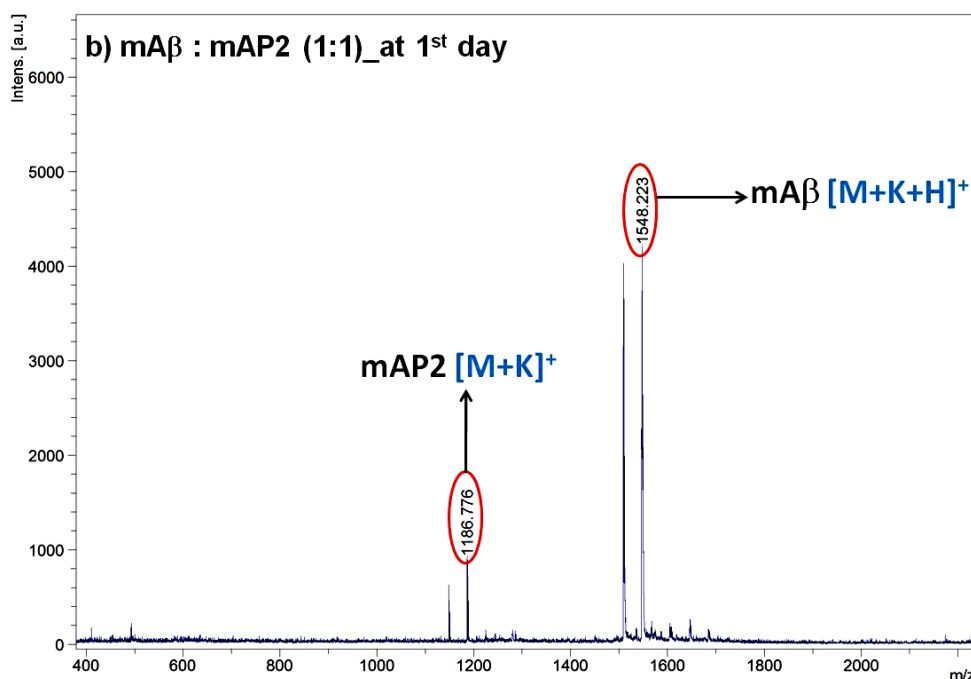
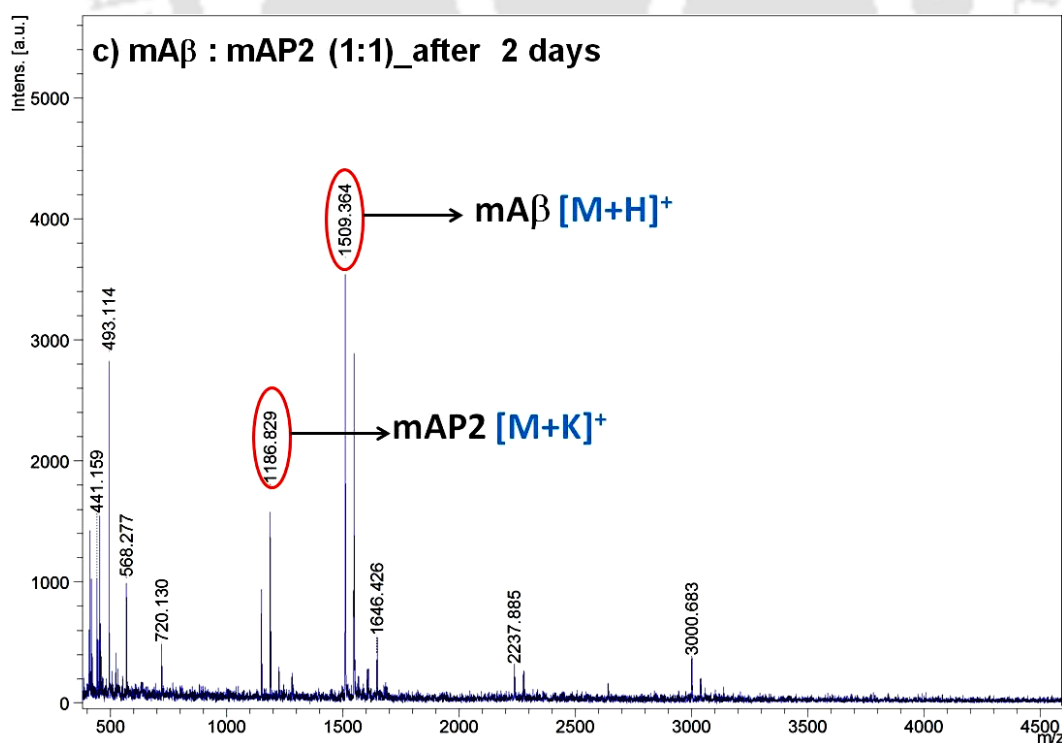
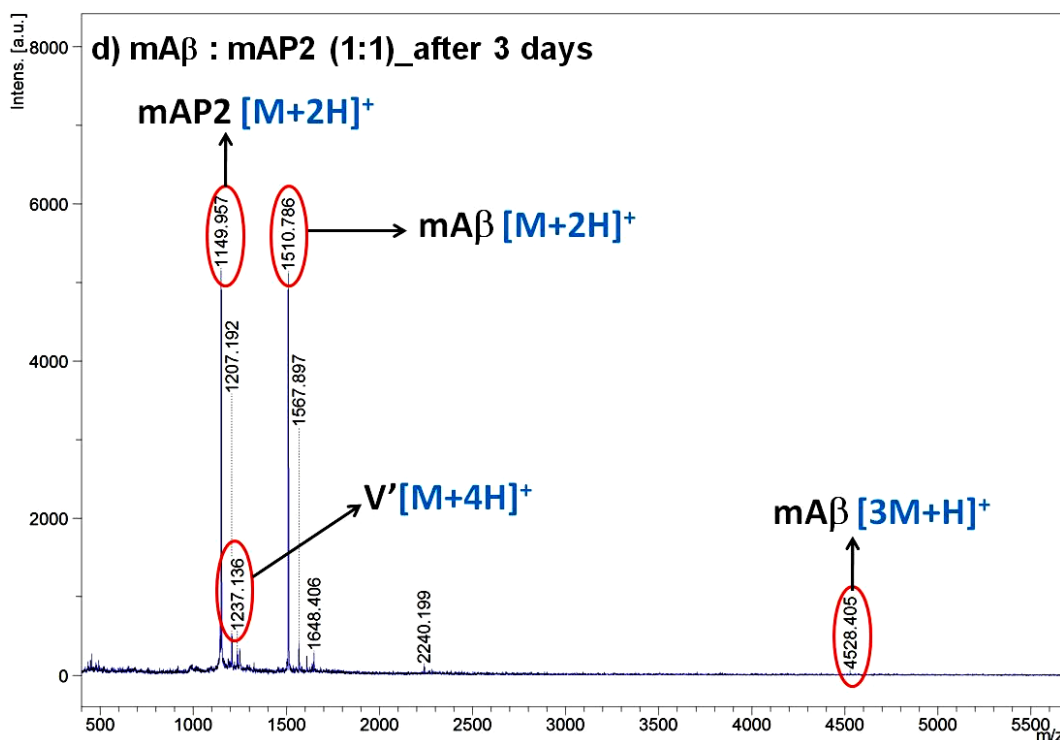
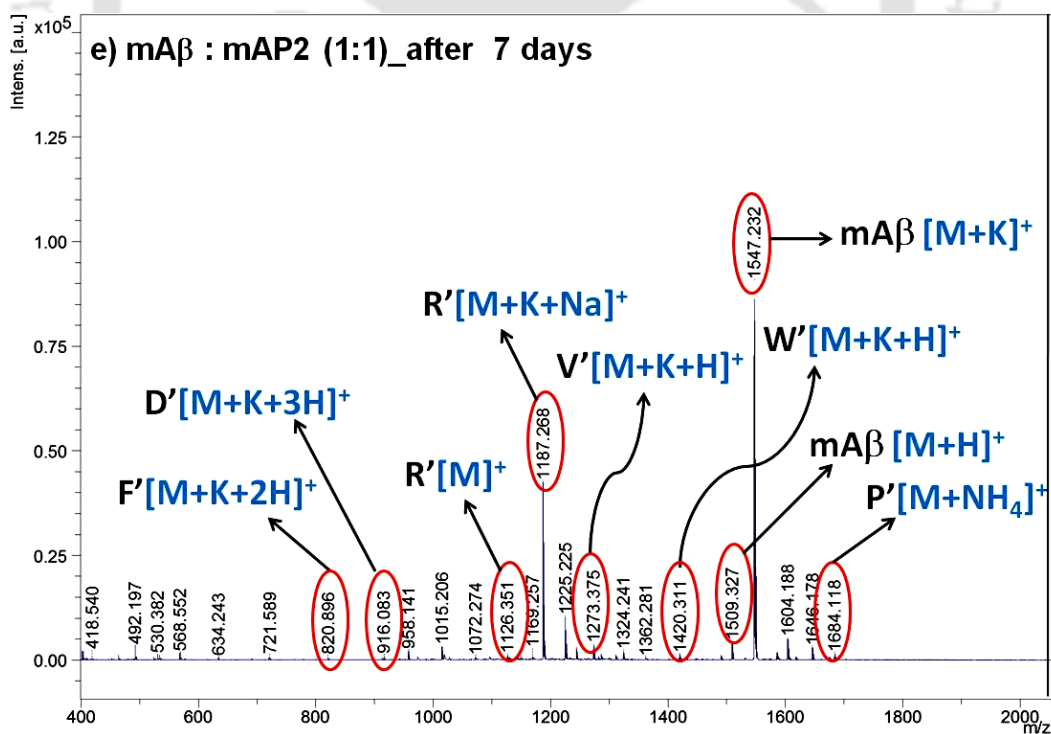
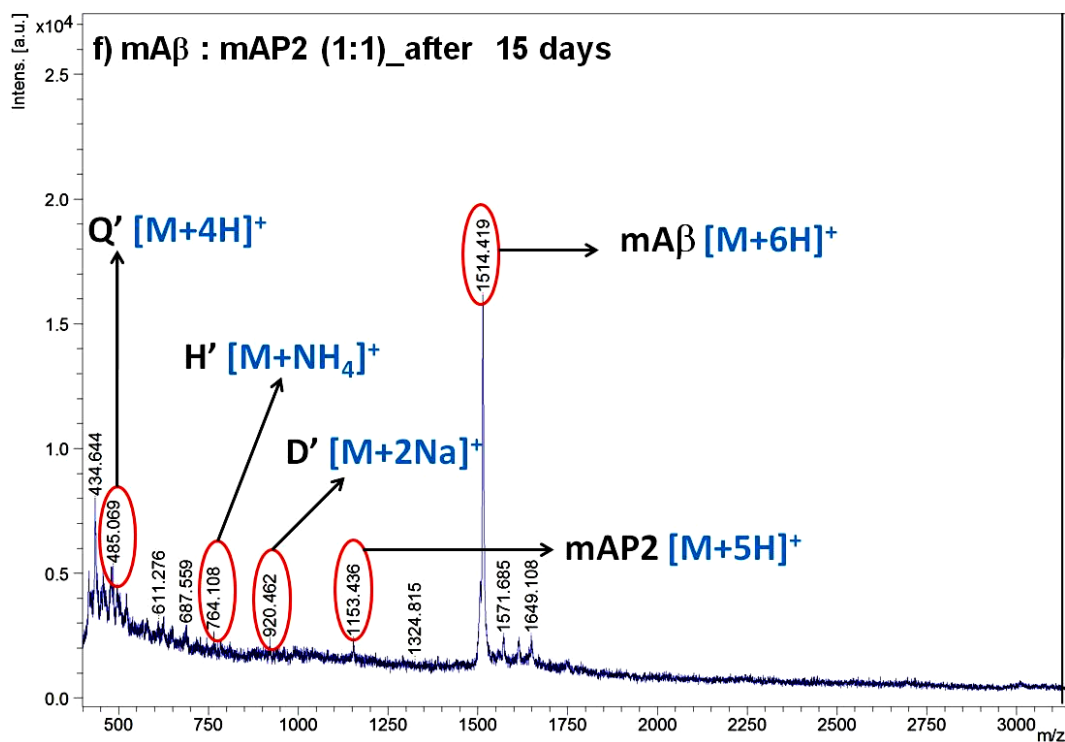
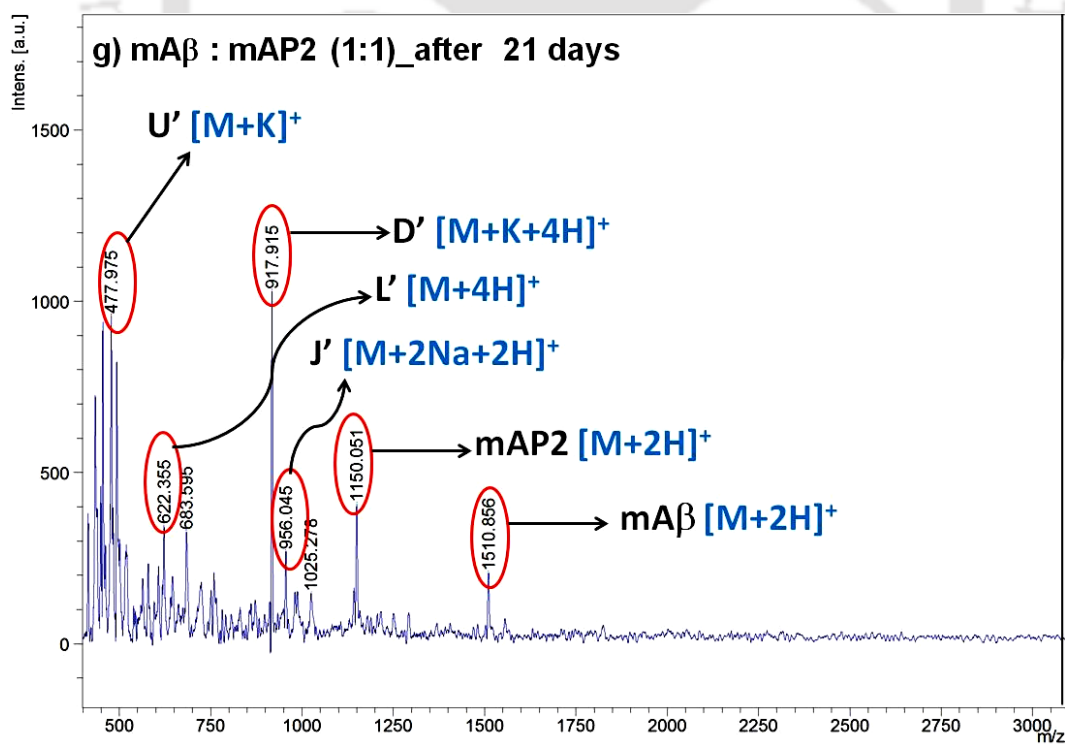


Figure 3.28. MALDI-TOF mass spectrum of mA β in the presence of mAP2 (1:1) at the 1st h.**Figure 3.29.** MALDI-TOF mass spectrum of mA β in the presence of mAP2 (1:1) at the 1st day.**Figure 3.30.** MALDI-TOF mass spectrum of mA β in the presence of mAP2 (1:1) after two days.

Figure 3.31. MALDI-TOF mass spectrum of mAP β in the presence of mAP2 (1:1) after three days.Figure 3.32. MALDI-TOF mass spectrum of mAP β in the presence of mAP2 (1:1) after seven days.

Figure 3.33. MALDI-TOF mass spectrum of mAP β in the presence of mAP2 (1:1) after 15 days.Figure 3.34. MALDI-TOF mass spectrum of mAP β in the presence of mAP2 (1:1) after 21 days.

3.5.6. Self-degradation of mAP3:

The plausible mechanism of the self-degradation of mAP3 was drawn (Figure 3.35-3.37) from the fragments getting after 15 days of incubation, though few fragments found after seven days are considered as a normal degraded product. The time-dependent MALDI-TOF mass analyses indicated mAP3 cleaved itself in a site-selective manner between Gly-Ser to generate mAP3-F1 peptide fragment following Route 1^{SD-mAP3}. It was observed that one molecule of mAP3 attacked the second molecule by its Ser side-chain as described in-details in Route 1^{SD-mAP2} and cleaved the second molecule between Gly-Ser to produce mAP3-F1 fragment, and finally reproduced itself pointing to the catalytic activity of mAP3.

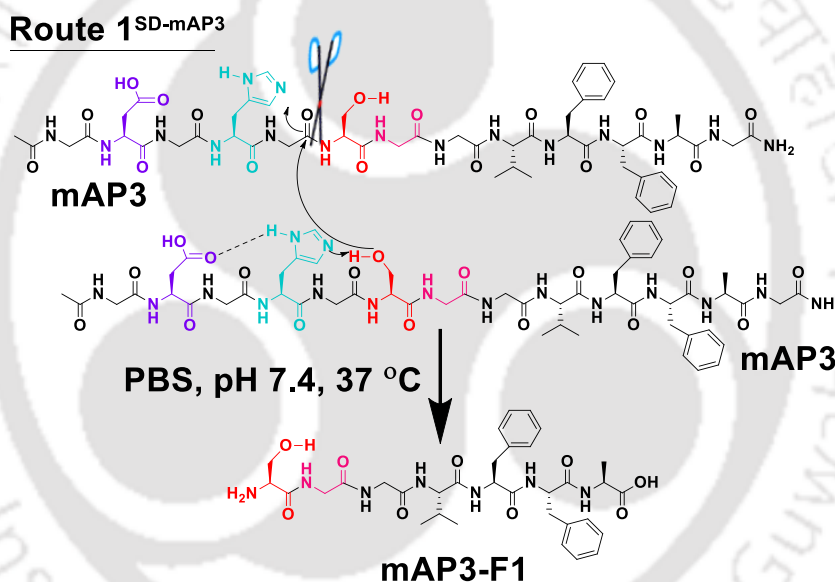


Figure 3.35. Plausible route of the self-degradation of mAP3 into various fragments (Route 1^{SD-mAP3}).

Similarly, mAP3 cleaved itself in a site-selective manner between Gly-Asp to generate mAP3-F3 peptide fragment probably following Route 2^{SD-mAP3}. Plausible routes were drawn concerning cleavage sites, but the plausible mechanism remains the same as described in-details in Route 1^{SD-mAP2} for each of the routes.

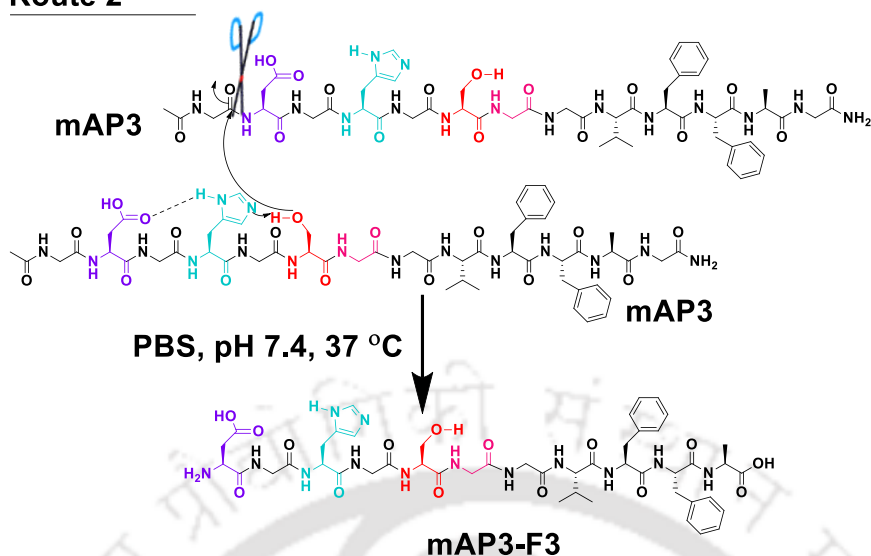
Route 2^{SD-mAP3}

Figure 3.36. Plausible route of the self-degradation of mAP3 into various fragments (Route 2^{SD-mAP3}).

Similarly, mAP3 cleaved itself in a site-selective manner between Gly-His to generate mAP3-F4 peptide fragment probably following Route 3^{SD-mAP3}. Plausible routes were drawn concerning cleavage sites, but the plausible mechanism remains the same as described in-details in Route 1^{SD-mAP2} for each of the routes.

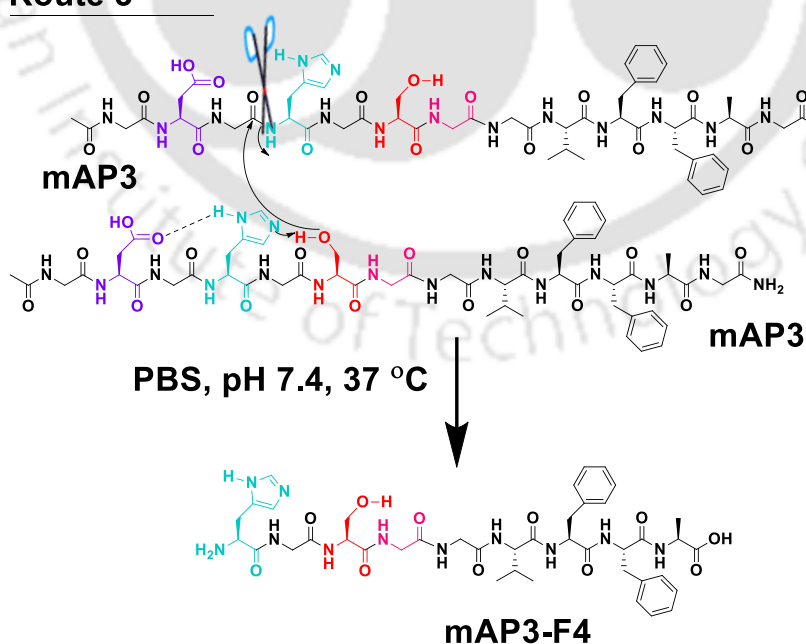
Route 3^{SD-mAP3}

Figure 3.37. Plausible route of the self-degradation of mAP3 into various fragments (Route 3^{SD-mAP3}).

Most of the logically drawn intermediates and final fragments could be identified by time-dependent MALDI-TOF mass spectrometric analyses of the mAP3 incubated in the absence of mA β (Figure 3.38-3.41). It was noticed that mAP3 was stable even after 21 days of incubation at the physiological condition.

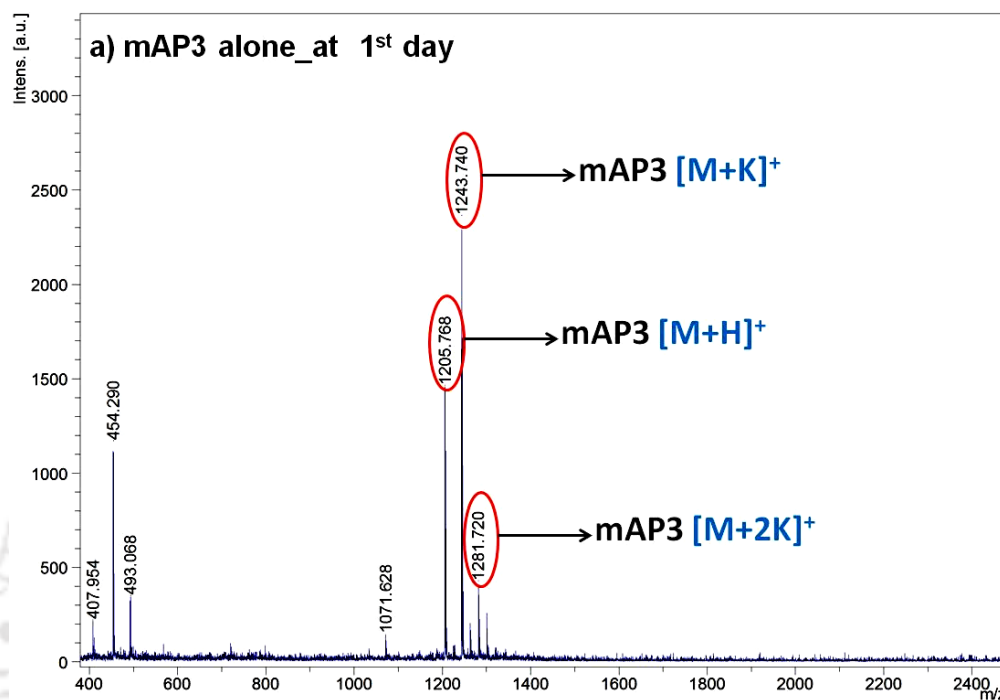


Figure 3.38. MALDI-TOF mass spectrum of mAP3 at the 1st day of incubation in PBS.

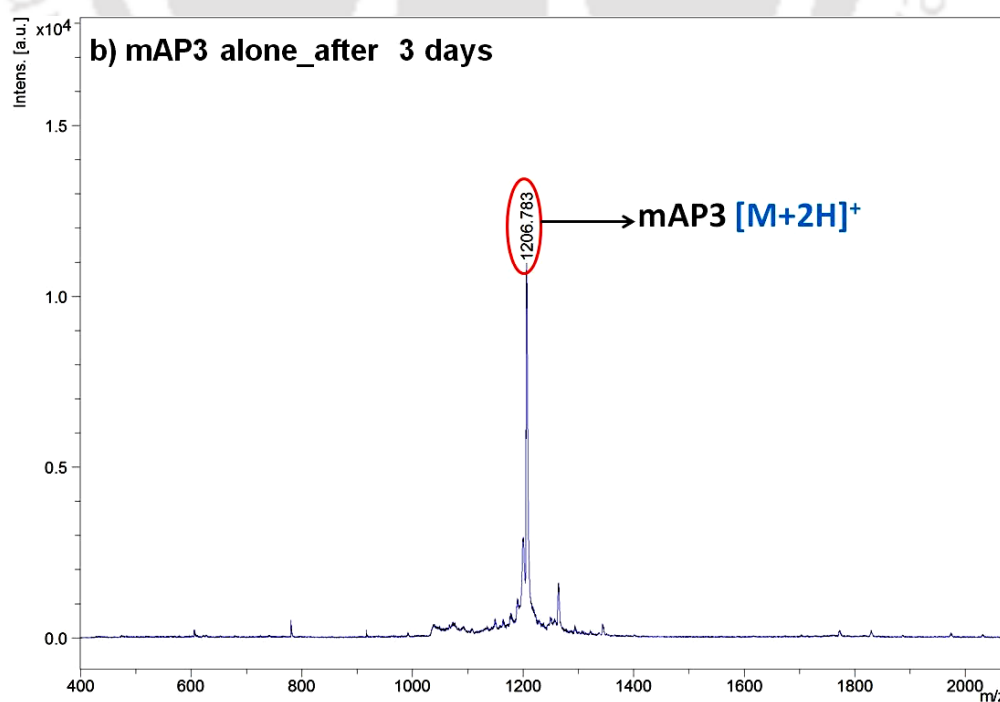


Figure 3.39. MALDI-TOF mass spectrum of mAP3 after three days of incubation in PBS.

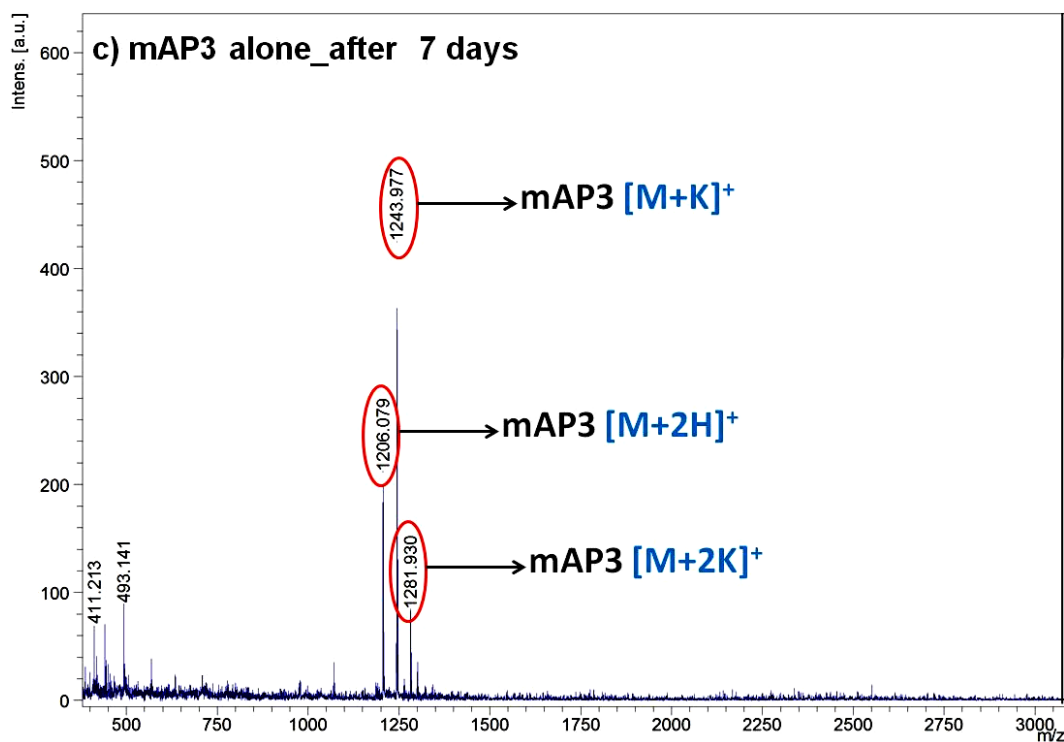


Figure 3.40. MALDI-TOF mass spectrum of mAP3 after seven days of incubation in PBS.

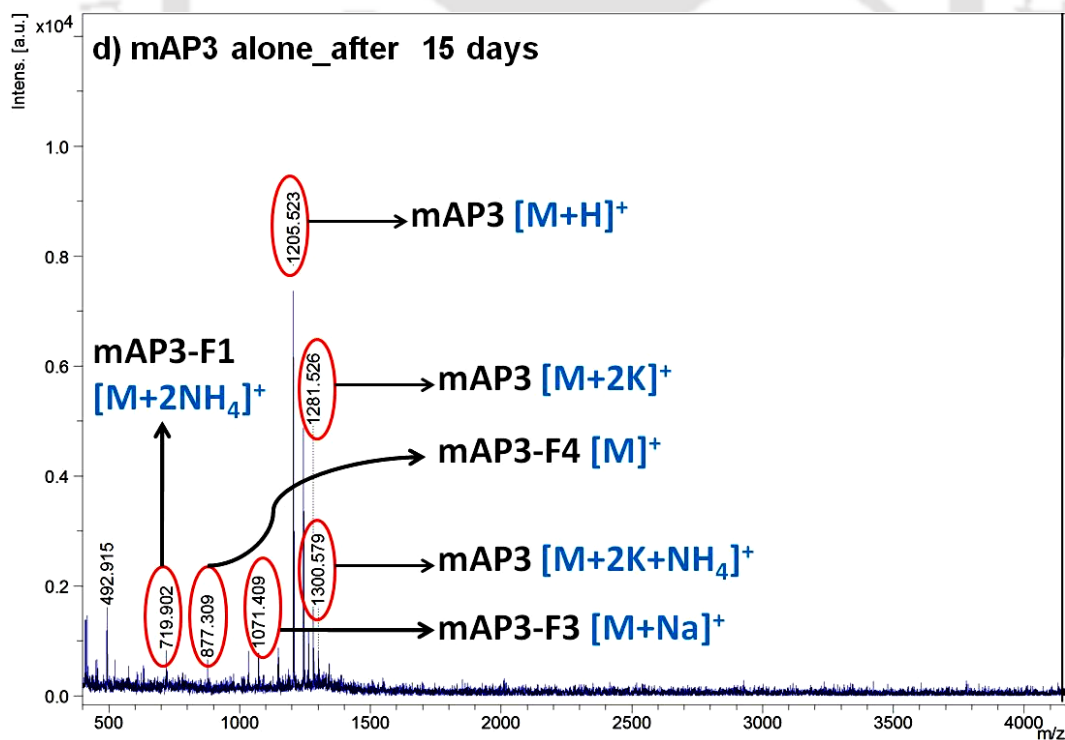


Figure 3.41. MALDI-TOF mass spectrum of mAP3 after fifteen days of incubation in PBS.

3.5.7. Plausible routes of the proteolytic cleavage of mA β by mAP3:

Peaks corresponding to the mass of various fragments and intermediates were detected for mA β after seven days of incubation in the presence of mAP3 (Figure 3.51-3.55). Plausible routes of the proteolytic cleavage of mA β by mAP3 were drawn (Figure 3.42-3.50 for mAP3) based on the MALDI-TOF mass values. These results indicated mAP3 cleaved the mA β in a site-selective manner (between His-His, His-Gln, Gln-Lys, Lys-Leu, Leu-Val, Val-Phe, Phe-Phe, and Phe-Ala). Plausible routes were drawn concerning cleavage sites, but the plausible mechanism remains the same for each of the routes.

It seems, at first the backbone of mA β binds adjacent to triad residues of mAP3 with the help of recognition segments. Through a hydrogen bond, Asp helps His to act as a general base to withdraw a proton from Ser as represented in route 1^{mA β -mAP3} (Figure 3.42). By this way, DHS residues of the mAP3 work altogether to facilitate nucleophilic attack by Ser on the carbonyl carbon of the peptide bond of mA β adjacent to the pocket created by DHS residues of mAP3, and a covalent mA β -mAPs (A' or A'') is formed. The donation of a proton from His (of mAP3) to the peptide's amide nitrogen (of mA β to be cleaved) produces a short-lived protonated tetrahedral amine intermediate which further facilitated by the negative charge on oxygen to cleave the subsequent peptide bond of mA β . On the other hand, the newly generated ester bond (formed by Ser oxygen) was simply hydrolysed to release mAP and produced another fragment of mA β .

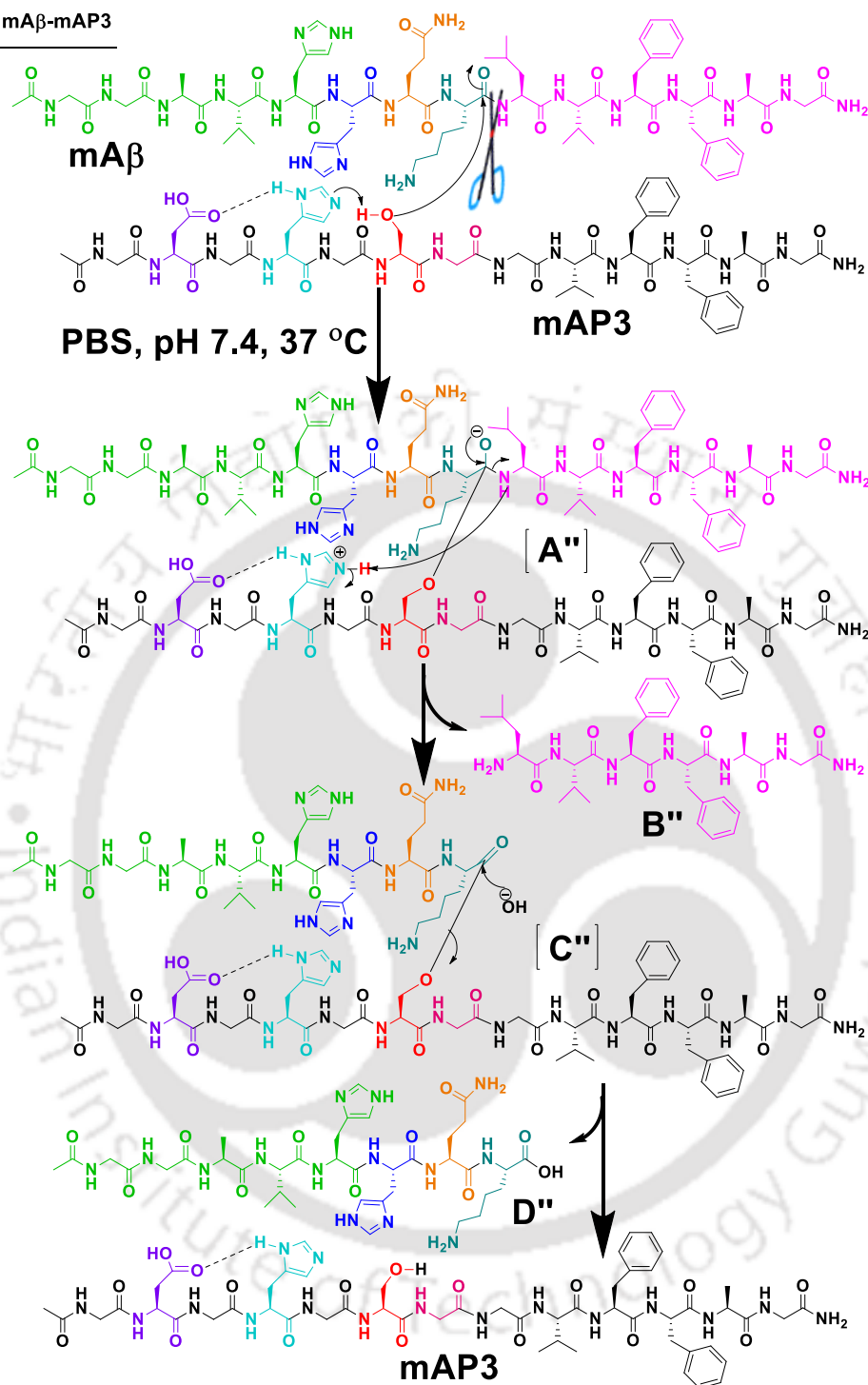
Route 1^{mA β -mAP3}

Figure 3.42. Plausible route for proteolysis of $mA\beta$ by $mAP3$ into various fragments (Route 1 ^{$mA\beta$ - $mAP3$}).

Similarly, $mAP3$ cleaved $mA\beta$ in a site-selective manner between Gln-Lys to generate peptide fragments F'' and H'' probably following Route 2 ^{$mA\beta$ - $mAP3$} (Figure 3.43). Plausible routes were drawn concerning cleavage sites, but the plausible mechanism remains the same as described in-details in Route 1 ^{$mA\beta$ - $mAP3$} for each of the routes.

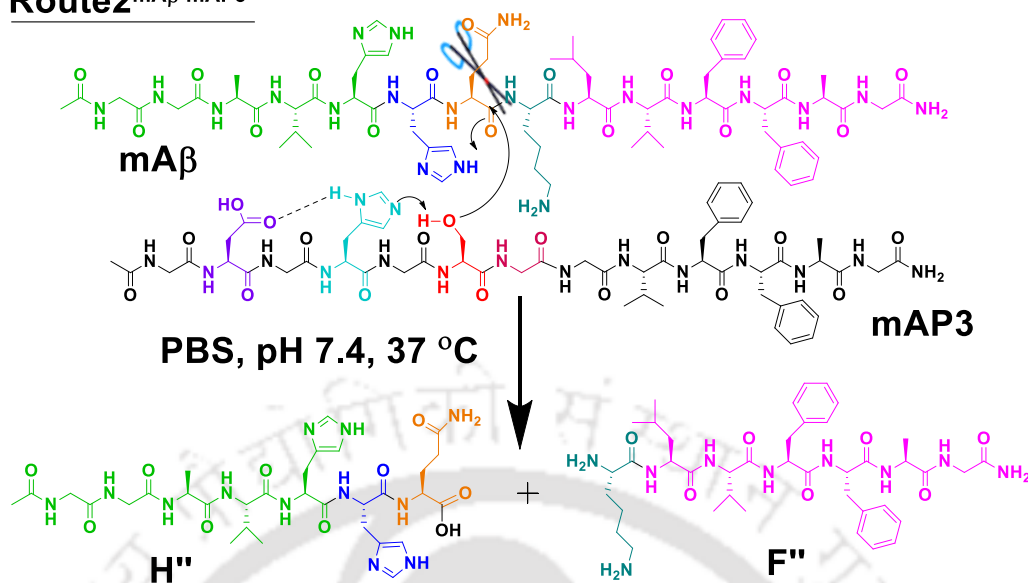
Route₂^{mA β -mAP3}

Figure 3.43. Plausible route for proteolysis of mA β by mAP3 into various fragments (Route 2^{mA β -mAP3}).

Similarly, mAP3 cleaved mA β in a site-selective manner between His-Gln to generate peptide fragments J'' and L'' probably following Route 3^{mA β -mAP3} (Figure 3.44). Plausible routes were drawn concerning cleavage sites, but the plausible mechanism remains the same as described in-details in Route 1^{mA β -mAP3} for each of the routes.

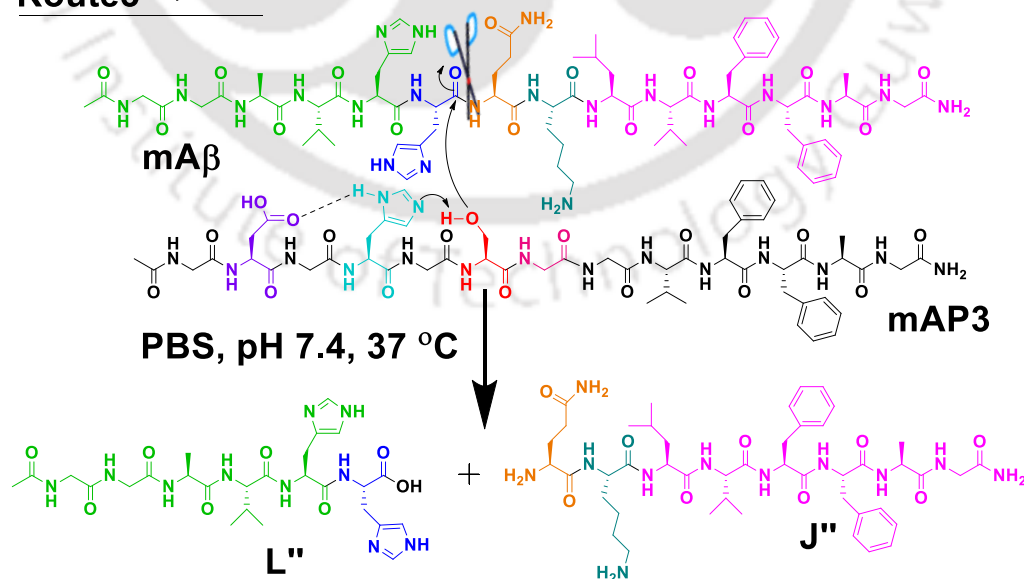
Route₃^{mA β -mAP3}

Figure 3.44. Plausible route for proteolysis of mA β by mAP3 into various fragments (Route 3^{mA β -mAP3}).

Similarly, mAP3 cleaved mA β in a site-selective manner between His-His to generate peptide fragments O'' and Q'' probably following Route 4^{mA β -mAP3} (Figure 3.45).

Route 4^{mA β -mAP3}

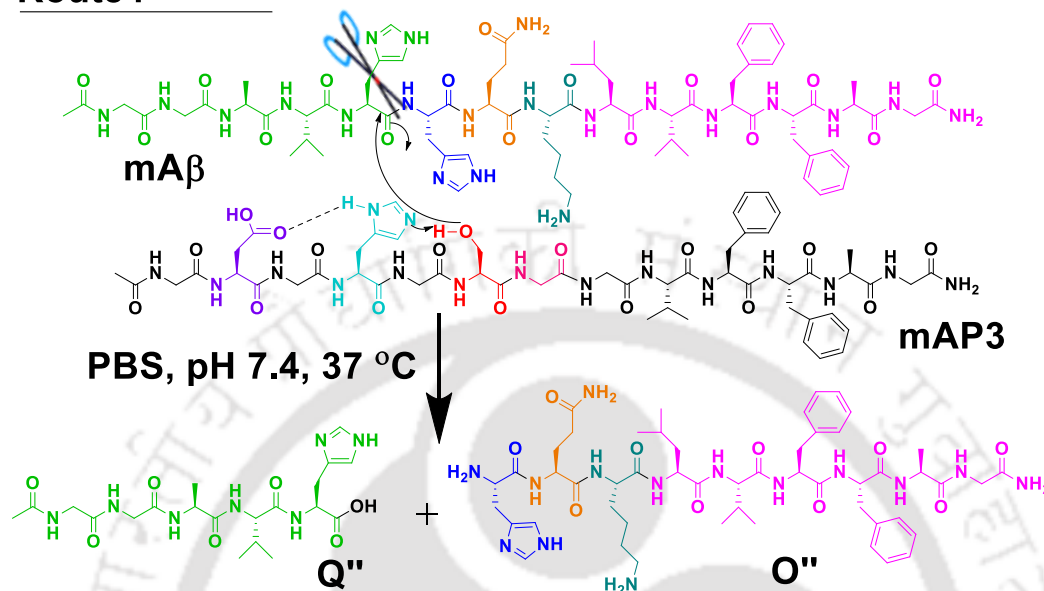


Figure 3.45. Plausible route for proteolysis of mA β by mAP3 into various fragments (Route 4^{mA β -mAP3}).

Similarly, mAP3 also cleaved mA β in a site-selective manner between Val-His to generate peptide fragment R'' probably following a reaction sequence as delineated in Route 5^{mA β -mAP3} (Figure 3.46).

Route 5^{mA β -mAP3}

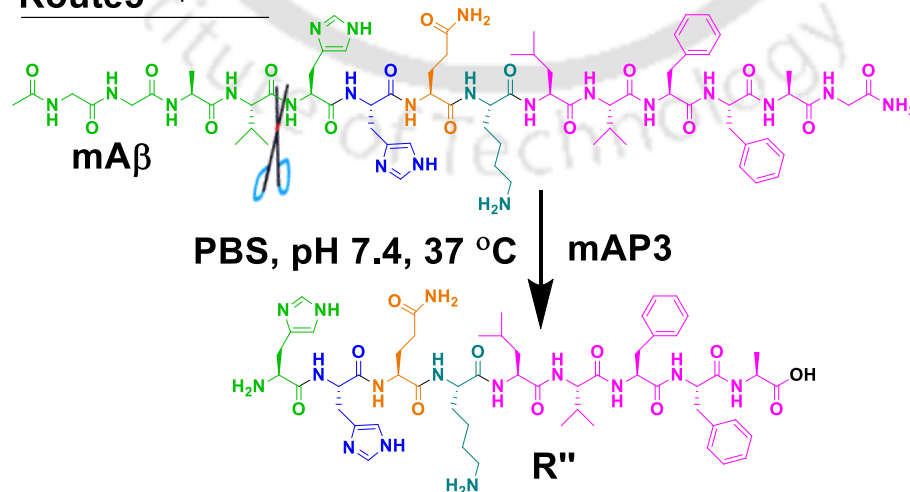


Figure 3.46. Plausible route for proteolysis of mA β by mAP3 into various fragments (Route 5^{mA β -mAP3}).

In a similar manner, mAP3 cleaved mA β between Leu-Val to generate peptide fragment S'' probably following the reaction pathway as depicted in Route 6^{mA β -mAP3} (Figure 3.47).

Route 6^{mA β -mAP3}

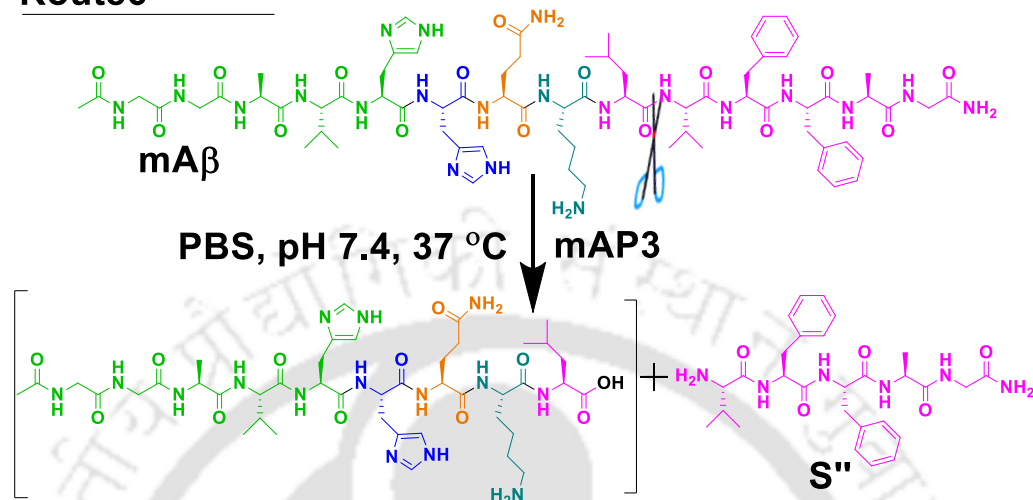


Figure 3.47. Plausible route for proteolysis of mA β by mAP3 into various fragments (Route 6^{mA β -mAP3}).

Also, mAP3 cleaved mA β in a site-selective manner between Val-Phe to generate peptide fragments T'' and U'' probably following Route 7^{mA β -mAP3} (Figure 3.48). Plausible routes were drawn concerning cleavage sites, but the plausible mechanism remains the same as described in-details in Route 1^{mA β -mAP3} for each of the routes.

Route 7^{mA β -mAP3}

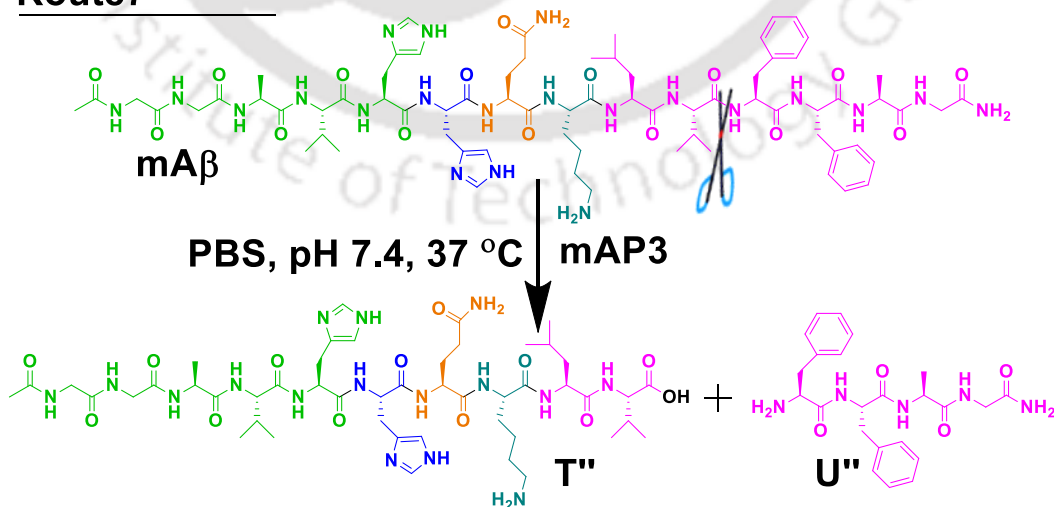


Figure 3.48. Plausible route for proteolysis of mA β by mAP3 into various fragments (Route 7^{mA β -mAP3}).

Similarly, mAP3 cleaved mA β in a site-selective manner between Phe-Phe to generate peptide fragment V'' probably following Route 8^{mA β -mAP3} (Figure 3.49). Plausible routes were drawn concerning cleavage sites, but the plausible mechanism remains the same as described in-details in Route 1^{mA β -mAP3} for each of the routes.

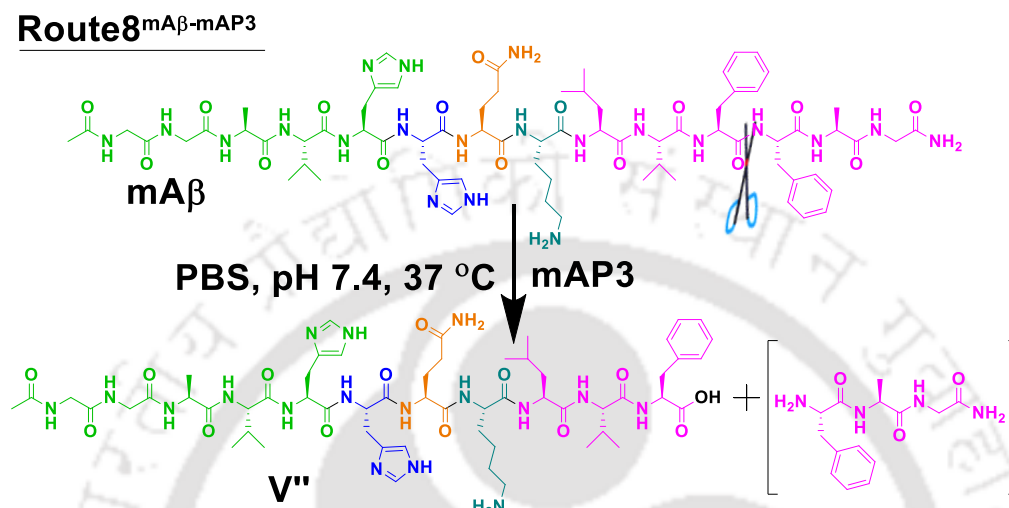


Figure 3.49. Plausible route for proteolysis of mA β by mAP3 into various fragments (Route 8^{mA β -mAP3}).

Similarly, mAP3 cleaved mA β in a site-selective manner between Phe-Ala to generate peptide fragment W'' probably following Route 9^{mA β -mAP3} (Figure 3.50).

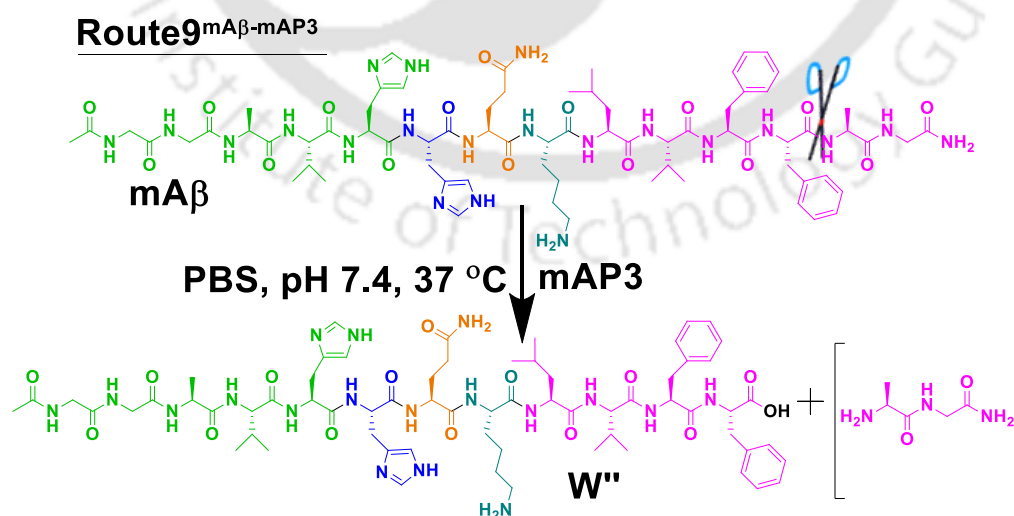


Figure 3.50. Plausible route for proteolysis of mA β by mAP3 into various fragments (Route 9^{mA β -mAP3}).

Most of the logically drawn intermediates and final fragments could be identified by time-dependent MALDI-TOF mass spectrometric analyses of the mAP3 incubated in the presence of mA β , except those in parentheses (Figure 3.51-3.55).

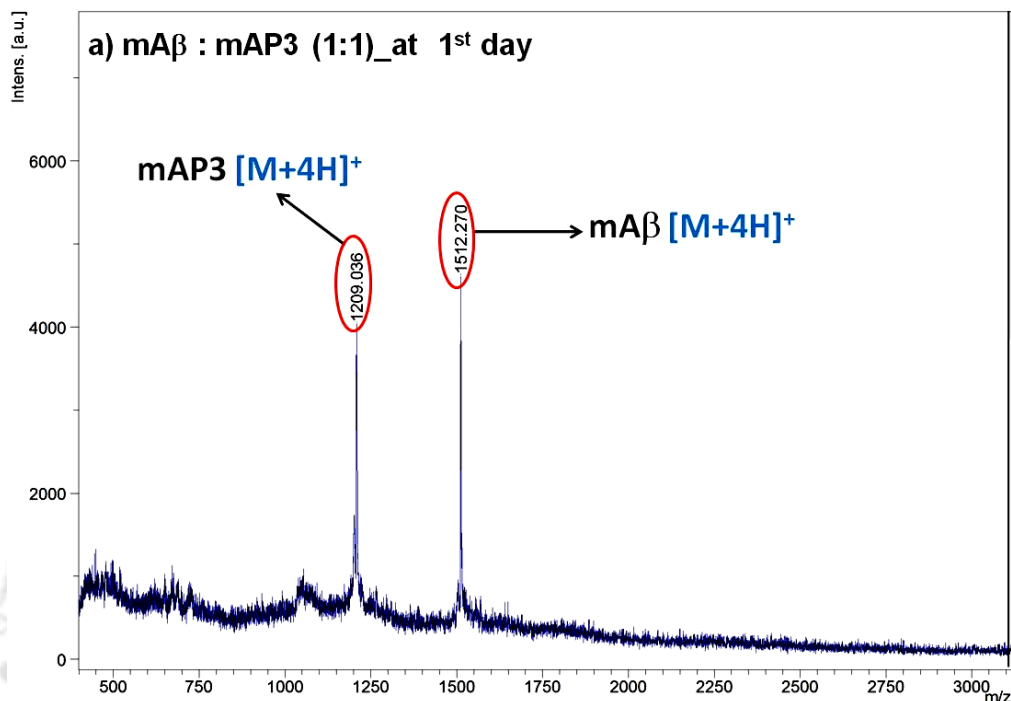


Figure 3.51. MALDI-TOF mass spectrum of mA β in the presence of mAP3 (1:1) at the 1st day.

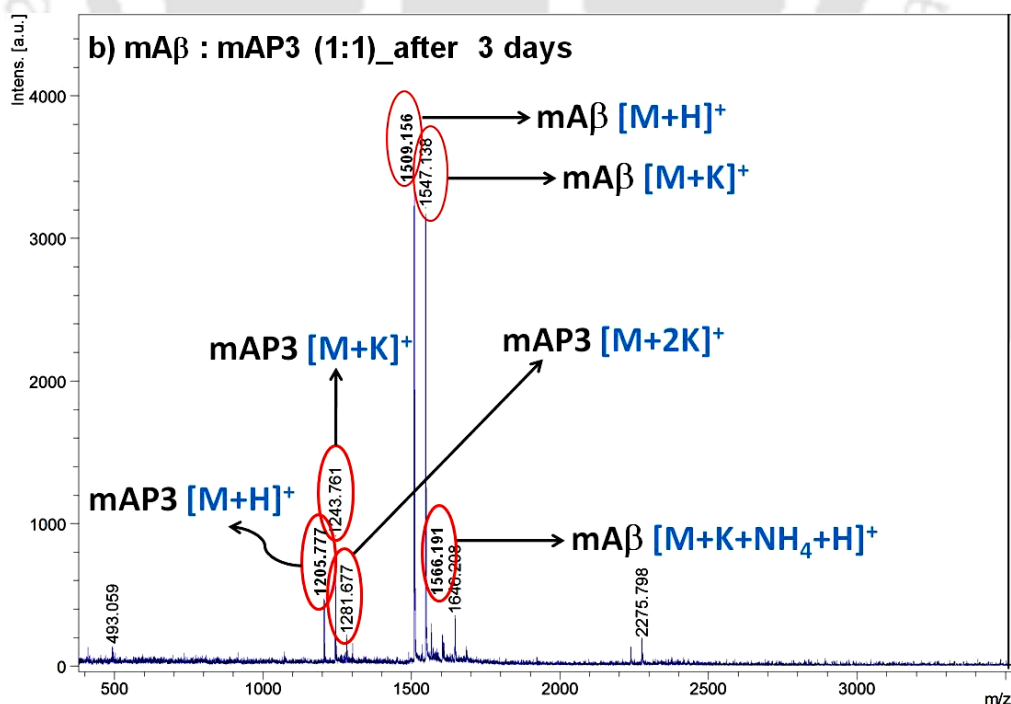


Figure 3.52. MALDI-TOF mass spectrum of mA β in the presence of mAP3 (1:1) after three days.

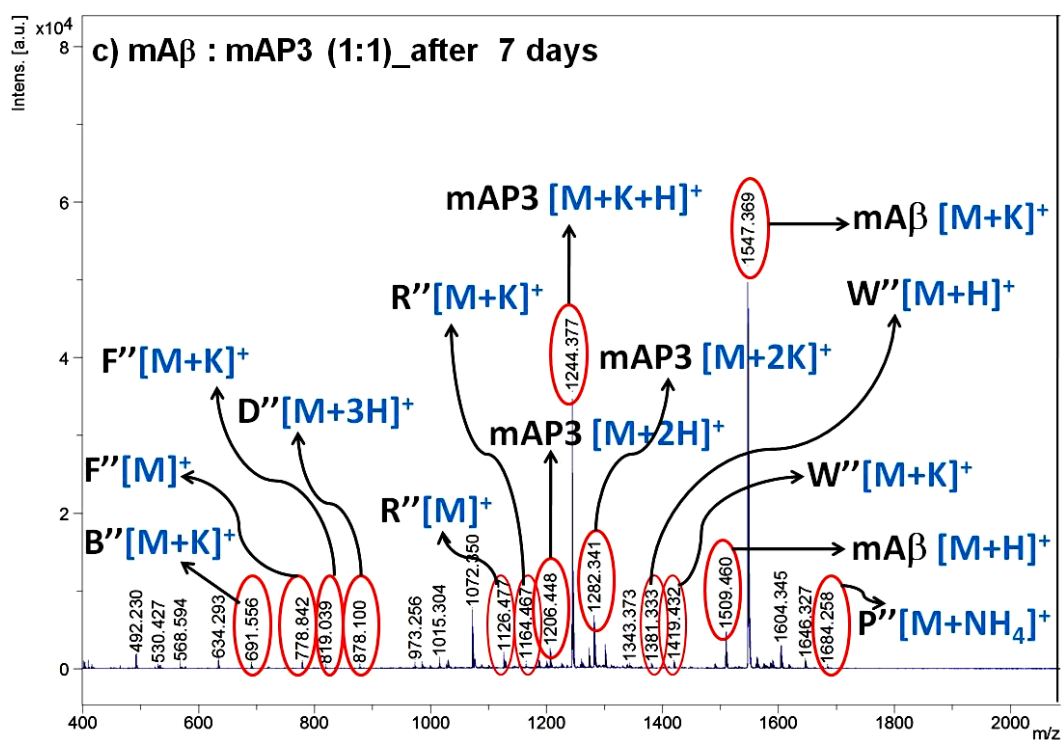


Figure 3.53. MALDI-TOF mass spectrum of mAP β in the presence of mAP3 (1:1) after seven days of incubation in PBS.

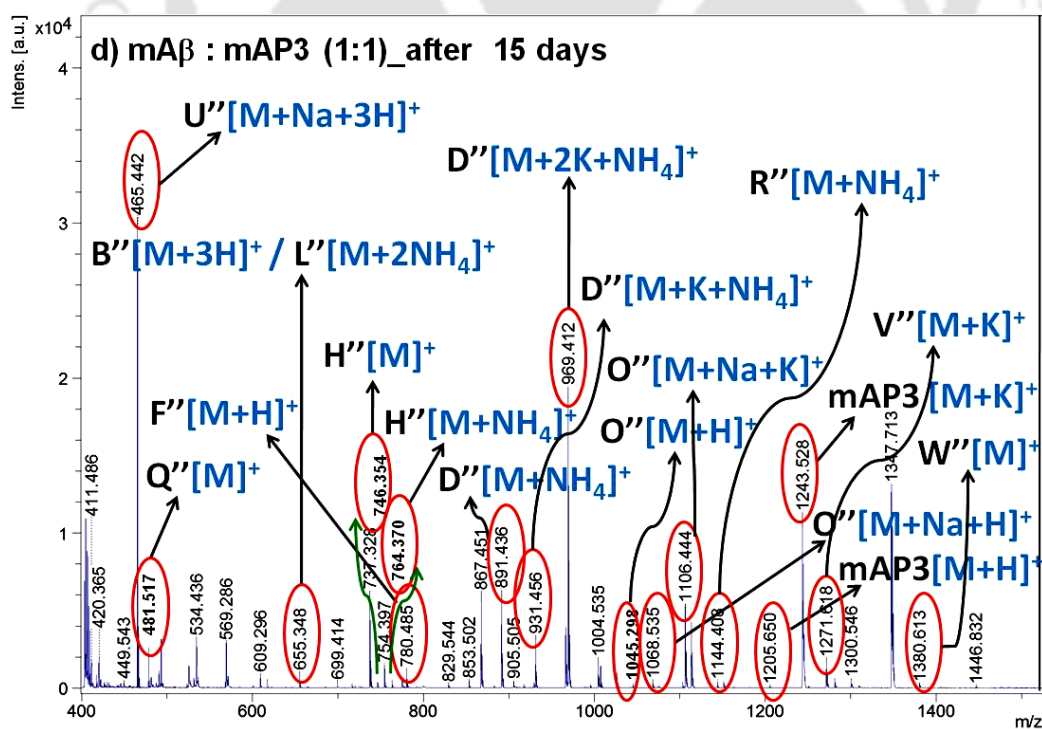


Figure 3.54. MALDI-TOF mass spectrum of mAP β in the presence of mAP3 (1:1) after fifteen days of incubation in PBS.

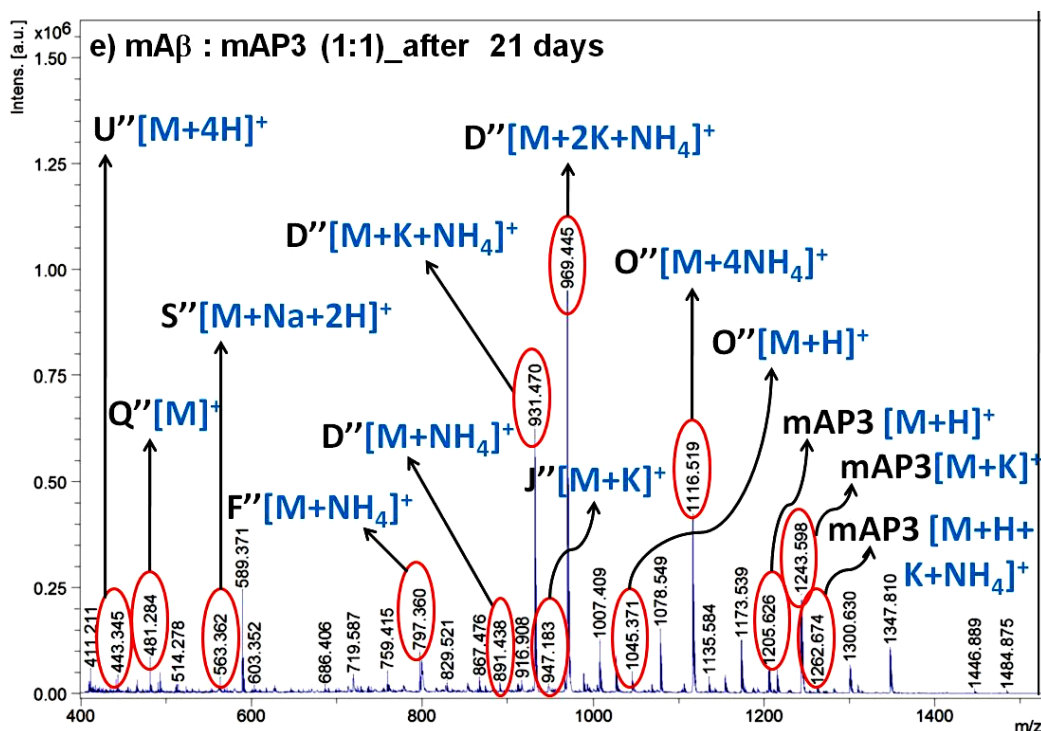


Figure 3.55. MALDI-TOF mass spectrum of mAbeta in the presence of mAP3 (1:1) after 21 days.

3.6. Time-dependent existence map of various fragments of mAbeta:

Though mAP2 and mAP3 cleaved mAbeta at the same sites in a similar fashion, the proteolytic reactivity of mAP3 was found to be better than that of mAP2; and it was confirmed from the time-dependent existence map of various fragments (Figure 3.56) and also from the intensity of mAbeta peak found from MALDI-TOF mass spectra.

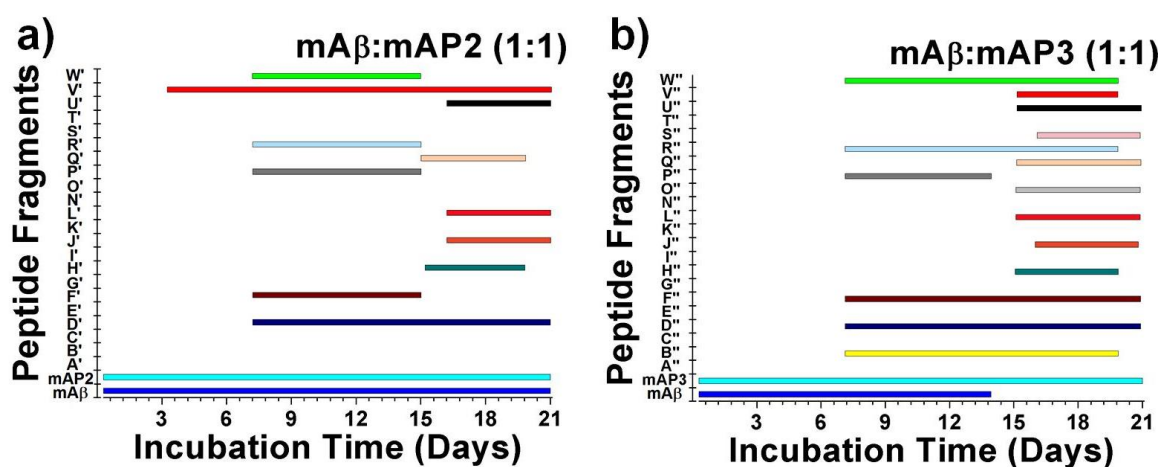


Figure 3.56. Time-dependent persistence map of various fragments of mAbeta in the presence of (a) mAP2, and (b) mAP3.

3.7. DFT calculation of the intermediate A'':

As we didn't find the mass (MALDI) for most of the intermediates, the most stable conformation of A'', one of the intermediates, was drawn after DFT calculation (Figure 3.57). A cage-like structure of A'' was obtained in which His proton (of mAP) is very close to the peptide's amide nitrogen (of mA β to be cleaved) to produce a short-lived protonated tetrahedral amine intermediate which further facilitated by the negative charge on oxygen to cleave the subsequent peptide bond of mA β .

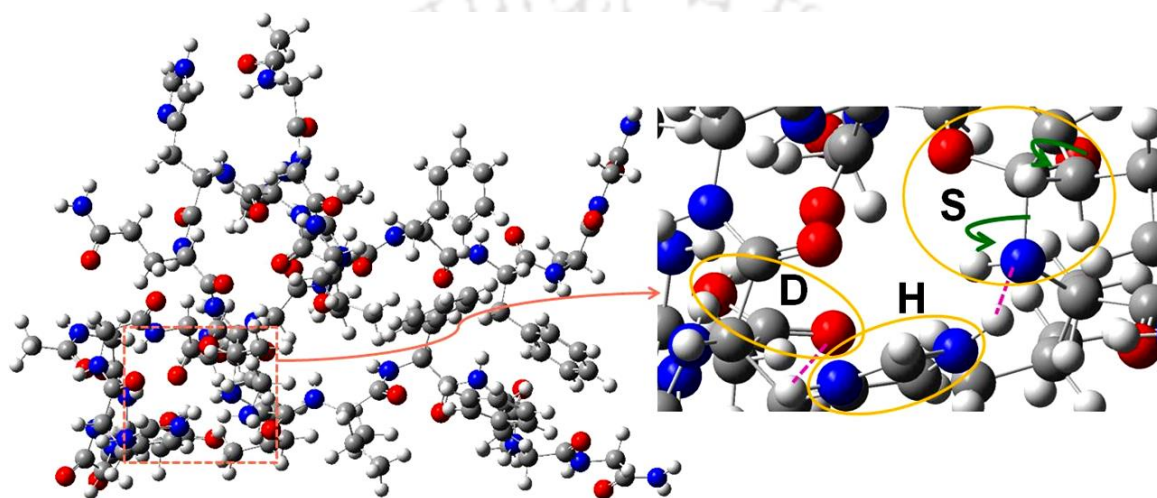


Figure 3.57. The most stable conformation of the intermediate A'' (Int_mA β -mAP3) [image generated by Gaussian 09W obtained from DFT calculation [Calculation Method: B3LYP, Basis Set: 6-31G, Total Energy -9336.72070930 a.u., RMS Gradient Norm 0.01447785 a.u., Dipole Moment 16.6163 Debye]. Nitrogen & Oxygen atoms are represented in blue & red, respectively.

3.8. HPLC kinetics study of mA β in the presence of mAP3 (1:1):

A time-dependent HPLC kinetics was performed (Figure 3.58) to investigate the proteolytic activity of mAP3 on mA β . As reactions were progressing with time, new peaks corresponding to various fragments were observed. The intensity of the peak [retention time (rt) 13.0 min] corresponding to mAP3 diminished with time but strongly appeared at ten days indicates its stability. The peak corresponding to mA β (rt 12.5 min) did not shift but reduced and became broader (extended to rt 12.6-12.8 min) in such a way that it looks like one peak climbed to the shoulder of another peak.

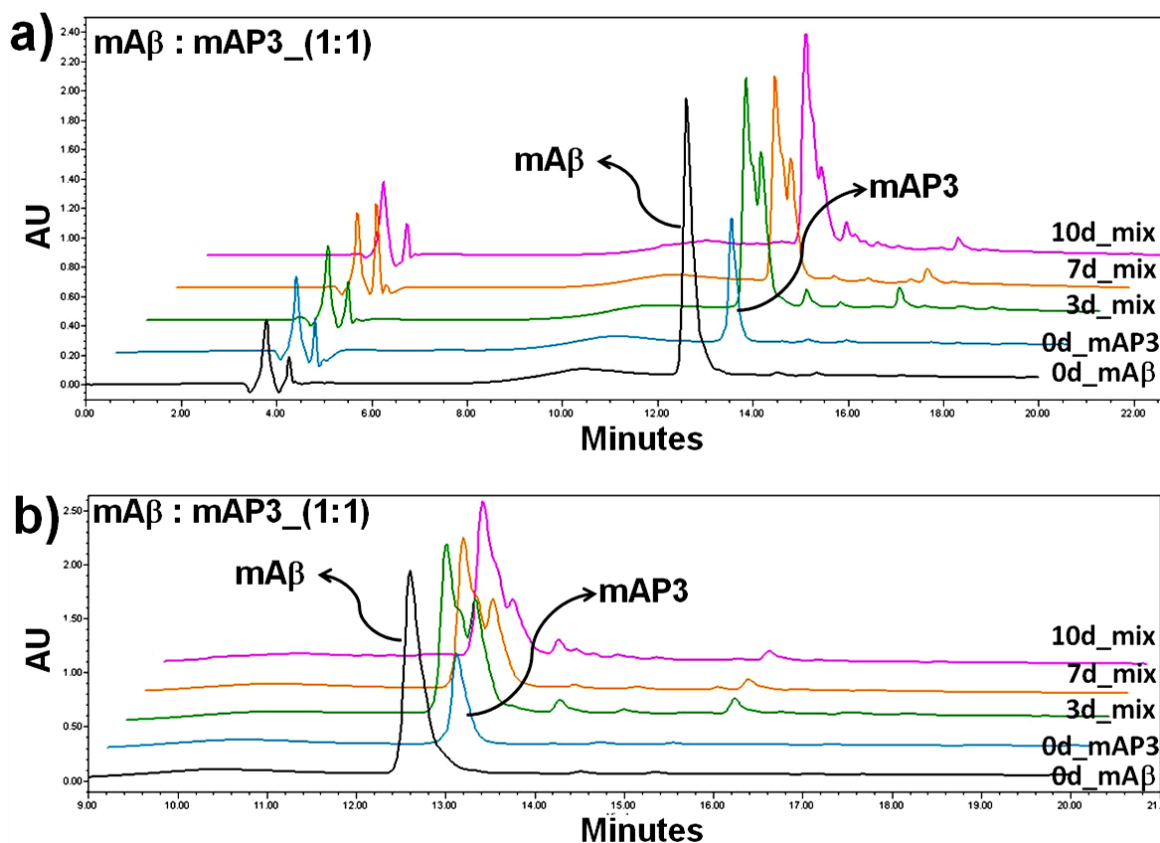


Figure 3.58. Time-dependent kinetics study of mAb β in the presence of mAP3 (1:1), up to ten days of incubation in PBS pH 7.4 at 37 °C by RP-HPLC (Kinetics study was done using Waters 600E analytical HPLC system. An Ascentis C18 analytical column, the flow rate of 0.8 ml/min, a linear gradient of 5-100% CH₃CN over 0-20 minutes in a total run time of 20 min were used. Dual-wavelength was selected at 214 nm and 254 nm).

The peak corresponding to rt 12.6-12.8 min were collected and further analyzed with MALDI-TOF mass (Figure 3.59-3.63) which revealed the existence of B'', D'', J'', L'' (after 3 days); B'', D'', F'', J'', Q'' (after 7 days); and B'', D'', F'', H'', J'', L'' (after 10 days). Also, mAb β and mAP3 were present at their same position (rt 12.5 min, and 13.0 min respectively). Though some unknown peaks at rt 13.4 min to 14.0 min were appeared but unable to identify them.

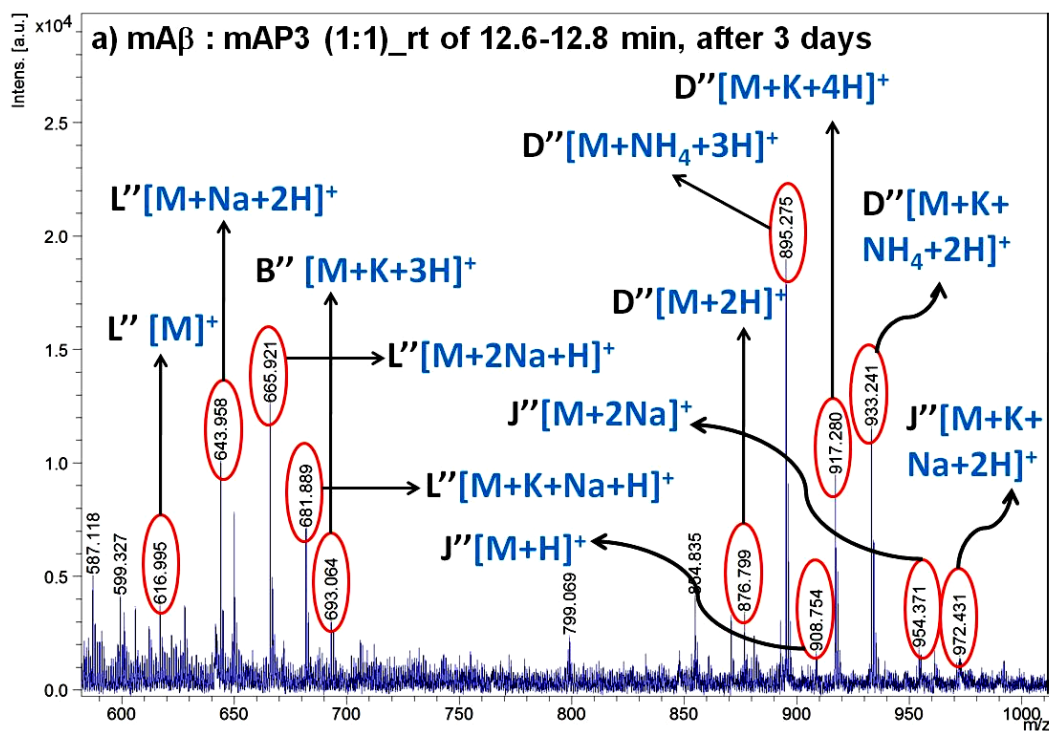


Figure 3.59. MALDI-TOF mass spectrum of the HPLC fragments corresponding to retention time of 12.6-12.8 min from the solution of mA β in the presence of mAP3 (1:1) after three days of incubation.

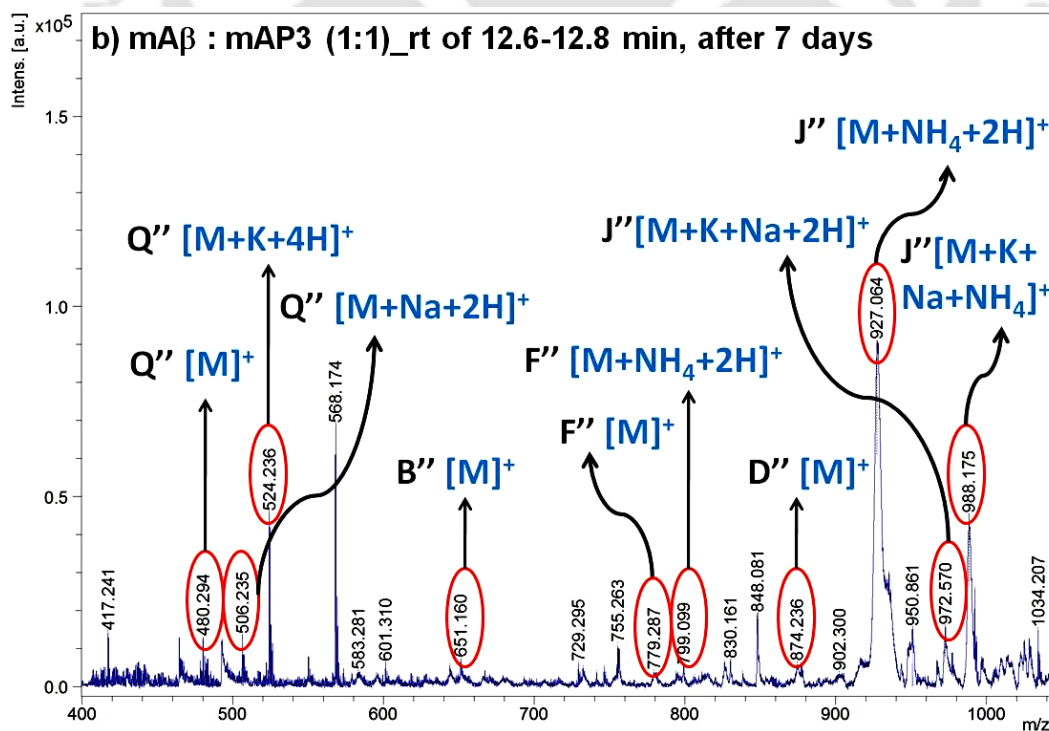


Figure 3.60. MALDI-TOF mass spectrum of the HPLC fragments corresponding to retention time of 12.6-12.8 min from the solution of mA β in the presence of mAP3 (1:1) after seven days of incubation.

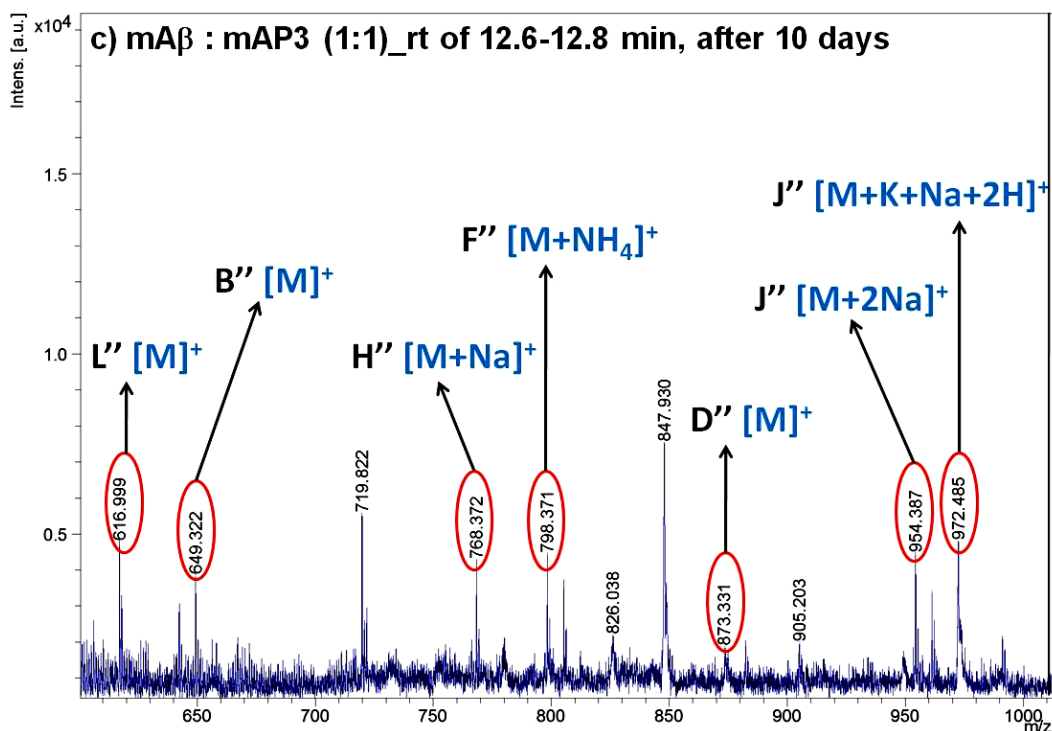


Figure 3.61. MALDI-TOF mass spectrum of the HPLC fragments corresponding to retention time of 12.6-12.8 min from the solution of mA β in the presence of mAP3 (1:1) after ten days of incubation.

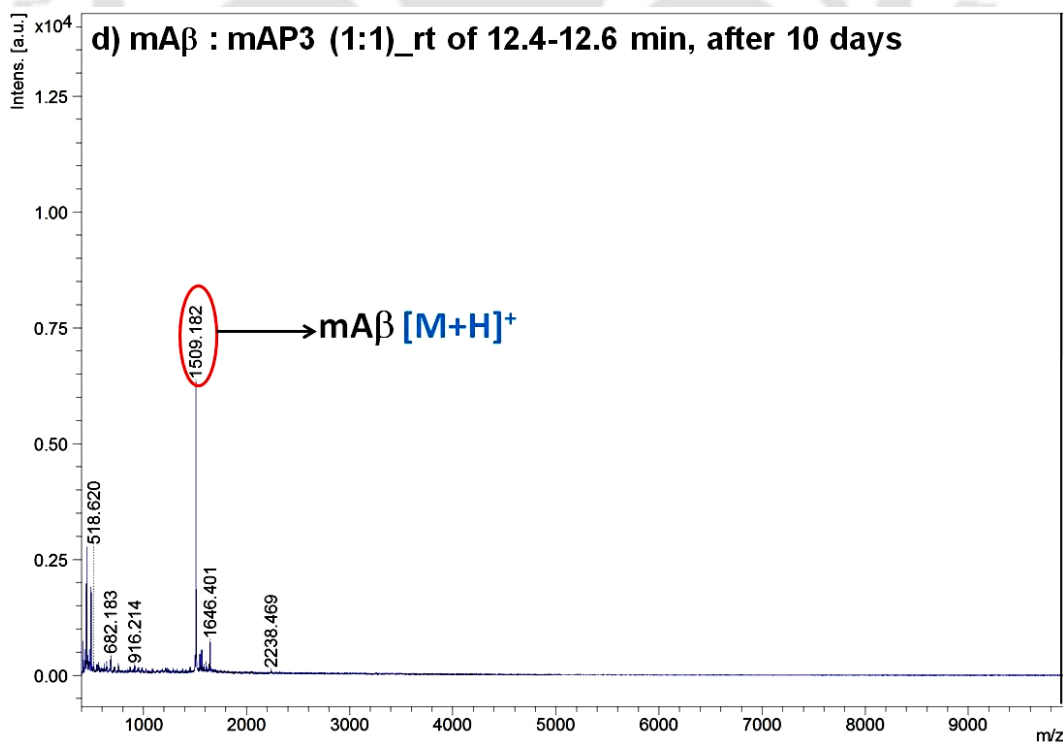


Figure 3.62. MALDI-TOF mass spectrum of the HPLC fragments corresponding to retention time of 12.4-12.6 min from the solution of mA β in the presence of mAP3 (1:1) after ten days of incubation.

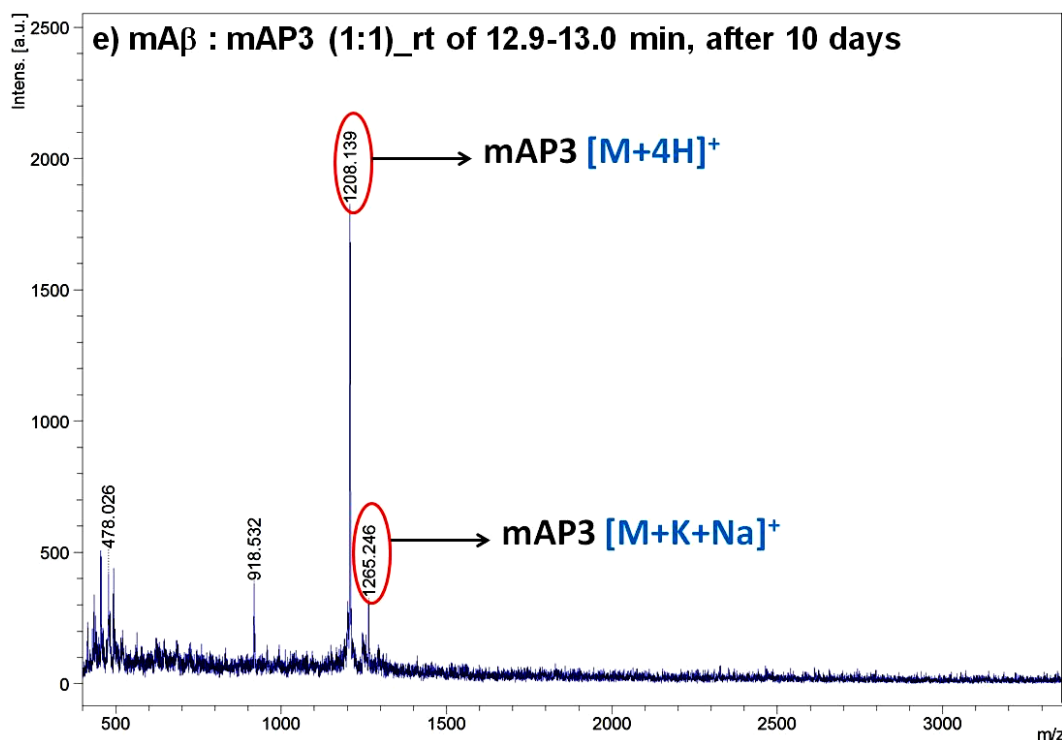


Figure 3.63. MALDI-TOF mass spectrum of the HPLC fragments corresponding to retention time of 12.9-13.0 min from the solution of mAb in the presence of mAP3 (1:1) after ten days.

3.9. Fluorescence resonance energy transfer (FRET) assay:

We have studied peptide degradation through FRET, and we designed the peptides accordingly. We designed and synthesized three derivatives of the Fluorescence labeled model Amyloid β (FmA β), FmA β 1, FmA β 2, and FmA β 3. Then we performed FRET assay. FmA β 1 contained a tryptophan (FRET donor) at C-terminus of the peptide, and N-terminus was kept free from the fluorophore. FmA β 2 contained a dansyl group (FRET acceptor) at N-terminus (opposite to the tryptophan in FmA β 1) of the peptide, and C-terminus was kept free from the fluorophore. We also synthesized FmA β 3, which contains both the donor and acceptor at C- and N-terminus of the peptide, respectively.

Table 3.3. List of fluorescence-labeled model Amyloid β (FmA β).

Sl. no.	Name	Peptide sequence	Role of the Peptide
1	FmA β 1	Ac-GGAVHHQKL VFFAG-W-NH ₂	FRET donor
2	FmA β 2	Dansyl-GGAVHHQKL VFFAG-NH ₂	FRET acceptor
3	FmA β 3	Dansyl-GGAVHHQKL VFFAG-W-NH ₂	FRET donor-acceptor

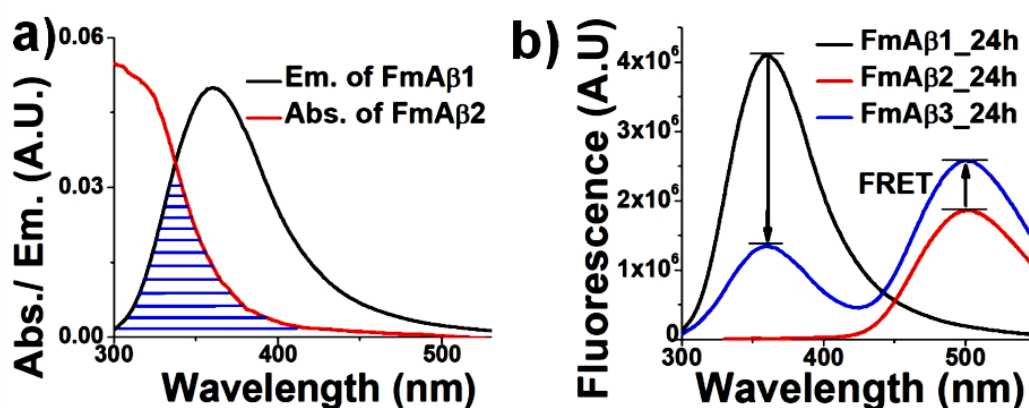


Figure 3.64. (a) Overlap of the emission spectrum of donor-peptide FmA β 1 (black) and the absorbance spectrum of acceptor-peptide FmA β 2 (red). (b) Fluorescence emission spectra of peptide FmA β 1 (black), FmA β 2 (red), and FmA β 3 (blue) after 24h of incubation. Spectra were recorded with 50 μ M solutions of the peptides in PBS (50 mM) pH 7.4. FmA β 1 and FmA β 3 were excited at 290 nm, whereas the peptide FmA β 2 was excited at 320 nm.

For the FRET study, we prepared $\sim$ 50 μ M solutions of FmA β 1-3 in PBS pH 7.4 (50 mM) and performed various photo-physical studies in the absence and presence of five-fold mAP3. The UV-visible and fluorescence spectra of the individual donor and acceptor revealed that the fluorescence spectrum of tryptophan in FmA β 1 (donor, Figure 3.64a) overlapped significantly with the absorption spectrum of dansyl in FmA β 2 (acceptor, Figure 3.64a). FmA β 3 contained both the donor (tryptophan) and acceptor (dansyl), which can be selectively excited at 290 nm (the absorbance maximum of the donor, FmA β 1) where there is very low absorbance of the acceptor (the absorbance maximum of dansyl, FmA β 2). Therefore, these two fluorophores (tryptophan and dansyl) should form a FRET pair in the designed peptide FmA β 3 as expected from the literature, and hence we proceeded for the FRET analysis.

To observe FRET, one of the important criteria is the energy transfer from the donor to the acceptor and the decrease of the fluorescence intensity of the donor, whereas the fluorescence intensity of the acceptor is expected to be increased. In figure 3.64b, we observed that after 24h of incubation, the fluorescence intensity of the donor (tryptophan in FmA β 3, $\lambda_{max}^{Em} = 360$ nm) in the presence of acceptor decreased from that of the emission of donor alone (FmA β 1, $\lambda_{max}^{Em} = 360$ nm) when it was excited at the maximum absorbance of the donor ($\lambda_{max}^{Abs} = 290$ nm). Simultaneously, the fluorescence intensity of

the acceptor (in the presence of donor) in FmA β 3 increased that of the acceptor (FmA β 2, $\lambda_{max}^{Em} = 500$ nm). This change in fluorescence intensity provided visual evidence of the FRET from the donor (tryptophan) to the acceptor (dansyl). But 72 h onwards, no such FRET was observed indicating the proteolytic degradation of the peptide FmA β 3 due to the presence of mAP3.

Time-dependent fluorescence emission spectra:

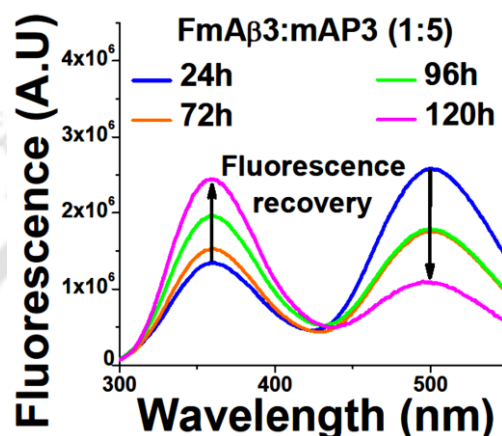


Figure 3.65. Time-dependent fluorescence emission spectra of peptide FmA β 3 after 24h (blue), after 72h (orange), after 96h (green), and after 120h (magenta) in the presence of five-fold mAP3. Spectra were recorded with 50 μ M solution of the peptide FmA β 3 in PBS (50 mM) pH 7.4. FmA β 3 was excited at 290 nm.

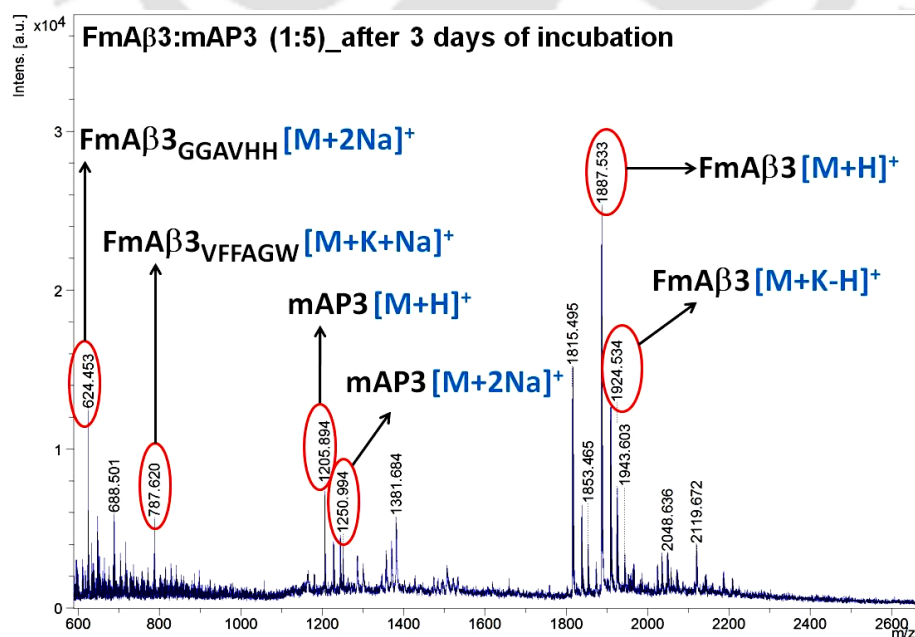


Figure 3.66. MALDI-TOF mass spectrum of FmA β 3 in the presence of five-fold mAP3 after three days of incubation in PBS pH 7.4 at 37 °C. mAP3 cleave FmA β 3 in-between Leu₁₇-Val₁₈ to produce FmA β 3_{VFFAGW}. mAP3 also cleave FmA β 3 in-between His₁₄-Gln₁₅ to produce FmA β 3_{GGAVHH}.

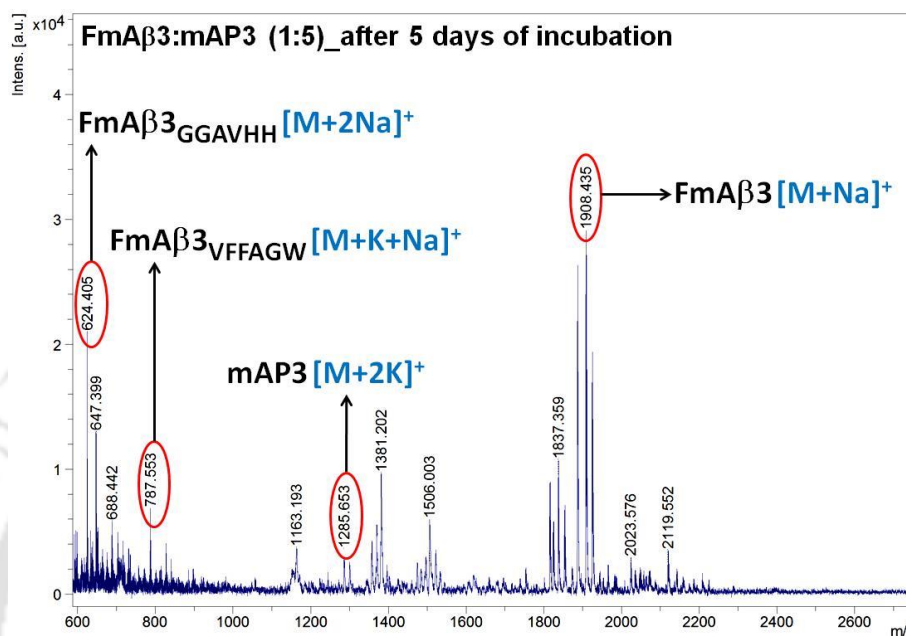
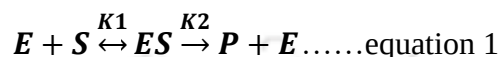


Figure 3.67. MALDI-TOF mass spectrum of FmA β 3 in the presence of five-fold mAP3 after five days of incubation in PBS pH 7.4 at 37 °C. mAP3 cleave FmA β 3 in-between Leu₁₇-Val₁₈ to produce FmA β 3_{VFFAGW}. mAP3 also cleave FmA β 3 in-between His₁₄-Gln₁₅ to produce FmA β 3_{GGAVHH}.

3.10. Kinetics of the mAP3-catalyzed hydrolysis of p-Nitrophenyl Acetate (NPA):

The rate of an enzymatic reaction depends on many factors such as pH, temperature, and substrate concentration. The pH of the medium affects protein conformation mainly when the enzyme's active site contains such a residue which become protonated or deprotonated at different pH. With increasing temperature (up to certain limit beyond which the enzyme becomes denatured), molecular motion and hence collisions between enzyme and substrate increases. In the presence of more substrate molecules, the enzyme molecules will be able to collide more quickly to bind with them. The catalytic activity of mAP3 was determined in PBS buffer (containing 2 vol% ethanol) of pH 7.0 at 25 °C in terms of the Michaelis–Menten model, by monitoring the increase in absorbance at 400 nm caused by the liberation of p-nitrophenol in mAP3-catalyzed hydrolysis (equation 1) of p-nitrophenyl acetate (NPA) using UV-visible absorption (Figure 3.68) based on the protocol described in the literature.¹⁰ The concentration of NPA was one mM. Kinetic

measurements of NPA cleavage were calculated from the initial velocity (V_0) of mAP3 as a function of three different concentrations of mAP3 (E). The initial velocity (V_0) of the reaction, in millimoles of p-nitrophenol produced/minute, was calculated from the increase in absorbance at 400 nm measured at specific time intervals during the reaction. Michaelis constant (K_m) was determined using the Lineweaver–Burk double-reciprocal plot of $1/V$ versus $1/[E]$ (equation 2).



$$\frac{1}{V} = \frac{1}{K2[S_0]} + \frac{K_m}{K2[S_0][E]} \dots\dots \text{equation 2}$$

Michaelis constant (K_m) is an important catalytic parameter, because K_m is a means of characterizing an enzyme's affinity for a substrate, and can provide a quantitative estimate of the functionality of enzymes. With the change in conformation of an enzyme, the active site becomes pretty messed up, and the change in activity can be represented quantitatively by the change in K_m value. Figure 3.69b shows the Lineweaver-Burk plot, which has excellent linearity and the slopes give $K_m = 4.22 \times 10^2$ M. The Turn Over Number (T.O.N.) was calculated as moles hydrolyzed/min/moles of the enzyme. So, T.O.N. = $0.00202/0.002 = 1.01$ [As assay volume is 2 mL, no. of moles of mAP3 = $(1 \text{ mol/L}) \times 2 \times 10^{-3} \text{ L}$]. These results indicate that the catalytic efficacy of the mAP3 is comparable to the other such reported artificial amide hydrolytic enzymes.¹¹

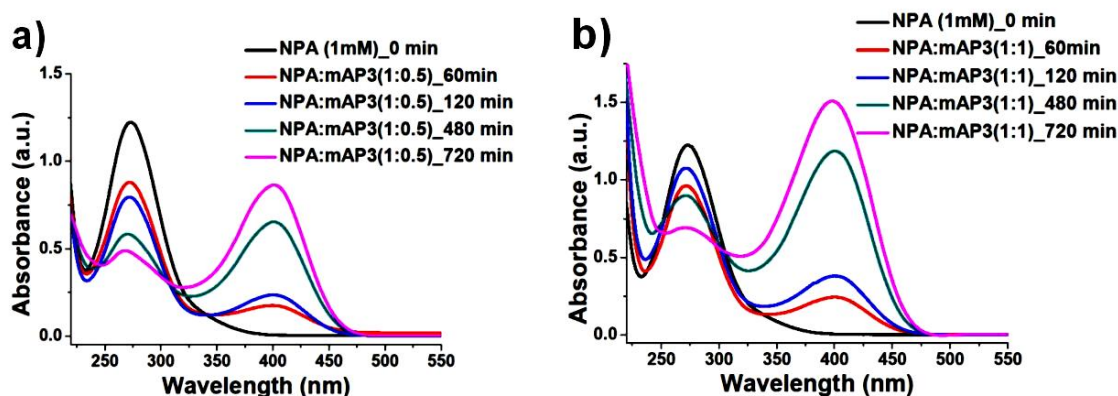


Figure 3.68. UV-visible absorption of NPA in the presence of a) 0.5 eq. and b) 1 eq of mAP3 in PBS buffer (containing 2 vol% ethanol) of pH 7.0 at 25 °C.

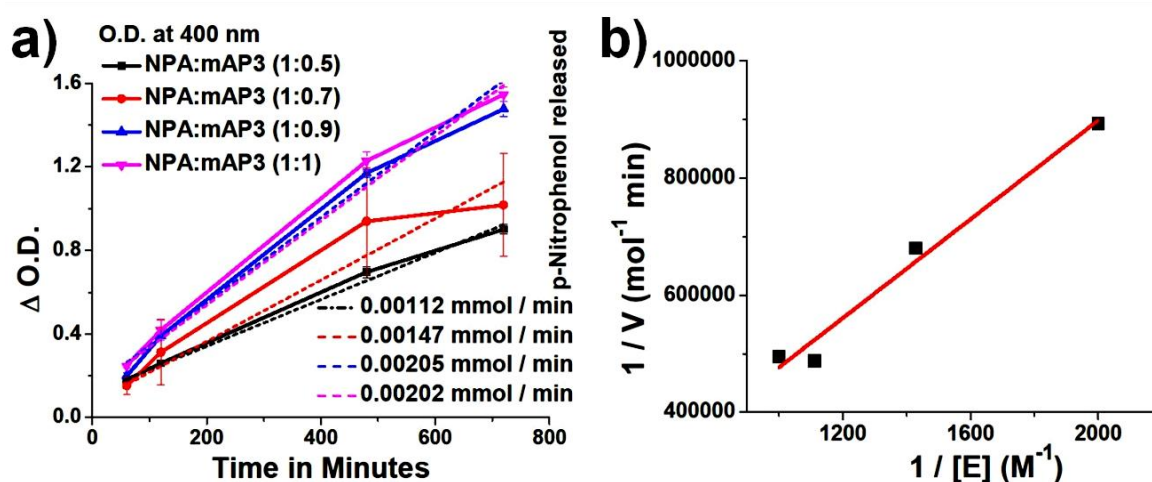


Figure 3.69. (a) The relationship between the increase in absorbance at 400 nm and time in the hydrolysis of NPA by mAP3 in PBS buffer (containing 2 vol% ethanol) of pH 7.0 at 25 °C. (b) The Lineweaver-Burk plot of mAP3-catalyzed hydrolysis of NPA.

3.11. Inhibition of amyloid accumulation of mA β by mAPs:

After finding the proteolytic activity of mAPs, we proceeded further to check the inhibition potential of mAPs. The kinetics of the amyloid accumulation of mA β was checked using different biophysical tools in absence and presence of a varied amount of mAPs.¹² In the beginning, the purified solid peptide samples were dissolved in PBS (50 mM, pH 7.4) completely, after consecutive treatment with TFA and HFIP followed by complete evaporation by purging nitrogen gas, to obtain the stock solutions of mA β (200 μ M) and mAPs (1 mM). mAPs were co-incubated with mA β in PBS pH 7.4 at 37 °C in separate vessels on a water bath till 30 days in parallel at the desired proportions. To investigate dose-dependent inhibitory efficacy of mAP3, equivalent amount and a five-fold molar excess of mAP3 were co-incubated with 200 μ M of mA β in PBS (50mM, pH 7.4) at 37 °C *in vitro*. To get a result more precisely, another two replicate solutions of each portion were prepared, and amyloid accumulation was monitored up to 30 days by different biophysical tools.

3.11.1. ThT fluorescence assay of mA β :

The fluorescent dye Thioflavin-T (ThT) is used to monitor amyloid fibril formation *in vitro* because of the large enhancement of its fluorescence emission (at around 485 nm when excited at 445 nm) upon binding to amyloid fibrils, and a linear correlation was

reported between the ThT fluorescent intensity with amyloid concentration over a wide range of ThT concentrations.

At first, dose-dependent ThT fluorescence assay of $\text{mA}\beta$ was performed with mAP3 for its prominent proteolytic activity among all mAPs . From the ThT assay (Figure 3.70a), it was found that the fluorescence intensity of $\text{mA}\beta$ (black, Figure 3.70a) increased with time in the absence of mAPs and became static after seven days after slight lowering in intensity. But in the presence of mAP3 , the fluorescence intensity of the $\text{mA}\beta$ decreased with time as the doses were increased from one-fold (red, Figure 3.70a) to five-fold (blue, Figure 3.70a) molar excess due to the formation of various $\text{mA}\beta$ fragments cleaved by mAP3 , indicating the excellent inhibitory efficacy of mAP3 against aggregation of $\text{mA}\beta$ (Figure 3.70a). In the case of mAP3 alone (dark cyan, Figure 3.70a), the fluorescence intensity was not increased with time implying mAP3 as a non-aggregating peptide. Next, a time-dependent ThT fluorescence assay of $\text{mA}\beta$ was performed with mAP1 - mAP3 (Figure 3.70b). From the result, it was clear that mAP1 (red, Figure 3.70b) is not efficient to inhibit the amyloid formation of $\text{mA}\beta$, as no such differences in fluorescence intensity were observed compare to $\text{mA}\beta$ alone. But five-fold molar excess of mAP2 (blue, Figure 3.70b) was found to inhibit $\text{mA}\beta$ aggregates with time. In case of mAP1 alone (magenta, Figure 3.70b), the fluorescence intensity was increased very slightly probably with time due to self-assembling VFFA segment; but no such increase in fluorescence intensity was observed in case of mAP2 alone (dark yellow, Figure 3.70b) indicating its non-aggregating nature. In conclusion, five-fold mAP2 or one-fold mAP3 have good inhibitory efficacy, but the more pronounced result was found in the case of five-fold mAP3 to inhibit $\text{mA}\beta$ aggregates.

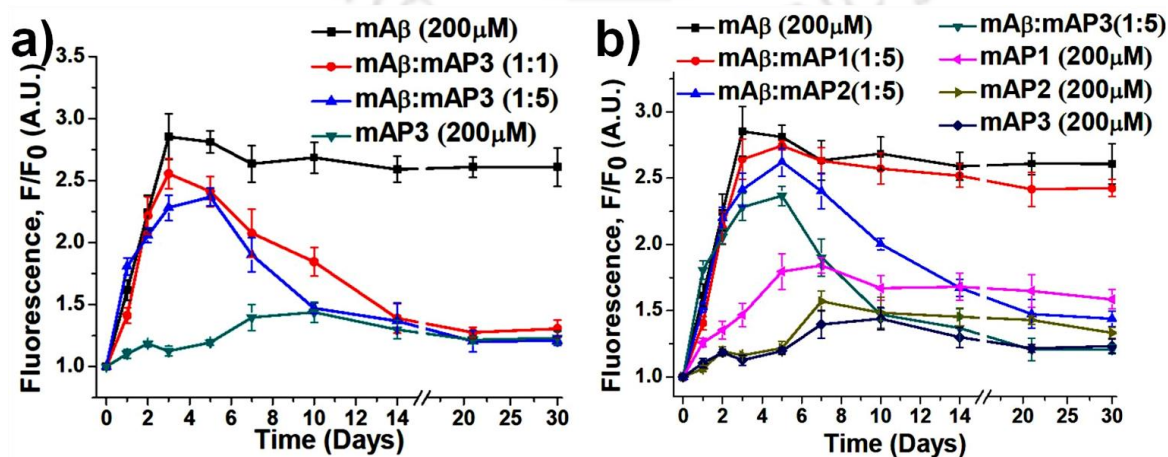


Figure 3.70. Normalized profiles of (a) dose-dependent, and (b) time-dependent ThT fluorescence assay. (a) Spectra of mA β in the absence (black), presence of one-fold mAP3 (red), five-fold mAP3 (blue), and mAP3 alone (dark cyan). (b) Spectra of mA β in absence (black), presence of five-fold mAP1 (red), five-fold mAP2 (blue), five-fold mAP3 (dark cyan), mAP1 alone (magenta), mAP2 alone (dark yellow), and mAP3 alone (navy blue). Error bars represent standard deviations of at least three independent measurements. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

3.11.2. Circular dichroism (CD) experiments of mA β :

Circular dichroism (CD) is the most important and validated technique to analyse the secondary and tertiary structure and conformation of protein molecules.¹³ The conformational changes of mA β (Figure 3.71a) were noticed from random coil (a negative band at ~202 nm) to α -helix (two negative bands at ~205 nm and ~230 nm) to finally β -sheet (a positive band at ~202 nm with a negative band at ~223 nm) structure over time, confirmed the aggregation process. Time-Dependent conformational changes of mAP1 (Figure 3.71b) from the random coil (a negative band at ~205 nm) to a mixture of mainly α -helix and β -sheet (a positive band at ~192 nm with two negative bands at ~205 nm and ~225 nm) structure were observed. Replacement of alanine of the DHS triad segments of mAP1 into glycine results in conformational changes of mAP2 (Figure 3.71c) over time, from a mixture of more α -helix and random coil into a mixture of predominantly random coil and α -helix conformation. Incorporation of another glycine in-between the DHS triad segments and the recognition motif of mAP2 results in time-dependent conformational changes of mAP3 (Figure 3.71d), from α -helix into a mixture of predominantly α -helix and random coil conformation. The presence of one-fold mAP3 inhibited fibril formation of mA β (Figure 3.71e) over time, and a mixture of more α -helices and random coil conformations were obtained throughout the kinetics.

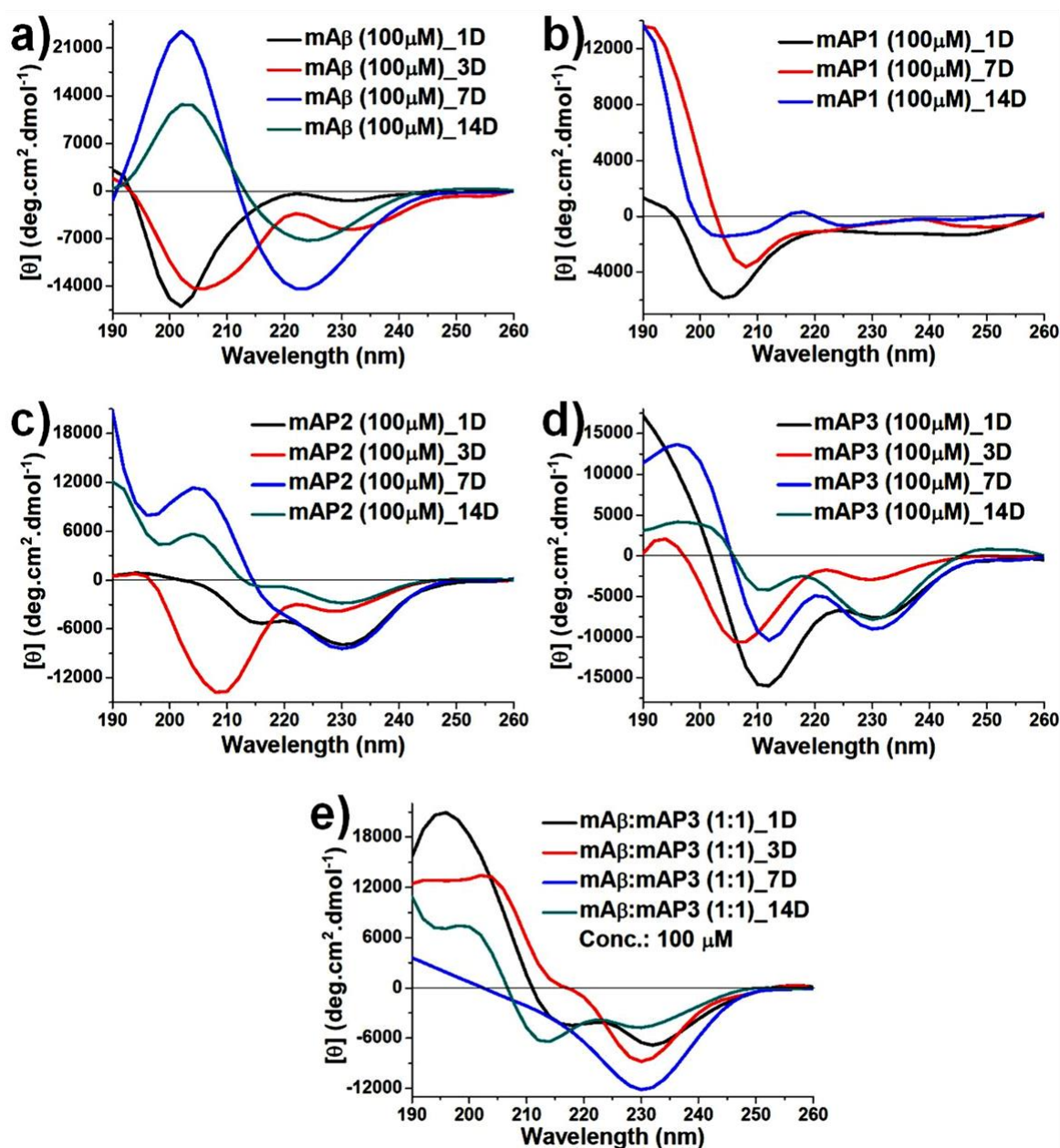


Figure 3.71. Time-dependent CD experiments of (a) mAP alone, (b) mAP1 alone, (c) mAP2 alone, (d) mAP3 alone, and (e) of mAP β in the presence of one-fold mAP3. (a) Conversion of mAP β from the random coil into β -sheet structure. (e) Inhibition capability of mAP3 to prevent the β -sheet structure of mAP β . Spectra were recorded at 1st day (black), after three days (red), after seven days (blue), and after fourteen days (dark cyan) of incubation. The peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C.

Already it was demonstrated that mAP β fibrils adopt a β -sheet conformation (black, Figure 3.72). In the presence of one-fold mAP3 (red, Figure 3.72a), the propensity of mAP β to form an α -helix (a positive band at ~200 nm with two negative bands at ~213 nm and

~ 228 nm) was increased, which confirms the reduction in aggregation. Random coil conformation of mA β (blue, Figure 3.72a) was confirmed (a negative band at ~ 204 nm) in the presence of five-fold molar excess of mAP3. Indeed, CD spectra confirmed the formation of more α -helices and reduced formation of β -sheets (a positive band at ~ 193 nm with two negative bands at ~ 212 nm and ~ 232 nm) for mA β (red, Figure 3.72b) when co-incubated along with five-fold mAP2. The other peptides also display other secondary structures described as earlier (Figure 3.71).

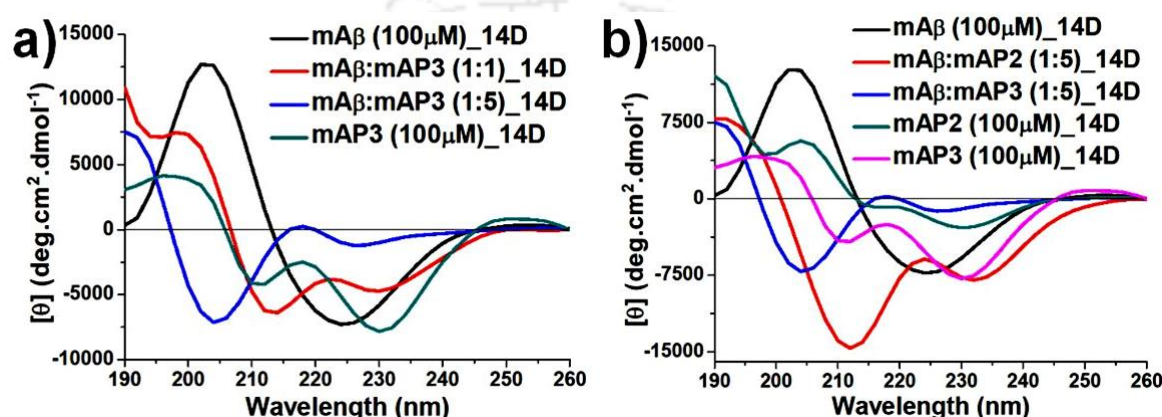


Figure 3.72. CD spectra of mA β in absence and presence of mAPs after 14 days of incubation. (a) Spectra of mA β in the absence (black), presence of one-fold mAP3 (red), five-fold mAP3 (blue), and mAP3 alone (dark cyan). (b) CD Spectra of mA β in the absence (black), presence of five-fold mAP2 (red), five-fold mAP3 (blue), mAP2 alone (dark cyan), and mAP3 alone (magenta). The peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C.

3.11.3. FTIR spectra of mA β :

The secondary structure of the peptides was monitored by the Fourier transformation infrared (FTIR) spectroscopic analysis. In FTIR spectra, the amide I band was observed (a) at 1634 cm⁻¹ for mA β alone (Figure 3.73a), (b) at 1644 cm⁻¹ for mA β in presence of one-fold mAP3 (Figure 3.73b), (c) at 1651 cm⁻¹ for mA β in presence of five-fold mAP3 (Figure 3.73c), (d) at 1656 cm⁻¹ for mAP3 alone (Figure 3.73d), (e) at 1641 cm⁻¹ for mA β in the presence of five-fold mAP1 (Figure 3.73e), (f) at 1648 cm⁻¹ for mA β in presence of five-fold mAP2 (Figure 3.73f), (g) at 1651 cm⁻¹ for mAP1 alone (Figure 3.73g), and (h) at 1652 cm⁻¹ for mAP2 alone (Figure 3.73h). These results indicated β -sheet rich structure for mA β alone and mA β along with mAP1; but non- β -sheet conformations mainly random coil or α -helix for rest of the peptides.

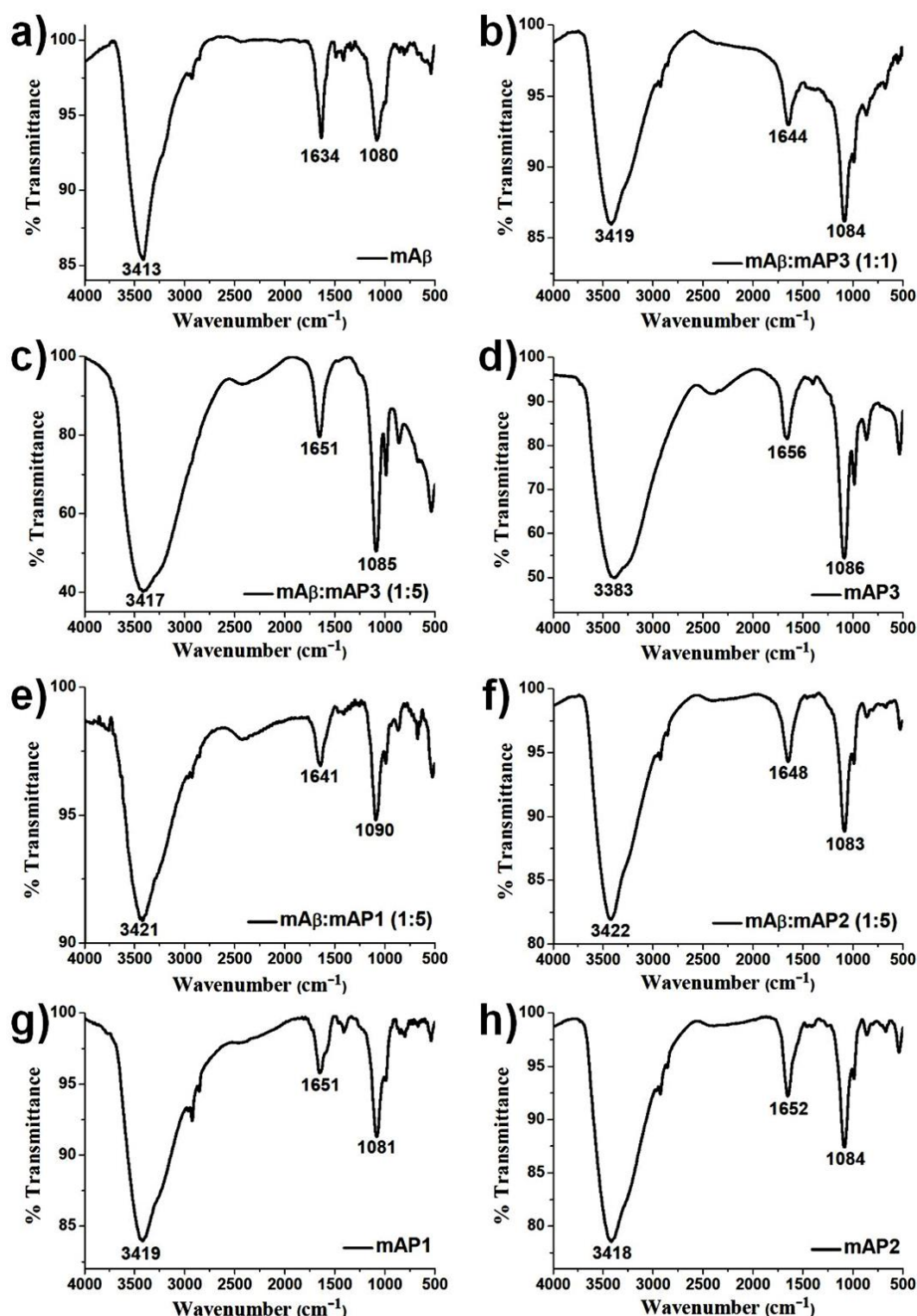


Figure 3.73. FTIR spectra of mAb β in the absence and presence of mAPs after fourteen days of incubation. FTIR Spectra of mAb β (a) in the absence, presence of (b) one-fold mAP3, (c) five-fold mAP3, and (d) mAP3 alone. FTIR Spectra of mAb β in presence of (e) five-fold mAP1, (f) five-fold mAP2, (g) mAP1 alone, and (h) mAP2 alone. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C.

3.11.4. TEM images and Congo red stained birefringence images of amyloid fibrils formed by mA β :

The presence of fibre, when viewed under the transmission electron microscope (TEM), is a characteristic property of amyloid formed by a peptide. The mA β formed clear fibrillar aggregates in the absence of mAPs (Figure 3.74. a-i) under TEM, but in the presence of one-fold mAP3 (Figure 3.74. a-ii) and five-fold mAP3 (Figure 3.74. a-iii), no such fibre was observed rather amorphous structures were noticed. The mAP3 alone also did not show any fibre (Figure 3.74. a-iv). Though fibre was detected from the solution of mA β along with five-fold mAP1 (Figure 3.74. c-i), amorphous structures along with very few tiny fibres were found for mA β along with five-fold mAP2 (Figure 3.74. c-ii). The mAP1 (Figure 3.74. c-iii) and mAP2 (Figure 3.74. c-iv) alone also did not show any fibre like mAP3. Therefore it can be concluded that mAPs are non-aggregating peptides, but among them, mAP2 and mAP3 can inhibit aggregation of mA β .

The expression of green gold birefringence of a peptide under cross-polarised light after staining with Congo red dye is another characteristic property of amyloid present in the sample. The clear green-gold birefringence was observed for mA β alone under cross-polarized light (Figure 3.74. b-i), but when mA β was incubated in the presence of one-fold mAP3 (Figure 3.74. b-ii) and five-fold mAP3 (Figure 3.74. b-iii) separately, no such green gold birefringence were observed. Such characteristic birefringence was not shown by mAP3 alone (Figure 3.74. b-iv). Though in the presence of five-fold mAP1, mA β showed green gold birefringence (Figure 3.74. d-i); but such characteristic birefringence was not observed either for mA β along with five-fold mAP2 (Figure 3.74. d-ii), or for the mAP1 (Figure 3.74. d-iii) and mAP2 (Figure 3.74. d-iv) alone when viewed under cross-polarized light. These results support that mAPs are non-amyloidogenic peptides, and both mAP2 and mAP3, unlike mAP1, significantly inhibited amyloid aggregation of mA β . The images were taken after fourteen days of incubation.

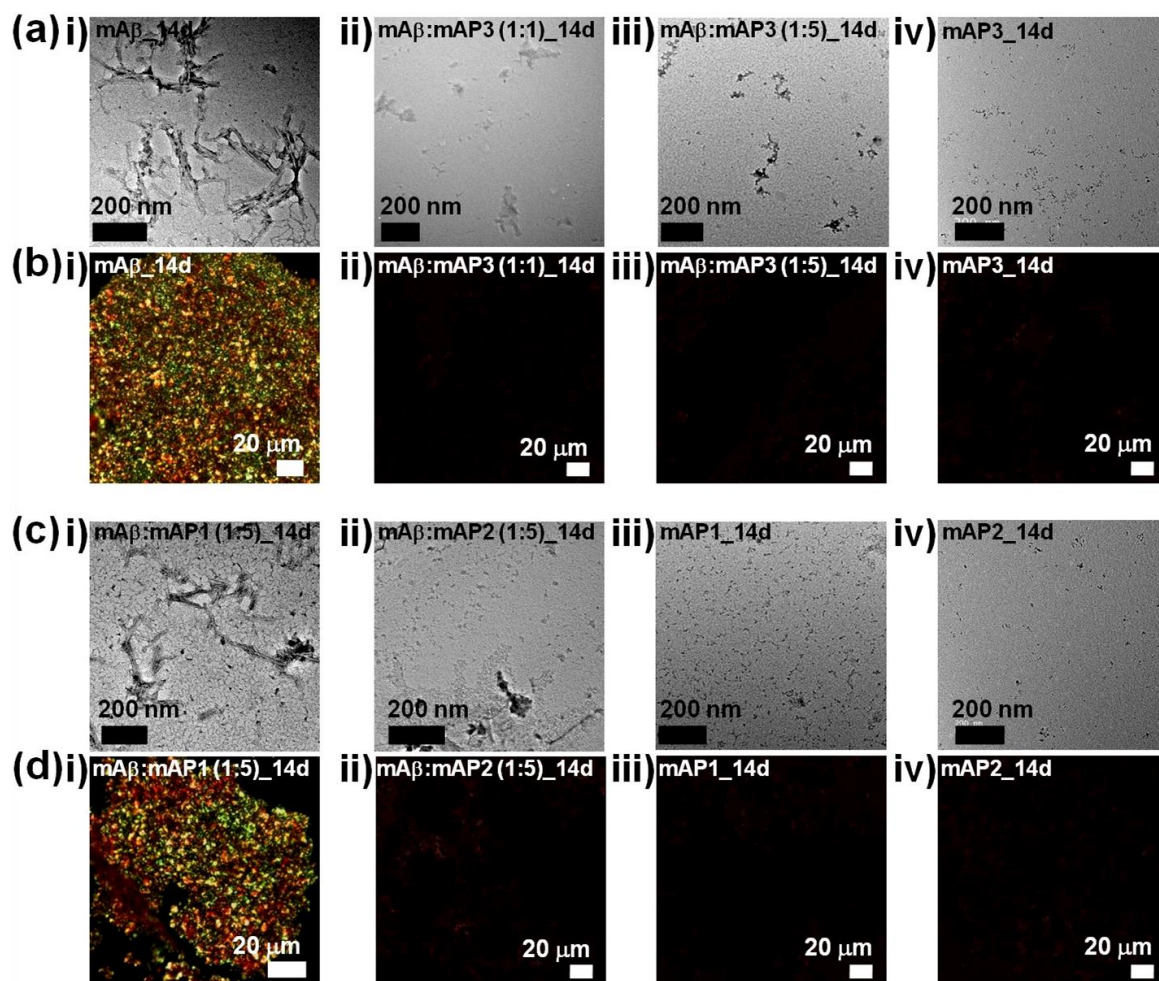


Figure 3.74. (a) TEM images, and (b) Congo red stained birefringence images of mAbeta (i) in the absence, and presence of (ii) one-fold mAP3, (iii) five-fold mAP3, and (iv) mAP3 alone. (c) TEM images, and (d) Congo red stained birefringence images of mAbeta in the presence of (i) five-fold mAP1, (ii) five-fold mAP2, (iii) mAP1 alone, and (iv) mAP2 alone. Scale bars are indicated as 200 nm for TEM images, and 20 μ m for birefringence images. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 $^{\circ}$ C in parallel. All the images were captured after fourteen days of incubation.

3.12. Disruption of amyloid accumulation of mAbeta by mAPs:

We noted that the growth phase for the fibrillization of mAbeta was from 24 h to 72 h. Therefore, mAPs were added to the mAbeta at 72 h (PBS pH 7.4 at 37 $^{\circ}$ C), assuming the presence of existing fibrillar assembly and the kinetics of amyloid disruption was monitored further by various biophysical tools up to 30 days.

3.12.1. ThT fluorescence assay of mA β :

The fluorescence intensity of mA β (black) increased with time in the absence of mAPs as described earlier (inhibition section). However, on the addition of one-fold mAP3 (red, Figure 3.75a) and five-fold mAP3 (blue, Figure 3.75a) at 72 h, the fluorescence intensity of mA β increased (for five-fold mAP3) initially due to self-assembling VFFA segment and suppressed after that due to the formation of various mA β fragments cleaved by mAP3. A similar type of result was observed for five-fold mAP2 (red, Figure 3.75b). The amount of preformed fibril of mA β at 72 h was found to be reduced by five-fold mAP3 (blue, Figure 3.75a/b) more significantly than one-fold mAP3 (red, Figure 3.75a) and five-fold mAP2 (red, Figure 3.75b).

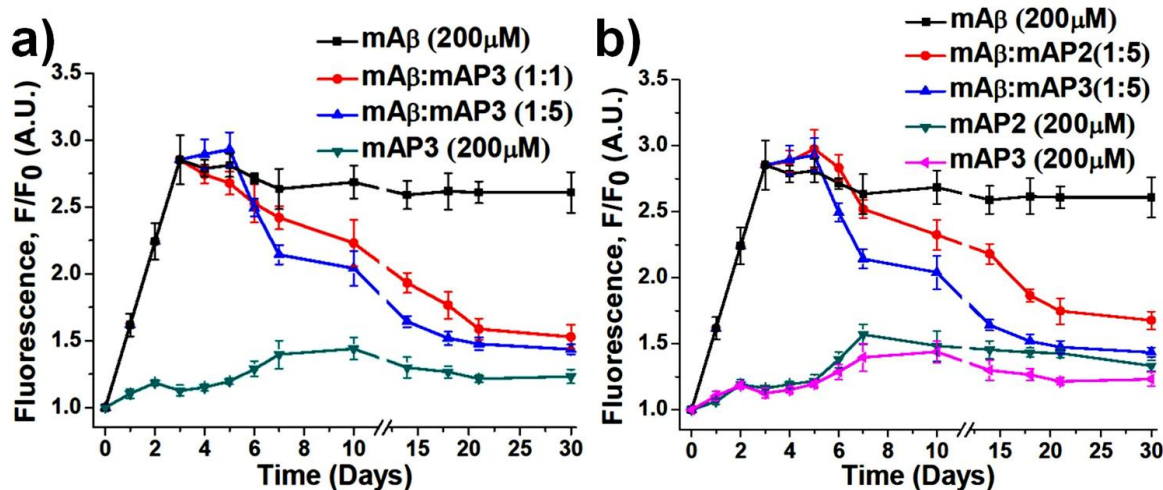


Figure 3.75. Normalized profiles of (a) dose-dependent, and (b) time-dependent ThT fluorescence assay where mAPs were added after three days to the preformed fibril of mA β . (a) Spectra of mA β in the absence (black), presence of one-fold mAP3 (red), five-fold mAP3 (blue), and mAP3 alone (dark cyan). (b) Spectra of mA β in absence (black), presence of five-fold mAP2 (red), five-fold mAP3 (blue), mAP2 alone (dark cyan), and mAP3 alone (magenta). Error bars represent standard deviations of at least three independent measurements. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

3.12.2. CD experiments of mA β :

β -Sheet conformation of mA β fibrils (black, Figure 3.76) was concluded from a positive band at ~ 203 nm with a negative band at ~ 225 nm. The tendency of mA β to form an α -helix (a positive band at ~ 205 nm with two negative bands at ~ 216 nm and ~ 231 nm) was

increased in the presence of one fold mAP3 (red, Figure 3.76a), which confirms the reduction in aggregation. Random coil conformation of mA β (blue, Figure 3.76a) was confirmed in the presence of five-fold molar excess of mAP3. Without a doubt, CD spectra confirmed the formation of a mixture of predominantly α -helices and reduced β -sheets (a positive band at ~ 199 nm with two negative bands at ~ 214 nm and ~ 232 nm) for mA β (red, Figure 3.76b) when co-incubated along with five-fold mAP2. The secondary structures of the control peptides were also displayed as described earlier (Figure 3.71).

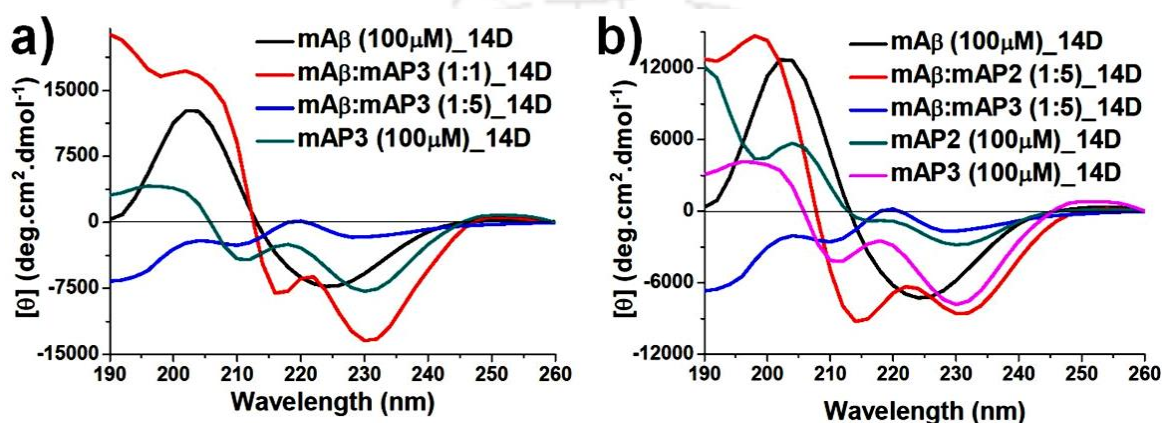


Figure 3.76. CD spectra of mA β in absence and presence of mAPs after 14 days of incubation. (a) Spectra of mA β in the absence (black), presence of one-fold mAP3 (red), five-fold mAP3 (blue), and mAP3 alone (dark cyan). (b) CD Spectra of mA β in the absence (black), presence of five-fold mAP2 (red), five-fold mAP3 (blue), mAP2 alone (dark cyan), and mAP3 alone (magenta). The peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C.

3.12.3. FTIR spectra of mA β :

The conformation of the peptides was checked by FTIR spectroscopic analysis. The amide I band was observed (a) at 1634 cm⁻¹ for mA β alone (Figure 3.77a), (b) at 1648 cm⁻¹ for mA β in presence of one-fold mAP3 (Figure 3.77b), (c) at 1656 cm⁻¹ for mA β in presence of five-fold mAP3 (Figure 3.77c), and (d) at 1643 cm⁻¹ for mA β in presence of five-fold mAP2 (Figure 3.77d). From the results, it was concluded that mA β exhibited β -sheet rich structure in the absence of mAPs, but exhibited random coil or α -helix conformation in the presence of either mAP2 or mAP3.

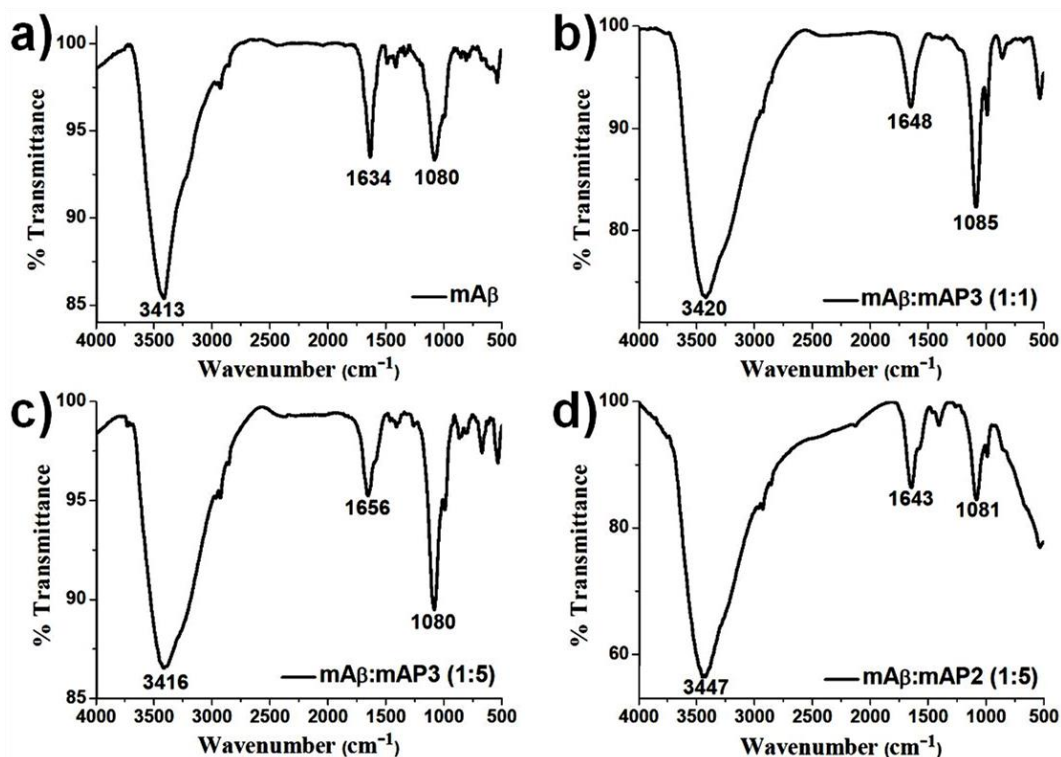


Figure 3.77. FTIR spectra of mAb β in the absence and presence of mAPs after fourteen days of incubation. FTIR Spectra of mAb β (a) in the absence, presence of (b) one-fold mAP3, (c) five-fold mAP3, and (d) five-fold mAP2.

3.12.4. TEM images and Congo red stained birefringence images of amyloid fibrils formed by mAb β :

The tendency of amyloidogenicity of mAb β was characterized by TEM and Congo red stained birefringence studies. The clear fibrillar aggregates (Figure 3.78. a-i) and green gold birefringence (Figure 3.78. b-i) were observed for mAb β in the absence of mAPs when viewed under TEM and cross-polarized light, respectively. However, in the presence of one-fold mAP3, very few tiny fibres (Figure 3.78. a-ii) and little amount of green gold birefringence (Figure 3.78. b-ii) were observed under TEM and cross-polarised light respectively indicates one-fold mAP3 is not sufficient to disrupt the aggregation of mAb β . When the dose was increased from one-fold to five-fold, mAP3 disrupted amyloid of mAb β totally as no such fibre (Figure 3.78. a-iii) or green gold birefringence (Figure 3.78. b-iii) was noticed. Also, five-fold mAP2 was not sufficient to disrupt the aggregation of mAb β , because mAb β in the presence of five-fold mAP2 showed few fibres (Figure 3.78. a-iv) and less amount of green gold birefringence (Figure 3.78. b-

iv) under TEM and cross-polarized light respectively. The images were taken after fourteen days of incubation.

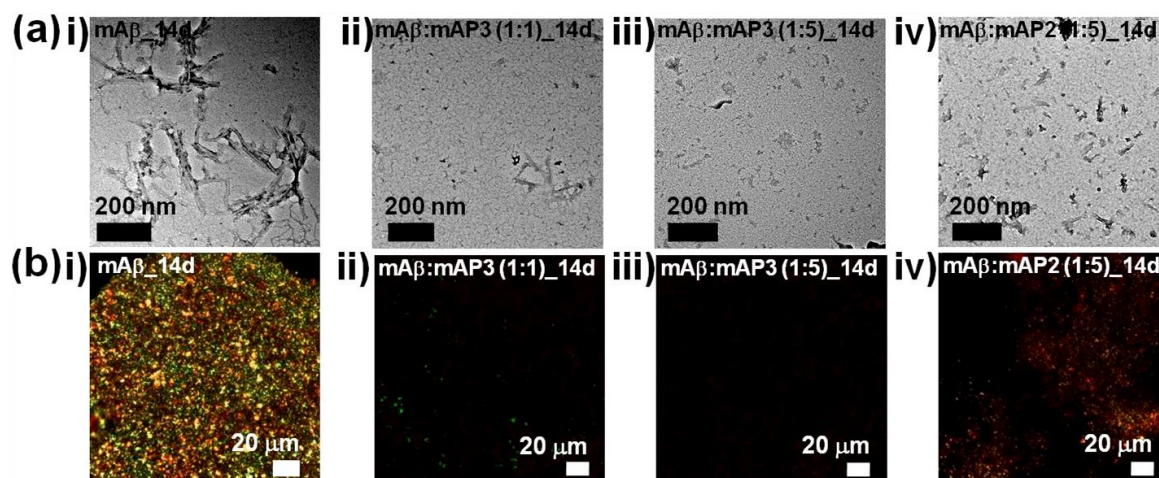


Figure 3.78. (a) TEM images, and (b) Congo red stained birefringence images of mAbeta (i) in absence, and presence of (ii) one-fold mAP3, (iii) five-fold mAP3, and (iv) five-fold mAP2. Scale bars are indicated as 200 nm for TEM images, and 20 μ m for birefringence images. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel. All the images were captured after 14 days of incubation.

3.13. Examination of the proteolytic activity of mAPs on A β ₁₋₄₀:

To find out the cleavage sites of A β ₁₋₄₀ by mAPs; comparable experiments were performed using commercially available A β ₁₋₄₀ peptide. Samples were prepared in the same way to that for mAbeta.

3.13.1. Self-degradation of A β ₁₋₄₀:

MALDI-TOF mass spectra of A β ₁₋₄₀ were analysed in a time-dependent manner (Figure 3.79-3.81) in the absence of mAPs. This was necessary to check and mark the peptide fragments appeared due to self-degradation of A β ₁₋₄₀. However, no degradation product was observable in MALDI-TOF mass spectra probably due to its solvent inaccessibility, as A β ₁₋₄₀ got aggregated after three days of incubation and it was confirmed from the MALDI mass data. The aliquot was taken from one the replicate solutions of A β ₁₋₄₀, which was incubated as a common sample of two different works.

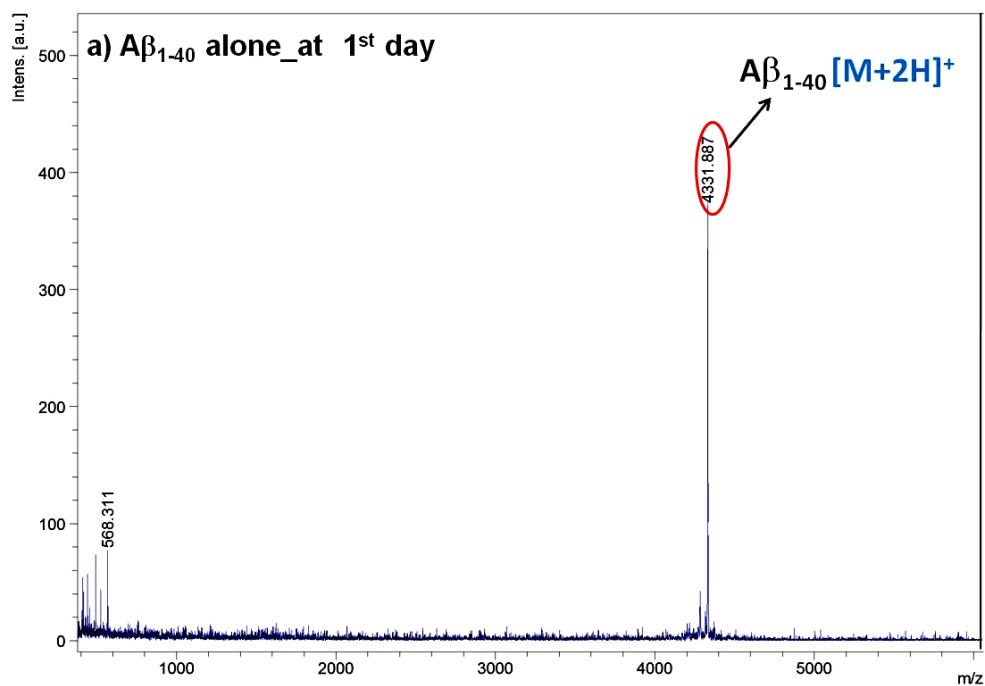


Figure 3.79. MALDI-TOF mass spectrum of $A\beta_{1-40}$ at the 1st day of incubation in PBS pH 7.4 at 37 °C.

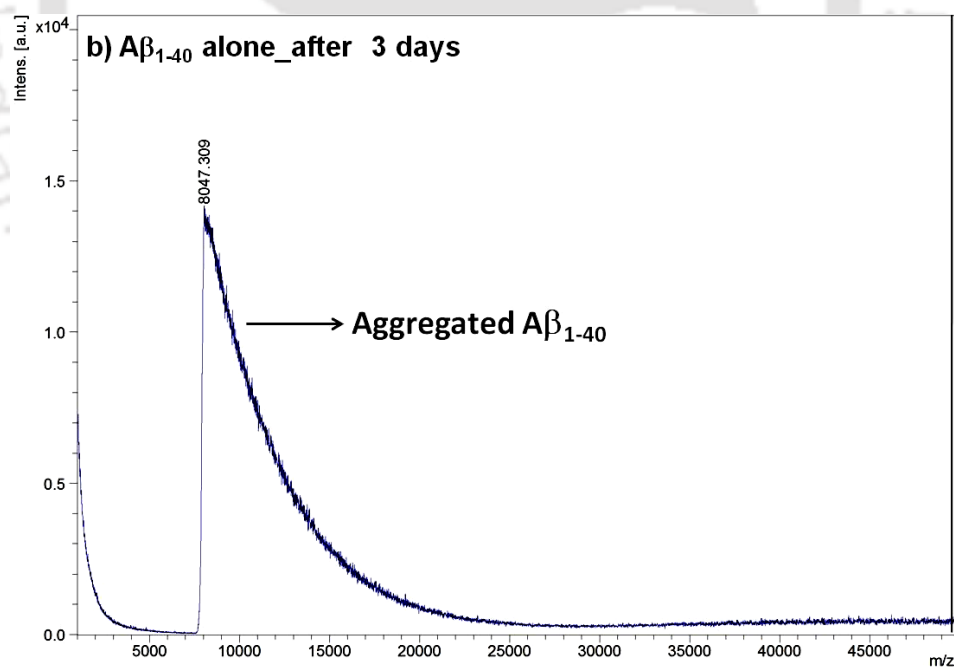


Figure 3.80. MALDI-TOF mass spectrum of $A\beta_{1-40}$ after three days of incubation in PBS.

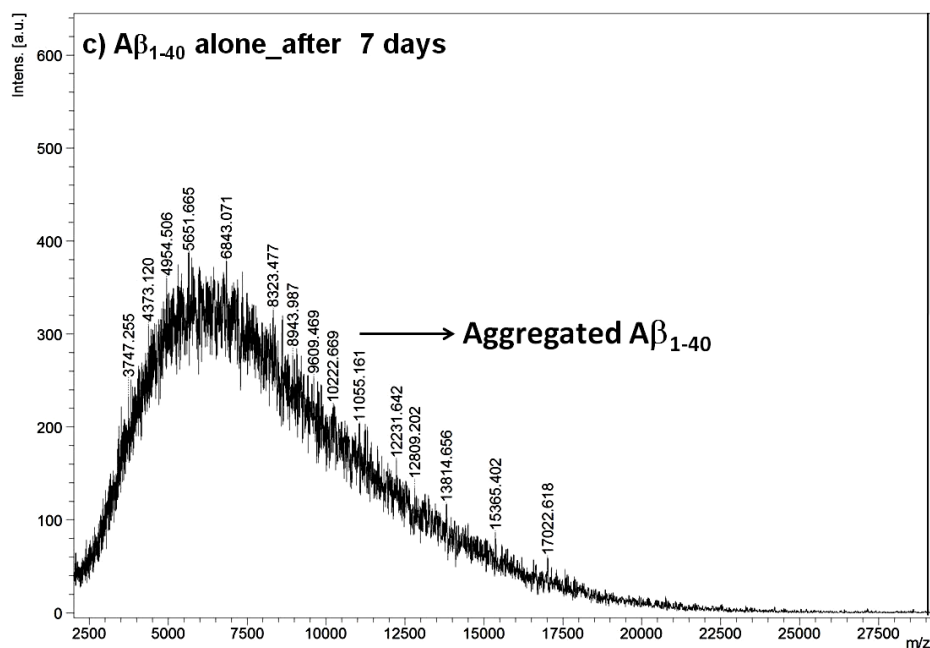


Figure 3.81. MALDI-TOF mass spectrum of $A\beta_{1-40}$ after seven days of incubation in PBS.

3.13.2. Proteolytic activity of mAP1 on $A\beta_{1-40}$:

No peptide fragment was found even after ten days, when $A\beta_{1-40}$ was incubated in the presence of five-fold mAP1 (Figure 3.82-3.83), indicating the inability of the proteolytic activity of mAP1. However, $A\beta$ -like aggregated peak was also not observed, pointing to the amyloid inhibiting nature of mAP1 against $A\beta$.

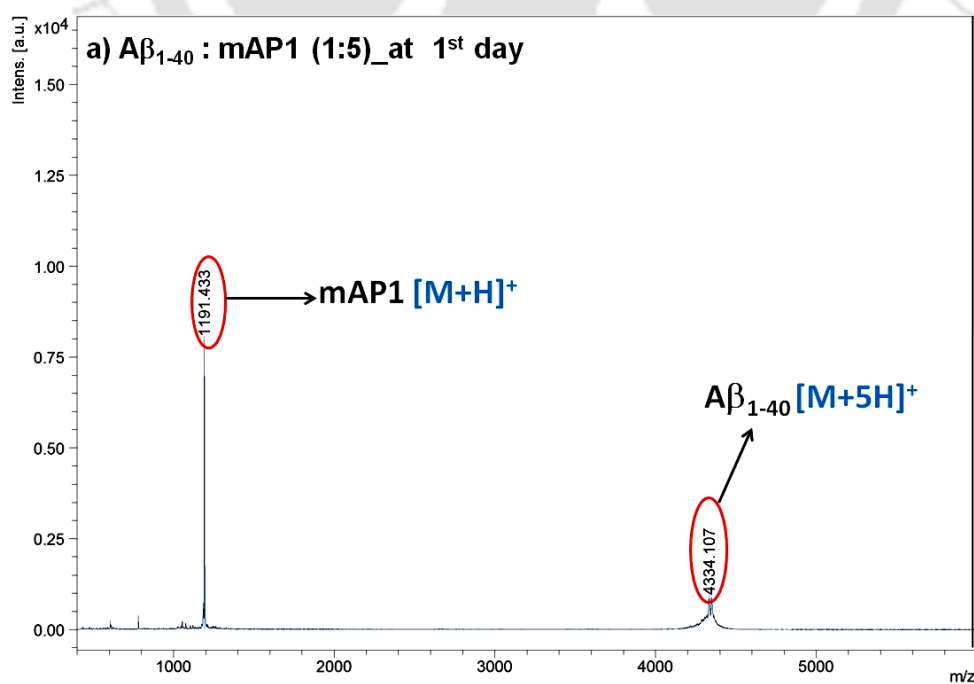


Figure 3.82. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of mAP1 (1:5) at the 1st day.

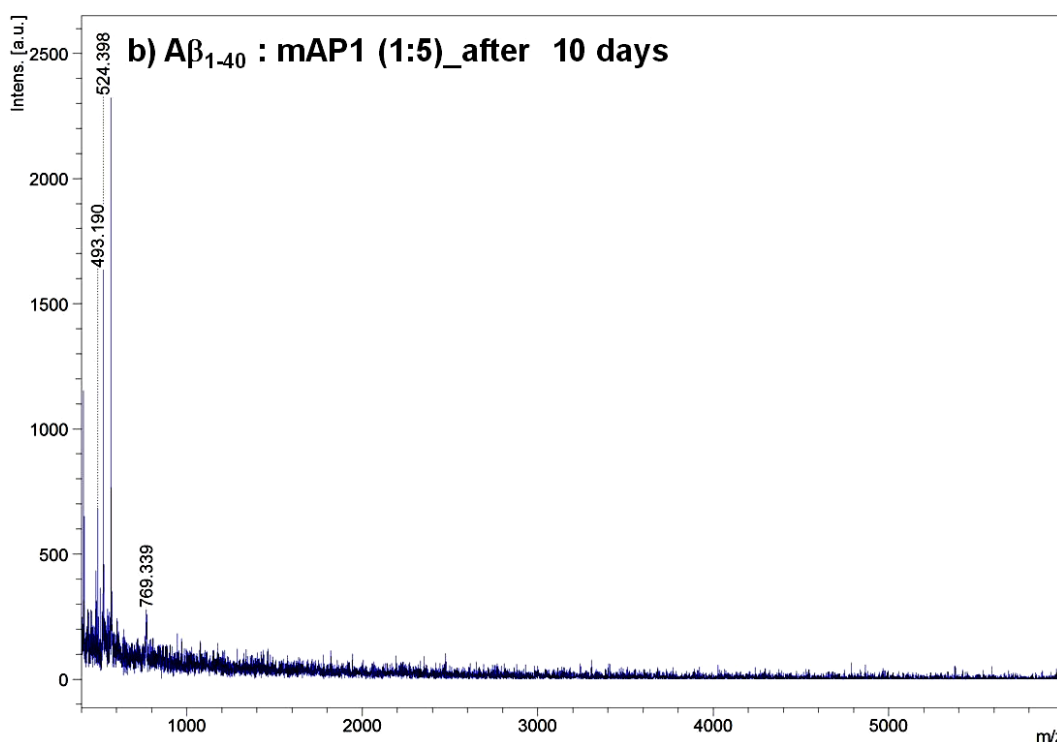


Figure 3.83. MALDI-TOF mass spectrum of A β ₁₋₄₀ in the presence of mAP1 (1:5) after ten days.

3.13.3. Plausible routes of the proteolytic cleavage of A β ₁₋₄₀ by mAP2 and mAP3:

However, after second day onwards, it was observed that mAP2 and mAP3 cleaved A β ₁₋₄₀ in a site-selective manner. Various peptide fragments and intermediates of different range of lifetimes were also found. Plausible routes of proteolytic cleavage of A β ₁₋₄₀ by either mAP2 or mAP3 (Figure 3.84) were explained from MALDI-TOF mass (Figure 3.85-3.90 for mAP2, and Figure 3.91-3.96 for mAP3) analyses. Enzymes such as neprilysin (NEP), endothelin-converting enzyme (ECE), insulin-degrading enzyme (IDE), Plasmin (P), angiotensin-converting enzyme (ACE), Matrix Metalloproteinases (MMPs) cleave aggregated A β ₁₋₄₀ in a site-selective manner for facilitating excretion.¹⁴ mAP3 cleaved A β ₁₋₄₀ in-between Lys16 & Leu17 probably via route 1^{A β -mAP3}, which is the specific cleavage site of α -secretase and site-selective for ECE, MMP2, MMP9 & P. mAP2 and mAP3 individually cleaved A β ₁₋₄₀ between Gln15 & Lys16 (unlike any known proteolytic enzymes) to form A β ₁₋₁₅ probably via route 2^{A β -mAPs}, between Leu17 & Val18 (ECE cleavage site) to form A β ₁₋₁₇ & A β ₁₈₋₄₀ probably via route 6^{A β -mAPs}, between Val18 & Phe19 (ECE, & IDE cleavage site) to form A β ₁₋₁₈ probably via route 7^{A β -mAPs}, between

Phe19 & Phe20 (IDE, & NEP cleavage site) to form $A\beta_{1-19}$ probably via route 8 $A\beta$ -mAPs, between Phe20 & Ala21 (IDE cleavage site) to form $A\beta_{1-20}$ probably via route 9 $A\beta$ -mAPs, and between Glu22 & Asp23 (unlike any known proteolytic enzymes) to form $A\beta_{1-22}$ probably via route 10 $A\beta$ -mAPs. They further sliced $A\beta_{1-40}$ into $A\beta_{9-40}$ at 7 days, and various fragments like $A\beta_{19-25}$, $A\beta_{13-18}$, $A\beta_{13-19}$, $A\beta_{14-20}$, $A\beta_{17-20}$, $A\beta_{15-19}$, $A\beta_{19-34}$, $A\beta_{19-23}$, $A\beta_{20-23}$, $A\beta_{21-29}$ etc. by prolonged incubation (for mAP2 in between 21 days to 30 days, and for mAP3 in between 10 days to 15 days). Interestingly, $A\beta_{1-40}$ like aggregation was not noted in the presence of mAP2 and mAP3, indicating amyloid solubilizing nature of mAP2 and mAP3.

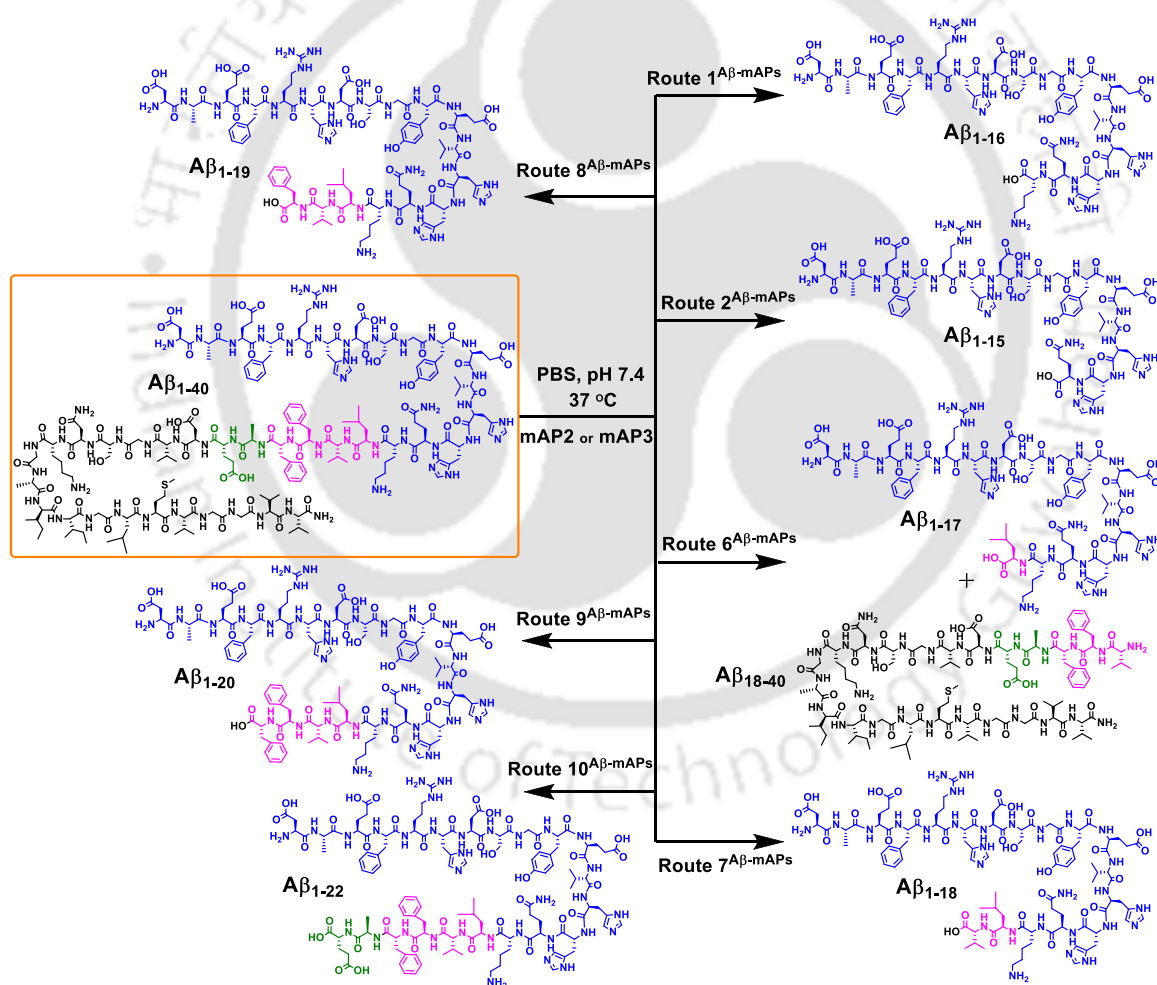


Figure 3.84. Plausible routes of the proteolysis of $A\beta_{1-40}$ into various fragments by mAPs (Route 1 $A\beta$ -mAP2, Route 2 $A\beta$ -mAP2, Route 6 $A\beta$ -mAP2 - Route 10 $A\beta$ -mAP2).

3.13.4. Proteolytic activity of mAP2 on $A\beta_{1-40}$:

mAP2 sliced $A\beta_{1-40}$ into various fragments (Figure 3.85-3.90) as described in proteolytic routes. Interestingly, $A\beta_{1-40}$ like aggregated peak was not observed in the presence of mAP2, indicating amyloid solubilising nature of mAP2.

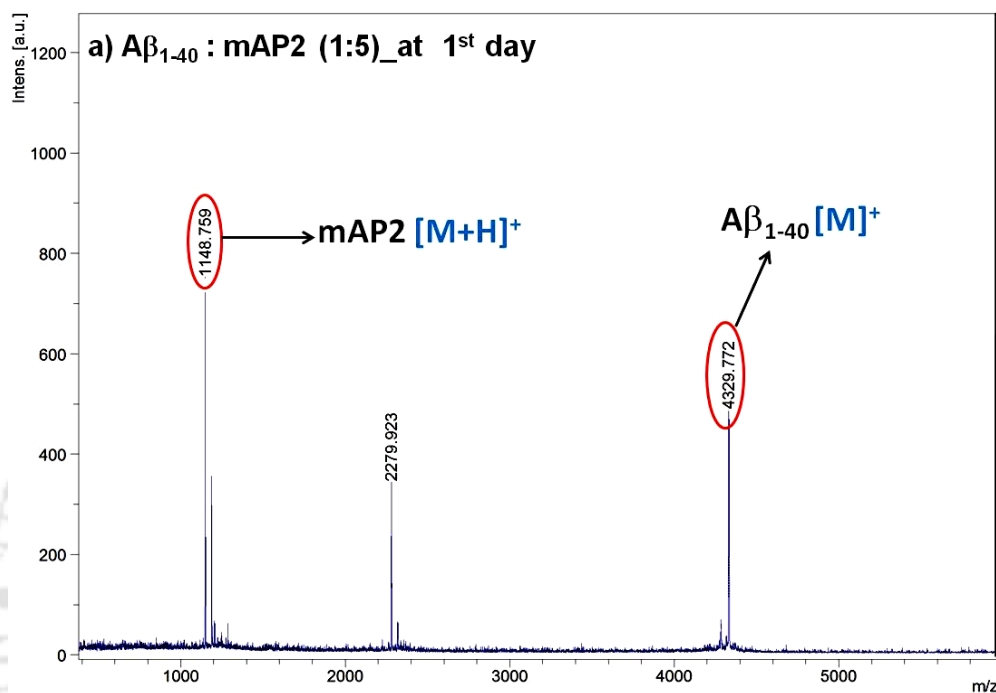


Figure 3.85. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of mAP2 (1:5) at the 1st day.

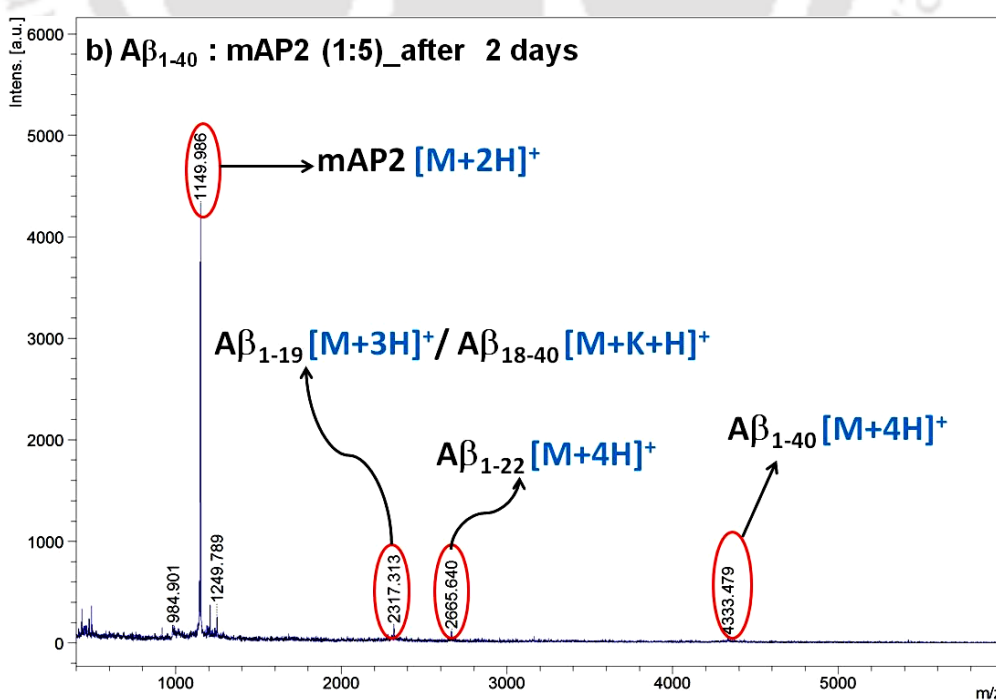
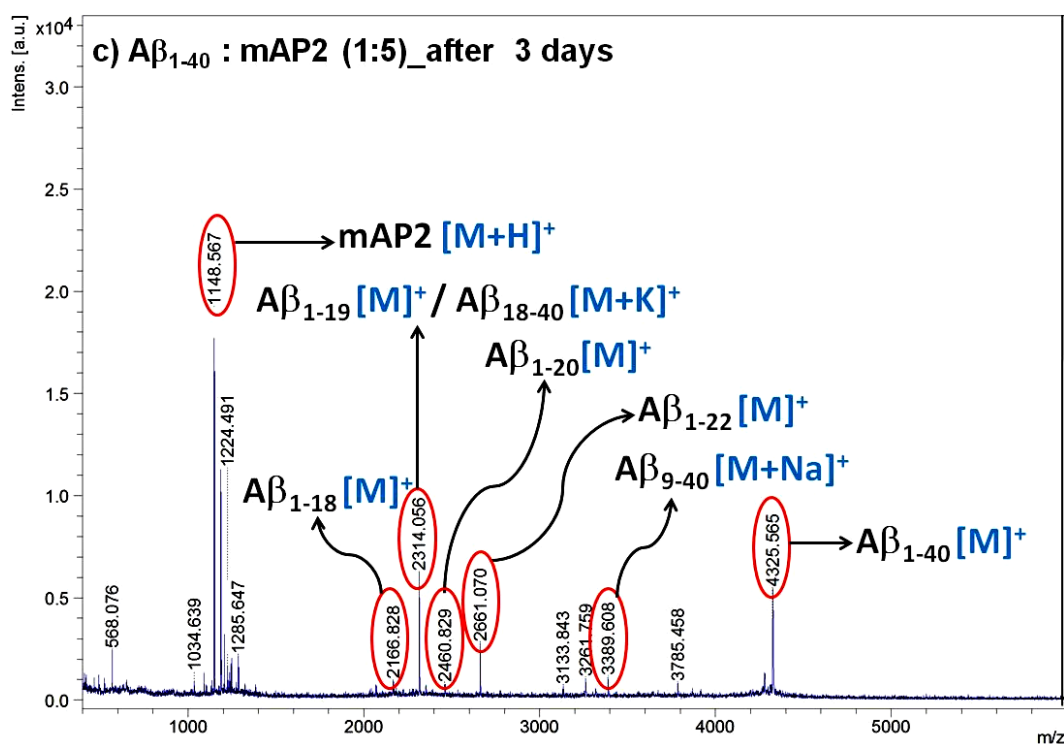
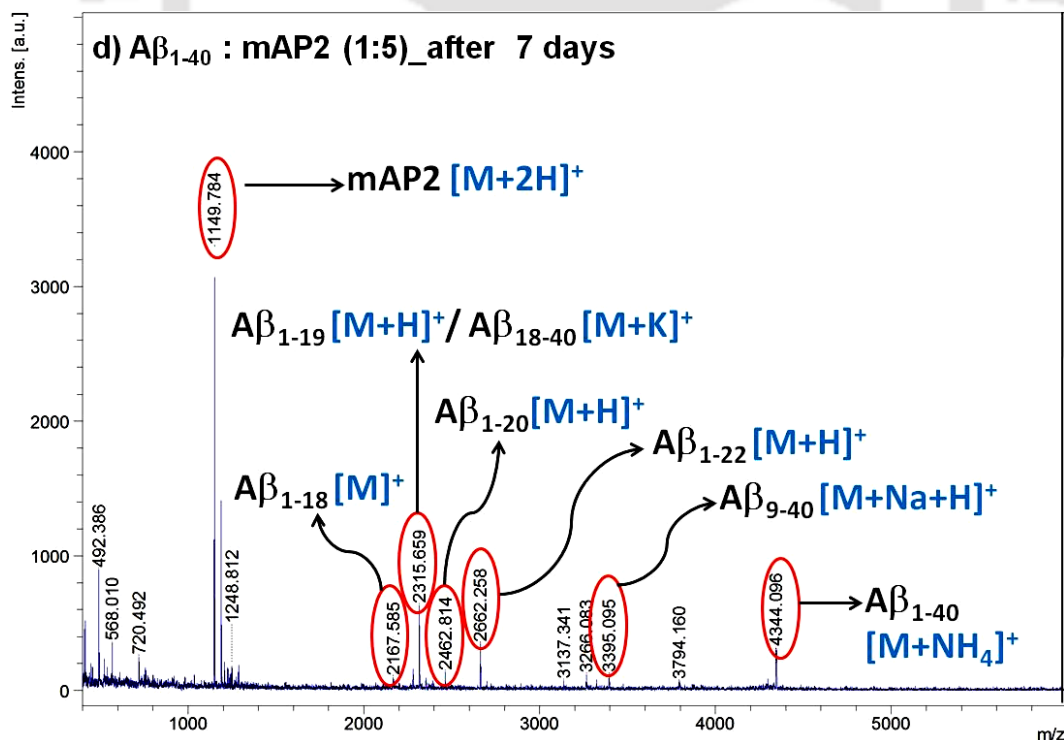


Figure 3.86. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of mAP2 (1:5) after two days.

Figure 3.87. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of mAP2 (1:5) after three days.Figure 3.88. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of mAP2 (1:5) after seven days of incubation in PBS.

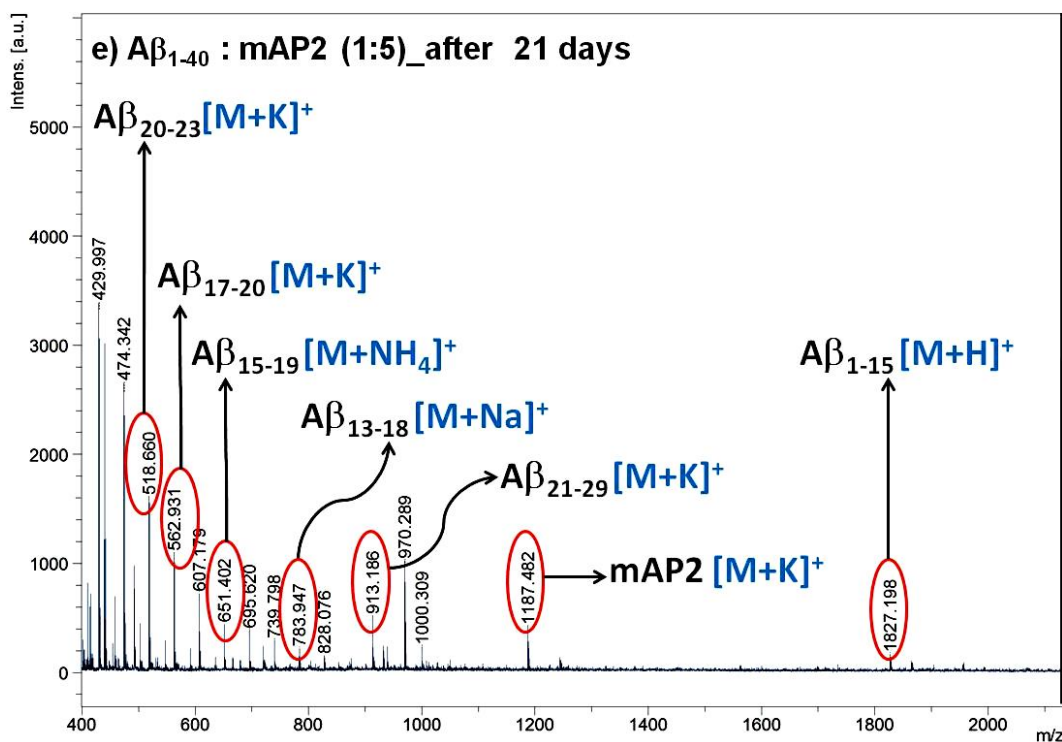


Figure 3.89. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of mAP2 (1:5) after twenty-one days of incubation in PBS.

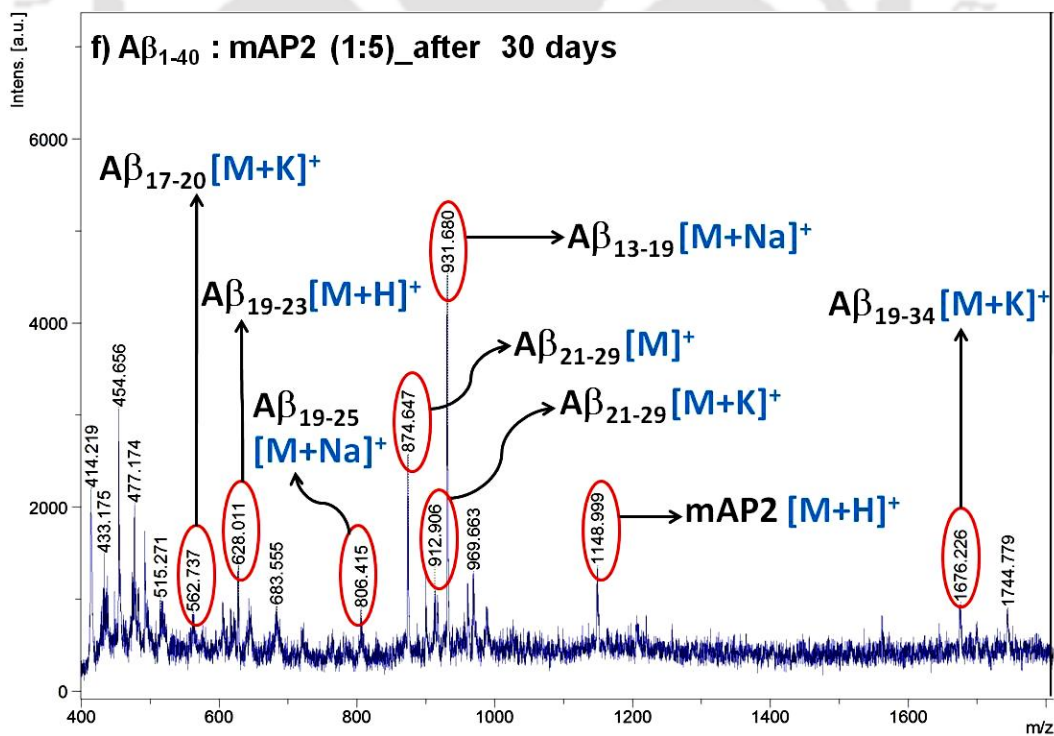


Figure 3.90. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of mAP2 (1:5) after thirty days of incubation in PBS.

3.13.5. Proteolytic activity of mAP3 on $A\beta_{1-40}$:

mAP3 sliced $A\beta_{1-40}$ into various fragments (Figure 3.91-3.96) as described in proteolytic routes. Interestingly, $A\beta_{1-40}$ like aggregation was not noted in the presence of mAP3, indicating amyloid solubilizing nature of mAP3.

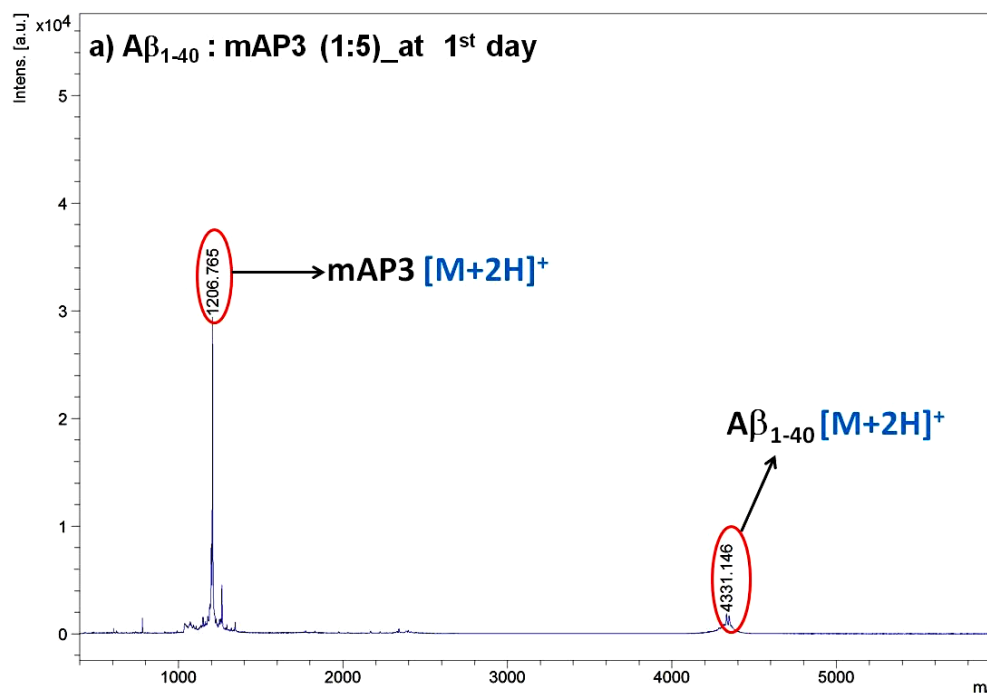


Figure 3.91. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of mAP3 (1:5) at the 1st day.

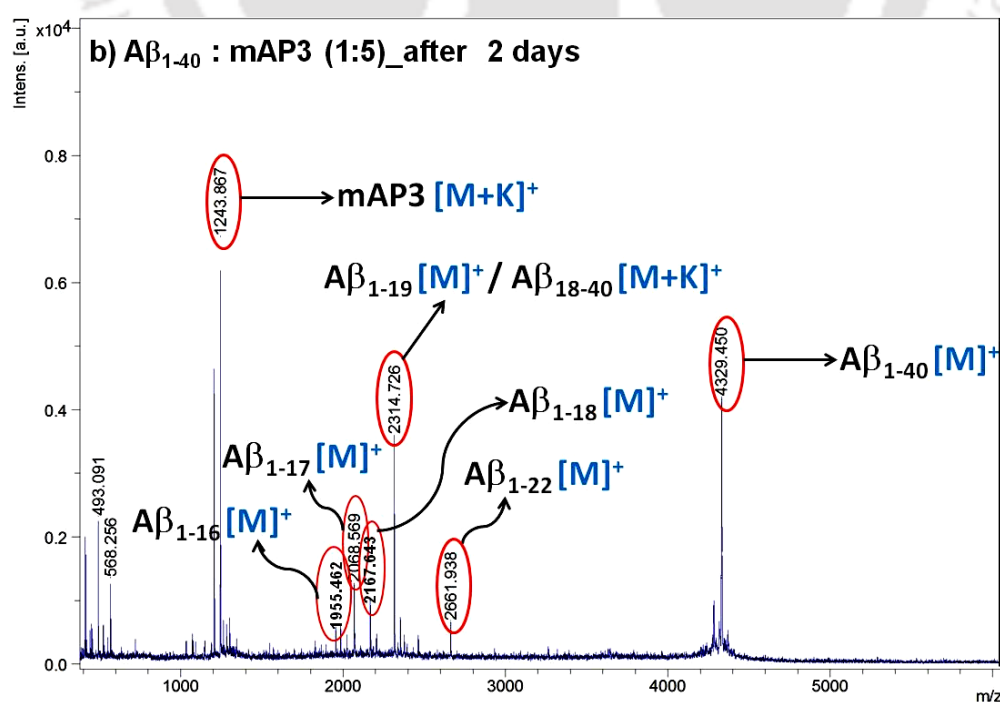


Figure 3.92. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of mAP3 (1:5) after two days.

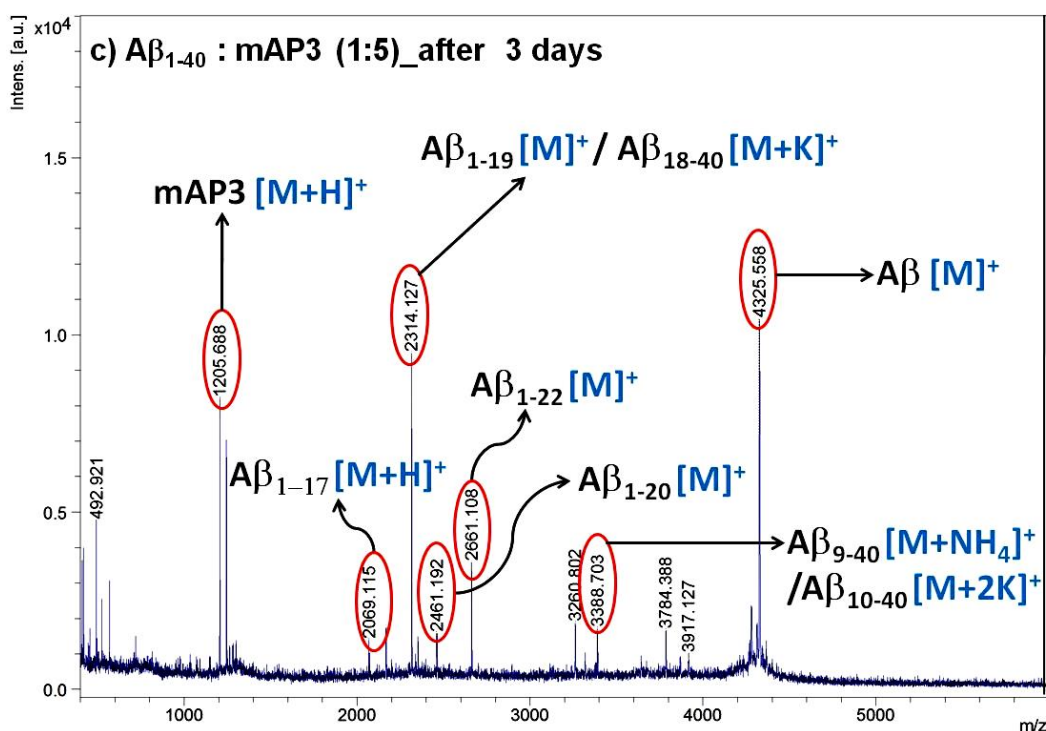


Figure 3.93. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of mAP3 (1:5) after three days of incubation in PBS.

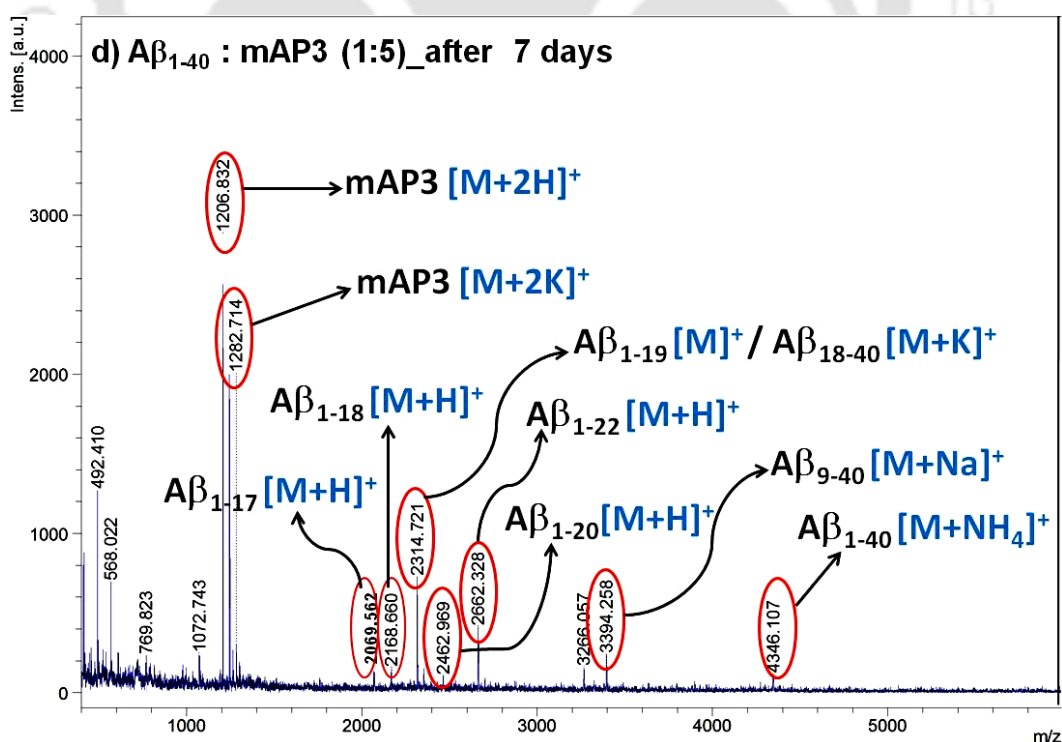


Figure 3.94. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of mAP3 (1:5) after seven days of incubation in PBS.

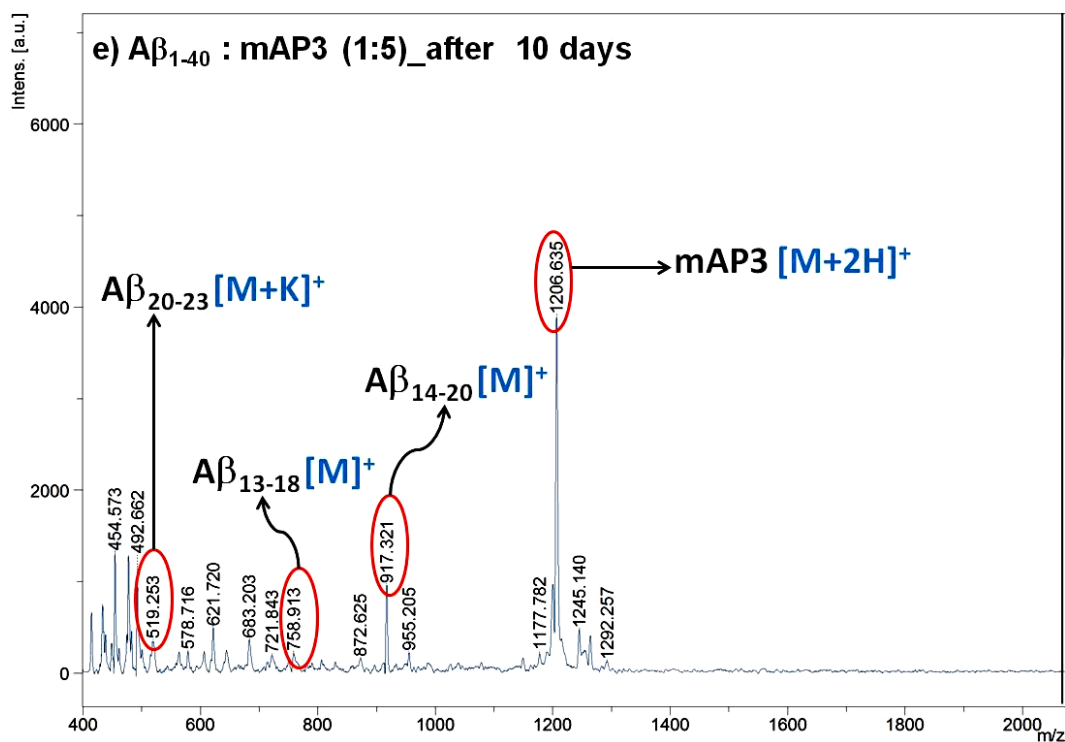


Figure 3.95. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of mAP3 (1:5) after ten days of incubation in PBS.

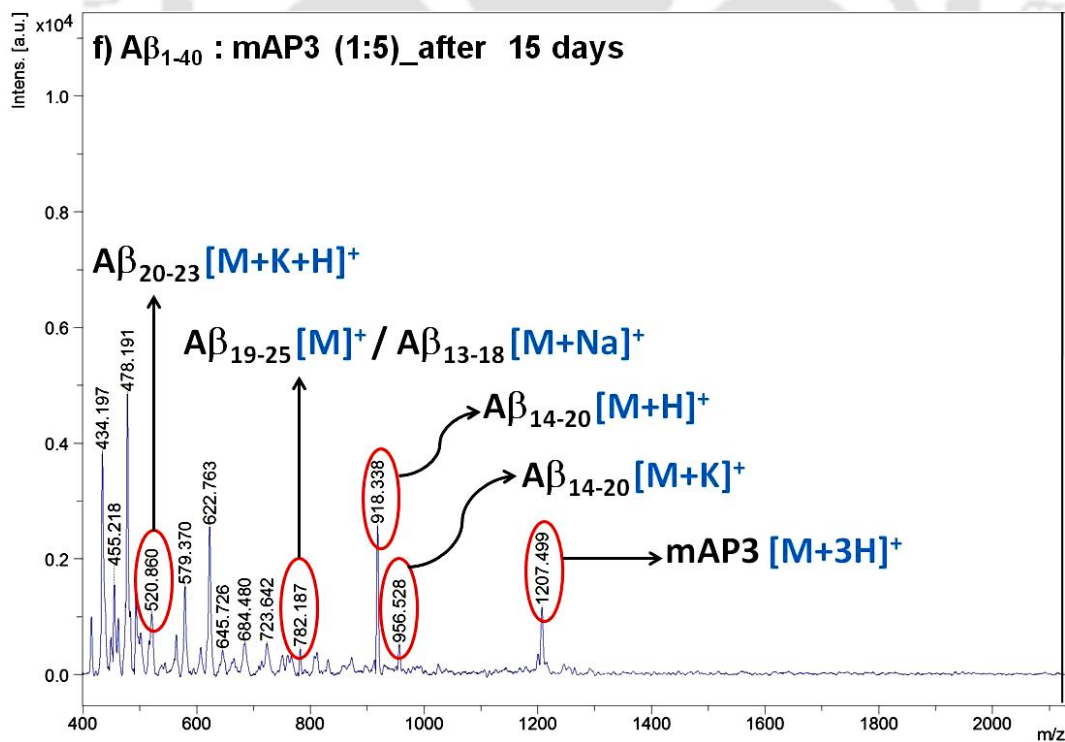


Figure 3.96. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of mAP3 (1:5) after fifteen days of incubation in PBS.

3.14. Time-dependent existence map of various fragments of $A\beta_{1-40}$:

Time-dependent persistence map of the $A\beta$ fragments (Figure 3.97) reveals the time-resolved manner of the programmed proteolysis of the designed mAPs.

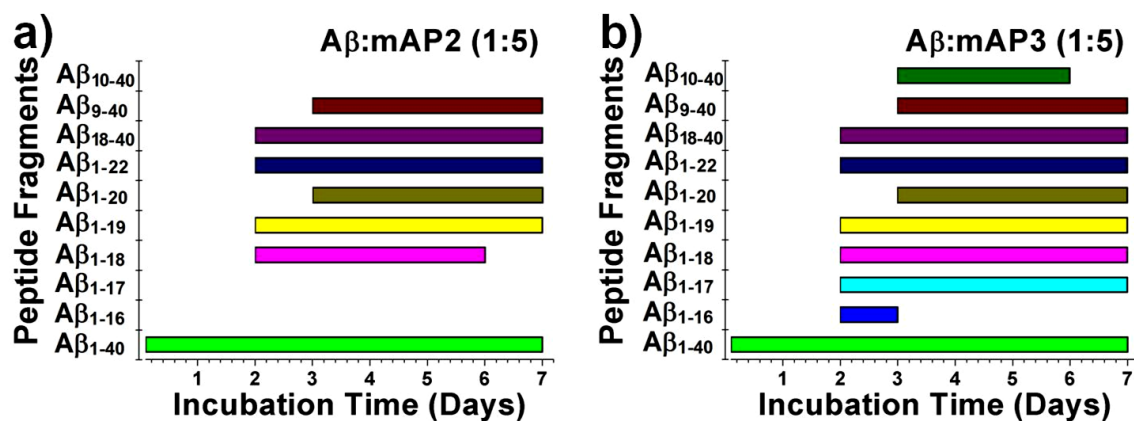


Figure 3.97. Time-dependent persistence map of various fragments of $A\beta_{1-40}$ in the presence of (a) mAP2, and (b) mAP3.

3.15. Multiple proteolytic enzyme activities of mAP2 and mAP3:

Thus, both mAP2 and mAP3 are capable to cleave $A\beta_{1-40}$ at various sites (Figure 3.98), mimicking the proteolytic action of multiple enzymes (NEP, ECE, IDE, P, ACE, MMPs).

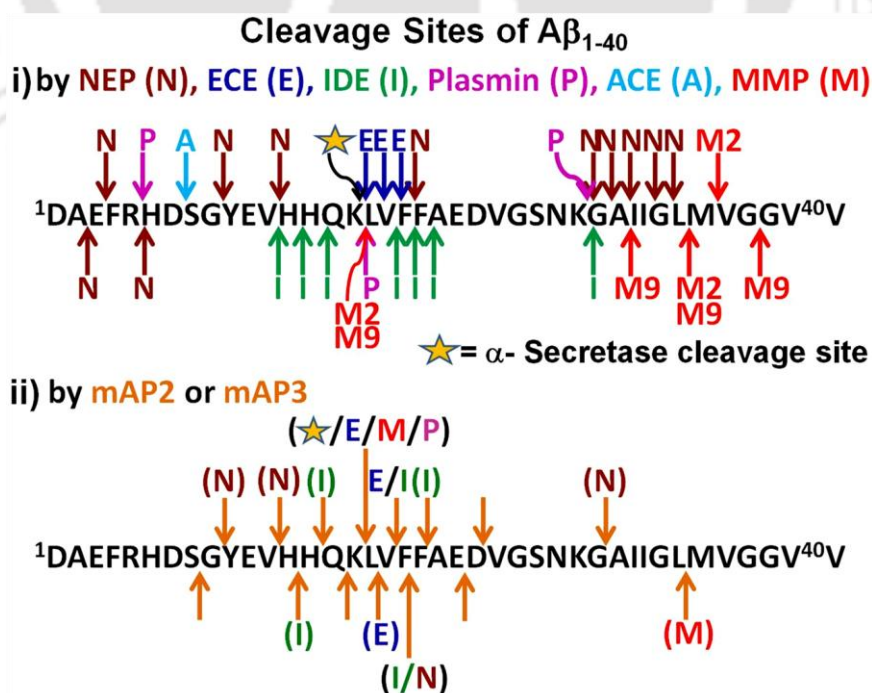


Figure 3.98. Multiple proteolytic enzyme activities of mAP2 and mAP3.

3.16. Inhibitory ability of KLVFF on $A\beta_{1-40}$:

MALDI-TOF mass spectra of $A\beta_{1-40}$ in the presence of KLVFF were analysed in a time-dependent manner (up to 15 days). However, mAPs mediated no such degradation product was observed in MALDI-TOF mass spectra, a few self-degraded peptide fragments were appeared due to the inhibition of $A\beta_{1-40}$ by KLVFF.

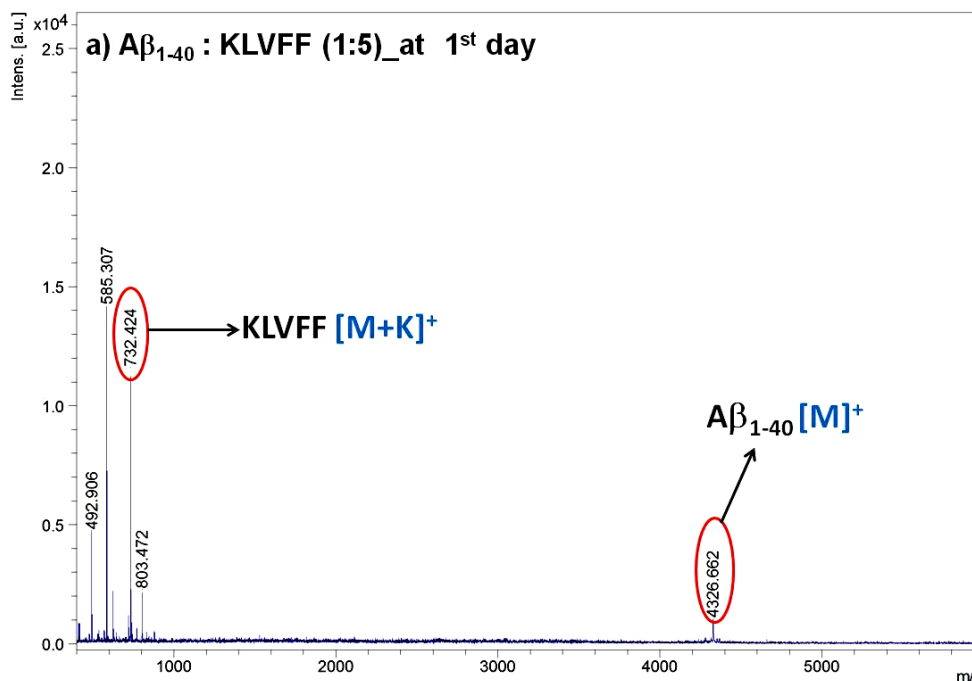


Fig. 3.99. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of KLVFF (1:5) on the first day of incubation in PBS pH 7.4 at 37 °C.

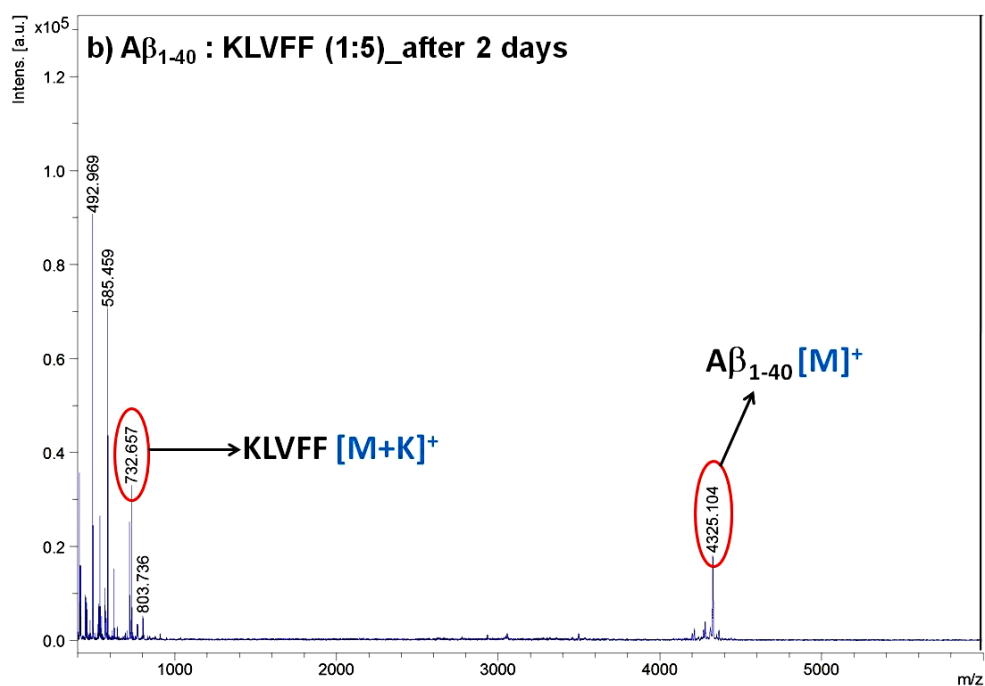


Fig. 3.100. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of KLVFF (1:5) after two days of incubation in PBS.

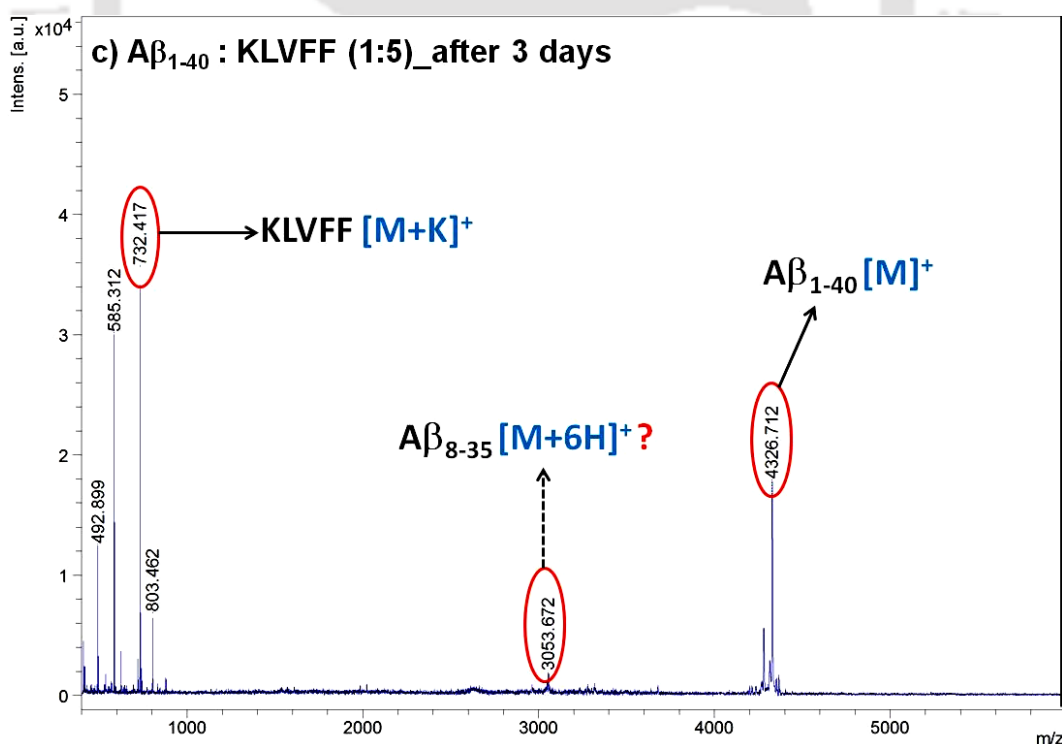


Fig. 3.101. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of KLVFF (1:5) after three days of incubation in PBS due to Ser-mediated proteolysis.

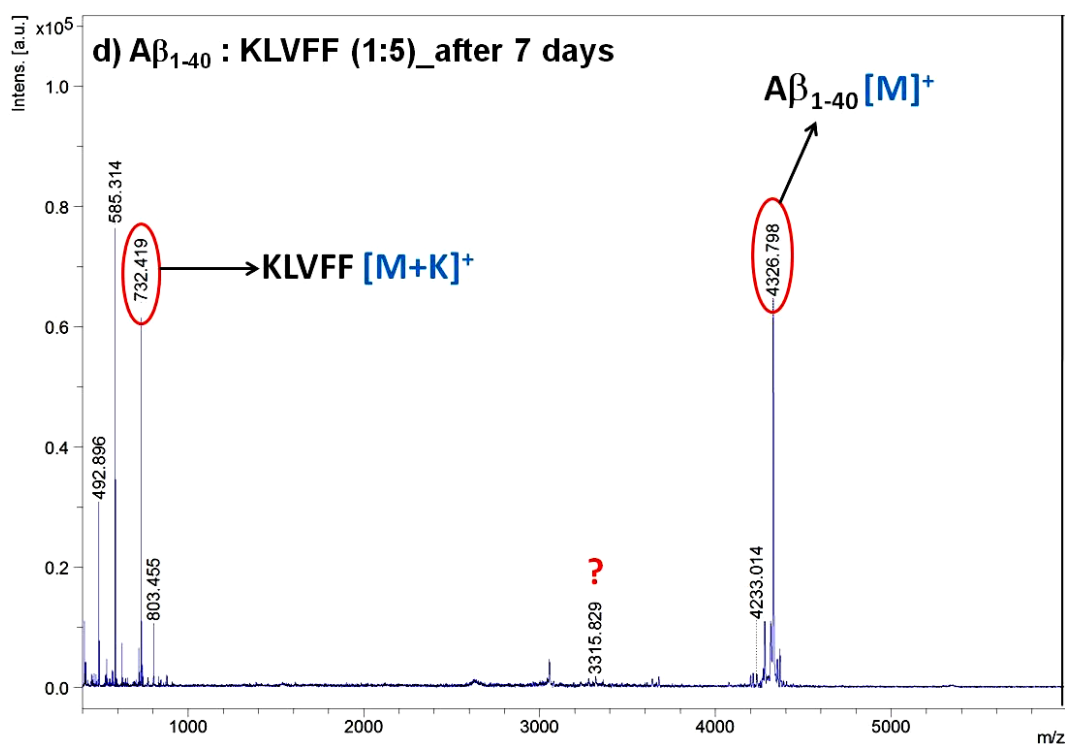


Fig. 3.102. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of KLVFF (1:5) after seven days.

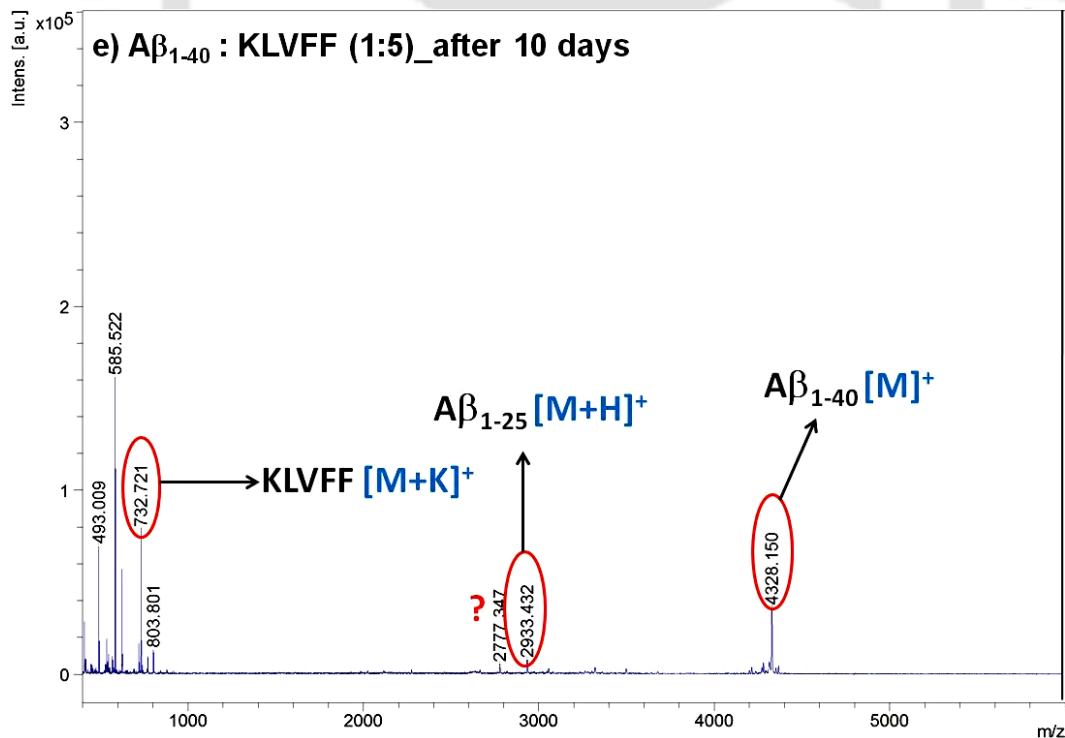


Fig. 3.103. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of KLVFF (1:5) after ten days.

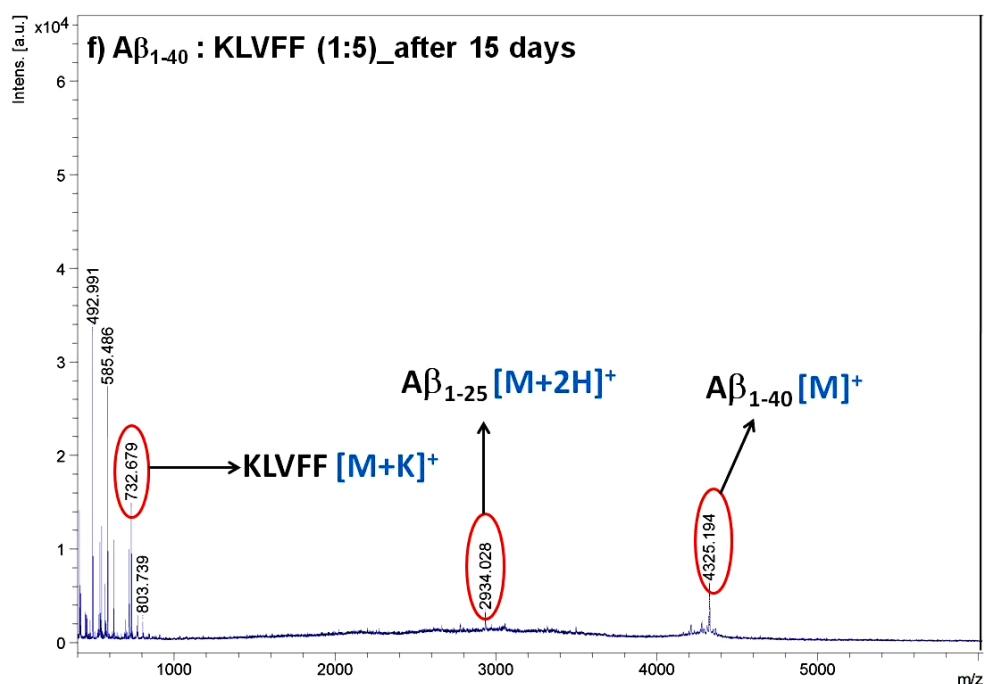


Fig. 3.104. MALDI-TOF mass spectrum of A β ₁₋₄₀ in the presence of KLVFF (1:5) after fifteen days.

3.17. Inhibitory ability of EGCG on A β ₁₋₄₀:

MALDI-TOF mass spectra of A β ₁₋₄₀ in the presence of EGCG were analysed in a time-dependent manner (up to 15 days). However, mAPs mediated no such degradation product was observed in MALDI-TOF mass spectra, a few self-degraded peptide fragments were appeared due to the inhibition of A β ₁₋₄₀ by EGCG.

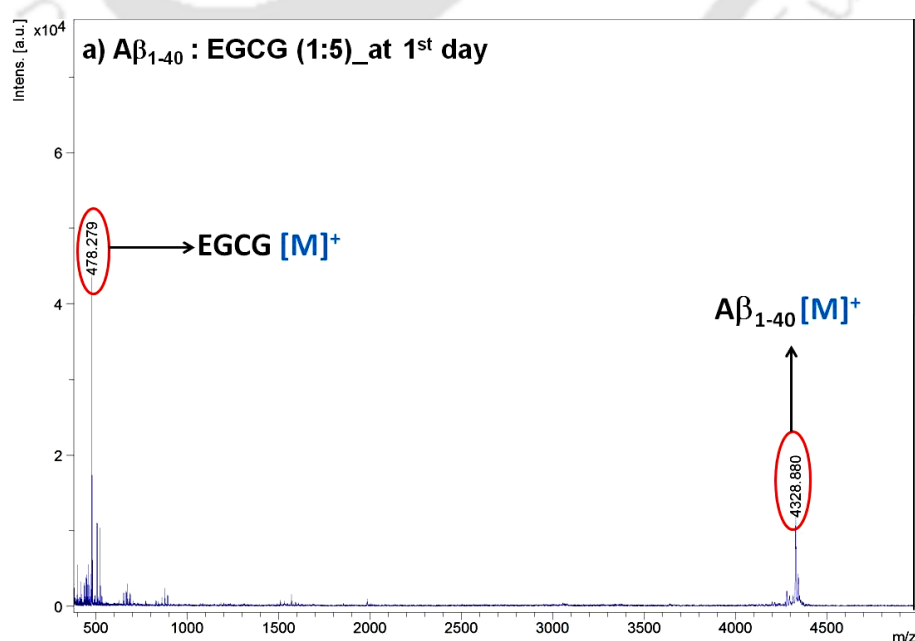


Fig. 3.105. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of EGCG (1:5) on the first day of incubation in PBS pH 7.4 at 37 °C.

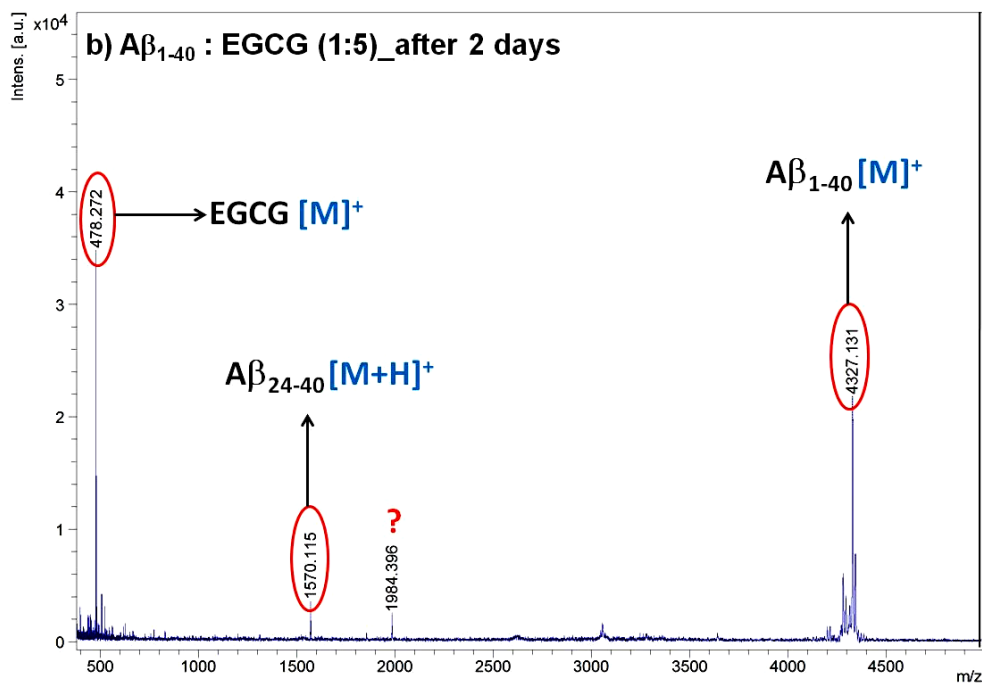


Fig. 3.106. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of EGCG (1:5) after two days.

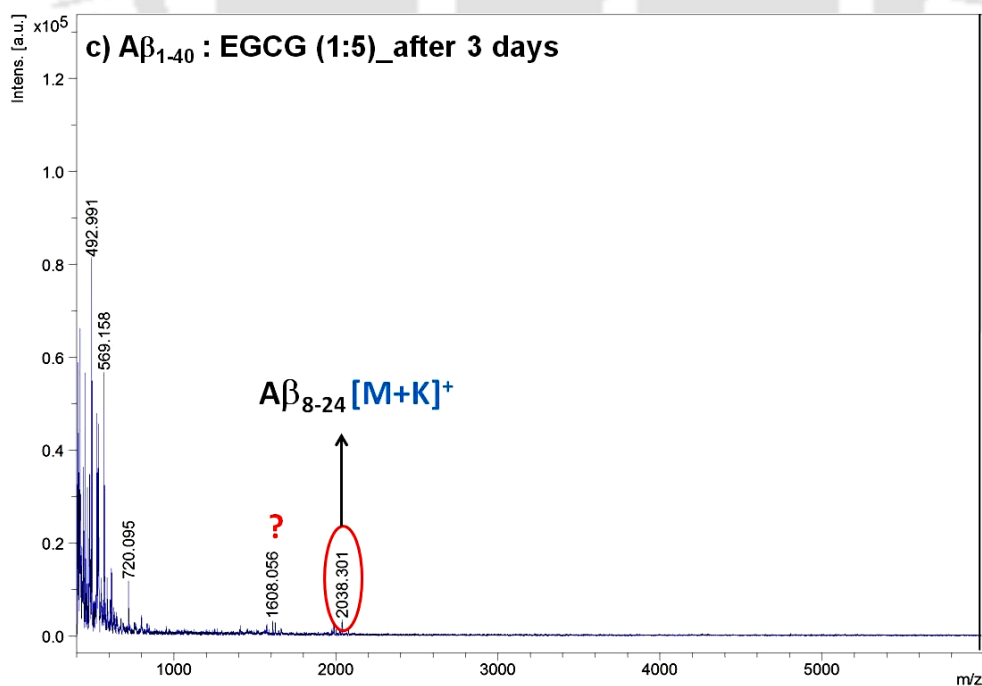


Fig. 3.107. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of EGCG (1:5) after three days of incubation in PBS due to Ser-mediated proteolysis.

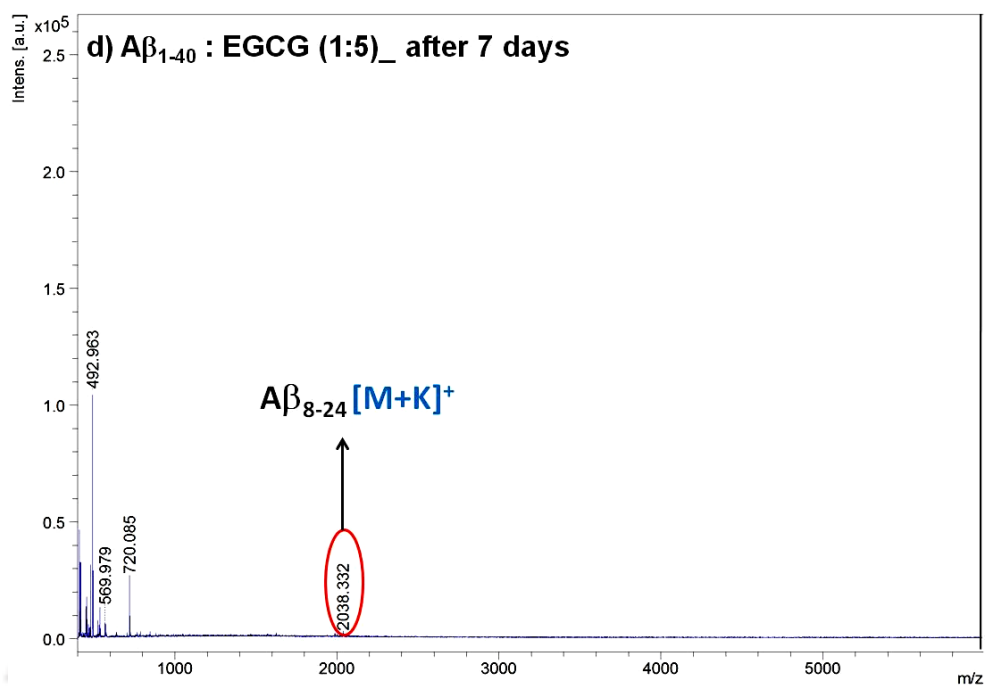


Fig. 3.108. MALDI-TOF mass spectrum of A β ₁₋₄₀ in the presence of EGCG (1:5) after seven days of incubation in PBS due to Ser-mediated proteolysis.

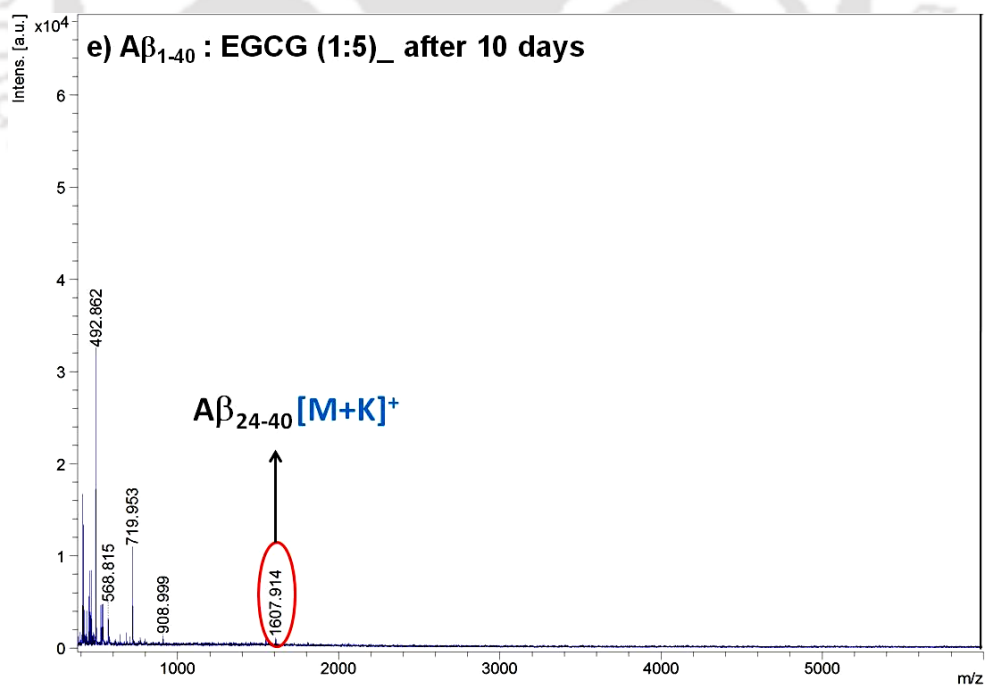


Fig. 3.109. MALDI-TOF mass spectrum of A β ₁₋₄₀ in the presence of EGCG (1:5) after ten days of incubation in PBS due to Ser-mediated proteolysis.

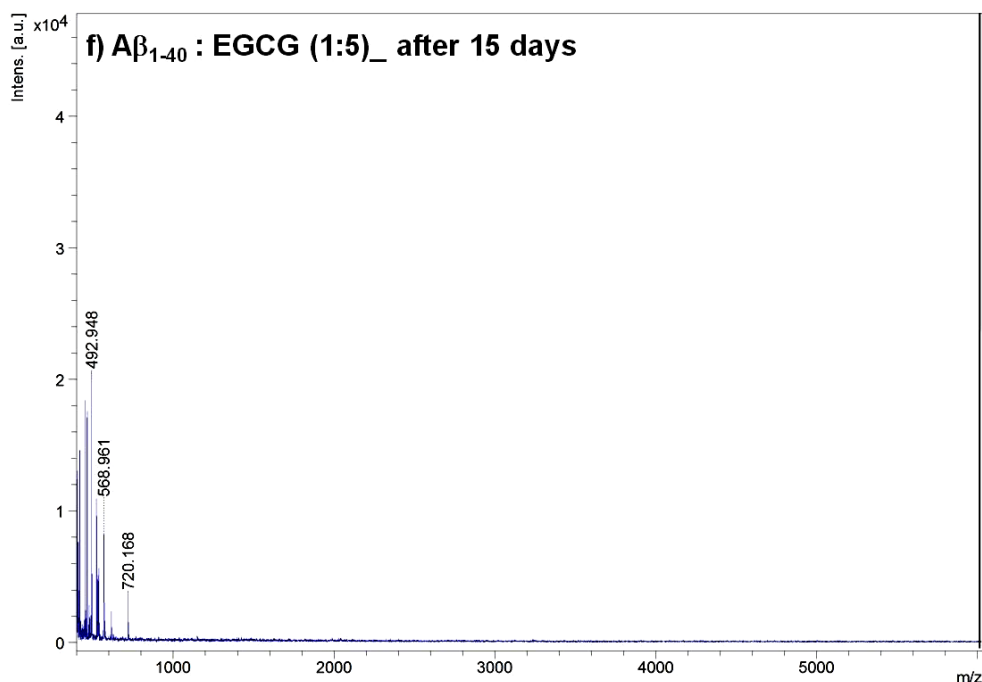


Fig. 3.110. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of EGCG (1:5) after fifteen days of incubation in PBS.

3.18. Examination of the proteolytic activity of mAP3 on DTP28:

Finally, to examine the selective nature of mAP3, a designed negative control (NC) peptide was co-incubated with mAP3 in 1:5 molar ratio at physiological pH and temperature and followed by time-dependent MALDI mass analyses. A 28 residue long partial sequence of the diphtheria toxin protein (DTP) was chosen as the negative control peptide as its sequence was unrelated to both $A\beta$ and the mAP3.

3.18.1. Self-degradation of the negative control (NC) peptide, DTP28:

NC underwent self-degradation at its Ser and Thr positions, which was confirmed by a parallel experiment with NC in the absence of mAP3 (Figure 3.111-3.115). Such cleavage may proceed via route 1^{NC} (Figure 3.111), which is unrelated to mAP3. The co-incubated sample did not show any of the expected fragments (Figure 3.116-3.117) due to cleavage by mAP3 unlike $A\beta_{1-40}$ even after ten days.

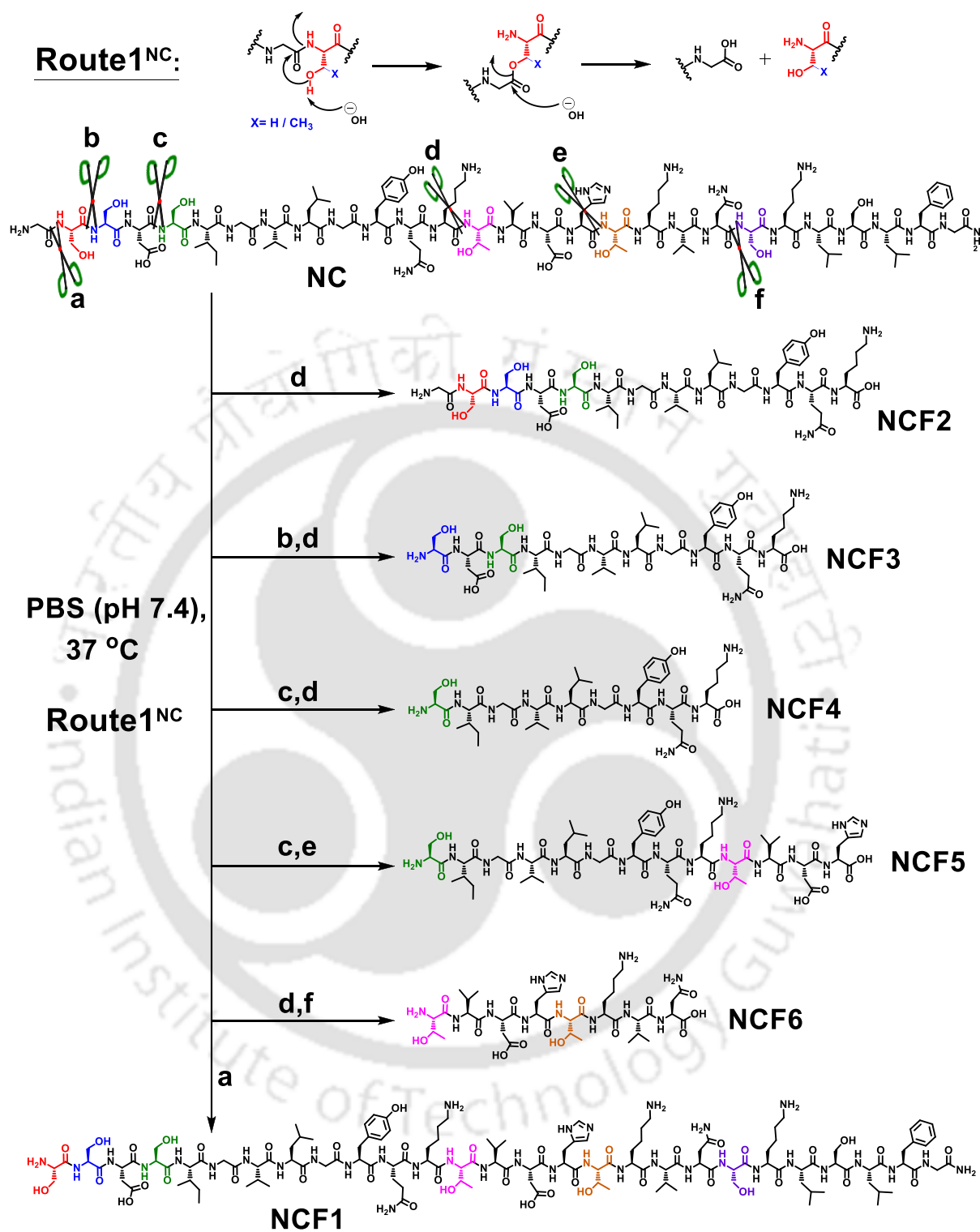


Figure 3.111. Plausible route of self-degradation of NC into various fragments (NCFs).

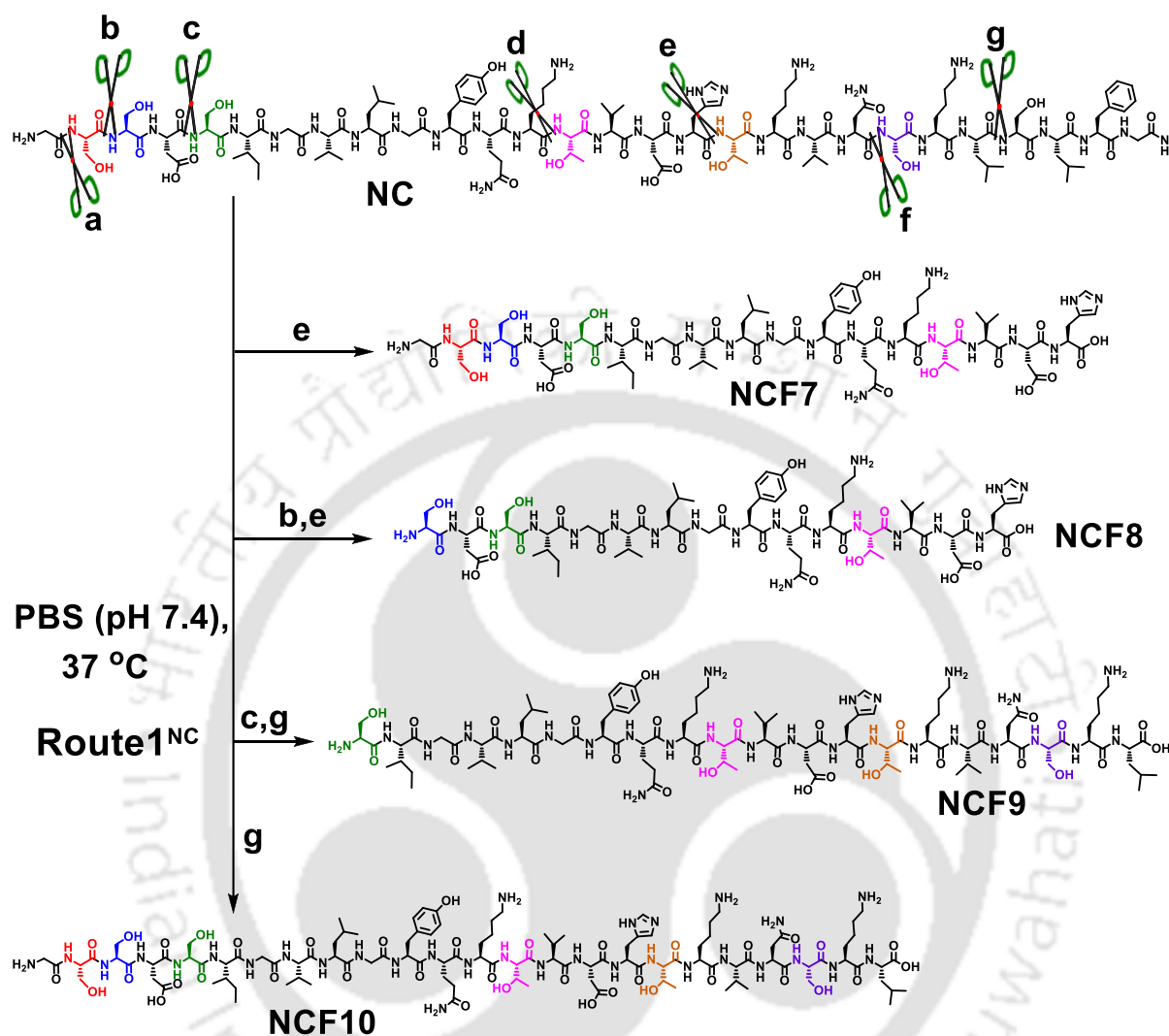
Route1^{NC} (continue):

Figure 3.112. Plausible route of self-degradation of NC into various fragments (NCFs).

Most of the logically drawn intermediates and final fragments could be identified by time-dependent MALDI-TOF mass spectrometric analyses of the NC peptide incubated in the absence of mAPs (Figure 3.113-3.115). The fragments are considered as self-degraded products at its Ser and Thr positions as described in route 1^{NC}.

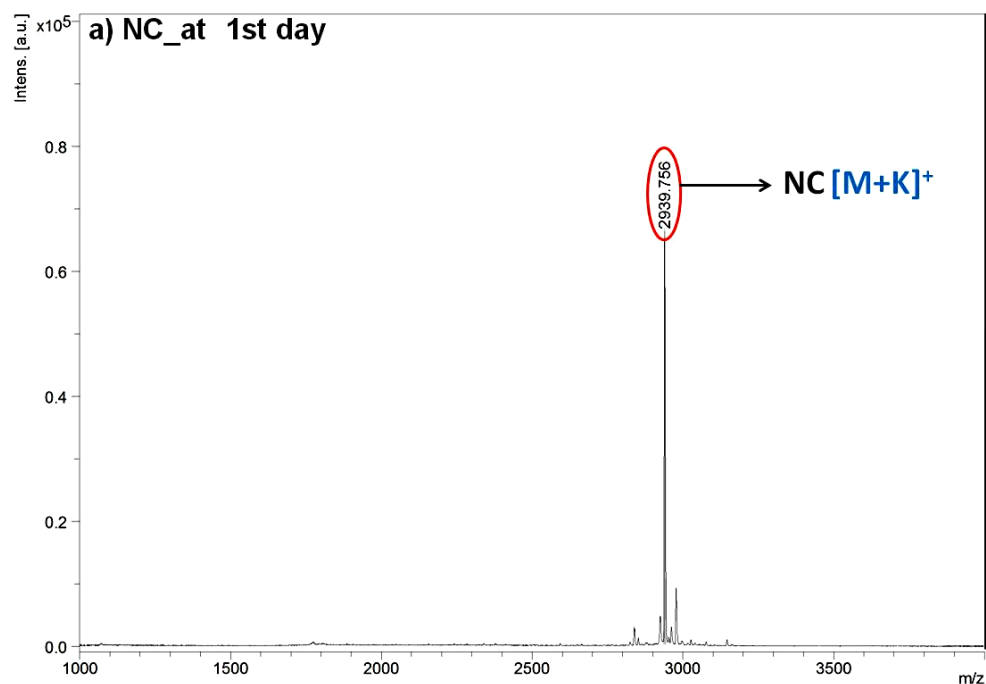


Figure 3.113. MALDI-TOF mass spectrum of NC in the absence of mAP3 at the 1st day of incubation in PBS.

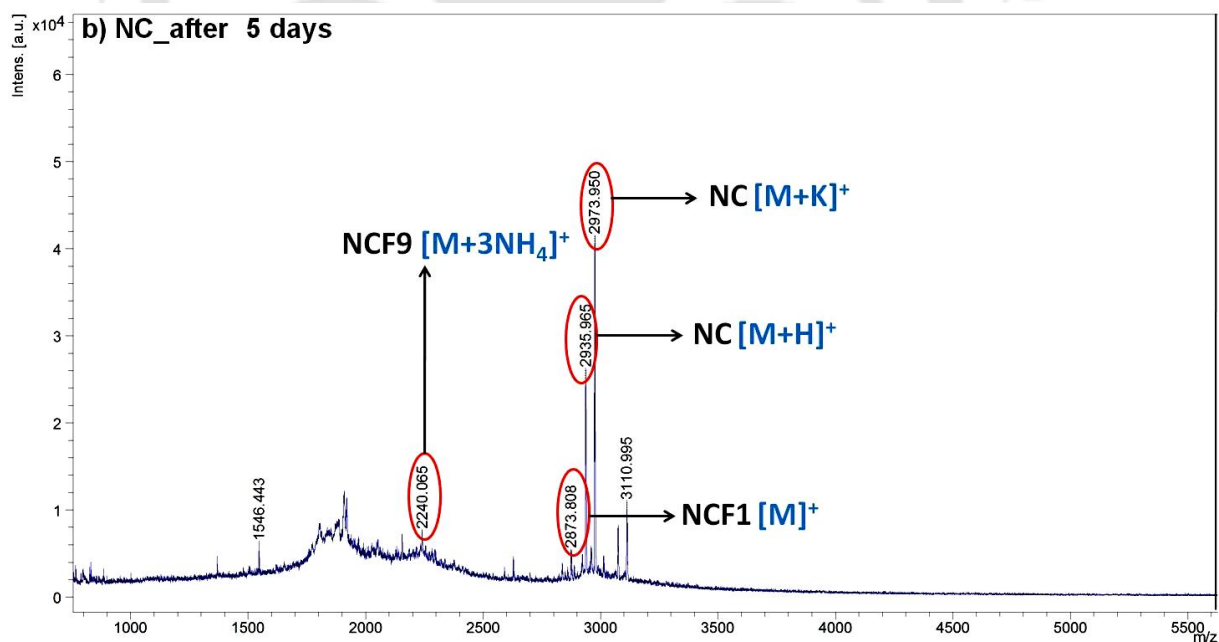


Figure 3.114. MALDI-TOF mass spectrum of NC in the absence of mAP3 after five days of incubation in PBS.

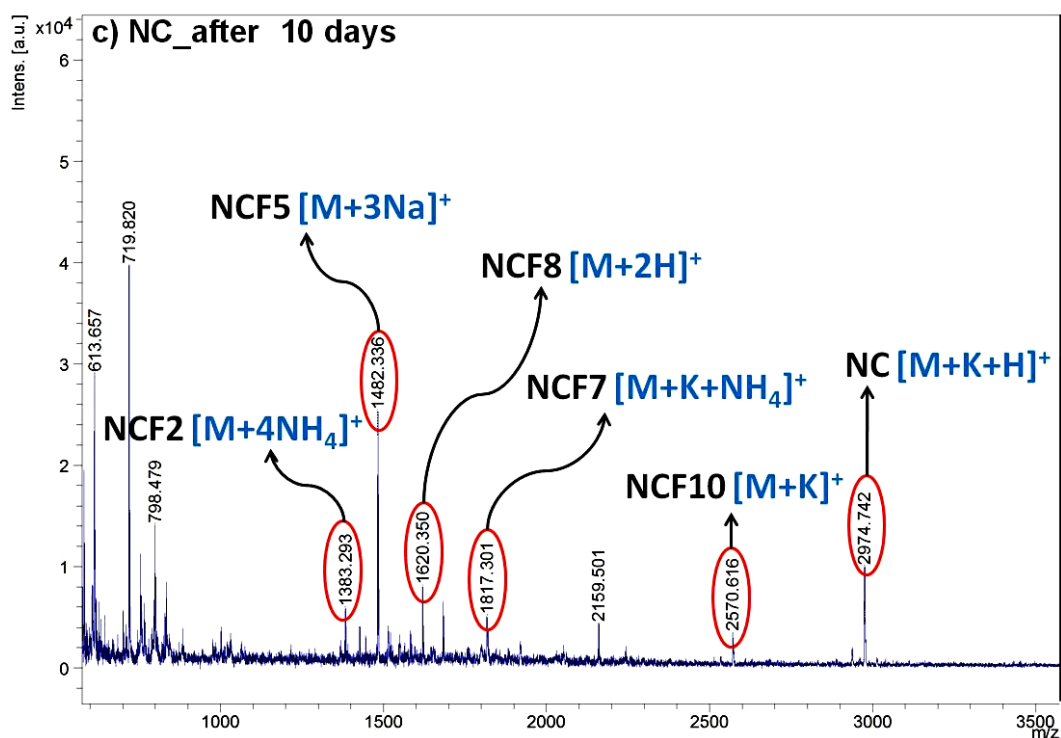


Figure 3.115. MALDI-TOF mass spectrum of NC in the absence of mAP3 after ten days of incubation in PBS.

3.18.2. Effect of mAP3 on the negative control (NC) peptide, DTP28:

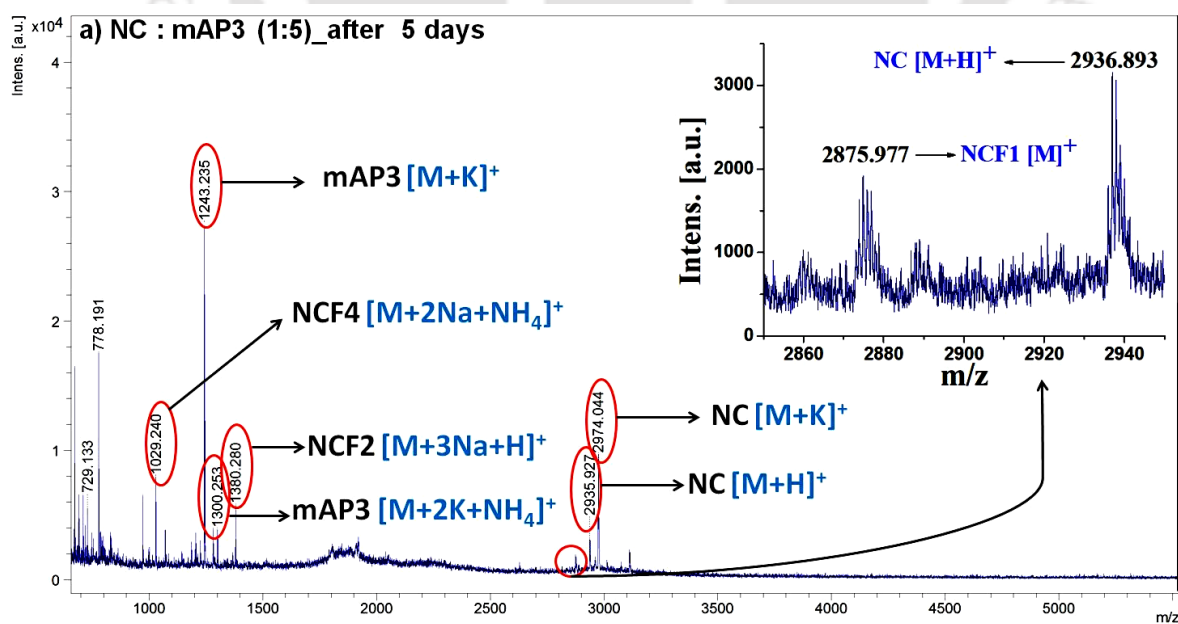


Figure 3.116. MALDI-TOF mass spectrum of NC in the presence of mAP3 (1:5) after five days of incubation in PBS.

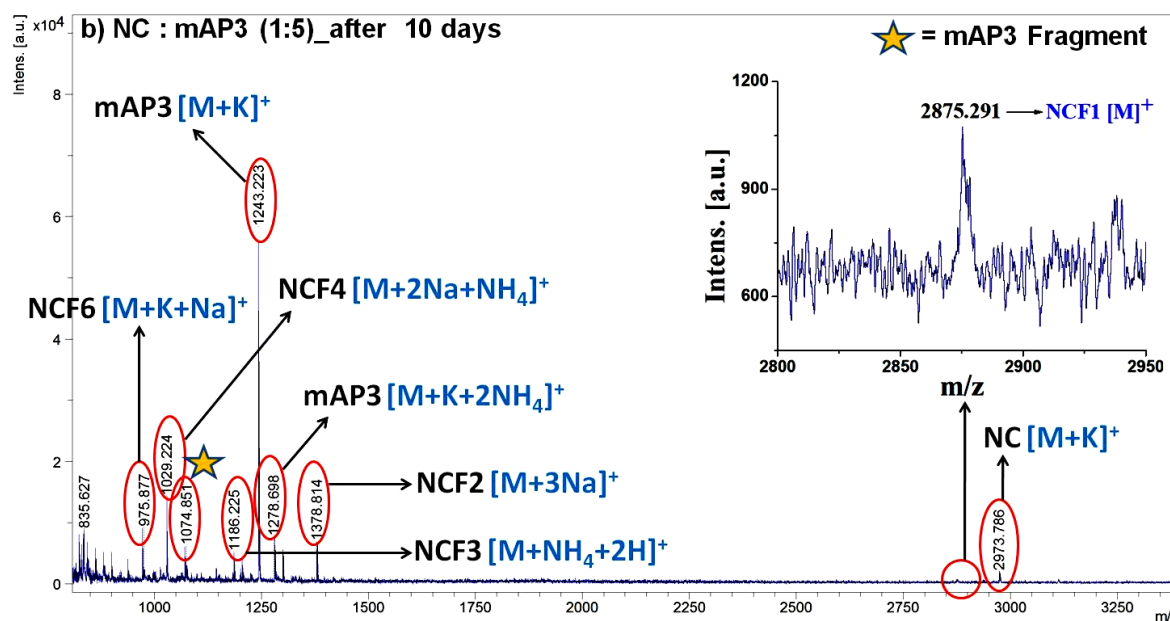


Figure 3.117. MALDI-TOF mass spectrum of NC in the presence of mAP3 (1:5) after ten days of incubation in PBS.

When the NC peptide was co-incubated separately in the absence and presence of mAP3, all of the fragments found for individual sets are not identical but were generated via the same mechanism; where mAP3 has no significant role without producing very few self-degraded fragments (as most of the self-degraded fragments of mAP3 appeared after 15 days as described earlier).

3.19. Inhibition of amyloid accumulation of $A\beta_{1-40}$ by mAPs:

After finding the proteolytic activity of mAPs against $A\beta_{1-40}$, we continued further to check the inhibition potential of mAPs. The kinetics of the amyloid accumulation of $A\beta_{1-40}$ was checked using different biophysical tools in absence and presence of mAPs in a dose-dependent manner. Samples were prepared mentioned before (mAP section) to obtain the stock solutions of $A\beta_{1-40}$ (50 μ M) and mAPs (1 mM). mAPs were co-incubated with $A\beta_{1-40}$ in PBS pH 7.4 at 37 $^{\circ}$ C in separate vessels on a water bath till 30 days in parallel at the desired proportions. To investigate dose-dependent inhibitory efficacy of mAP3, one-fold and a five-fold molar excess of mAP3 were co-incubated with 50 μ M of $A\beta_{1-40}$ at the physiological condition. To get the result more accurately another two replicate solutions of each portion were prepared and amyloid accumulation was monitored by different biophysical tools for up to 30 days.

3.19.1. ThT fluorescence assay of $A\beta_{1-40}$:

Dose-dependent ThT fluorescence assay of $A\beta_{1-40}$ was performed with mAP3 because of its prominent proteolytic activity among all mAPs. From the result (Figure 3.118a), it was found that the fluorescence intensity of $A\beta_{1-40}$ (black) increased with time in the absence of mAPs and became static up to 15 days after slight lowering in intensity, and further decreased later as there is a probability for mature fibril to come out from the solution. But in the presence of mAP3, the fluorescence intensity of the $A\beta_{1-40}$ decreased with time as the doses were increased from one-fold (red, Figure 3.118a) to five-fold (blue, Figure 3.118a) molar excess due to the formation of various $A\beta_{1-40}$ fragments cleaved by mAP3, indicating the excellent inhibitory efficacy of mAP3 against amyloid formation of $A\beta_{1-40}$ (Figure 3.118a). Later time-dependent ThT fluorescence assay of $A\beta_{1-40}$ was performed with mAP1-mAP3 (Figure 3.118b). From the result, it was cleared that mAP1 (red, Figure 3.118b) is not efficient to inhibit the amyloid formation of $A\beta_{1-40}$, as no such differences in fluorescence intensity were observed compare to $A\beta_{1-40}$ alone. But five-fold molar excess of mAP2 (blue, Figure 3.118b) was found to inhibit $A\beta_{1-40}$ aggregates with time. In conclusion, five-fold mAP2 or one-fold mAP3 was able to inhibit $A\beta_{1-40}$, but the more pronounced result was found in case of 5 fold mAP3 to inhibit the amyloid formation of $A\beta_{1-40}$.

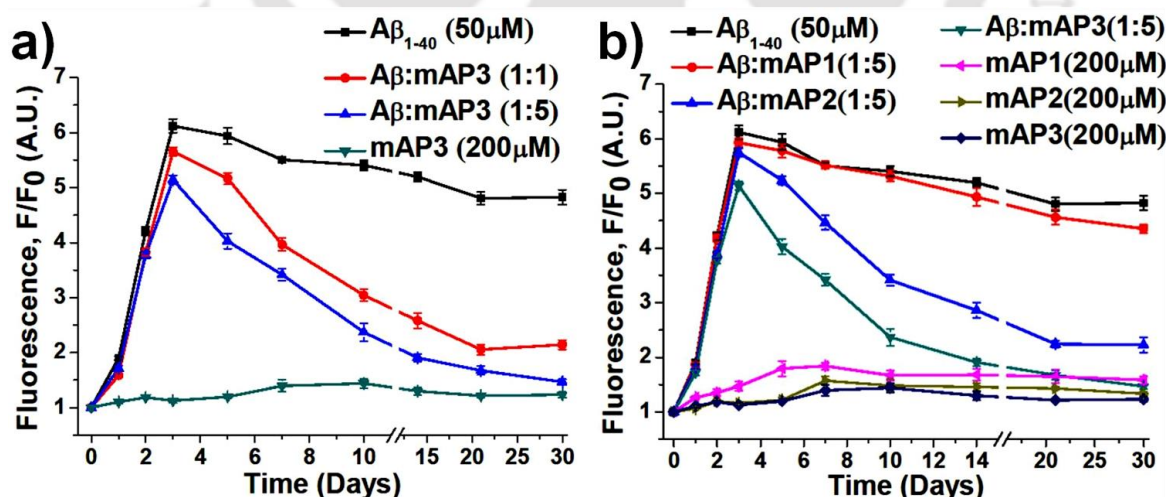


Figure 3.118. Normalized profiles of (a) dose-dependent, and (b) time-dependent ThT fluorescence assay. (a) Spectra of $A\beta_{1-40}$ in the absence (black), presence of one-fold mAP3 (red), five-fold mAP3 (blue), and mAP3 alone (dark cyan). (b) Spectra of $A\beta_{1-40}$ in the absence (black), presence of five-fold mAP1 (red), five-fold mAP2 (blue), five-fold mAP3 (dark cyan), mAP1 alone (magenta), mAP2

alone (dark yellow), and mAP3 alone (navy blue). Error bars represent standard deviations of at least three independent measurements. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

3.19.2. CD experiments of $A\beta_{1-40}$:

Time-dependent conformational changes of $A\beta_{1-40}$ (Figure 3.119a) were noticed from the random coil (a negative band at ~ 204 nm) to β -sheet (a positive band at ~ 204 nm with a negative band at ~ 225 nm) structure, confirmed the aggregation process. In the presence of one fold molar excess of mAP3, fibril formation of $A\beta_{1-40}$ (Figure 3.119b) was inhibited over time, and a mixture of more α -helices and random coil conformations were obtained throughout the kinetics.

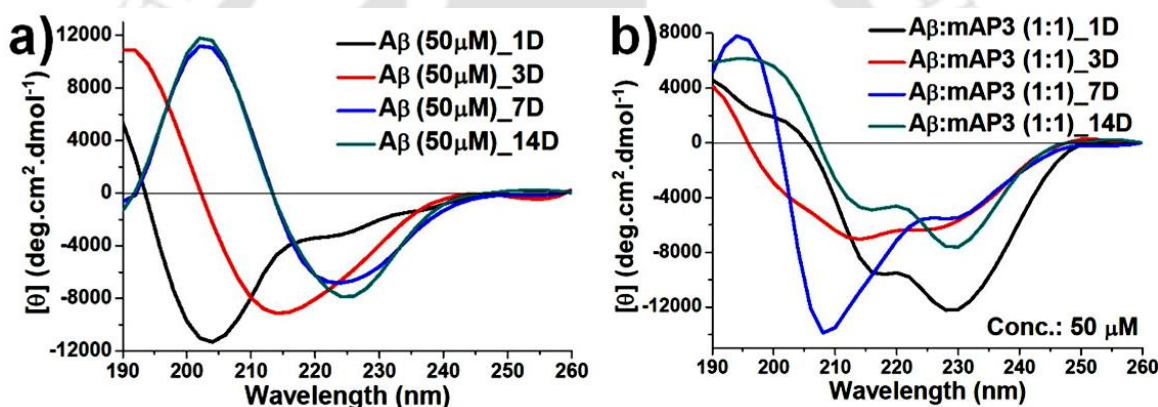


Figure 3.119. Time-dependent CD experiments of $A\beta_{1-40}$ (a) in absence, and (b) presence of one-fold mAP3. (a) Conversion of $A\beta_{1-40}$ from the random coil into β -sheet structure. (b) Inhibition capability of mAP3 to prevent the β -sheet structure of $A\beta_{1-40}$. Spectra of $A\beta_{1-40}$ at the 1st day (black), after three days (red), after seven days (blue), and after fourteen days (dark cyan) of incubation. The peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C.

Already it was demonstrated that $A\beta_{1-40}$ fibrils adopt a β -sheet conformation (black, Figure 3.120). In the presence of one fold mAP3 (red, Figure 3.120a), the propensity of $A\beta_{1-40}$ to form an α -helix (a positive band at ~ 200 nm with two negative bands at ~ 214 nm and ~ 229 nm) was increased, which confirms the reduction in aggregation. Random coil conformation of $A\beta_{1-40}$ (blue, Figure 3.120a) was confirmed (a negative band at ~ 199 nm) in the presence of five-fold molar excess of mAP3. Indeed, a mixture of predominantly α -helices and random coil (two negative bands at ~ 210 nm and ~ 235 nm) structure were obtained for $A\beta_{1-40}$ (red, Figure 3.120b) when co-incubated along with

five-fold molar excess of mAP2. The other peptides also display other secondary structures described as earlier (Figure 3.71).

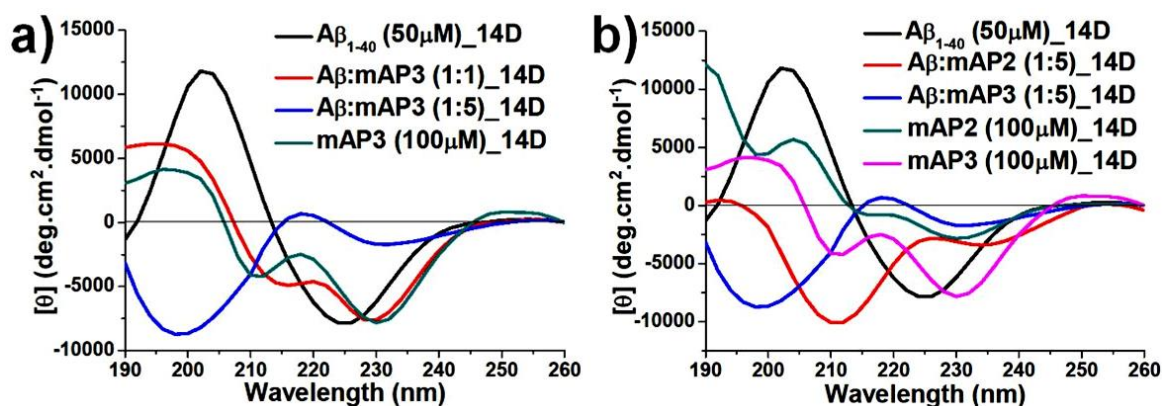


Figure 3.120. CD spectra of A β_{1-40} in the absence and presence of mAPs after fourteen days of incubation. (a) Spectra of A β_{1-40} in the absence (black), presence of one-fold mAP3 (red), five-fold mAP3 (blue), and mAP3 alone (dark cyan). (b) CD Spectra of A β_{1-40} in the absence (black), presence of 5 fold mAP2 (red), five-fold mAP3 (blue), mAP2 alone (dark cyan), and mAP3 alone (magenta). The peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C.

3.19.3. FTIR spectra of A β_{1-40} :

FTIR is also an important technique to monitor the secondary structure of the peptides and proteins. In FTIR spectra, the amide I band was detected (a) at 1634 cm^{-1} for A β_{1-40} alone (Figure 3.121a), (b) at 1646 cm^{-1} for A β_{1-40} in the presence of one-fold mAP3 (Figure 3.121b), (c) at 1651 cm^{-1} for A β_{1-40} in the presence of five-fold mAP3 (Figure 3.121c), (d) at 1656 cm^{-1} for mAP3 alone (Figure 3.121d), (e) at 1640 cm^{-1} for A β_{1-40} in the presence of five-fold mAP1 (Figure 3.121e), (f) at 1654 cm^{-1} for A β_{1-40} in presence of five-fold mAP2 (Figure 3.121f), (g) at 1651 cm^{-1} for mAP1 alone (Figure 3.121g), and (h) at 1652 cm^{-1} for mAP2 alone (Figure 3.121h). These results indicated that A β_{1-40} when incubated alone exhibited β -sheet structure, but in the presence of either mAP2 or mAP3, A β_{1-40} displayed mainly random coil or α -helix conformations.

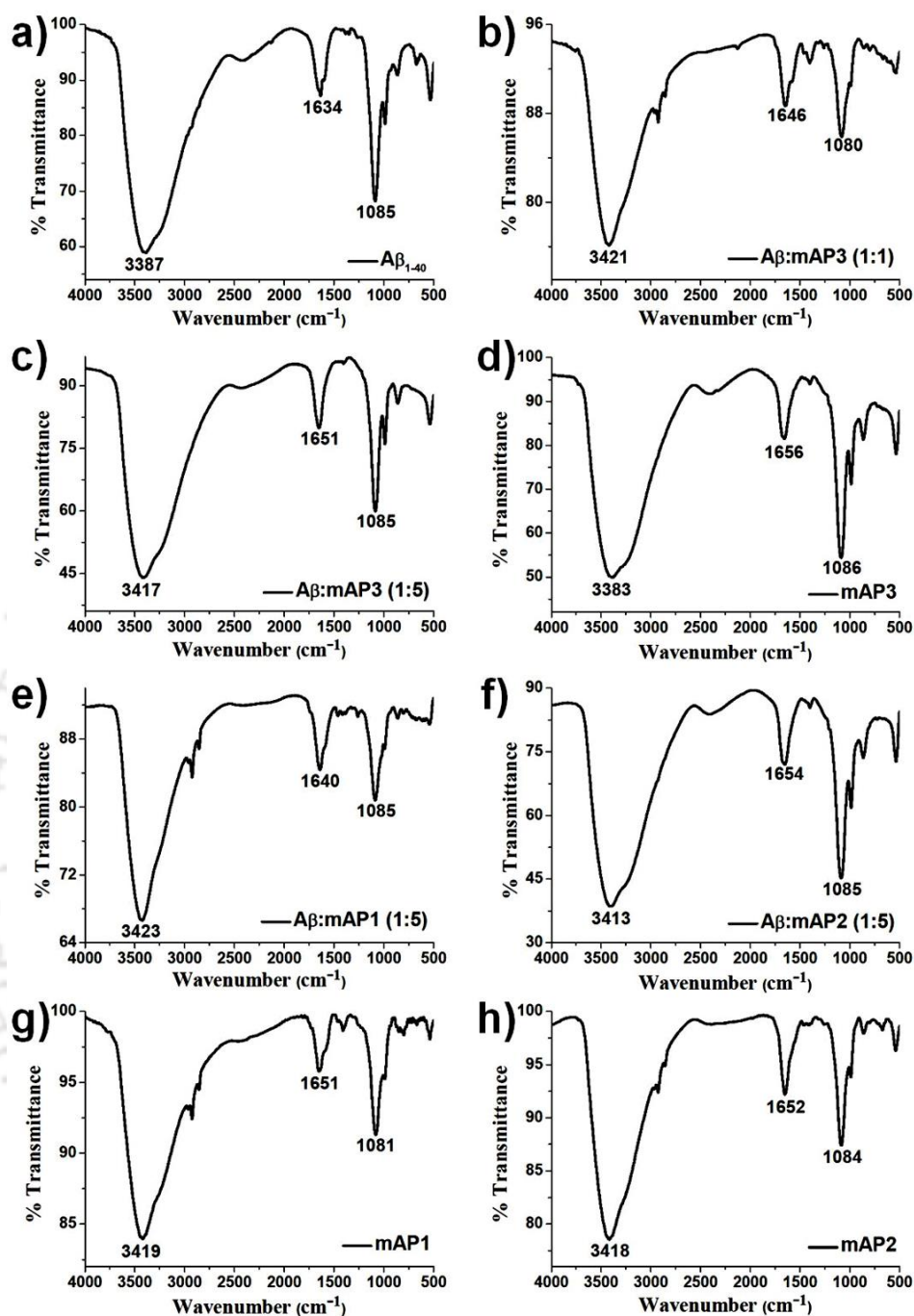


Figure 3.121. FTIR spectra of $A\beta_{1-40}$ in the absence and presence of mAPs after fourteen days of incubation. FTIR Spectra of $A\beta_{1-40}$ (a) in the absence, presence of (b) one-fold mAP3, (c) five-fold mAP3, and (d) mAP3 alone. FTIR Spectra of $A\beta_{1-40}$ in presence of (e) five-fold mAP1, (f) five-fold mAP2, (g) mAP1 alone, and (h) mAP2 alone. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C.

3.19.4. TEM images, Congo red-stained birefringence images, and AFM images of amyloid fibrils formed by $A\beta_{1-40}$:

The presence of fibril structure under TEM is one of the important features of amyloid formed by a peptide. The $A\beta_{1-40}$ alone formed clear fibrillar aggregates (Figure 3.122. a-i) under TEM, but in the presence of one-fold mAP3 (Figure 3.122. a-ii) and five-fold mAP3 (Figure 3.122. a-iii), no such fibre was observed rather amorphous structures were noticed. Though fibre was detected from the sample of $A\beta_{1-40}$ in the presence of five-fold mAP1 (Figure 3.122. d-i), amorphous structures along with very few tiny fibres were noticed for $A\beta_{1-40}$ when incubated along with five-fold mAP2 (Figure 3.122. d-ii). The mAP1 (Figure 3.122. d-iii) and mAP2 (Figure 3.122. d-iv) alone also did not show any fibre like mAP3 (Figure 3.122. a-iv). Therefore it can be concluded mAP2 and mAP3 have the capability to inhibit aggregation of $A\beta_{1-40}$, unlike mAP1.

The appearance of green gold birefringence of a peptide under cross-polarized light after staining with Congo red dye is another characteristic feature of amyloid present in the sample. The bright green-gold birefringence was observed for $A\beta_{1-40}$ alone under cross-polarized light (Figure 3.122. b-i), but when $A\beta_{1-40}$ was incubated in the presence of one-fold mAP3 (Figure 3.122. b-ii) and five-fold mAP3 (Figure 3.122. b-iii) separately, no such green gold birefringence were observed. Though in the presence of five-fold mAP1, $A\beta_{1-40}$ showed green gold birefringence (Figure 3.122. e-i), but such characteristic birefringence was not observed for $A\beta_{1-40}$ along with five-fold mAP2 (Figure 3.122. e-ii) when viewed under cross-polarized light. These results support that both mAP2 and mAP3, unlike mAP1, significantly inhibited amyloid aggregation of $A\beta_{1-40}$. From AFM images, the same morphologies (Figure 3.122. c, f) were obtained as found in TEM images. The images were taken after fourteen days of incubation.

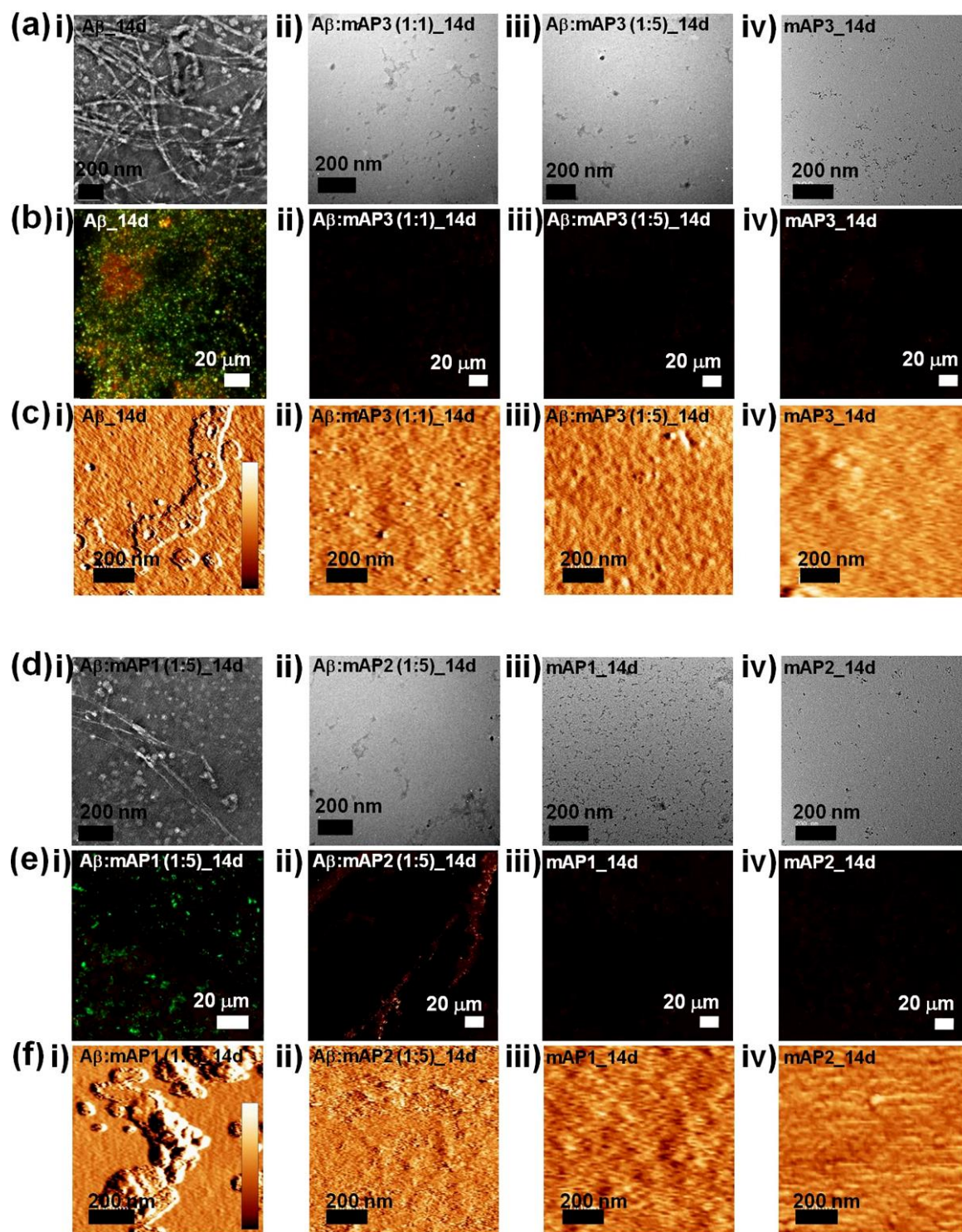


Figure 3.122. (a) TEM images, (b) Congo red-stained birefringence images, and (c) AFM images of A β ₁₋₄₀ (i) in the absence, and presence of (ii) one-fold mAP3, (iii) five-fold mAP3, and (iv) mAP3 alone. (d) TEM images, (e) Congo red-stained birefringence images, and (f) AFM images of A β ₁₋₄₀ in the presence of (i) five-fold mAP1, (ii) five-fold mAP2, (iii) mAP1 alone, and (iv) mAP2 alone. Scale bars are indicated as 200 nm for TEM and AFM images [height data (inset), scan size 1 μ m, Z range 90nm for c-i & f-i, and 20 nm for (c, f)-(ii-iv)], and 20 μ m for birefringence images. All the peptide

solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel. All the images were captured after fourteen days of incubation.

3.20. Disruption of amyloid accumulation of $A\beta_{1-40}$ by mAPs:

Earlier we found that when $A\beta_{1-40}$ was co-incubated along with mAP3, it inhibited the amyloid formation of $A\beta_{1-40}$ in a dose-dependent manner. At the diseased condition, the AD-affected brain is rich in toxic oligomers and amyloid plaques due to the presence of preformed fibrils. So, it is necessary to check whether mAPs can disrupt the preformed fibrillar aggregates of $A\beta_{1-40}$ or not, *in vitro*. We noted that the growth phase for fibrillization of $A\beta_{1-40}$ was from 24 h to 72 h. Therefore, mAPs were added in a dose-dependent manner to the $A\beta_{1-40}$ at 72 h (PBS pH 7.4 at 37 °C), assuming the presence of existing fibrillar assembly and the kinetics of amyloid disruption was monitored further by various biophysical tools up to 30 days and compared with the inhibition results.

3.20.1. ThT fluorescence assay of $A\beta_{1-40}$:

The time-dependent increase in fluorescence intensity of $A\beta_{1-40}$ (black) was noticed in the absence of mAPs as described earlier (inhibition section). But on the addition of one-fold mAP3 (red, Figure 3.123a) and five-fold mAP3 (blue, Figure 3.123a) to the $A\beta_{1-40}$ at 72 h *in vitro*, the fluorescence intensity of $A\beta_{1-40}$ suppressed significantly with time in a dose-dependent manner due to the formation of various $A\beta_{1-40}$ fragments cleaved by mAP3. The decrease in fluorescence intensity of $A\beta_{1-40}$ was also observed after addition of 5 fold mAP2 (red, Figure 3.123b) with time. The amount of preformed fibril of $A\beta_{1-40}$ at 72 h was found to be reduced by five-fold mAP3 (blue, Figure 3.123a/b) more significantly than that of one-fold mAP3 (red, Figure 3.123a) and five-fold mAP2 (red, Figure 3.123b).

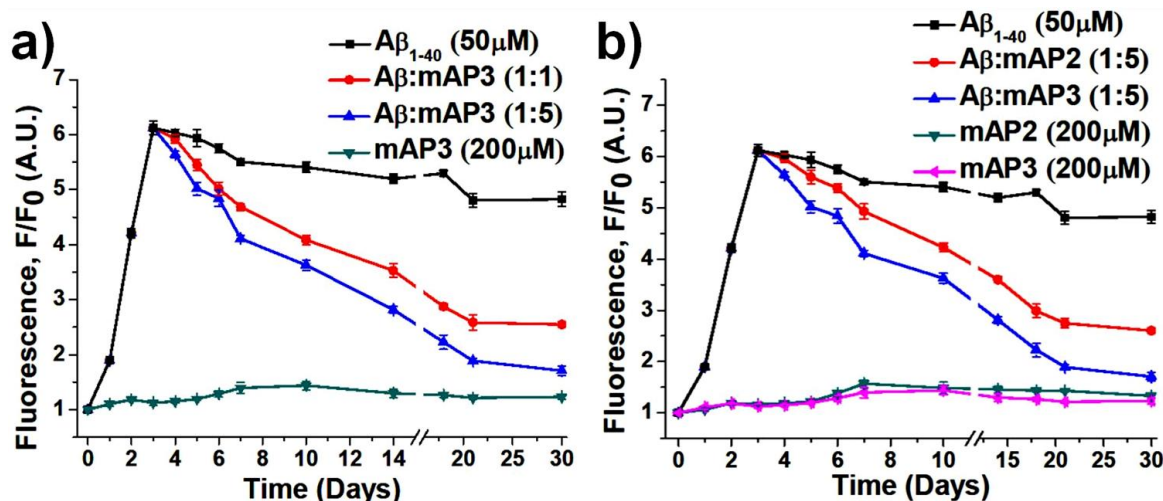


Figure 3.123. Normalized profiles of (a) dose-dependent, and (b) time-dependent ThT fluorescence assay where mAPs were added after three days to the preformed fibril of $A\beta_{1-40}$. (a) Spectra of $A\beta_{1-40}$ in the absence (black), presence of one-fold mAP3 (red), five-fold mAP3 (blue), and mAP3 alone (dark cyan). (b) Spectra of $A\beta_{1-40}$ in absence (black), presence of five-fold mAP2 (red), five-fold mAP3 (blue), mAP2 alone (dark cyan), and mAP3 alone (magenta). Error bars represent standard deviations of at least three independent measurements. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

3.20.2. CD experiments of $A\beta_{1-40}$:

β -Sheet structure of fibril $A\beta_{1-40}$ (black, Figure 3.124) was concluded from a positive band at ~ 204 nm with a negative band at ~ 225 nm. CD spectra confirmed the random coil conformation (a negative band at ~ 205 nm) of $A\beta_{1-40}$ (red, Figure 3.124a) when co-incubated along with one fold mAP3. Random coil conformation (a negative band at ~ 198 nm) of $A\beta_{1-40}$ (blue, Figure 3.124a) was also concluded in the presence of five-fold molar excess of mAP3. The tendency of $A\beta_{1-40}$ to form an α -helix (a positive band at ~ 202 nm with two negative bands at ~ 215 nm and ~ 230 nm) was increased in the presence of five-fold mAP2 (red, Figure 3.124b), which confirms the reduction in aggregation. The secondary structures of the control peptides were also displayed as described earlier (Figure 3.71).

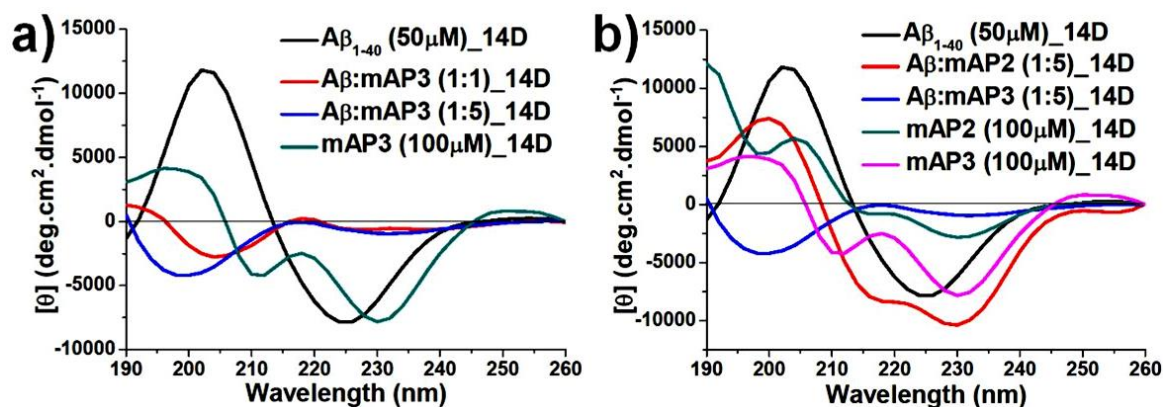


Figure 3.124. CD spectra of $\text{A}\beta_{1-40}$ in the absence and presence of mAPs after fourteen days of incubation. (a) Spectra of $\text{A}\beta_{1-40}$ in the absence (black), presence of one-fold mAP3 (red), five-fold mAP3 (blue), and mAP3 alone (dark cyan). (b) CD Spectra of $\text{A}\beta_{1-40}$ in the absence (black), presence of five-fold mAP2 (red), five-fold mAP3 (blue), mAP2 alone (dark cyan), and mAP3 alone (magenta). The peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C.

3.20.3. FTIR spectra of $\text{A}\beta_{1-40}$:

The conformation of the peptides was verified by FTIR spectroscopic analysis also. The amide I band was observed (a) at 1634 cm^{-1} for $\text{A}\beta_{1-40}$ alone (Figure 3.125a), (b) at 1650 cm^{-1} for $\text{A}\beta_{1-40}$ in presence of one-fold mAP3 (Figure 3.125b), (c) at 1651 cm^{-1} for $\text{A}\beta_{1-40}$ in presence of five-fold mAP3 (Figure 3.125c), and (d) at 1645 cm^{-1} for $\text{A}\beta_{1-40}$ in presence of 5 fold mAP2 (Figure 3.125d). From the results, it was confirmed that $\text{A}\beta_{1-40}$ displayed β -sheet structure in the absence of mAPs; but in the presence of either mAP2 or mAP3, $\text{A}\beta_{1-40}$ exhibited random coil or α -helix conformation.

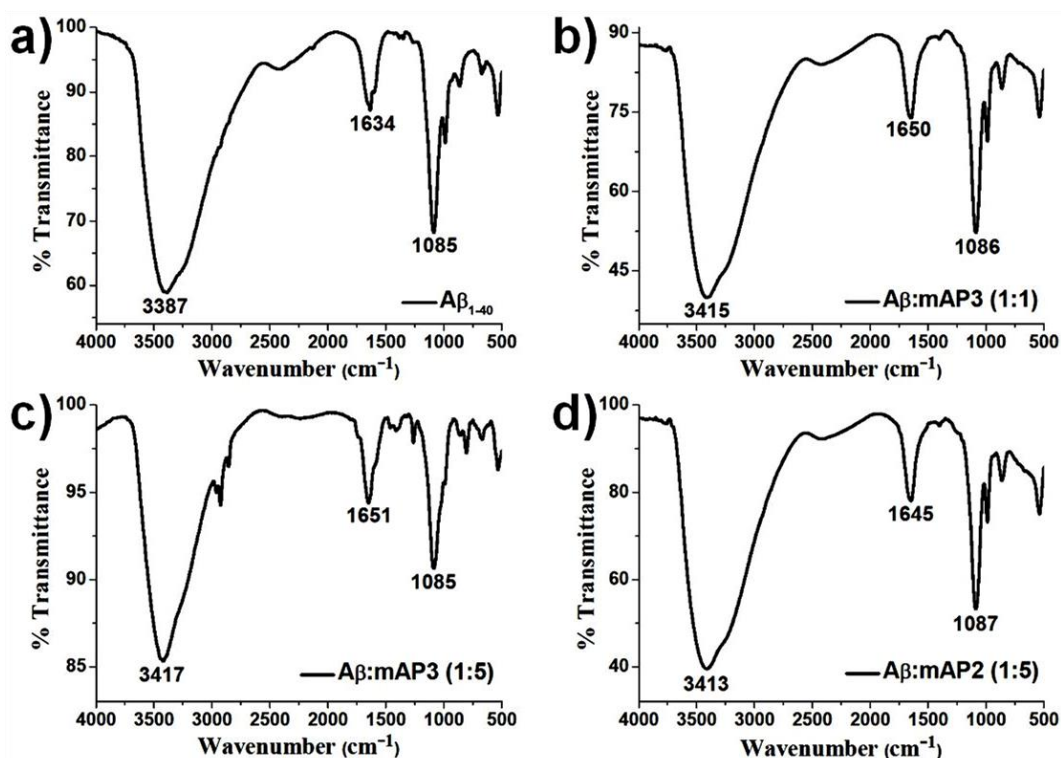


Figure 3.125. FTIR spectra of $A\beta_{1-40}$ in the absence and presence of mAPs after fourteen days of incubation. FTIR Spectra of $A\beta_{1-40}$ (a) in the absence, presence of (b) one-fold mAP3, (c) five-fold mAP3, and (d) five-fold mAP2. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C.

3.20.4. TEM images, Congo red-stained birefringence images, and AFM images of amyloid fibrils formed by $A\beta_{1-40}$:

The propensity of amyloidogenicity of $A\beta_{1-40}$ was characterized by TEM, Congo red-stained birefringence, and AFM studies. When $A\beta_{1-40}$ was incubated in the absence of mAPs, the clear fibrillar aggregates (Figure 3.126. a-i) and green gold birefringence (Figure 3.126. b-i) were observed from the sample when viewed under TEM and cross-polarized light respectively. But in the presence of one-fold mAP3, no such fibre (Figure 3.126. a-ii) or green gold birefringence (Figure 3.126. b-ii) was observed, indicates one-fold mAP3 is sufficient to disrupt the aggregation of $A\beta_{1-40}$. When the dose was increased from one-fold to five-fold, mAP3 disrupted amyloid of $A\beta_{1-40}$ totally as amorphous structure (Figure 3.126. a-iii), and no such birefringence (Figure 3.126. b-iii) was noticed under TEM and cross-polarized light respectively. But fivefold mAP2 was found to be not sufficient to disrupt the aggregation of $A\beta_{1-40}$, a few fibres (Figure 3.126. a-iv) and

little amount of green gold birefringence (Figure 3.126. b-iv) was noticed for the $A\beta_{1-40}$ in the presence of five-fold mAP2 under TEM and cross-polarised light respectively. From AFM images, the same morphologies (Figure 3.126. c) were obtained as found in TEM images. The images were taken after fourteen days of incubation.

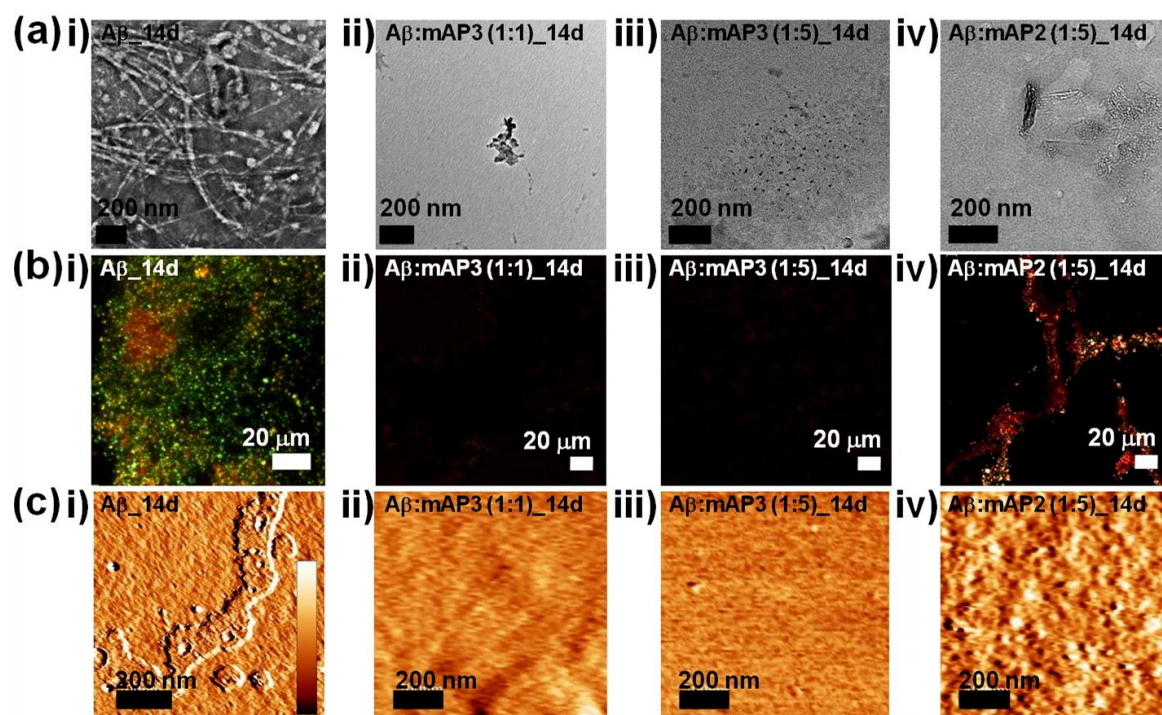


Figure 3.126. (a) TEM images, (b) Congo red-stained birefringence images, and (c) AFM images of $A\beta_{1-40}$ (i) in absence, and presence of (ii) one-fold mAP3, (iii) five-fold mAP3, and (iv) five-fold mAP2. Scale bars are indicated as 200 nm for TEM and AFM images [height data (inset), scan size 1 μ m, Z range 90nm for c-i, and 20 nm for c-(ii-iv)], and 20 μ m for birefringence images. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 $^{\circ}$ C in parallel. All the images were captured after fourteen days of incubation.

3.21. Time-dependent TEM images and Congo red stained birefringence images of amyloid fibrils formed by $A\beta_{1-40}$:

The time-dependent TEM (Figure 3.127. a-c) and Congo red stained birefringence (Figure 3.127. d-f) studies were performed to get an idea regarding the time scale of amyloid inhibition and disruption. It was observed that while the fibrillar structure and green-gold birefringence continued for $A\beta_{1-40}$ (Figure 3.127. a, d) during the entire duration of the experiment, they gradually decreased and finally disappeared at fourteen days of incubation in the presence of five-fold mAP3 (Figure 3.127. b, e for inhibition, and Figure

3.127. c, f for disruption). All the investigations were carried out under near-physiological conditions in vitro (PBS of pH 7.4 at 37 °C).

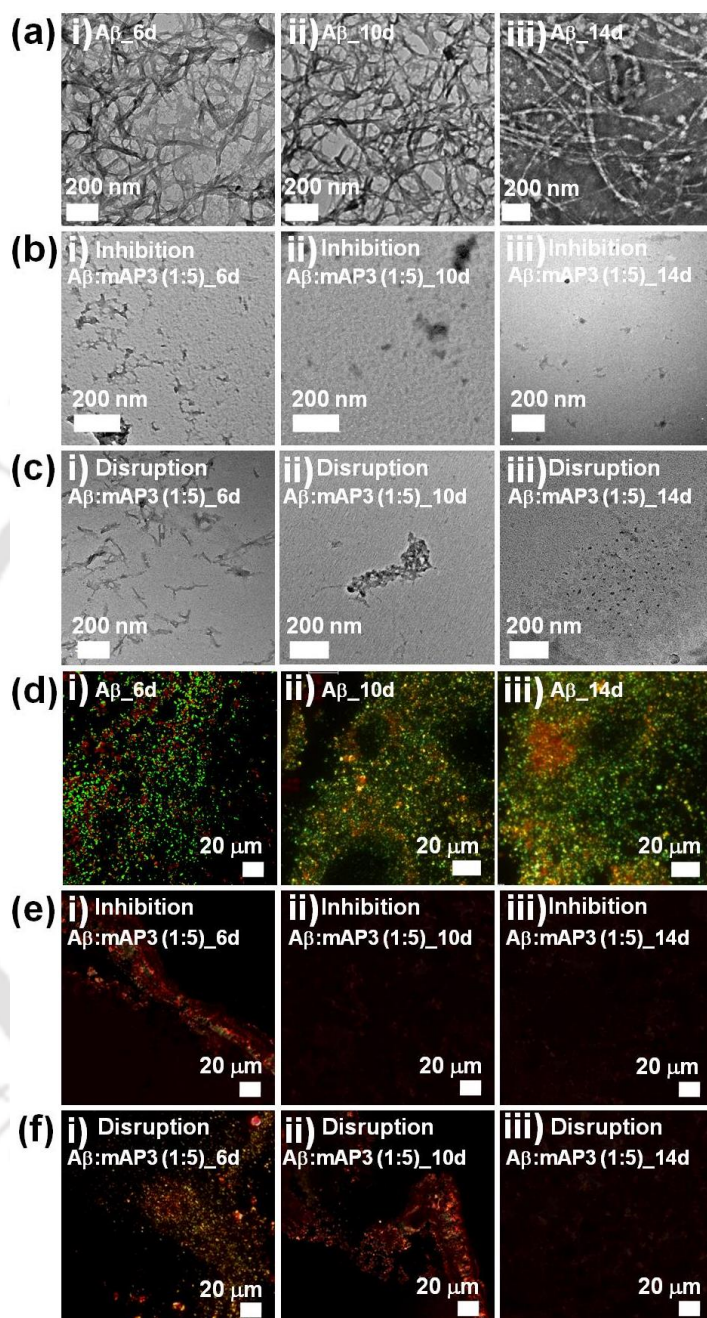


Figure 3.127. Time-dependent (a-c) TEM images, and (d-f) Congo red-stained birefringence images of $A\beta_{1-40}$ (a, d) in the absence, and presence of (b-c, e-f) five-fold molar mAP3. Images were recorded after (i) six days, after (ii) ten days, and after (iii) fourteen days of incubation [(b, e) for inhibition studies, and (c, f) for disruption studies where mAP3 was added after 3 days to the preformed fibril of $A\beta_{1-40}$]. Scale bars are indicated as 200 nm for TEM images, and 20 μ m for birefringence images. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

3.22. DLS analysis of inhibiting $A\beta_{1-40}$ fibril formation by mAP3:

From dynamic light scattering (DLS) study, it is possible to determine qualitatively about the aggregated state of $A\beta_{1-40}$.¹⁵ After 24 h of incubation, large particles with a diameter of ~ 900 nm (along with a small peak of ~ 5500 nm) were observed for $A\beta_{1-40}$ alone (black, Figure 3.128). But when $A\beta_{1-40}$ was incubated along with one-fold mAP3 (red, Figure 3.128) and five-fold mAP3 (blue, Figure 3.128), the peak was shifted to ~ 400 nm and ~ 300 nm respectively corresponding to smaller molecular-weight aggregates. The mAP3 in the absence of $A\beta_{1-40}$ exhibited a peak ~ 60 nm (dark cyan, Figure 3.128) after 24 h. So, mAP3 can reduce the size of $A\beta$ aggregates to different extents in a dose-dependent manner.

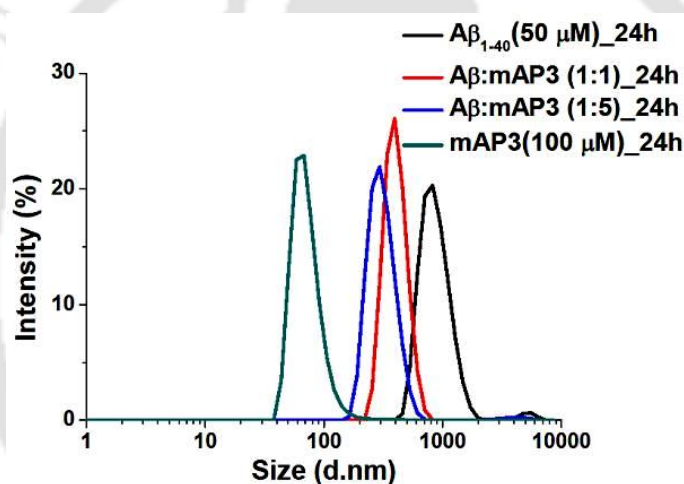


Figure 3.128. Overlay of DLS profiles of $A\beta_{1-40}$ after 24 h of incubation in the absence (black), and presence of one-fold molar excess of mAP3 (red), five-fold molar excess of mAP3 (blue); and mAP3 alone (dark cyan).

3.23. Vesicle leakage study:

Oligomeric species can form pores in the cell membrane resulting in membrane disruption.¹⁶ Therefore soluble oligomers of $A\beta_{1-40}$ are considered to be more toxic than insoluble fibrillar aggregates of $A\beta_{1-40}$. From the vesicle leakage study,¹¹ it is possible to measure cell membrane disruption capability by additives and therefore were performed using carboxyfluorescein dye entrapped large unilamellar vesicles (LUVs, cell membrane mimic) to prove the existence of toxic oligomeric species in the solution of $A\beta_{1-40}$ co-incubated along with mAPs at the physiological condition. Prepared LUVs were

characterized by transmission electron microscopy (TEM, Figure 3.129a). From the result, it was observed that the solution A ($A\beta_{1-40}$ incubated for 24h, red, Figure 3.129. b-c) caused more dye leakage implies the presence of toxic oligomeric species. Again, the solution B ($A\beta_{1-40}$ incubated for ten days, blue, Figure 3.129. b-c) caused very less LUV leakage in comparison to solution A. The results confirmed that soluble oligomers have greater ability to make pore formation on LUVs and hence more toxic in comparison to the mature fibrils of $A\beta_{1-40}$. The $A\beta_{1-40}$ treated with mAP2 (solution C) caused little more dye leakage (dark cyan, Figure 3.129. b-c) in comparison to solution B. Lesser extent of dye leakage was observed when fivefold mAP3 (magenta, Figure 3.129. b-c) was co-incubated with $A\beta_{1-40}$ (solution D), and that leakage was almost comparable with that of the untreated LUVs (black, Figure 3.129. b-c). The results confirmed that mAP2 and mAP3 disrupted the preformed amyloid aggregates of $A\beta_{1-40}$ into non-toxic species.

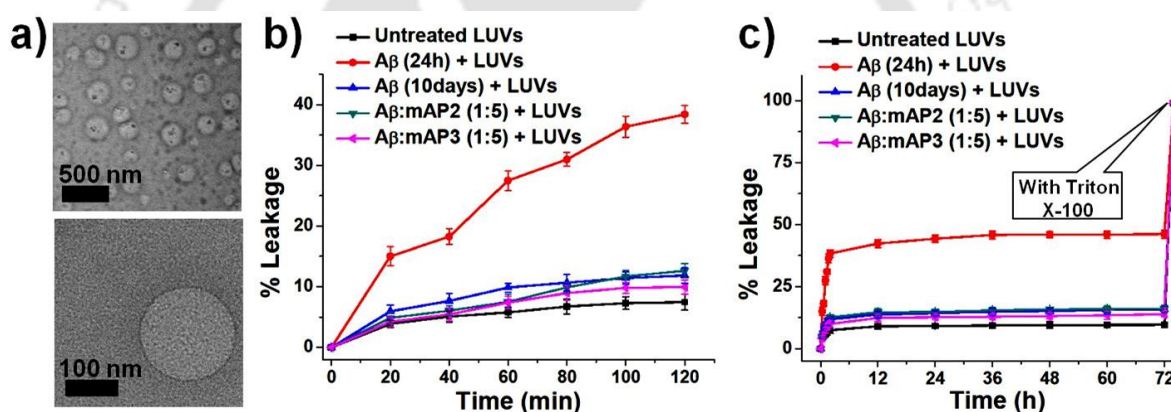


Figure 3.129. (a) TEM images of negatively stained LUVs. The concentration of the sample was 100 μ M. Scale bars are indicated as 500 nm (for upper one) and 100 nm (for lower one). % dye (carboxyfluorescein) leakage from LUVs (b) up to 120 min and (c) up to 72 h. % of dye leakage by untreated LUVs (black), LUVs treated with $A\beta_{24h}$ (red), LUVs treated with $A\beta_{10d}$ (blue), LUVs treated with $A\beta$:mAP2(1:5)_{10d} (dark cyan), and LUVs treated with $A\beta$:mAP3(1:5)_{10d} (magenta) [mAPs were added after 3 days to the preformed fibril of $A\beta_{1-40}$]. Error bars represent standard deviations of at least three independent measurements.

3.24. Conclusions:

Chymotrypsin is a Ser protease which hydrolyzes the peptide bonds specifically adjacent to an aromatic amino acid residues (Trp, Phe, Tyr). We have designed and synthesized Asp-His-Ser (DHS) triad containing peptides named mAPs in such a way that the active site of the peptide has a pocket where Asp, His, and Ser are linked via intramolecular hydrogen bonding so that Ser participates the hydrolysis of a peptide bond adjacent to the triad. Keeping in view of the importance of α -secretase site specificity, triad segment was attached with self-assembling core hydrophobic region of $A\beta$ V₁₈FFA₂₁. Grippingly, incorporation of Gly in between triad (DHS) residues is significant but crucial in-between triad and VFFA segments; and higher proteolysis rate was observed in case of two consecutive Gly rather than one Gly in the particular cases. The desired pocket made by the triad (DHS) of mAP3 was determined from DFT calculation, and the binding sites of mAP that recognize the target peptide have been searched by molecular docking. It was found that newly designed mAPs cleave a smaller $A\beta$ fragment ($A\beta_{12-21}$ or APP₆₈₃₋₆₉₂, m $A\beta$) and $A\beta$ also in a site-selective manner; and it cleaves every amide bond in-between Gln₁₅-Ala₂₁ initially at the physiological condition, which was confirmed from MALDI-TOF mass analyses. Results obtained from several biophysical tools (ThT-assay, CD, FTIR, TEM, AFM, Congo red birefringence, and DLS) suggest that mAPs are non-amyloidogenic and significantly inhibited as well as disrupted the preformed amyloid of $A\beta_{1-40}$ into non-toxic metabolites *in vitro*. After analysing various experiments it was concluded that 1 fold mAP3 or 5 fold mAP2 was able to inhibit and disrupt the amyloid formation of aggregated $A\beta_{1-40}$, but more pronounced results were found in case of 5 fold mAP3. So, a lower dosage of mAP3 may reduce the side effects. Therefore newly developed mAP is a drug-like molecule that cleaves the peptide backbone of $A\beta$ selectively and may cure AD, and hence the designed mAPs offer a new therapeutic option against amyloidoses (e.g. AD, type 2 diabetes mellitus, Parkinson's disease, etc.).

3.25. References:

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3.26. Supporting data:

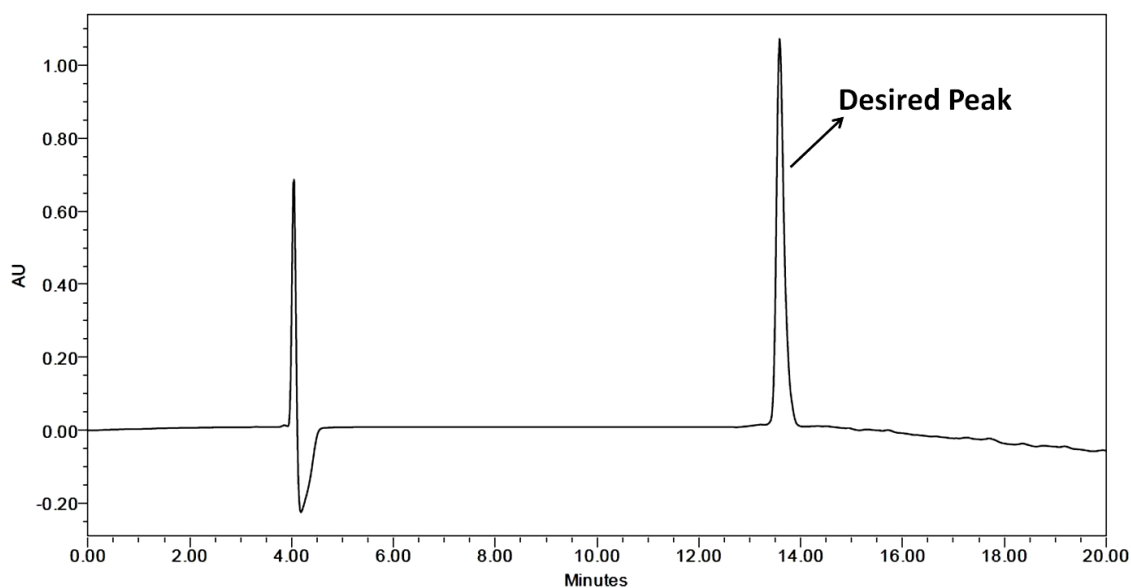


Figure 3.130. HPLC profile picture of the pure mAP1.

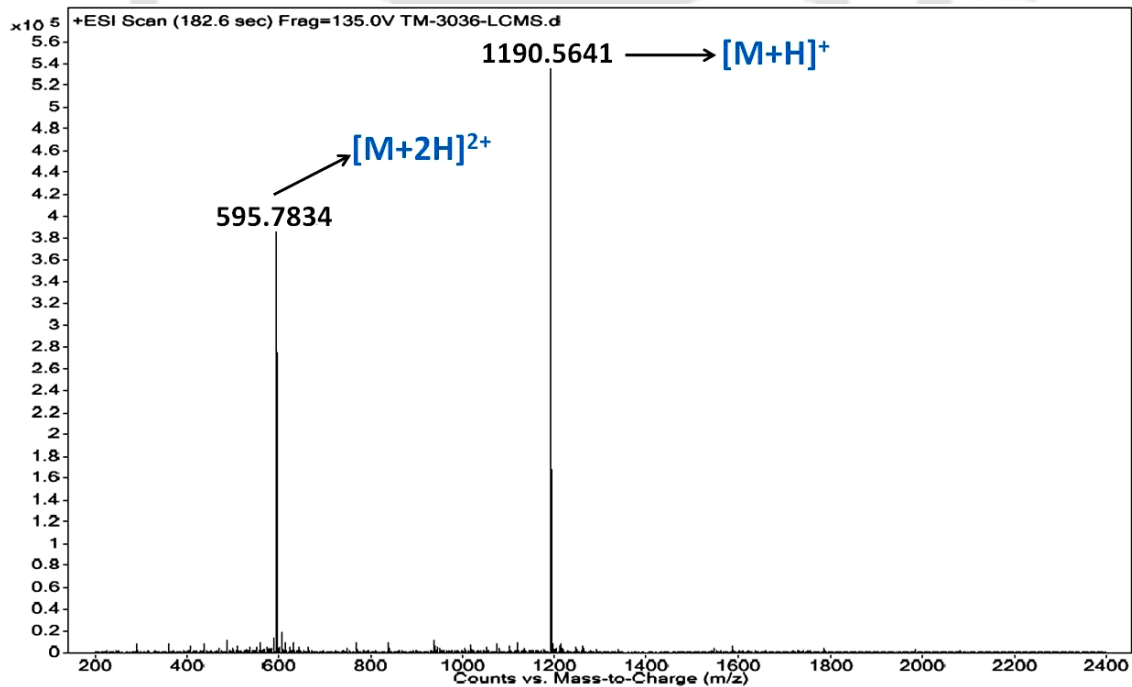


Figure 3.131. ESI-MS profile picture of the pure mAP1. Calculated m/z for $C_{54}H_{75}N_{15}O_{16}$ $[M+H]^+$ is 1190.5594, observed 1190.5641, calculated m/z for $C_{54}H_{75}N_{15}O_{16}$ $[M+2H]^{2+}$ is 595.7836, observed 595.7834.

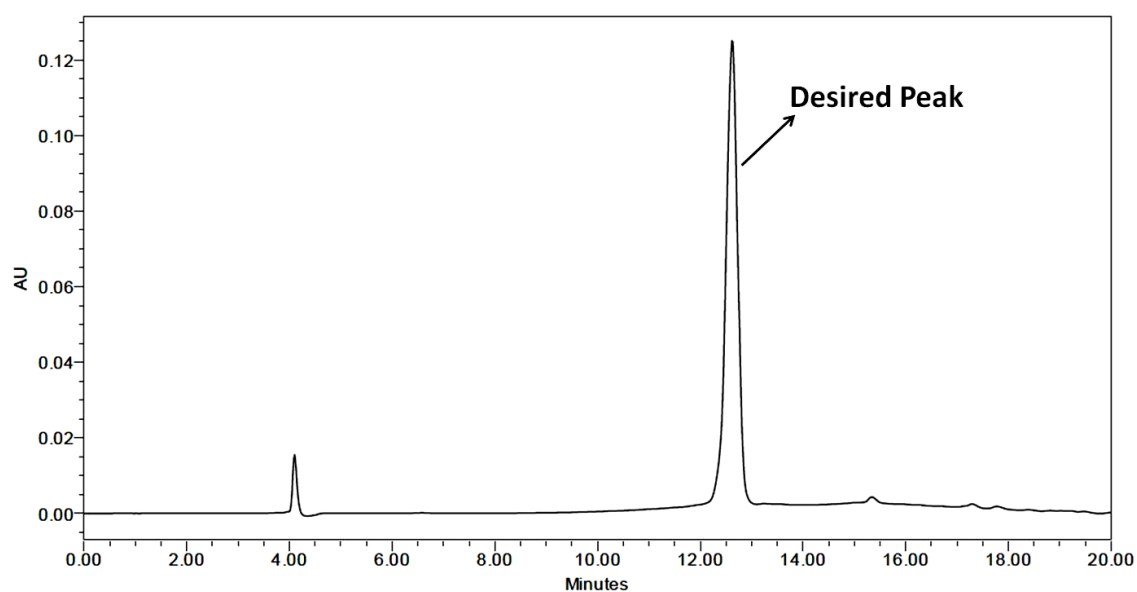


Figure 3.132. HPLC profile picture of the pure mAP2.

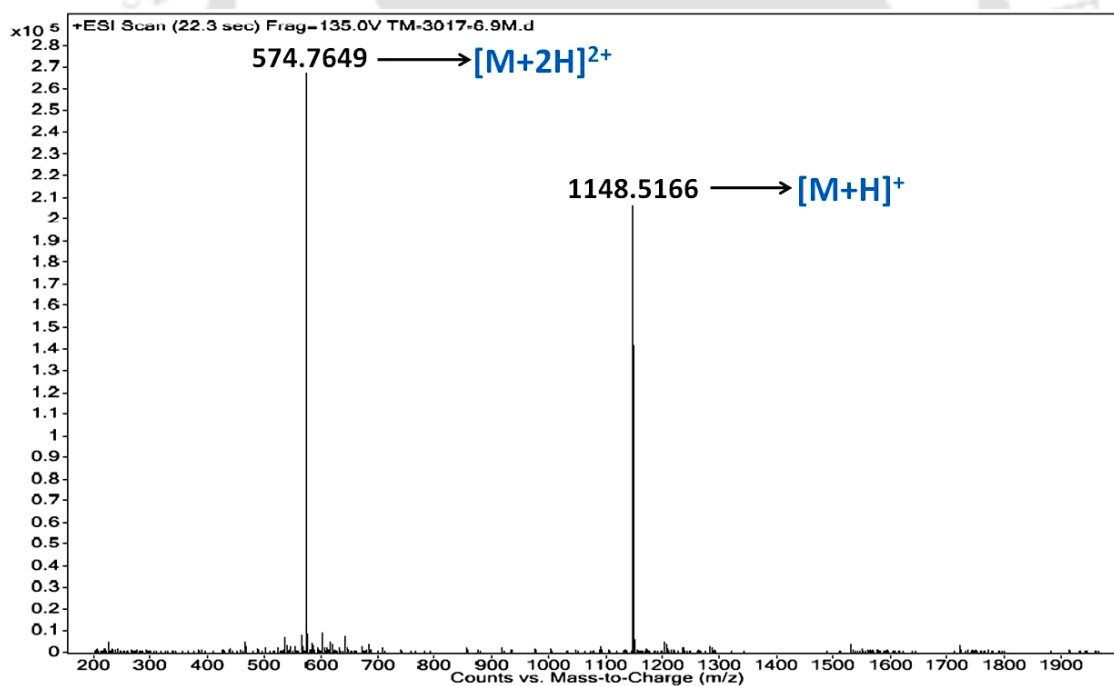


Figure 3.133. ESI-MS profile picture of the pure mAP2. Calculated m/z for $C_{51}H_{69}N_{15}O_{16}$ $[M+H]^+$ is 1148.5125, observed 1148.5166, calculated m/z for $C_{51}H_{69}N_{15}O_{16}$ $[M+2H]^{2+}$ is 574.7601, observed 574.7649.

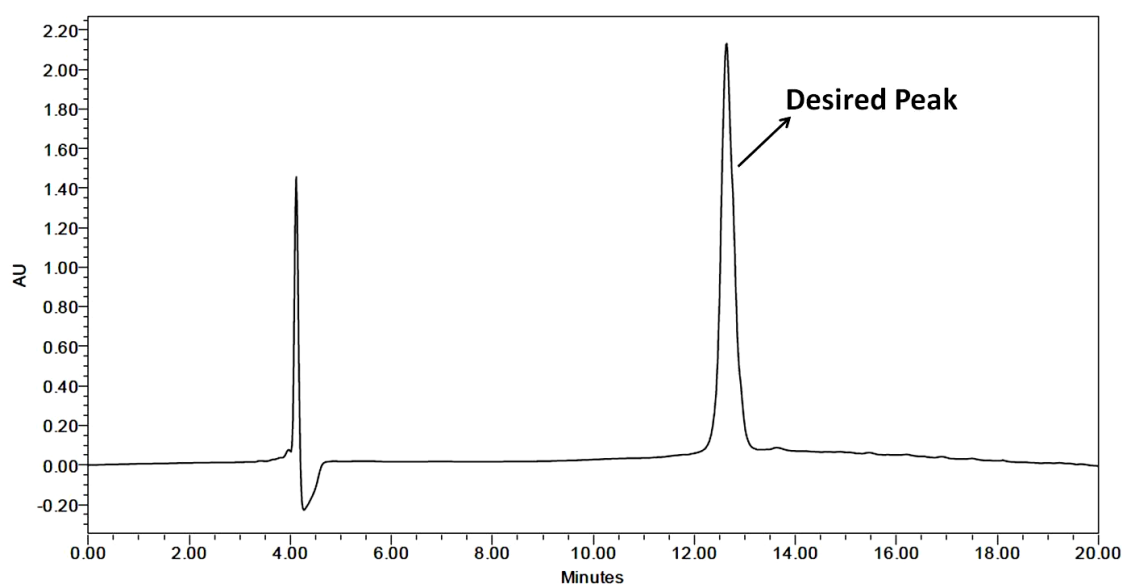


Figure 3.134. HPLC profile picture of the pure mAP3.

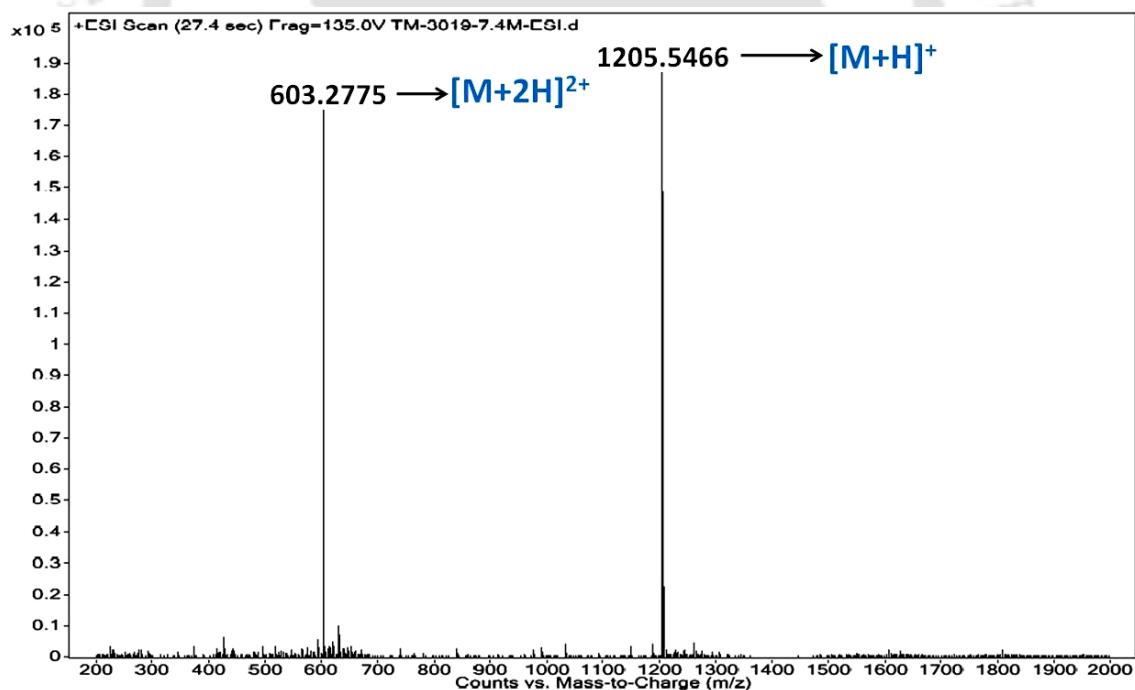


Figure 3.135. ESI-MS profile picture of the pure mAP3. Calculated m/z for $C_{53}H_{72}N_{16}O_{17}$ $[M+H]^+$ is 1205.5339, observed 1205.5466, calculated m/z for $C_{53}H_{72}N_{16}O_{17}$ $[M+2H]^{2+}$ is 603.2708, observed 603.2775.

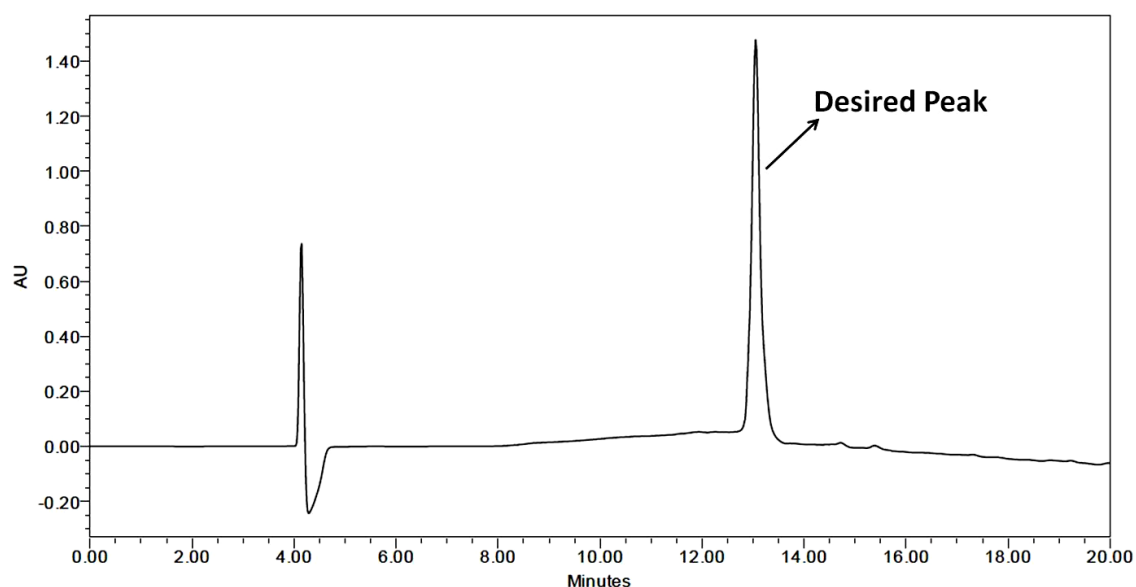


Figure 3.136. HPLC profile picture of the pure mA β peptide.

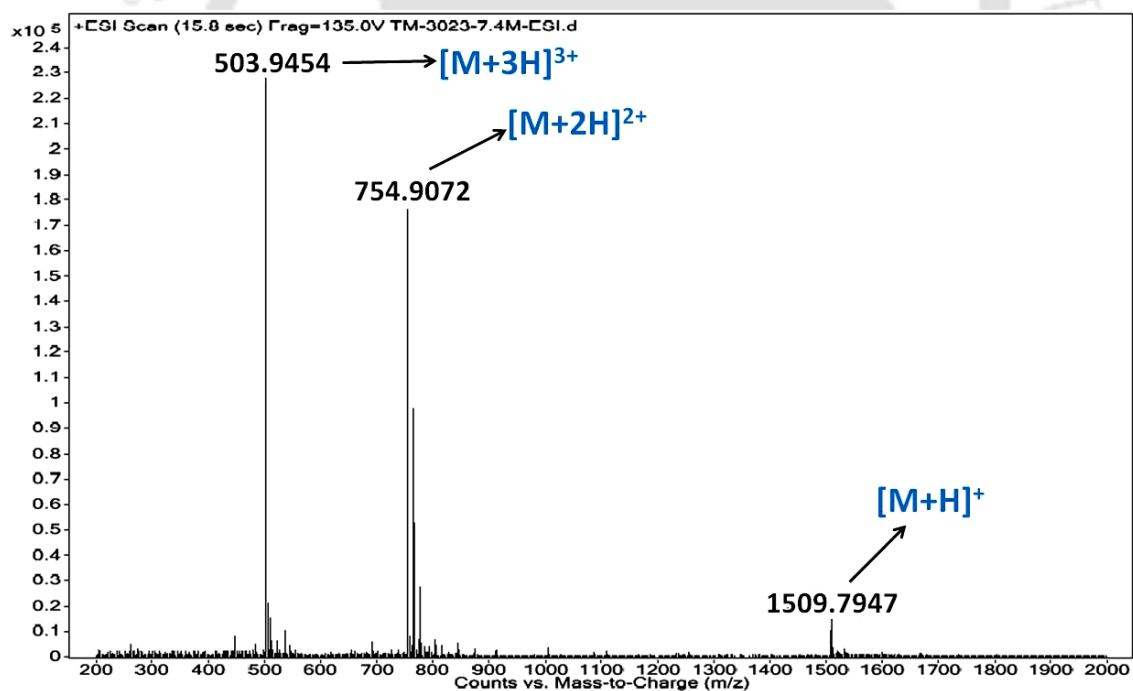


Figure 3.137. ESI-MS profile picture of the pure mA β peptide. Calculated m/z for $C_{71}H_{105}N_{21}O_{16}$ $[M+H]^+$ is 1509.7323, observed 1509.7947, calculated m/z for $C_{71}H_{105}N_{21}O_{16}$ $[M+2H]^{2+}$ is 755.4422, observed 754.9072 (Mass difference of 0.5 indicates a doubly charged peak), calculated m/z for $C_{71}H_{105}N_{21}O_{16}$ $[M+3H]^{3+}$ is 503.9161, observed 503.9454.

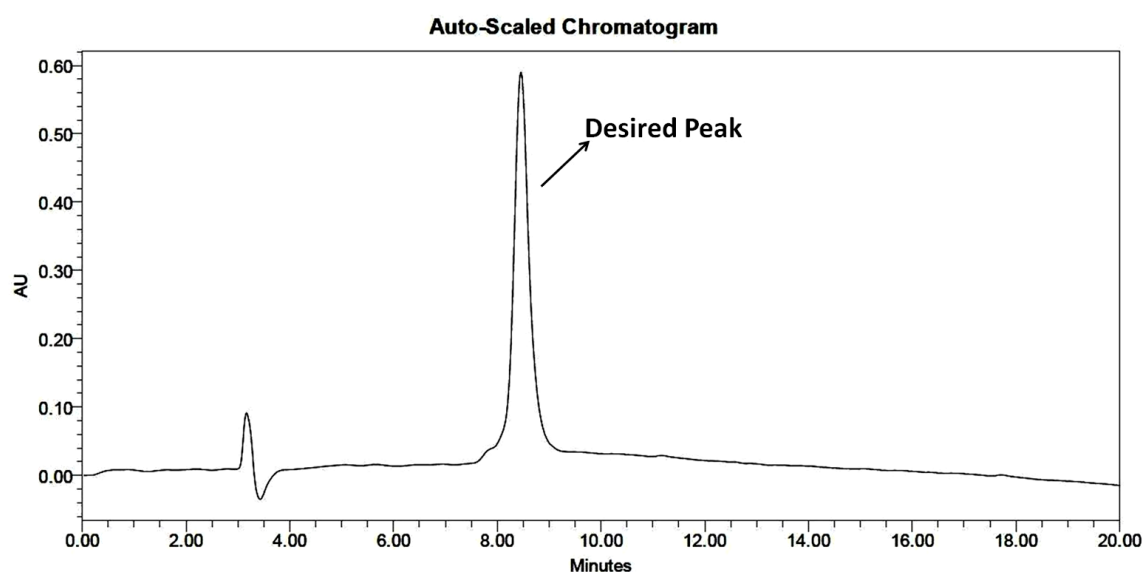


Figure 3.138. HPLC profile picture of the pure DTP28.

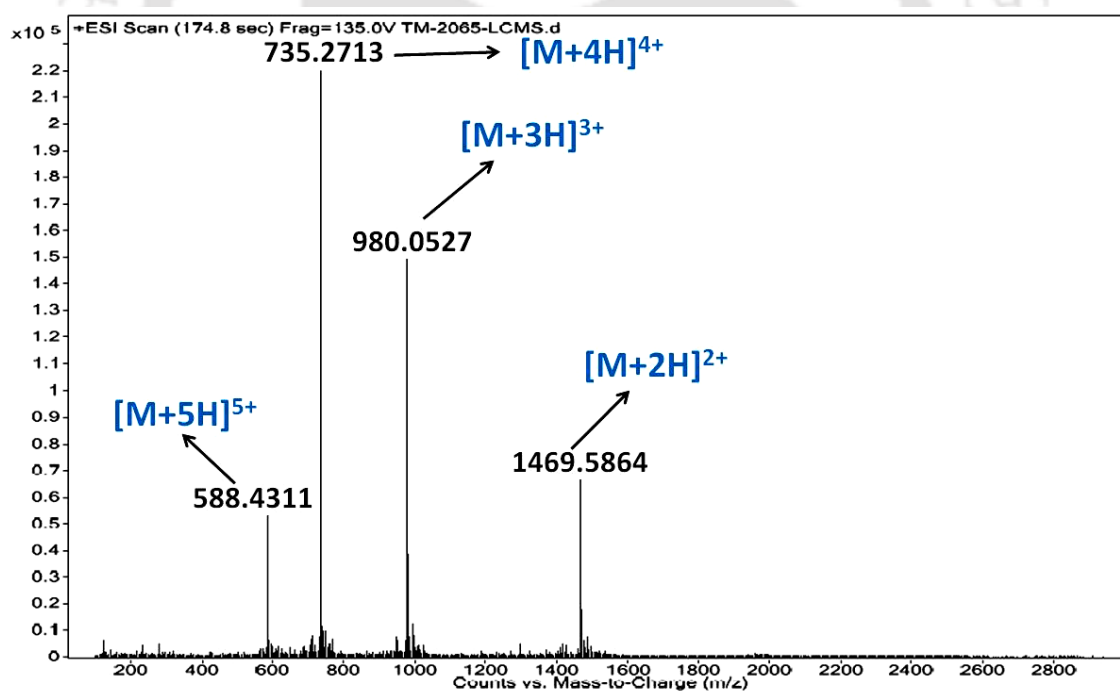


Figure 3.139. ESI-MS profile picture of the pure DTP28. Calculated m/z for $C_{129}H_{210}N_{36}O_{42}$ $[M+2H]^{2+}$ is 1469.6318 and observed 1469.5864, calculated m/z for $C_{129}H_{210}N_{36}O_{42}$ $[M+3H]^{3+}$ is 980.0879 and observed 980.0527, calculated m/z for $C_{129}H_{210}N_{36}O_{42}$ $[M+4H]^{4+}$ is 735.3159 and observed 735.2713, calculated m/z for $C_{129}H_{210}N_{36}O_{42}$ $[M+5H]^{5+}$ is 588.4527 and observed 588.4311.



Chapter 4: Native and non-native triad based protease mimics digest Amyloid β

4.1. Introduction:

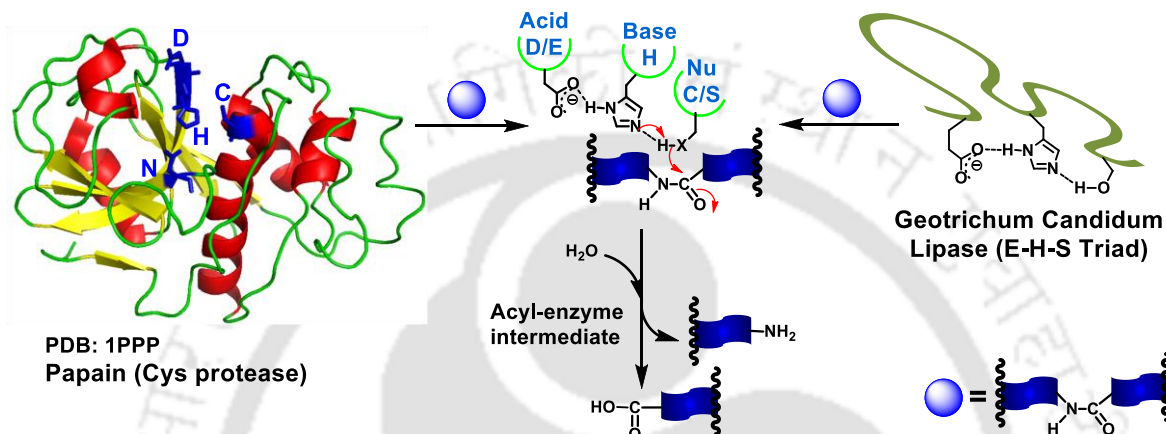
Targeted protein degradation is very important for both chemical biology and drug design research. Though new well-designed techniques namely, endosome targeting chimeras (ENDTAC)¹ and lysosome targeting chimeras (LYTACs)² have been revealed to internalize and degrade extracellular proteins, no proteolysis targeting chimeras (PROTACs)³ or photoswitchable PROTACs (PHOTACs)⁴ is known until now for extracellular A β degradation.

4.2. Design of the PMs and other relevant peptides:

Inspiring from the proteolytic activities of mAPs, described in Chapter 3, a series of native and non-native triad based peptide-conjugates are designed (Table 4.1) in this chapter linking a target protein recognizing unit (here, VFFA for the site-specific recognition to A β) with a small proteolytic unit, termed as “Protease Mimic (PM)”. The key feature of the design is that the proteolytic activity of a catalytic triad of Papain (cysteine protease) or *Geotrichium Candidum* Lipase (serine protease) active site could be mimicked by integrating the triad residues by flexible Gly between each of them (similarly designed as mentioned in Chapter 3) that acted as the small proteolytic unit. Next, by involving the thiol/disulfide exchange cleavage⁵ mechanism, we wanted to design a new catalytic site based on the active site of some of the native protease. The aim of designing PMs was to boost their proteolytic activity. We also designed a model aggregating peptide (a partial sequence of A β , A β ₁₂₋₂₁ or APP₆₈₃₋₆₉₂) which can act similar to the larger A β (hereafter denoted as mA β) to understand the mechanism of its proteolysis easily. It contains both the self-assembling core hydrophobic region,

KLVFFA and the α -secretase cleavage site (Lys₁₆-Leu₁₇) of A β also. Further, we designed a control peptide DTP28, which contains 26 residues of R domain of Diphtheria toxin protein from S505 to F530, and Gly at both N- and C-terminus as spacers. Characterization data of the synthesized peptides were attached in the supporting data (Figure 4.39-4.44).

a) Proteolysis by Papain and Geotrichum Candidum Lipase:



b) Proteolysis by Protease Mimic (PM):

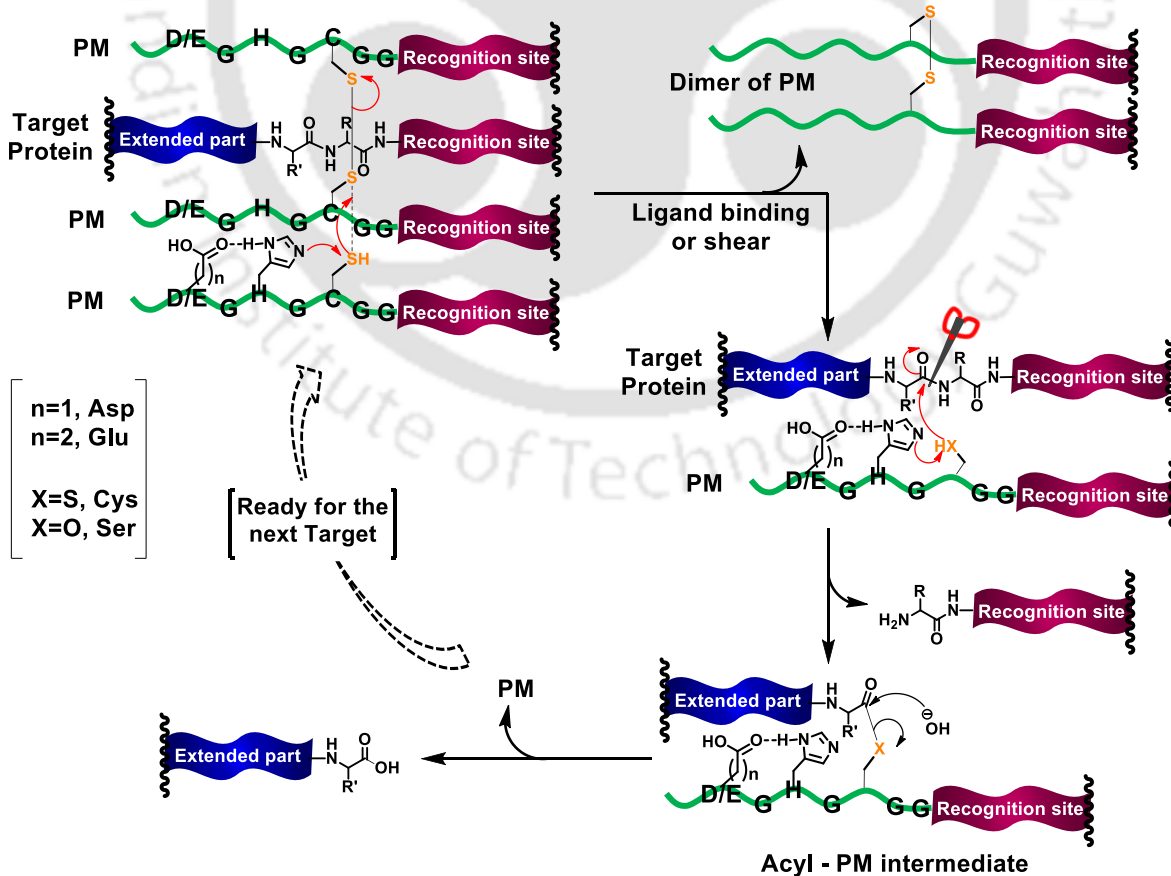


Figure 4.1. Design principles of protease mimic (PM). a) Proteolysis of a polypeptide substrate by Papain (Cys protease) and Geotrichium Candidum Lipase (Ser protease). b) Plausible mode of action of PM: target specific proteolysis by mimicking the catalytic triad of Cys or Ser protease.

Table 4.1. Sequences of the peptides and their roles in the current study.

Sl. no.	Name of the peptide (short name)	Peptide sequence (one-letter code)	Role of the peptide
1	Protease mimic 1 (PM1)	Ac-GADGHGCGGVFFAG-NH ₂	Proteolytic cleavage
2	Protease mimic 2 (PM2)	Ac-GAEGHGS GG VFFAG-NH ₂	Proteolytic cleavage
3	Protease mimic 3 (PM3)	Ac-GAEGHGC GG VFFAG-NH ₂	Proteolytic cleavage
4	A segment of A β (mA β)	Ac-GGAV ₁₂ HHQKL VFFA ₂₁ G-NH ₂	Prototype of A β
5	A β ₁₋₄₀	D ₁ AEFRHDSGYEVHHQKL VFFAEDVGSN KGAIIGLMVGGVV ₄₀ -NH ₂	Target peptide
6	A segment of Diphtheria toxin protein (DTP28)	GS ₅₀₅ SDSIGVLGYQKTVDHTKVNSKLSLF 530G-NH ₂	Control peptide

4.3. The most stable conformation of PMs:

By DFT based calculation, we obtained the most stable conformation of the peptides (PMs) (Figure 4.2) by using B3LYP as energy functional and 6-31G as a basis set through Gaussian 5.0.9 program. In all the cases, it was found that His, the base in triad residues, was very close to the active nucleophilic center Ser or Cys. Similar active site structure was found in Papain. Hence, PMs are believed to act as artificial protease.

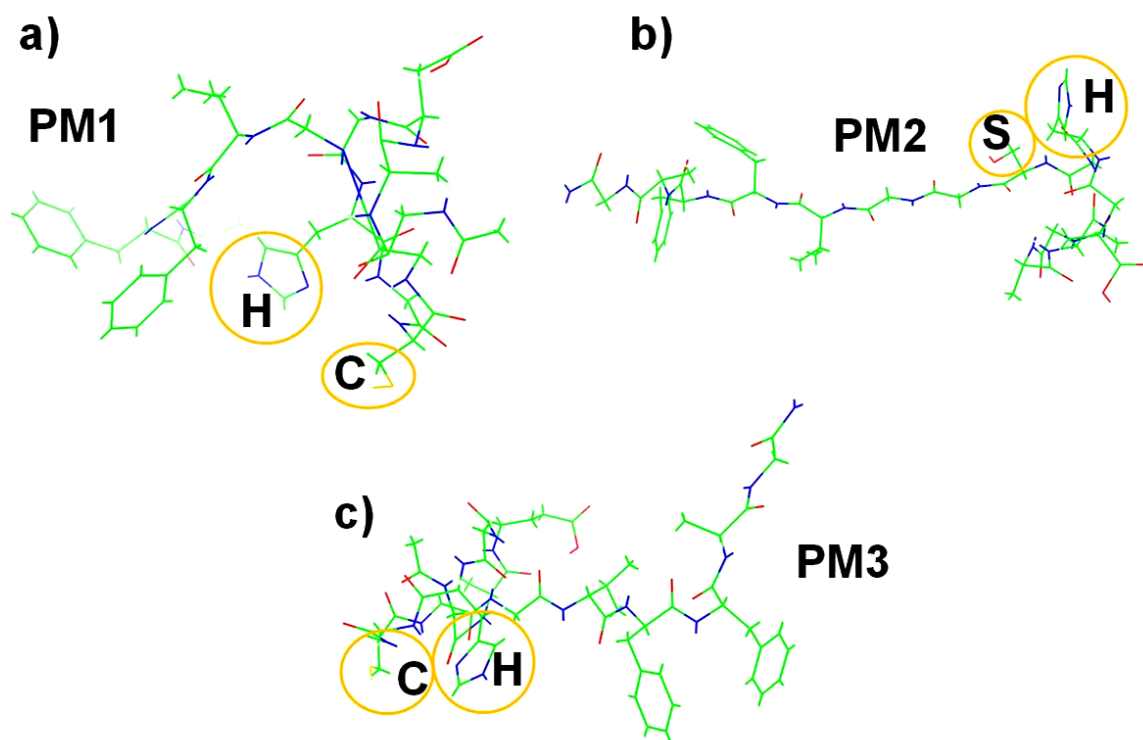


Figure 4.2. (a-c) The most stable conformation of PM1-PM3 respectively [image generated by PyMOL (Line representation)] obtained from DFT calculation [Calculation Method: B3LYP, Basis Set: 6-31G; Total Energy -4786.93277873 a.u. for PM1, -4503.42688684 a.u. for PM2, and -4826.21060632 a.u. for PM3]. Nitrogen & Oxygen atoms are represented in blue & red, respectively.

4.4. Molecular Docking:

It was noticed that (Figure 4.5), PMs interact well in the helical $A\beta_{1-40}$ binding pocket having a binding affinity -6.1 kcal/mol (for PM1), -6.1 kcal/mol (for PM2), and -6.2 kcal/mol (for PM3). Also, PMs binds well (Figure 4.6) in the fibril $A\beta_{1-40}$ binding pocket having a binding affinity -4.8 kcal/mol (for PM1), -4.0 kcal/mol (for PM 2), and -5.2 kcal/mol (for PM3). We observed that interacting amino acids in the $A\beta_{1-40}$ binding pocket are the hydrophobic region, *i.e.*, Leu17, Val18, Phe19, Phe20, and Ala21 in most of the cases. Finally, PMs were docked into the helical C-terminal domain of APP (C99, residues 683-728) [PDB ID: 2LP1], and it was found that (Figure 4.7) PMs fitted well in the APP binding pocket also having binding affinity -4.4 kcal/mol (for PM1), -4.7 kcal/mol (for PM2), and -5.5 kcal/mol (for PM3) where the interacting amino acids in the pocket were L₆₈₈VFF₆₉₁. From the docking results, it was also noticed that in all the cases,

a pocket with the close proximity of triad residues was found, as desired. Interestingly, PM3 has shown the maximum binding affinity among others.

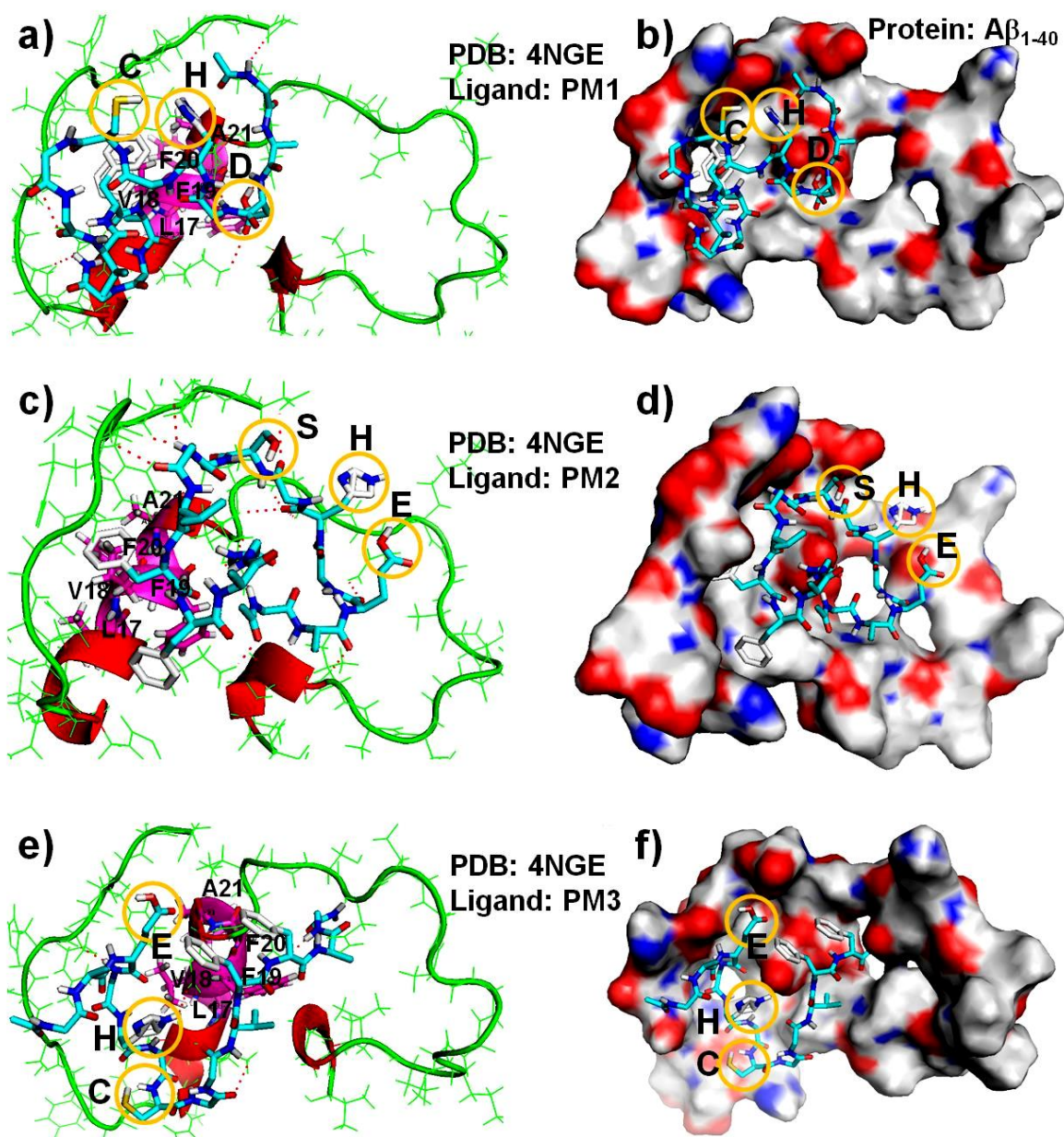


Figure 4.3. Molecular docking images of PM1 (a, b), PM2 (c, d), and PM3 (e, f) into helical $A\beta_{1-40}$ (PDB ID 4NGE) reveals that PMs bind at the binding site (V18FFA21) with binding affinity -6.1 kcal/mol, -6.1 kcal/mol, and -6.2 kcal/mol, respectively. Structures are shown as line and cartoon representation (for a, c, e), and surface representation (for b, d, f). The surface of $A\beta_{1-40}$ is coloured according to the charges of the atoms where negatively and positively charged zones are represented in red and blue, respectively.

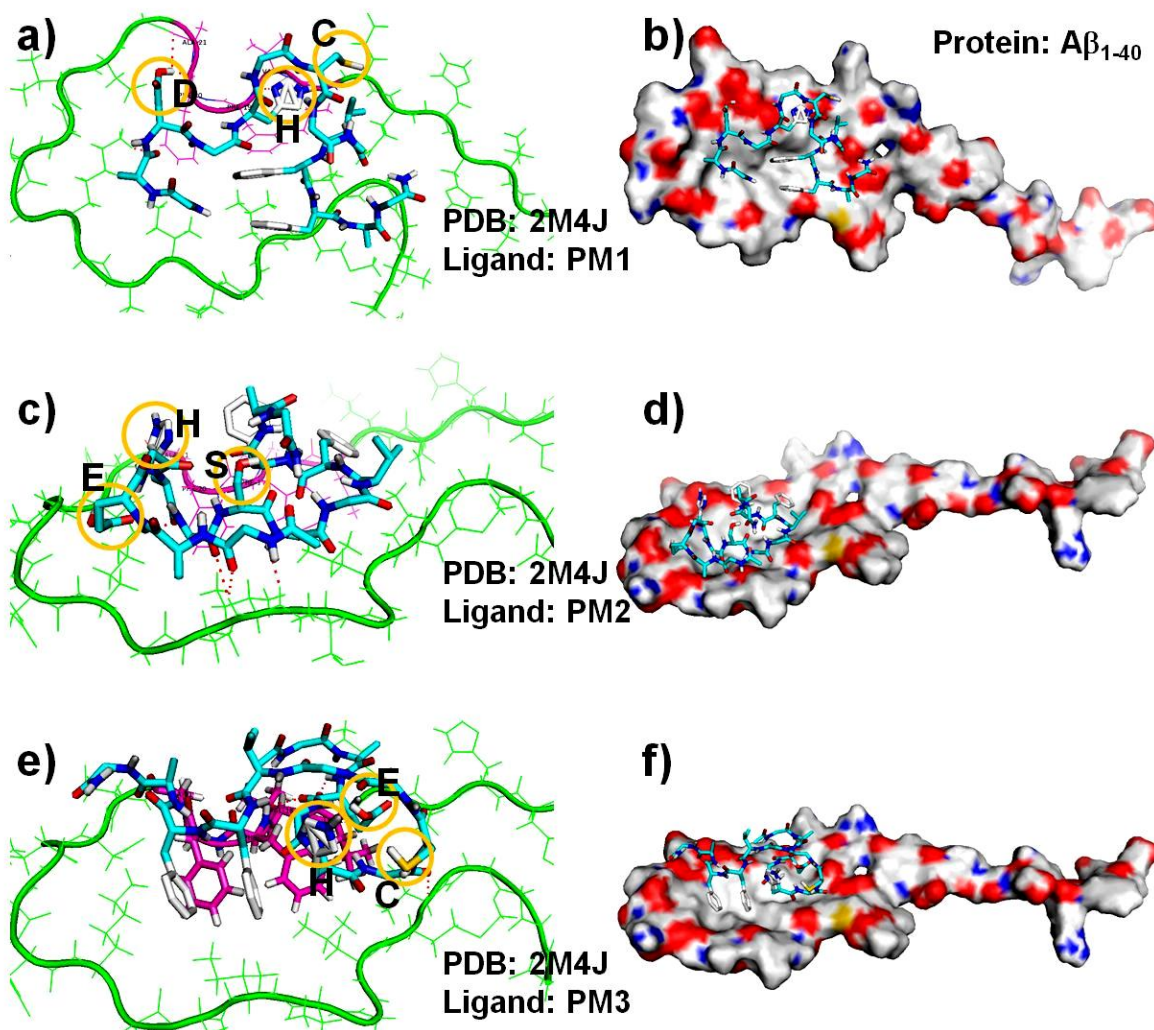


Figure 4.4. Molecular docking images of PM1 (a, b), PM2 (c, d), and PM3 (e, f) into fibril $A\beta_{1-40}$ (PDB ID 2M4J) reveals that PMs bind at the binding site (V₁₈FFA₂₁) with binding affinity -4.8 kcal/mol, -4.0 kcal/mol, and -5.2 kcal/mol, respectively. Structures are shown as line and cartoon representation (for a, c, e), and surface representation (for b, d, f). The surface of $A\beta_{1-40}$ is coloured according to the charges of the atoms where negatively and positively charged zones are represented in red and blue, respectively.

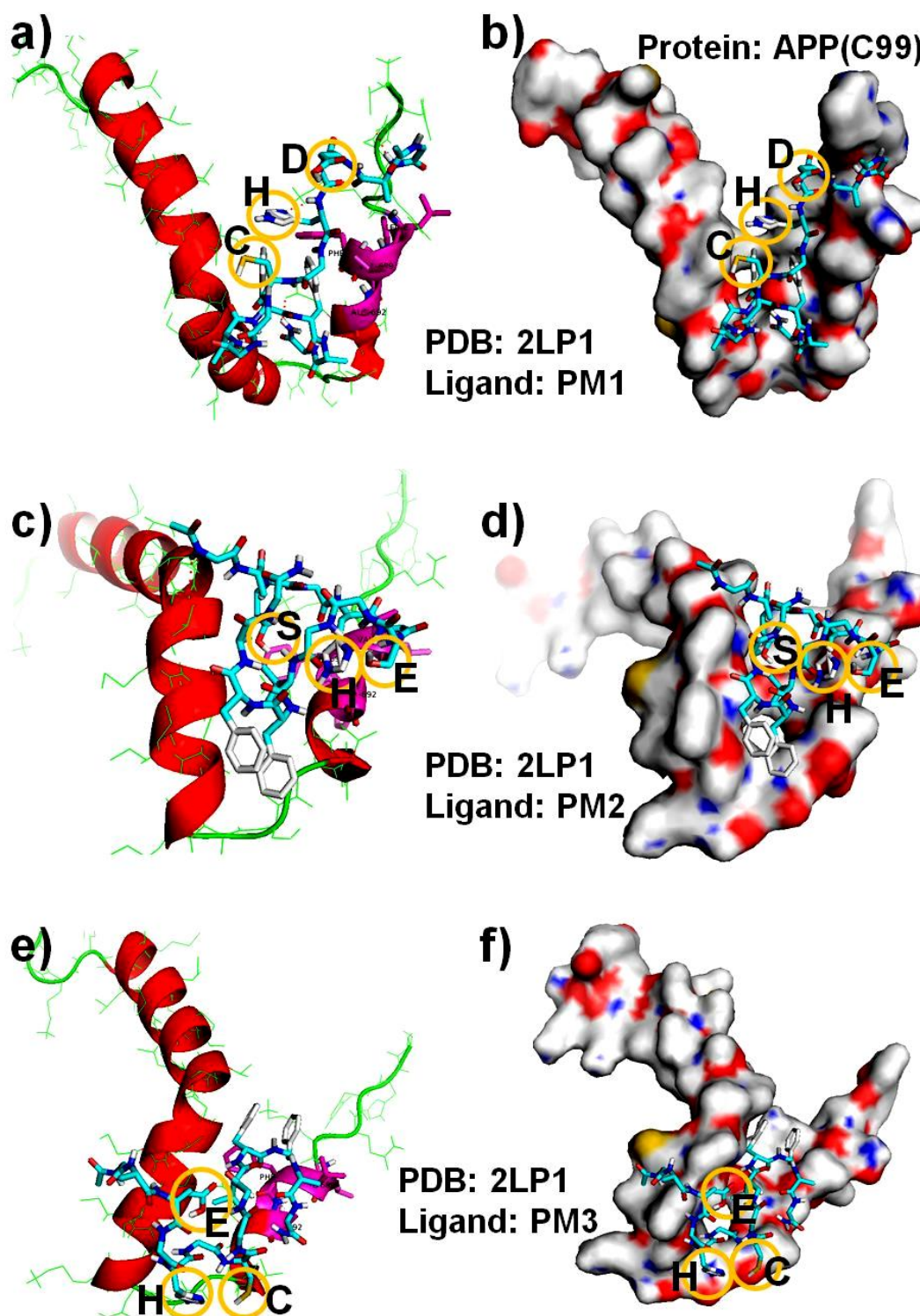


Figure 4.5. Molecular docking images of PM1 (a, b), PM2 (c, d), and PM3 (e, f) into helical 99 residues C-terminal domain (PDB ID: 2LP1) of APP(C99) reveals that PMs bind at the binding site (V₁₈FFA₂₁) with binding affinity -4.4 kcal/mol, -4.7 kcal/mol, and -5.5 kcal/mol, respectively. Structures are shown as line and cartoon representation (for a, c, e), and surface representation (for

b, d, f). The surface of $A\beta_{1-40}$ is coloured according to the charges of the atoms where negatively and positively charged zones are represented in red and blue, respectively.

4.5. Examination of the proteolytic activity of the PMs on $mA\beta$:

To obtain a primary idea on the proteolytic efficacy of the PMs, we examined the proteolytic activity of the PMs on the $mA\beta$. For that, we dissolved the purified peptides in PBS (50 mM, pH 7.4) to make suitable stock solutions and co-incubated in 1:1 molar ratio of the $mA\beta$ at physiological pH and temperature on a water bath. Aliquots were tested by a MALDI-TOF mass spectrometer in a time-dependent way.

4.5.1. Self-degradation of $mA\beta$:

From a time-dependent MALDI-TOF mass spectrometric analyses of the $mA\beta$, dimer was found after two days of incubation, but no such degraded peak was detected after five days of incubation for its solvent in-accessibility due to the aggregation. This control experiment was performed to check self-degraded fragments, and to confirm the proteolytic activity of PMs against $mA\beta$ while performing co-incubation in the presence of PMs. MALDI-TOF mass spectra were shown in Chapter 3 (Figure 3.8-3.12).

4.5.2. Self-degradation of PM1:

The catalytic mechanism of the cysteine-based triad is boosted by thiol/disulfide exchange cleavage. The thiolate of Cys attacks the intermolecular disulfide bond (formed by PM) in a nucleophilic substitution reaction resulting in a target protein bound intermediate (by hydrophobic interaction in-between PM and $A\beta$) with a new thiolate which can shear the adjacent protein.

The results of MALDI-TOF mass values indicated PM1 cleaved itself in a site-selective manner following Route 1^{SD-PM1}. It was observed that one molecule of PM1 became covalently attached with the second molecule by the nucleophilic attack of Cys side-chain as described in Route 1^{SD-PM1} and cleaved the second molecule to produce PM2-F1 peptide fragment, and finally reproduced itself pointing to the catalytic activity of PM1.

Similarly, PM1 cleaved itself in a site-selective manner probably following Route 2^{SD-PM1}, and Route 3^{SD-PM1} to produce PM2-F2 and PM2-F3 respectively. Plausible routes were drawn considering the cleavage sites, but the plausible mechanism remains the same for each of the routes.

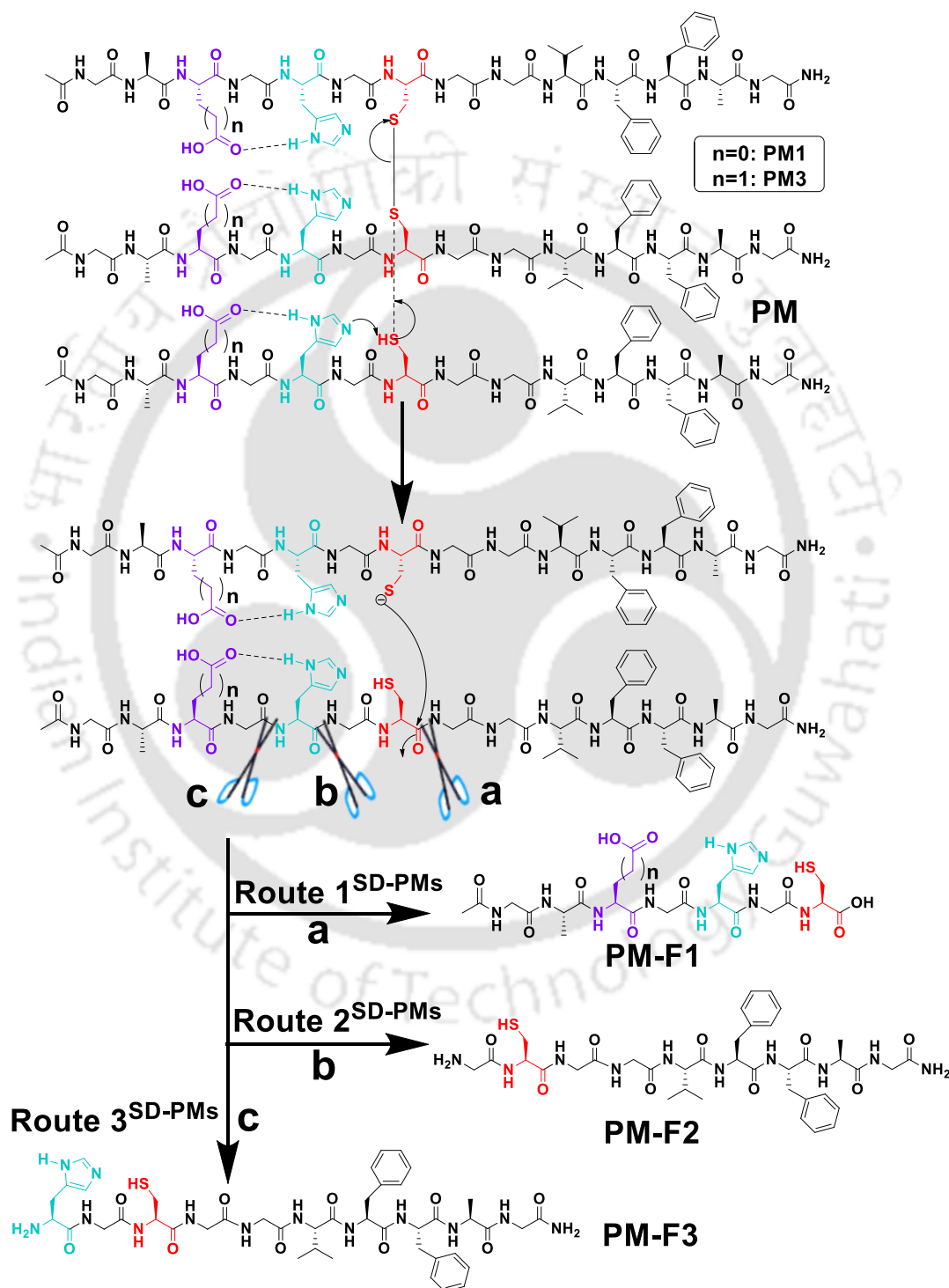


Figure 4.6. Plausible routes of the self-degradation of PM1.

Most of the logically drawn intermediates and final fragments could be identified by time-dependent MALDI-TOF mass spectrometric analyses of the PM1 incubated in the absence of mA β (Figure 4.7-4.10).

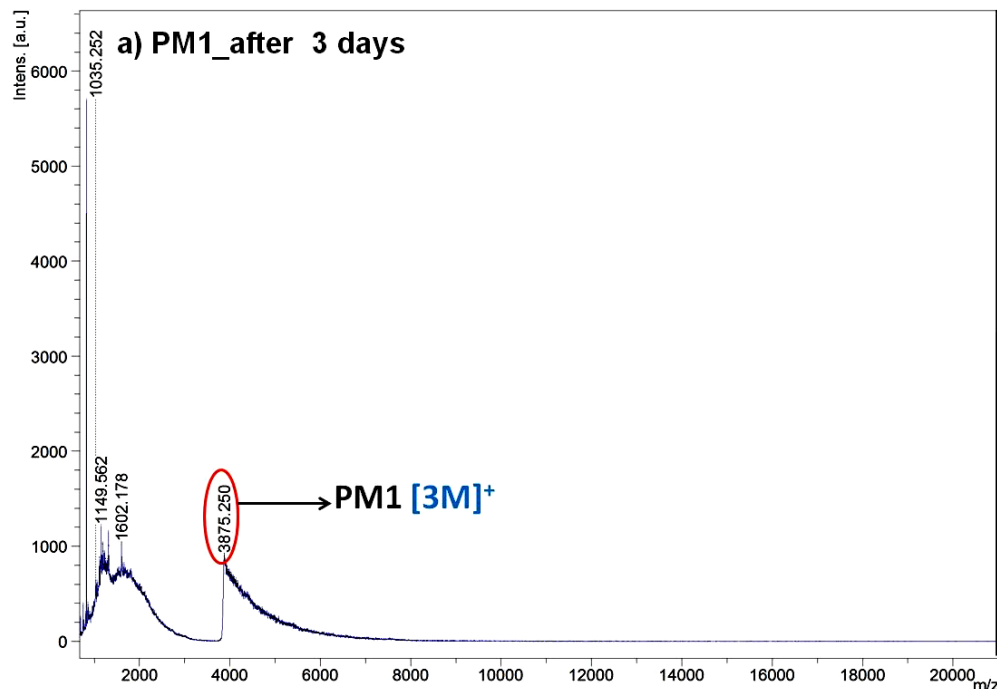


Figure 4.7. MALDI-TOF mass spectrum of PM1 after three days of incubation.

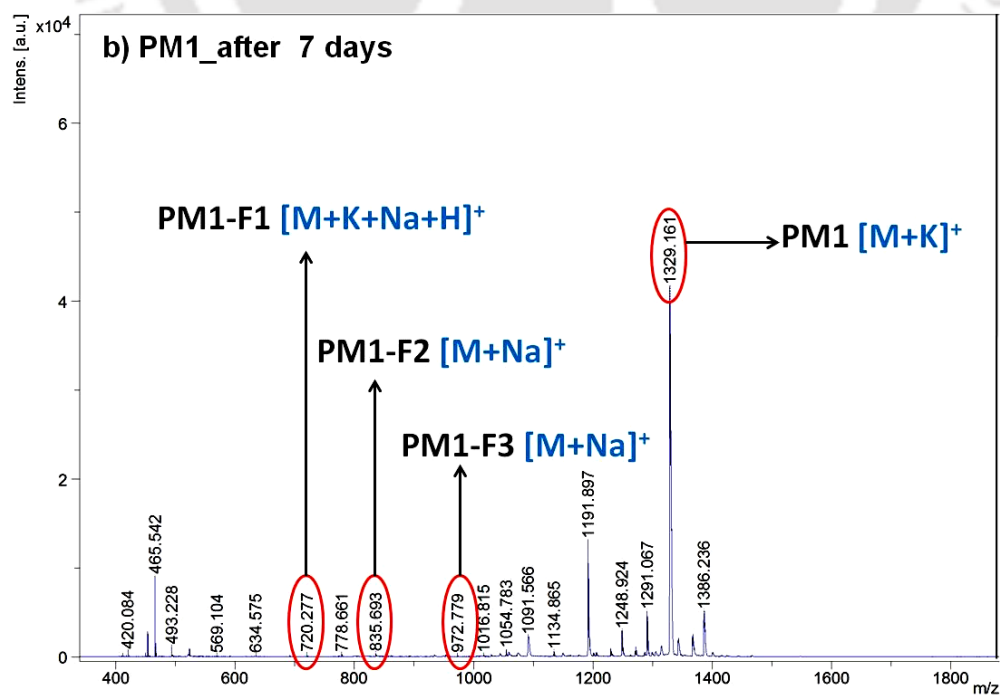
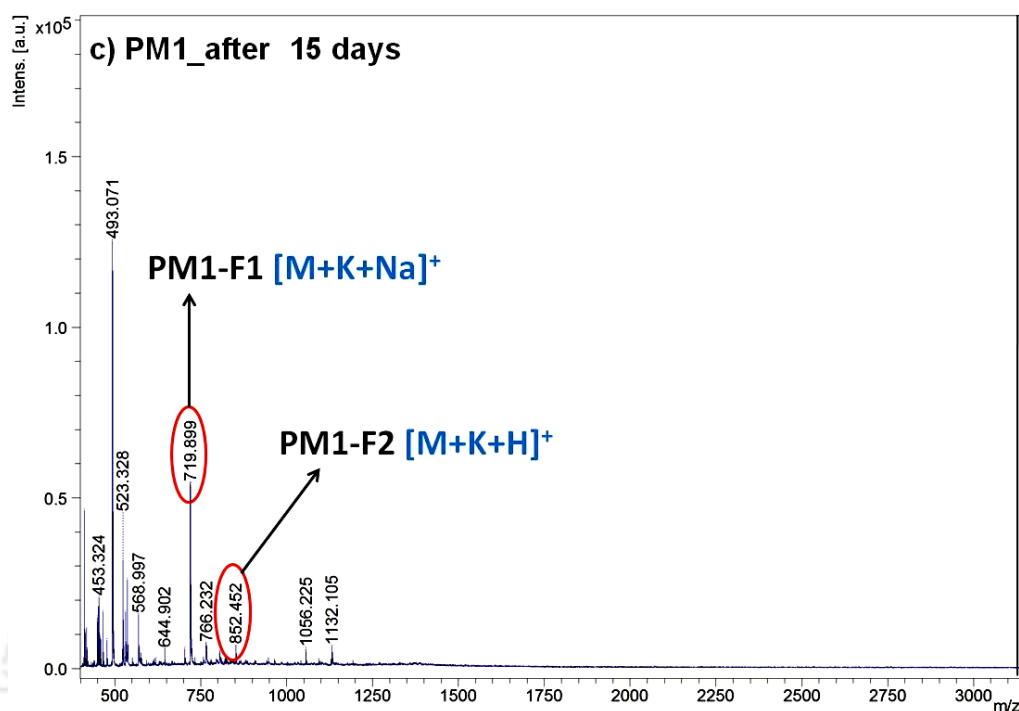


Figure 4.8. MALDI-TOF mass spectrum of PM1 after seven days of incubation.**Figure 4.9. MALDI-TOF mass spectrum of PM1 after 15 days of incubation.**

4.5.3. Proteolytic activity of PM1 on mA β :

Peaks corresponding to the mass of various fragments and intermediates were detected for mA β after seven days of incubation in the presence of PM1 (Figure 4.12-4.13). Plausible routes of the proteolytic cleavage of mA β by PM1 were drawn (Figure 4.10-4.11) for PM1) based on the MALDI-TOF mass values. These results indicated PM1 cleaved the mA β in a site-selective manner (between His-His, His-Gln, Gln-Lys, and Lys-Leu). Plausible routes were drawn concerning cleavage sites, but the plausible mechanism remains the same for each of the routes.

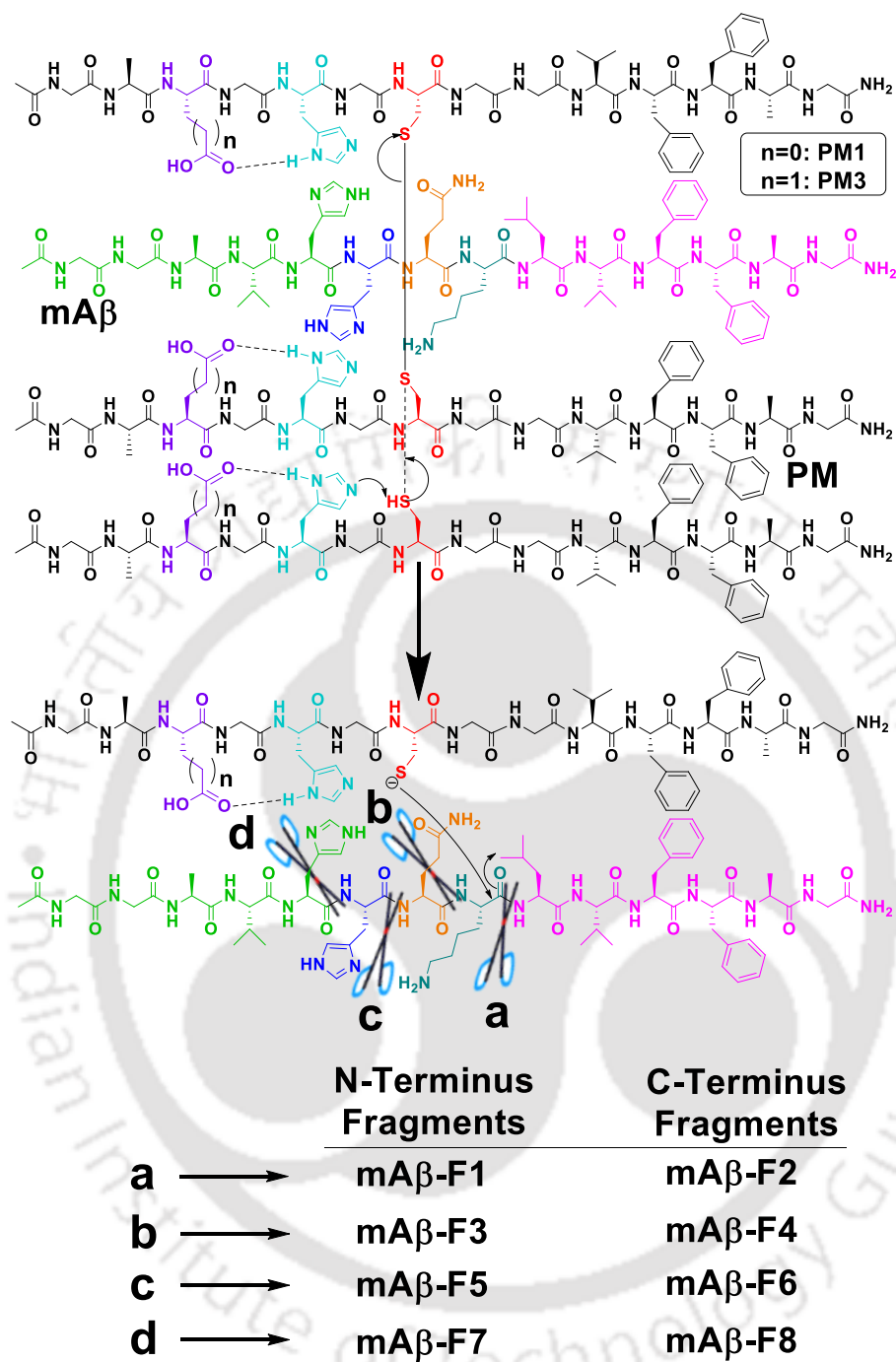


Figure 4.10. Plausible routes of the proteolysis of mA β into various fragments by PMs (Route 1^{mA β -PMs}).

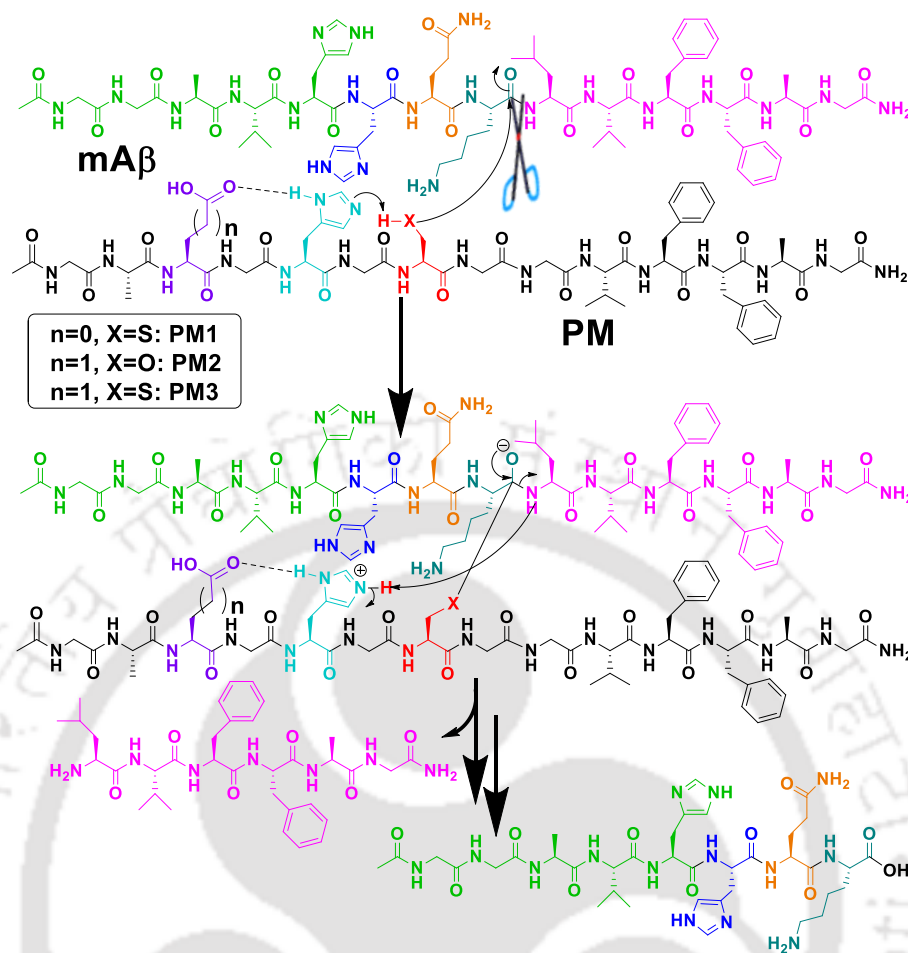


Figure 4.11. Plausible routes of the proteolysis of mAbeta into various fragments by PMs (Route 1^{mAbeta}-PMs, continued).

Most of the logically drawn intermediates and final fragments could be identified by time-dependent MALDI-TOF mass spectrometric analyses of the PM1 incubated in the presence of mAbeta (Figure 4.12-4.13).

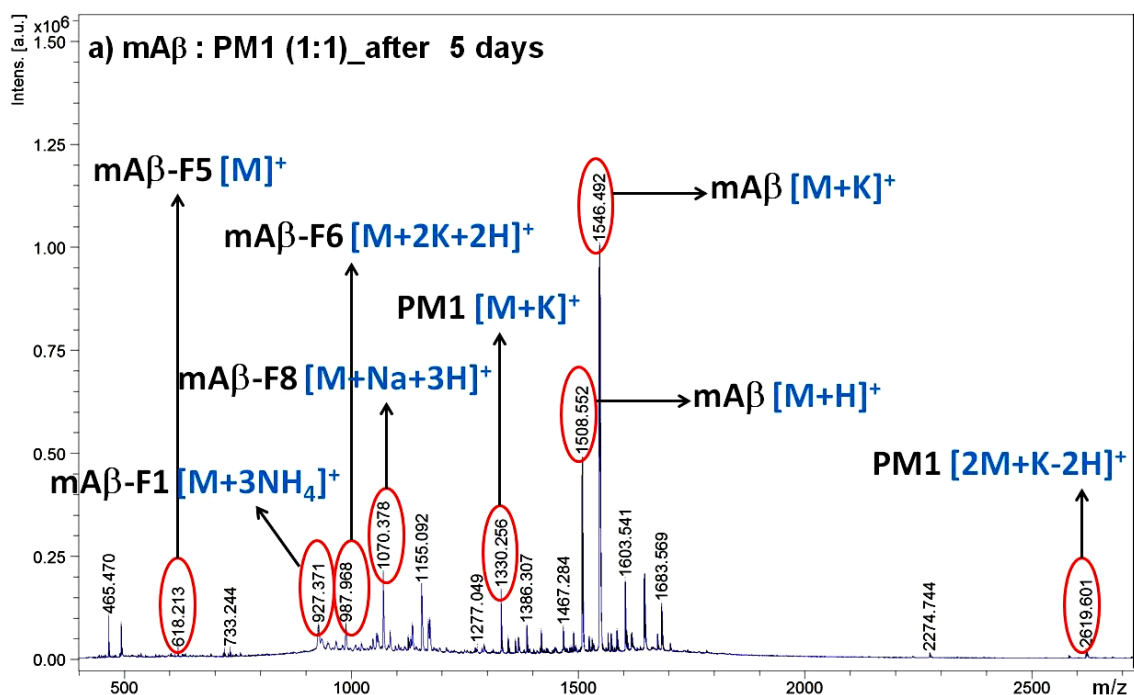


Figure 4.12. MALDI-TOF mass spectrum of mAbeta in the presence of PM1 (1:1) after five days of incubation.

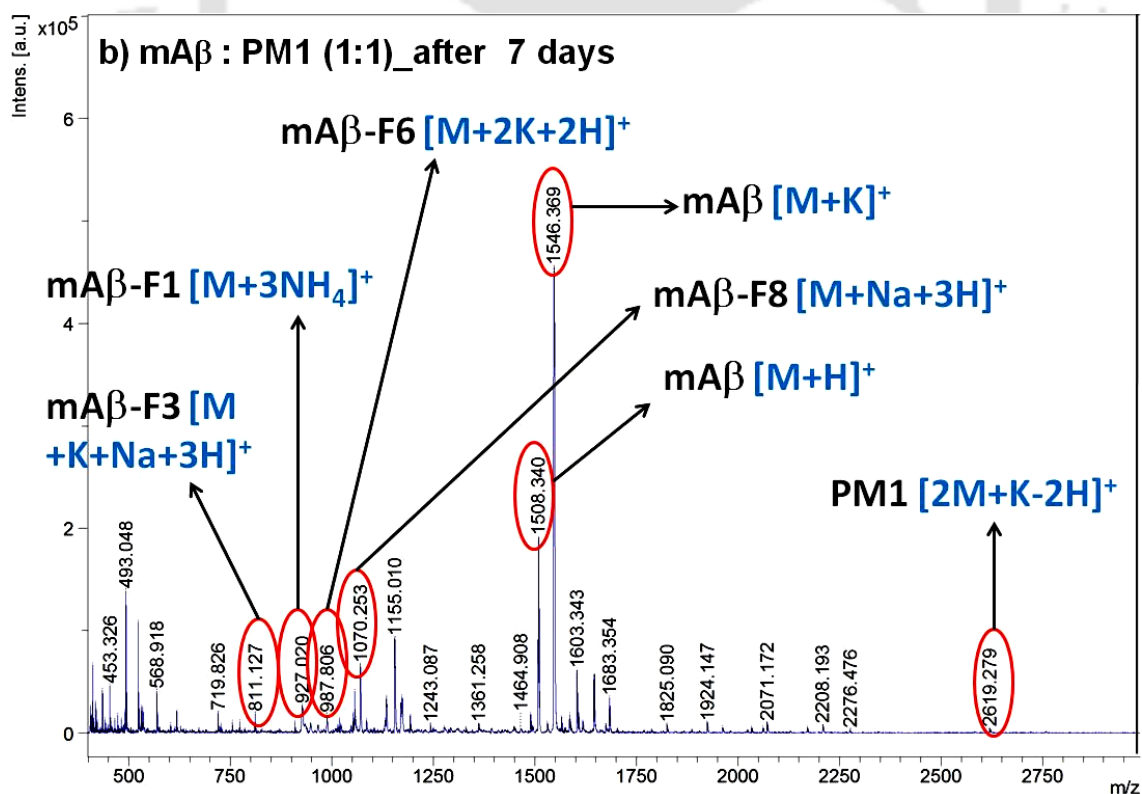


Figure 4.13. MALDI-TOF mass spectrum of mAbeta in the presence of PM1 (1:1) after five days of incubation.

4.5.4. Proteolytic cleavage of mA β by PM2:

MALDI-Mass data analysis is not done yet for PM2. However, the peaks corresponding to the mass of various fragments and intermediates were detected for mA β in the presence of a mimic of a serine protease in Chapter 3 (Figure 3.28-3.34), which closely resembles with PM2. The results of MALDI-TOF mass values indicated miniature serine protease cleaved the mA β in a site-selective manner (between His-His, His-Gln, Gln-Lys, Lys-Leu, Leu-Val, Val-Phe, and Phe-Phe).

4.6. Non-aggregating property of PMs:

After finding the proteolytic activity of PMs, we continued further to check the non-aggregating property of PMs. At first, the purified solid peptide samples were dissolved in PBS (50 mM, pH 7.4) completely to obtain the stock solutions of PMs (500 μ M) and incubated at different molar concentrations (after proper dilution) at the physiological condition in separate vessels on a water bath till 30 days in parallel. The amyloid accumulation was monitored by Thioflavin-T (ThT) fluorescence assay. It was observed that the fluorescence intensity of PMs was increased initially due to the presence of hydrophobic segment VFFA, but decreased thereafter due to their self-degradation. In the case of PM1 and PM3, comparatively higher intensity (initially) was noticed probably due to the presence of dimer or trimer formed by Cys. So, PMs are non-aggregating in nature.

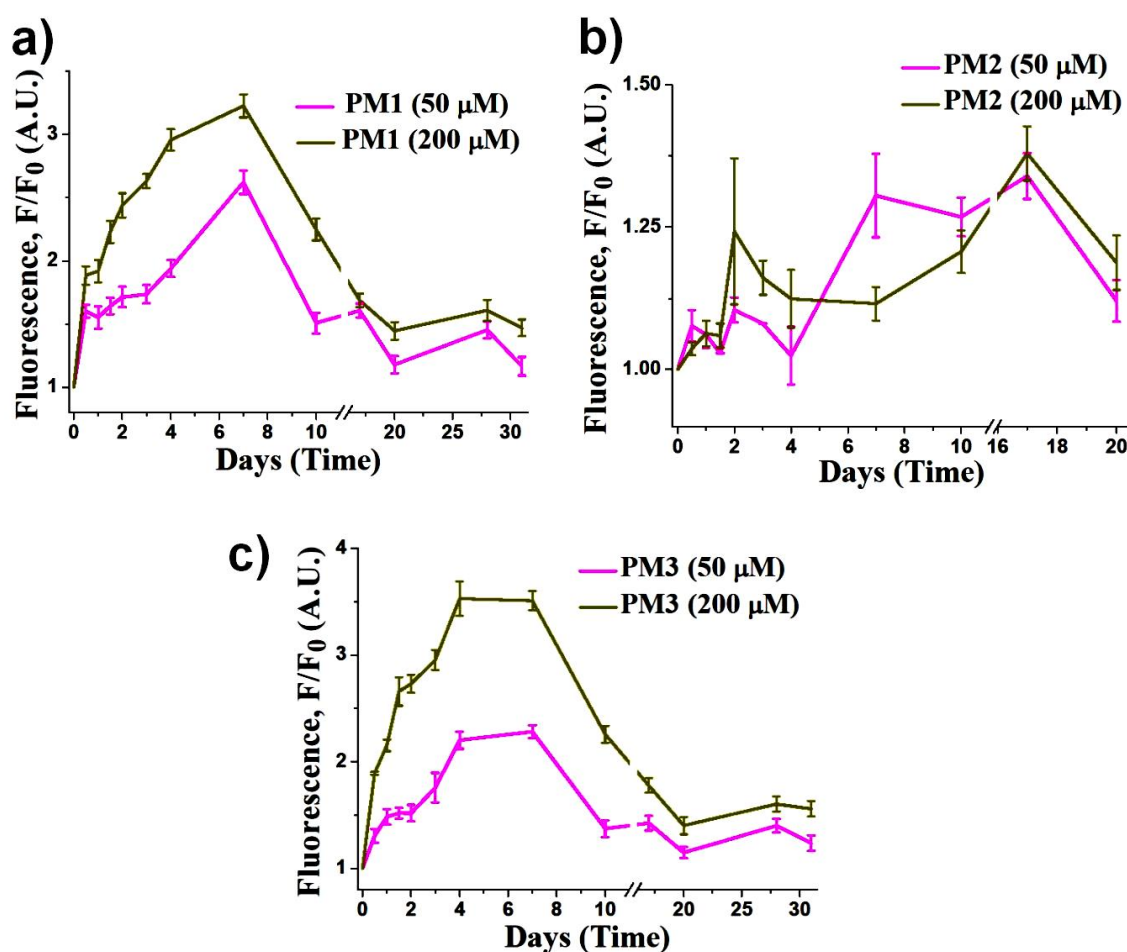


Figure 4.14. Normalized profiles of time-dependent ThT fluorescence assay. (a-c) Spectra of PM1-PM3 respectively of 50 μM (magenta) and 200 μM (dark yellow). Error bars represent standard deviations of at least three independent measurements. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

4.7. Inhibition of amyloid accumulation of mA β by PM1:

The kinetics of the amyloid accumulation of mA β was checked in the absence and presence of PMs. The stock solution of mA β (200 μM) was obtained by dissolving in PBS (50 mM, pH 7.4) completely, after consecutive treatment with TFA and HFIP followed by complete evaporation by purging nitrogen gas, and other peptide solutions were prepared as described above. PM1 was co-incubated with mA β in separate vessels on a water bath up to 30 days in parallel at the desired proportion (1:1) *in vitro*. To get a result more precisely, another two replicate solutions of each portion were prepared, and different biophysical tools were used to monitor amyloid accumulation.

4.7.1. ThT fluorescence assay, CD experiments, and FTIR spectra of mA β .^{6,7}

At first, the time-dependent ThT fluorescence assay of mA β was performed with PM1. From the ThT assay (Figure 4.15-a), it was found that the fluorescence intensity of mA β (black) increased with time in the absence of PM1 and became static after three days of incubation. But in the presence of one-fold PM1 (red), the fluorescence intensity of the mA β increased more with time due to co-aggregation, but slowly decreased from seven days onwards due to the formation of various mA β fragments cleaved by PM1, indicating the inhibitory efficacy of PM1 against aggregation of mA β .

From CD spectra (Figure 4.15-b), it was observed that mA β adopt a β -sheet conformation (black, a negative band at ~ 218 nm with a positive band at ~ 200 nm). In the presence of one-fold PM1 (red, two negative bands at ~ 198 nm, and at ~ 230 nm of low intensity), the non- β -sheet structure of mA β was obtained. Random coil conformation of PM1 (magenta) was confirmed from a negative band at ~ 190 nm.

In FTIR spectra (Figure 4.15-c), the amide I band was observed at 1636 cm^{-1} for mA β alone (black), at 1643 cm^{-1} for mA β in the presence of one-fold PM1 (red), and at 1655 cm^{-1} for PM1 alone (magenta). These results indicated β -sheet rich structure for mA β alone; but mA β along with PM1, and PM1 alone form a random coil or α -helix.

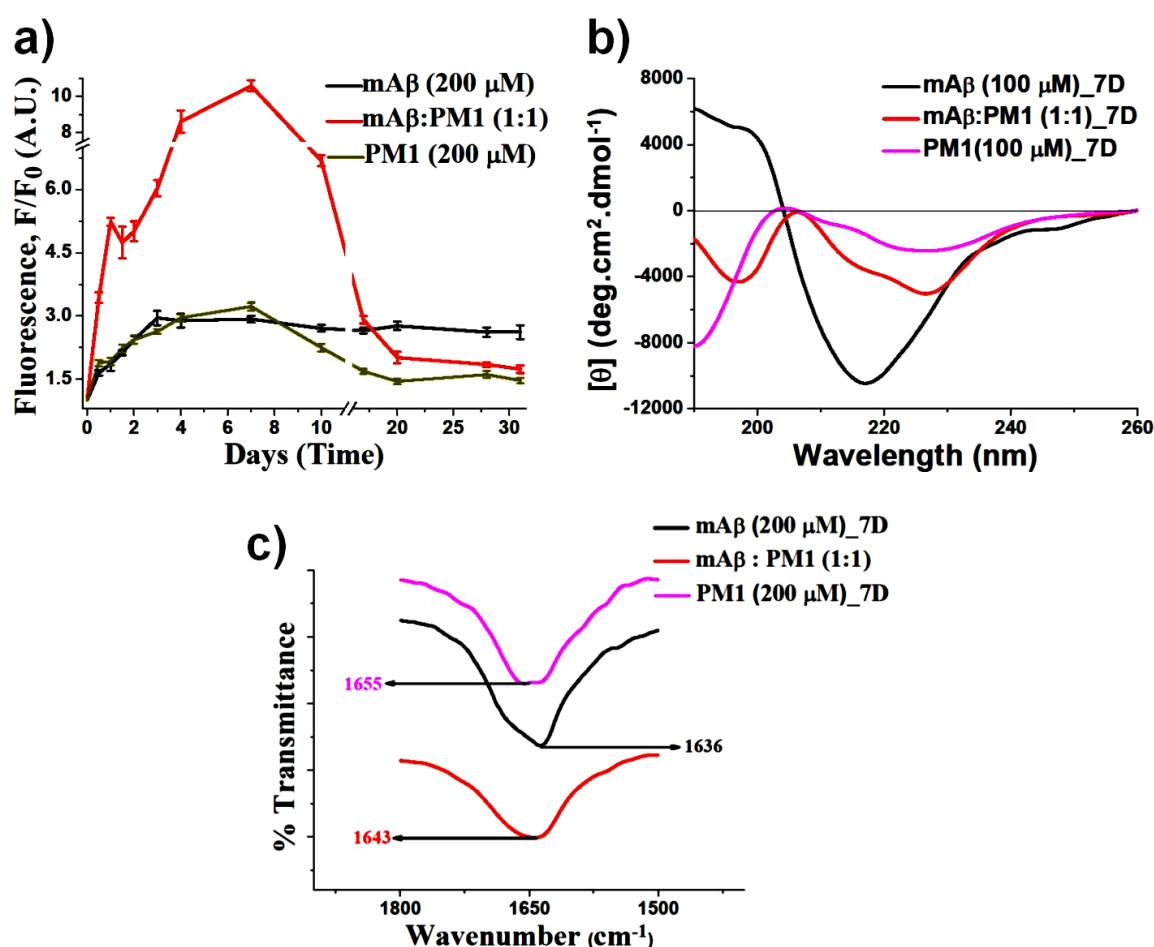


Figure 4.15. (a) Normalized profiles of time-dependent ThT fluorescence assay. Spectra of mAbeta in the absence (black), presence of one-fold PM1 (red), and PM1 alone (dark yellow). Error bars represent standard deviations of at least three independent measurements. (b) CD spectra, and (c) FTIR spectra of mAbeta in the absence (black), and presence of one-fold PM1 (red), and PM1 alone (magenta). All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

4.7.2. TEM images and Congo red stained birefringence images of amyloid fibrils formed by mAbeta:

The mAbeta formed clear fibrillar aggregates in the absence of PM1 (Figure 4.16. a-i) under TEM, but in the presence of one-fold PM1 (Figure 4.16. a-ii) amorphous structures were noticed. The PM1 alone also did not show any fibre (Figure 4.16. a-iii). Therefore it can be concluded that PM1 can inhibit aggregation of mAbeta.

The clear green-gold birefringence was viewed for mA β alone under cross-polarized light (Figure 4.16. b-i), but no such birefringence was viewed either when mA β was incubated in the presence of one-fold PM1 (Figure 4.16. b-ii) or for PM1 alone (Figure 4.16. b-iii) after ten days of incubation. These results support that PM1 is non-amyloidogenic peptides, and inhibited amyloid aggregation of mA β .

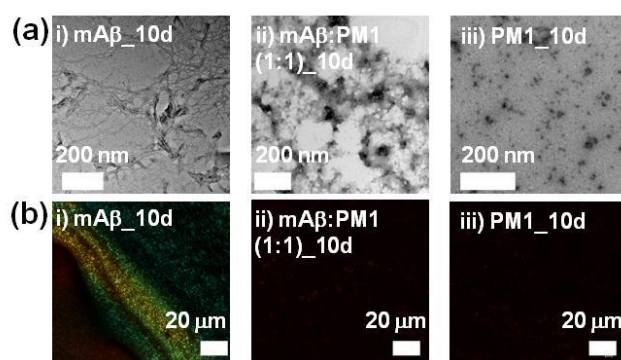


Figure 4.16. (a) TEM images, and (b) Congo red-stained birefringence images of mA β (i) in the absence, and presence of (ii) one-fold PM1, and (iii) PM1 alone. Scale bars are indicated as 200 nm for TEM images, and 20 μ m for birefringence images. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel. All the images were captured after ten days of incubation.

4.8. Inhibition of amyloid accumulation of mA β by PM2:

The kinetics of the amyloid accumulation of mA β was also checked in the absence and presence of PM2. PM2 was co-incubated with mA β in separate vessels on a water bath up to 30 days in parallel at the desired proportion (1:1) *in vitro*. To get a result more precisely, another two replicate solutions of each portion were prepared, and different biophysical tools were used to monitor amyloid accumulation.

4.8.1. ThT fluorescence assay, CD experiments, and FTIR spectra of mA β :

The fluorescence intensity of mA β (black) increased with time in the absence of PM2 as described earlier. However, in the presence of one-fold PM2 (red, Figure 4.17-a) the fluorescence intensity of mA β decreased from the initial hour. This result indicates that PM2 significantly inhibited the amyloid accumulation of mA β .

The β -Sheet conformation of mA β (black) converted to a random coil (red, two negative bands at ~ 195 nm, and at ~ 224 nm of low intensity) in the presence of one-fold PM2 (magenta, Figure 4.17-b). The random coil structure was found for PM2 alone (magenta, two negative bands at ~ 195 nm, and at ~ 226 nm of low intensity) also.

In Figure 4.17-c, the amide I band was observed at 1646 cm^{-1} for mA β in the presence of one-fold PM2 (red), and at 1649 cm^{-1} for PM2 alone (magenta). So, it was concluded that mA β exhibited β -sheet rich structure in the absence of PM2, but in the presence of PM2 exhibited random coil or α -helix conformation.

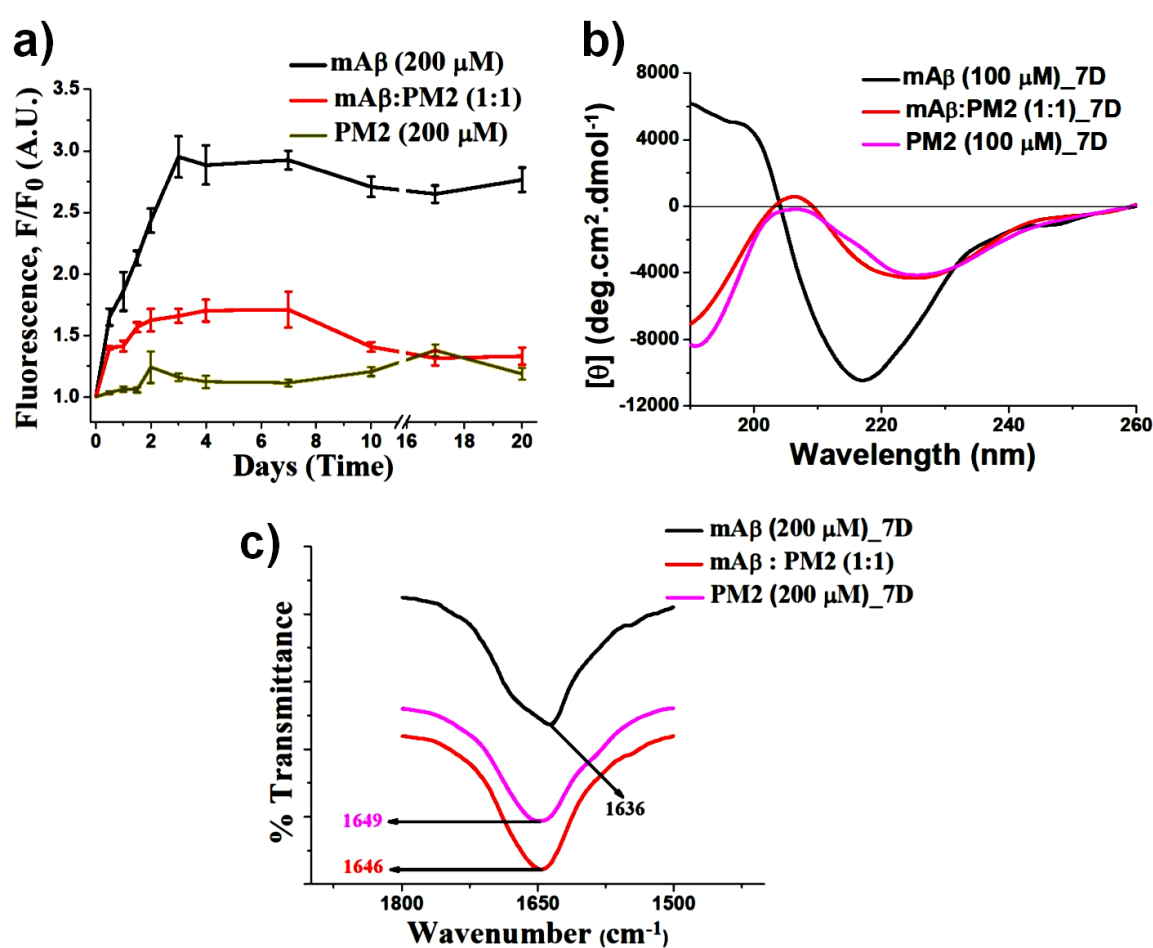


Figure 4.17. (a) Normalized profiles of time-dependent ThT fluorescence assay. Spectra of mA β in the absence (black), presence of one-fold PM2 (red), and PM2 alone (dark yellow). Error bars represent standard deviations of at least three independent measurements. (b) CD spectra, and (c) FTIR spectra of mA β in the absence (black), and presence of one-fold PM2 (red), and PM2 alone (magenta). All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

4.8.2. TEM images and Congo red stained birefringence images of amyloid fibrils formed by mA β :

The clear fibrillar aggregates (Figure 4.18. a-i) and green gold birefringence (Figure 4.18. b-i) were observed for mA β in the absence of PM2 when viewed under TEM and cross-polarized light, respectively. However, in the presence of one-fold PM2, no fibres (Figure 4.18. a-ii) or birefringence (Figure 4.18. b-ii) were observed after ten days of incubation under TEM, and cross-polarised light respectively indicates one-fold PM2 is sufficient to inhibit the aggregation of mA β . PM2 alone (Figure 4.18. b-iii) also did not form any fibre or birefringence confirms its non-aggregating and non-amyloidogenic property.

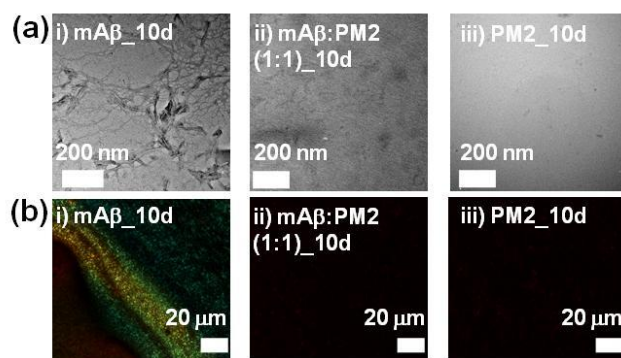


Figure 4.18. (a) TEM images, and (b) Congo red-stained birefringence images of mA β (i) in the absence, and presence of (ii) one-fold PM2, and (iii) PM2 alone. Scale bars are indicated as 200 nm for TEM images, and 20 μ m for birefringence images. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel. All the images were captured after ten days of incubation.

4.9. Inhibition of amyloid accumulation of mA β by PM3:

Next, the kinetics of amyloid accumulation of mA β was checked in the absence and presence of PM3. PM3 was co-incubated along with mA β in separate vessels on a water bath up to 30 days in parallel at the desired proportion (1:1) *in vitro*. Another two replicate solutions of each portion were prepared to get a result more precisely, and various biophysical tools were used to monitor amyloid accumulation.

4.9.1. ThT fluorescence assay, CD experiments, and FTIR spectra of mA β :

Next, a time-dependent ThT fluorescence assay of mA β was performed with PM3 (Figure 4.19-a). From the result, a one-fold molar excess of PM3 (blue, Figure 3.66b) was found to inhibit mA β aggregates with time. In the presence of PM3, the fluorescence intensity of mA β was increased slightly at the 1st day due to self-assembling VFFA segment, but after one day it decreased gradually with time probably due to the proteolytic activity of PM3. In conclusion, one-fold PM3 have good inhibitory efficacy against mA β aggregates.

Indeed, CD spectra confirmed the formation of a predominantly random coil and reduced form of β -sheets (two negative bands at ~ 195 nm, and ~ 222 nm of very low intensity) for mA β (Figure 4.19-b) when co-incubated along with one-fold PM3 which confirms the reduction in aggregation. Random coil conformation of PM3 alone was confirmed from two negative bands at ~ 195 nm and ~ 222 nm of weak intensity.

In Figure 4.19-c, the amide I band was noticed at 1660 cm^{-1} for mA β in the presence of one-fold PM3 (red), and at 1652 cm^{-1} for PM3 alone (magenta). So, in conclusion, mA β exhibited random coil or α -helix conformation in the presence of PM3.

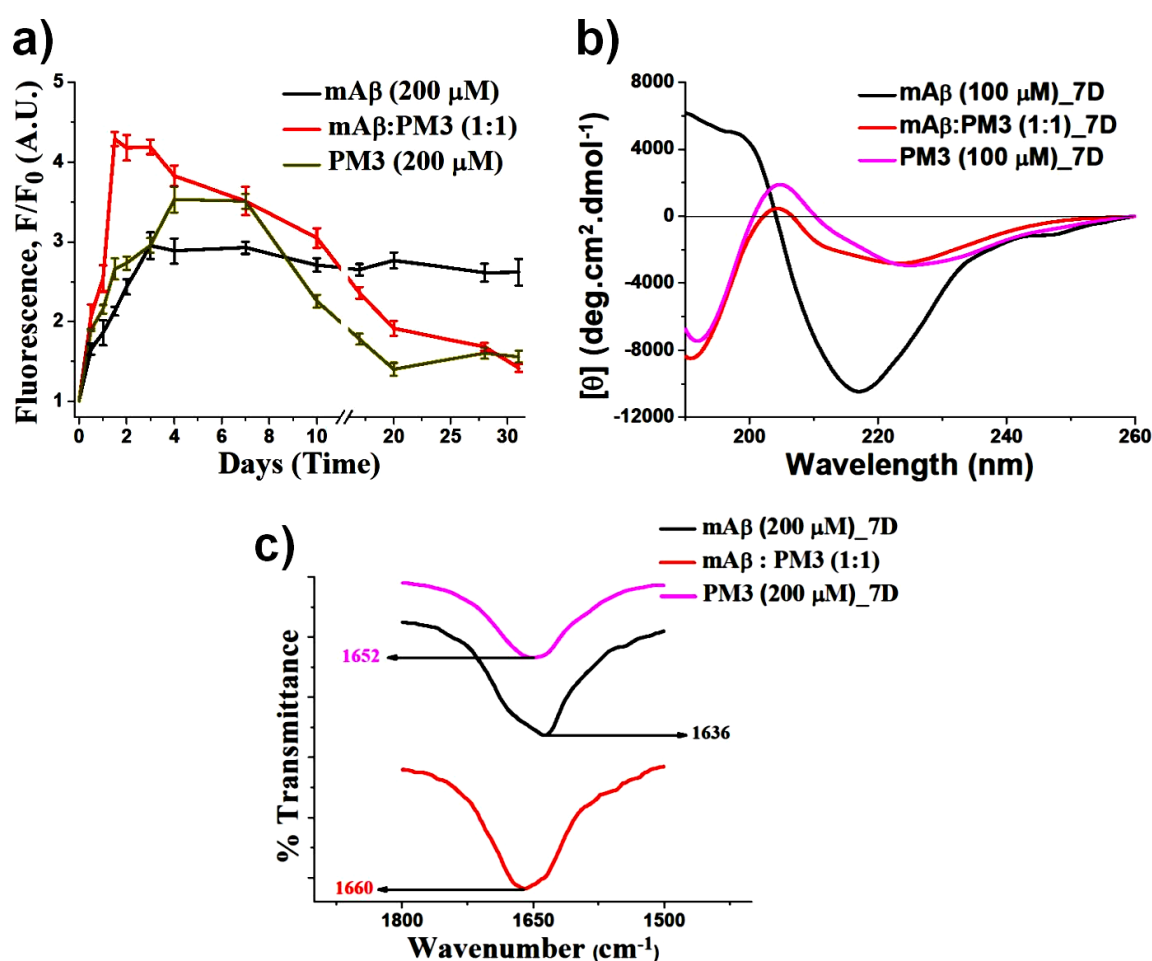


Figure 4.19. (a) Normalized profiles of time-dependent ThT fluorescence assay. Spectra of mAb β in the absence (black), presence of one-fold PM3 (red), and PM3 alone (dark yellow). Error bars represent standard deviations of at least three independent measurements. (b) CD spectra, and (c) FTIR spectra of mAb β in the absence (black), and presence of one-fold PM3 (red), and PM3 alone (magenta). All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

4.9.2. TEM images and Congo red stained birefringence images of amyloid fibrils formed by mAb β :

In the presence of one-fold PM3, amorphous structures (Figure 4.20. a-ii) and no characteristic birefringence (Figure 4.20. b-ii) were observed after ten days of incubation under TEM and cross-polarised light respectively indicates one-fold PM3 can inhibit the aggregation of mAb β . PM3 alone (Figure 4.20. b-iii) also didn't form any fibre or birefringence confirms its non-aggregating and non-amyloidogenic property.

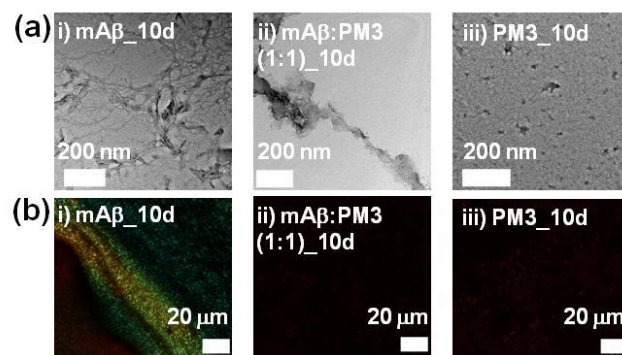


Figure 4.20. (a) TEM images, and (b) Congo red-stained birefringence images of mAb β (i) in the absence, and presence of (ii) one-fold PM3, and (iii) PM3 alone. Scale bars are indicated as 200 nm for TEM images, and 20 μ m for birefringence images. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 $^{\circ}$ C in parallel. All the images were captured after ten days of incubation.

4.10. Examination of the proteolytic activity of PMs on A β ₁₋₄₀:

To find out the cleavage sites of A β ₁₋₄₀ by PMs; similar experiments were performed using commercially available A β ₁₋₄₀ peptide. Samples were prepared in the same way to that for mAb β .

4.10.1. Self-degradation of A β ₁₋₄₀:

MALDI-TOF mass spectra of A β ₁₋₄₀ were analysed in a time-dependent manner in the absence of PMs. This was necessary to check and mark the peptide fragments appeared due to self-degradation of A β ₁₋₄₀. However, no degradation product was observable in MALDI-TOF mass spectra probably due to its solvent inaccessibility, as A β ₁₋₄₀ got aggregated after three days of incubation and it was confirmed from the MALDI mass data. The aliquot was taken from one the replicate solutions of A β ₁₋₄₀, which was incubated as a common sample of two different works, and the spectra were shown in Chapter 3 (Figure 3.75-3.77).

4.10.2. Plausible routes of the proteolytic cleavage of $A\beta_{1-40}$ by PMs:

After third day onwards, it was observed that PMs cleaved $A\beta_{1-40}$ in a site-selective manner. Various peptide fragments and intermediates of different range of lifetimes were also found. Plausible routes of proteolytic cleavage of $A\beta_{1-40}$ by either PMs (Figure 4.21) were explained from MALDI-TOF mass (Figure 4.22-4.24 for PM1, and Figure 4.26-4.28 for PM3) analyses. Enzymes such as neprilysin (NEP), endothelin-converting enzyme (ECE), insulin-degrading enzyme (IDE), Plasmin (P), angiotensin-converting enzyme (ACE), Matrix Metalloproteinases (MMPs) cleave aggregated $A\beta_{1-40}$ in a site-selective manner for facilitating excretion.⁸ PM3 cleaved $A\beta_{1-40}$ in-between Lys16 & Leu17 probably via route 1 ^{$A\beta$ -PM3}, which is the specific cleavage site of α -secretase and site-selective for ECE, MMP2, MMP9 & P. PM3 individually cleaved $A\beta_{1-40}$ between Leu17 & Val18 (ECE cleavage site) to form $A\beta_{1-17}$ & $A\beta_{18-40}$ probably via route 2 ^{$A\beta$ -PM3}, between Val18 & Phe19 (ECE, & IDE cleavage site) to form $A\beta_{1-18}$ probably via route 3 ^{$A\beta$ -PM3}, between Phe19 & Phe20 (IDE, & NEP cleavage site) to form $A\beta_{1-19}$ probably via route 4 ^{$A\beta$ -PM3}, between Phe20 & Ala21 (IDE cleavage site) to form $A\beta_{1-20}$ probably via route 5 ^{$A\beta$ -PM3}, and between Glu22 & Asp23 (unlike any known proteolytic enzymes) to form $A\beta_{1-22}$ probably via route 7 ^{$A\beta$ -PM3}. Interestingly, $A\beta_{1-40}$ like aggregation was not noted in the presence of PM1 and PM3, indicating amyloid solubilizing nature of PM1 and PM3.

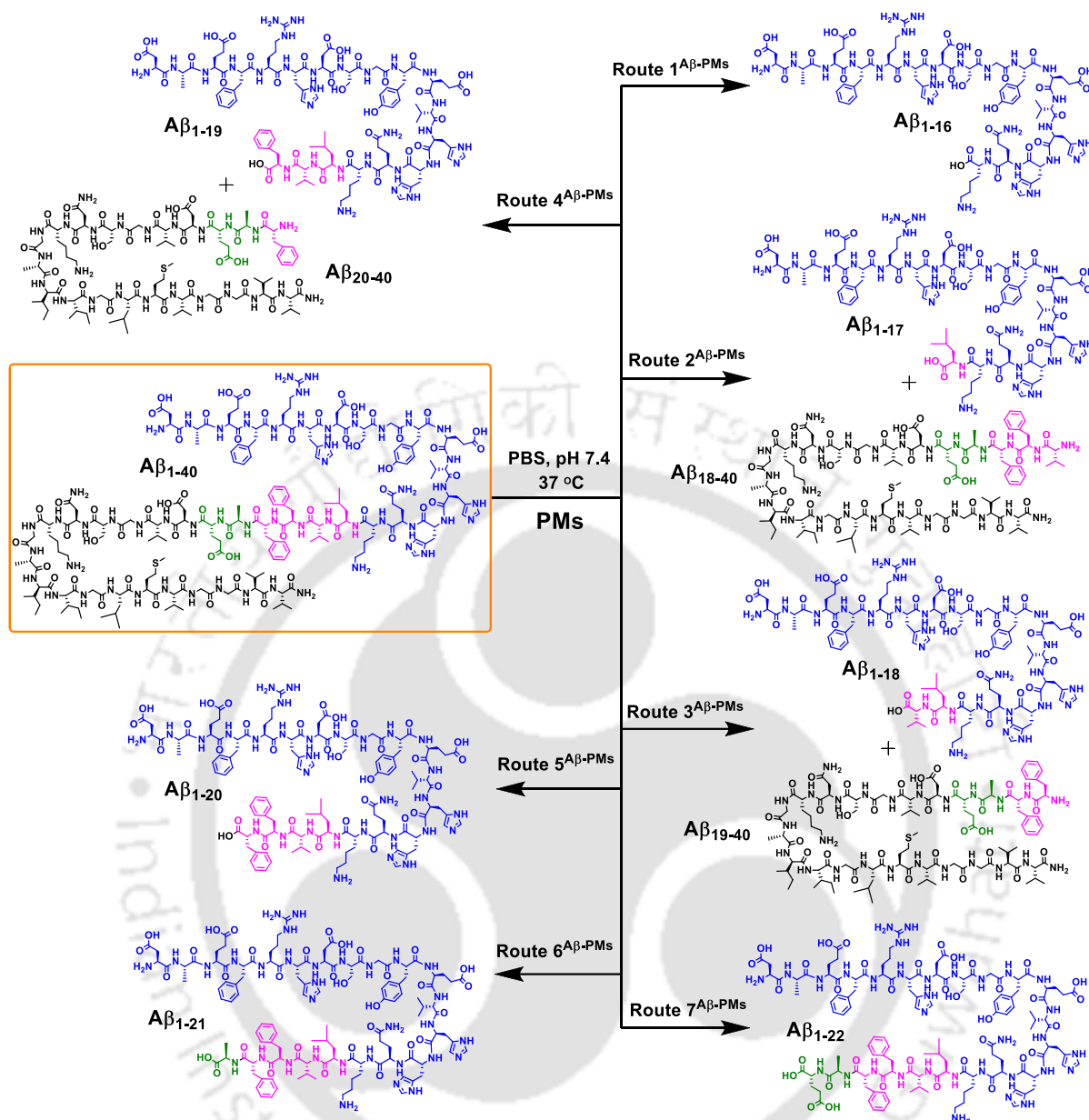


Figure 4.21. Plausible routes of the proteolysis of $A\beta_{1-40}$ into various fragments by PMs (Route 1 $^{A\beta-PMs}$ - Route 7 $^{A\beta-PMs}$).

4.10.3. Proteolytic activity of PM1 on $A\beta_{1-40}$:

Mainly three peptide fragment was found, when $A\beta_{1-40}$ was incubated in the presence of one-fold PM1 (Figure 4.22-4.24), indicating the ability of the proteolytic activity of PM1. Also, $A\beta$ -like aggregated peak was not observed, pointing to the amyloid solubilising nature of PM1 against $A\beta$.

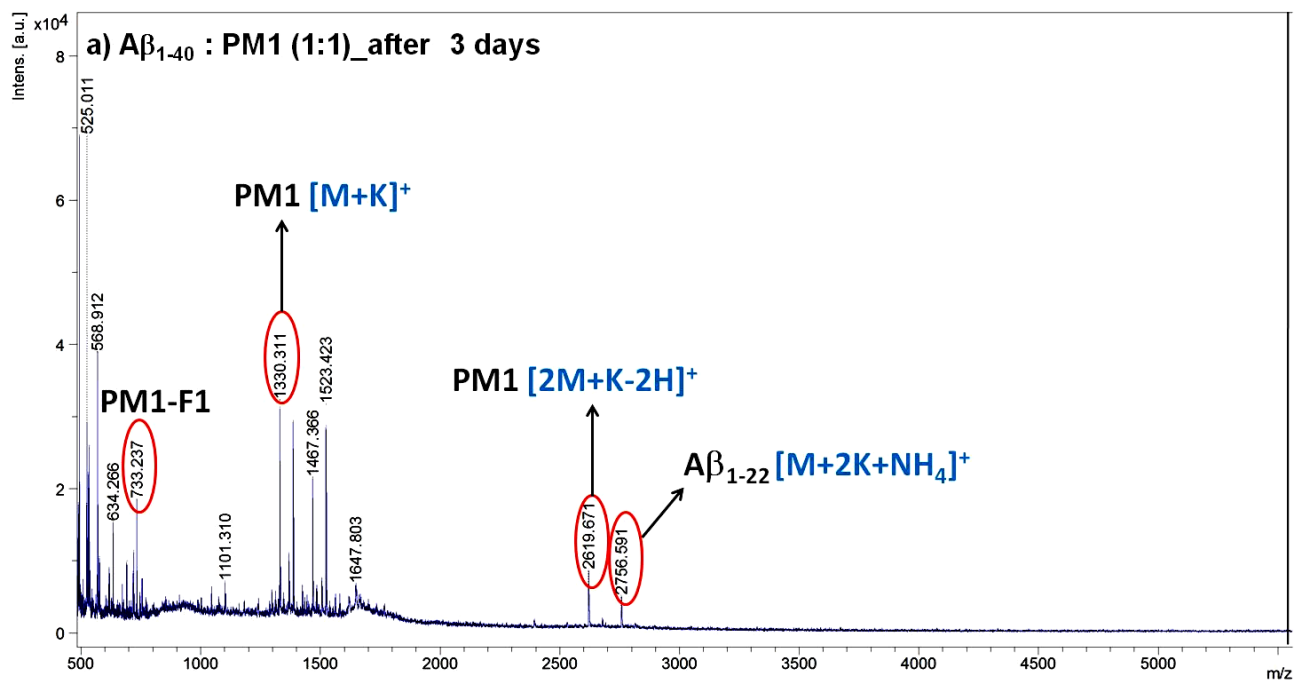


Figure 4.22. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of PM1 (1:1) after three days of incubation in PBS.

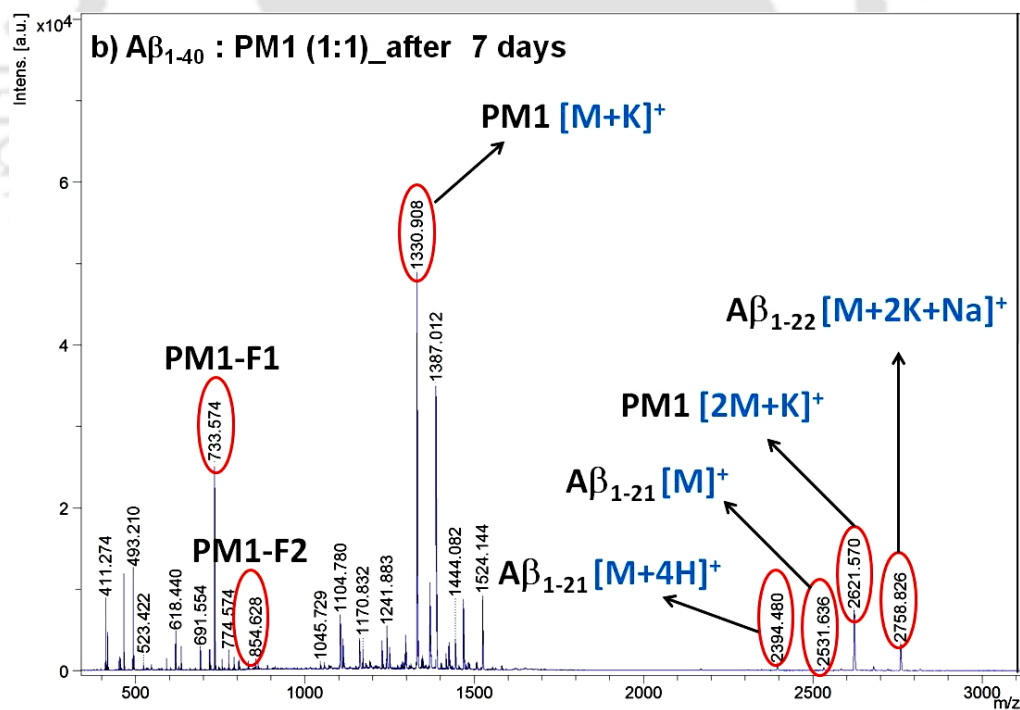


Figure 4.23. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of PM1 (1:1) after seven days of incubation in PBS.

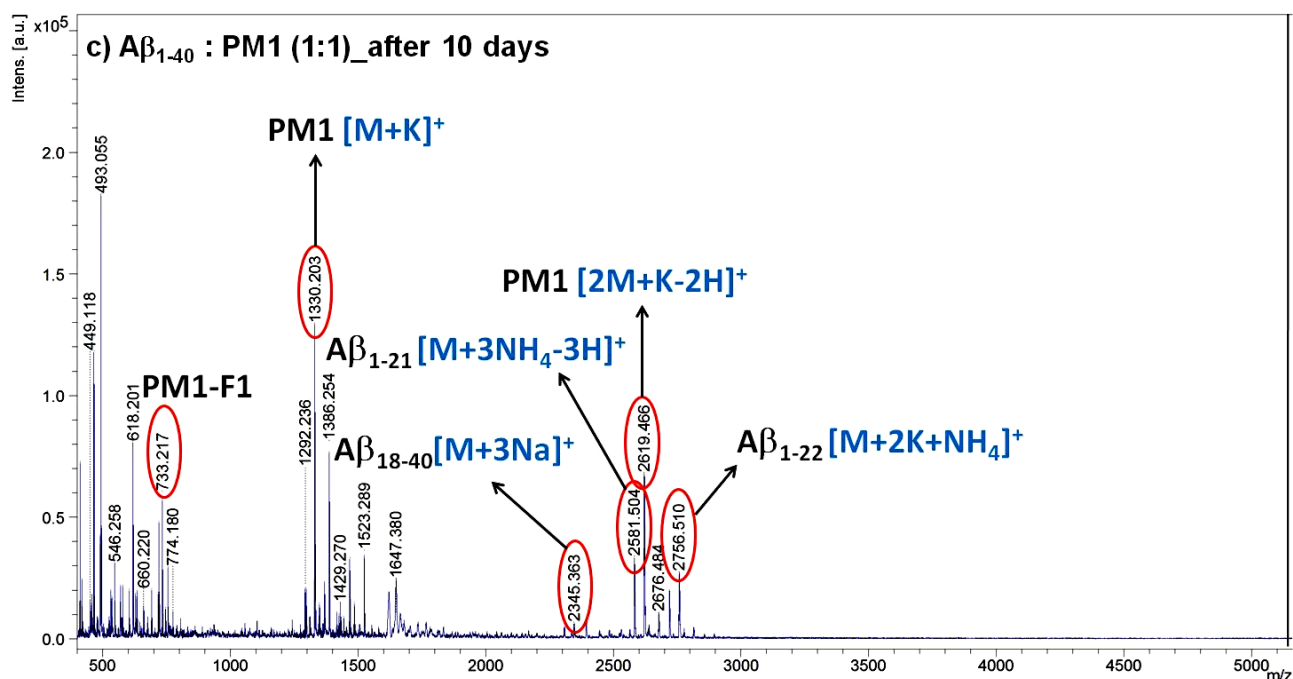


Figure 4.24. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of PM1 (1:1) after seven days of incubation in PBS.

4.10.4 Proteolytic enzyme activities of PM1:

Thus, PM1 is capable to cleave $A\beta_{1-40}$ at various sites (Figure 4.25), mimicking the proteolytic action of ECE.

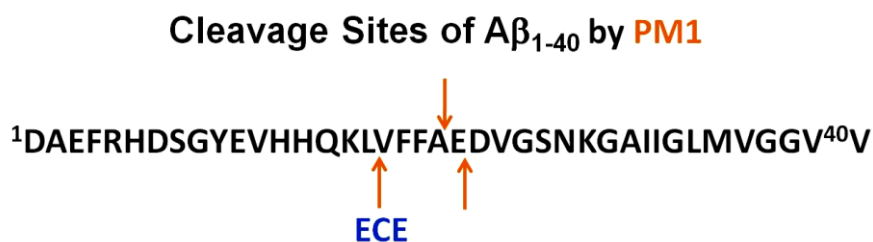


Figure 4.25. Multiple proteolytic enzyme activities of PM1.

4.10.5. Proteolytic activity of PM3 on $A\beta_{1-40}$:

PM3 sliced $A\beta_{1-40}$ into various fragments (Figure 4.26-4.28) as described in proteolytic routes. Interestingly, $A\beta_{1-40}$ like aggregation was not noted in the presence of PM3, indicating amyloid solubilizing nature of PM3.

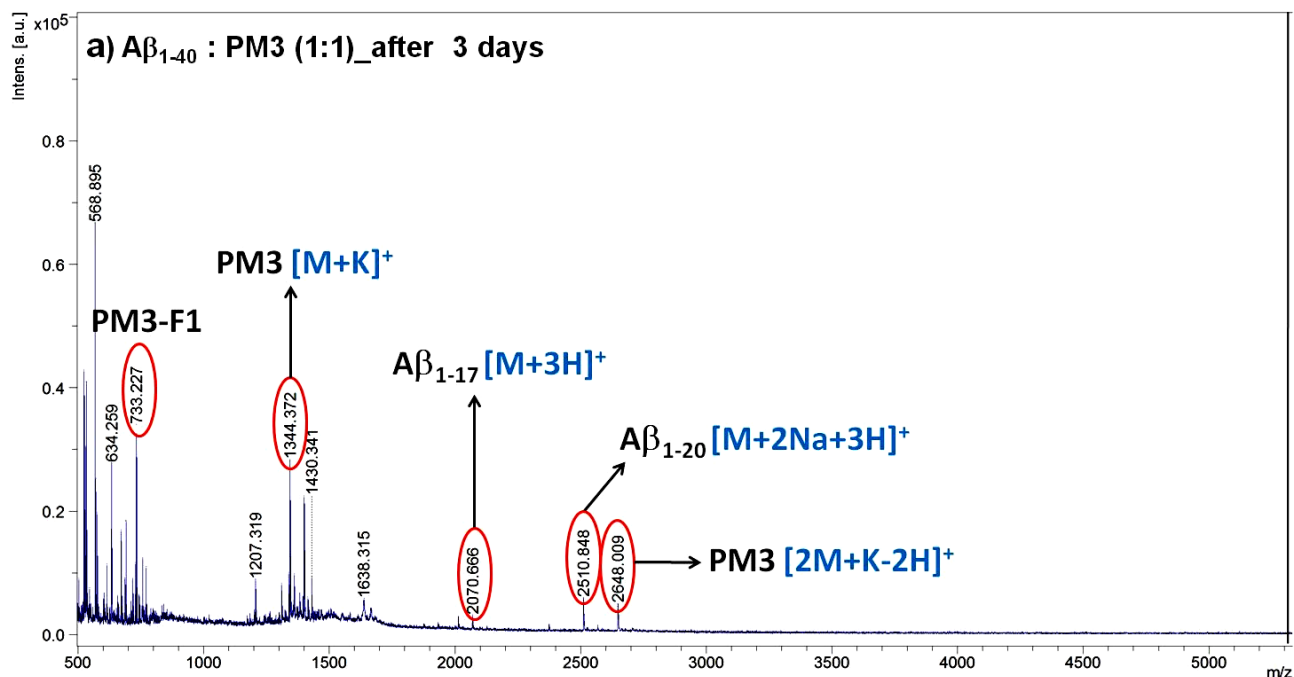


Figure 26. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of PM3 (1:1) after three days of incubation in PBS.

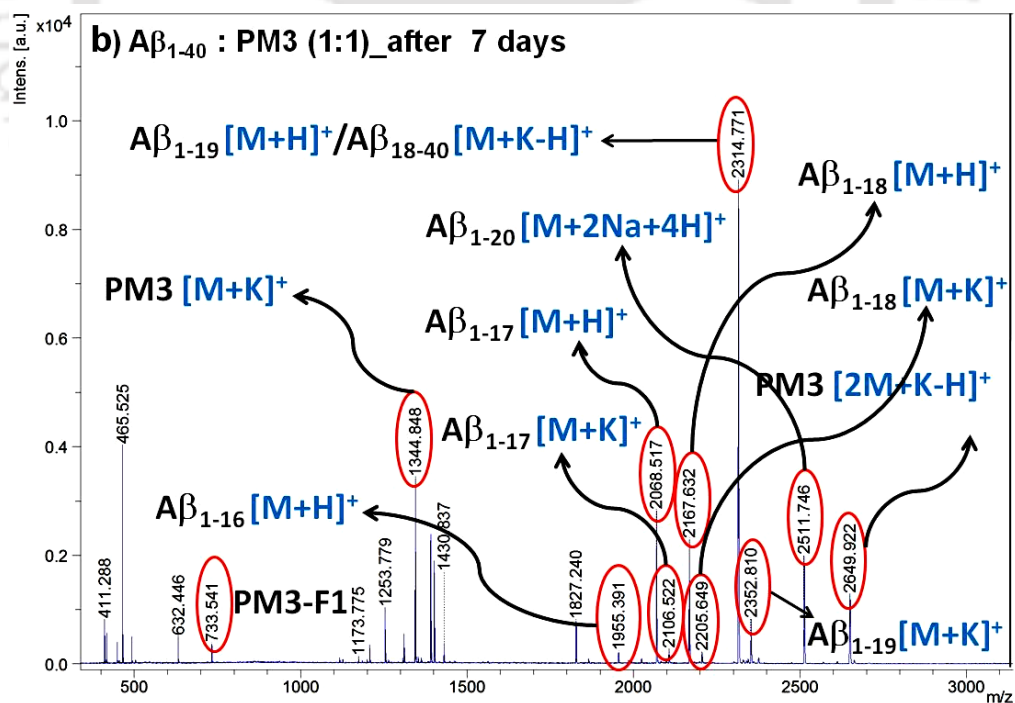


Figure 4.27. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of PM3 (1:1) after seven days of incubation in PBS.

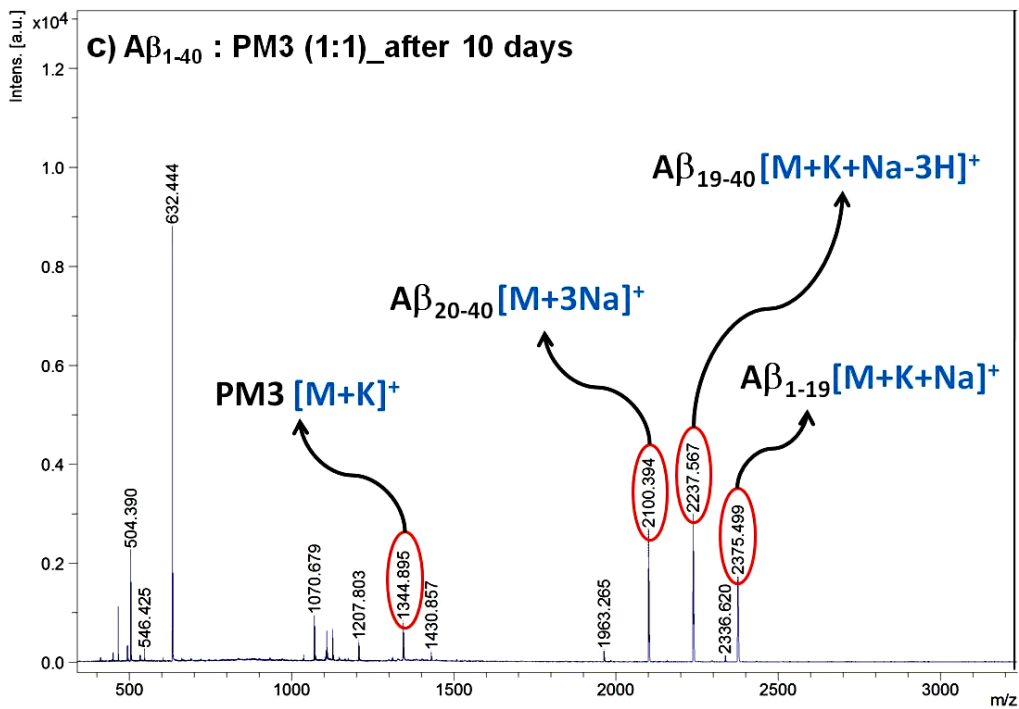


Figure 28. MALDI-TOF mass spectrum of $A\beta_{1-40}$ in the presence of PM3 (1:1) after ten days of incubation in PBS.

4.10.6. Time-dependent existence map of various fragments of $A\beta_{1-40}$:

Time-dependent persistence map of the $A\beta$ fragments (Figure 4.29) reveals the time-resolved manner of the programmed proteolysis of the designed PM3.

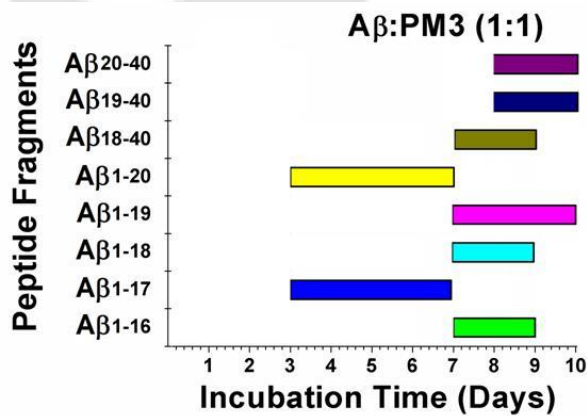


Figure 4.29. Time-dependent persistence map of various fragments of $A\beta_{1-40}$ in the presence of PM3.

4.10.7. Multiple proteolytic enzyme activities of PM3:

Thus, PM3 is capable to cleave $A\beta_{1-40}$ at various sites (Figure 4.30), mimicking the proteolytic action of multiple enzymes (NEP, ECE, IDE, P, ACE, MMPs).

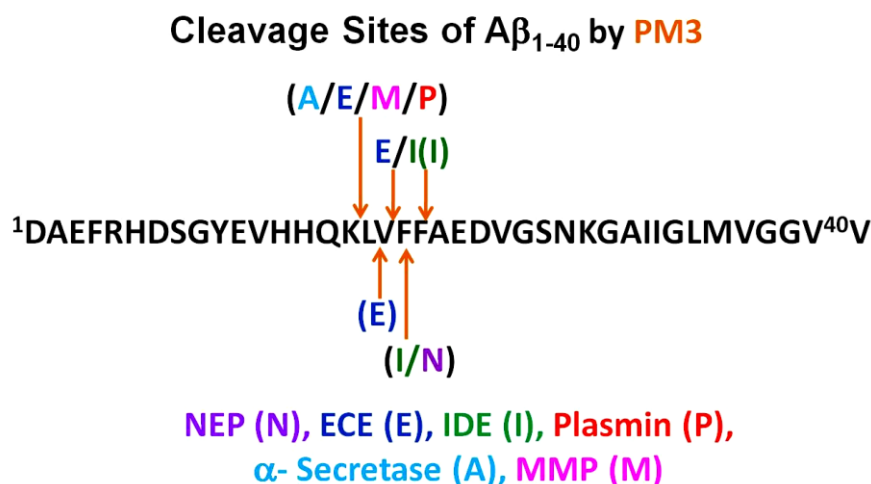


Figure 4.30. Multiple proteolytic enzyme activities of PM3.

4.11. Examination of the proteolytic activity of PM3 on DTP28:

Finally, to examine the selective nature of PM3, a designed negative control (NC) peptide was co-incubated with PM3 in 1:1 molar ratio at physiological pH and temperature and followed by time-dependent MALDI mass analyses. A 28 residue long partial sequence of the diphtheria toxin protein (DTP) was chosen as the negative control peptide as its sequence was unrelated to both $A\beta$ and the PM3.

4.11.1. Effect of PM3 on the negative control (NC) peptide, DTP28:

NC underwent self-degradation at its Ser and Thr positions, which was confirmed by a parallel experiment with NC in the absence of PM3 (Figure 3.95-3.99). Such cleavage may proceed via route 1^{NC} (Figure 3.95), which is unrelated to PM3. The co-incubated sample did not show any of the expected fragments (Figure 4.31) due to cleavage by PM3 unlike $A\beta_{1-40}$ even after seven days.

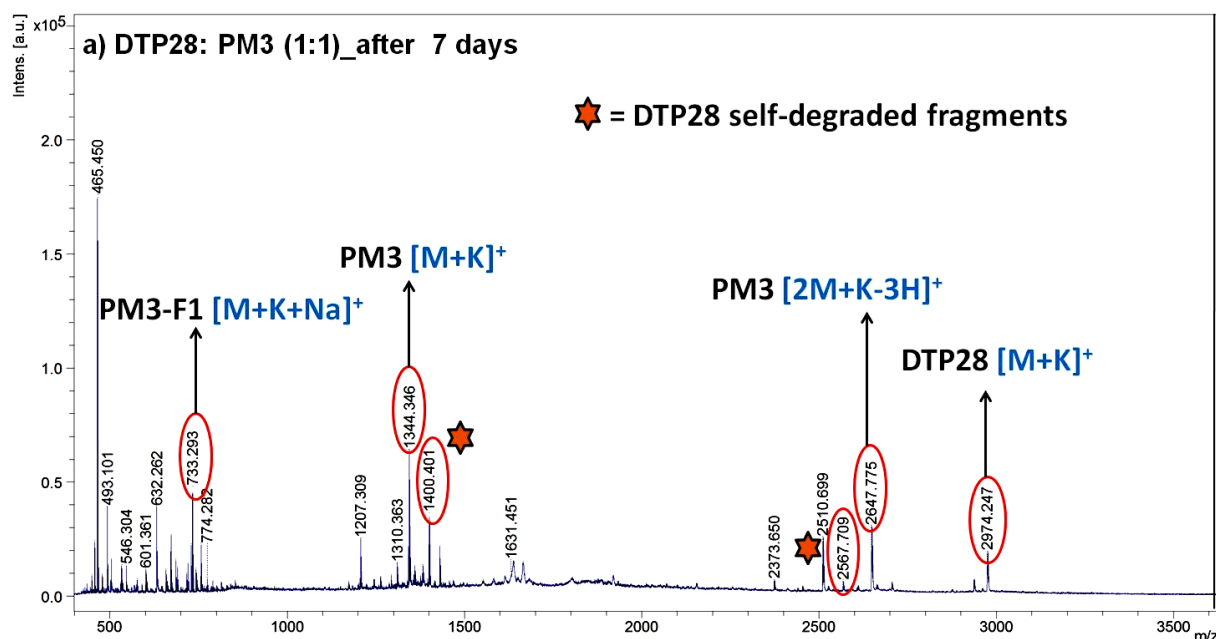


Figure 4.31. MALDI-TOF mass spectrum of NC (DTP28) in the presence of PM3 (1:1) after seven days of incubation in PBS.

4.12. Inhibition of amyloid accumulation of $A\beta_{1-40}$ by PM1:

After finding the proteolytic activity of PMs against $A\beta_{1-40}$, we carried on further to verify the inhibition potential of PMs against $A\beta_{1-40}$. The kinetics of the amyloid accumulation of $A\beta_{1-40}$ was checked using various biophysical tools in the absence and presence of PMs in a dose-dependent manner. Samples were prepared mentioned in the experimental method chapter to obtain the stock solutions of $A\beta_{1-40}$ (50 μ M) and PMs (500 μ M). PMs were co-incubated with $A\beta_{1-40}$ in PBS pH 7.4 at 37 $^{\circ}$ C at the desired proportions in separate vessels on a water bath till 30 days in parallel. To investigate the dose-dependent inhibitory efficacy of PM1, one-fold and a five-fold molar excess of PM1 were co-incubated with 50 μ M of $A\beta_{1-40}$ at the physiological condition. To get the result more accurately another two replicate solutions of each portion were arranged.

4.12.1. ThT fluorescence assay, CD experiments, and FTIR spectra of $A\beta_{1-40}$:

Dose-dependent ThT fluorescence assay of $A\beta_{1-40}$ was performed with PM1 because of its proteolytic activity. From the result (Figure 4.32-a), it was found that the fluorescence intensity of $A\beta_{1-40}$ (black) increased with time in the absence of PM1 and became static after slight lowering in intensity, and further decreased later as there is a probability for mature fibril to come out from the solution. In the presence of one-fold PM1 (red), the fluorescence intensity of the $A\beta_{1-40}$ decreased with time. But, with increasing the dose from one-fold to five-fold (blue) molar excess, the fluorescence intensity of the $A\beta_{1-40}$ increased more for Cys induced co-aggregation, and after seven days it was found to be decreased gradually due to the formation of various $A\beta_{1-40}$ fragments cleaved by PM1, indicating the inhibitory efficacy of PM1 against amyloid formation of $A\beta_{1-40}$.

It was observed that $A\beta_{1-40}$ fibrils adopt a β -sheet conformation (black, Figure 4.32-b, a positive band at ~ 200 nm with a negative band at ~ 222 nm). In the presence of one-fold PM1 (red), $A\beta_{1-40}$ converted to a mixture of predominantly random coil and β -sheet structure (two negative bands at ~ 190 nm and ~ 222 nm) which confirms the reduction in aggregation. A mixture of predominantly α -helix and β -sheet structure conformation of $A\beta_{1-40}$ (dark cyan) was confirmed (two negative bands at ~ 202 nm and ~ 228 nm) in the presence of five-fold molar excess of PM1. Indeed, a random coil structure (a negative band at ~ 190 nm) was obtained for PM1 alone (magenta). The spectra were taken after seven days of incubation.

In FTIR spectra (Figure 4.32-c), the amide I band was observed at 1633 cm^{-1} for $A\beta_{1-40}$ alone (black), at 1641 cm^{-1} for $A\beta_{1-40}$ in the presence of one-fold PM1 (red), at 1644 cm^{-1} for $A\beta_{1-40}$ in the presence of five-fold PM1 (blue), and at 1655 cm^{-1} for PM1 alone (magenta). From the results, it was confirmed that $A\beta_{1-40}$ displayed β -sheet structure in the absence of PM1; but in the presence of PM1, $A\beta_{1-40}$ exhibited random coil or α -helix conformation.

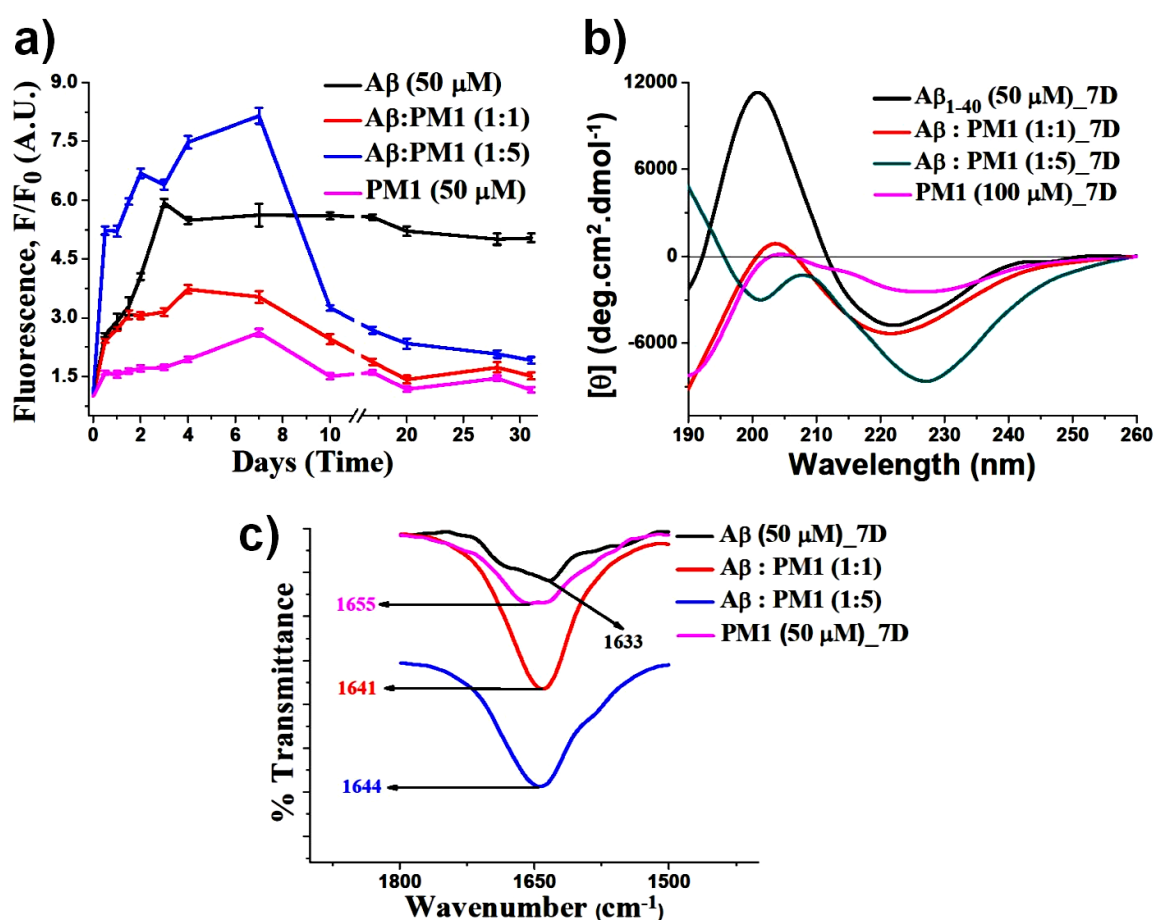


Figure 4.32. Normalized profiles of (a) dose-dependent ThT fluorescence assay. Spectra of A β 1-40 in the absence (black), presence of one-fold PM1 (red), five-fold PM1 (blue), and PM1 alone (magenta). Error bars represent standard deviations of at least three independent measurements. (b) CD spectra of A β 1-40 in the absence and presence of PM1 after seven days of incubation. Spectra of A β 1-40 in the absence (black), presence of one-fold PM1 (red), five-fold PM1 (dark cyan), and PM1 alone (magenta). (c) FTIR spectra of A β 1-40 in the absence and presence of PM1 after seven days of incubation. Spectra of A β 1-40 in the absence (black), presence of one-fold (red) PM1, five-fold PM1 (blue), and PM1 alone (magenta). All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C.

4.12.2. TEM images and Congo red-stained birefringence images of amyloid fibrils formed by A β 1-40:

The A β 1-40 alone showed clear fibrillar aggregates (Figure 4.33. a-i) under TEM, but in the presence of one-fold PM1 (Figure 4.33. a-ii) and five-fold PM1 (Figure 4.33. a-iii),

amorphous structures and tiny fibres were noticed respectively after ten days of incubation. The PM1 (Figure 4.106. a-iii) alone also did not show any fibre. Therefore it can be concluded PM1 can inhibit aggregation of $A\beta_{1-40}$.

The bright green-gold birefringence was observed for $A\beta_{1-40}$ alone under cross-polarized light (Figure 4.33. b-i), but when $A\beta_{1-40}$ was incubated in the presence of one-fold PM1 (Figure 4.106. b-ii) and five-fold PM1 (Figure 4.106. b-iii) separately, no such birefringence was observed. The PM1 (Figure 4.106. a-iii) alone also did not show any birefringence. The images were taken after ten days of incubation.

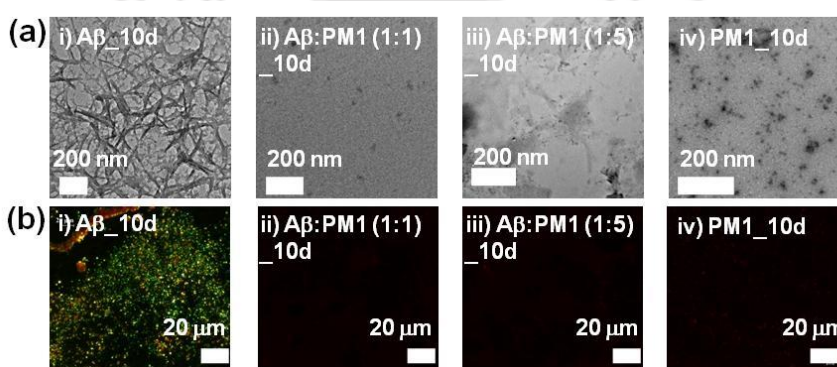


Figure 4.33. (a) TEM images, and (b) Congo red-stained birefringence images of $A\beta_{1-40}$ (i) in the absence, and presence of (ii) one-fold PM1, (iii) five-fold PM1, and (iv) PM1 alone. Scale bars are indicated as 200 nm for TEM images, and 20 μ m for birefringence images. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel. All the images were captured after ten days of incubation.

4.13. Inhibition of amyloid accumulation of $A\beta_{1-40}$ by PM2:

A one-fold and a five-fold molar excess of PM2 were co-incubated with 50 μ M of $A\beta_{1-40}$ at the physiological condition to investigate dose-dependent inhibitory efficacy of PM2.

4.13.1. ThT fluorescence assay, CD experiments, and FTIR spectra of $A\beta_{1-40}$:

The time-dependent increase in fluorescence intensity of $A\beta_{1-40}$ (black) was noticed in the absence of PM2 as described in the case of PM1. But in the presence of one-fold PM2

(red, Figure 4.34-a) and five-fold PM2 (blue, Figure 4.34-a), the fluorescence intensity of $A\beta_{1-40}$ suppressed significantly with time in a dose-dependent manner due to the formation of various $A\beta_{1-40}$ fragments proteolysis by PM2.

CD spectra (Figure 4.34-b) confirmed the conversion of β -Sheet structure (black) into a random coil conformation (two negative bands at ~ 190 nm, and ~ 225 nm of low intensity) of $A\beta_{1-40}$ when co-incubated along with one-fold PM2 (red) and five-fold PM2 (dark cyan). Random coil conformation of PM2 (two negative bands at ~ 192 nm, and ~ 227 nm of low intensity) was also displayed (magenta).

In FTIR spectra, the amide I band was observed at 1633 cm^{-1} for $A\beta_{1-40}$ alone (black), at 1645 cm^{-1} for $A\beta_{1-40}$ in the presence of one-fold PM2 (red), at 1641 cm^{-1} for $A\beta_{1-40}$ in the presence of five-fold PM2 (blue), and at 1649 cm^{-1} for PM2 alone (magenta). From the results, it was confirmed that in the presence of either PM2, $A\beta_{1-40}$ exhibited random coil or α -helix conformation.

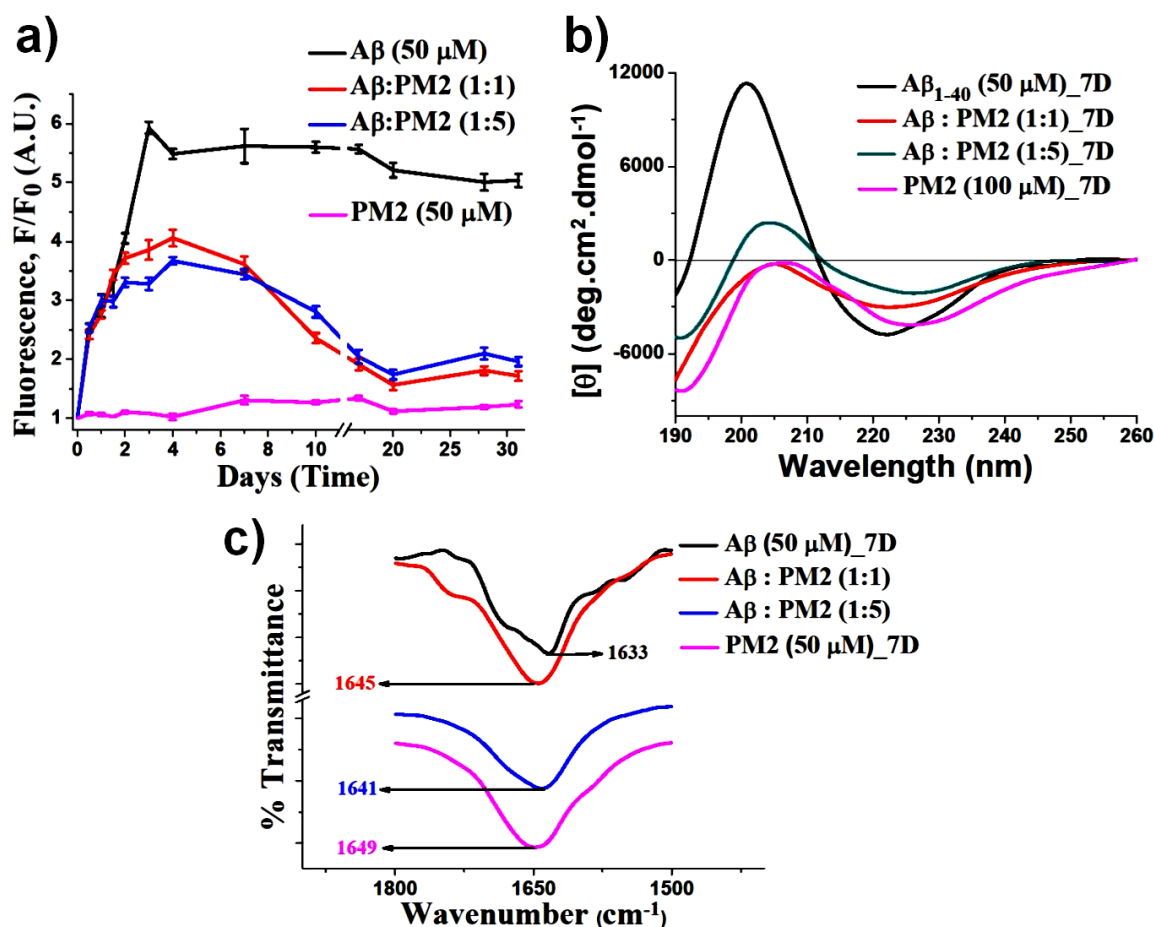


Figure 4.34. Normalized profiles of (a) dose-dependent ThT fluorescence assay. Spectra of $A\beta_{1-40}$ in the absence (black), presence of one-fold PM2 (red), five-fold PM2 (blue), and PM2 alone (magenta). Error bars represent standard deviations of at least three independent measurements. (b) CD spectra of $A\beta_{1-40}$ in the absence and presence of PM2 after seven days of incubation. Spectra of $A\beta_{1-40}$ in the absence (black), presence of one-fold PM2 (red), five-fold PM2 (dark cyan), and PM2 alone (magenta). (c) FTIR spectra of $A\beta_{1-40}$ in the absence and presence of PM2 after seven days of incubation. Spectra of $A\beta_{1-40}$ in the absence (black), presence of one-fold (red) PM2, five-fold PM2 (blue), and PM2 alone (magenta). All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C.

4.13.2. TEM images, and Congo red-stained birefringence images of amyloid fibrils formed by $A\beta_{1-40}$:

When $A\beta_{1-40}$ was incubated in the absence of PM2, the clear fibrillar aggregates and green gold birefringence were observed from the sample when viewed under TEM and cross-polarized light respectively. However, in the presence of one-fold and five-fold PM2, no such fibre (Figure 4.35. a-ii) or green gold birefringence (Figure 4.35. b-ii) was observed, indicates one-fold PM2 has the ability to inhibit significantly the aggregation of $A\beta_{1-40}$. No fibre (Figure 4.35. a-iv) or green gold birefringence (Figure 4.35. b-iv) was noticed for PM2 alone. The images were taken after ten days of incubation.

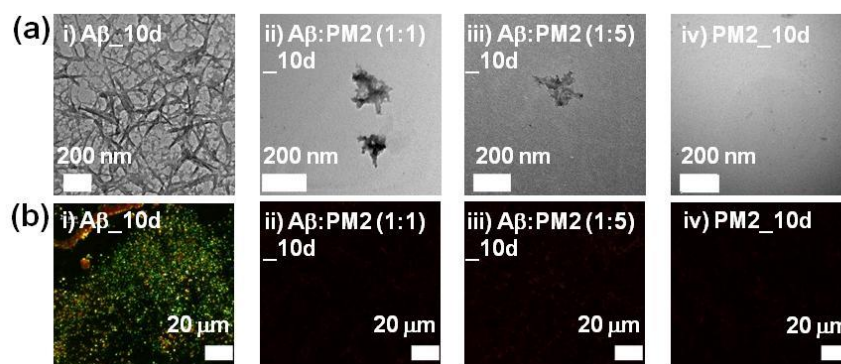


Figure 4.35. (a) TEM images, and (b) Congo red-stained birefringence images of $A\beta_{1-40}$ (i) in the absence, and presence of (ii) one-fold PM2, (iii) five-fold PM2, and (iv) PM2 alone. Scale bars are indicated as 200 nm for TEM images, and 20 μ m for birefringence images. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel. All the images were captured after ten days of incubation.

4.14. Inhibition of amyloid accumulation of $A\beta_{1-40}$ by PM3:

A one-fold and a five-fold molar excess of PM3 were co-incubated with 50 μ M of $A\beta_{1-40}$ at the physiological condition to investigate dose-dependent inhibitory efficacy of PM3.

4.14.1. ThT fluorescence assay, CD experiments, and FTIR spectra of $A\beta_{1-40}$:

A dose-dependent ThT fluorescence assay of $A\beta_{1-40}$ was performed with PM3 (Figure 4.36-a). From the result, it was cleared that one-fold PM3 (red) is efficient to inhibit the amyloid formation of $A\beta_{1-40}$, as the fluorescence intensity was found to be decreased with time compare to $A\beta_{1-40}$ alone. But in the presence of five-fold molar excess of PM3 (blue) the fluorescence intensity of $A\beta_{1-40}$ increased initially due to co-aggregation but after four days it decreased with time due to the proteolytic activity of PM3. In conclusion, PM3 was able to inhibit the amyloid formation of $A\beta_{1-40}$, but the more pronounced result was found in the case of one-fold PM3.

In the presence of PM3, a conformational changes of $A\beta_{1-40}$ (Figure 4.36-b) was noticed from the β -sheet (a positive band at ~ 200 nm with a negative band at ~ 222 nm) structure to a mixture of random coil and β -sheet conformation (two negative bands at ~ 195 nm and ~ 220 nm) confirmed the inhibition of aggregation process. In the case of PM3 alone, random coil structure (a negative band at ~ 195 nm) was obtained.

In FTIR spectra (Figure 4.36-c), the amide I band was observed at 1633 cm^{-1} for $A\beta_{1-40}$ alone (black), at 1641 cm^{-1} for $A\beta_{1-40}$ in the presence of one-fold PM3 (red), at 1644 cm^{-1} for $A\beta_{1-40}$ in the presence of five-fold PM2 (blue), and at 1652 cm^{-1} for PM3 alone (magenta). From the results, it was confirmed that in the presence of either PM3, $A\beta_{1-40}$ exhibited random coil or α -helix conformation.

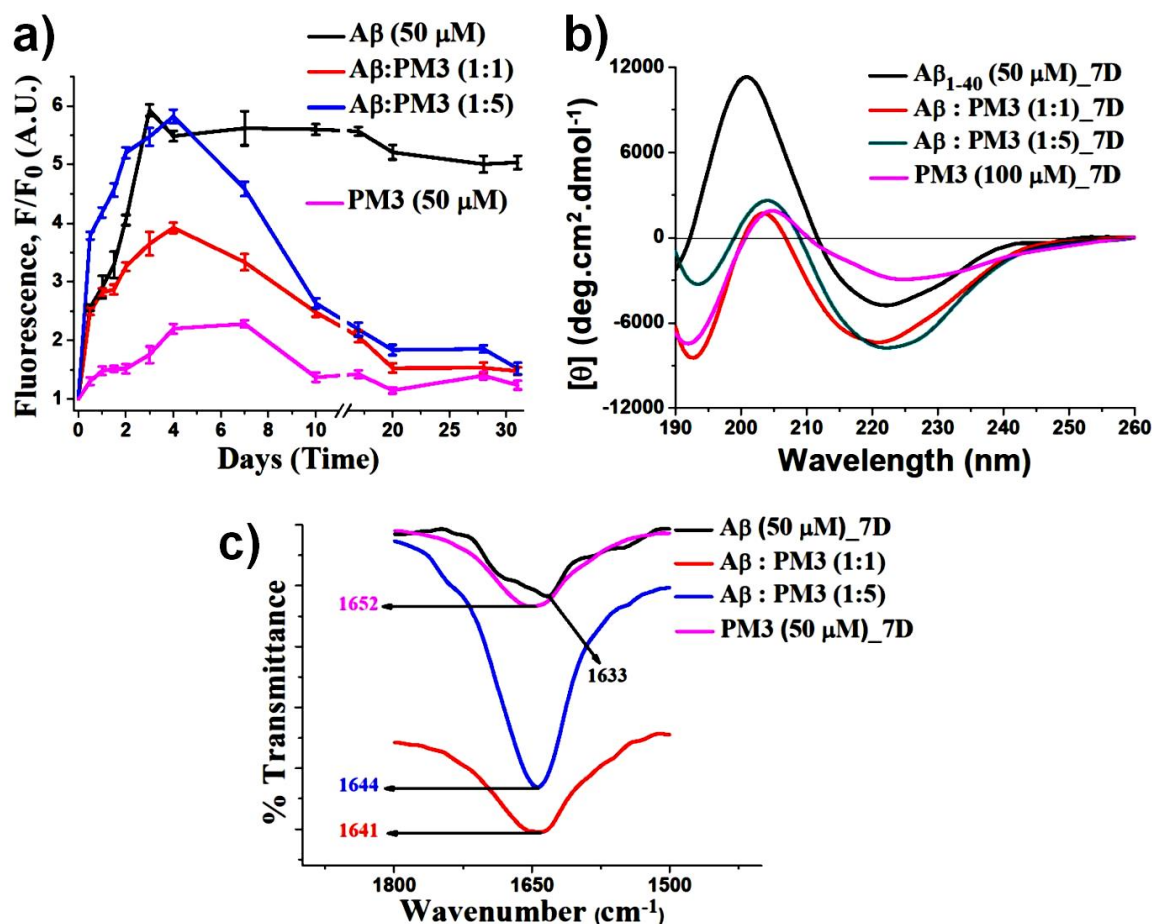


Figure 4.36. Normalized profiles of (a) dose-dependent ThT fluorescence assay. Spectra of A β 1-40 in the absence (black), presence of one-fold PM3 (red), five-fold PM3 (blue), and PM3 alone (magenta). Error bars represent standard deviations of at least three independent measurements. (b) CD spectra of A β 1-40 in the absence and presence of PM3 after seven days of incubation. Spectra of A β 1-40 in the absence (black), presence of one-fold PM3 (red), five-fold PM3 (dark cyan), and PM3 alone (magenta). (c) FTIR spectra of A β 1-40 in the absence and presence of PM1 after seven days of incubation. Spectra of A β 1-40 in the absence (black), presence of one-fold (red) PM3, five-fold PM3 (blue), and PM3 alone (magenta). All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C.

4.14.2. TEM images, and Congo red-stained birefringence images of amyloid fibrils formed by A β 1-40:

Though fibre and green gold birefringence were detected from the sample of A β 1-40 alone (Figure 4.37. a-i, and 4.37. b-i), no such fibre and characteristic birefringence were

observed for $A\beta_{1-40}$ incubated along with one-fold PM3 (Figure 4.37. a-ii, and 4.37. b-ii) when viewed under TEM and cross-polarized light respectively. Amorphous structures along with very few tiny fibres were noticed for $A\beta_{1-40}$ when incubated along with five-fold PM3 (Figure 4.37. a-iii) after ten days of incubation, but it didn't show any birefringence (Figure 4.37. b-iii). PM3 alone also didn't show such fibre or birefringence (Figure 4.37. a-iv, and 4.37. b-iv). These results support that PM3 significantly inhibited amyloid aggregation of $A\beta_{1-40}$. The images were taken after ten days of incubation.

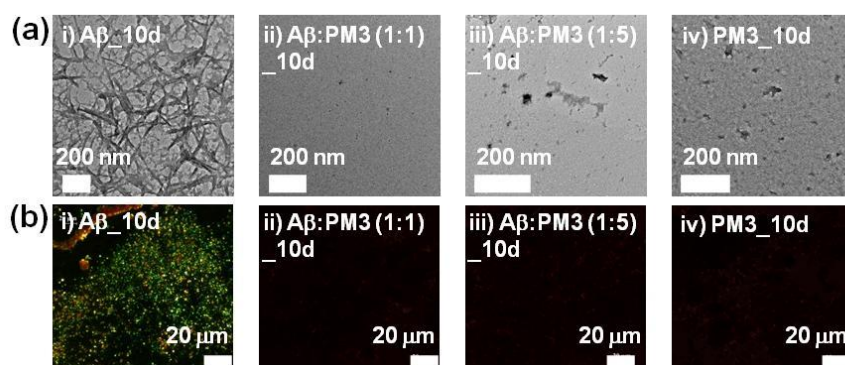


Figure 4.37. (a) TEM images, and (b) Congo red-stained birefringence images of $A\beta_{1-40}$ (i) in the absence, and presence of (ii) one-fold PM3, (iii) five-fold PM3, and (iv) PM3 alone. Scale bars are indicated as 200 nm for TEM images, and 20 μ m for birefringence images. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel. All the images were captured after ten days of incubation.

4.15. Time-dependent TEM images and Congo red stained birefringence images of amyloid fibrils formed by $A\beta_{1-40}$:

The time-dependent TEM (Figure 4.38. a-c) and Congo red stained birefringence (Figure 4.38. d-f) studies were carried out to obtain an idea regarding the time scale of amyloid inhibition and disruption. It was noticed that while the fibrillar structure and bright green-gold birefringence continued for $A\beta_{1-40}$ (Figure 4.38. a, d) during the entire period of the experiment, they gradually decreased and finally disappeared at fourteen days of incubation in the presence of one-fold PM3 (Figure 4.38. b, e for inhibition, and Figure 4.38. c, f for disruption). All the investigations were carried out under near-physiological conditions in vitro (PBS of pH 7.4 at 37 °C).

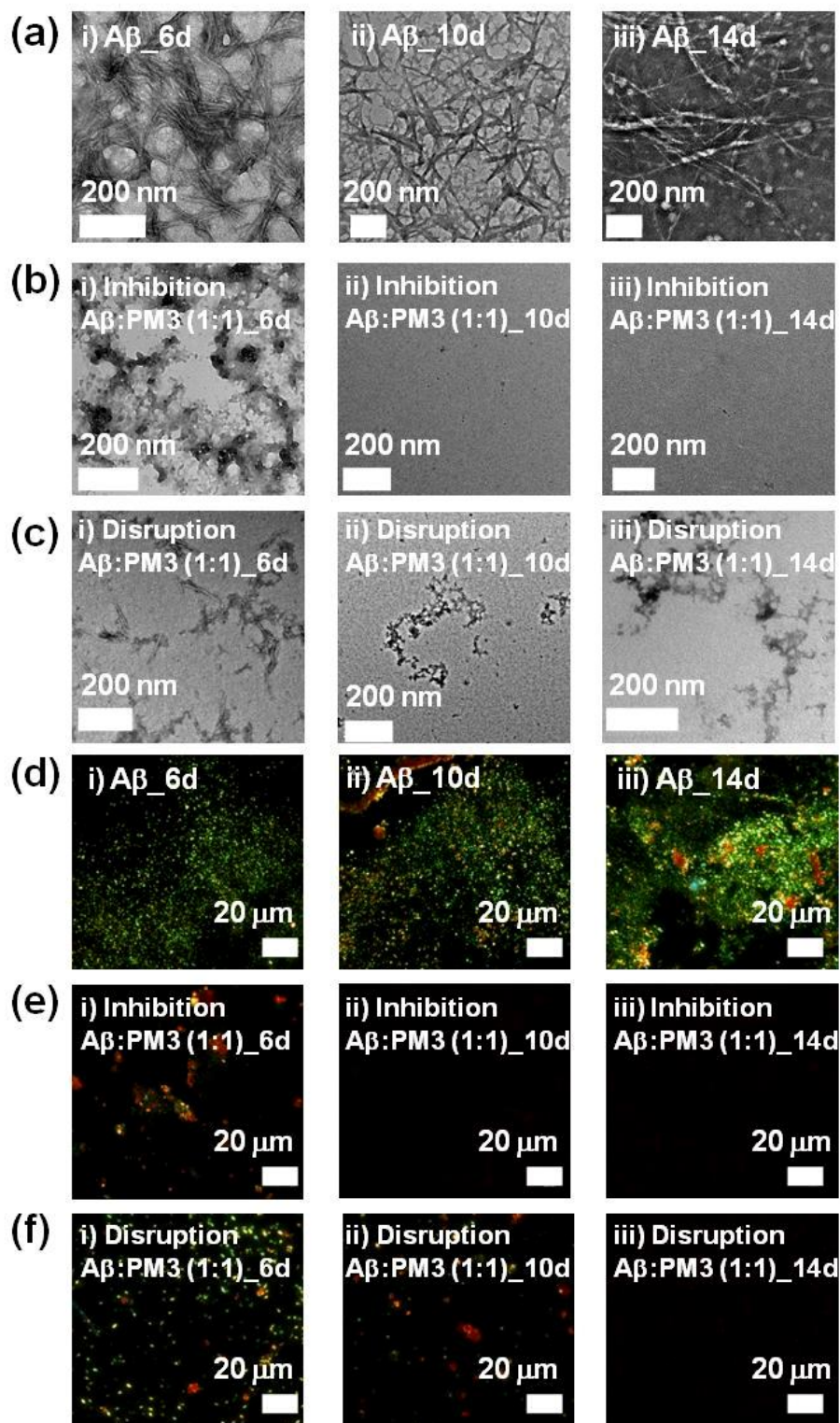


Figure 4.38. Time-dependent (a-c) TEM images, and (d-f) Congo red-stained birefringence images of $A\beta_{1-40}$. (a, d) in the absence, and presence of (b-c, e-f) one-fold molar PM3. Images were recorded after (i) six days, after (ii) ten days, and after (iii) fourteen days of incubation [(b, e) for inhibition studies, and (c, f) for disruption studies where PM3 was added after 3 days to the preformed fibril of

A β ₁₋₄₀]. Scale bars are indicated as 200 nm for TEM images, and 20 μ m for birefringence images. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

4.16. Conclusions:

In this chapter, a series of native and non-native triad based peptide-conjugate are designed linking a target protein recognizing unit with a small proteolytic unit, termed as “Protease Mimic (PM)”. The critical feature of the design is that the proteolytic activity of a catalytic triad of Papain (cysteine protease) or *Geotrichium Candidum* Lipase (serine protease) active site could be mimicked by integrating the triad residues by flexible Gly between each of them that acted as the small proteolytic unit. It was found that newly designed PMs cleave a smaller A β fragment (A β ₁₂₋₂₁ or APP₆₈₃₋₆₉₂, mA β) and A β also in a site-selective manner at the physiological condition, which was confirmed from MALDI-TOF mass analyses. Results obtained from several biophysical tools (ThT-assay, CD, FTIR, TEM, and Congo red birefringence) suggest that PMs are non-amyloidogenic and significantly inhibited the amyloid accumulation of A β ₁₋₄₀ *in vitro*. After analysing various experiments it was concluded that one-fold PM3 can inhibit the amyloid formation of aggregated A β ₁₋₄₀. So, a lower dosage of PM3 may reduce the side effects. Therefore newly developed PM is a drug-like molecule that cleaves the peptide backbone of A β selectively and may cure AD, and hence the designed PMs offer a new therapeutic option against amyloidoses (e.g. AD, type 2 diabetes mellitus, Parkinson’s disease, etc.).

4.17. References:

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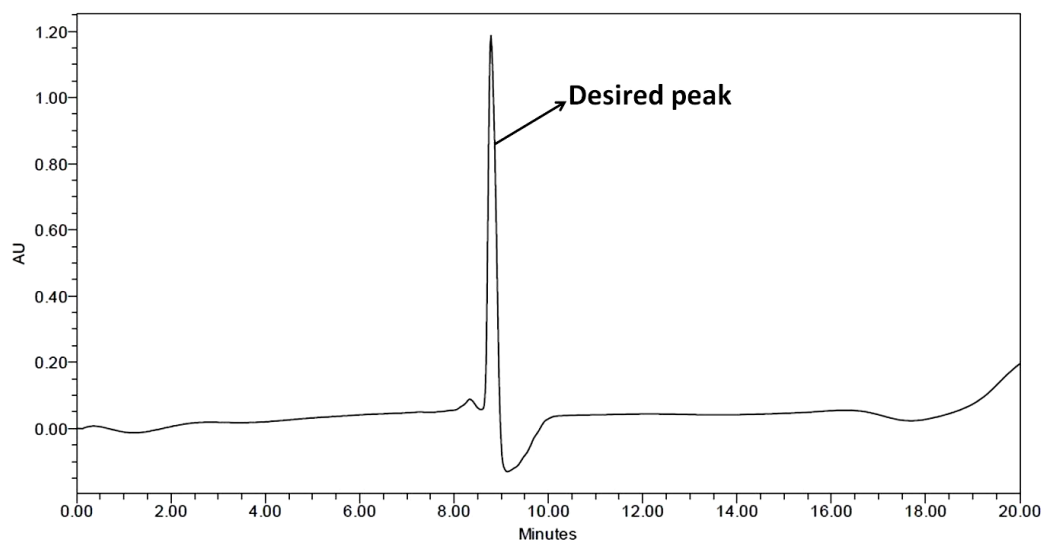
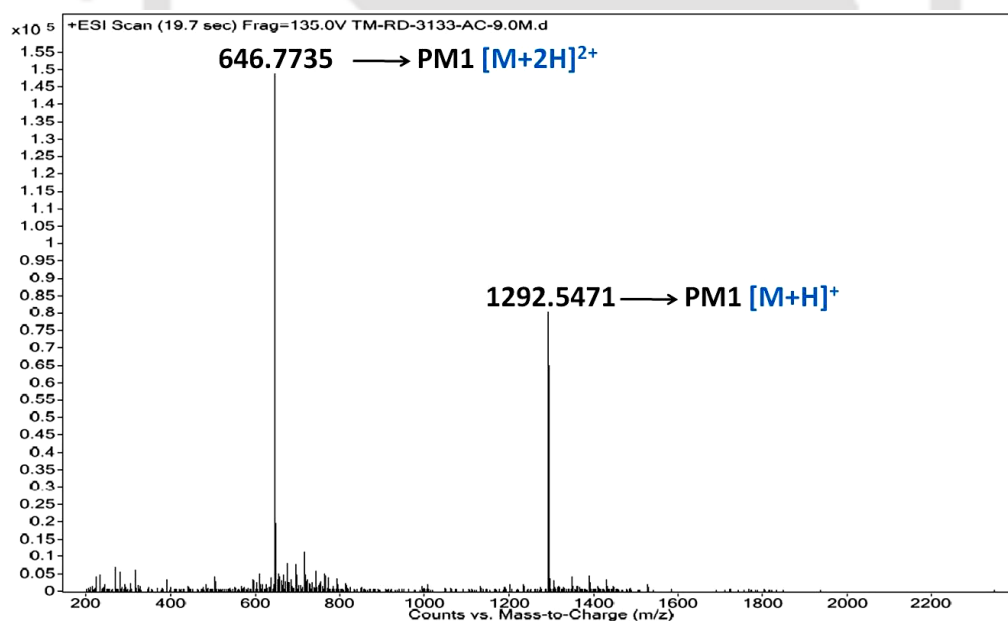
4.18. Supporting data:**Figure 4.39. HPLC profile picture of the PM1.**

Figure 4.40. ESI-MS profile picture of purified PM1. Calculated m/z for $C_{56}H_{77}N_{17}O_{17}S$ $[M+H]^+$ is 1292.5404, observed 1292.5471; Calculated m/z for $C_{56}H_{77}N_{17}O_{17}S$ $[M+2H]^{2+}$ is 646.7702, observed 646.7735.

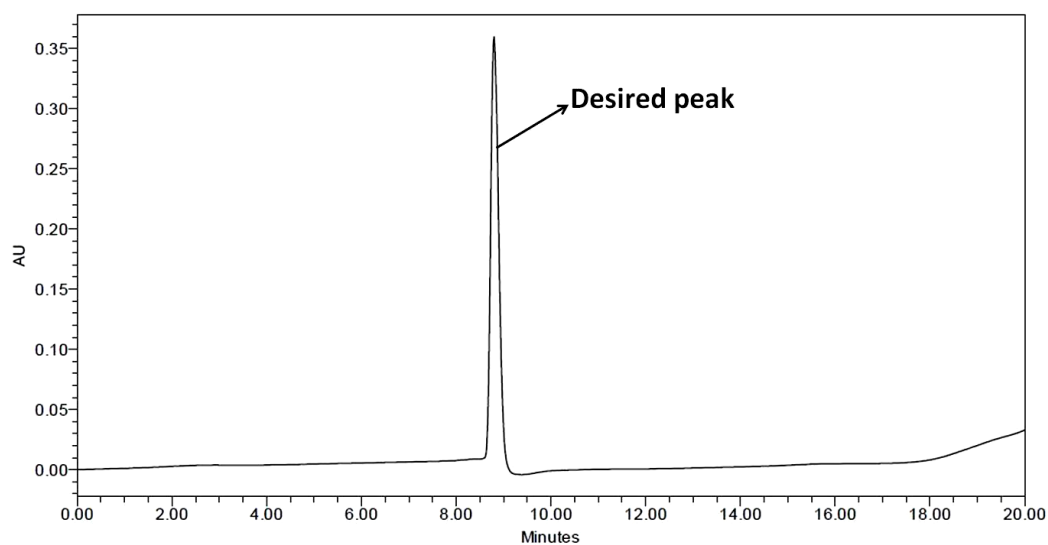


Figure 4.41. HPLC profile picture of the PM2.

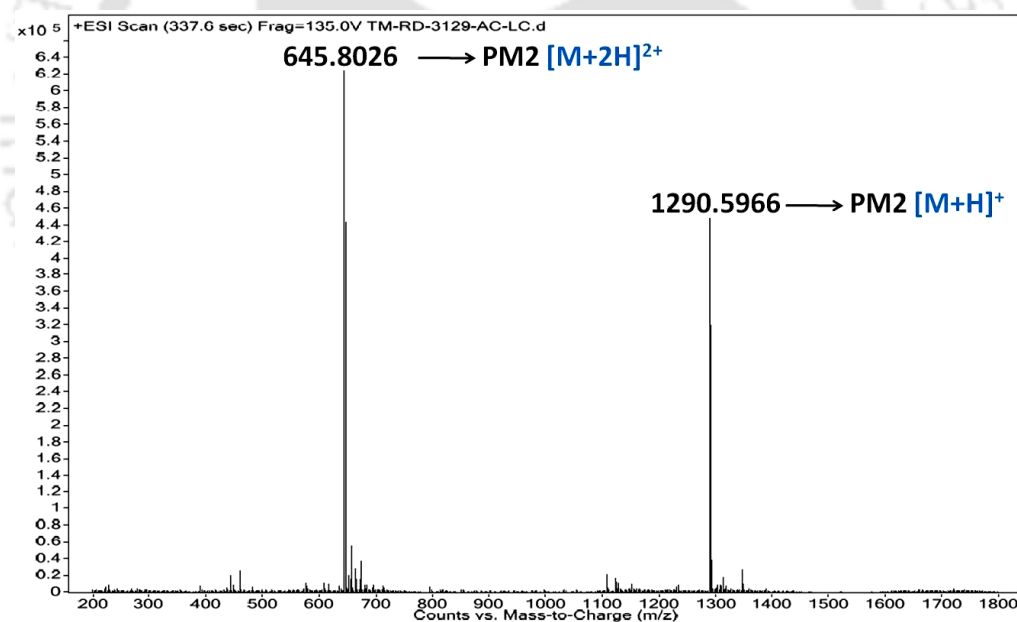


Figure 4.42. ESI-MS profile picture of purified PM2. Calculated m/z for $C_{57}H_{79}N_{17}O_{18}$ $[M+H]^+$ is 1290.5789, observed 1290.5966; Calculated m/z for $C_{56}H_{77}N_{17}O_{17}S$ $[M+2H]^{2+}$ is 645.7894, observed 645.8026.

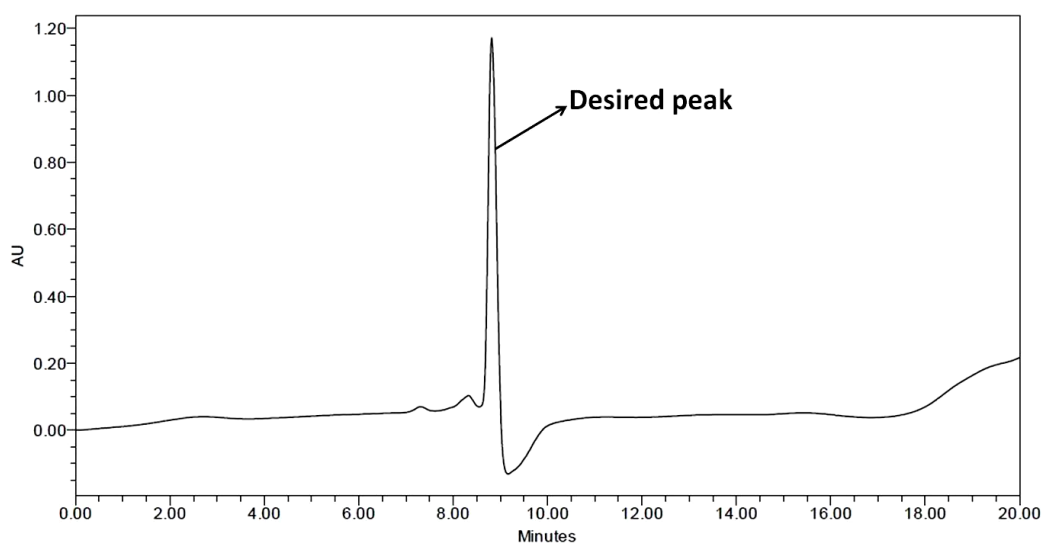


Figure 4.43. HPLC profile picture of the PM3.

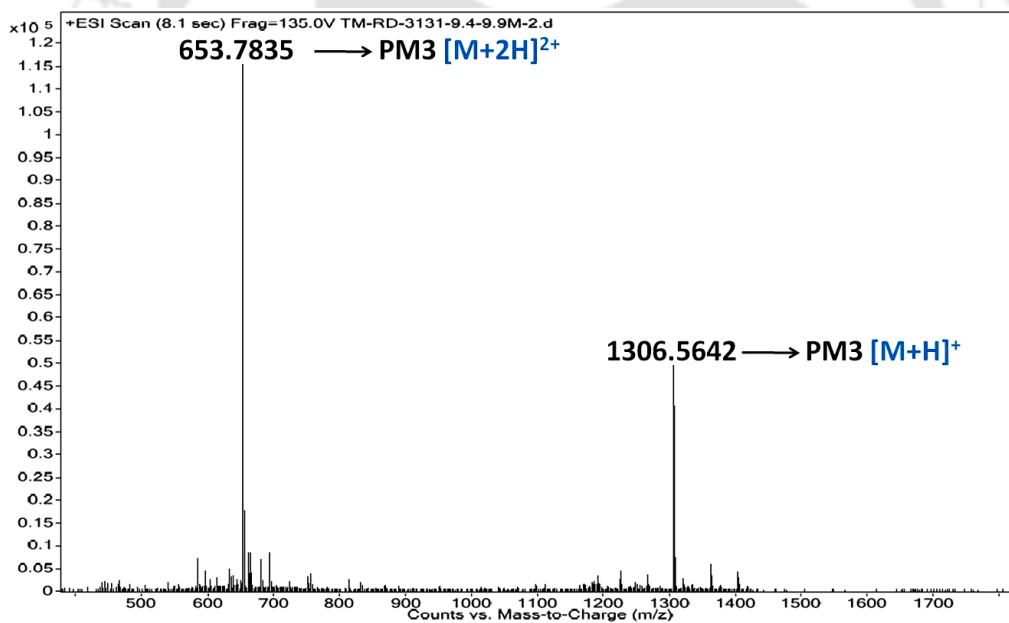


Figure 4.44. ESI-MS profile picture of purified PM3. Calculated m/z for $C_{57}H_{79}N_{17}O_{17}S$ $[M+H]^+$ is 1306.5561, observed 1306.5642; Calculated m/z for $C_{56}H_{77}N_{17}O_{17}S$ $[M+2H]^{2+}$ is 653.7780, observed 653.7835.

Chapter 5: Design and development of a taurine containing peptide conjugate and its application in aggregation inhibition

5.1. Introduction:

Alzheimer's disease (AD) is the most common form of neurodegenerative disease, and the extracellular deposition of A β in the form of senile plaques is considered to play a crucial role in AD.¹ According to the amyloid cascade hypothesis, some transition-metal ions, mainly Cu²⁺, Zn²⁺, and Fe³⁺ can bind to A β and influence to aggregate which finally accumulated in amyloid plaques in AD brain, contributing to metal homeostasis dysregulation.^{2,3,4} Such metal-bound A β complexes cause oxidative stress and induce severe toxicity via the production of reactive oxygen species (ROS). The senile plaques formed by A β contain a comparatively higher concentration of redox-active metal ions like Cu²⁺, Zn²⁺, *etc.*, promoting oxidative damage and directly involved in the physiological toxicity to the adjacent neurons.⁵ A β peptides coordinate with Cu²⁺, and Zn²⁺ ions mostly via its first 16 amino acid residues to form the corresponding metal-bound A β complex.⁶ It is known that Zn²⁺ and Cu²⁺ bind to three His residues (His₁₃, His₁₄, and His₆) of A β . The fourth ligand may be aspartate at position 1; otherwise it may be either the N-terminus or carboxylate side chain. Hence, metal chelation therapy should be considered as one of the valuable strategies for the treatment against AD. Peptides are considered as the simplest biological recognition elements for metal binding.⁷ Currently, peptides attracted much attention to the development of drugs due to their good biocompatibility.

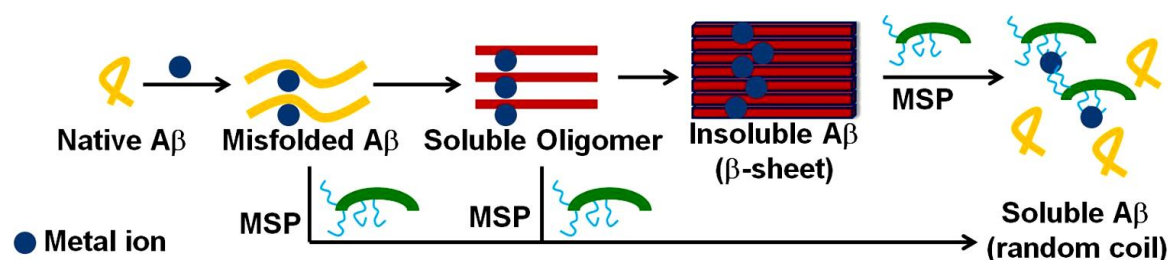


Figure 5.1. Concept of the inhibition of aggregation by metal abstraction.

5.2. Design of the peptide:

Deprotonation of the sulphonic acid is energetically more favorable than that of a similar carboxyl group, and the resulting sulphonate group acts as a vital ligand (especially for Cu) in proteins.⁸ From the results of a comparison of chelating agents like DMPS (2, 3-dimercapto-1-propanesulfonic acid), EDTA (ethylene diamine tetraacetic acid), and DMSA (dimercaptosuccinic acid), it was found that DMPS has the strongest Cu binding affinity, and also good binding affinity towards Zn ion due to the presence of sulphonic acid.⁹ As, the sulfonate group can coordinate to metal ion strongly, in this work we have used Taurine (2-amino-ethanesulphonic acid) by attaching its amine group with the side-chain carboxyl group of Glutamic acid. Metal scavenger peptide (MSP) was designed (Table 5.1) by connecting the repeated sequence of -E(Taurine)-G- (Glu-side-chain modified taurine containing staff separated by Gly) to the self-recognition motif VFFA, for the site-specificity concerning Aβ. Gly was incorporated for the flexibility purpose so that the sulfonate group of Taurine units can come close together for their metal-chelating affinity. [E(Taurine)-G]₄ part of MSP is used as the active site to chelate metal-ion. It was reported that Cu²⁺, Zn²⁺ ions form salt bridge predominantly through ¹³His-Metal-¹⁴His conformation as well as bridges with ⁶His of Aβ.¹⁰ MSP was designed in such a way that the sulfonate groups lie around His₁₃-His₁₄ of Aβ, as [E(Taurine)-G]₄ part was attached with the recognition domain 18-21 of Aβ.

Table 5.1. Sequences of the peptides and their roles in the current study.

Sl. No.	Name of the peptide (short name)	Peptide sequence	Role of the peptide
1	Metal Scavenger Peptide (MSP)	Ac-G-[E(Taurine)-G] ₄ -VFFAG-NH ₂	Metal-ion chelator
2	Aβ ₁₋₄₀ peptide	DAEFRHDSGYEVHHQKLVFFAEDVG SNKGAIIGLMVGGVV-NH ₂	Aggregating peptide

5.3. The most stable conformation of MSP:

The most stable conformation of MSP was achieved (Figure 5.2) after calculating DFT based methods using B3LYP as energy functional and 6-31G as a basis set through Gaussian 5.0.9 program. In the case of MSP, it was found that three sulfonate groups out of four lie close to each other for which it could be possible to chelate metal ions from the metal-A β complex.

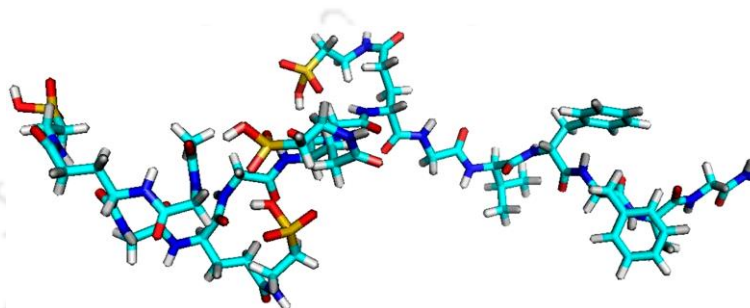


Figure 5.2. The most stable conformation of MSP [image generated by PyMOL (Line representation)] obtained from DFT calculation [Calculation Method: B3LYP, Basis Set: 6-31G, Total Energy - 7615.98581693 a.u.].

5.4. Molecular docking study:

It was observed that (Figure 5.3), MSP bind well in the fibril A β ₁₋₄₀ (PDB ID: 2M4J) binding pocket having a binding affinity -4.9 kcal/mol. We observed that interacting amino acids in the A β ₁₋₄₀ binding pocket are the hydrophobic region, *i.e.*, Val18, Phe19, Phe20, and Ala21. It was also noted that two sulfonate groups of MSP lie around His₁₃ and His₁₄ of A β ₁₋₄₀. MSP also interact well with the Zn induced dimer of the metal-binding domain 1-16 of human A β ₁₋₄₀ (PDB ID 2MGT) having a binding affinity -4.4 kcal/mol. In this case also two sulfonate groups of MSP lie very close to His₁₃-His₁₄ and Zn²⁺ ion.

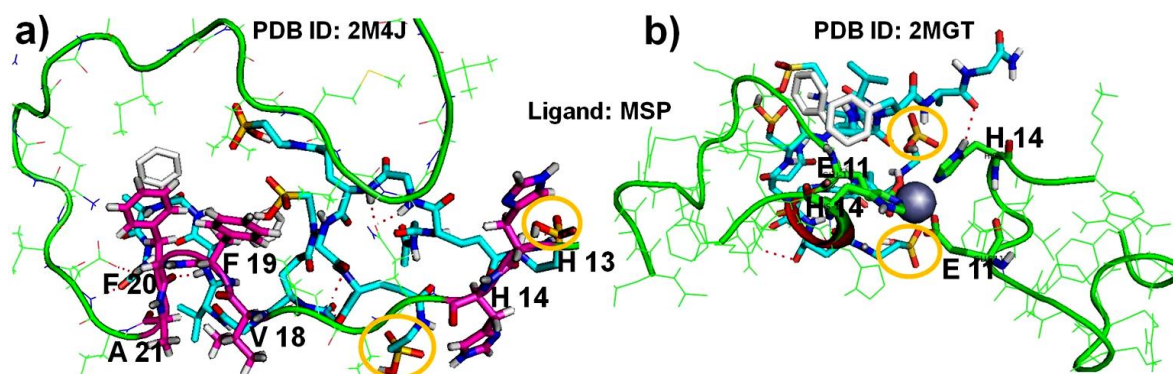


Figure 5.3. Molecular docking images of MSP into (a) fibril Aβ₁₋₄₀ (PDB ID 2M4J) reveals that MSP bind at the binding site (V₁₈FFA₂₁) with binding affinity -4.9 kcal/mol. Molecular docking images of MSP into (b) Zn induced dimer of the metal-binding domain 1-16 of human Aβ₁₋₄₀ (PDB ID 2MGT) reveals that MSP bind with binding affinity -4.4 kcal/mol. Structures are shown as line and cartoon representation.

5.5. Metal ion chelation effect of MSP by Isothermal Titration Calorimetry (ITC) titration:

ITC experiments can be used preferably to study the binding between a ligand and a receptor. From this experiment, three parameters can be obtained directly, and the parameters are the binding constant (K), enthalpy change (ΔH), and binding stoichiometry (n) of the interaction in solution. In this work, ITC measurements were carried out to study the chelating thermodynamics of MSP to redox-active metal ions (Fe^{3+} , Cu^{2+} , and Zn^{2+}) under specific conditions.¹¹

ITC titrations were conducted on a MicroCal iTC200 (GE Healthcare) at 37 °C. Measurements were carried out in 50 mM PBS buffer of pH = 7.4. All the solutions were degassed for around 15 min to remove dissolved gases before titration. Aβ₁₋₄₀ peptide or MSP was loaded in the cell, and metal chloride salts were placed in the syringe. For Aβ, stirring speed was kept at 250 rpm, the pre-titration delay was 150 seconds, an initial volume of 0.4 μL and others of 2 μL were adopted (for MSP, the values are 300 rpm, 150 seconds, 0.4 μL, and 1.3 μL respectively). The ITC data were corrected for the heat of dilution of the titrant and subtracted by injecting the metal ion solution into the sample

buffer. Finally, the sequential binding sites model was selected to analyze data by Origin 7.0 software.

ITC titration of Fe^{3+} added separately to 0.025 mM $\text{A}\beta_{1-40}$ and 0.1 mM MSP in 50 mM PBS at pH 7.4 are shown in Figure 5.4. The binding process was exothermic. From the measurement, the binding constant of MSP ($K: 1.34\text{E}5 \pm 3.8\text{E}4 \text{ M}^{-1}$) to Fe^{3+} ion was found to be higher than that of $\text{A}\beta_{1-40}$ ($K: 1.25\text{E}4 \pm 7.5\text{E}3 \text{ M}^{-1}$). Thus it can be concluded that MSP can bind and extract Fe^{3+} ion from the Fe^{3+} - $\text{A}\beta_{1-40}$ complex.

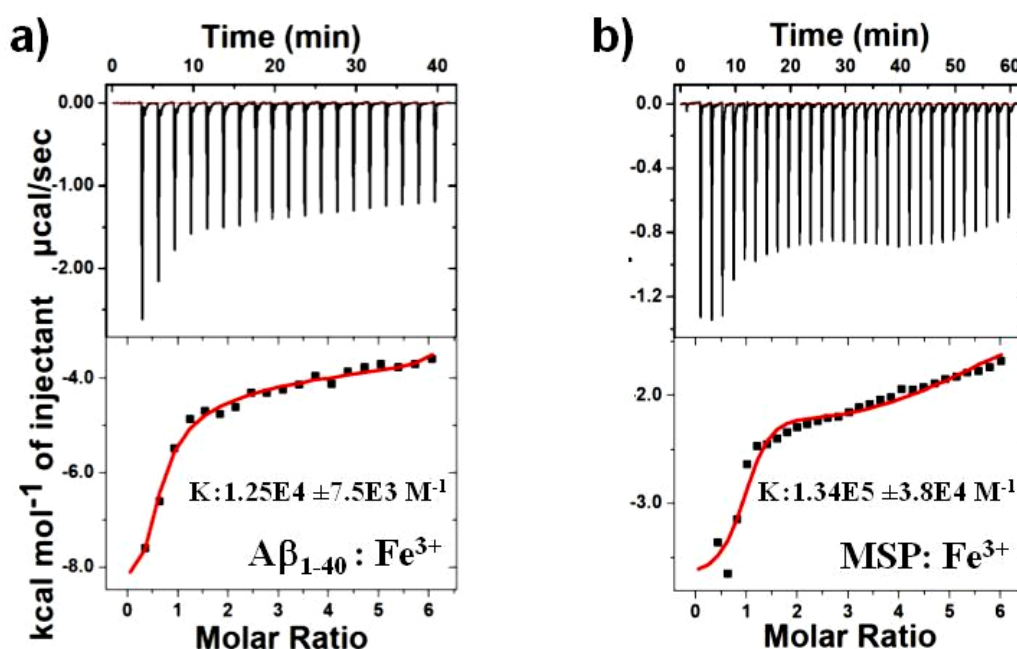


Figure 5.4. ITC of Fe^{3+} titration to (a) $\text{A}\beta_{1-40}$ and (b) MSP in 50 mM PBS (pH = 7.4) at 37 °C. For (a), $[\text{Fe}^{3+}] = 0.75 \text{ mM}$, $[\text{A}\beta_{1-40}] = 0.025 \text{ mM}$. For (b), $[\text{Fe}^{3+}] = 3 \text{ mM}$, $[\text{MSP}] = 0.1 \text{ mM}$. Top panels indicate the power signal experienced for each injection. Bottom panels indicate integrated peak areas corresponding to the measured heat released for each injection. The red lines show least-squares fits according to the sequential binding sites model.

In Figure 5.5, ITC titration of Cu^{2+} added separately to 0.025 mM $\text{A}\beta_{1-40}$ and 0.1 mM MSP in 50 mM PBS at pH 7.4 are shown. The binding process was found to be exothermic as a whole. From the measurement, it was found that the binding constant of MSP ($K: 9.93\text{E}4 \pm 8.6\text{E}4 \text{ M}^{-1}$) to Cu^{2+} ion was higher marginally than that of $\text{A}\beta_{1-40}$ ($K: 9.25\text{E}4 \pm 6.4\text{E}4 \text{ M}^{-1}$). According to Irving-Williams series, when the major coordination sites are N atoms, Cu(II) complexes are the most stable complexes among other divalent

first-row transition metal ions.¹² In this particular case, three His residue of A β is the competitor against sulphonate of MSP. Hence from the marginally higher value of binding constant, it can be said that MSP could be able to bind and extract Cu²⁺ ion from the Cu²⁺-A β ₁₋₄₀ complex.

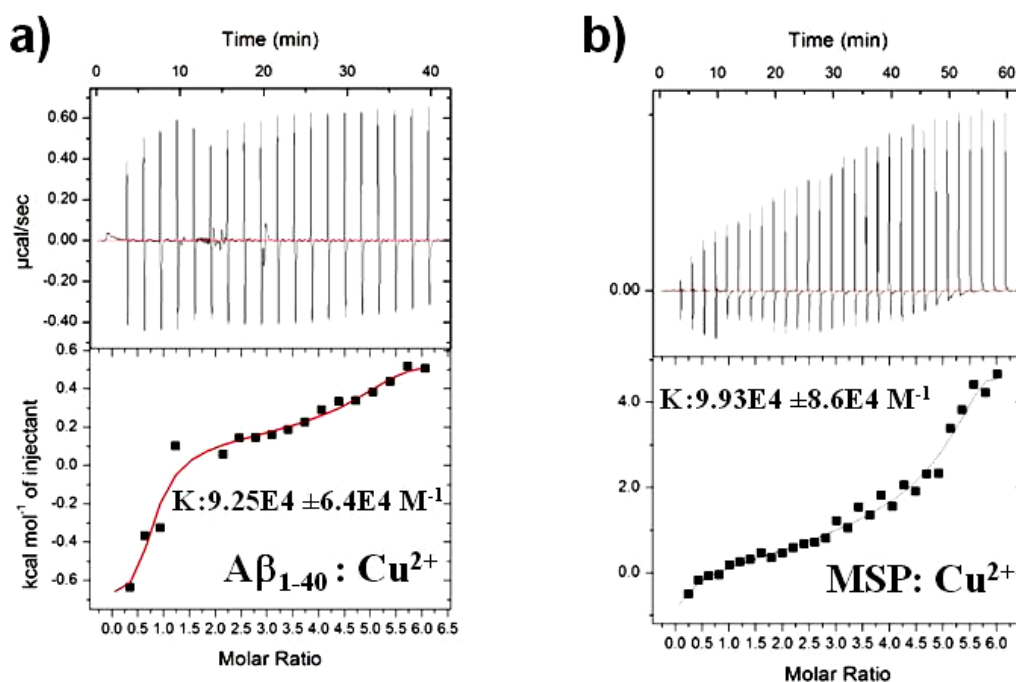


Figure 5.5. ITC of Cu²⁺ titration to (a) A β ₁₋₄₀ and (b) MSP in 50 mM PBS (pH = 7.4) at 37 °C. For (a), [Cu²⁺] = 0.75 mM, [A β ₁₋₄₀] = 0.025 mM. For (b), [Cu²⁺] = 3 mM, [MSP] = 0.1 mM. Top panels indicate the power signal experienced for each injection. Bottom panels indicate integrated peak areas corresponding to the measured heat released for each injection. The lines show least-squares fits according to the sequential binding sites model.

In Figure 5.6, ITC titration of Zn²⁺ added separately to 0.025 mM A β ₁₋₄₀ and 0.1 mM MSP in 50 mM PBS at pH 7.4 are shown. The overall binding process was exothermic. From the measurement, it was found that the binding constant of MSP (K: 1.71E5 ± 4.9E4 M⁻¹) to Zn²⁺ ion was higher than that of A β ₁₋₄₀ (K: 3.87E4 ± 4.73E3 M⁻¹). Thus it can be concluded that MSP can bind and extract Zn²⁺ ion from the Zn²⁺-A β ₁₋₄₀ complex.

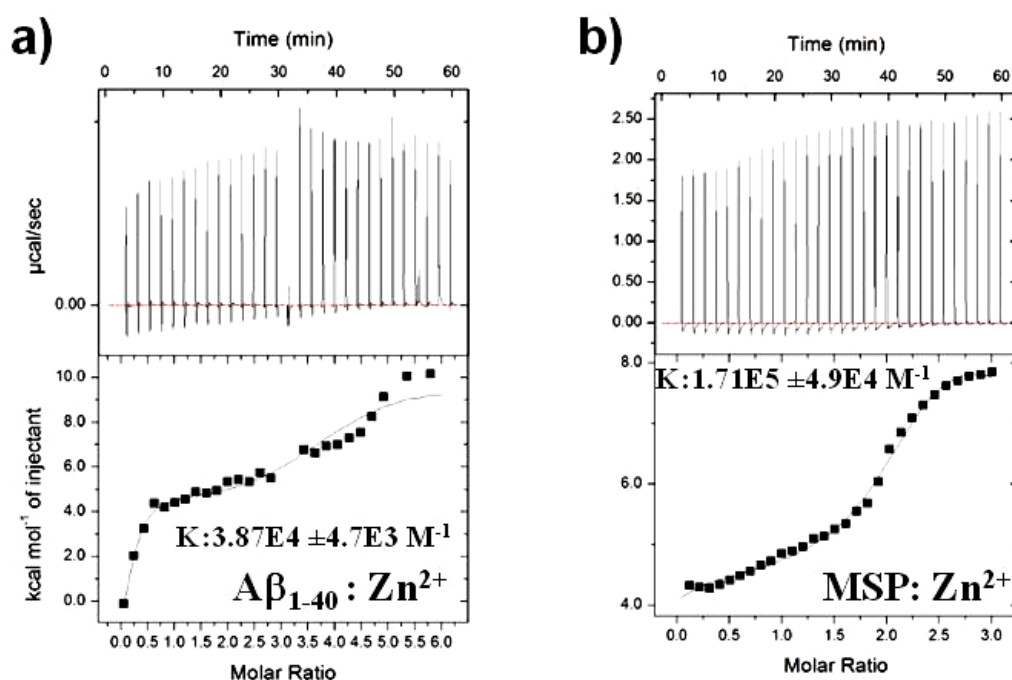


Figure 5.6. ITC of Zn^{2+} titration to (a) $\text{A}\beta_{1-40}$ and (b) MSP in 50 mM PBS (pH = 7.4) at 37 °C. For (a), $[\text{Zn}^{2+}] = 0.75 \text{ mM}$, $[\text{A}\beta_{1-40}] = 0.025 \text{ mM}$. For (b), $[\text{Zn}^{2+}] = 1.5 \text{ mM}$, $[\text{MSP}] = 0.1 \text{ mM}$. Top panels indicate the power signal tested for each injection. Bottom panels indicate integrated peak areas corresponding to the measured heat released for each injection. The lines show least-squares fits according to the sequential binding sites model.

5.6. Inhibition of amyloid accumulation of $\text{A}\beta_{1-40}$ by MSP:

After getting the higher binding constant of MSP against $\text{A}\beta_{1-40}$ towards redox-active metal ions, we continued further to check the inhibition potential of MSP. The kinetics of the amyloid accumulation of $\text{A}\beta_{1-40}$ was tested using different biophysical tools in the absence and presence of MSP in a dose-dependent manner. Samples were prepared as mentioned in the experimental method chapter to obtain the stock solutions of $\text{A}\beta_{1-40}$ (50 μM) and MSP (0.5 mM). MSP was co-incubated with $\text{A}\beta_{1-40}$ and metal- $\text{A}\beta_{1-40}$ (1:1) complex in PBS of pH 7.4 at 37 °C in separate vessels on a water bath up to seven days in parallel at the desired proportions. To investigate the dose-dependent inhibitory efficacy of MSP, two-fold, and a ten-fold molar excess of MSP was co-incubated with 50 μM of metal- $\text{A}\beta_{1-40}$ complex *in vitro*. To get the result more precisely another two replicate solutions of each portion were prepared and amyloid accumulation was monitored by different biophysical tools for up to seven days.

5.6.1. Inhibition of Fe^{3+} -bound $\text{A}\beta$ (Fe^{3+} - $\text{A}\beta$) by MSP:

i) ThT fluorescence assay of $\text{A}\beta_{1-40}$:

Dose-dependent ThT fluorescence assay of Fe^{3+} - $\text{A}\beta_{1-40}$ complex was performed with MSP, along with metal-free $\text{A}\beta_{1-40}$ separately. From the result (Figure 5.7), it was found that the fluorescence intensity of $\text{A}\beta_{1-40}$ (black) incubated alone increased with time and became static after four days after slight lowering in intensity. For Fe^{3+} - $\text{A}\beta_{1-40}$ complex (red), the fluorescence intensity was increased rapidly after one day than that of $\text{A}\beta_{1-40}$. But in the presence of MSP, the fluorescence intensity of the Fe^{3+} - $\text{A}\beta_{1-40}$ increased initially due to probable co-aggregation, but decreased with time as the doses were increased from two-fold (magenta) to ten-fold (dark yellow) molar excess due to the metal chelation ability of MSP, indicating the inhibitory efficacy of MSP against amyloid formation of the Fe^{3+} - $\text{A}\beta_{1-40}$ complex and metal-free $\text{A}\beta_{1-40}$ as well. It was noticed that the fluorescence intensity of MSP (blue) alone is not increased with time showing its non-aggregating property.

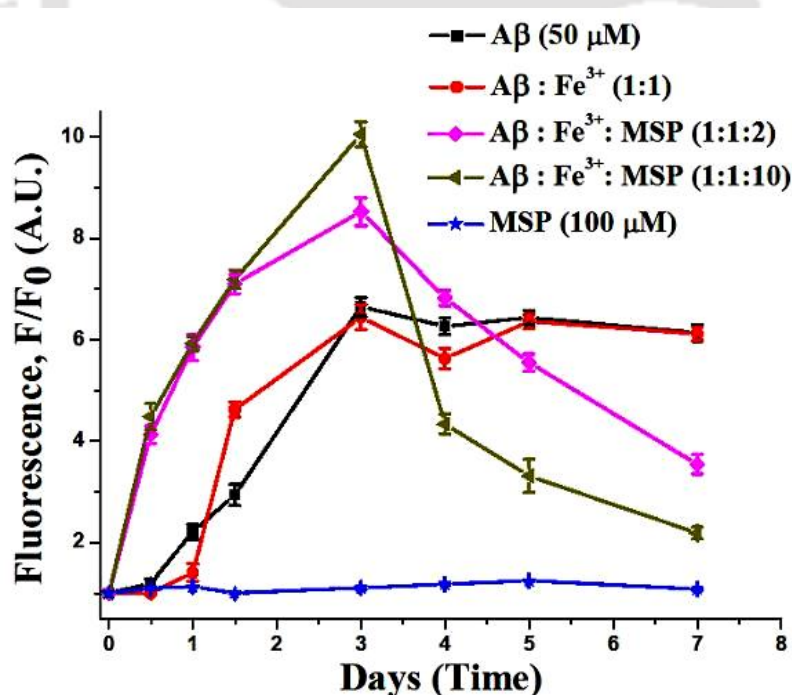


Figure 5.7. Normalized profiles of dose-dependent ThT fluorescence assay. Spectra of metal-free $\text{A}\beta_{1-40}$ alone (black), Fe^{3+} - $\text{A}\beta_{1-40}$ (1:1) complex in the absence (red), presence of two-fold MSP (magenta), ten-fold MSP (dark yellow), and MSP alone (blue). Error bars represent standard deviations of at

least three independent measurements. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

ii) CD experiments of A β ₁₋₄₀:

In Figure 5.8, the β -sheet structure of A β ₁₋₄₀ alone (black) was confirmed from a positive band at ~200 nm with a negative band at ~220 nm. The increased intensity of the negative band at ~220 nm was noticed for the Fe³⁺-A β ₁₋₄₀ complex (red) confirms the β -sheet structure. In the presence of two-fold MSP (magenta) and ten-fold MSP (dark yellow), the negative band at ~220 nm shifted to 228 nm and 235 nm respectively with decreased intensity, confirms the non- β -sheet structure of Fe³⁺-A β ₁₋₄₀. MSP (blue) itself displayed the non- β -sheet structure which was confirmed from a positive band at ~215 nm with a negative band at ~235 nm.

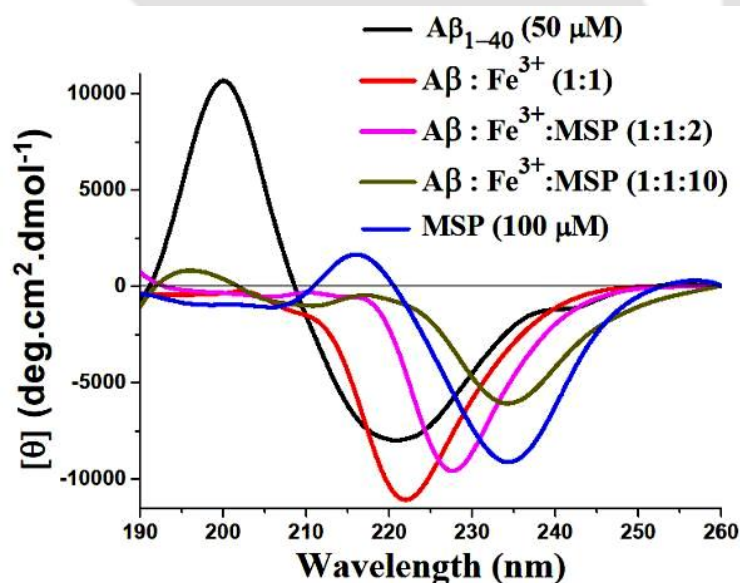


Figure 5.8. CD spectra of A β ₁₋₄₀ in the absence and presence of MSP after seven days of incubation. Spectra of metal-free A β ₁₋₄₀ alone (black), Fe³⁺-A β ₁₋₄₀ (1:1) complex in the absence (red), presence of two-fold MSP (magenta), ten-fold MSP (dark yellow), and MSP alone (blue). All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

iii) TEM images, and Congo red-stained birefringence images of amyloid fibrils formed by A β ₁₋₄₀:

After addition of inhibitor or disruptor, TEM images of the metal-free and metal-bound A β ₁₋₄₀ aggregates was found to be smaller and amorphous aggregates and considered to be less toxic.¹³ The metal-free A β ₁₋₄₀ and Fe³⁺-A β ₁₋₄₀ formed clear fibrillar aggregates (Figure 5.9. a-i,ii) under TEM, but in the presence of two-fold MSP (Figure 5.9. a-iii) and ten-fold MSP (Figure 5.9. a-iv), no such fiber was observed. MSP (Figure 5.9. a-v) alone also did not show any fiber. Therefore it can be concluded MSP can inhibit aggregation of Fe³⁺-A β ₁₋₄₀.

The bright green-gold birefringence was observed for metal-free A β ₁₋₄₀ and Fe³⁺-A β ₁₋₄₀ under cross-polarized light (Figure 5.9. b-i,ii), but when A β ₁₋₄₀ was incubated in the presence of two-fold MSP (Figure 5.9. b-iii) and ten-fold MSP (Figure 5.9. b-iv) separately, no such green gold birefringence were observed. MSP (Figure 5.9. b-v) alone also did not show any birefringence. These results support that MSP significantly inhibited amyloid aggregation of Fe³⁺-A β ₁₋₄₀.

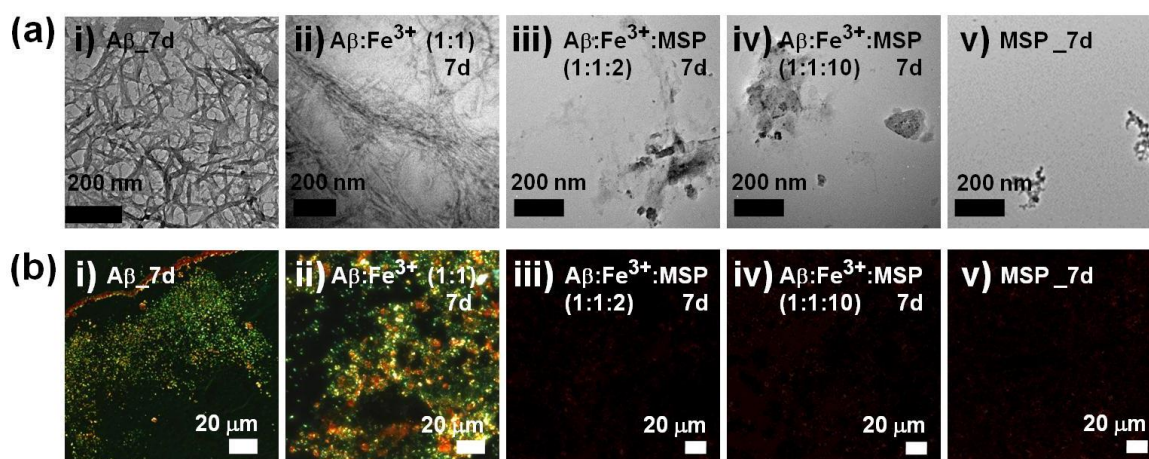


Figure 5.9. (a) TEM images, and (b) Congo red-stained birefringence images of (i) metal-free A β ₁₋₄₀, (ii) Fe³⁺-A β ₁₋₄₀; Fe³⁺-A β ₁₋₄₀ in the presence of (iii) two-fold MSP, (iv) ten-fold MSP, and (v) MSP alone. Scale bars are indicated as 200 nm for TEM and 20 μ m for birefringence images. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel. All the images were captured after seven days of incubation.

5.6.2. Inhibition of Cu^{2+} -bound $\text{A}\beta$ (Cu^{2+} - $\text{A}\beta$) by MSP:

i) ThT fluorescence assay of $\text{A}\beta_{1-40}$:

Dose-dependent ThT fluorescence assay of Cu^{2+} - $\text{A}\beta_{1-40}$ complex was performed with MSP, along with metal-free $\text{A}\beta_{1-40}$ separately. From the result (Figure 5.10), it was observed that the fluorescence intensity of Cu^{2+} - $\text{A}\beta_{1-40}$ complex (red) was increased more rapidly from the first hour than that of $\text{A}\beta_{1-40}$ and became static after three days after lowering in intensity as there is a probability for mature fibril to come out from the solution. However, in the presence of MSP, the fluorescence intensity of the Cu^{2+} - $\text{A}\beta_{1-40}$ decreased with time from the starting hour and found to be dose-independent (two-fold (magenta), and ten-fold (dark yellow) molar excess of MSP), indicating the excellent inhibitory efficacy of MSP against amyloid formation of the Cu^{2+} - $\text{A}\beta_{1-40}$ complex and metal-free $\text{A}\beta_{1-40}$ also.

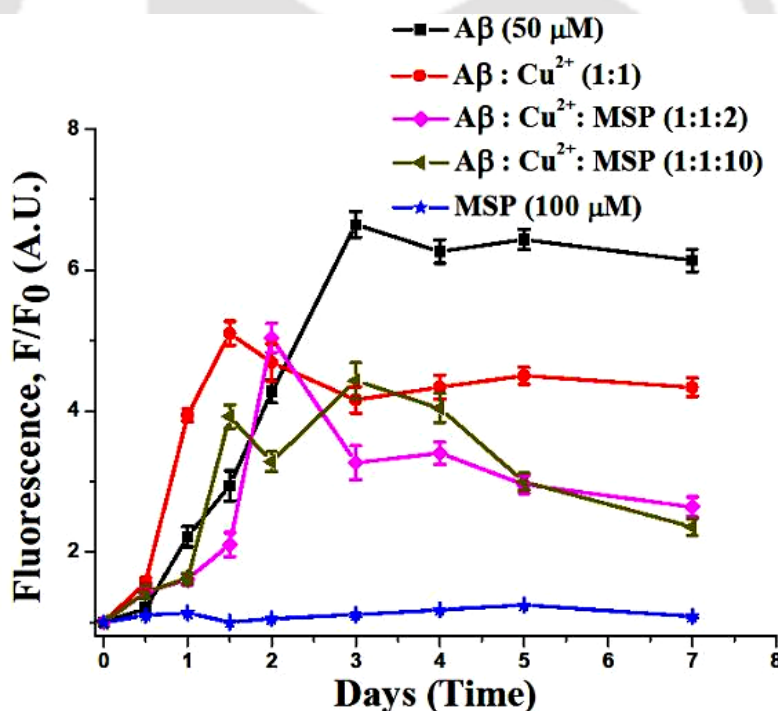


Figure 5.10. Normalized profiles of dose-dependent ThT fluorescence assay. Spectra of metal-free $\text{A}\beta_{1-40}$ alone (black), Cu^{2+} - $\text{A}\beta_{1-40}$ (1:1) complex in the absence (red), presence of two-fold MSP (magenta), ten-fold MSP (dark yellow), and MSP alone (blue). Error bars represent standard deviations of at least three independent measurements. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

ii) CD experiments of A β ₁₋₄₀:

Already it was demonstrated that A β ₁₋₄₀ (black) fibrils adopt a β -sheet conformation. Cu²⁺-A β ₁₋₄₀ complex (red, Figure 5.11) also exhibited the β -sheet structure and confirmed from the intense negative band at $\sim$ 220 nm. In the presence of two-fold MSP (magenta, Figure 5.11) and ten-fold MSP (dark yellow, Figure 5.11), the negative band at $\sim$ 220 nm shifted to 230 nm and 235 nm respectively with lowering in intensity, confirms the non- β -sheet structure of Cu²⁺-A β ₁₋₄₀. MSP (blue) exhibited the non- β -sheet structure as described earlier.

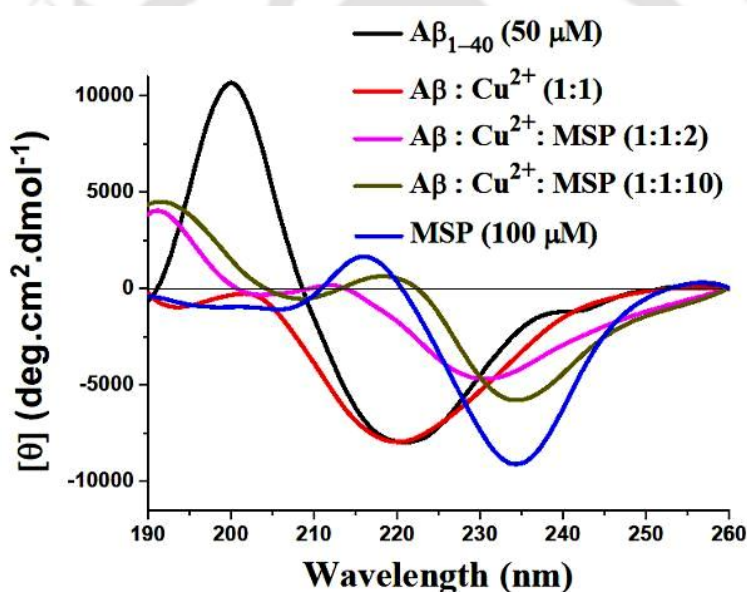


Figure 5.11. CD spectra of metal-free A β ₁₋₄₀, Cu²⁺-A β ₁₋₄₀ in the absence and presence of MSP after seven days of incubation. Spectra of metal-free A β ₁₋₄₀ alone (black), Cu²⁺-A β ₁₋₄₀ (1:1) complex in the absence (red), presence of two-fold MSP (magenta), ten-fold MSP (dark yellow), and MSP alone (blue). All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

iii) TEM images and Congo red-stained birefringence images of amyloid fibrils formed by A β ₁₋₄₀:

Cu²⁺-A β ₁₋₄₀ formed clear fibrillar aggregates (Figure 5.12. a-i) under TEM, but in the presence of two-fold MSP (Figure 5.12. a-ii) and ten-fold MSP (Figure 5.12. a-iii), no

such fiber was noticed, somewhat amorphous structures were noticed. Therefore it can be concluded MSP can inhibit aggregation of Cu^{2+} - $\text{A}\beta_{1-40}$.

The bright green-gold birefringence was observed for Cu^{2+} - $\text{A}\beta_{1-40}$ under cross-polarized light (Figure 5.12. b-i), but when $\text{A}\beta_{1-40}$ was incubated in the presence of two-fold MSP (Figure 5.12. b-ii) and ten-fold MSP (Figure 5.12. b-iii) separately, no such green gold birefringence were noticed. These results support that MSP significantly inhibited amyloid aggregation of Cu^{2+} - $\text{A}\beta_{1-40}$.

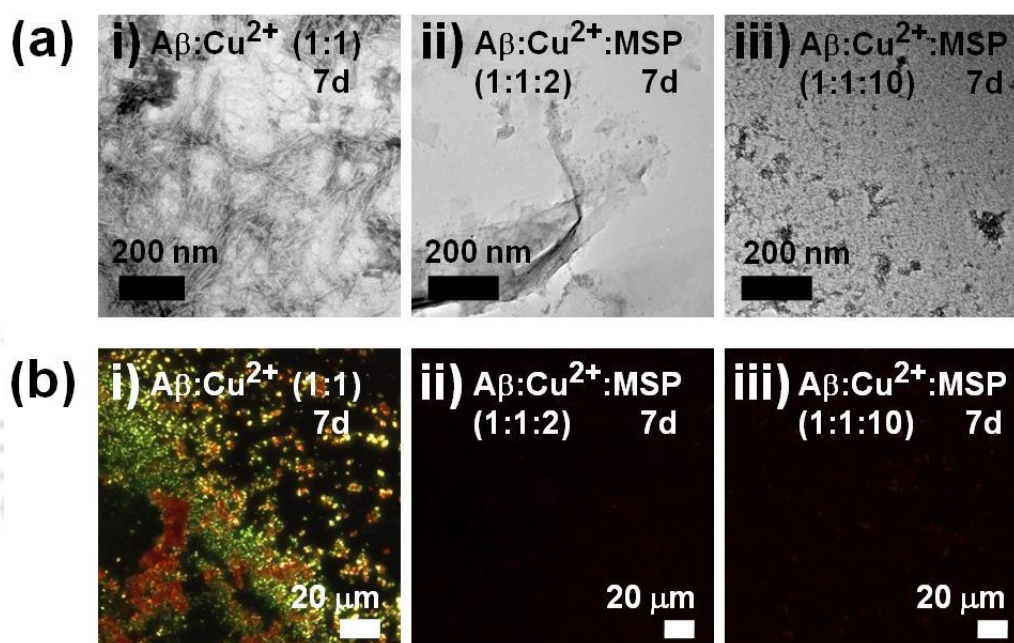


Figure 5.12. (a) TEM images, and (b) Congo red-stained birefringence images of Cu^{2+} - $\text{A}\beta_{1-40}$ (i) in the absence, presence of (ii) two-fold MSP, and (iii) ten-fold MSP. Scale bars are indicated as 200 nm for TEM and 20 μm for birefringence images. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel. All the images were captured after seven days of incubation.

5.6.2. Inhibition of Zn^{2+} -bound $\text{A}\beta$ (Zn^{2+} - $\text{A}\beta$) by MSP:

i) ThT fluorescence assay of $\text{A}\beta_{1-40}$:

Dose-dependent ThT fluorescence assay of Zn^{2+} - $\text{A}\beta_{1-40}$ complex was performed with MSP, along with metal-free $\text{A}\beta_{1-40}$ separately. From the result (Figure 5.13), it was observed that the fluorescence intensity of Zn^{2+} - $\text{A}\beta_{1-40}$ complex (red) was increased but lower than that of $\text{A}\beta_{1-40}$ (black) and decreased after four days as there is a probability for

mature fibril to come out from the solution. This is because Zn binding to A β induces rapid aggregation to form Zn-A β aggregates, for comparatively higher Zn-binding affinity towards A β .¹⁴ In the presence of two-fold (magenta) molar MSP, the fluorescence intensity was found to be higher than Zn²⁺-A β ₁₋₄₀, but lower than the intensity of metal-free A β ₁₋₄₀. But in the presence of ten-fold (dark yellow) molar excess of MSP, the fluorescence intensity of the Zn²⁺-A β ₁₋₄₀ was found to be lower than that of Zn²⁺-A β ₁₋₄₀ and metal-free A β ₁₋₄₀ as well, indicating the excellent inhibitory efficacy of MSP against amyloid formation of the Zn²⁺-A β ₁₋₄₀ complex and metal-free A β ₁₋₄₀ also.

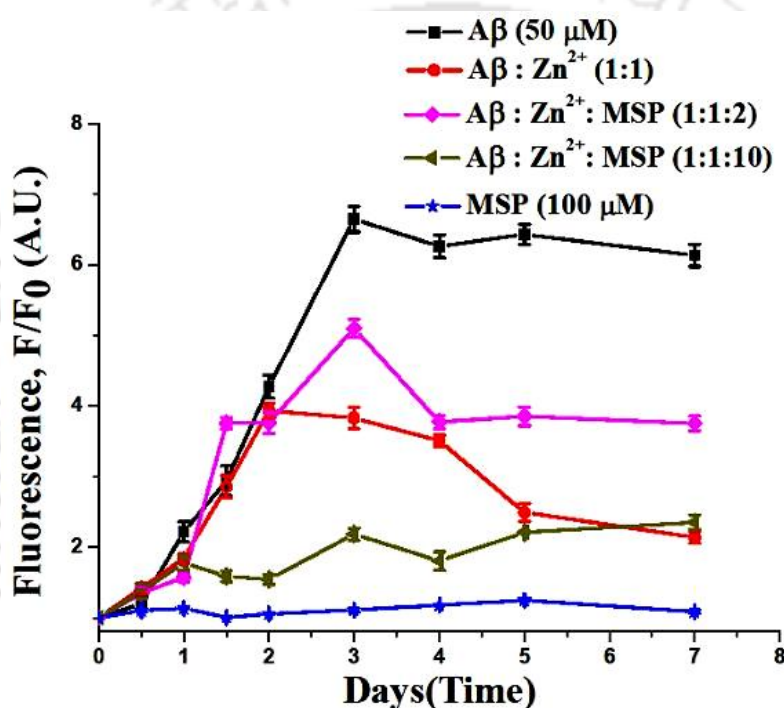


Figure 5.13. Normalized profiles of dose-dependent ThT fluorescence assay. Spectra of metal-free A β ₁₋₄₀ alone (black), Zn²⁺-A β ₁₋₄₀ (1:1) complex in the absence (red), presence of two-fold MSP (magenta), ten-fold MSP (dark yellow), and MSP alone (blue). Error bars represent standard deviations of at least three independent measurements. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

ii) CD experiments of A β ₁₋₄₀:

Already it was demonstrated that A β ₁₋₄₀ (black) fibrils adopt a β -sheet conformation. Zn²⁺-A β ₁₋₄₀ complex (red, Figure 5.14) also displayed the β -sheet structure and

confirmed from the strong negative band at ~ 220 nm. But when $\text{Zn}^{2+}\text{-A}\beta_{1-40}$ was co-incubated along with two-fold MSP (magenta, Figure 5.14) and ten-fold MSP (dark yellow, Figure 5.14), the negative band at ~ 220 nm shifted to 230 nm and 237 nm respectively with lowering in intensity, confirms the tendency of $\text{Zn}^{2+}\text{-A}\beta_{1-40}$ to form non- β -sheet structure. MSP (blue) itself displayed the non- β -sheet structure, as explained earlier.

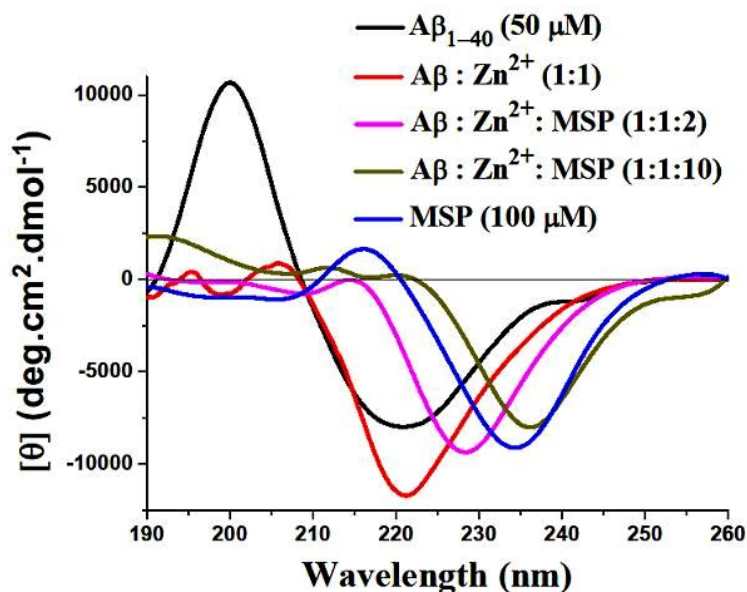


Figure 5.14. CD spectra of metal-free $\text{A}\beta_{1-40}$, $\text{Zn}^{2+}\text{-A}\beta_{1-40}$ in the absence and presence of MSP after seven days of incubation. Spectra of metal-free $\text{A}\beta_{1-40}$ alone (black), $\text{Zn}^{2+}\text{-A}\beta_{1-40}$ (1:1) complex in the absence (red), presence of two-fold MSP (magenta), ten-fold MSP (dark yellow), and MSP alone (blue). All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

iii) TEM images, and Congo red-stained birefringence images of amyloid fibrils formed by $\text{A}\beta_{1-40}$:

$\text{Zn}^{2+}\text{-A}\beta_{1-40}$ formed clear fibrillar aggregates (Figure 5.15. a-i) under TEM, but in the presence of two-fold MSP (Figure 5.15. a-ii) and ten-fold MSP (Figure 5.15. a-iii), no such fiber was observed somewhat amorphous structures were observed. Therefore it can be concluded MSP can inhibit aggregation of $\text{Zn}^{2+}\text{-A}\beta_{1-40}$.

The bright green-gold birefringence was observed for $\text{Zn}^{2+}\text{-A}\beta_{1-40}$ under cross-polarized light (Figure 5.15. b-i), but when $\text{A}\beta_{1-40}$ was incubated in the presence of two-fold MSP

(Figure 5.15. b-ii) and ten-fold MSP (Figure 5.15. b-iii) separately, no such green gold birefringence was observed. These results support that MSP significantly inhibited amyloid aggregation of Zn^{2+} - $\text{A}\beta_{1-40}$.

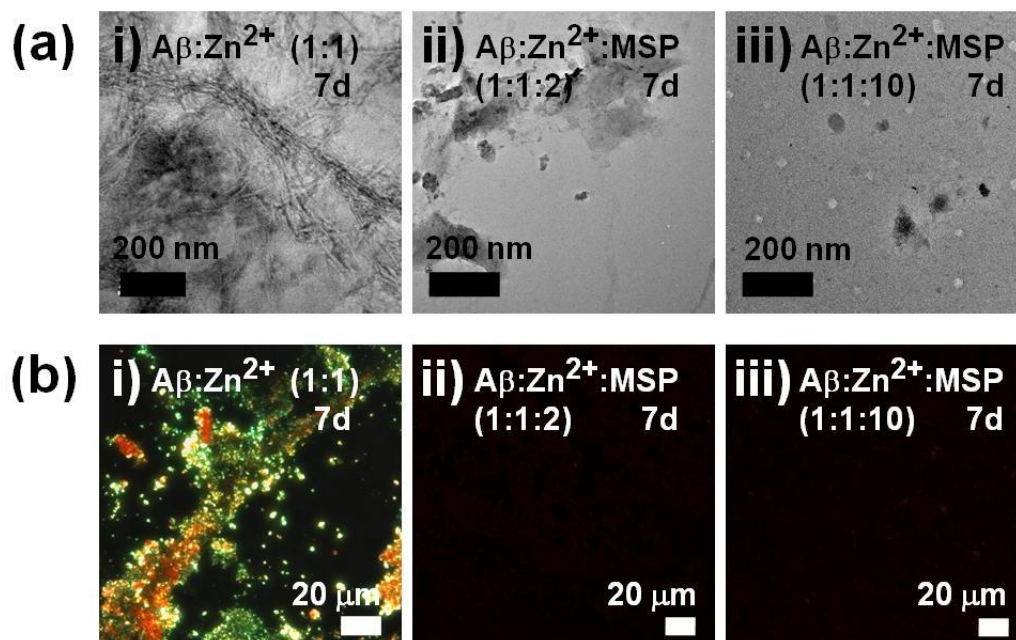


Figure 5.15. (a) TEM images, and (b) Congo red-stained birefringence images of Zn^{2+} - $\text{A}\beta_{1-40}$ (i) in the absence, presence of (ii) two-fold MSP, and (iii) ten-fold MSP. Scale bars are indicated as 200 nm for TEM and 20 μm for birefringence images. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel. All the images were captured after seven days of incubation.

5.7. Dynamic Light Scattering (DLS) study:

From the DLS study¹⁵, it was observed that the diameter of the dissolved particles of $\text{A}\beta_{1-40}$ aggregates (black, Figure 5.16) appeared at around 1100 nm after two days of incubation. Two peaks with the diameter of around 1000 nm and 5500 nm were observed in the case of Fe^{3+} - $\text{A}\beta_{1-40}$ complex (blue, Figure 5.16) after two days of incubation confirms its rapid aggregation propensity over metal-free $\text{A}\beta_{1-40}$. However, in the presence of two-fold (red, Figure 5.16) MSP, smaller molecular-weight aggregates with the diameter of 120 nm (lower intensity), and 800 nm (higher intensity), and higher molecular-weight aggregates with the diameter of 5500 nm (higher intensity) were noticed after five days of incubation. However, in the presence of ten-fold (green, Figure 5.16) MSP, smaller molecular-weight aggregates with the diameter of 480 nm (higher

intensity), were noticed after five days of incubation along with an extra peak around 5500 nm of very lower intensity. The results indicate that the fibril network was present in all the cases after five days of incubation, but MSP could reduce the size of A β aggregates to different extents in a dose-dependent manner. Ten-fold molar excess MSP showed more prominent effect than two-fold.

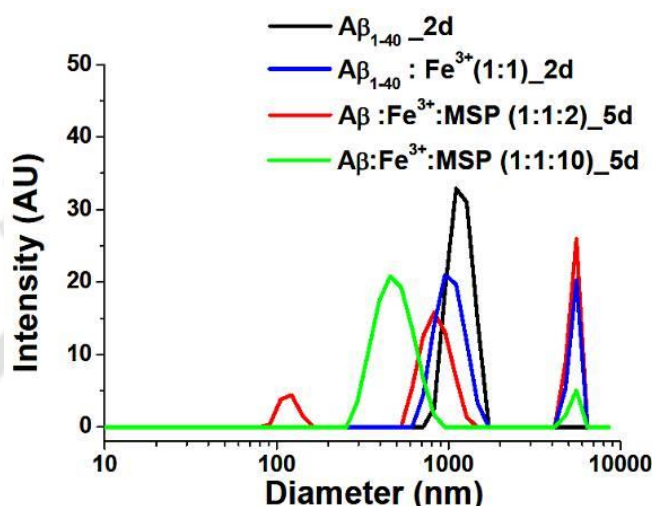


Figure 5.16. Overlay of DLS profiles of A β ₁₋₄₀ (black, 50 μ M) after two days, Fe³⁺-A β ₁₋₄₀ (blue, 1:1, 50 μ M) after two days of incubation. Overlay of DLS profiles of Fe³⁺-A β ₁₋₄₀ in the presence of two-fold (red), and a five-fold (green) molar excess of MSP after five days of incubation. All the peptide solutions were incubated in PBS pH 7.4 (50 mM) at 37 °C in parallel.

5.8. Vesicle leakage study:

Soluble oligomers of A β ₁₋₄₀ can form pores in the cell membrane resulting in membrane disruption,¹⁶ and hence oligomeric species are considered as more toxic than insoluble fibrillar aggregates of A β ₁₋₄₀. From the vesicle leakage study, we can measure cell membrane disruption capability by additives. So, we prepared carboxyfluorescein dye entrapped large unilamellar vesicles (LUVs) which is well-known as the cell membrane mimic. Next, we performed the vesicle leakage study to confirm the existence of toxic oligomeric species in the solution of metal-A β ₁₋₄₀ co-incubated along with MSP at the physiological condition. LUVs were prepared as described in the experimental method chapter and characterized by transmission electron microscopy (TEM, Figure 5.17). The diameter of prepared LUVs was found to be around 200 nm.

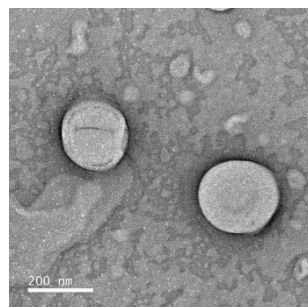


Figure 5.17. TEM images of negatively stained LUVs. The concentration of the sample was 100 μ M. Scale bars are indicated as 200 nm.

Three sets of solution were prepared to perform the study, and those are i) solution A: LUVs treated with $A\beta$ _incubated for one day, solution B: LUVs treated with Fe^{3+} - $A\beta$ (1:1)_incubated for seven days, and solution C: LUVs treated with Fe^{3+} - $A\beta$:MSP (1:1:10)_incubated for seven days. MSP were added after three days to the preformed fibril of Fe^{3+} - $A\beta$. Entrapped dye releases by membrane rupture of LUVs in the solutions were monitored by time-dependent fluorescence assay. From the result, it was monitored that solution A (red, Figure 5.18) caused more dye leakage indicates the presence of toxic oligomers. Again, the solution B (blue, Figure 5.18) caused very less LUV leakage in comparison to solution A. The results confirmed that soluble oligomers have greater ability to make pore formation on LUVs and hence more toxic in comparison to the mature fibrils of Fe^{3+} - $A\beta_{1-40}$. The Fe^{3+} - $A\beta_{1-40}$ treated with MSP (solution C) caused slight more dye leakage (green, Figure 5.18) in comparison to solution B. In-short, lesser extent of dye leakage was viewed when ten-fold MSP was co-incubated along with Fe^{3+} - $A\beta_{1-40}$, and that leakage was almost comparable with that of the untreated LUVs (black, Figure 5.18). Hence, it can be concluded that MSP disrupted the preformed amyloid aggregates of $A\beta_{1-40}$ into non-toxic metabolites.

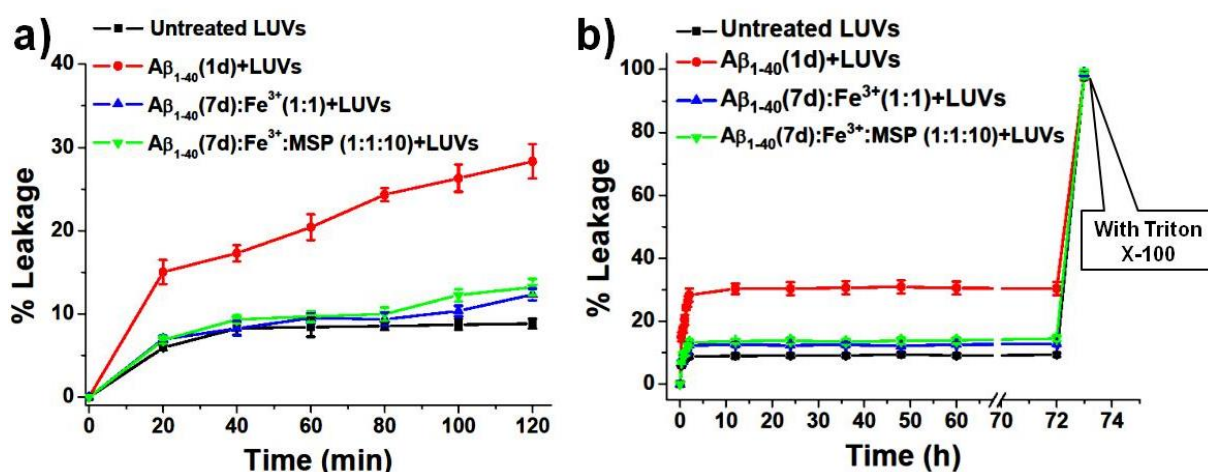


Figure 5.18. % dye (carboxyfluorescein) leakage from LUVs (a) up to 120 min and (b) up to 72 h. % of dye leakage by untreated LUVs (black), LUVs treated with Aβ_{one day} (red), LUVs treated with Fe³⁺-Aβ (1:1)_{seven days} (blue), and LUVs treated with Fe³⁺-Aβ:MSP (1:1:10)_{seven days} (green), [MSP were added after three days to the preformed fibril of Fe³⁺-Aβ₁₋₄₀]. Error bars represent standard deviations of at least three independent measurements.

5.9. Conclusions:

Upon development of AD, several pathogenic features, for example, metal ion dyshomeostasis, Aβ aggregation, oxidative stress, *etc.*, are recognized, indicating metal ions and Aβ to be considered as probable pathological factors.¹⁷ These factors interact with each other via various pathways; like Aβ bind to metal ions such as redox-active metal ions (Cu²⁺, Zn²⁺, Fe³⁺ *etc.*) followed by facilitation of Aβ aggregation to generate toxic Aβ oligomeric species.¹⁸ Modulation of metal dyshomeostasis in a number of neurodegenerative diseases can be controlled by metal-ion chelation therapy, and hence metal ion chelators have been extensively used for AD treatment.^{19,20} In the case of AD, aggregation of Aβ in the presence of redox-active metal ions (Cu²⁺, Zn²⁺, Fe³⁺) are responsible for the cellular toxicity.^{21,22} Thorough experimental and computational studies have recommended that multiple binding modes of metal ions in Aβ peptides could be present simultaneously. Herein, taurine containing metal scavenger peptide (MSP) has been presented as the inhibitor of Aβ aggregation *in vitro*. We have designed the peptide conjugate which contains VFFA as recognition part and taurine unit attached with side chain of Glu, in such a way that –SO₃H groups of taurine unit are believed to lie around

¹³His and ¹⁴His of A β to coordinate metal ion with metal-bound A β , because Cu²⁺, Zn²⁺ ions form salt bridge predominantly through ¹³His-Metal-¹⁴His conformation as well as bridges with ⁶His of A β . From the ITC measurements, the binding constant of MSP to the metal ions (Fe³⁺, Cu²⁺, and Zn²⁺) was found to be higher than that of A β ₁₋₄₀. Thus it can be concluded that MSP can bind and extract such metal ions from the metal-A β ₁₋₄₀ complex. From various biophysical studies (ThT Fluorescence assay, CD, TEM, Cong Red birefringence, and DLS studies), it was concluded that in the presence of MSP, metal-A β ₁₋₄₀ complex was not able to form fibrillar aggregates or amyloid. The prominent metal quenching ability of MSP was found in Zn²⁺-A β ₁₋₄₀ aggregates among other metal-bound A β ₁₋₄₀, which was concluded from the Th.T assay. Cell membrane disruption capability by additives was examined by the vesicle leakage study and the results confirmed that MSP disrupted the preformed amyloid of metal-bound A β ₁₋₄₀ into non-toxic metabolites. So, in conclusion, MSP has noticeable anti-aggregating and anti-amyloidogenic ability towards metal-bound A β , and this is because unfolded structure of aggregated protein converted to the folded protein by metal extraction of MSP.

5.10. References:

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5.11. Supporting data:

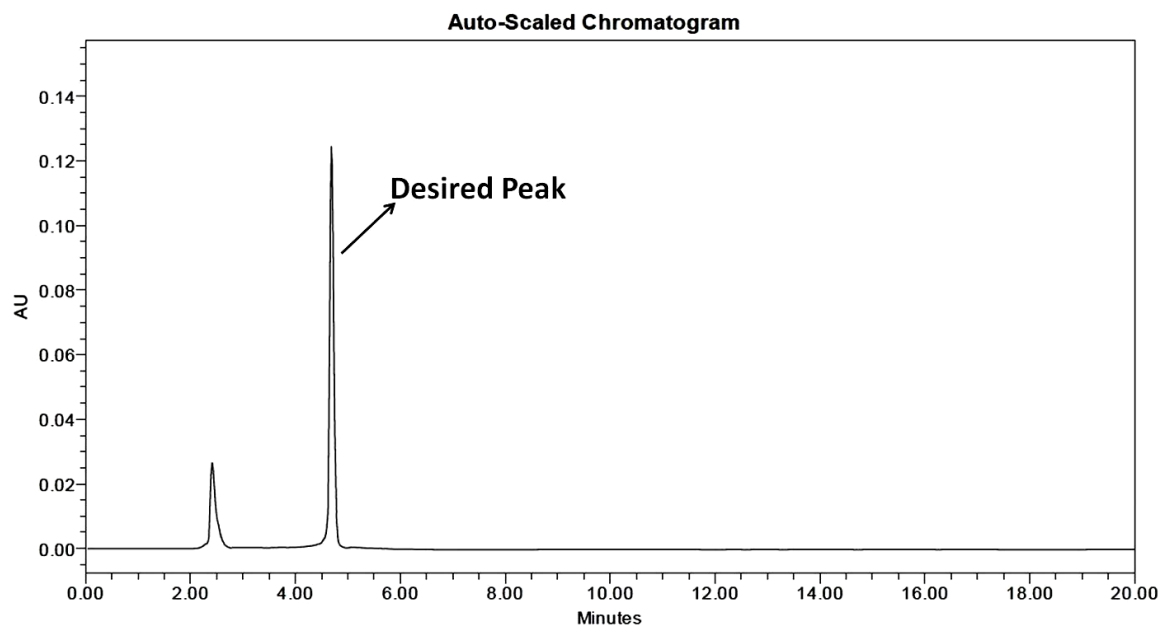


Figure 5.19. HPLC profile picture of the metal scavenger peptide (MSP).

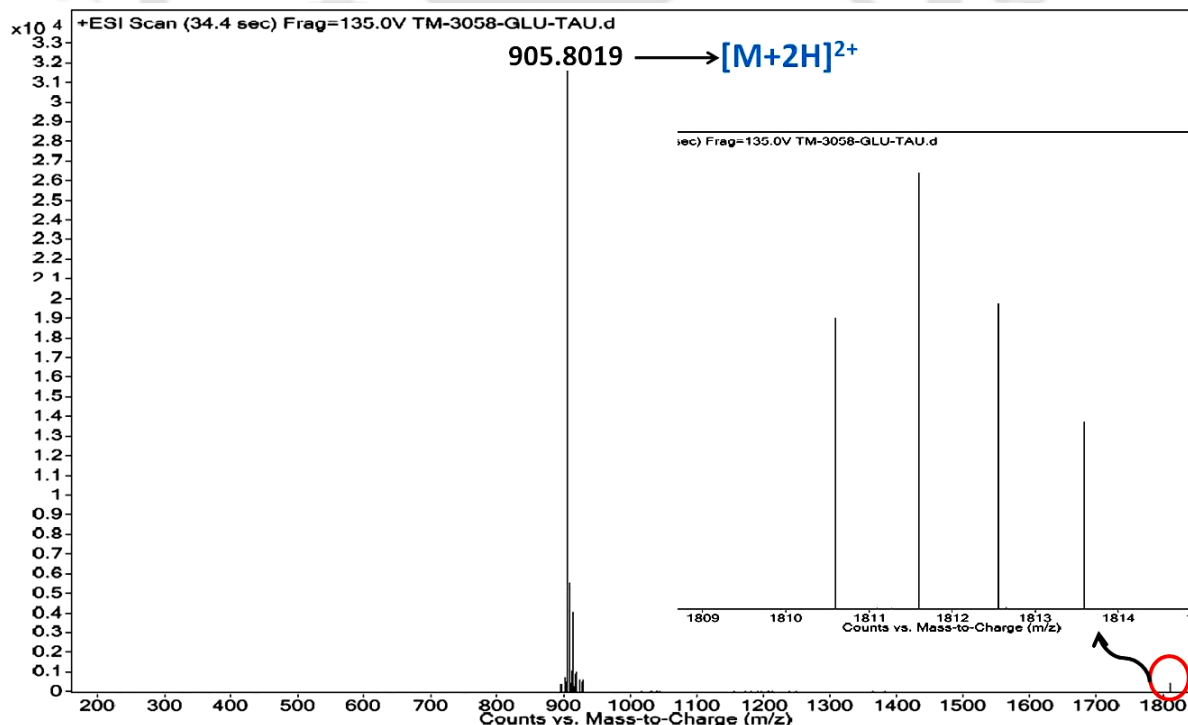


Figure 5.20. ESI-MS profile picture of purified MSP. Calculated m/z for $C_{68}H_{103}N_{19}O_{31}S_4$ $[M+2H]^{2+}$ is 905.7975, observed 905.8019. [inset: Calculated m/z for $C_{68}H_{103}N_{19}O_{31}S_4$ $[M+H]^+$ is 1810.5950, observed 1810.7.]



Patent and Publication

List of Patents:

1. Indian patent application (complete specifications), filed on 23.01.2019; Title: “Design and Development of Functional Mimics of α -Secretase and their Application in Drug Design against Amyloidoses”; Application No. **201931002772**; Inventors: Dr. Bhubaneswar Mandal and **Tanmay Mondal**.

List of Publications:

1. **Tanmay Mondal** and Bhubaneswar Mandal*. “Engineered peptidic constructs metabolize amyloid β by self-assembly-driven reactions” *ChemComm*. 2019, 55, 4933-4936. *(Invited for a feature back cover of ChemComm)*

2. **Tanmay Mondal** and Bhubaneswar Mandal*. “Targeted Amyloid Degradation by Miniature Artificial Protease”. *ChemComm*. 2020, 56, 2348-2351. *(Invited for a feature back cover of ChemComm)*

3. **Tanmay Mondal** and Bhubaneswar Mandal*. “Conventional and Unconventional Triad Based Protease Mimic Digest Amyloid” (Manuscript to be submitted)

4. **Tanmay Mondal**, Sujan Kalita, Rinku Dutta, and Bhubaneswar Mandal*. Design and development of a novel taurine containing peptide conjugate and their application in drug design against Alzheimer’s Disease and related disorders. (Manuscript to be submitted)

5. **Tanmay Mondal**, Rinku Dutta, and Bhubaneswar Mandal*. “Designed peptide conjugates metabolize model Amyloid β ”. (Manuscript under preparation)

6. Ashim Paul, Krishna Chaitanya Nadimpally, **Tanmay Mondal**, Kishore Thalluri, and Bhubaneswar Mandal*. Inhibition of Alzheimer’s Amyloid- β Peptide Aggregation and its Disruption by a Conformationally Restricted α/β Hybrid Peptide. *ChemComm.*, 2015, 51, 2245-2248.

7. Ashim Paul, Bhanita Sharma, **Tanmay Mondal**, Kishore Thalluri, Sandip Paul, and Bhubaneswar Mandal*. Amyloid β Derived Switch-Peptides as a Tool for Investigation of Early Events of Aggregation: A Combined Experimental and Theoretical Approach. *MedChemComm.* 2016, 7, 311-316.

8. Mahesh Agarwal, **Tanmay Mondal**, and Biplab Bose*. “Peptides Derived From A Short Stretch of The Receptor-Binding Domain of Diphtheria Toxin Bind To Its Receptor”. *Toxicon*, 2019, 169, 109-116.

9. Dharm Dev, Tapasi Kalita, **Tanmay Mondal**, and Bhubaneswar Mandal*. “Synthesis of Amide from Ketoxime via Beckmann Rearrangement using Catalytic amount of Ethyl 2-cyano-2-(2-nitrobenzenesulfonyloxyimino)acetate (o-NosylOXY)”. (Manuscript under review)

Abstracts Published in Conference Proceedings:

1. **Tanmay Mondal** and Bhubaneswar Mandal*, Development of Functional Mimic of α -secretase towards the Drug Design against Alzheimer's Disease, Frontier in Chemical Sciences (**FICS 2018**), 6th-8th December 2018, IIT Guwahati, Assam-781039, Page-443 (P-299) [**Poster Presentation**].
2. **Tanmay Mondal** and Bhubaneswar Mandal*, Development of Novel Scorpion Peptides towards the Drug Design against Alzheimer's Disease, 23rd CRSI National Symposium in Chemistry (**CRSI NSC-23**), 13th-15th July 2018, IISER Bhopal, Bhopal-462066, Page-179 (PA-107) [**Poster Presentation**].
3. **Tanmay Mondal** and Bhubaneswar Mandal*, Design and Development of Artificial α -Secretases and its Application in Drug Design against Alzheimer's Disease, A Satellite Symposium on Peptides in Biology & Material Sciences (**sIPS-2018**), 22th-23th February 2018, Shankarpur, West Bengal, Page-26 (ST4) [**Oral Presentation**].
4. **Tanmay Mondal** and Bhubaneswar Mandal*, Design and Development of a Novel class of Metal Scavenger Peptides and their Application in Drug Design against Alzheimer's Disease and Related Disorders, International Conference on Chemistry for Human Development (**ICCHD-2018**), 8th-10th January 2018, Heritage Institute of Technology, Kolkata, Page-127 (P-104) [**Poster Presentation**].
5. **Tanmay Mondal**, Rinku Dutta, Sujan Kalita, and Bhubaneswar Mandal*, Biophysical Methods for Studying the Kinetics of Amyloid Aggregation and Fibrillogenesis in Alzheimer's Disease and Related Disorders, International Conference on Sophisticated Instruments in Modern Research (**ICSIMR-2017**), 30thJune-1stJuly 2017, Central Instruments Facility, IIT Guwahati, Page-123 (PP-62) [**Poster Presentation**].



Curriculum Vitae

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1.	University of Calcutta, Kolkata	B.Sc.(Hons.)	2008	Chemistry (Hons.), Physics, Math.
2.	Vidyasagar University, Midnapore	M.Sc.	2010	Organic (Special), Physical, Inorganic
3.	IIT Guwahati, Assam	Ph.D. Fellow (Continue)	24.07.2013 - Continue	Peptide Chemistry & Alzheimer's disease
3.	HONORS/ AWARDS			
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4.	PROFESSIONAL EXPERIENCE
	Laboratory and Instrumentation: During my research, I gained experience in Peptide syntheses (solid phase & solution phase), Organic reactions & rearrangements, HPLC, Mass (ESI-LCMS, APCI, MALDI), Fluorescence, CD, TRPL, TEM, AFM, FESEM, Polarizable microscope, NMR, FTIR, UV, DLS. Computational Chemistry: Molecular Docking and DFT Calculation. Software: MGL Tools, Gaussian, Origin, Adobe Illustrator, Adobe Photoshop, MS-Office, WSxM, Spectra Manager, Chem Draw, WinCoot, MestReNova. Teaching Experience: Worked as Teaching Assistant (TA) in B.Tech. Chemistry Laboratory (2014 July-2014 Nov) and Departmental Operator of ESI-LCMS & HRMS (2014 Dec-2017 Dec) at IIT Guwahati, India. I have guided a project fellow (duration: 6 months), a master student (duration: 4 months), 3 summer trainees (duration: 2 months), and 2 junior research fellows (duration: 1 year).