

Exploring the Structural Properties of α,γ -Hybrid Peptide Foldamers to Design Antimicrobials, $A\beta_{42}$ -Oligomers Aggregation Inhibitors and Nanotubes

**A thesis
Submitted in partial fulfillment of the requirements
of the degree of
Doctor of Philosophy**

**By
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ID: 20163455**



Indian Institute of Science Education and Research, Pune

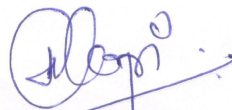
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Dedicated to my beloved teacher's....

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled “**Exploring the Structural Properties of α,γ -Hybrid Peptide Foldamers to Design Antimicrobials, $A\beta_{42}$ -Oligomers Aggregation Inhibitors and Nanotubes**” submitted by **Puneeth Kumar D. R.** (ID No. **20163455**) carried out by the candidate at the Indian Institute of Science Education and Research (IISER), Pune, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or Institution.

Date: 22/08/23



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Declaration

I hereby declare that the thesis entitled “**Exploring the Structural Properties of α,γ -Hybrid Peptide Foldamers to Design Antimicrobials, $A\beta_{42}$ -Oligomers Aggregation Inhibitors and Nanotubes**” submitted for the degree of Doctor of Philosophy in Chemistry at Indian Institute of Science Education and Research (IISER), Pune has not been submitted by me to any other University or Institution. This work was carried out at Indian Institute of Science Education and Research (IISER), Pune, India under the supervision of Prof. Hosahudya N. Gopi.

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Acknowledgment

I owe a special debt of gratitude to my supervisor, Dr. Hosahudya N. Gopi. He patiently gave me the direction, inspiration, and guidance I needed to move forward with the doctoral programme and finish my dissertation. Throughout the duration of my PhD, he has been a powerful and encouraging advisor to me. Throughout my graduate studies, I have always been motivated by his guiding ideas and work ethic. I want to sincerely thank my supervisor for his valuable advice, suggestions, and efforts to help me grow as a researcher.

I want to express my gratitude for the excellent infrastructure and research facilities provided by IISER Pune's former directors, Profs. Jayant B. Udgaonkar and K. N. Ganesh. Their passion for science served as a constant source of motivation. I am also thankful to the IISER-Pune for my five years research fellowship for Ph.D.

My deepest gratitude goes out to Prof. V. G. Anand and Prof. H. V. Thulasiram of my research advisory committee for their insightful comments made at my RAC meetings, which greatly influenced the direction of my research efforts. I also like to express my gratitude for all of the chemistry professors at IISER, Pune, for their support and direction.

I thank Prof. S. Raghothama, Dr. Dilip K. Senapathi (IISc, Bangalore), Gijo George for NMR modelling structures and Dr. T. S. Mahesh, Dr. Jeetender Chugh for their help in 2D NMR experiments, Dr. Sidhrath Chopra, Grace Kaul and Abdul Akhir from CDRI, Lucknow for Antimicrobial Activity studies, Dr. Muhammed Musthafa and Dr. Zahid Bhat from IISER-Pune for Impedance measurements, Prof. Arnab Mukherjee and Uddipan Bhattacharya from IISER-Pune for Molecular Dynamics calculations, special thanks to Prof. Srinivas Hotha, Prof. Amritha Hazra and Prof. Raghavendra Kikkeri and their lab members (Sumith Sen, Roopali and Deepak Ganesh) to access their lab facilities.

I must acknowledge the help from Sandip and Nithin for NMR analysis, Mahesh and Suresh Prajapathi for MALDI analysis, Yathis for FE-SEM study, Prashanth for HR-TEM analysis, Ravindra for SC-XRD analysis, Sandip Kanade for HRMS study, Megha for AFM analysis, Vijaya Vital for Confocal Microscope study. I am very thankful to Mayuresh, Thushar Kulkarni, Ganesh Dimber and Sanjay meena for the administrative help.

I am fortunate to work with excellent blend of talented and unique lab mates who helped me a lot in solving my research problems. Their moral support and helping nature gave me strength to move forward during my critical time. I have very special appreciation for Dr. Susheel Benke, Dr. Rajkumar Misra, Dr. Rahi M. Reja, Dr. Kuruva Veersh, Dr. Anindita Adak and Dr. Sanjith Dey who helped me a lot in my research career. I also thank my other lab mates Sachin A. Nalawade, Manjith Singh, Saikat Pahan, Souvik Roy, Souvik Panda, Sandip Toraskar, Devanand, Vivek Kumar, Abhijith S. A and Rajat Patel for their direct or indirect inputs in my research.

Without my excellent pals Drs. Chethan D. S., Ashok Kumar B., Uday H. S., and Anand Kumar T., life would be boring. We've been together for a very long time and have shared all of life's experiences. They were always available to help me whenever I needed them. They always stood by me in my difficult times with their helpful and caring attitudes. I would like to express my sincere gratitude to you all for bearing with me for so long. I also want to express my gratitude for the cooperation of all of my chemistry department buddies. My five years at IISER flew by due to all of these people in the space of a few days.

Without the assistance of my family, I might not have been able to finish my PhD study. I would want to express my deepest gratitude to my family, especially my parents and my wife, for their unwavering love and support. My brother love and support was always there for me as I pursued my PhD.

As a final prayer, I'd want to thank Lord Shiva for providing me the courage and blessing I needed to finish my research and turn in this thesis.

Puneeth Kumar D. R.

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Abbreviations

Ac₂O = Acetic anhydride

AD = Alzheimer's Disease

ACN = Acetonitrile

AcOH = Acetic acid

Aib = α -Amino isobutyric acid

Aic = 4-amino isocaproic acid

aq. = Aqueous

Bn = Benzyl

Boc = tert-Butoxycarbonyl

(Boc)₂O = Boc anhydride

^tBu = tertiary Butyl

Calcd. = Calculated

CCDC no. = Cambridge Crystallographic Data Centre number

CD = Circular Dichroism

CIF = Crystallographic Information File

COSY = COrrrelation SpectroscopY

CV = Cyclic Voltammetry

d γ = dehydro gamma

DAPI = 4',6-DiAmidino-2-PhenylIndole

DCC = N, N' -Dicyclohexylcarbodiimide

DCM = Dichloromethane

DiPEA = Diisopropylethyl Amine

DMAP = 4-Dimethylaminopyridine

DMF = Dimethylformamide

DMSO = Dimethylsulfoxide

ELISA = Enzyme-Linked Immuno Sorbent Assay

Et = Ethyl

EtOAc = Ethyl acetate

EtOH = Ethanol

FACS = Fluorescence-Activated Cell Sorting

FITC = Fluorescein IsoThioCyanate

Fmoc = 9-Fluorenylmethoxycarbonyl

Fmoc-OSu = N-(9-Fluorenylmethoxycarbonyloxy) succinimide

g = gram

h = hours

H-bond = Hydrogen bond

HBTU = 2-(1H-benzotriazol-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate

HCl = Hydrochloric acid

HOBt = Hydroxybenzotriazole

HSQC = Heteronuclear Single Quantum Coherence

IBX = 2-Iodoxybenzoic acid

LAH = Lithium Aluminium Hydride

M = Molar

MALDI-TOF/TOF = Matrix-Assisted Laser Desorption/Ionization-Time of Flight

Me = Methyl

MeOH = Methanol

mg = Milligram

min = Minutes

μ L = Micro liter

mL = Milliliter

mM = MilliMolar

mmol = millimoles

MS = Mass Spectroscopy

N = Normal

nM = NanoMolar

NHS = N-hydroxy succinimide

NMP = N-methyl pyrrolidone

NMR = Nuclear Magnetic Resonance

NOE = Nuclear Overhauser Effect

NPN = N-Phenyl-1-Naphthylamine

ORTEP = Oak Ridge Thermal-Ellipsoid Plot Program

PG = Protecting Group

PI = Propidium Iodide

pM = PicoMolar

PMBN = PolyMyxin B Nonapeptide

ppm = Parts per million

Py = Pyridine

R_f = Retention factor

ROESY = Rotating-frame nuclear Overhauser Effect correlation Spectroscopy

RP- HPLC = Reversed Phase-High Performance Liquid Chromatography

R_t = Retention time

RT = Room Temperature

TFA = Trifluoroacetic acid

THF = Tetrahydrofuran

ThT = Thioflavin T

TOCSY = T^Otal Correlation Spectroscop^Y

UV = Ultraviolet–Visible Spectroscopy

Abstract

The structure and functions of proteins have inspired the design of new foldamers from organic templates and unnatural amino acid building blocks. In comparison to the foldamers derived from homogenous sequences of beta- and gamma-amino acid building blocks, heterogeneous sequences composed of α -, β - and γ -amino acids offered a variety of H-bonded protein secondary structure mimetics. Among the mixed peptide sequences, α,γ - and $\alpha\alpha\gamma$ -hybrid peptides have been shown to adopt stable 12- and 10/12-helical conformations. We have explored the combination of α - and γ -amino acid to design different types of protein secondary structure mimetics. The present work is mainly focusing on the design of α,γ -hybrid peptides as $A\beta_{42}$ aggregation inhibitors, proteolytically stable peptide antibiotics, novel β -double helix, and hybrid peptide nanotubes to measure protein-small molecule interactions. The designed amphiphilic cationic α,α,γ -helical peptides displayed potent broad-spectrum antimicrobial activity (AMA) across the panel of Gram-positive, and Gram-negative bacteria including MRSA and VRSA. The results of Time Kill Analysis, Post Antibiotic Effect, Skin Infection Model, and other biophysical assays suggested that these α,α,γ -helical peptides efficiently control the growth of the bacteria through instant membrane depolarization. In addition, we have also designed cationic amphiphilic peptides and $\alpha\alpha\gamma$ -hybrid peptides as $A\beta_{42}$ aggregation inhibitors. Among various $A\beta$ -oligomers, $A\beta_{1-42}$ ($A\beta_{42}$) is known to be highly aggregation-prone and also toxic. The designed peptides not only inhibit the soluble $A\beta_{42}$ into aggregated fibrils but also disintegrate matured fibrils of $A\beta_{42}$ into smaller assemblies. The biophysical analysis comprising the Thioflavin T assay, CD, 2D-NMR HSQC, DLS, and ELISA assays revealed that upon interaction the peptide induces a conformational change in the $A\beta_{42}$. Further, the cell assay experiments revealed a significant reduction in the toxicity of the $A\beta_{42}$ in the presence of cationic peptides. We further noticed that hydrophobic α,γ -hybrid peptide helical foldamers spontaneously self-assembled into nanotubes and we explored these nanotubes for label-free detection of small molecule-protein interactions. The small molecules were immobilized on the surface of the functionalized nanotubes and measured the protein interactions using nanoscale capacitance and impedance spectroscopy. In comparison to the 12-helix structures of 1:1 α,γ -hybrid peptides, we have shown the novel β -double helix structures from 1:1 alternating α - and α,β -unsaturated γ -amino acids. The 2D NMR analysis and molecular simulations studies revealed that the peptides adopted stable anti-parallel β -double helix conformations in solution. Overall, we have demonstrated the antimicrobial activities including drug resistant bacteria, inhibition of the aggregation of $A\beta_{42}$ of Alzheimer's disease, nanotubes as means to detect protein-small molecule interactions without labels, and novel β -double helix structures from the mixed α,γ -hybrid peptides.

Publications

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2. **Puneeth Kumar, D. R.;** Bhat, Z. M.; Dey, S.; Roy, S.; Panda Mahapatra, S.; Pahan, S.; Thotiyl, M. O.; Gopi, H. N. Foldamer Nanotubes Mediated Label-Free Detection of Protein-Small Molecule Interactions. *Chem.Eur. J.* **2023**, *29*, e2023004.
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4. **Puneeth Kumar, D. R.;** George, G.; Bhattacharya, U.; Pahan, S.; Dey, S.; Mukherjee, A.; Gopi, H. N. Designing β -Double-Helix Structures from α,γ -Hybrid Foldamers. *Under preparation*.
5. **Puneeth Kumar, D. R.;** Akhir, A.; Kaul, G.; Chopra, S.; Gopi, H. N. Mechanistic Study of Protease Resistant Antimicrobial Peptides against Drug Resistive Bacteria. *Under preparation*.
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Chapter 1

General Introduction of Peptide Foldamers Composed of Unnatural Amino Acids and their Folding Behavior

1.1. Introduction

Proteins are substantial, intricate molecules that have a variety of vital functions in the body.¹ They are essential to the majority of the tasks carried out by cells and are necessary for the development, maintenance, and control of the body's tissues and organs. Proteins three-dimensional architectures are crucial to how they work.² Due to their capacity to fold into distinct and three dimensional rigid conformations, proteins are specifically chosen by nature to perform various functions (Figure 1 and 2). The numerous proteins with distinct 3D shapes and activities that Nature has created using the 20 ribosomal amino acids are called riboproteins. Generally, there are four stages at which protein structures are described as primary, secondary, tertiary and quaternary structures as shown in Figure 1. Examining protein three-dimensional structures reveals their intricate tertiary patterns and connections in the quaternary structure. Hypothetically, the breakdown of tertiary structure in protein results in a small number of secondary structural components, like helices, turns, and β -sheets, which are put together using roughly organized loops. The super secondary arrangements, such as coiled-coils, hairpins (β - β motifs), β -meanders, helix-turn-helix, Greek key motifs, β - α - β motifs, etc., are formed when these secondary constructions in the protein are further coupled by flexible loops. In order to create protein structures from scratch, it is crucial to comprehend how proteins fold.

Typically, four levels of description are used to characterise protein structures as shown in Figure 1., where primary structure is the simplest level of protein structure, is represents the number and sequence of amino acids linked together to form a polypeptide chain. Each amino acid is linked to the next amino acid through peptide bonds created during the protein biosynthesis process. The secondary structure arises from the hydrogen bonds formed between atoms of the polypeptide backbone in a specific three-dimensional conformation. Tertiary structure of a protein refers the overall three-dimensional arrangement of its polypeptide chain in space. It is generally stabilized by hydrogen and ionic bond interactions and internal hydrophobic interactions between nonpolar amino acids side chains and also from disulphide linkage. Whereas quaternary structure of a protein defines the association of several protein chains or subunits into a closely packed arrangement. Each of subunits has its own primary, secondary, and tertiary structure. All these subunits are held together by hydrogen bonds and van-der Waals forces between nonpolar side chains. De novo creation of protein secondary structures is crucial for understanding protein folding and function, but it also opens up a world of possibilities for structure-based drug design.³

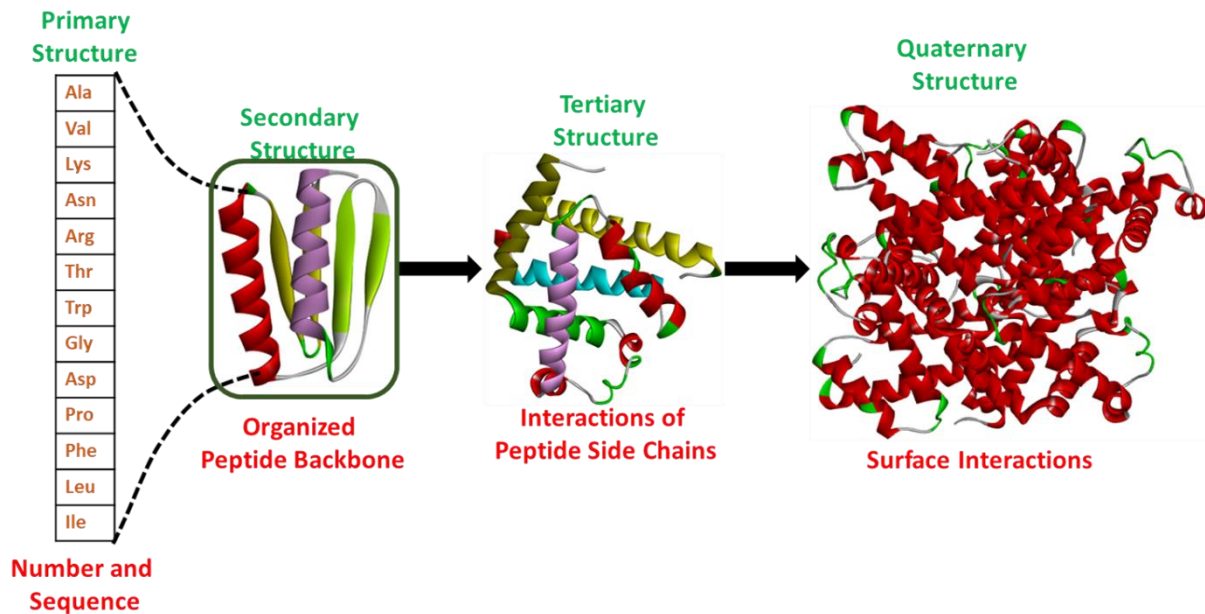


Figure 1: The four levels of the structural arrangement of proteins are shown schematically. (PDB codes: 6MRS for secondary structure,^{4a} 1MBO for tertiary and quaternary structures^{4b}).

1.2. Protein Secondary Structures

Helices, reverse turns, and β -sheets, make up the majority of secondary structures in the protein. The α -helix and β -sheet structures were first described by Linus Pauling and colleagues in their pioneering work. The distinct pattern of H-bonds between the NH and CO groups of the main chain defines these secondary structures. Secondary structures are combined and joined by loops of various lengths to form protein structures. Reverse turns are the loop provinces that join two neighbouring β -strands that are anti-parallel to each other. G. N. Ramachandran and colleagues⁵ in their seminal work, used the two degrees of torsional freedom ϕ and ψ to outline the stereo chemically permitted conformations of polypeptide secondary structures (Figure 2C). Below are some concise descriptions of secondary structures.

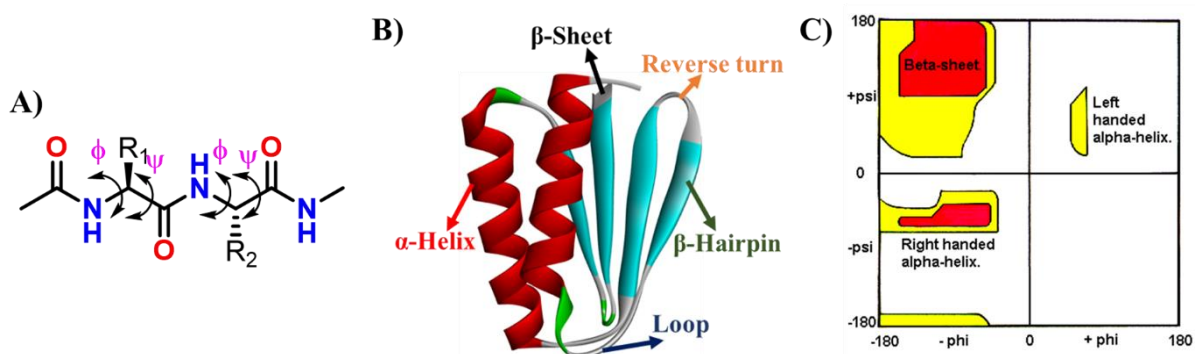


Figure 2: A) α -amino acid-based peptide backbone with torsion angles of ϕ and ψ . B) A ribbon representation of a protein illustrating many secondary structural components (PDB code: 6MRS).^{4a} C) Traditional Ramachandran plot shows the permitted area for various secondary structures torsional angles.

Helices: Helices are divided into the 2.2_7 (γ -helix), 3_{10} , 3.6_{13} (α -helix), and 4.4_{16} (π)-helices on the basis of hydrogen bonding arrangement and total number of residues involved per turn. The α -helix is the most frequent motif among the several kinds of helices and the most common secondary structure seen in proteins. In 1951, Linus Pauling proposed the structure of the α -helix.² The α -helix is a right-handed screw-like structure with 3.6 residues per turn and 13 atoms in the H-bond pseudo cycle (M and P are indicated Left-handedness and Right-handedness, respectively). The average ϕ and ψ values for the 3.6_{13} -P-helix, which represents the general nomenclature of the α -helix, are -60° and -45° , respectively. Proteins rarely include the 3_{10} -helix, in contrast to the α -helix. The 3_{10} -P helix's average ϕ and ψ values are -49° and -26° , respectively. The 10-atom H-bond pseudo cycle and three residues on each turn are described by the name 3_{10} -helix. Likewise, the π -helix is represented by the 4.4_{16} -P-helix ($\phi = -55^\circ$, $\psi = -70^\circ$), while the γ -helix is represented by the 2.2_7 helix ($\phi = -70^\circ$, $\psi = 70^\circ$).

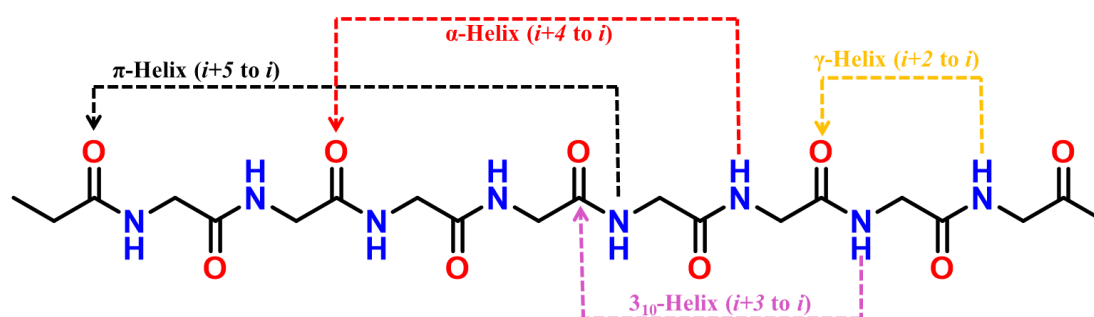


Figure 3: The hydrogen bonding arrangement in various protein helices is shown schematically.

β -Sheets: The second most important structural component in proteins is β -Pleated sheets. Typically 5-10 amino acid residues length, prolonged structure β -sheets. The ϕ and ψ values are found to be in the upper side of the left quadrant of the Ramachandran plot large structurally permissible zone.⁵ The first β -sheet structure was proposed by William Astbury in 1930.⁶ However, in 1951 Linus Pauling and Robert Corey proposed the revised version of β -sheet structure.⁷ On the basis of the directionality of the β -strands, β -pleated sheets further divided into (i) parallel and (ii) anti-parallel β -sheets (Figure 4). It is more common for β -sheets to have a right-handed twist ($\phi = -135^\circ$, $\psi = 135^\circ$) rather than a perfectly extended nature ($\phi = -180^\circ$, ψ

= 180°), which is encouraged by interstrand geometric restrictions and intrastrand non-bonded interactions. Layers of β -sheets are typically orientated to one another at a minor angle ($\sim 30^\circ$) in aligned β -sheet packing or close to 90° in orthogonal β -sheet packing at the tertiary structural level.

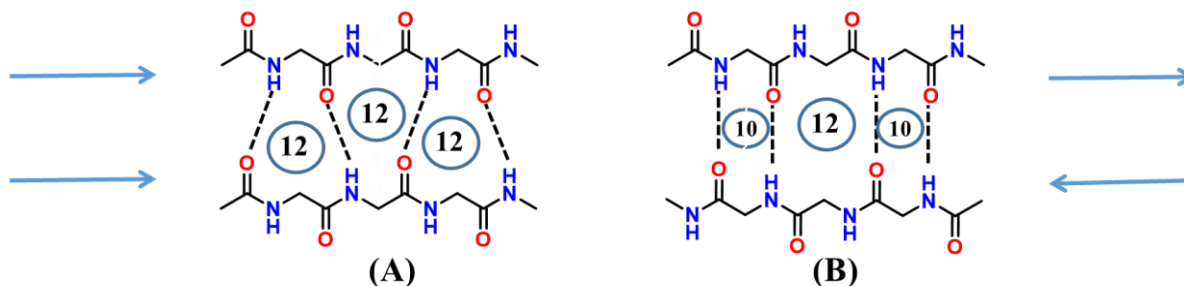


Figure 4: H-bonding and β -sheet directionality are displayed in (A) parallel and (B) anti-parallel β -sheets, respectively.

Reverse Turns: In the proteins secondary structures, reverse turns are also included as one of the structure. The number of residues required to induce the polypeptide chain's reverse directionality is used to categorize reverse turns. They are divided into the following categories: α -turn (5 amino acids), β -turn (4 amino acids), γ -turn (3 amino acids), and π -turn (6 amino acids). Further, the frequently arising β -turns are categorized into type I, type II, and type III along with their reflected images, type I', type II', and type III' turns, based on the dihedral angles ϕ and ψ of the $i+1$ and $i+2$ residues.⁸

1.3. Mimicking protein structures

Recent advances in the study of protein structure and function have given us a mechanistic understanding of a wide range of biological activities. Understanding the principles governing protein structure and stability is essential for de novo design of current or new protein folds. Over the past few decades, designing folded structures using sterically constrained α -amino acid templates has received an enormous amount of attention.⁹ In addition to enabling us to understand the structure, function, and folding of proteins, the de novo synthesis of secondary structural elements in the protein brings out numerous opportunities for structure-based drug design.¹⁰ In addition to the α -amino acid, a wide range of non-natural amino acid residues and synthetic building blocks have been discovered for use as templates in the proposal of secondary structures in the protein. The most extensively investigated peptide folding structures are made from the γ - and β -amino acids, which are among the various non-natural amino acids and organic templates.¹¹ These synthetic γ - and β -peptide folding structures offered a distinctive opportunity to develop various protein structural mimics. Several sorts of

structure adopted by the γ - and β -peptides and foldamers from hybrid peptides made up of α,β -, α,γ - and β,γ -systems are explained further down.

1.4. β -Peptide Foldamers

Prof. Samuel H. Gellman introduced the word "Foldamers" which he defined as "any oligomer which can adopt a rigid conformation in solution."^{11b} The β -amino acids are the homologated species of α -amino acids. They can be either β^3 or β^2 amino acids, depending on where the side chain is located. Double homologation of α -amino acids results in the formation of γ^2 -, γ^3 -, and γ^4 - amino acids. β -Peptides and higher homologous oligomers, in contrast to α -peptides, have shown to be metabolically and proteolytically stable.^{11a,11c} These characteristics mark β -peptide foldamers and other higher homologous oligomers more striking from a biological standpoint. Various β -amino acids utilised in the proposal of a β -peptide sequence is presented in Figure 5. In β -amino acids, the extra torsion variable θ , together with ϕ and ψ , can be used to distinguish between the backbone fluxionality. Figure 5 depicts the local configuration of β -residues. During the previous 20 years, foldamers from β -peptides were composed of β -amino acid residues became incredibly effective tools for building protein secondary structures.

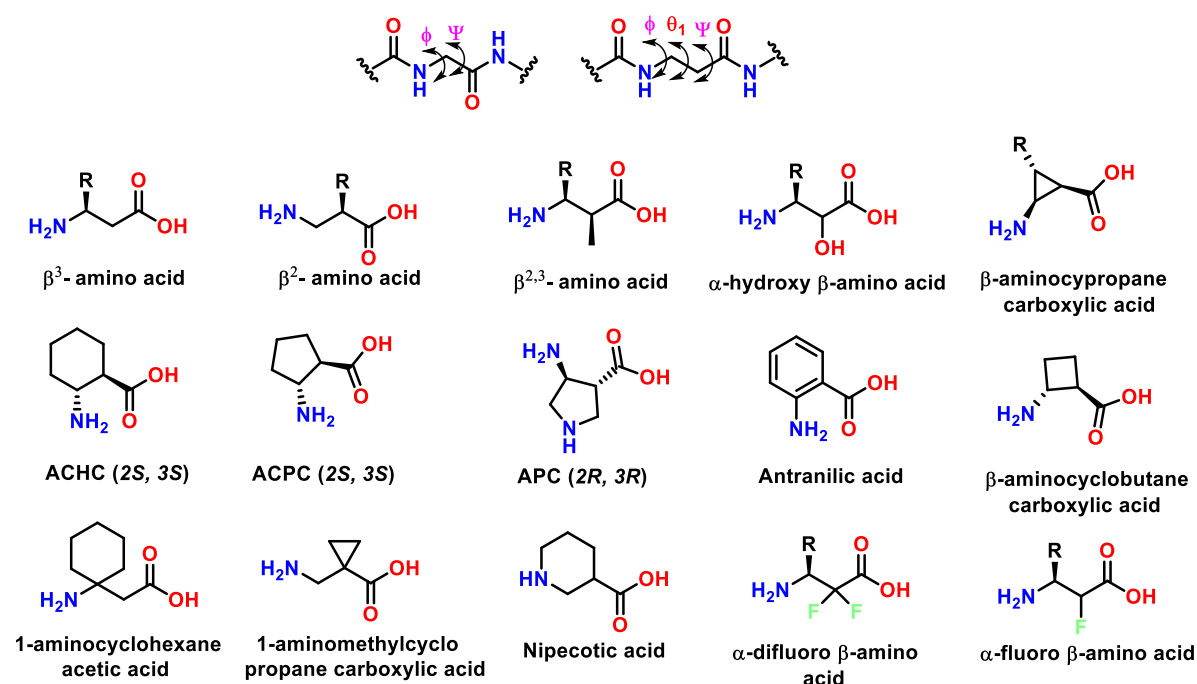


Figure 5: Different β -amino acids structure utilized in the proposal of foldamers.

Gellman¹² and Seebach¹³ presented cyclic and acyclic chiral β - amino acid residues, respectively, in their pioneering work and studied the structural characteristics of polypeptides

derived from these modified amino acid residues. Gellman and colleagues reported the ACPC (cyclic *trans*-2-amino cyclopentanecarboxylic acid) and ACHC (*trans*-2-amino cyclohexanecarboxylic acid) homooligomers in their respective 12-membered hydrogen-bond and 14-membered hydrogen-bond pseudo cycles helical structures.¹² Intriguingly, it has been demonstrated that C₈- and C₁₂-helices adopt a hydrogen bond directionality (C←N) that is comparable to that of an α -helix, whereas in C₁₀ – and C₁₄-structures, the hydrogen bond directions (N←C) are switched. Similar to this, Seebach and colleagues demonstrated that oligomers of acyclic β^3 amino acids can form left-handed 14-helical conformations. Additionally, they demonstrated how the oligomers of β^2 -amino acids can form the right-handed 14-helix shape. It has been demonstrated that β -peptide foldamers made of alternating β^2 and β^3 residues (1:1) adopt 12/10-helical structures with alternate H-bond directionality as opposed to the 14-helical conformations.¹³ Hofman and co-workers derived a number of foldameric forms available to the β -peptides through rigorous ab initio calculations.¹⁴ The several helices that can be used by the β -peptide, as anticipated by Hofmann and associates are presented in Figure 6. Additionally, Sharma and colleagues demonstrated miscellaneous 10/12 or 12/10 folding patterns and thoroughly examined the structures of the β -peptide oligomers constructed from carbo- β -amino acids.¹⁵ Reiser et al was demonstrated incorporation of cyclic β -amino acids in the design of β -peptides.¹⁶

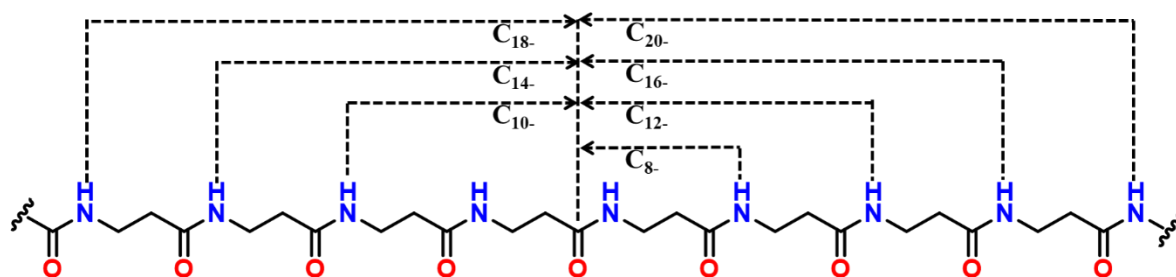


Figure 6: Systematic quantum calculations were used to identify possible H-bonding patterns in β -peptide oligomers.

Further, oligomers of *trans*-2-aminocyclobutane carboxylic acid were reported by Aitken and colleagues to adapt into a well-defined 12-helix.¹⁷ and Fulop *et al.* described the different cyclic β -amino acid units were used in the generation of β -peptides.¹⁸ The crystal structures of 14-helix from the β -peptide composed of *trans*-ACHA, 12-helices from the homooligomers of *trans*-ACPC and cyclobutane β -amino acid, hairpin from α,β -disubstituted β -amino acids and turn from β^3 -amino acid are shown in Figure 7. Further, β -amino acid residues have also been utilized for the design of reverse turns and structures resembling β -sheets. In addition to

observing the production of antiparallel polar β -sheets, Gellman and his colleagues also reported β -hairpin conformations employing various β -turn inducing segments.¹⁹ Later, Balaram and his co-workers investigated the β -hairpin structures by introducing β -amino acid residues in the peptide sequence.²⁰ Hybrid peptide foldamers were also designed as a result of the construction of hybrid β -hairpins using α - and β -amino acids in various combinations. The $\text{NH}\cdots\text{O}=\text{C}$ hydrogen bonds directionality is the primary distinction between the β -sheets produced by β^3 -amino acids and α -amino acids. The H-bonds in α -peptide β -sheets alternate in direction, but the H-bonds in β -residues have been shown to run in the same direction, resulting in polar sheets. Furthermore, Ortono *et al.* described the *cis*-ACBC (*cis*-2-aminocyclobutane-1-carboxylic acid) residues homo-oligomers with strand-like arrangements supported by the intramolecular six-membered H-bonds. Interesting supramolecular interactions and self-assembly are facilitated by these strand-like structures, resulting in nanoscale fibers or gels.²¹

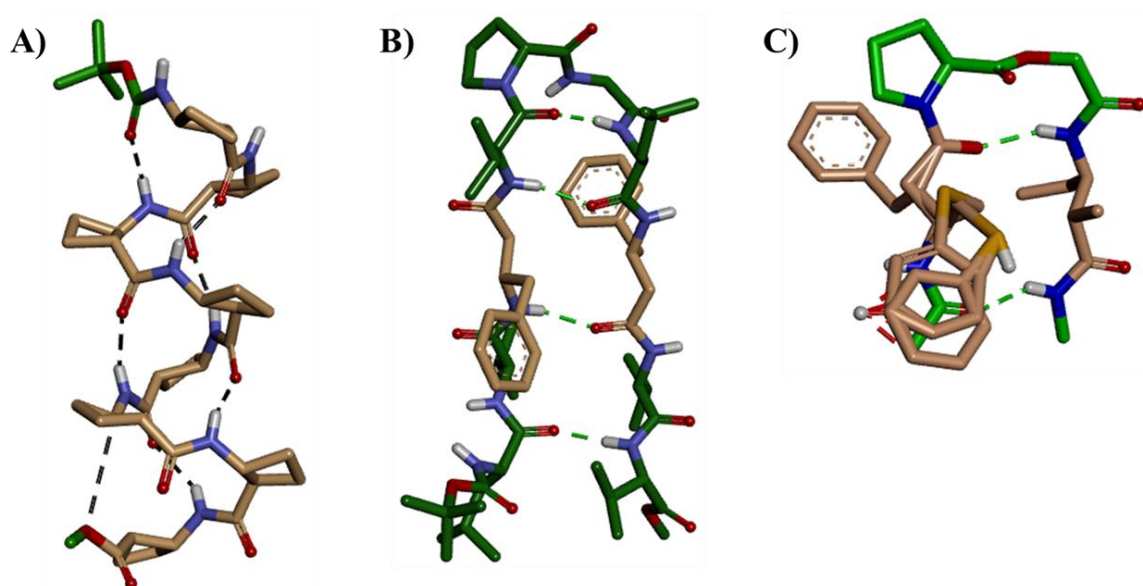


Figure 7: Crystal structure of A) C_{12} -helix of cyclobutane β -amino acid.¹⁷ Conformation β -hairpin fabricated from (B) β^3 -amino acids²⁰ and (C) α,β -disubstituted β -amino acids.^{19b} (Tan or wheat color represented the β -amino acids).

1.5. γ -Peptide Foldamers

Gamma-amino acids are double homologated amino acid residues with two $-\text{CH}_2-$ moieties between the $\text{C}=\text{O}$ and C^α carbons. Along with the regular torsional variables ϕ and ψ , these two $-\text{CH}_2-$ groups also bring together θ_1 and θ_2 torsional variables. Contrary to β -amino acids, γ -amino acids have not been extensively studied, likely as a result of the challenges associated

with the the synthesis of stereochemically pure amino acids. However, Seebach and colleagues²² and Hanessian *et al.*²³ recognised the 14-helix of the γ^4 -amino acids oligomers as well as modified 2, 3, and 4 γ -amino acids in their initial research. The H-bonds between the NH group of residue ($i+3$) and carbonyl group of residue (i) allow the γ^4 -peptide to adopt a 14-helix with right-handedness (C_{14} - pseudo cycles), in contrast to the β^3 -peptides. Hofmann and his co-workers anticipated different types of helices for γ -peptide foldamers through extensive quantum calculations.²⁴ They claimed that the C_{14} - and C_9 - helices are the most stable among all possible helices. Later, the C-linked carbo- γ^4 -amino acid and γ -aminobutyric acid (GABA) by alternating strategy Sharma and co-workers group experimentally confirmed the expected C_9 -helices structure.²⁵ In Figure 8, various hydrogen-bonded rings on helices are illustrated. Several types of γ -amino acid building blocks investigated in the scheme of γ -peptides are shown in Figure 9.

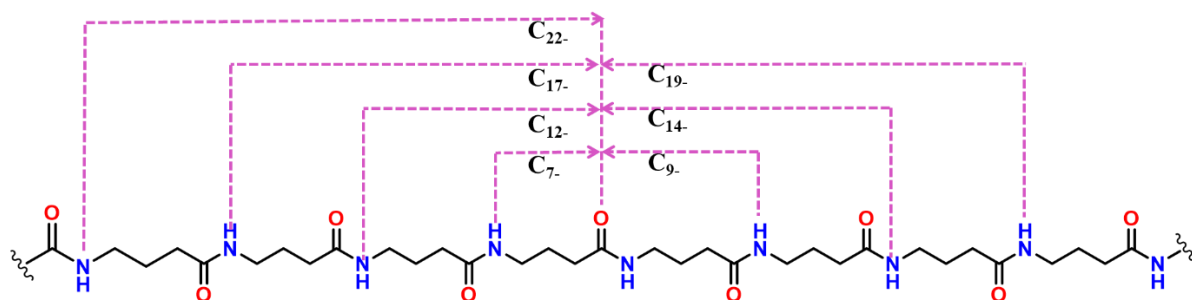


Figure 8: Systematic quantum calculations demonstrate the possible H-bonding pattern in γ -peptide oligomers.

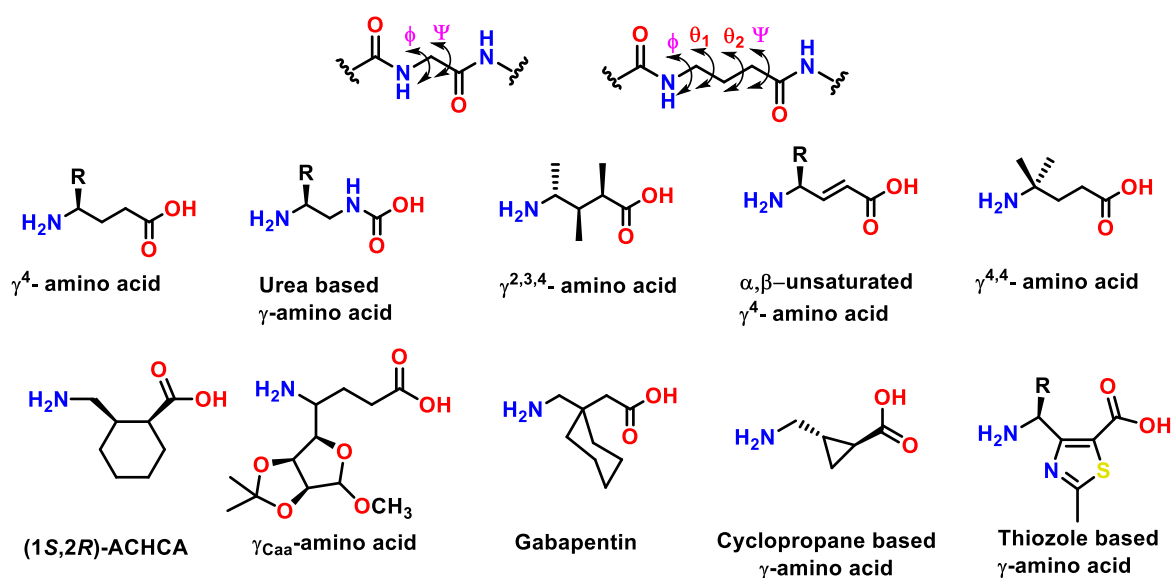


Figure 9: The chemical structure of γ -amino acids is utilised in the construction of foldamers.

In their pioneering work, Balaram and colleagues identified C₉ helix from the single crystal oligomers of gabapentin. By forming H-bonds between the *i* to *i* + 2 residues, the C₉ helical structure is stabilized.²⁶ Balaram and coworkers described the C₁₄ helical shape of an oligomer of γ^4 -amino acids with proteinogenic side chains (γ^4 -Val, γ^4 -Leu, γ^4 -Ile) in single crystals in addition to gabapentin.²⁷ The stable C₁₄ helices from the cyclic γ -amino acids were described by Gellman and colleagues.²⁸ Seebach and colleagues demonstrated the trisubstituted $\gamma^{2,3,4}$ -peptides 14-helical confirmation in addition to γ^4 -amino acids.²⁹ Figure 10 demonstrates the crystal structures of the γ -peptides 9 and 14 helices.

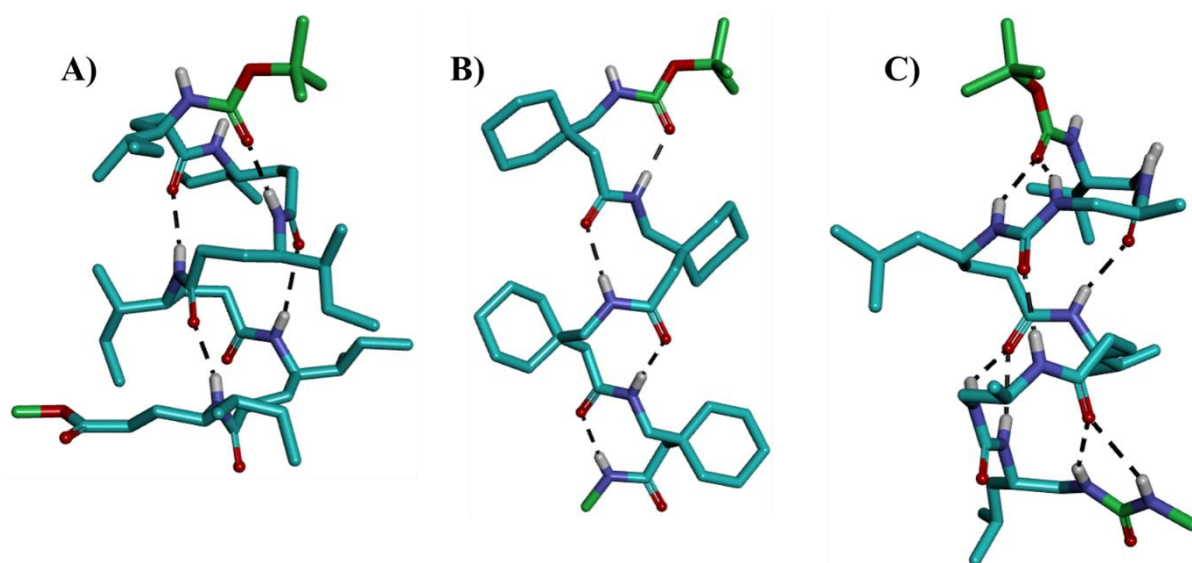


Figure 10: Crystal structure of A) C₁₄-helix of γ^4 -peptide,²⁷ (Boc-(γ^4 (R)Ile)₆-OMe), B) C₉-helix of gabapentin oligomers,²⁶ (Boc-(Gpn)₄-NHMe) and C) C₁₄-helical conformation of oligoureas,³² (Light sea green color represents γ -amino acids).

In addition, Smith and colleagues designed the cyclopropane γ -peptides and investigated their structural behavior.³⁰ The endless parallel sheet confirmation was adopted by the short oligomer, which was stabilized by diverged H-bonding between one CH of the cyclopropane ring and the carbonyl oxygen of the amide NH group. This is in contrast to the helical hallmark. They subsequently utilized a non-peptidic reverse turn to design a hairpin conformation using this β -sheet promoting feature.³⁰ The $\gamma^{2,3}$ -*trans*-dioxolane homooligomers have been demonstrated to form C₇ helix in solution, in contrast to extended sheets.³¹ There are several reports on the use of γ -amino acids with backbone heteroatoms such as O and N in addition to these acyclic and cyclic γ -amino acids. A variety of γ -peptide oligoureas were examined for their structural characteristics by Guichard and his associates. Similar to other γ -peptide helices, these γ -peptide oligoureas acquire a 14-helical conformation.³² Recently, Millard and

his colleagues demonstrated the existence of fine-defined 9-helix from γ -amino acid residues with thiazole backbones in both the solution and solid states.³³ The structural preference of both β - and α -aminoxy peptides was demonstrated by Yang and colleagues.³⁴ They observed C_8 helical conformations in the case of α -aminoxy peptide foldamers, and turns and C_9 helices in the case of β -aminoxy peptide foldamers. LeGrel *et al.* also found that the NH moiety is inserted into the backbone of β^3 -amino acids between the amine and β -carbon, resulting in aza-amino acids.³⁵ The conformations of azapeptides are stabilised by small range turns similar to H-bonds, like amino oxypeptides.

1.6. Heterogeneous Foldamers containing β - and γ -Amino Acids

In contrast to their homogeneous backbone counterparts, mixed heterogeneous peptides have recently attracted a lot of attention. This is due to the fact that altering the alignment and amino acids order in the sequence of the peptide allows one to produce a range of helical foldamers with various H-bonding patterns. Balaram and his co-workers revealed the structures of hybrid peptide made of higher homologous amino acids and β -amino acids into host alpha-peptide helices and β -hairpins in their pioneering study.³⁶ Hofmann's groups anticipated numerous types of helices that could be found in mixed α,β -hybrid peptides using systematic quantum chemical calculations.³⁷ Additionally, researchers found that glycine and β -alanine containing α,β -peptides can able to fold into 8 distinct stabilized helix, including 9-, 9/11, 11/9, 11, 12/13, 15/14, 18/16, and 16/18 helices. The dynamic stability in solution between 14/15 and 11 helical conformations was described by Jagadeesh *et al.*³⁸ using an α,β -hybrid peptide composed L-alpha-Ala and *cis*- β -furanoid sugar amino acid (*cis*-FSAA). Raiser and colleagues designed the hybrid peptides consists of L-alpha-Ala and *cis*-2-aminocyclopropanecarboxylic acid (*cis*-Acc), and also demonstrated that, 3_{13} -helical structure stabilised by $i \rightarrow i+2$ H-bonds.³⁹ In a fascinating work, Gellman and colleagues revealed the crystallographic conformation of *trans* 2-aminocyclopentane carboxylic acid and its derivatives (*trans*-ACPC amino acids) and Aib in short α,β -hybrid peptides with backbone constraints. These hybrid peptides fold into an 11-helix, but when the N-terminal amino acid Aib was replaced with an α -amino acid alanine, they transformed into a 14/15-helices, indicating that between these two helices having a delicate borderline energies.⁴⁰ Sharma and colleagues revealed that, from the hybrid α,β -sequence consists of α -amino acid and β^3 -amino acid adopt 11/9 helices with alternate H-bonding directionality.⁴¹ Recently, Amblard *et al.* demonstrated the conformational shift between 18/16-helix and 9/11-helix from α,β -hybrid peptides composed of tri-substituted β -amino acid

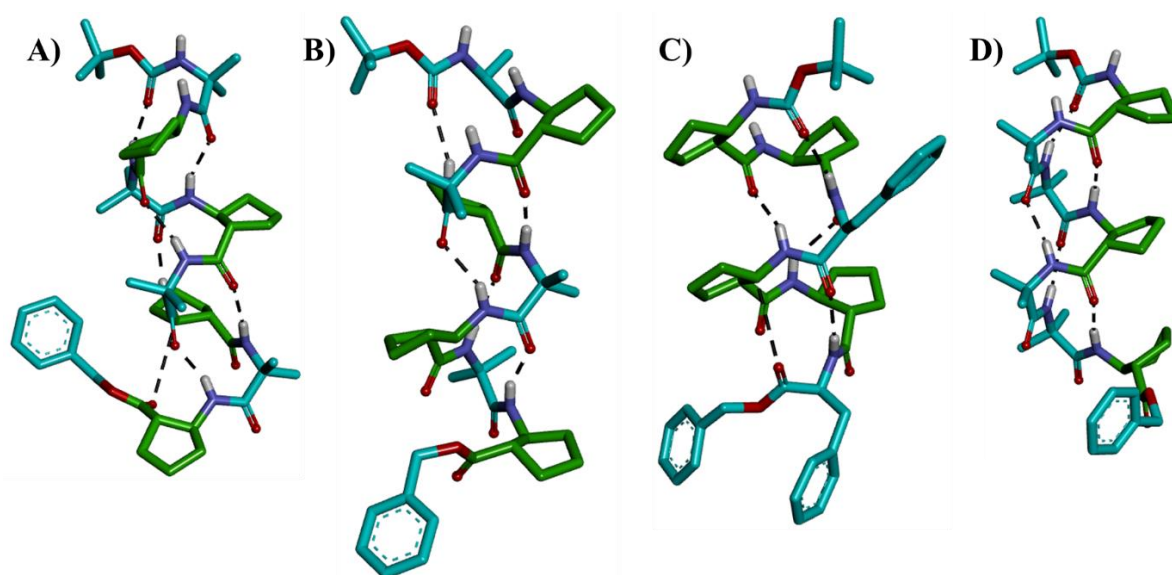


Figure 11: Crystal structure of A) 11-helix from α,β -hybrid peptide,^{40a} B) 14/15-helix from α,β -hybrid peptide,^{40b} C) 12/11/11-helix from β,β,α -hybrid peptide,⁴³ D) 10/11/11-helix from α,α,β -hybrid peptide.⁴³ (Green color represented the β -amino acids).

[(S)-ABOC].⁴² Further, $C_{10/11/11}$ -helix and $C_{11/11/12}$ -helix formation in 2:1 and 1:2 α,β -hybrid peptides were shown by Gellman and his co-workers.⁴³ In addition to this, Gellman and colleagues demonstrated remarkable helix bundle quaternary arrangements in oligomers consists of repeats with $\alpha-\alpha-\beta-\alpha-\beta$ and $\alpha-\beta-\beta-\alpha-\beta-\beta$.⁴⁴ The stereo chemical arrangements in the confirmations of α,β -hybrid peptide foldamers were examined by Fulop and coworkers.⁴⁵ Figure 11 illustrates various sorts of β -amino acids used to mimic protein structures with various H-bonding patterns.

The structural characteristics of α,γ -hybrid peptides have also been studied, in part as a result of the conformational diversity shown in α,β -hybrid peptides. Hofmann and his co-workers anticipated various helical forms that would be accessible to the oligomers of alternating (1:1) α - and γ -amino acids by quantum chemical calculations. They suggested the 12-helix conformation as the utmost stable helical conformation, followed by mixed 18/20 or 12/10 helices.⁴⁶ Further, Balaram and colleagues showed that α,γ -hybrid composed of 1:1 Aib and Gabapentin residue formed a 12 helix.^{47a} In a hybrid peptide with a 1:1 ratio of α - and γ^4 -amino acids, stable C_{12} -helix structure was observed and the structure was stabilised through intramolecular hydrogen bonding between the i and $i+3$ residues.^{47b} Continued from the previous section, Sharma and his co-workers described the 12/10-helical mixed conformations

from numerous α,γ -peptides obtained from reiterating entity with alternate L- α -Ala and C-linked carbo- γ -amino acid from D-mannose (γ -Caa).⁴⁸ Further evidence of the design, synthesis, and characterization of several α,γ -peptides containing sterically restricted cyclic γ -amino acids was provided by Gellman's group.⁴⁹ In addition to the crystallographic characterization, the stable 12-helix structures in an aqueous buffer were also demonstrated by NMR research.⁵⁰ Gopi and his co-workers revealed co-existence of 12- and 15/17-helices from achiral α,γ -hybrid peptide in their recent work.^{51a} But the 12-helix changes into a 12/10-mixed helical form when the Aib residue is replaced by α -amino acids.^{51b} Furthermore, Gopi and his colleagues demonstrated how base-mediated $\alpha,\beta \rightarrow \beta,\gamma$ double-bond migration creates a stable C₁₂ helix from an unstructured α,γ -hybrid peptide.⁵² In addition to helical conformations, γ -amino acids were used to construct β -strands of β -hairpins and β -turns segments. The crystal structure of the β -hairpin from Boc-Leu-Phe-Val-Aib-Gpn-Leu-Phe-Val-OMe was reported by Balaram and his co-workers and also they showed the β -hairpin is stabilized through four cross-strand H-bonds with the Aib-Gpn fragment forms a 12-membered non-helical turn.⁵³ Additionally, Roy *et al.* showed the anti-parallel β -strands have a β -hairpin conformation from the hybrid peptide (Boc-Leu-Val- γ Abu-Val-^DPro-Gly-Leu- γ Abu-Val-OMe) composed of unsubstituted γ -amino acids.⁵⁴ There are limited publications on the structural characteristics of 1:1 alternating hybrid β,γ -peptides in addition to hybrid α,γ -peptides. Hofmann and colleagues systematic quantum calculations suggested that the stable 13-, 11-, mixed 20/22- or 11/13-, helices would be available to the hybrid β/γ -helices.⁵⁵ Figure 12 depicts the H-bonding pattern of hybrid β/γ -peptides.

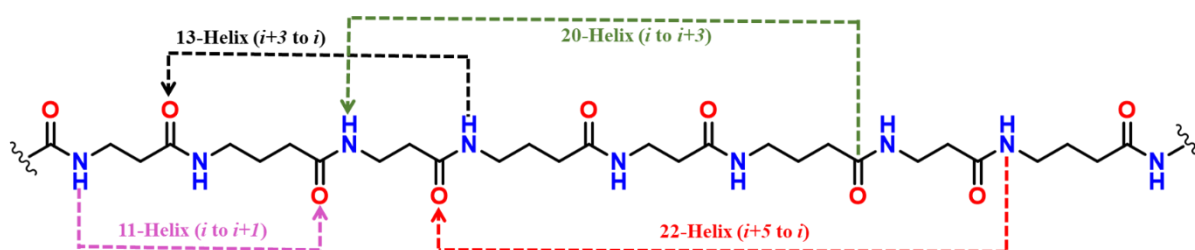


Figure 12: Based on quantum mechanical calculations, the possible H-bonding pattern in the β,γ -hybrid peptide.

Kunwar and Sharma have also demonstrated the 11/13 mixed helical conformations in solution from hybrid β/γ -peptides made of C-linked carbo- γ - and β -amino acids.⁵⁶ Gellman and his co-workers successfully demonstrated continuous 13-helix in hybrid β/γ -peptides, which is

identical to the α -helix, in both solution and in single crystals.⁵⁷ In addition, the 13-helix in the single crystal structure of the hybrid β,γ -peptide Boc- β Leu-Gpn-Val-OMe is described by Balaram and colleagues.⁵⁸ Similar helical characteristics to those of the α -helix can be seen in the 13-helical conformations of the β,γ -hybrid peptides. The structural characteristics of β,γ -hybrid peptides offered a unique chance to develop α -helix H-bond mimics. Additionally, Kokschi and associates have shown that β,γ -hybrid peptide fragments are incorporated into the α -peptide coiled coil sequences.⁵⁹ Aitken and colleagues also investigated the 13-helix from the unconstrained γ^4 -amino acids and the 9/8-ribbon structure from the hybrid β,γ -peptide with stereochemically controlled β -amino acids.⁶⁰ Various sorts of γ -amino acids used to mimic protein structures with various H-bonding patterns are presented in Figure 13.

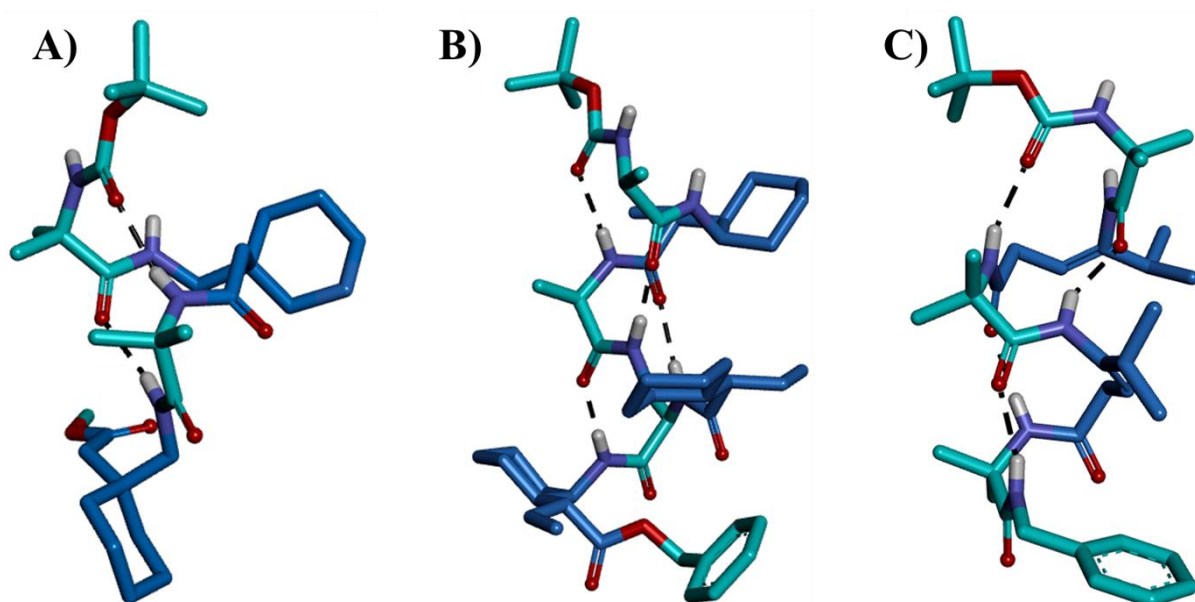


Figure 13: Crystal structure of A) 12-helix from α,γ -hybrid peptide Boc-Aib-Gpn-Aib-Gpn-OMe,^{47a} B) 12-helix from α,γ -hybrid peptide Boc- D Ala- Et ACHA- D Ala- Et ACHA- D Ala- Et ACHA-OBn,^{49a} C) C_{12} -helix from α,γ^4 -hybrid peptide BocNH-[Aib-(Z)- β,γ -unsaturated γ^4 -Val]₂-Aib-OBn.⁵² (Steel blue color represents γ -amino acids).

1.7. Biologically Active Peptide Foldamers

Although α -peptides are simple to develop to inhibit various kinds of protease inhibitors, protein-protein interactions, and antibacterial candidates, their applicability was limited by their proteolytic susceptibility. The fields of medicinal chemistry and chemical biology have given β -, γ - and the $\alpha\beta$ -, $\alpha\gamma$ -hybrid peptide foldamers a lot of interest because of their proteolytic stability and ability to fold rapidly into precise structures.⁶¹ Amphiphilic cationic 14-helical peptides designed from β -amino acids were described by Gellman and colleagues,

who also demonstrated their potency and bacterial selectivity.^{62a} In addition to that, they designed HCMV (Human Cytomegalovirus) entrance inhibitors based on β -peptides.^{62b} Further, Gellman and colleagues displayed amphiphilic hybrid α,β -peptides consists of β -amino acids in addition to homooligomers, and they exhibited strong antimicrobial activity against diverse gram-positive and gram-negative bacteria.^{62c} In addition, the development of inhibitors against the Bak/Bcl-xL interactions has also been investigated using, α,β -hybrid peptides.^{62d} The substance that blocks Bak/Bcl-xL communications most effectively adopts a 15/14 helix conformation in solution form. Further, Schepartz and colleagues developed p53 mimics from the β^3 -peptides and used them as p53-MDM2 interaction inhibitors.^{63a,63b} They also disclosed the design and synthesis of inhibitors for fusion caused by HIV-1 gp41.^{63c} Although they were not as thoroughly studied as β -peptide foldamers, foldamers formed from γ -peptide and their analogs have demonstrated various potential biological applications. Different γ -amino acids, including γ^2 -, γ^3 -, γ^4 -, and $\gamma^{2,3,4}$ - were used to design the peptide that demonstrated proteolytic stability against 15 proteolytic enzymes and did not degrade after 48 hours.^{11c} The helical γ -peptide foldamers as dual inhibitors of A β_{42} and IAPP oligomarization and fibrillation were demonstrated by Onger and her coworkers.⁶⁴ Hoyer and his co-workers demonstrated that, small γ -peptides exhibit submicro molar affinities with a variety of human somatostatin receptor types.⁶⁵ In addition, Bucci and his colleagues showed the oligonucleotide analogs derived from γ -peptides and γ/ϵ -peptides have a significant affinity for binding to DNA or RNA.⁶⁶ Royo and his colleagues have also investigated the cellular intake of *N*-functionalized hexamers of *cis*- γ -aminoproline.⁶⁷ Further, Gopi and co-workers demonstrated the design and synthesis of unsaturated^{68a} and saturated^{68b} α,γ -hybrid lipo peptides and also they explored their antimicrobial activity. Guichard and his colleagues showed the potent antimicrobial activity from the short α,γ -hybrid peptides.⁶⁹ Further Millard and his co-workers designed the thiazole based γ -peptide foldamers as a potent antimicrobial candidate.⁷⁰

1.8. Foldamers of α,β -unsaturated γ -amino acids

Vinylogous amino acid building block are α,β -unsaturated counterparts of the γ^4 -amino acids previously mentioned. α,β -Unsaturated (*E*)- Vinylogous amino acids are often found in various peptide natural products, such as miraziridine A, cyclotheonamide (A, B, C, D, and E), and gallinamide A, in contrast to saturated γ^4 -amino acids.⁷¹ These naturally arising peptides with vinylogous building blocks have the same cysteine and serine protease inhibitory properties as their synthesized analogs.⁷² (*E*)-vinylogous building blocks have been used as preliminary materials for several chemical reactions like epoxidation, Diels-Alder reaction, and

Michael addition reactions to yield a diversity of γ -amino acids in addition to their possible protease inhibitory function.⁷³ By their biological and chemical characteristics, several chemists were inspired to explore the conformational behavior of (*E*)-vinyllogous building blocks in monomers and different peptides. In their earlier study, Schreiber and colleagues described the unusual helical structure of the hybrid tetrapeptide and the extended β -sheet conformation from the dipeptides of (*E*)-vinyllogous building blocks.⁷⁴ In addition, Chakraborty *et al.* revealed how (*E*)-vinyllogous building blocks can be utilized to design β -hairpins with reverse turns.⁷⁵ Further, 9-helical conformations of (*Z*)-vinyllogous amino acid dipeptides were reported by Claude and colleagues.⁷⁶ In parallel, calculations using the ab initio MO theory on the oligomers of (*E*)- and (*Z*)-vinyllogous homooligomers have been reported by Hofmann and colleagues.⁷⁷ According to these results, larger H-bonded cycles with 14- to 27-members are preferred in higher oligomers of (*E*)-vinyllogous amino acids. However, (*Z*)-vinyllogous amino acid oligomers prefer the smaller hydrogen-bonded cycles, such as 9- and 12-helices. These findings also indicated that the bigger hydrogen-bonded helical structures of (*E*)-vinyllogous amino acids have an inner diameter big enough to pass the ions and molecules. The helical structures that were discovered through calculations using the ab initio MO theory are shown in Figure 14.

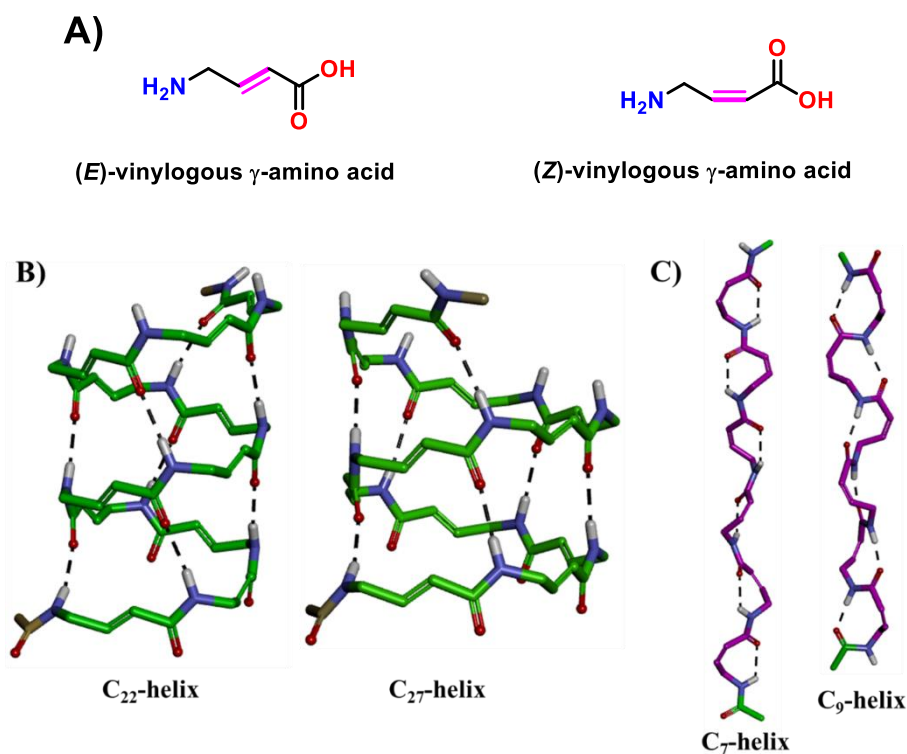


Figure 14: (A) The chemical structures of (*E*)- and (*Z*)-vinyllogous γ -amino acids. (B) Theoretically predicted structures of C_{22} - and C_{27} -helical homooligomers of (*E*)-vinyllogous γ -

amino acids.⁷⁷ (C) Theoretically predicted structures of C₇- and C₉-helical homooligomers of (Z)-vinyllogous γ -amino acids.⁷⁷

From our group recently showed anti-parallel β -strands by introducing (*E*)-vinyllogous γ -amino acids at the facing positions, reported the stable β -hairpin single conformations.^{78a} In addition to the β -hairpins, we have also designed miniature β -meanders,^{78b} and several β -sheet structures.^{78c} Additionally, we have used (Z)-vinyllogous γ -amino acids to create 12-helical structures from α,γ -hybrid peptide.^{78d} Recently, we also described the use of homo-oligomers of the 4,4-gem dimethyl, α,β -unsaturated γ -amino acid to create β -double-helical structures.^{78e,78f} Figure 15 displays the crystal structures of various α,β -unsaturated γ -amino acid foldamers.

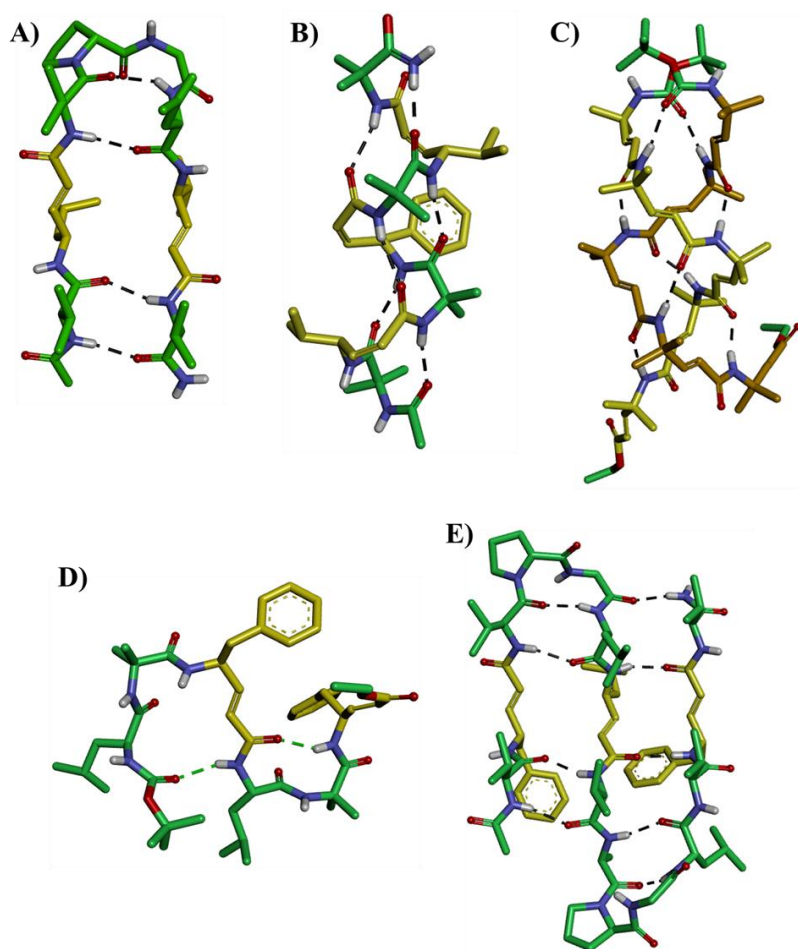


Figure 15: Crystal structure of various foldamers from vinyllogous γ -amino acids. (A) Stable β -hairpins from α,γ -hybrid peptide Ac-Val-d γ Leu-Val-DPro-Gly-Leu-d γ Val-Val-NH₂ composed of (*E*)-vinyllogous γ -amino acid,^{78a} (B) C₁₂-helical confirmation from α,γ -hybrid peptide (Ac-Aib-dgVal-Aib-dgPhe-Aib-dgVal-Aib-CONH₂) composed of (Z)-vinyllogous γ^4 -amino acids.^{78d} (C) β -double helical structures from γ -poly peptide (Boc-[dgAic]₄-OEt) composed of (*E*)-vinyllogous $\gamma^{4,4}$ -amino acid homooligomer.^{78e} (D) Miniature β -meanders,

super secondary structures from $\alpha\gamma$ -hybrid peptide Boc-Leu-Aib-dgPhe-Leu-Aib-dgPhe-OEt composed of (*E*)-vinyllogous γ - amino acid,^{78b} (E) Three stranded β -sheets from the peptide Ac-Val-dgPhe-Val-^DPro-Gly-Leu-dgLeu-Val-Ala-^DPro-Gly-Leu-Val-dgPhe-Val-NH₂ composed of (*E*)-vinyllogous amino acids.^{78c}

1.9. References

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Chapter 2

Mechanistic Study of Protease Resistant Antimicrobial Peptides against Drug Resistive Bacteria

2.1 Introduction

The increasing emergence and prevalence of antibiotic resistance among bacterial pathogens especially with Gram-negative bacteria has become a global public health challenge.¹ The discovery of new drugs at the time of the golden age of antibiotics became a revolution in biological treatment of infections. Nevertheless, the increasing resistance to these antibiotics leads to ineffective against most of the pathogens.² The misuse and/or overuse of any antibiotics will develop microbial resistance.²

It modifies the physiological channels that the antibiotic targets. The mutation makes the bacteria develop strain resistant to antibiotics.³ In the year 1943, it was first noted in the case of penicillin. Since then, practically all of the antibiotics that were used have been shown to be resistant to use. If we look at the development since 1980, we can see that the resistance time has substantially decreased to just one and a half years from the initial 10-15 years it used to take for bacteria to acquire antibiotic resistance.⁴ Some situations saw the emergence of the resistance in the same year the antibiotic was released. Figure 1 displays the timeline of the evolution of antibiotic resistance for different antibiotics.

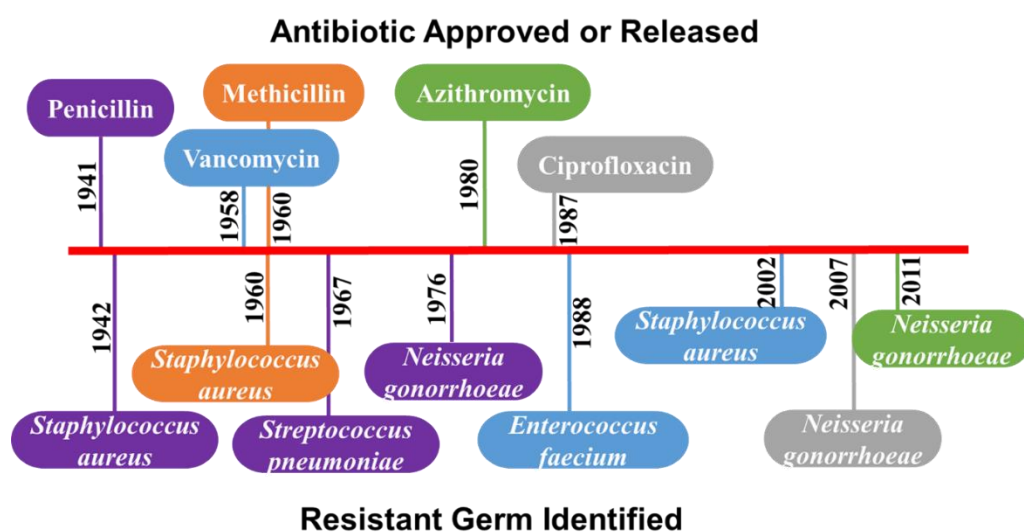


Figure 1: A timeline illustrating the development of antibiotic resistance.

Therefore, in present days there is an urgent need to developing and/or searching of new antibiotics to resolve this major problem. The results from the literature over the past several years suggested that, the antimicrobial peptides (AMPs) are isolated from almost all living organisms ranging from bacteria to humans and these peptides can able to show their role as a first line of defence against virulent pathogens.⁵ In general, the AMPs which are naturally available have a length ranging from 12-50 residues with great diversity of different amino acid

combinations to maintain cationic and hydrophobic nature, size and structure.⁶ These cationic based AMPs can effectively kill the bacteria through membrane lysis, this mechanism of action significantly having less chance to induce antibiotic resistivity.⁷ Indeed, AMPs can provide a great alternative strategy to kill the multidrug resistance pathogens.⁸ However, AMPs were lagging due to few drawbacks like their structural instability, poor selectivity, less bioavailability, toxicity, and week stability towards proteases.^{7c} To overcome these drawbacks, several strategies have been made to get better or to retain its activity by mimicking the amphiphilic and cationic character of natural AMPs including peptides with alternating L- and D- amino acids⁹ and other synthetic peptides,¹⁰ stapled peptides¹¹ β -peptides¹² and lipo β -peptides,¹³ γ -peptides,¹⁴ peptoids,¹⁵ peptidopolysaccharides,¹⁶ lipopeptides.¹⁴

The extensive literature survey on AMPs suggests that, due poor biocompatibility and proteolytic lability limit their potential for clinical usage. To overcome these major drawbacks to get good antimicrobial activity from the peptides is by making proteolytic resistance peptides with insertion of β - or γ - amino acids in the peptide backbone.^{14,17}

In that contest, γ -amino acids have demonstrated a greater inclination for helical structure, according to recent developments in the structural analysis of γ - and hybrid γ -peptides.¹⁸ The homooligomers of γ -amino acids have demonstrated the ability to adopt 14-helical conformations (14-membered H-bonds) without any restrictions on stereochemistry.¹⁹

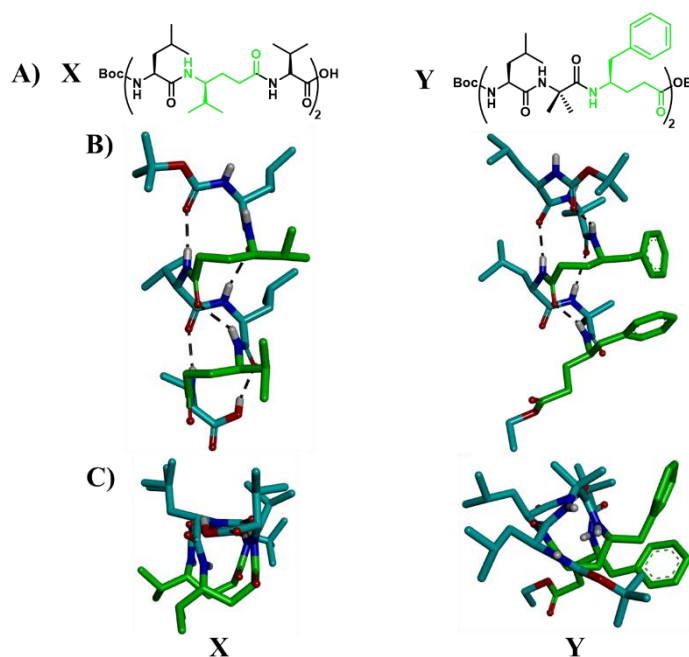


Figure 2: $\alpha\gamma$ -hybrid peptides. (A) Chemical structure of hybrid peptides X and Y. (B) Crystal structure of 10/12-helix from $\alpha\gamma$ -hybrid peptide X: Boc-(Leu- γ^4 (R)Val-Val)₂-OH and 10/12-

helix from $\alpha\alpha\gamma$ -hybrid peptide Y: Boc-(Leu-Aib- γ -Phe)₂-OEt. (C) Top views of the $\alpha\alpha\gamma$ -hybrid peptides.

The 12-helical (12-membered H-bonds) conformations of the hybrid peptides with 1:1 alternating α - and γ -amino acids have been proven to be stable.²⁰ Balaram and colleagues and we recently reported the 10/12-helical shape of a hybrid peptide made up of 2:1 α - and γ -amino acids in single crystals.^{21,22} Figure 2A displays the peptides structures and amino acid sequences. The side-chains of the amino acids are positioned at the three sides of the helical cylinder, which is an intriguing aspect of the 10/12-helix shown in Figure 2B and 2C. These foldamers based peptides will be conformationally rigid and stable. Due to their stability in the conformation we can able to predict their secondary structures for further modification. With this knowledge we propose that foldamer based AMPs can provide great potential to kill the Multi-Drug Resisitive (MDR) bacterial pathogens by rupturing the bacterial membrane.

2.2. Classification of AMPs:

It is challenging to classify natural AMPs due to their diversity. According to their (1) source, (2) activity, (3) structural characteristics, and (4) amino acid-rich species, AMPs are categorised, as shown in Figure 3.

2.2.1 Based on Sources: According to statistical information in antimicrobial peptide database (APD3), the origins of AMPs can be classified as mammals (of which human host defence peptides make up a significant share), amphibians, microbes, and insects. The AMPs discovered in oceans have also drawn a lot of interest.

2.2.2 Based on Activity: According to the statistics of the ADP3 database, there are 18 categories in which AMP activity can be classified. Antiviral, antibacterial, antiparasitic, anti-human immunodeficiency virus (HIV), anti-tumor and antifungal peptides can be used to describe these categories.

2.2.3 Based on Amino Acid-Rich Species

Proline-Rich Antimicrobial Peptides (PrAMPs): Basically proline is a non-polar amino acid. PrAMPs behave differently from other AMPs in that they kill bacteria by entering the bacterial cytoplasm via the inner membrane transporter SbmA rather than rupturing membranes.²³

Tryptophan (Trp)- and Arginine (Arg)-Rich Antimicrobial Peptides: As a non-polar amino acid, tryptophan (Trp) has a notable impact on the lipid bilayer's interface, whereas arginine (Arg), a basic amino acid, confers peptide charge and hydrogen bond interactions, which are crucial qualities to combine with the bacterial membranes abundant anionic component. Trp residues appear to act as Arg-rich AMPs natural aromatic activators through ion-pair- π interactions, which enhance peptide-membrane interactions.^{24,25} In addition to that, indolicidin and triptpticin, two well-known AMPs that are abundant in Arg and Trp residues.

Histidine-Rich Antimicrobial Peptides: Histidine is a basic amino acid, Histidine-rich AMPs exhibit strong membrane penetration activity. Based on $RR(XH)_2XDPGX(YH)_2RR-NH_2$ (where X stands for I, W, V, and F), HV2 is a histidine-rich AMP. This peptide makes bacterial cell membranes more permeable, which results in cell membrane rupture and death. In addition to that, by preventing the production of tumor necrosis factor α (TNF- α), HV2 exhibits a potent anti-inflammatory effect and restricts bacterial movement in a concentration-dependent manner.²⁶

Glycine-Rich Antimicrobial Peptides: Attacins and Diptericins, are majorly present in the nature as a glycine-rich Antimicrobial Peptides.^{27,28} These peptides have a significant impact on the tertiary structure of the peptide chain since they include 14% to 22% glycine residues. In contrast to typical AMPs, glycine-rich AMPs from salmonid cathelicidins stimulate phagocyte-mediated microbicidal processes.²⁹ Additionally, the centrally symmetrical glycine-rich GG3 is great potential for a commercial antibiotic against clinical Gram-negative bacteria.³⁰

2.2.4 Based on AMPs Structures: Based on their structural characteristics, antimicrobial peptides can be categorized into four groups: linear α -helical peptides, β -sheet peptides, linear extension structures, and both α -helix and β -sheet peptides.³¹ In addition, more complicated cyclic peptides and AMPs (such as lasso peptides and thioether bridged structures) are observed.³²

Linear AMPs: If the linear AMPs are not in contact with the hydrophobic bacterial membranes, they do not fold into a regular secondary structure. The peptides length can range from 10 to 50 residues, and it frequently contains high concentrations of specific amino acids like Trp, Arg, or Pro along with balance in their hydrophobicity and cationicity. They interact with intracellular proteins as well as disrupt the membrane. This group includes Histatin 5 from humans,³³ Proline-Arginine rich PR39 from pigs,³⁴ Apidaecin from honeybees,³⁵ and

Indolicidin, which is rich in tryptophan from cow.³⁶ Once they are in contact with the bacterial membrane, they develop a secondary α -helical shape.

β -Sheet AMPs: Defence miniproteins and various β -hairpin peptides contribute to this class of AMPs. They have also been discovered to prevent the synthesis of DNA, RNA, and proteins in addition to rupturing the bacterial cell membrane. By forming toroidal pores, the peptides damage the bacterial membranes. Disulfide bridges (one, two, or more) between the Cys residues stabilize the β -sheet structures.³⁷ The largest 45 residue peptide known as human β -defensin-3 contains a three-stranded antiparallel β -sheet. Insect defensins,³⁸ Bovine lactoferricin,³⁹ Protegrin I⁴⁰ and Human defensins⁴¹ are examples of sheet AMPs.

Helical AMPs: Cationic amphipathic helical peptides are a significant type of AMPs that have undergone extensive research. To achieve amphipathicity, the hydrophobic and cationic residues are uniformly distributed along the axis of the α -helix. The peptides cluster around the Ca^{2+} binding sites on the surface of the microbial membrane, travel into the cellular membrane, and ultimately break the membrane. African clawed frog's Magainin,⁴² Cecropin from silk moth's,⁴³ human AMP LL-37,⁴⁴ Alamethicin,⁴⁵ Buforin II from vertebrates⁴⁶ and synthetic peptide pexiganan⁴⁷ belong within this category. New synthetic antimicrobial peptides are created using the structure of α -helical peptides as a model.

Lipid tagged AMPs: Only bacteria and fungi are capable of producing lipopeptides when they are cultivated.⁴⁸ They are made up of a short linear or cyclic peptide sequence that has a long chain fatty acid covalently attached at the N-terminus. The antibacterial and antifungal properties of lipopeptides are both present.⁴⁹ Both cationic and anionic compounds, such as Polymyxin⁵⁰ and Daptomycin,⁵¹ can be used. In order to damage the bacterial membrane, lipopeptides frequently self-aggregate on its surface. Native lipopeptides, sadly, are harmful to both mammalian and non-mammalian cells since they are not cell-selective. Although the antimicrobial peptides exhibit a wide range of structural variations, disruption of the cellular membrane has been identified as a common mechanism of action.

2.3. Mechanism of Action of AMPs

By disrupting the bacterial membrane, the AMPs cause permanent harm. There are numerous ways to accomplish this. Additionally, they are discovered to enter the microbial cell and disrupt a number of internal processes that result in cell death. The antimicrobial peptides have a dual mechanism of action as a result. They affect both intracellular targets and the bacterial

membrane. The amphipathic structure of AMPs is utilized for interaction with the membrane. Below, it is covered in more detail.

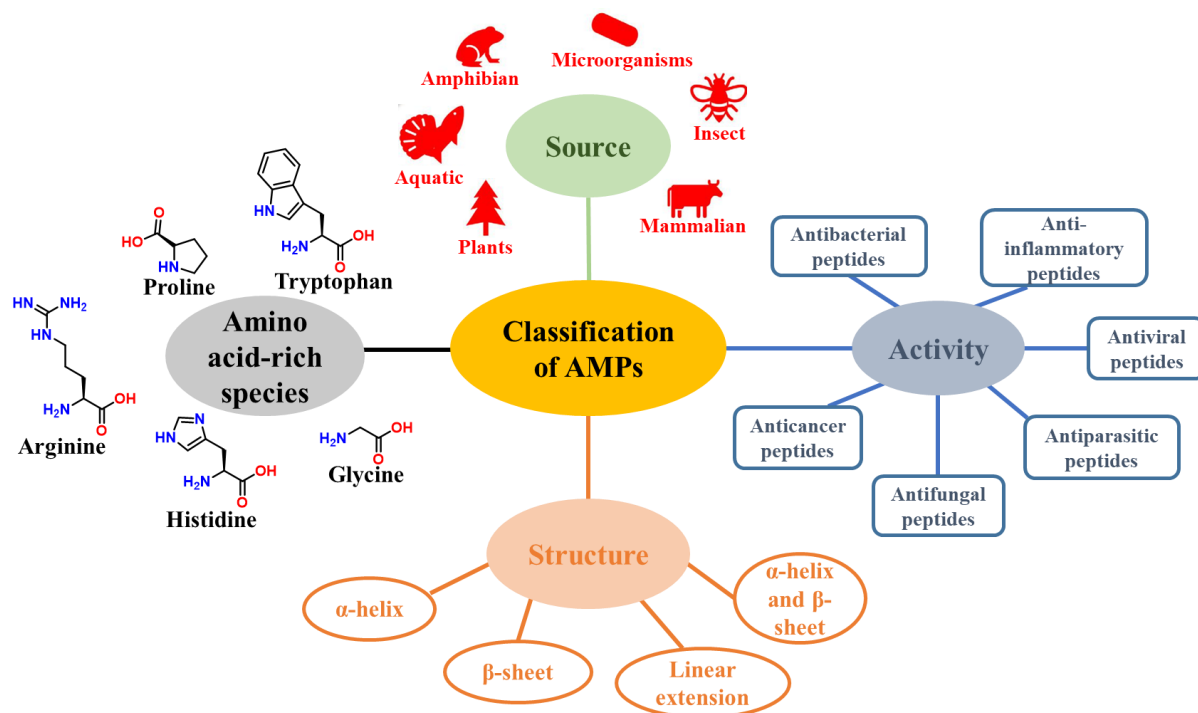


Figure 3: Antimicrobial peptide classification.⁵²

2.3.1. Membrane Targeting Mechanism

Most of the antimicrobial peptides specifically target bacterial cell membranes, because bacterial cell membranes are designed differently than plant and animal cell membranes.⁵³ The bacterial cell membrane outer layer is densely packed with phospholipids that are negatively charged. As a result, the cationic AMPs have a significant interaction with the bacterial membrane. However, the negatively charged head groups of animal and plant cells are projected inside the bilayer and are not visible on the surface, leaving the outer membrane of these cells neutral. Additionally, animal cell cholesterol stabilizes the lipid bilayer and reduces the effectiveness of the antimicrobial peptide.⁵⁴ Figure 4 depicts the interaction of the antimicrobial peptide with the mammalian membrane and the bacterial cell membrane.

The direct activity of AMPs is largely dependent on contact with the membrane. Numerous AMPs cause the microbial membrane's physical activity to be disrupted and/or move through the membrane into the bacterial cytoplasm to act on an intracellular target. With the help of

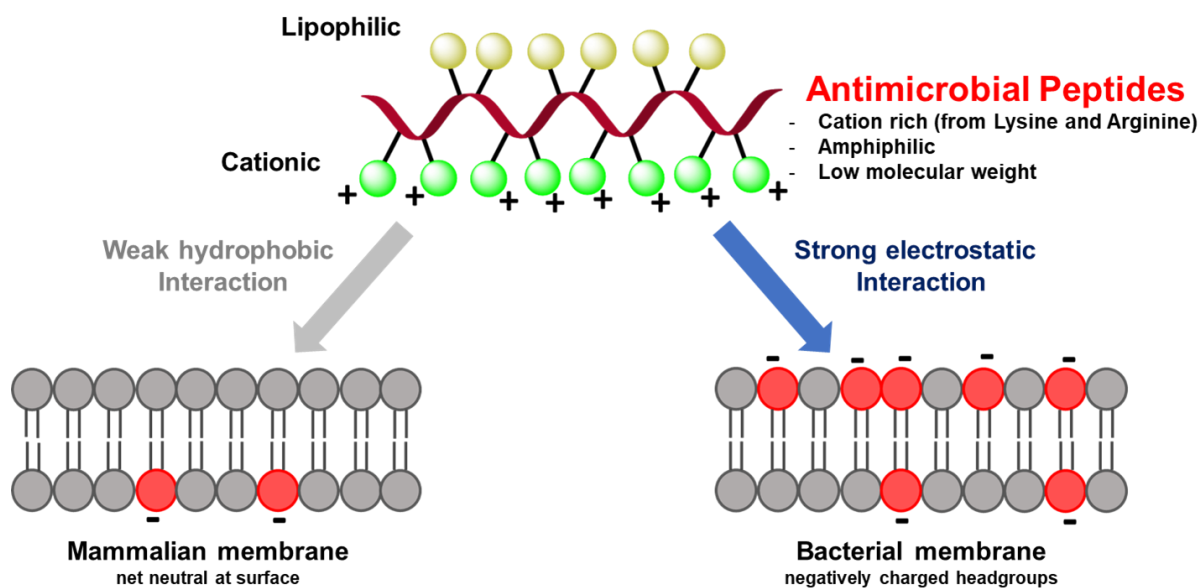


Figure 4: Schematic representation of the positively charged antimicrobial peptides (AMPs) interactions with bacterial and mammalian cell membranes.

membrane lysis (from colistin,⁵⁵ bacitracin,⁵⁶ daptomycin,⁵⁷ and polymyxin B⁵⁸), membrane poration (from gramicidin D⁵⁸ and tyrothricin⁵⁹), or the inhibition of cell wall formation (from teicoplanin⁶⁰ and vancomycin⁶¹), AMPs can destroy bacterial membrane integrity and cause death. Bacteria having a negative surface charge, such as Gram-negative and Gram-positive bacteria, can interact electrostatically with cationic peptides. Thus, lipopolysaccharides in the outer membrane of Gram-negative bacteria and teichoic acids in the cell walls of Gram-positive bacteria contribute extra electronegative charge to the bacterial surface. To describe how AMPs interact with the bacterial membrane, various models have been proposed. Models such as the pole and carpet models, as well as the toroidal pore and barrel-stave variants of the pole model, can be used to explain the membrane-targeting mechanisms of AMPs. So far, three approaches for rupturing the inner membrane have been described: (i) **In the barrel-stave model**,^{62a,62b} peptides can enter the membrane perpendicularly, encouraging peptide-peptide interactions due to the amphipathic structure of the AMP and leading to the formation of a peptide-lined transmembrane pore is shown in Figure 5 (A), (ii) Regarding the **toroidal pore model**,^{63(a-c)} a pore is formed by both the peptide and the phospholipid head group, and a curvature in the lipid layer triggers the insertion of the peptides is shown in Figure 5 (B), and (iii) As a result of the peptide's complete coverage of the membrane in the **carpet model**,^{64a,64b} the membrane integrity is lost and leads to the formation of micelles are shown in Figure 5 (C),

2.3.2. Non-Membrane Targeting Mechanism

AMPs enter cells either directly or by endocytosis. AMPs will recognize and interact with the target after entering the cytoplasm. By interacting with specific proteins involved in the biological process, peptides can translocate into the cytoplasm and directly block the synthesis of cell walls⁶⁵ and proteins,⁶⁶ bacterial cell division,⁶⁷ or DNA replication⁶⁸ is shown in Figure 5 (D). Histatins penetrate the cytoplasm and cause cells that are actively respiring to lose non-lytic amounts of ATP, which disrupts the cell cycle.⁶⁹ Mersadycin combines with lipid II to prevent the production of the cell wall.⁶⁵ *S. typhimurium* becomes filamentous when PR-39 is added, which causes the cells to elongate and further unable to undergo cell division.⁷⁰ Additionally, it prevents the production of proteins and causes the degradation of several proteins necessary for DNA replication. Tachyplesin⁷¹ and buforin II⁷² stick to DNA and RNA and regulate the proteins biosynthesis. AMPs can quickly destroy a variety of bacteria because of their multiple modes of action.

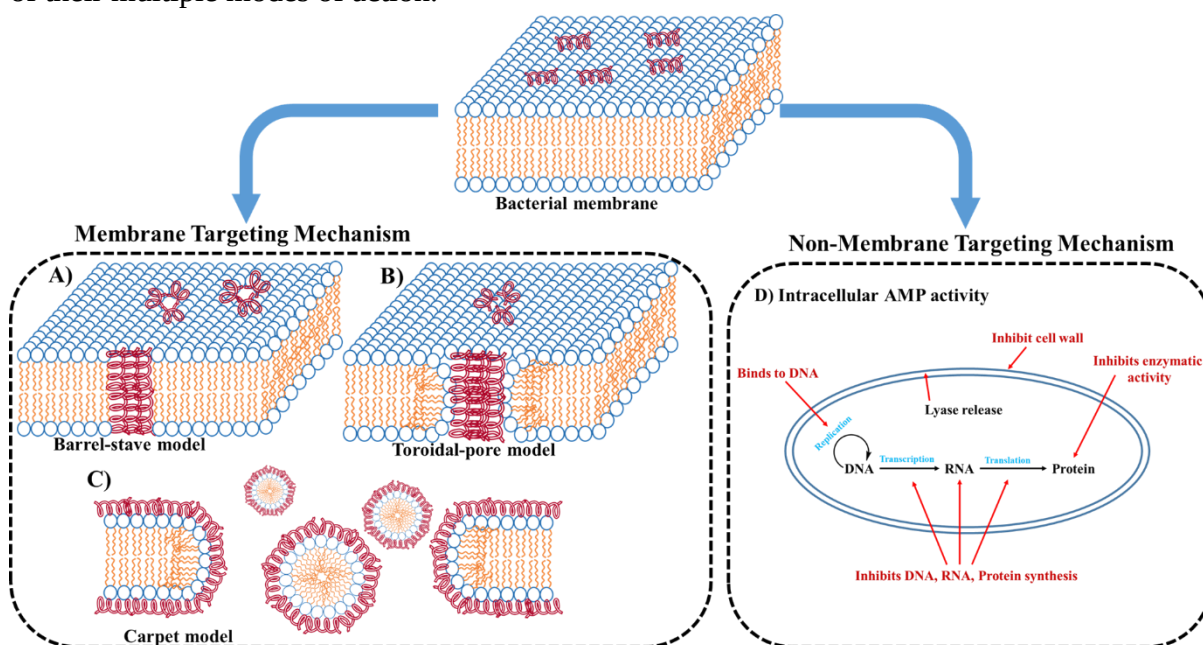


Figure 5: Models of action for extracellular AMP activity. (A) Barrel-Stave model, (B) Toroidal pore model, (C) Carpet model. (D) Model of action for intracellular AMP activity.

2.4 Aim and rational of the present work

From the previous studies, on the basis of structural evidence of $\alpha\gamma$ -hybrid peptides, we sought to investigate the antimicrobial activity from the cationic based AMPs by screening the newly designed $\alpha\gamma$ -hybrid peptides by incorporating non-natural amino acids in the peptide sequence along with α -peptides. Broad spectrum of AMA of the newly designed peptide foldamers against virulent ESKAPE pathogens was conducted to understand the antibacterial activity of

these designed amphiphilic foldamers. The activity of new peptides to control the growth of bacteria was determined by analyzing the Minimum Inhibitory Concentration (MIC) values and also with Methicillin-sensitive *Staphylococcus aureus* (MSSA), Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant *Staphylococcus aureus* (VRSA) were tested. Along with antimicrobial activity, their Time Kill Kinetic study, Post Antibiotic Study (PAE), haemolytic activity, cytotoxicity were also investigated. In addition, to check their activity in the *in-vivo* studies we performed Murine Skin Infection Model and also with Murine Neutropenic Thigh Model. Further, to know the mechanism of action of designed peptide to kill the growth of the bacteria, we tested the morphology of the bacteria with peptides by taking FE-SEM and confocal images. In continuation, to know the specific interaction of the peptides with bacteria we performed the different membrane assays (1-NPN assay, β -galactosidase assay) and also Fluorescence-activated cell sorting (FACS) analysis was carried out to know the exact mechanism action of peptide to kill the bacteria. Further, we showed the proteolytic stability of these $\alpha\gamma$ -hybrid AMPs.

2.5 Results and Discussions

2.5.1 Peptide design and synthesis

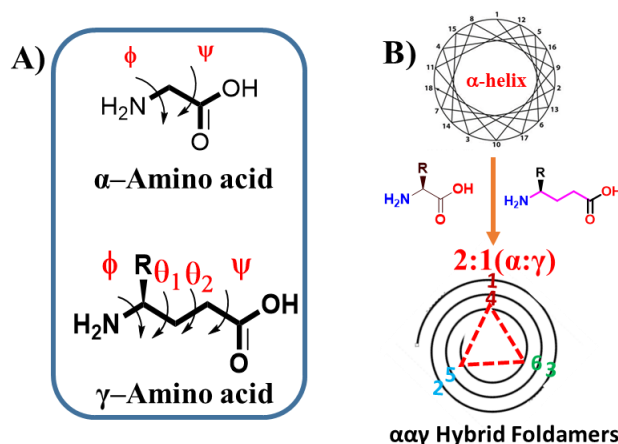


Figure 6: A) Structure of the monomeric building blocks of α - and γ^4 -amino acid. B) Helical wheel diagram depicting side chain projection in α/γ^4 -hybrid peptides. γ^4 -amino acids are shown in green.

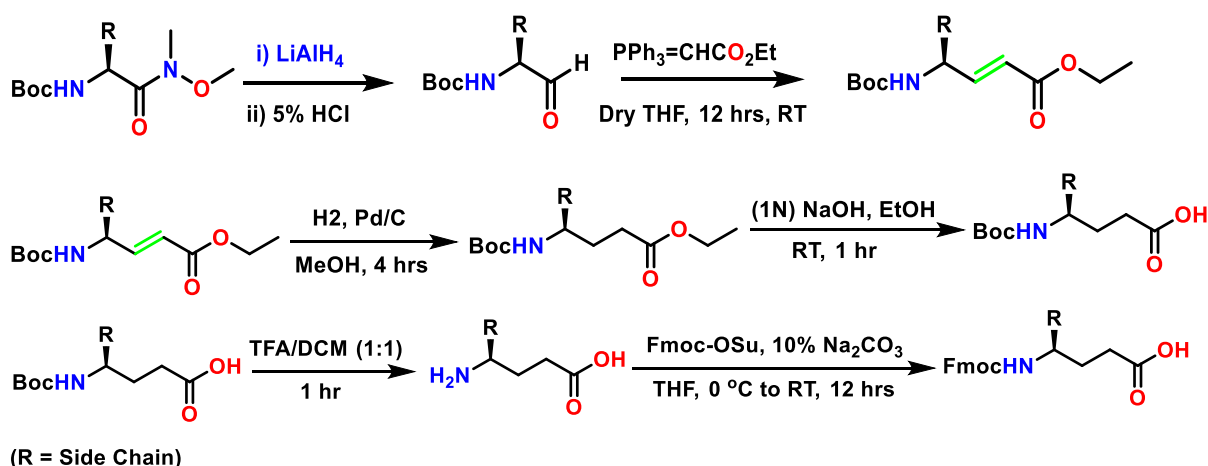
Hybrid foldamers are receiving greater attention because of their stable helical peptidomimetic scaffold because the alpha helix lacks stability and residues are diffusing throughout the helical cylinder.²² Balaram and his co-workers and also from our group reported the hybrid peptide with the combination of α - and γ -amino acids in the ratio of 2:1 and showed the 10/12-helical conformation in a single crystal.^{21,22} The interesting property of the 10/12-helix is that the

residues or side chain of the amino acids are projected in three positions of the helical cylinder shown in the Figure 6. This type of special arrangement of side chain of the residues in the helical cylinder provides an opportunity to design unique amphiphilic helices by consuming less number of γ -amino acids. In this way we can accommodate the cationic moiety majorly at one face of the helical cylinder to have more electrostatic interaction with negatively charged membrane to control the bacterial growth. In this work we showed the screening of newly designed $\alpha\gamma$ -hybrid peptides along with α -peptides to find good antimicrobial peptides. In Table 1, the sequence of the designed $\alpha\gamma$ -hybrid peptides and α -peptides is displayed. In the beginning we designed the peptides **P1-P4** by keeping the Lys-Leu-Ala as a repeating unit in both alpha and hybrid peptides. Later we replaced all the Lys with Arg in the peptides **P6-P7**. Further, for more screening purposes we synthesised peptides **P8-P22** by changing the position of Arg, by providing more hydrophobicity and also by changing the peptide length. In addition to this we synthesized peptides **P23-P29** by attaching different lipid tails at the N-terminal of the active peptides. After screening all these peptides, for comparative study with highly active peptide **P9**, we synthesised peptides **P30** and **P31** by replacing all Arg with Lys in the peptide **P9**. All gamma amino acid was synthesised by using the known protocol explained in Scheme 1.²⁰ Further, these modified γ^4 -amino acids will be protected with Fmoc-Osu to use in SPPS. All of the peptides were synthesized using conventional Fmoc-Chemistry on Knorr Amide resin. All peptides were purified on a C₁₈ column using reversed-phase HPLC. The mass of the purified peptides was further confirmed by MALDI-TOF/TOF analysis. The lyophilized pure peptides was used to check their AMA.

Table 1: Sequence of the designed α - and $\alpha\gamma$ -hybrid peptides (**P1-P31**).

SL. No	Peptide Code	Sequence
1	P1	H ₂ N-Lys-Leu-Ala-Lys-Leu-Ala-Lys-Lys-Leu-Ala-Lys-Leu-Ala-Lys-Leu-CONH ₂
2	P2	Ac-Lys-Leu-Ala-Lys-Leu-Ala-Lys-Lys-Leu-Ala-Lys-Leu-Ala-Lys-Leu-CONH ₂
3	P3	H ₂ N-Lys-Leu- γ Ala-Lys-Leu- γ Ala-Lys-Lys- γ Leu-Ala-Lys- γ Leu-Ala-Lys- γ Leu-CONH ₂
4	P4	Ac-Lys-Leu- γ Ala-Lys-Leu- γ Ala-Lys-Lys- γ Leu-Ala-Lys- γ Leu-Ala-Lys- γ Leu-CONH ₂
5	P5	Ac-Arg-Leu-Ala-Arg-Leu-Ala-Arg-Arg-Leu-Ala-Arg-Leu-Ala-Arg-Leu-CONH ₂
6	P6	Ac-Arg-Leu- γ Ala-Arg-Leu- γ Ala-Arg-Arg- γ Leu-Ala-Arg- γ Leu-Ala-Arg- γ Leu-CONH ₂
7	P7	H ₂ N-Arg-Leu-Leu-Arg-Leu-Leu-Arg-Arg-Leu-Leu-Arg-Leu-Leu-Arg-Leu-CONH ₂
8	P8	Ac-Arg-Leu-Leu-Arg-Leu-Leu-Arg-Arg-Leu-Leu-Arg-Leu-Leu-Arg-Leu-CONH ₂
9	P9	H ₂ N-Arg-Leu- γ Leu-Arg-Leu- γ Leu-Arg-Arg- γ Leu-Leu-Arg- γ Leu-Leu-Arg- γ Leu-CONH ₂
10	P10	Ac-Arg-Leu- γ Leu-Arg-Leu- γ Leu-Arg-Arg- γ Leu-Leu-Arg- γ Leu-Leu-Arg- γ Leu-CONH ₂
11	P11	H ₂ N-Arg-Leu-Leu-Arg-Leu-Leu-Arg-Arg-Leu-Leu-Arg-Leu-CONH ₂
12	P12	Ac-Arg-Leu-Leu-Arg-Leu-Leu-Arg-Arg-Leu-Leu-Arg-Leu-CONH ₂
13	P13	H ₂ N-Arg-Leu- γ Leu-Arg-Leu- γ Leu-Arg-Arg- γ Leu-Leu-Arg- γ Leu-CONH ₂

14	P14	Ac-Arg-Leu- γ Leu-Arg-Leu- γ Leu-Arg-Arg- γ Leu-Leu-Arg- γ Leu-CONH ₂
15	P15	H ₂ N-Leu-Arg-Leu-Leu-Arg-Leu-Leu-Arg-Leu-Leu-Arg-Leu-Leu-Arg-Leu-CONH ₂
16	P16	Ac-Leu-Arg-Leu-Leu-Arg-Leu-Leu-Arg-Leu-Leu-Arg-Leu-Leu-Arg-Leu-CONH ₂
17	P17	H ₂ N-Leu-Arg-Leu-Leu-Arg-Leu-Leu-Arg-Leu-Leu-Arg-Leu-CONH ₂
18	P18	Ac-Leu-Arg-Leu-Leu-Arg-Leu-Leu-Arg-Leu-Leu-Arg-Leu-CONH ₂
19	P19	H ₂ N-Leu-Arg-Ala-Leu-Arg-Ala-Leu-Arg-Ala-Leu-Arg-Ala-Leu-Arg-Ala-CONH ₂
20	P20	Ac-Leu-Arg-Ala-Leu-Arg-Ala-Leu-Arg-Ala-Leu-Arg-Ala-Leu-Arg-Ala-CONH ₂
21	P21	H ₂ N-Leu-Arg-Ala-Leu-Arg-Ala-Leu-Arg-Ala-Leu-Arg-Ala-CONH ₂
22	P22	Ac-Leu-Arg-Ala-Leu-Arg-Ala-Leu-Arg-Ala-Leu-Arg-Ala-CONH ₂
23	P23	C ₁₂ -Arg-Leu-Ala-Arg-Leu-Ala-Arg-Arg-Leu-Ala-Arg-Leu-Ala-Arg-Leu-CONH ₂
24	P24	C ₁₂ -Arg-Leu- γ Leu-Arg-Leu- γ Leu-Arg-Arg- γ Leu-Leu-Arg- γ Leu-Leu-Arg- γ Leu-CONH ₂
25	P25	C ₁₀ -Arg-Leu- γ Leu-Arg-Leu- γ Leu-Arg-Arg- γ Leu-Leu-Arg- γ Leu-Leu-Arg- γ Leu-CONH ₂
26	P26	C ₈ -Arg-Leu- γ Leu-Arg-Leu- γ Leu-Arg-Arg- γ Leu-Leu-Arg- γ Leu-Leu-Arg- γ Leu-CONH ₂
27	P27	C ₁₂ -Arg-Leu- γ Leu-Arg-Leu- γ Leu-Arg-Arg- γ Leu-Leu-Arg- γ Leu-CONH ₂
28	P28	C ₁₀ -Arg-Leu- γ Leu-Arg-Leu- γ Leu-Arg-Arg- γ Leu-Leu-Arg- γ Leu-CONH ₂
29	P29	C ₈ -Arg-Leu- γ Leu-Arg-Leu- γ Leu-Arg-Arg- γ Leu-Leu-Arg- γ Leu-CONH ₂
30	P30	H ₂ N-Lys-Leu- γ Leu-Lys-Leu- γ Leu-Lys-Lys- γ Leu-Leu-Lys- γ Leu-Leu-Lys- γ Leu-CONH ₂
31	P31	Ac-Lys-Leu- γ Leu-Lys-Leu- γ Leu-Lys-Lys- γ Leu-Leu-Lys- γ Leu-Leu-Lys- γ Leu-CONH ₂



Scheme 1: Monomeric building block of γ^4 -amino acid synthetic procedure.

2.5.2. *In vitro* antimicrobial activity

2.5.2.1. Determination of MIC ($\mu\text{g/mL}$) of designed peptides against ESKAP pathogen panel

The *in vitro* antimicrobial activity of peptides was assessed by measuring the minimum inhibitory concentrations (MICs) using the broth microdilution method on the clinically significant pathogenic bacteria in humans (ESKAP pathogen panel) consisting of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Klebsiella pneumoniae* (BAA-1705), *Acinetobacter baumannii* (BAA-1605) and *Pseudomonas aeruginosa* (ATCC 27853) and the results are outlined in Table 2. The Clinical Laboratory Standards Institute (CLSI)

criteria were followed while determining the MIC.⁷³ Levofloxacin was employed as the reference standard, and the peptide concentration used was between 64 and 0.03 µg/mL.

The lysine contained peptides **P1** to **P4** (Table 1, **P1**, **P2** are alpha peptides and **P3**, **P4** are $\alpha\alpha\gamma$ -hybrid peptides) displayed the weakest antimicrobial activity (MIC = $\geq$ 64 µg/mL) across the stains tested in this study. Further, all lysine residues (**P2** and **P4**) were replaced by arginine (**P5** and **P6**) (Table 1), interestingly peptide **P5** (15 mer-alpha peptide) exhibited good inhibitory activity against **ESKAP** pathogen panel (MIC = 1 to 16 µg/mL) with a greater than

Table 2: MIC (µg/mL) of designed peptides against ESKAP pathogen panel.

Peptides	Gram +ve	Gram -ve			
	<i>S. aureus</i> ATCC 29213	<i>E. coli</i> ATCC 25992	<i>K. pneumoniae</i> BAA-1705	<i>A. baumannii</i> BAA 1605	<i>P. aeruginosa</i> ATCC 27853
P1	>64	>64	>64	>64	>64
P2	>64	>64	>64	>64	>64
P3	>64	>64	>64	>64	>64
P4	>64	>64	>64	>64	>64
P5	4	16	16	1	>64
P6	>64	64	>64	>64	>64
P7	NT	NT	NT	NT	NT
P8	>64	16	>64	16	>64
P9	2	2	8	4	16
P10	64	64	64	64	64
P11	NT	NT	NT	NT	NT
P12	NT	NT	NT	NT	NT
P13	NT	NT	NT	NT	NT
P14	2	4	16	4	32
P15	>64	>64	>64	>64	>64
P16	16	>64	>64	64	>64
P17	4	8	16	8	32
P18	NT	NT	NT	NT	NT
P19	>64	>64	>64	>64	>64
P20	>64	>64	>64	>64	>64
P21	>64	>64	>64	>64	>64
P22	NT	NT	NT	NT	NT
P23	8	8	16	8	16
P24	8	4	32	4	32
P25	8	8	64	16	64
P26	16	8	32	16	32
P27	16	16	32	16	32
P28	16	16	64	16	64
P29	8	8	32	16	32
P30	4	4	8	4	32
P31	32	16	>64	8	>64
Levofloxacin	0.125	0.03	64	8	0.5

NT = Not Tested and the MIC values $\leq$ 16 were mentioned with bold.

4 to 64-fold reduction in MICs for **ESKAP** pathogens compared to peptide **P2** except *P. aeruginosa* (MIC = > 64 µg/mL). In contrast, peptide **P6** ($\alpha\alpha\gamma$ -hybrid peptide) exhibited poor inhibitory activity against the **ESKAP** pathogen panel (MIC = > 64 µg/mL). By considering peptide **P5** as a standard control/reference to synthesis series of peptides for further screening by varying the hydrophobicity, and position of arginine and also by varying the length of the peptides to find better (potent) antimicrobial peptide against the **ESKAP** pathogen panel. All alanine residues (**P5** and **P6**) were replaced with leucine to provide more hydrophobicity in the peptides **P7** to **P14** (Table 1). Out of **P7** to **P10** 15-mer peptides, **P7**, **P8** are alpha peptides and **P9**, **P10** are $\alpha\alpha\gamma$ -hybrid peptides and also out of **P11** to **P14** 12 mer peptides, **P11**, **P12** are alpha peptides and **P13**, **P14** are $\alpha\alpha\gamma$ -hybrid peptides. Among peptides **P7** to **P14**, **P9** displayed highly potent antimicrobial activity against **ESKAP** pathogen panel (MIC = 2 to 6 µg/mL). Surprisingly peptide **P10** which is acylated version of peptide **P9** showed weak antimicrobial activity, indicates that very optimised/balanced hydrophobicity or hydrophilicity needs to be there to get better antimicrobial active peptide. In continuation peptide **P8**, which is alpha version of the peptide **P9/P10** exhibited poor antimicrobial activity against **ESKAP** pathogens panel (MIC = > 64 µg/mL) except *S. aureus* and *A. baumannii* showed modest inhibitory activity (MIC = 16 µg/mL). Peptide **P14** (12 mer, $\alpha\alpha\gamma$ -hybrid peptide) shorter version of **P9/P10** exhibited the good antimicrobial activity as same as **P9** against **ESKAP** pathogens panel (MIC = 2 to 16 µg/mL) except *P. aeruginosa* (MIC = 32 µg/mL), showed moderate inhibitory activity. Peptides **P7**, **P11**, **P12** and **P13** was not tested. For further screening with respect to peptide **P5**, all alanine residues were replaced by leucine along with that position of arginine in peptides **P15** to **P18** (Table 1), **P15** and **P16** are 15 mer alpha peptides exhibited very poor antimicrobial activity (MIC = > 64 µg/mL). Surprisingly, peptide **P17** (12 mer alpha peptide) displayed potent antimicrobial activity against **ESKAP** pathogens (MIC = 4 to 16 µg/mL) but showed moderate activity with *P. aeruginosa* (MIC = 32 µg/mL). In addition to this Peptides **P19** to **P22** synthesized by replacing all leucine which is attached to C-terminal of the arginine with alanine residue in the peptides **P15** to **P18** (Table 1) displayed very poor antimicrobial activity against **ESKAP** pathogens panel (MIC = > 64 µg/mL) except **P22**, which was not tested. For further screening peptides **P5**, **P9** and **P14** were chosen to modify by attaching different lipids (dodecanoic acid-C₁₂, decanoic acid-C₁₀ and octanoic acid-C₈) at the N-terminal to get peptide **P23** to **P29**. Peptide **P23** showed potent antimicrobial activity against **ESKAP** pathogens panel (MIC = 8 to 16 µg/mL), rest of all lipopeptides exhibited similar type of potent antimicrobial activity against *E. coli*, *S. aureus*, and *A. baumannii* (MIC = 4 to 16

µg/mL), but showed moderate antimicrobial activity against *K. pneumoniae* and *P. aeruginosa* (MIC = 32 to 64 µg/mL).

In our continued efforts, arginine (**P9**, **P10**) was replaced by lysine (**P30**, **P31**) for further modification. Surprisingly **P30** displayed potent antimicrobial activity similar to **P9** against **ESKAP** pathogens panel (MIC = 4 to 8 µg/mL), but showed moderate activity against *P. aeruginosa* (MIC = 32 µg/mL). On the other hand **P31**, which is acylated version of **P30** showed potent antimicrobial activity against *E. coli*, and *A. baumannii* (MIC = 8 to 16 µg/mL), moderate activity against *S. aureus* (MIC = 32 µg/mL) and very weak activity against *K. pneumoniae* and *P. aeruginosa* (MIC = >64 µg/mL).

2.5.2.2. Time and concentration dependent killing kinetics of P5 and P9 against *S. aureus* ATCC 29213

The reduction in *S. aureus* bacteria after treatment with peptides **P5** and **P9** at 1 × MIC and 10 × MIC was calculated as a function of time to examine the peptide effectiveness in eliminating bacterial cells.⁷⁴ Vancomycin was employed in this investigation as a positive control at 1 × MIC and 10 × MIC. The data reported in Figure 7 showed that *S. aureus* was not entirely eradicated even after 24 hours of treatment with 1 × MIC of the peptides **P5** or **P9**. The same characteristic was seen with vancomycin. When treated with 10 × MIC of peptide **P5** and/or **P9**, *S. aureus* was fully eradicated (≥99.99%) in 1 hour. It's interesting to note that, the same action is seen at 10 × MIC with vancomycin only after 24 hrs of exposure. At 10 × MIC of peptide **P5** and/or **P9**, *S. aureus* was shown to kill more quickly, yielding > 99.99% bacterial death after 60 min of peptide exposure. These results show that both the peptides **P5** and **P9** displayed high efficiency in killing *S. aureus* at 10 × MIC with 100% bacterial killing after 1 hr of incubation. In contrast, after 6 hrs of continuous incubation/exposure with peptides, the *S. aureus* growth was observed in the case of peptide **P5**. However, surprisingly we did not observe any further growth in the case of peptide **P9**. This study also suggests that the peptide **P9** strong hydrophobicity and arginine content both led to a higher sterilisation rate.⁷⁵ We

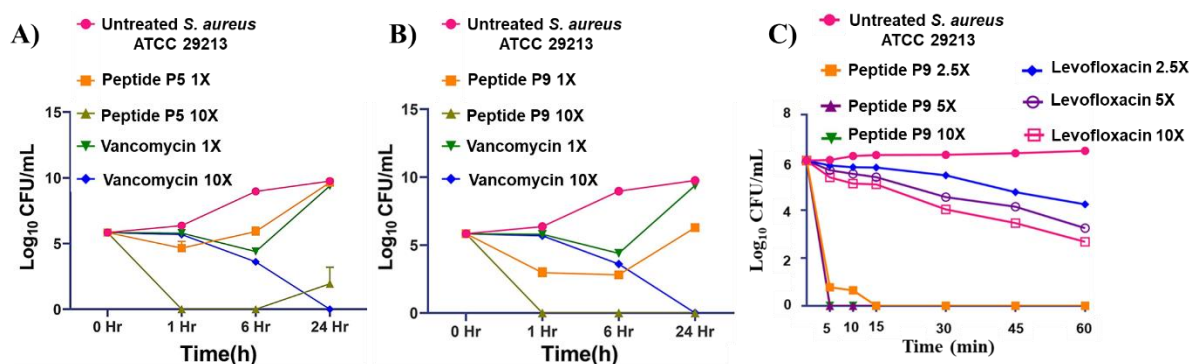


Figure 7. Killing kinetics of peptides against *S. aureus* ATCC 29213 with vancomycin and levofloxacin as standard references. A) With **P5** for 24 hrs at $1 \times \text{MIC}$ and $10 \times \text{MIC}$, B) With **P9** for 24 hrs at $1 \times \text{MIC}$ and $10 \times \text{MIC}$ and C) With **P9** for 60 min at $2.5 \times \text{MIC}$, $5 \times \text{MIC}$, and $10 \times \text{MIC}$. The error bars show the standard deviations obtained from triplicate samples that were used to test the peptides or antibiotics.

Repeated the experiment with *S. aureus* cells cultured for up to one hour (60 min), using levofloxacin as a positive control, to further examine the effectiveness of the peptide **P9**. Here, we incubated *S. aureus* with peptide **P9** and/or levofloxacin at $2.5 \times \text{MIC}$, $5 \times \text{MIC}$, and $10 \times \text{MIC}$ and then assessed their effectiveness as a function of time. Figure 6C demonstrates that *S. aureus* was entirely eradicated after 15 minutes of treatment even with $2.5 \times \text{MIC}$ of peptide **P9**. More rapid eradication of *S. aureus* was also seen at $5 \times \text{MIC}$ or $10 \times \text{MIC}$ of peptide **P9**, resulting in 100% bacterial destruction after 5 min of peptide exposure. In contrast, even after one hour, we did not observe any bacterial killing effect from positive control levofloxacin at $2.5 \times \text{MIC}$, $5 \times \text{MIC}$, or $10 \times \text{MIC}$. This type of bacterial cell killing propensity from peptides makes it more intricate or challenging for bacteria to acquire resistance to the chosen/designed peptides.

2.5.2.3. Protease Resistance of Peptide **P9** against Trypsin Hydrolysis

Because most AMPs are so sensitive to protease degradation, therapeutic usage of most of them has been unsuccessful.⁷⁶ The resistance to proteolysis of designed alpha and/or hybrid peptides was therefore assessed before *in vivo* investigations using trypsin enzyme,⁷⁷ and the effects of trypsin enzyme at various exposure periods on the peptides were studied using RP-HPLC. The peptide bonds next to positively charged (Lys or Arg) residues are broken by trypsin. The RP-HPLC profile demonstrated trypsin protease degradation of the peptides at various time points, as illustrated in Figure 8. We used the active peptides **P5** and **P9** in this work. **P5** was an alpha peptide and another was **P9**, a hybrid peptide. Arg residues were present in both peptides. Alpha-peptide (**P5**) was entirely digested by trypsin enzyme in less than 60 minutes, as shown in Figure 8B. Under identical circumstances, hybrid peptide (**P9**), even after a 12-hrs incubation with the trypsin enzyme, displayed a highly comparable chromatographic peak made up of samples that hadn't been treated with a protease (0-hr) as shown in Figure 8A. According to the results, hybrid peptide **P9** was discovered to be entirely immune to the trypsin protease, with no sign of breakdown even after 12 hrs. Figure 8C displayed the percentage of intact peptide that was still present after trypsin digestion. The hybrid peptide **P9** was almost

entirely maintained even after a 12-hrs trypsin protease treatment, which is consistent with the data above. Alpha peptide **P5**, on the other hand, almost totally disappeared after treatment with trypsin protease for 60 minutes. These findings suggested that highly stable peptide backbones in hybrid peptides were designed to be resistant to trypsin digestion.

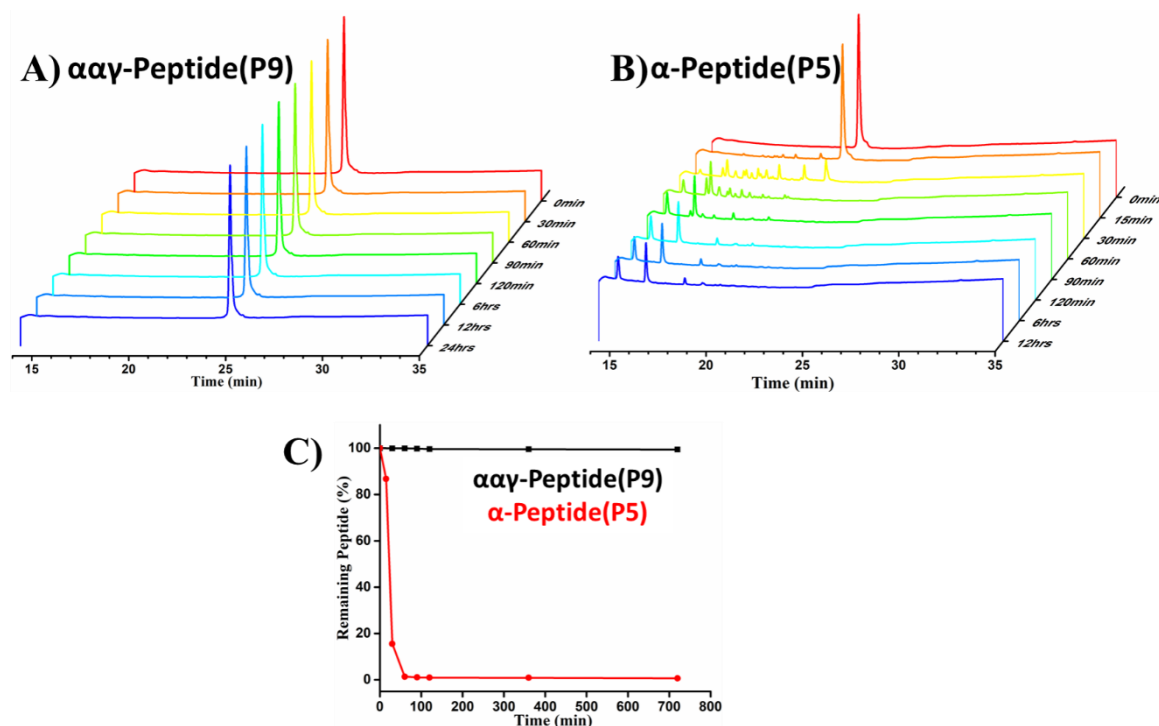


Figure 8: The peptides protease stability was examined using an RP-HPLC chromatograph at various time intervals after incubation with trypsin at 37 °C. A) With peptide **P9** and B) with control peptide **P5**. C) Integration of the peak from the RP-HPLC profile was used to determine the retention rate of the peptides **P5** and **P9** in the presence of trypsin.

2.5.2.4. Biocompatibility of designed peptides: Hemolytic activity and Cytotoxicity

Mammalian cells can still be lysed even though their composition differs from that of bacterial cells. Since AMPs exert their antimicrobial activity through a non-receptor mediated membrane penetration mechanism, which may have decreased their biocompatibility to normal cells, therefore the toxicity is one of the major barriers preventing the AMPs use in clinical treatment.^{78,79} To address this, the haemolytic behaviour of peptides at concentrations ranging from 1 to 250 μ M towards human red blood cells (hRBCs) was investigated in order to assess the cell selectivity of designed hybrid-AMPs along with alpha-peptides. The haemolytic activities of the peptides at different concentrations are shown in Figure 9A, and Figure 9B displayed the minimal peptide concentrations (HC_{10} and HC_{50}) that resulted in 10% and 50%

hemolysis, respectively, of the designed peptides. With the exception of **P9** and **P23**, which caused 50% hemolysis at 159 $\mu\text{g/mL}$ and 130 $\mu\text{g/mL}$, respectively, the majority of the designed peptides did not generate 50% hemolysis even at the highest taken concentration (250 $\mu\text{g/mL}$). Despite the fact that **P9** and **P23** had a very small concentration-dependent toxicity against hRBCs, the concentration needed to cause hemolysis was much higher than the MIC values (table 3). The ratio of the minimum haemolytic concentration for 10% hemolysis (MHC) to the minimum inhibitory concentration (MIC) for *S. aureus* bacterial cells, which was reported as the therapeutic index (TI), is listed in table 3 to help further assess the alpha and hybrid-AMPs' ability to select cells. Among the designed peptides, **P5**, **P9**, **P14**, **P17** and **P30** showed the good TI of 20.5, 22.5, 64, 34.25 and 37.5 for *S.aureus* bacteria, indicating that these peptides has larger potential applications as good antibiotic. **P2** and **P4**, in contrast, exhibited the lowest cell selectivity. Interesting results are shown in Table 6 for peptide **P9** and **P30**, which further shown superior TI even for drug-resistant bacterial strains (MRSA, VRSA).

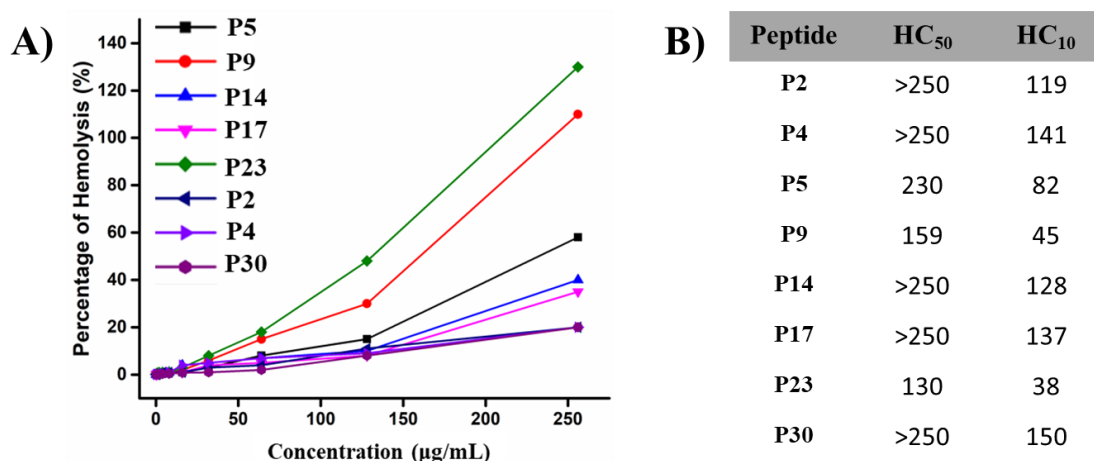


Figure 9: A) The designed AMPs hemolytic activity towards hRBCs, B) The HC₅₀ and HC₁₀ concentrations of the AMPs ($\mu\text{g/mL}$).

Table 3: MHC of the designed AMPs against hRBCs and peptides therapeutic indexes (TIs).

Peptide	MIC ^a ($\mu\text{g/mL}$)	MHC ₁₀ ^b ($\mu\text{g/mL}$)	TI ^c
P2	>64	119	1.85
P4	>64	141	2.20
P5	4	82	20.5
P9	2	45	22.5
P14	2	128	64
P17	4	137	34.25
P23	8	38	4.75
P30	4	150	37.5

^aThe MIC ($\mu\text{g/mL}$) values for the specified AMPs against *S. aureus* ATCC 29213 are given in column. ^bThe peptide concentration that results in 10% hemolysis of human red blood cells (hRBCs) is known as the minimum haemolytic

concentration (MHC). ^c The therapeutic index (TI) is determined by dividing HC₁₀ by MIC. (Larger values show a higher cell selectivity).

Following that, using the MTT assay, we assessed Vero cells cytotoxicity's (obtained from the kidney cell line of an African green monkey; ATCC CCL-81) in response to the antimicrobial active peptides **P5**, **P9**, and **P30**. The findings of the cytotoxicity are displayed in Table 4. The outcomes showed that the peptide **P5**, with a CC₅₀ value of 320 µg/mL, did not exhibit any toxicity even at large concentrations. Cells were unharmed by peptide **P9** up to 60 µg/mL concentration, but higher concentrations render it toxic. This had a CC₅₀ value of >20, <40 µg/mL. With peptide **P30**, we saw the same outcomes, this also had a CC₅₀ value of >20, <40 µg/mL. The selectivity index (SI), which is defined as the CC₅₀/MIC ratio, measures how biocompatible a peptide is.⁸⁰ Table 4 contains SI values. **P5** had the highest SI value of the three peptides, which was 40. **P9** and **P30** had SI values of >10, <20 and >5, <10, respectively, which were relatively lower than **P5** SI value. This suggests that **P5** had the best structural parameter balance and was more cell selective than Vero cells (mammalian cells) against *S. aureus* ATCC 29213 bacteria. Any compound with SI ≥10 is a good point to start when trying to find new drugs. In accordance with the aforementioned claim, peptide **P9** was not cytotoxic to mammalian cells with a high SI (≥10), allowing for continued preclinical development. We can't use the peptide **P5** for any further study or in vivo experiments due to its sensitivity to proteolysis (Trypsin), and also because the bacterial growth improved after 6 hours of incubation with **P5**, which we confirmed by time kill kinetic study, in spite of the fact that **P9** displayed less SI when compared to **P5**, which exhibits negligible toxicity against Vero cells. Due to its robust, broad-spectrum efficacy, low cytotoxicity, and excellent selectivity against bacteria in contrast to mammalian cells, peptide **P5** may still be used topically. Surprising results are shown in Table 6 for peptides **P9** and **P30**, which further shown excellent SI even for drug-resistant bacterial strains (MRSA, VRSA).

Table 4: The **P5**, **P9**, and **P30** AMPs' cytotoxicity towards Vero cells and the Selectivity Index (SI).

Peptide	MIC ^a (µg/mL)	CC ₅₀ ^b (µg/mL)	SI ^c
P5	8	320	40
P9	2	>20, <40	>10, <20
P30	4	>20, <40	>5, <10

^aThe MIC (µg/mL) values for the specified AMPs against *S. aureus* ATCC 29213 are given in column. ^bAccording to the dose-response curve, CC₅₀ is the lowest peptide concentration at which 50% of the cell population dies. ^cThe Selectivity Index (SI) is determined by dividing CC₅₀ by MIC. (Larger values show a higher cell selectivity).

2.5.2.5. Determination of MIC ($\mu\text{g/mL}$) of peptide P9 and P30 against MRSA and VRSA

The development of viable therapeutic candidates for the treatment of MDR-*S. aureus* infections is an important unmet need since MDR-*S. aureus* constitute a worldwide danger to human health. According to the established CLSI recommendations, peptide P9 and P30 were tested against a variety of well-defined and described clinical isolates of MRSA and VRSA in order to assess their range of activity against various strains of MDR-*S. aureus*.⁸¹ Table 5 provides a list of the findings. Reference standards were vancomycin, meropenem, and levofloxacin. It's interesting to note that both peptides P9 and P30 showed same potency (MIC= 2 to 4 $\mu\text{g/mL}$) against various kinds of MDR-*S. aureus* strains. These strains, in contrast, showed resistance to a wide range of antibiotics, including vancomycin, levofloxacin, and meropenem, and they also possessed a wide range of drug-resistance mechanisms. These findings in Table 5 made it abundantly evident that a different, unique mechanism might be at work behind the peptide P9 and P30 antibacterial effects.

Table 5: MIC ($\mu\text{g/mL}$) of peptides P9 and P30 against clinical multidrug resistant *S. aureus* strains including MRSA and VRSA along with control antibiotics.

Bacteria	Strain	P9	P30	Levofloxacin	Meropenem	Vancomycin
<i>S. aureus</i>	ATCC 29213	2	2	0.125	0.5	1
Methicillin resistant <i>S. aureus</i> (MRSA)	NRS 100	2	2	0.25	>64	2
	NRS 119	2	4	16	>64	2
	NRS 129	2	2	0.23	32	1
	NRS 186	2	2	4	64	1
	NRS 191	2	2	16	>64	2
	NRS 192	2	2	8	64	1
	NRS 193	2	4	32	>64	2
	NRS 194	2	2	0.125	8	1
	NRS 198	2	2	32	>64	2
	VRS 1	2	2	32	>64	>64
Vancomycin resistant <i>S. aureus</i> (VRSA)	VRS 4	2	2	>64	>64	>64
	VRS 12	2	2	32	32	>64

Table 6: MIC, SI and TI values of the peptides P9 and P30.

Bacteria	Strain	MIC ^a ($\mu\text{g/mL}$)		SI ^b		TI ^c	
		P9	P30	P9	P30	P9	P30
<i>S. aureus</i>	ATCC 29213	2	2	>10, <20	>10, <20	22.5	75
Methicillin resistant <i>S. aureus</i> (MRSA)	NRS 100	2	2	>10, <20	>10, <20	22.5	75
	NRS 119	2	4	>10, <20	>5, <10	22.5	37.5
	NRS 129	2	2	>10, <20	>10, <20	22.5	75
	NRS 186	2	2	>10, <20	>10, <20	22.5	75
	NRS 191	2	2	>10, <20	>10, <20	22.5	75
	NRS 192	2	2	>10, <20	>10, <20	22.5	75
	NRS 193	2	4	>10, <20	>5, <10	22.5	37.5
	NRS 194	2	2	>10, <20	>10, <20	22.5	75
	NRS 198	2	2	>10, <20	>10, <20	22.5	75
	VRS 1	2	2	>10, <20	>10, <20	22.5	75
Vancomycin resistant <i>S. aureus</i> (VRSA)	VRS 4	2	2	>10, <20	>10, <20	22.5	75
	VRS 12	2	2	>10, <20	>10, <20	22.5	75

^a Is the MIC (µg/mL) values for the peptides **P9** and **P30** against MRSA and VRSA. ^b The Selectivity Index (SI) is determined by dividing CC₅₀ by MIC. ^c The therapeutic index (TI) is determined by dividing HC₁₀ by MIC. (Larger values show a higher cell selectivity). HC₁₀ (µg/mL) Values: for **P9**: 45, for **P30**: 150 and CC₅₀ (µg/mL) Values: for **P9**: >10, <20, for **P30**: >10, <20.

2.5.2.6. MIC (µg/mL) of Peptide **P9** for various *Enterococcus* species including clinical strains of *Enterobacter cloacae* complex

According to Table 5, the peptide **P9** had extremely strong anti-MDR-*S. aureus* action. Our investigation of the peptide **P9** activity against numerous *Enterococcus* species, including clinical strains of the *Enterobacter cloacae* complex, was driven by the results we had already seen. Compared to *E. faecalis*, *E. faecium* is intrinsically more resistant to antibiotics, with more than half of its pathogenic isolates displaying resistance to vancomycin, ampicillin, and high concentrations of aminoglycosides.^{82,83} Peptide **P9** shown negligible effect against various kinds of VSE, as shown in Table 7. The extraordinarily potent efficacy of peptide **P9**, in contrast, was demonstrated against numerous VRE strains. In contrast, the reference standards vancomycin, minocycline, and levofloxacin showed substantial action for VSE but not for VRE. Additionally, using levofloxacin as a standard reference, the peptide **P9** activity was tested against numerous clinical strains of the *Enterobacter cloacae* complex. The prevalence of carbapenem-resistant infections is rising, and *Enterobacter* Spp., the second most prevalent carbapenem-resistant Enterobacteriaceae (CRE) in the United States, is a major contributor.⁸³ Interest in these organisms has grown as a result of the development of multidrug resistance (MDR), especially resistance to the last-resort carbapenems, meropenem, imipenem, and estapenem. In this context, we evaluated **P9** efficacy against *Enterobacter* spp., and the findings are summarised in Table 8. In accordance with the results, peptide **P9** had a potent effect against several *Enterobacter cloacae* bacteria, with MIC values ranging from 8 to 16 µg/mL (Table 8). In contrast, we found a wide range of MIC values for the reference standard drug levofloxacin (0.5 to 32 µg/mL).

Table 7: MIC (µg/mL) of peptide **P9** against various *Enterococcus* panel.

Bacteria	Strain	P9	Levofloxacin	Minocycline	Vancomycin
MSSA	<i>S. aureus</i> ATCC 29213	2	0.25	0.25	1
<i>Enterococcus</i> panel					
VSE (<i>E. faecalis</i>)	NR 31884	64	0.5	0.5	2
	NR 31885	64	0.5	0.5	2
	NR 31886	64	0.5	0.5	2
	NR 31887	16	0.5	16	1
	NR 31888	64	0.5	0.5	2
VRE (<i>E. faecium</i>)	NR 31903	1	>64	8	>64
	NR 31909	0.5	>64	8	>64
	NR 31912	0.25	64	8	>64

Table 8: MIC ($\mu\text{g/mL}$) of peptide **P9** against clinical strains of *Enterobacter cloacae* complex.

Bacteria	Strain	P9	Vancomycin
<i>S. aureus</i>	ATCC 29213	2	1
<i>Enterobacter cloacae</i> complex (ECC)	NR 50394	8	0.5
	NR 50399	16	16
	NR 50401	8	32

2.5.2.7. Emergence of bacterial resistance in *S. aureus* ATCC 29213 with Peptide **P9** at sub-MIC concentration

The evolution of drug resistance can be sped up by exposing microorganisms to a drug over an extended period of time at concentrations below the lethal threshold.⁸⁴ Although it is believed that there is little chance that bacteria will become resistant to AMPs. Resistance is still a crucial study criterion since certain resistant bacteria have evolved AMP resistance.^{85,86,87} As a result, the time-kill kinetics study's findings about the peptide **P9** ability to instantly kill bacteria inspired us to employ the serial passage assay to look into the emergence of microbial resistance to the designed hybrid peptide. Figure 10 displays *S. aureus* drug resistance to peptide **P9** or levofloxacin under serial passaging at sub-MIC doses. In accordance with the results, *S. aureus* was serially exposed to peptide **P9** for 29 passages, generating stains that were still extremely sensitive to the hybrid peptide and their antibacterial activity was unaffected during the test. Levofloxacin, on the other hand, showed signs of resistance as early as passage 11, and after 29 passages, its MIC value had grown by 128-fold, showing that resistance to levofloxacin might develop quickly with subtherapeutic therapy. It's interesting to note that the methicilin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) still responded well to peptide **P9** as an antimicrobial killing agent. According to the aforementioned findings, AMPs' unique non-specific membrane-disruptive action may be superior in terms of developing penetrating resistance.⁸⁸ To evaluate the biocompatibility of the designed peptides, additional arginine residues and excessive hydrophobicity may result in greater cell toxicity.

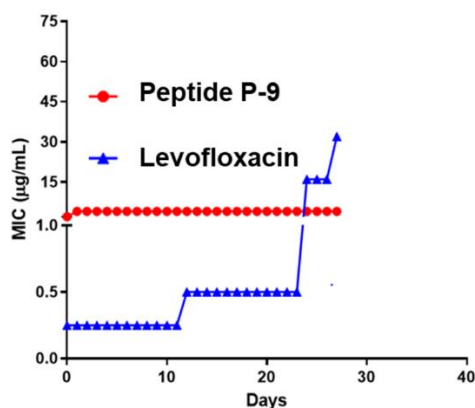


Figure 10. Bacterial resistance after 29 passes of treatment with peptide **P9** and levofloxacin at a concentration below MIC, as determined by the sequential passaging method, on *S. aureus* ATCC 29213. Plotting was done between the number of passes and the fold change in the MIC of the peptide **P9** or levofloxacin.

2.5.2.8. Cross-Drug Resistance of Peptide **P9** against the levofloxacin-resistant *S. aureus* ATCC 29213

By evaluating the antibacterial activity of the peptides and antibiotics against the antibiotic-resistant strain isolated from 29 passages in the resistant development assay, the cross-drug resistance was evaluated. Table 9 provides a summary of the findings. Even after 29 passes in the presence of **P9**, there were no increases in the peptide MIC, indicating that the *S. aureus* bacterial cells were unable to develop resistance. The MICs of the other antibiotics indicated in Table 9 also did not alter. On the other hand, the evidence that the levofloxacin resistant strain was successfully isolated was supported by the 128-fold increases in the MIC of levofloxacin after 29 passes in the presence of levofloxacin. The linezolid MIC was slightly elevated by two fold when compared to the laboratory strain *S. aureus* ATCC 29213, while the MICs for the other antibiotics indicated in Table 9 remained the same against both common and levofloxacin-resistant bacteria. Surprisingly, peptide **P9** showed similar MIC values against both common and strains that were resistant to levofloxacin.

The fact that the levofloxacin-resistant isolates did not exhibit cross-resistance to the peptide **P9** indicates that **P9** has a different target site or antimicrobial mechanism than levofloxacin, which works by inhibiting the two essential bacterial enzymes DNA gyrase and topoisomerase IV.^{89,90} Levofloxacin probably inhibits these enzymes by complexing with the topoisomerase enzymes.⁹¹ In the end, DNA replication is prevented, which prevents cell division and leads to cell death. On the whole, the quick bacterial death demonstrated by the peptide **P9** is probably

another distinguishing feature of membrane-disruptive antimicrobials like the membrane-active antibiotic polymyxin-B.

Table 9: Cross-Drug Resistance or Antibigram of Peptide **P9** and antibiotics against the levofloxacin-resistant *S. aureus* obtained after 29 passages treatment with levofloxacin or peptide **P9** at sub-MIC concentration.

Compounds	MIC (µg/mL)			Fold change in MIC	
	<i>S. aureus</i> ATCC 29213	<i>S. aureus</i> ATCC 29213 after 29 th Passage			
		With P9	With Levofloxacin	P9 non-resistant strain	Levofloxacin-resistant strain
P9	4	4	4	1	1
Ceftazidime	16	16	16	1	1
Daptomycin	1	1	1	1	1
Gentamycin	0.25	0.25	0.25	1	1
Levofloxacin	0.25	0.25	32	1	128
Linezolid	2	2	4	1	2
Meropenem	0.125	0.125	0.125	1	1
Minocycline	0.25	0.25	0.25	1	1
Rifampicin	0.0078	0.0078	0.0078	1	1
Vancomycin	1	1	1	1	1

2.5.2.9. Determination of Post antibiotic effect (PAE) of Peptide **P9** against *S. aureus* ATCC 29213

When selecting antibiotic dose regimens, the post antibiotic effect (PAE), a pharmacodynamic parameter, may be taken into account. After a brief period of exposure to an antimicrobial agent, PAE refers to the ongoing suppression of bacterial growth.⁹² Any molecule that has a long PAE has an advantage since it reduces the dosages needed for therapeutic clearance. Vancomycin was used as the control in this case to determine the PAE of peptide **P9** against *S. aureus*. Table 10 shows that peptide **P9** had a prolonged PAE of ~9 hrs at 10 X MIC and ~2 hrs at 1 X MIC. Vancomycin's PAE, on the other hand, was found to be ~2 hours at 10 X MIC and ~1 hour at 1 X MIC. These findings demonstrate that peptide **P9**, which exhibits concentration-dependent bacterial action with prolonged PAE, can be a potent antibacterial candidate.

Table 10: *In vitro* Post antibiotic effect (PAE) of Peptide **P9** against *S. aureus* ATCC 29213.

Treatments	Time for 1 log ₁₀ (h)	PAE (h)
untreated <i>S. aureus</i> ATCC 29213	~2	0
P9 1X	~4	~2
P9 10X	~11	~9
Vancomycin 1X	~3	~1
Vancomycin 10X	~4	~2

2.5.2.10. Anti-biofilm activity of Peptide P9 against *S. aureus* ATCC 29213

Biofilms produced by bacteria that are growing on a surface can frequently lead to a number of illnesses. Biofilms may offer protection against eradication by antimicrobial agents, impeding treatment and escalating drug resistance^{93,94}. Interestingly, the majority of medications that have been authorised have very little effect against bacteria that are protected by biofilms; therefore, it is crucial to assess molecules' anti-biofilm activity during the drug development process.⁹⁵ Therefore, using a biofilm model adopting *S. aureus* ATCC 29213 cells, we further assessed the anti-biofilm capabilities of the designed peptide **P9** and comparison medications (vancomycin and levofloxacin). Figure 11 illustrates how peptide **P9** demonstrated strong anti-biofilm action by reducing the biofilm by 6% at 1 X MIC or 10 X MIC in comparison to the control. In contrast, levofloxacin only produced a 1% or 1.5% reduction in biofilm at 1 X MIC or 10 X MIC, while vancomycin produced a 2% or 4% reduction at both MIC levels. However, disulfiram (DSF) produced a 7% reduction at both MIC levels. Therefore, peptide **P9** is more effective than vancomycin and levofloxacin at reducing the biofilm at 1 X MIC value, similar to disulfiram.

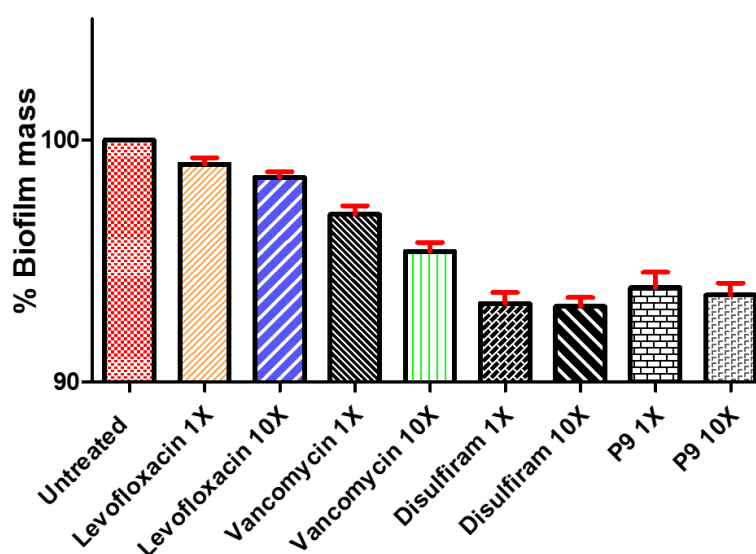


Figure 11: Anti-biofilm activity of peptide **P9** and comparison medicines against the *S. aureus* ATCC 29213 biofilm at their 1 X and/or 10 X MIC. Crystal violet staining was used to measure a biofilm's biomass at 600 nm. The outcomes were expressed as the percentage of biofilm formation, with the control well's maximum percentage being the percentage in the absence of peptides or comparators.

2.5.2.11. Checkerboard Assay: Determination of synergy of peptide P9 with FDA approved antibiotics

Determining a drug's activity when combined with other FDA-approved antibiotics is crucial because the treatment of MDR infections frequently requires a combination of medications to achieve therapeutic clearance. In this situation, the activity of the peptide **P9** was assessed in the presence of a panel of antibiotics that are used in clinical staphylococcal infection treatment. Peptide **P9** did not synergize with or antagonize with any of the FDA-approved antibiotics, as reported in Table 11. It can therefore be used in a therapeutic combination.

Table 11: FDA-approved antibiotics combined with the peptide **P9** to combat *S. aureus* ATCC 29213. Fractional Inhibitory Concentration, abbreviated as FIC.

Drug	MIC (µg/mL)	MIC of "P9" in the presence of drug (µg/mL) "A"	MIC of drug in the presence of "P9" (µg/mL) "B"	FIC A	FIC B	ΣFIC (FIC A + FIC B)	Inference
P9	2						
Ceftazidime	16	2	16	1	1	2	No interaction
Daptomycin	1	2	1	1	1	2	No interaction
Gentamycin	0.25	2	0.25	1	1	2	No interaction
Levofloxacin	0.125	2	0.125	1	1	2	No interaction
Linezolid	2	2	2	1	1	2	No interaction
Meropenem	0.5	2	0.5	1	1	2	No interaction
Minocycline	0.0625	2	0.0625	1	1	2	No interaction
Rifampicin	0.0039	2	0.0039	1	1	2	No interaction
Vancomycin	1	2	1	1	1	2	No interaction

[ΣFIC= FIC A + FIC B. (FIC A= MIC of **P9** in the combination with antibiotics/MIC of **P9** alone, FIC B= MIC of antibiotics in the combination with **P9**/MIC of antibiotics alone). The combination is considered synergistic when the ΣFIC is ≤ 0.5, indifferent when the ΣFIC is > 0.5 to 4, and antagonistic when the ΣFIC is > 4.]⁹⁶

2.5.3. In vivo antimicrobial activity

2.5.3.1. In vivo Efficacy of peptide P9 in a Murine Skin Infection Model against *S. aureus* ATCC 29213

As peptide **P9** displayed excellent activity in an *in vitro* studies against MDR-*S. aureus* pathogens and showed less toxicity to hRBCs and Vero cells (mammalian cells) at the peptide MIC, these results encouraged us to check whether the peptide **P9** could be able to maintain their antimicrobial activity *in vivo*, using an established murine skin infection model against *S. aureus* ATCC 29213 along with fusidic acid as the comparator, and the results are plotted in Figure 12A. Six-week-old male BALB/c mice were divided into five groups (n = 8) to begin the formation of a skin wound. Each group of mice was administered a suspension of 2%

fusidic acid (positive control), 1% peptide **P9**, base or petroleum jelly (25–30 mg, used as a vehicle for topical delivery of the peptide **P9** or antibiotic-fusidic acid) twice daily for four days after the formation of an open wound (infected with *S. aureus* ATCC 29213) in the dorsal region of infected mice. A group of untreated mice was euthanized 4 hours after infection in order to measure the infectious dose in each experiment, and topical test treatments were started at the same time. After the end of the treatment, it was found that the bacterial burden in the wounds of the infected mice was reduced. To encourage effective wound repair and avoid inducing a significant inflammatory response that might impair the healing of injured tissues, it is essential to reduce the bacterial burden in infected wounds.⁹⁷ Figure 12A shows that in the murine skin infection model, twice daily treatment with 1% peptide **P9** significantly reduced the bacterial burden in the skin by $\sim 5.9 \log_{10}$ cfu/g, while treatment with 2% fusidic acid significantly reduced the bacterial burden in the skin by $\sim 5.0 \log_{10}$ cfu/g, as compared to the untreated mice, which showed a bacterial burden in the skin by $\sim 7.5 \log_{10}$ cfu/g. These findings suggested that the peptide **P9** potent in vitro antibacterial activity against *S. aureus* ATCC 29213 was further supported by *in vivo* tests using a murine skin infection model.

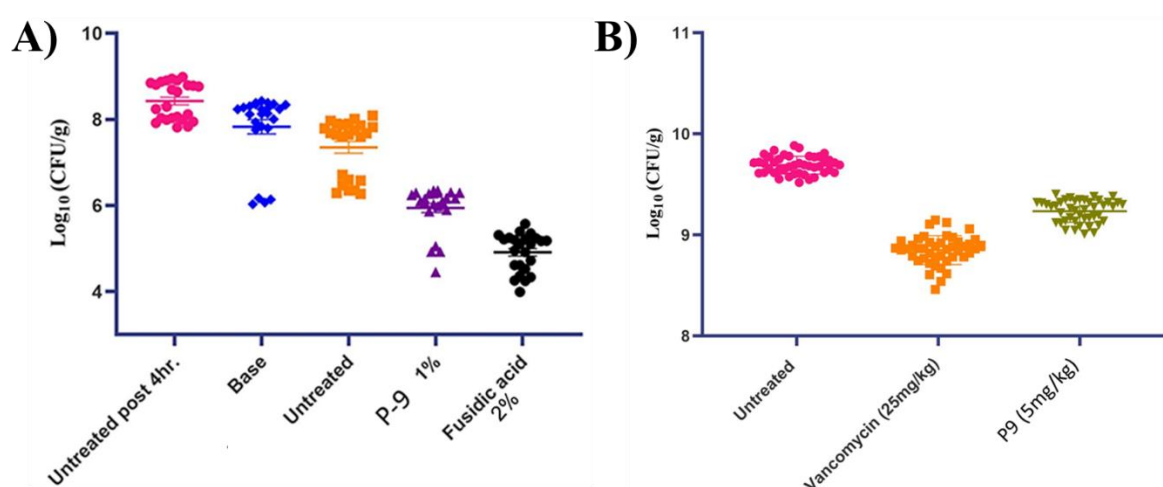


Figure 12: *In vivo* activity of **P9** against *S. aureus* ATCC 29213. A) Efficacy of the designed peptide **P9** in an *in vivo* *S. aureus* ATCC 29213-infected murine skin infection model. Plots show the decrease in cfu/g of tissue. 2% fusidic acid and 1% peptide **P9** were administered to mice twice daily. B) Efficacy of the designed peptide **P9** in an *in vivo* model of *S. aureus* ATCC 29213-infected murine neutropenic thigh infection in mice. Plots show the decrease in cfu/g of tissue. In this model, mice received two IP doses of peptide **P9** and vancomycin at 3 and 6 hours after infection. For each peptide **P9** or antibiotic under investigation, the error bars show the standard deviations obtained from triplicate samples.

2.5.3.2. *In vivo* Efficacy of Peptide P9 in a Murine Thigh Infection Model against *S. aureus* ATCC 29213

The above results confirmed that peptide **P9** displayed a potent antimicrobial activity against ESKAPE pathogens, including various MDR-*S. aureus* strains, and we also demonstrated the *in vivo* efficacy in a murine skin infection model (Figure 12A). Since the susceptibility of a pathogen to a drug *in vivo* can differ noticeably from the susceptibility *in vitro* and also from the above results we were inspired to see whether the peptide **P9** could still be able to maintain their antimicrobial activity in subsequent *in vivo* studies or not, to know this we tested the effectiveness of the peptide **P9** against *S. aureus* infections in a murine neutropenic thigh infection model. This model has been heavily used to assess the *in vivo* effectiveness of several compounds against *S. aureus*.^{104,105} In a nutshell, *S. aureus* was injected into the thigh of neutropenic mice, followed by two intraperitoneally (IP) injections of the peptide **P9** at 5 mg/kg, and two injections of the antibiotic vancomycin at 25 mg/kg. The mouse groups that received vancomycin treatment and saline treatment served as the negative and positive controls, respectively. Figure 12B shows that treatment with peptide **P9** at 5 mg/kg significantly decreased mean bacterial counts ($\sim 0.8 \log_{10}$ cfu/g) in the thigh compared to the control group, which is comparable to vancomycin at 25 mg/kg ($\sim 0.9 \log_{10}$ cfu/g) as compared to the no-drug control. According to these findings, peptide **P9**, even at a dosage of 1/5, is just as effective as vancomycin at reducing the bacterial load in infected mice.

2.5.3.3. Determination of MIC of peptide P9 against fusidic acid and mupirocin resistance *S. aureus* ATCC 29213

As a result of the fact that peptide **P9** shown superior or equivalent antimicrobial activity to fusidic acid against *S. aureus* ATCC 29213 in the murine skin infection model, we decided to test **P9** antimicrobial effectiveness against *S. aureus* ATCC 29213 that was resistant to both fusidic acid and mupirocin. One of the few remaining antibiotics, fusidic acid, can be applied topically to treat skin and soft tissue infections as well as methicillin-resistant *S. aureus* infections. However, resistance to this critical antibiotic has increased considerably in recent years.⁹⁸⁻¹⁰⁰ Also, since mupirocin is a key component of decolonization regimens and is frequently used in healthcare settings to control the spread of MRSA and eliminate *S. aureus* nasal colonisation, its effectiveness will decline as mupirocin resistance among *S. aureus* isolates emerges.¹⁰¹⁻¹⁰³ We intended to create *S. aureus* ATCC 29213 strains resistant to fusidic acid and mupirocin in order to test **P9** efficacy against those bacteria. The MIC for both fusidic

acid and mupirocin increased by 1028 folds as shown in Table 12, confirming the effective isolation of the fusidic acid and mupirocin resistant strains after a series of 18th passages. We tested the effectiveness of **P9** against resistant *S. aureus* strains after successfully isolating organisms that were resistant to fusidic acid and mupirocin, using these antibiotics as a comparator and levofloxacin as a reference standard. The findings, which are shown in Table 12, showed that the peptide **P9** was equally effective (MIC = 2 to 4 g/mL) against the fusidic acid- and mupirocin-resistant *S. aureus* ATCC 29213 strain, and that these bacteria did not show levofloxacin resistance. These strains, in contrast, showed resistance to mupirocin and fusidic acid. These findings strongly suggest that the strains that were fusidic acid and/or mupirocin resistant did not exhibit cross-drug resistance against peptide **P9**, suggesting that the peptide **P9** may be acting through an additional, unique mechanism.

Table 12: MIC (μg/mL) of peptide **P9** combined with control antibiotics against fusidic acid and mupirocin resistant *S. aureus* ATCC 29213.

Compounds	MIC (µg/mL)			Fold change in MIC	
	<i>S. aureus</i> ATCC 29213	<i>S. aureus</i> ATCC 29213 after 18 th Passage			
		With Fusidic acid	With Mupirocin	Fusidic acid-resistant strain	Mupirocin-resistant strain
P9	2	2	4	1	2
Fusidic acid	0.25	512	0.5	2048	2
Mupirocin	0.25	0.25	512	1	2048
Levofloxacin	0.25	0.25	0.25	1	1

Table 13: Cross-Drug Resistance of antibiotics against the fusidic acid and mupirocin resistant *S. aureus* ATCC 29213 obtained after 18th passages treatment with fusidic acid and mupirocin at sub-MIC concentration.

Compounds	MIC (µg/mL)			Fold change in MIC	
	<i>S. aureus</i> ATCC 29213	<i>S. aureus</i> ATCC 29213 after 18 th Passage			
		With Fusidic acid	With Mupirocin	Fusidic acid-resistant strain	Mupirocin-resistant strain
Ceftazidime	16	16	16	1	1
Daptomycin	1	1	1	1	1
Gentamycin	0.25	1	0.25	4	1
Levofloxacin	0.25	0.125	0.25	5	1
Linezolid	2	2	2	1	1
Meropenem	0.125	0.125	0.125	1	1
Minocycline	0.25	0.25	0.25	1	1
Rifampicin	0.0078	0.0078	0.0078	1	1
Vancomycin	1	2	1	2	1
Fusidic acid	0.25	512	0.25	2048	1
Mupirocin	0.25	0.25	512	1	2048

2.5.4. Analysis of Peptides Membrane-Active Mechanism

2.5.4.1. Identifying the Localization of peptides on the bacterial membrane surface

The effectiveness of peptide **P9** with bacterial cells was examined using confocal microscopy investigations (Figure 13). These tests were conducted using *E. coli* K12MG1655 cells. We also employed DAPI (4',6-diamidino-2-phenylindole) and PI (propidium iodide) in addition to the peptide. The nuclei of the cells will be stained with DAPI to help identify them, and PI, a membrane-impermeable dye, can pierce the membrane of a wounded cell to stain the nuclei.¹⁰⁶ Figure 13A shows the fluorescence increase of PI in the bacterial cells following incubation with peptide **P9**. In contrast, in the absence of the peptide **P9** depicted in Figure 13A, we did not notice any PI fluorescence. These findings strongly suggested that the bacterial cell membrane was being efficiently ruptured by peptide **P9**.

Additionally, fluorescein isothiocyanate (FITC) bound peptide **P30** was utilized along with PI and DAPI to investigate the peptide cellular localization and site of action using confocal laser scanning microscopy (CLSM) in *E. coli* K12MG1655 cells. The resulting images are presented in Figure 13B. The green fluorescence-emitting FITC was used to conjugate the peptide **P30**. The red fluorescence of PI was found inside the bacterial cell membrane, as we saw in the early tests, and the green fluorescence of the FITC-attached peptide **P30** was visibly accumulated on the bacterial membrane as shown in Figure 13B and also from the merged images illustrates the peptide localization more intuitively and demonstrates that it accumulated around the *E. coli* membrane and that it rapidly permeated the bacterial membrane. Additionally, the membrane integrity was damaged by peptide **P9** or **P30**, which led to the demise of the bacterial cells. These findings support the membrane penetration findings previously discussed as well as other membrane-disruptive antimicrobial peptide examples.¹⁰⁷ While AMPs rapidly disrupt the bacterial cell membrane through mechanical measures, conventional antibiotics inhibit key intracellular targets involved in biosynthetic pathways in microorganisms; it is believed that this helps overcome antibiotic resistance brought on by conventional antibiotics.^{108,109}

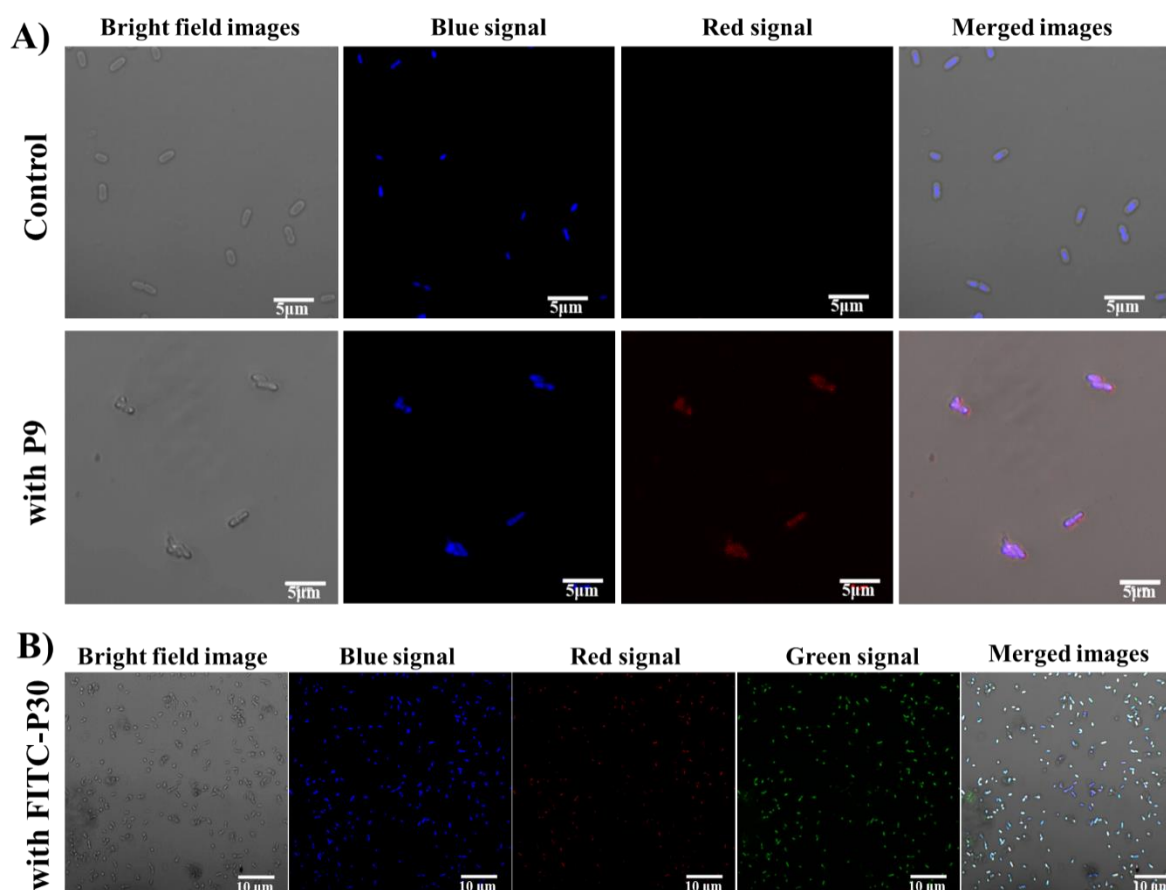


Figure 13: CLSM (Confocal Laser Scanning Microscopy) image analysis of *E. coli*. A) Images that have been treated with **P9** display, in order from left to right, bright-field images, blue signals from DAPI, red signals from PI, and combined images. Scale bar: 5 μm. B) Was given FITC-**P30** treatment. Bright-field pictures, blue signal from DAPI, green signal from FITC-**P30**, red signal from PI, and combined images are shown from left to right. Scale bar: 10 μm.

2.5.4.2. FE-SEM Images: Bacterial Morphological Observation

We all know that a lot of naturally occurring AMPs have been proven to disrupt membranes after binding due to differences in electrochemical potential.¹¹⁰ Most active peptides **P5**, **P9** and **P30** in the present investigation suppress the growth of Gram-positive and Gram-negative bacteria with MIC values that are nearly the same throughout the panel. This prompted us to investigate their mechanisms of action.

The impact of the peptides on the membrane morphology of *E. coli* K12MG1655 and *S. aureus* 29213 was further visually verified through field-emission scanning electron microscopy (FE-SEM). The FE-SEM images of *E. coli* K12MG1655 and *S. aureus* 29213 bacterial cells before and after treatment with peptides **P5**, **P9** and **P30** are shown in Figure 14. The bacterial membranes of *E. coli* K12MG1655 and *S. aureus* 29213 were smooth and had intact membrane morphology in the absence of peptide treatment (Figure 14A and 14E). When *E. coli* K12MG1655 and *S. aureus* 29213 were subjected to the peptides **P5**, **P9** and **P30**, their bacterial membranes significantly corrugated, disrupt, and fragmented, causing a significant amount of intracellular material to flow out of the bacterial cell (Figure 14B to 14D and 14F to 14H).

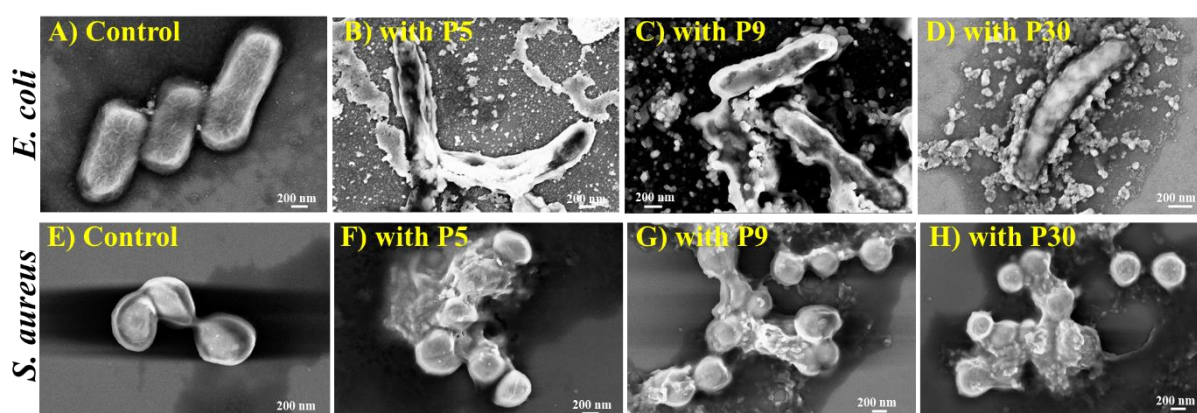


Figure 14: FE-SEM images of *E. coli* K12MG1655 and *S. aureus* 29213 after being exposed to AMPs at 2 X MIC for 1 hr. A to D represent *E. coli*: A) Control, without peptide, B) with **P5**, C) with **P9** and D) with **P30**; E to H represent *S. aureus*: E) Control, without peptide, F) with **P5**, G) with **P9** and H) with **P30**. Scale bar = 200 nm).

2.5.4.3. Membrane Integrity Assay by FACS Analysis

The effectiveness of the peptide to disrupt bacterial membranes was examined using flow cytometry (FCM) and the DNA intercalating dye propidium iodide (PI), which may pass through damaged cell membrane. Figure 15 presents the findings. As can be seen in Figure 15A, when peptide was not present (as a negative control), only 0.5% of *E. coli* K12MG1655 cells had PI fluorescence signals, signifying intact cell membranes. With the peptides **P5**, **P9**, and **P30**, the percentage of membrane rupture (PI fluorescence signal) increased. The increasing percentage of *E. coli* K12MG1655 cells that were PI-positive was evidence that the peptides **P5**, **P9**, and **P30** caused more than 90%, 97.5%, and 98.4% of *E. coli* K12MG1655

membranes to rupture at 1 X MIC. These findings support the theory that peptides kills bacteria by compromising the structural integrity of their membranes.

To determine the location of the peptide on the surface of *E. coli* K12MG1655, we repeated the experiment using peptide **P30** which was FITC-tagged. Ampicillin was utilized as a positive control in this case. Figure 16 displays the results. Only 0.5% and 0.4% of *E. coli* K12MG1655 cells had FITC and PI fluorescence signals in the absence of the peptide, indicating intact cell membranes (Figure 16A and 16B). *E. coli* K12MG1655 cells, with FITC-tagged peptide **P30** caused an increase in FITC and PI fluorescence. At 1 X MIC of the FITC-tagged peptide **P30**, 95% and 99.5%, respectively, of the *E. coli* K12MG1655 cells were FITC and PI-positive (Figure 16C and 16D). Notably, the positive control ampicillin demonstrated a lesser ability to cause cytoplasmic membrane depolarization and harm the integrity of the *E. coli* K12MG1655 cellular membrane when compared to peptide **P30** (Figure 16E and 16F).

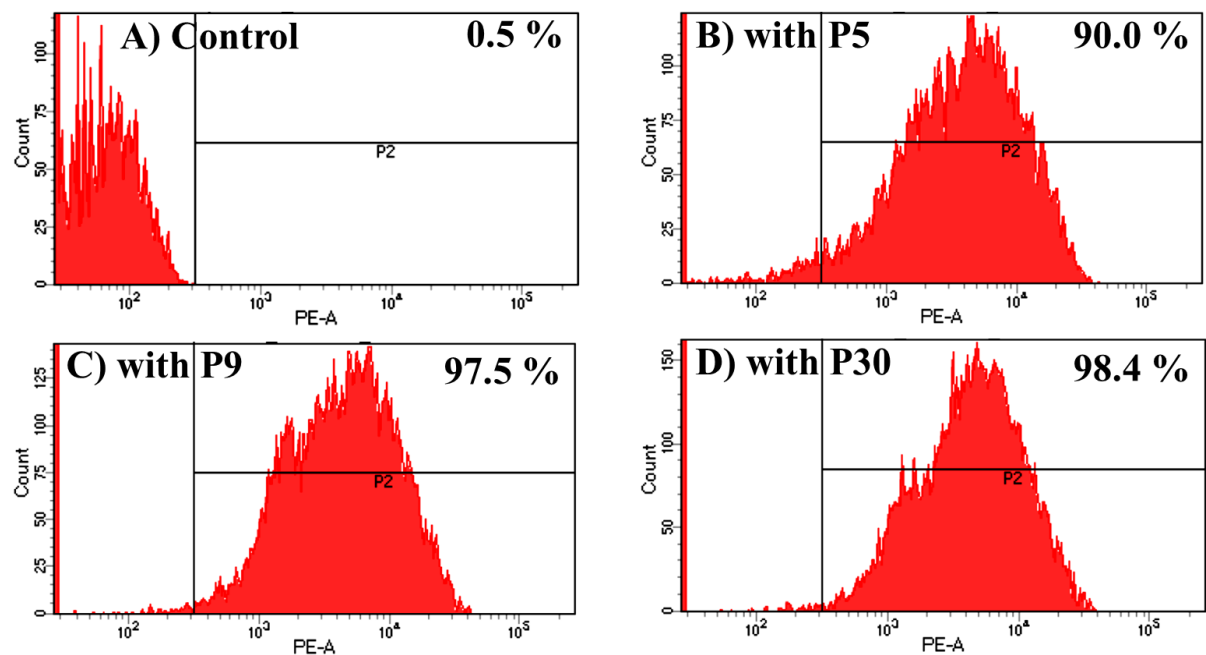


Figure 15: *E. coli* K12MG1655 cells exposed to AMPs at their 1 X MIC for 1 hr demonstrated membrane disintegration. The increments in cellular fluorescence intensity of PI (10 μ g/mL) were measured by flow cytometry after 1 hr. The peptides were not employed when processing the control. A) Without peptide (negative control 0.5%), B) with **P5** (90.0%), C) with **P9** (97.5%) and D) with **P30** (98.4%). 100% means that every cell was PI-strained and appeared fluorescent.

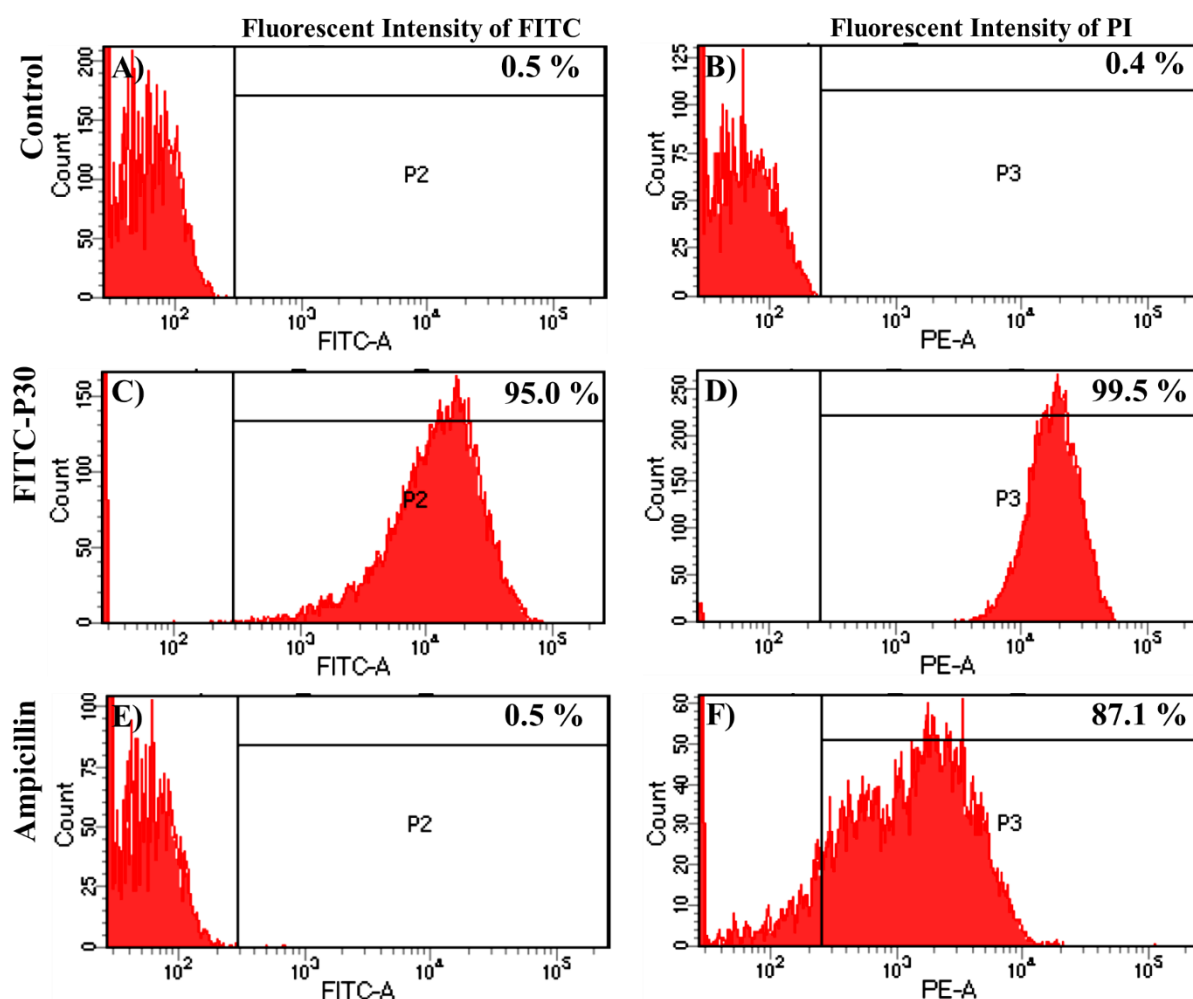


Figure 16: *E. coli* K12MG1655 flow cytometry analysis. By using FITC-**P30** and ampicillin (as a positive control) at 1 X MIC for 1 hr, the increase in cellular fluorescence intensity of PI (10 μ g/mL) and FITC were determined by flow cytometry. (A and B) without peptide (negative control), (C and D) with FITC-**P30**, (E and F) with ampicillin (positive control). (A, C, and E are responsible for Fluorescent Intensity of FITC) and (B, D, and F are responsible for Fluorescent Intensity of PI). The results of the flow cytometry further showed that the cell membrane's integrity was compromised by the hybrid peptide **P30**.

Additionally, we investigated the 1-NPN assay¹¹¹ to investigate the permeability of the outer cell membrane and the enzyme galactosidase leakage assay^{14,112} to investigate the permeability of the inner cell membrane using AMPs on *E. coli* K12MG1655 cells in order to understand the mechanism of action of these peptides.

2.5.4.4. Outer Membrane Permeabilization: 1-NPN assay

A distinctive feature of Gram-negative bacteria is the outer membrane. *E. coli* K12MG1655 cells were selected as the model bacterium to examine the outer membrane permeability of the peptide. The fluorescent probe 1-N-phenyl naphthylamine (1-NPN) fluoresces in a hydrophobic environment, and on the other-hand fluoresces quenched in an aqueous environment. As the peptide damages the *E. coli* K12MG1655 cells outer membrane, 1-NPN comes in contact with the hydrophobic environment and fluoresces more intensely. Here *E. coli* K12MG1655 cells were incubated with peptides at 1 X MIC values. The results suggested that, the peptides (**P5**, **P9**, and **P30**) which are more antimicrobial active showed the more bacterial outer membrane permeability like Melittin which we considered as a comparator shown in Figure 17. In contrast, we did not observe any much florescence in the case of peptides (**P1** and **P6**) which had poor antimicrobial activity indicates their poor bacterial outer membrane permeability. From the 1-NPN assay, the results indicates that, Peptides **P5**, **P9** and **P30** can able to permeabilize the bacterial outer membrane efficiently.

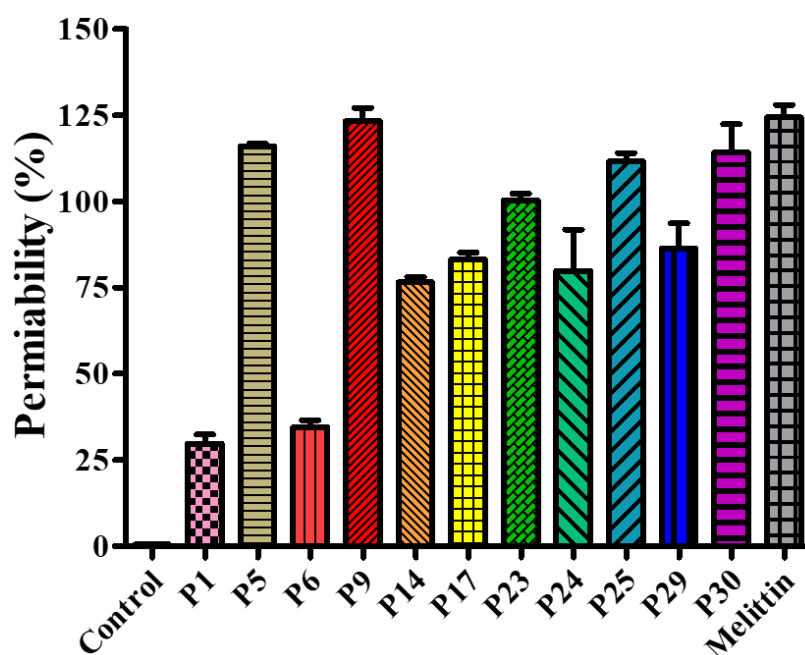


Figure 17: The peptides and melittin-induced increase in permeability of the outer membrane. The fluorescent dye (1-NPN) assay was used to assess the intake of 1-NPN by *E. coli* K12MG1655 cells in the presence of peptides and melittin at 1 X MIC concentration for 1 hr. The wells devoid of the test substance served as the control. Error bars have been set and the experiments were run in triplicate. (1-NPN: λ_{ex} = 350 nm, λ_{em} = 420 nm).

2.5.4.5. Inner Membrane Permeabilization: β -Galactosidase leakage from *E. coli* cells

To confirm in more detail how these peptides do really damage the bacterial membrane, we measured the release of cytoplasmic β -galactosidase enzyme from *E. coli* K12MG1655 cells carrying the LacZ reporter gene in order to better examine the cytoplasmic membrane permeability of peptides. A typical fluorogenic substrate, 4-methylumbelliferyl- β -galactosidase (MUG), was used to monitor the release of β -galactosidase from *E. coli* K12MG1655 cells, which will likely occur when the cell wall is disrupted. The release of β -galactosidase from the *E. coli* K12MG1655 cells after they were incubated with peptides is shown in Figure 18. After being treated with most antimicrobial active Peptides **P5**, **P9** and **P30**, β -galactosidase is released as evidenced by an increase in fluorescence intensity. In contrast, after treatment with poor antimicrobial active Peptides **P1** and **P6**, no β -galactosidase release was seen. When Melittin was used as a comparator in this experiment, more β -galactosidase was released; however, when peptide was not present, there was no β -galactosidase release considered as a negative control. The results of this experiment suggest that the peptides **P5**, **P9** and **P30** bind and rupture the bacterial cell membrane, which then facilitated the release of β -galactosidase.

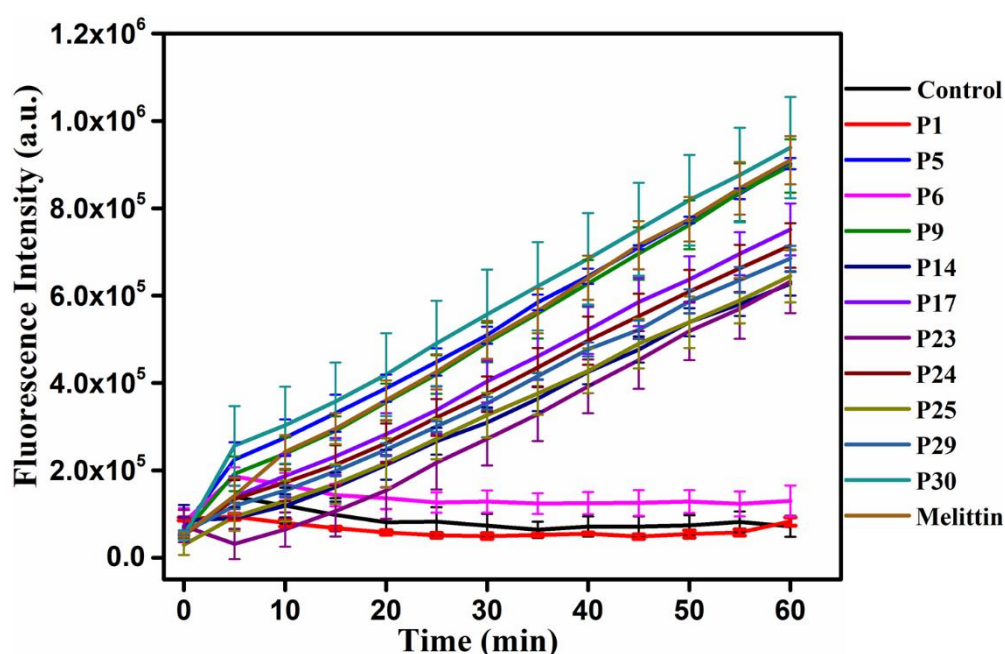


Figure 18: The peptide and melittin-induced increase in inner membrane permeability. The amount of 4-methylumbelliferyl- β -galactosidase (MUG) hydrolyzed as a result of the release of cytoplasmic β -galactosidase from *E. coli* K12MG1655 cells that had been exposed to peptides and melittin at a concentration of 1 X MIC for 1 hr was determined by monitoring the fluorescence of 4-methylumbelliferone (MUF). The wells devoid of the test substance served

as the control. Error bars have been set and the experiments were run in triplicate. (MUF: λ_{ex} = 368 nm, λ_{em} = 449 nm).

2.5.4.6. Determination of the Peptide **P9** non-restricted entry through the OM in GNB

We believed that designed peptides kill the bacteria by rapidly rupturing the membrane after examining all these membrane integrity results. We carried out a MIC ($\mu\text{g/mL}$) test in the presence of Polymyxin B Nonapeptide (PMBN) (Table 14) to further confirm more accurately whether the peptide **P9** disrupts the bacterial membrane or has any barrier to enter the outer membrane (OM). PMBN, which binds to the bacterial lipopolysaccharide (LPS), can improve the permeability of the Gram-negative bacteria (GNB) outer membrane towards antibiotics.¹¹³ PMBN is expected to still have the ability to permeabilize GNB outer membranes, making them susceptible to a number of antibiotics while being less toxic and having no bacterial activity.¹¹⁴ For this reason, peptide **P9** activity was determined in the presence of sub-inhibitory concentrations (10 $\mu\text{g/mL}$) of PMBN in order to verify the non-restricted entry through the GNB outer membranes, a known non-resistance mechanism that was responsible for **P9** activity against GNB pathogens. The results are tabulated in Table 14. Intriguingly, peptide **P9** displayed the same strong anti-*E. coli* ATCC 25922 and anti-*A. baumannii* BAA-1605 action in the presence and absence of PMBN, proving that the GNB OM did not hinder its entry.

Table 14: MIC ($\mu\text{g/mL}$) of peptide **P9** against GNB pathogens in the presence of PMBN.

Bacterial Strain	MIC (µg/mL)								
	PMBN	P9		Rifampicin		Vancomycin		Levofloxacin	
		PMBN		PMBN		PMBN		PMBN	
		(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)
<i>E. coli</i> ATCC 25922	>64	4	4	4	0.0625	512	64	0.0156	0.0156
<i>A. baumannii</i> BAA-1605	>64	2	2	4	0.0625	128	32	8	8

2.6. Conclusion

We have demonstrated the design and synthesis of series of cation rich amphiphilic 10/12 hybrid peptides along with alpha peptides. Surprisingly, $\alpha\alpha\gamma$ -helical peptide (**P9**) displayed potent broad-spectrum of antimicrobial activity (AMA) across the panel of Gram-positive, and Gram-negative bacteria including MRSA. Time kill analysis study suggests that $\alpha\alpha\gamma$ -helical peptides was the more potent candidate than α -peptides to kill the bacteria. AMA with the combination of various drugs reveals that peptide **P9** have a different mode of action than the

many other drugs including daptomycin. The mechanism of action peptides **P9** on *E. coli* using 1-NPN assay, β -galactosidase assay, FE-SEM, FACS, and confocal imaging results suggested that peptide **P9** inhibit the bacterial growth through membrane depolarization. Further bacterial passage assay studies revealed that *S. aureus* does not show any resistance to the peptide **P9** even after the 29th passage on the other hand *S. aureus* develops resistance to the levofloxacin within the same period, and also antibiogram of a 29th-day culture was not displayed resistance to other antibiotic tested. The Post Antibiotic Effect (PAE) of this peptide **P9** against *S. aureus* revealed that to reach 1 log₁₀ (CFU) *S. aureus* will take ~9 hrs but on other hand, Vancomycin will take ~2 hrs. The skin infection model suggested that the designed peptide antibiotic **P9** can control the growth of *S. aureus* at 1% concentration in topical cream and also surprisingly these peptide **P9** in the *in vivo* studies showed the reduced bacterial burden in mice was suggested by thigh infection model. Moreover, the peptide **P9** were found to be less hemolytic. As we anticipated the peptide **P9** showed very weak liability towards trypsin digestion. Overall, this study underlines the potential of $\alpha\alpha$ -hybrid peptide **P9** in the development of next-generation antimicrobial therapeutics.

2.7. Experimental Section

2.7.1. Material and Methods

All of the chemicals, fluorescent dyes, enzymes, and reagents were obtained from commercial providers with excellent purity grades and used directly. The 96-well polystyrene plates with flat bottoms were bought from Invitrogen Bio services. All of the N-Fmoc protected amino acids (>95% purity), Rink Amide Resin were bought from GL Biochem, China. 10 mM Tris-base, 150 mM NaCl, and 0.1 mM EDTA were used to make Tris-buffer, and either diluted HCl or NaOH was added to get the pH to 8.2. 10 mM sodium phosphate, 150 mM sodium chloride, and 1 mM EDTA were combined to make a sodium phosphate buffer. The pH was then adjusted to 7.4 by adding either diluted HCl or NaOH. Lipids, trypsin, FITC, 4-methylumbelliferyl- β -galactosidase, PI, DAPI, and 1-NPN were all purchased from Sigma-Aldrich along with all dry solvents and reagents.

All bacterial media and supplements, such as Mueller-Hinton agar, Mueller-Hinton cation supplemented broth II, and tryptic soy broth (TSB), were bought from Becton-Dickinson (Franklin Lakes, NJ, USA). Sigma-Aldrich (St. Louis, MO, USA), where all other compounds and antibiotics were purchased. Lonza (Lonza, USA) provided the Roswell Park Memorial

Institute Medium (RPMI) and Foetal Bovine Serum (FBS). Every technique used was carried out in accordance with the rules and regulations that applied.

2.7.2. Bacterial strains

All pure lyophilized peptides were screened against a bacterial panel, namely *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Klebsiella pneumoniae* (BAA-1705), *Acinetobacter baumannii* (BAA-1605), *Pseudomonas aeruginosa* (ATCC 27853) and *Enterococcus* sp. In addition to this, peptides **P9** and **P30** was screened against a bacterial panel consisting of ESKAPE pathogens, namely *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Klebsiella pneumoniae* (BAA-1705), *Acinetobacter baumannii* (BAA-1605), *Pseudomonas aeruginosa* (ATCC 27853) and *Enterococcus* sp. Drug-resistant clinical strains of *S. aureus* and *Enterococci*, including those that are resistant to vancomycin and other clinically-utilized antibiotics, were added to the panel. These strains were routinely cultivated on MHA and MHBII after being obtained from the Biodefense and Emerging Infections Research Resources Repository/Network on Antimicrobial Resistance in *Staphylococcus aureus*/American Type Culture Collection (BEI/NARSA/ATCC, USA). In order to develop the starter culture, a single colony was picked from the MHA plate, inoculated into MHBII, and incubated overnight at 37 °C with shaking for 18–24 h.

2.7.3. Solid Phase Peptide Synthesis (SPPS) and HPLC Purification

The solid phase peptide synthesis of the peptides **P1–P30** was performed manually on Rink amide resin at a scale of 0.2 mmol under standard Fmoc- conditions. NMP was employed as a coupling solvent, 20% piperidine in DMF was used to deprotect the Fmoc- group, and the combination of HBTU and HOBt was used as coupling reagents. The Kaiser test was used to monitor the coupling reactions. After the synthesis is finished, the resin has been stirred for around 3 hours in a cocktail of trifluoroacetic acid (TFA)/triisopropylsilane/water/phenol (90:5:5:5) to release the peptides from the solid support. Further, the resin was filtered. Under reduced pressure, the crude peptide-containing filtrate was evaporated, and the gummy residue was then triturated with cold diethyl ether (50 mL). Centrifugation was used to separate the precipitate. Further diluting the crude peptides in 10 mL of ACN/H₂O and then purified on a C₁₈ column by reversed-phase HPLC. The peptides were purified using an acetonitrile and water gradient system with a 0.1% TFA with 2 ml/min flow rate. By using MALDI-TOF/TOF analysis, the mass of the isolated peptides was confirmed. The pure peptides were lyophilized for further studies.

2.7.4. Antibiotic susceptibility testing

The broth microdilution technique was used to assess the antibiotic susceptibility of all peptides in accordance with the CLSI guidelines.¹¹⁵ Test compounds stock solutions at 10 mg/mL were prepared in DMSO. Bacterial cultures were inoculated in MHBII, optical density (OD) at 600 nm was measured, and dilution to achieve $\sim 10^6$ CFU/mL was subsequently carried out. In a two-fold serial dilution method, the compounds were tested at concentrations ranging from 64 to 0.5 mg/L, with 2.5 μ L of each concentration added to each well of a 96-well round bottom microtiter plate. After that, each well containing either the test compounds or the relevant controls received 97.5 μ L of bacterial solution. The plates were incubated at 37 °C for 18 to 24 hours before the MIC value was determined. The lowest concentration of the compound at which there is no discernible growth is known as the MIC. Using duplicate samples, MIC determinations were performed three times independently for each test compound.

2.7.5. Bacterial time kill kinetics with P5 and P9

The time-kill approach, which was previously reported,¹¹⁶ was used to determine the presence or absence of bactericidal activity. Briefly, *S. aureus* ATCC 29213 was diluted in MHBII to $\sim 10^6$ CFU/mL and treated with 1X and 10X MICs of peptides **P5**, **P9** and Vancomycin, and then incubated at 37 °C with shaking for 24 hours. 100 μ L samples were collected at 0, 1, 6, and 24 hours. They were serially diluted in PBS and plated on MHA, then incubated at 37 °C for 18 to 20 hours. By counting the colonies from plates and graphing the CFU/mL of surviving bacteria at each time point in the presence and absence of compound, the kill curves were generated. The mean values were plotted after each experiment was carried out three times in triplicate.

Experiment was repeated for the peptide **P9** for 60 min intervals shaking at 37 °C. Here, *S. aureus* ATCC 29213 was diluted in MHBII to $\sim 10^6$ CFU/mL and treated with 2.5 X, 5 X and 10 X MICs of peptide **P9** and Vancomycin, and then incubated at 37 °C with shaking for 60 mins. 100 μ L samples were collected at 0, 5, 10, 15, 30, 45 and 60 mins. They were serially diluted in PBS and plated on MHA, then incubated at 37 °C for 18 to 20 hours. By counting the colonies from plates and graphing the CFU/mL of surviving bacteria at each time point in the presence and absence of compound, the kill curves were generated. The mean values were plotted after each experiment was carried out three times in duplicate.

2.7.6. Proteolytic Stability of Peptide P5 and P9 against Trypsin Hydrolysis

Each Peptide (1 mg) was dissolved in 1 mL of Tris buffer (50 mM, pH 8.2). Here, we tested the proteolytic resistance of peptides using 0.05% trypsin in EDTA solution, which we bought from Sigma. In the present study, 200 μ L of the primary stock solution (1 mg/mL) of peptide was taken, and 2 μ L of 0.05% trypsin in EDTA were added. This mixture was then incubated at 37 °C to test the stability of the peptide. At various time intervals [0, 30, 60, 90, 120, 360, and 1440 min, respectively], further 25 μ L aliquots of the incubated solution were taken. This was further diluted by adding 25 μ L of Tris-buffer (50 mM). After this, the stability of the peptide against trypsin hydrolysis was examined using reverse phase HPLC with ACN/H₂O as the mobile phase.¹¹⁷

2.7.7. Haemolytic activity of AMPs

Fresh human red blood cells (hRBCs) were collected with anti-coagulating agent EDTA. The hRBCs were diluted to a final concentration of 4% v/v after four rounds of washing in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.2). A final volume of 100 μ L was used for the test in a sterile 96-well plate, and an aliquot of 50 μ L of hRBCs suspension was added to 50 μ L (100 μ g/mL) of 2-fold serially diluted peptides in Tris buffer. The plate was first incubated for one hour at 37 °C, followed by a 15-minute, 3000 rpm centrifugation. The supernatant (50 μ L) from each well was transferred to a new 96-well plate with 50 μ L of water, and the absorbance at 540 nm was measured to track the release of haemoglobin. The studies were carried out twice, and the negative and positive controls, respectively, were hRBC suspensions with Tris buffer and 1% Triton-X. The formula for calculating percentage of hemolysis was $(U - U_N) / (U_P - U_N) \times 100$, where U represents the test well's absorbance, U_N the negative control's absorbance, and U_P the positive control's absorbance (100 % percent hemolysis).¹⁴

2.7.8. Cell cytotoxicity of AMPs

The MTT assay was employed to test Vero cells for cell toxicity.¹¹⁸ A 96-well plate with $\sim 10^3$ cells/well was seeded, and it was incubated at 37 °C in a 5% CO₂ atmosphere. After 24 hours, the test compound was added and incubated for 72 hours at a concentration ranging from 100-12.5 μ g/mL. Following the incubation, MTT was added to each well, which was then incubated for a further 4 hours at 37 °C. The residual medium was then discarded, 0.1 mL of DMSO was added to solubilize the formazan crystals, and an OD measurement at 540 nm was taken to calculate the CC₅₀. The definition of CC₅₀ is the lowest concentration of a compound at which

a 50% reduction in cell viability occurs. Each experiment was carried out three times using a positive control substance called doxorubicin.

2.7.9. Drug interaction of P9 with FDA approved drugs

Interaction of **P9** with FDA approved drugs namely Vancomycin, Levofloxacin, Ceftazidime, Linezolid and Gentamycin was tested by the checkerboard method. Serial two-fold dilutions of each drug were freshly prepared prior to testing. **P9** was two-fold diluted along the abscissa ranging from 8-0.0008 mg/L (12 dilutions) while the antibiotics were serially diluted along the ordinate (8 dilutions) ranging from 2-0.03 mg/L for Vancomycin and Gentamycin; 4-0.06 mg/L for Linezolid; 1-0.015 mg/L for Levofloxacin and 32-0.25 mg/L for Ceftazidime in 96 well microtiter plate. 95 μ L of $\sim 10^5$ CFU/mL was added to each well and plates were incubated at 37°C for 24 h. After the incubation, the Σ FICs (fractional inhibitory concentrations) were calculated as follows: Σ FIC = FIC A + FIC B, where FIC A is the MIC of drug A in the combination/MIC of drug A alone and FIC B is the MIC of drug B in the combination/MIC of drug B alone. The combination is considered synergistic when the Σ FIC is ≤ 0.5 , indifferent when the Σ FIC is > 0.5 to 4, and antagonistic when the Σ FIC is > 4 .¹¹⁹

2.7.10. Induced Resistant Mutant Generation Studies Using P9 against *S. aureus* ATCC 29213

Serial passaging was started once the MIC was determined by harvesting bacterial cells that were growing at the highest concentration of the compound and inoculating them into fresh media containing one-half of the MIC of the test compound. Another MIC assay was performed on this inoculum. Cells from the previous passage that were growing in the compound's maximum concentration were again harvested and tested for MIC after a 24-hr incubation period. For 29 passages, the process was repeated. The fold increase in MIC was calculated by plotting the compound's MIC value against the number of passages.¹²⁰

2.7.11. Post antibiotic effect (PAE) of Peptide P9 against *S. aureus* ATCC 29213

In order to determine the PAE of **P9**, an overnight culture of *S. aureus* ATCC 29213 was diluted in MHBII to 105 CFU/mL, subjected to 1 X and 10 X MICs of Vancomycin and **P9**, and then incubated at 37°C for 1 h. After the incubation period, the culture was centrifuged and two times washed with pre-warmed MHBII to get rid of any antibiotic residue. Cells were then reconstituted in drug-free MHBII and continued to incubate at 37 °C. After every 1 hour, samples were taken, serially diluted, and plated on TSA for the enumeration of CFU. The PAE

was calculated using the formula $PAE = T - C$, where T is the difference in time needed for a 1 Log₁₀ rise in CFU versus the CFU that was observed immediately after the drugs was removed, and C is the CFU in a similarly treated drug-free control.¹²¹

2.7.12. Determination of activity of P9 against *S. aureus* biofilm

As previously mentioned,¹²² the determination of **P9** anti-biofilm activity was carried out. *S. aureus* ATCC 29213 was cultivated over night at 37 °C in 1% TSB with 180 RPM shaking. The overnight culture was diluted in fresh TSB broth (1:100) and 0.2 mL of the freshly diluted culture was added to a 96-well polystyrene flat bottom plate. The plate was then covered with an adhesive foil lid to maintain low oxygen levels and incubated for 48 hours at 37 °C in a static condition. After incubation, the media was decanted, and to get rid of the planktonic bacteria, the plate was gently rinsed three times with 1X PBS (pH 7.4). Refilled plates with TSB at a different drug concentration were then incubated for a further 24 hrs at 37 °C. The media was decanted after drug treatment, washed three times with 1X PBS (pH 7.4), and then the biofilm was fixed by incubating the plate at 60 °C for 1 hr. The biofilm is then fixed, stained with 0.06% crystal violet for 10 minutes, rinsed with PBS, and allowed to dry at room temperature. The bound crystal violet was eluted with 30% acetic acid (0.2 mL) to quantify the biofilm. To quantify the biofilm, an absorbance measurement at 600 nm on a microtiter plate reader was carried out. Along with **P9**, levofloxacin, vancomycin and disulfiram was used as a comparator.

2.7.13. Animals experiments

BALB/c mice that were six to eight weeks old were purchased from the National Laboratory Animal Facility of the Central Drug Research Institute of the CSIR in Lucknow. The Central Drug Research Institute of the CSIR in Lucknow's Institutional Animal Ethics Committee examined and approved the experimental protocols. The Committee for the Purpose of Control and Supervision of studies on Animals (CPCSEA, Govt. of India) criteria were followed when conducting animal studies.

2.7.13.1. Activity of P9 in murine skin infection model against *S. aureus* ATCC 29213

In this investigation, male BALB/c mice aged six weeks were used. The animals were housed in individual cages and kept in a controlled environment with a temperature of 25 ± 2 °C, a humidity level of 40%, and a light/dark cycle of 12/12 hrs. To begin with, an intradermal injection of anesthetic (a mixture of 60 mg/kg ketamine and 8 mg/kg xylazine, i.p.) was

administered to the mice. The skin was cleansed with sterile water after the dorsal hair was removed, and then *S. aureus* ATCC 29213 (10^8 cfu/ml) was injected to cause the infection. To assess the infectious dose in each experiment, a set of untreated mice were euthanized 4 hrs post-infection, and topical test treatments were started at the same time. Following the first treatment, the second dose was administered 16 hrs later, and from that point on, the drug regimen consisted of twice daily administrations (in the morning and evening, distanced by 8 hours), for a period of 4 days. The treatment groups included mice that were untreated, treated with 2% fusidic acid (positive control), 1% **P9** (test drug), and treated with base (vehicle). The mice received regular treatments of 25–30 mg of each of 2% fusidic acid, 1% **P9**, and base, as specified. To prevent carryover effects, all treatment groups were killed 18 hrs following the final topical treatment. After sacrifice, $\sim 2 \times 2$ cm² portions of injured skin were removed aseptically, and they were then homogenized with 500 μ L of 1 $\times$ PBS in 2 mL MP tissue grinding Lysing Matrix F tubes by using MP FastPrep-24 set at 4.0 m/s for 30 s (three cycles). Following homogenization, the appropriate dilutions were plated on TSA plates and overnight incubated at 37 °C to determine the quantity of surviving bacteria in each treatment group. Three times the experiment was conducted, the mean log₁₀ cfu/g for every experiment was calculated, and all three experiments average means and SEM were plotted.¹²³

2.7.13.2. Activity of P9 in murine neutropenic thigh infection model against *S. aureus* ATCC 29213

The murine neutropenic *S. aureus* infection model as described was used to assess the *in vivo* ability of peptide **P9** to eradicate *S. aureus* infection.¹¹⁶ In brief, male BALB/c mice weighing (~ 22 -24 g) were utilised throughout the investigation. The mice were rendered neutropenic by a series of intraperitoneal (IP) cyclophosphamide injections given 4 and 1 days before to injection.^{124a,124b} To establish infection, mice right thighs were then injected with *S. aureus* ATCC 29213 ($\sim 10^9$ CFU). Mice were intraperitoneally (IP) injected with peptide **P9** and vancomycin at doses of 5 mg/kg and 25 mg/kg of body weight, respectively, three hrs after the start of the infection. Saline was given to the control animals at the same dose and frequency as the medication. The mice were killed after 24 hrs, and the thigh tissue was removed, weighed, homogenised in 5 mL of saline, and serially diluted before plating on MHA plates for CFU analysis. CFU were counted after 18–24 hrs of incubation at 37 °C. The mean data is plotted after each experiment was carried out three times in duplicate.

2.7.14. Confocal Laser Scanning Microscopy (CLSM) Experiments

To understand the localization of peptide on the bacteria, *E. coli* K12MG1655 cells were incubated with Peptide **P9** and also **P30** which is tagged with FITC at the N-terminal of the peptide and propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) were used to counterstain the nucleic acids in cell and bacterial chromosome, respectively. Stock solutions of PI (1 mg/mL in water), DAPI (1 mg/mL in 5% 1,4-diazabicyclo[2.2.2]octane, 50% glycerol buffer), and peptides (1 mg/mL aqueous solution) were prepared. The *E. coli* K12MG1655 cells were grown in Luria-Bertani (LB) broth at 37 °C to an absorbance 0.2 at 600 nm then incubated with **P9** and FITC tagged peptide **P30** at 0.5× MIC values at 37 °C for 10 min. Cells in the absence of peptide considered as control. After incubation cells were centrifuged for 10 min at 4000 rpm (1073 g, rotor: Eppendorf Minispin), washed three times with media, finally cells were pelleted down and resuspended with PI (2 µL), DAPI (1µL) in PBS by maintaining 1 mL as a final volume. The unbound PI and DAPI dyes were removed by washing with excess PBS after a 15-minute incubation period at 4 °C. The sample (5µL) was drop casted on a glass slide that had already been cleaned, coated with a thin cover slip, and scanned using an OLYMPUS ZX81 laser scanning confocal microscopy by inverting the sample.¹¹¹

2.7.15. FE-SEM Study

100 µL of the peptides **P5**, **P9** and **P30** were pipetted into a 96-well cell culture plate at 2× MIC values. 100 µL of either the *S. aureus* or *E. coli* K12MG1655 bacterial solution in Luria-Bertani (LB) broth were added to this peptide solution. At 600 nm, the bacterial concentration was adjusted to 0.1–0.2 optical density. Approximately two hours were spent incubating the bacterial solution at 37 °C. The pellet was washed with PBS after centrifuging the cells at 4000 rpm for approximately 10 minutes at 25 °C. 2.5% glutaraldehyde was used to fix the cells, and PBS was used to wash them. The cells were then centrifuged again for approximately 10 minutes at 25 °C, washed with a graded EtOH solution of 30, 50, 70, 95, and 100%, and then finally washed with water. Finally, the dried bacterial cells were drop-casted on a silicon wafer and examined after the gold coating by taking the image under a field emission scanning electron microscope.^{14b}

2.7.16. Flow Cytometry to determine the Membrane Integrity

To understand the membrane integrity of peptides against *E. coli* K12MG1655 cells we performed through the fluorescence-assisted cell sorting (FACS) with a flow cytometer in the presence of peptides. The *E. coli* K12MG1655 cells were grown in Luria-Bertani (LB) broth at 37 °C to an absorbance 0.2 at 600 nm. Further, *E. coli* K12MG1655 cells were incubated with

peptides **P5**, **P9** and **P30** and also with FITC tagged peptide **P30** at $1 \times \text{MIC}$ values at 37°C for 15 min and also incubated with ampicillin as a positive control. After incubation cells were centrifuged for 10 min at 4000 rpm (1073 g, rotor: Eppendorf Minispin), washed three times with PBS, Then samples were resuspended with PI (10 $\mu\text{g/mL}$) in PBS. After 15 min incubation at room temperature in the dark, the unbound dye was removed by washing with an excess amount of PBS. The fluorescence-assisted cell sorting (FACS) with a flow cytometer (Becton-Dickinson, USA) was used to examine the cells stained by PI and FITC. The control sample was prepared by incubating the *E. coli* K12MG1655 cells in the absence of peptides.¹²⁵

2.7.16. Inner Membrane Permeability Assay

The *E. coli* K12MG1655 cells were grown in Luria-Bertani (LB) broth at 37°C to an absorbance 0.6 at 600 nm. Extracellular β -galactosidase was removed from the cells by centrifuging them three times and washing them three times with fresh media as reported earlier.¹⁴ Briefly, 10 μL of each of the peptides and melittin were added to 90 μL of Luria-Bertani broth containing *E. coli* K12MG1655 cells that produce β -galactosidase and the peptide and melittin final concentration in the solution was maintained as $1 \times \text{MIC}$. The bacterial solution was centrifuged for 10 min at 4000 rpm (1073 g, rotor: Eppendorf Minispin) after 1 hr incubation time. 20 μL (0.4 mg/mL) of 4-methylumbelliferyl- β -galactosidase indicator was added to 80 μL of the clear supernatant solution. By observing the fluorescence emission at 449 nm after excitation at 368 nm, the release of β -galactosidase was monitored up to 1 hr. The control experiment was carried out in the absence of the peptide.

2.7.17. Outer Membrane Permeabilization (1-NPN Assay)

The *E. coli* K12MG1655 cells were grown in Luria-Bertani (LB) broth at 37°C to an absorbance 0.2 at 600 nm. *E. coli* K12MG1655 cells were incubated with 1-N-phenylnaphthylamine (NPN) (10 μM) in LB broth for 30 min. For subtraction, the background fluorescence was recorded with multi-mode plate reader (excitation $\lambda = 350 \text{ nm}$, emission $\lambda = 420 \text{ nm}$). Then, in a sterile 96-well black plate, 100 μL of cell suspension was incubated with equal volumes of peptides and melittin solution at same $1 \times \text{MIC}$ concentrations. The fluorescence was recorded upto saturation level. Three biological replicates were used in each test, which was carried out separately in duplicate. Here polymyxin-B used as a positive control. The formula for calculating percentage of 1-NPN uptake was $(F - F_N) / (F_P - F_N) \times 100$, where F represents the test well's absorbance, F_N the negative control's absorbance, and F_P the positive control's absorbance (polymyxin-B: 100 % percent permeability).^{111b}

2.7.18. Outer Membrane Susceptibility Assay Using Polymyxin-B nonapeptide (PMBN)

The antibacterial susceptibility testing (Broth microdilution assay) procedure was used to determine the MIC of the compound and the control antibiotics (Rifampicin, Vancomycin, and levofloxacin) against Gram-negative bacteria (*E. coli* ATCC 25922 and *A. baumannii* BAA-1605) in the presence of polymyxin-B nonapeptide (PMBN). To make the outer membrane more permeable and make it more feasible for the compound to enter, MHB was given a sub lethal (10 µg/ml) dosage of PMBN.¹²⁶

2.8. References

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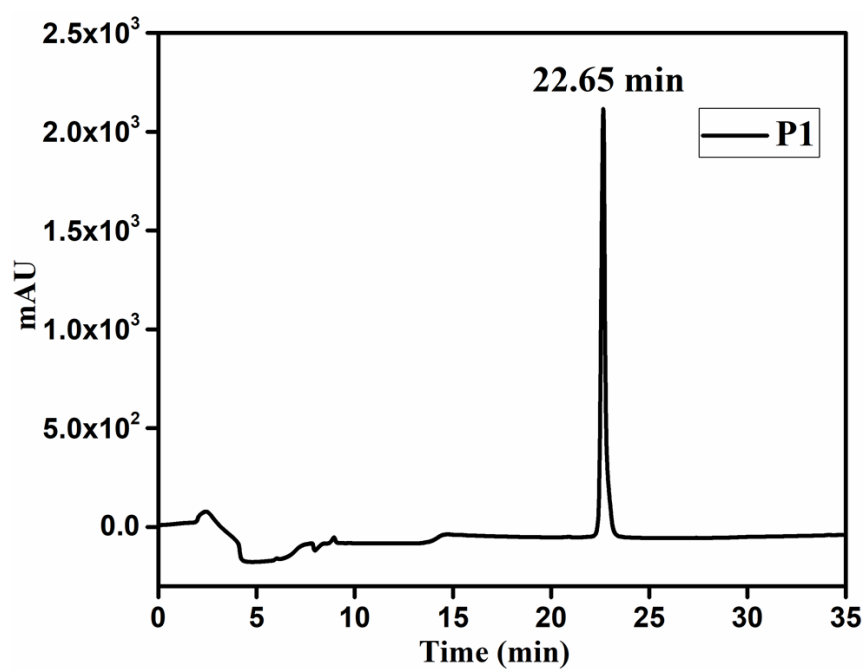
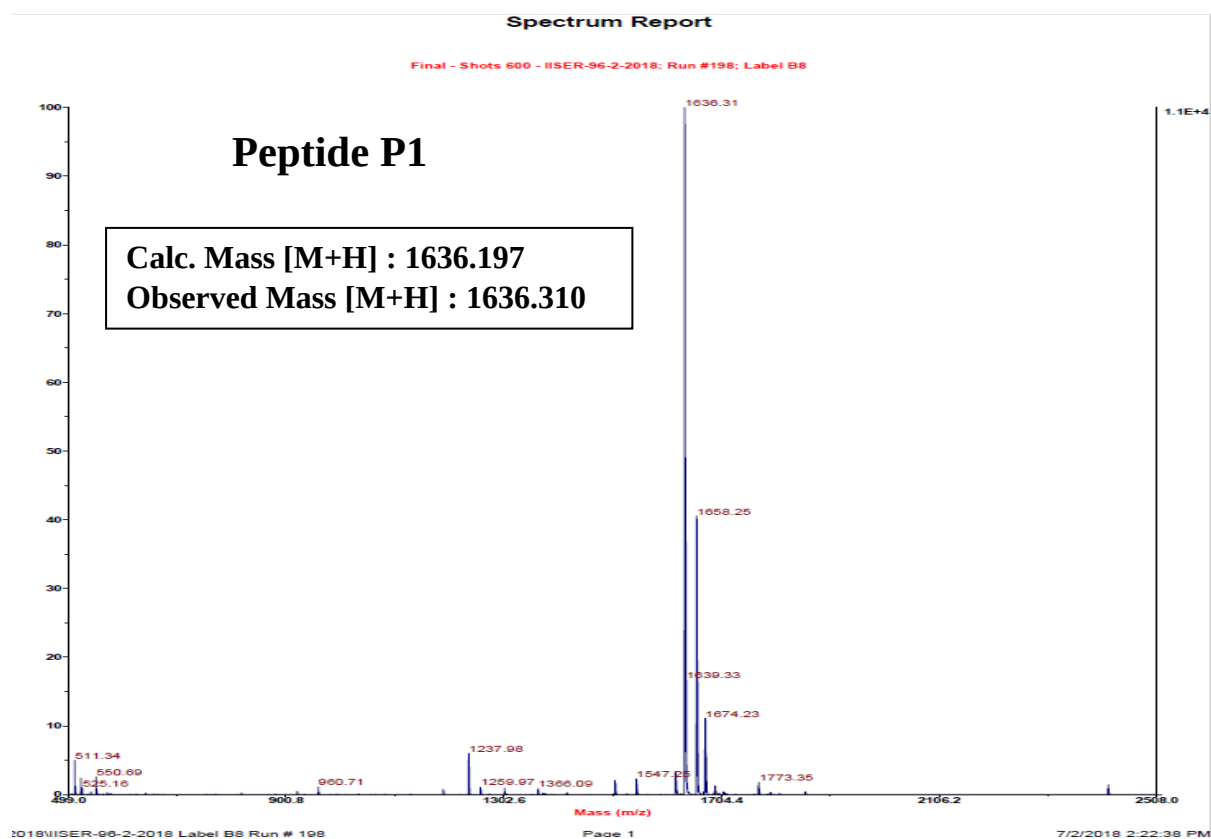
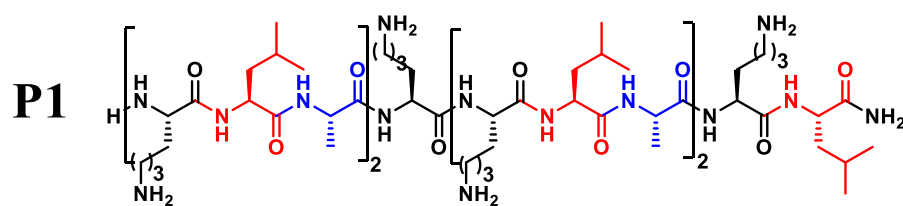
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2.9. Appendix II: Mass Spectra and RP-HPLC traces of Peptides P1-P32

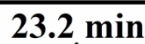




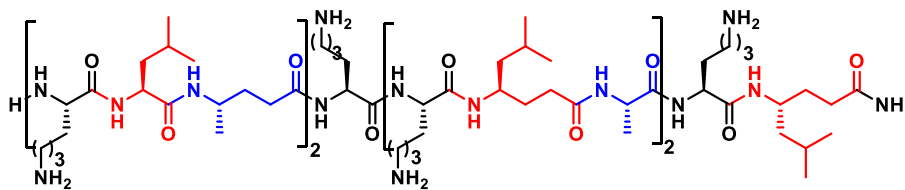
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Observed Mass [M+H] : 1679.410

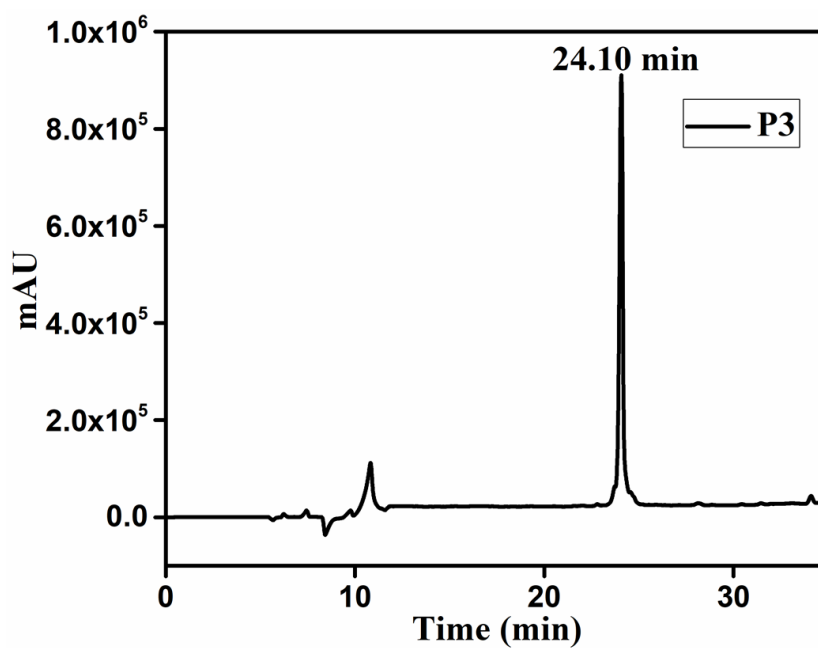
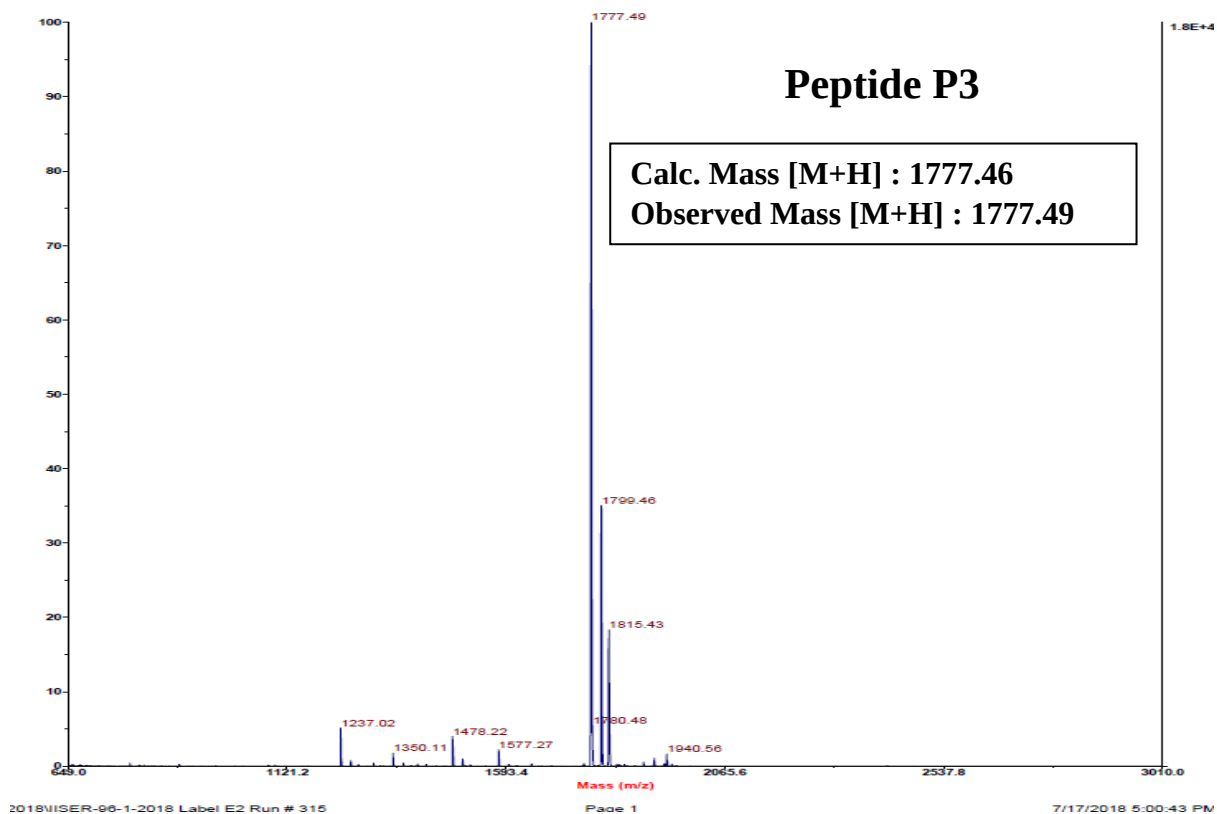


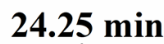
P3



Spectrum Report

Final - Shots 500 - IISER-96-1-2018; Label E2



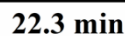




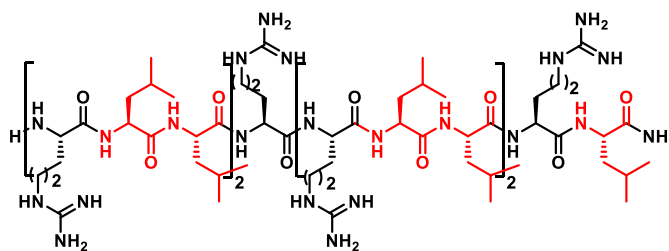
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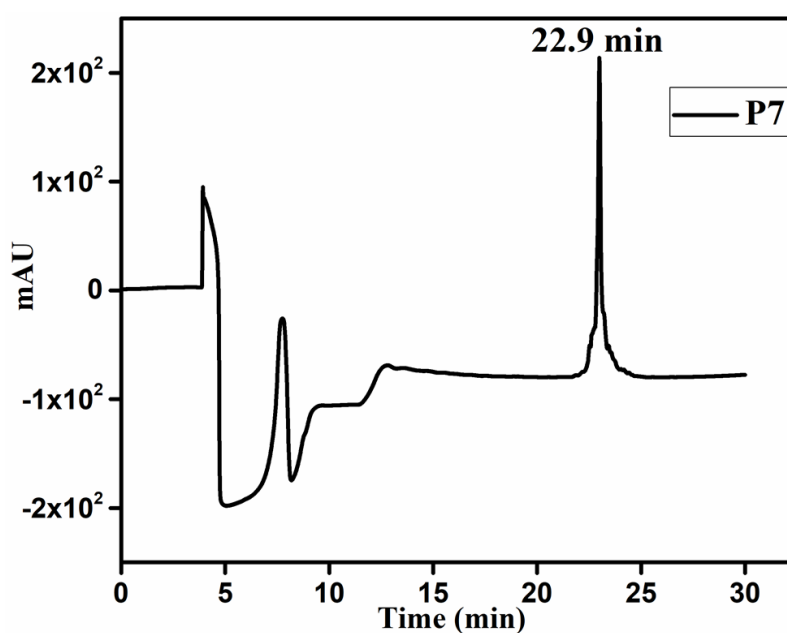
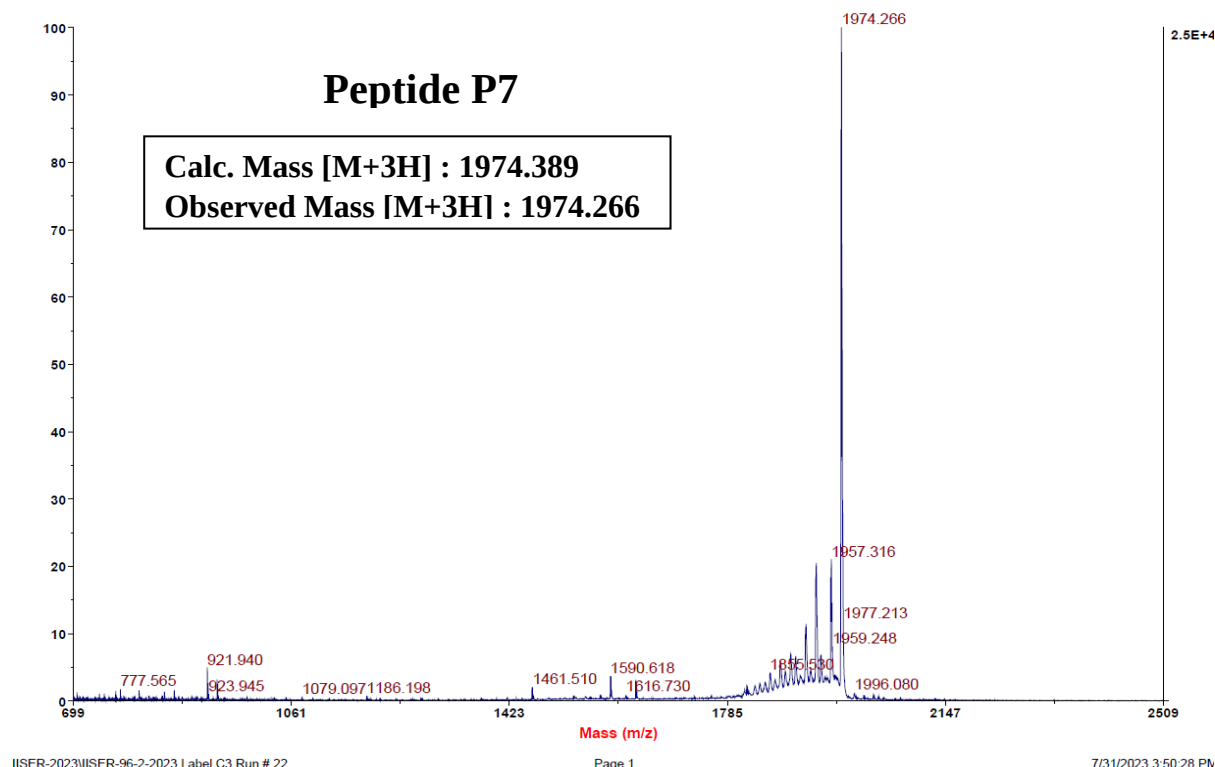


P7



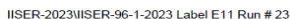
Spectrum Report

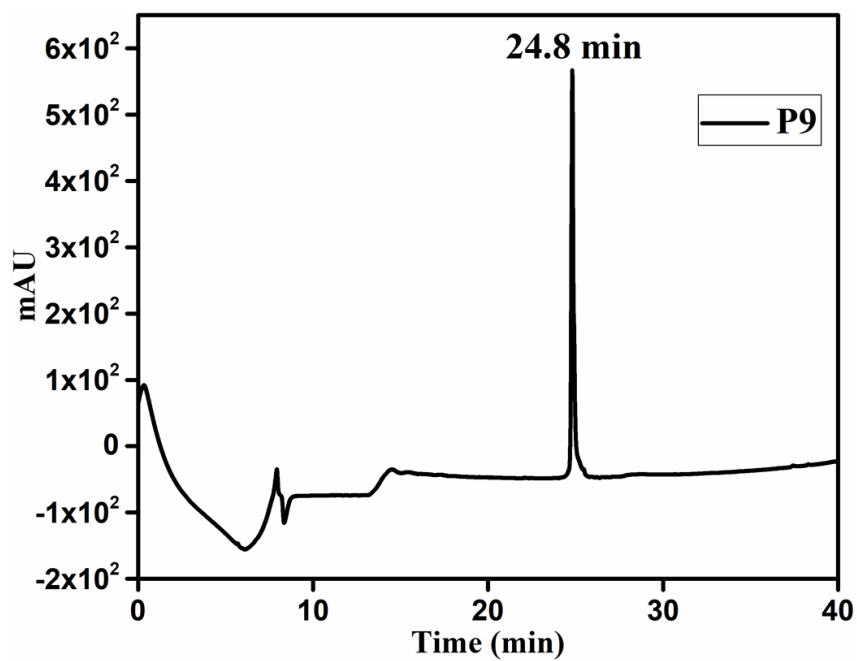
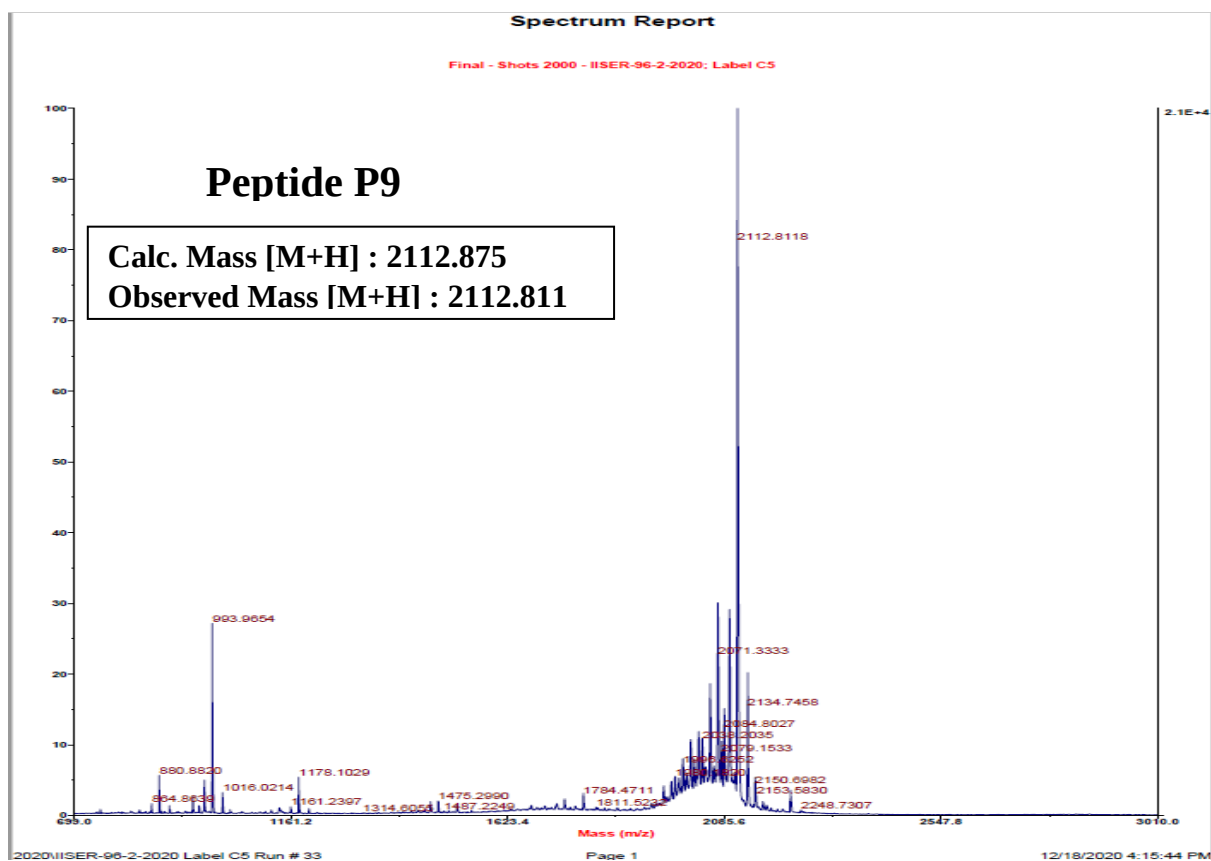
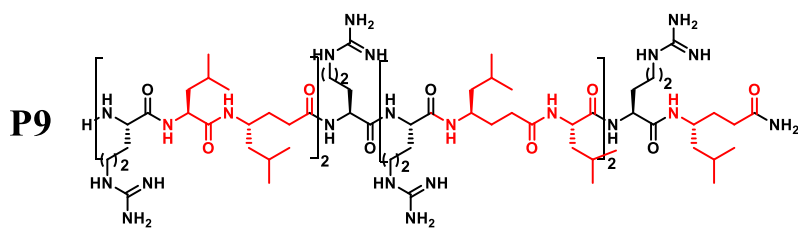
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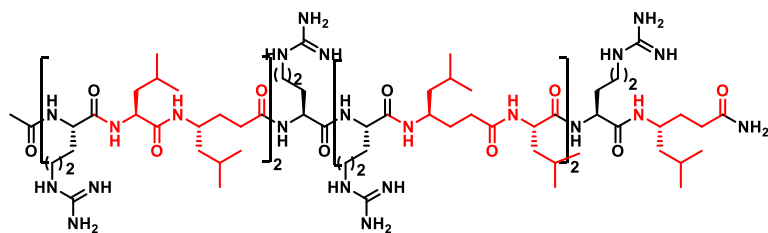


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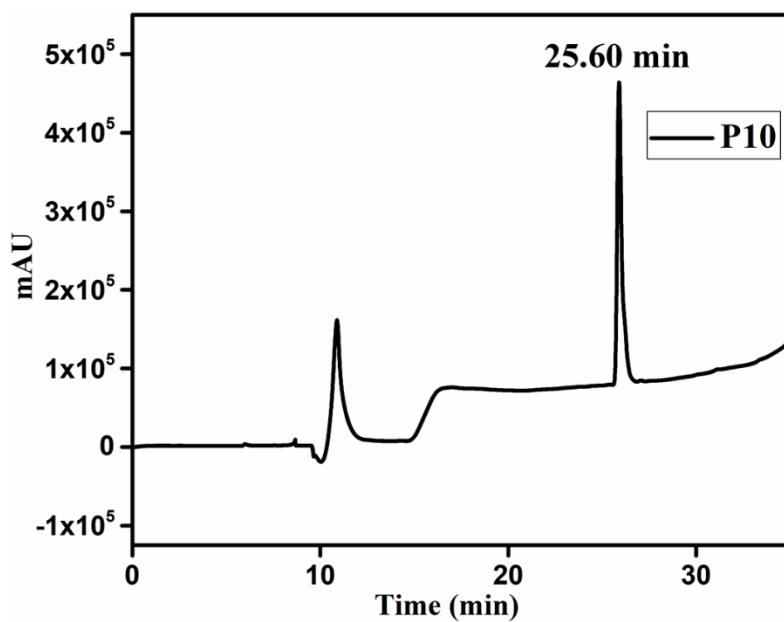
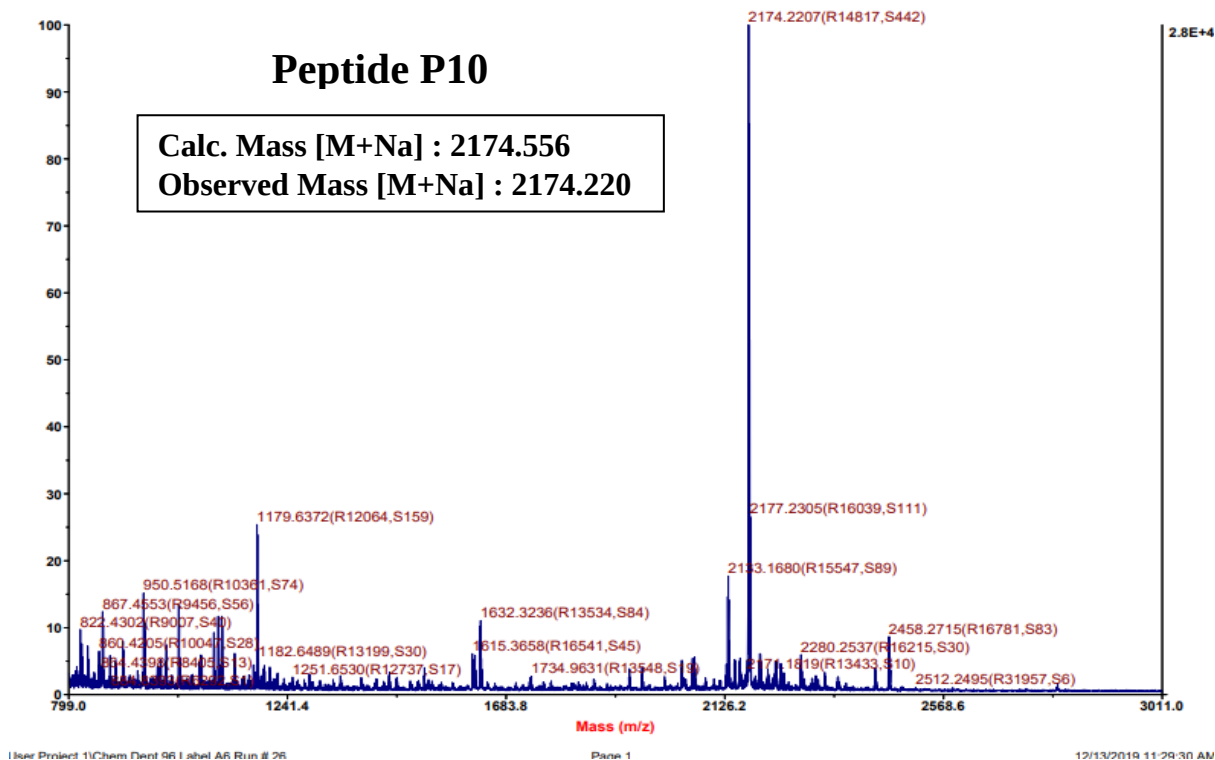


P10



Spectrum Report

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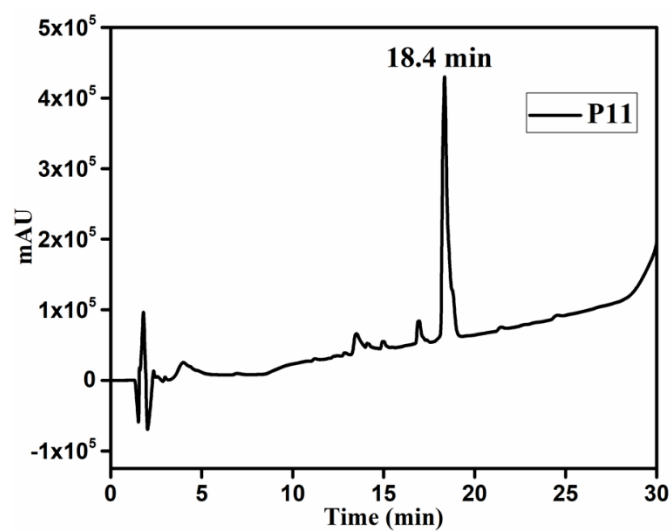




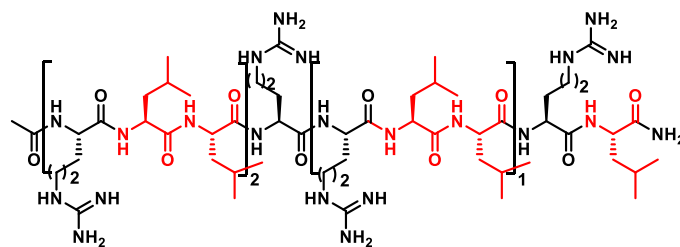
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Observed Mass [M+H] : 1590.371

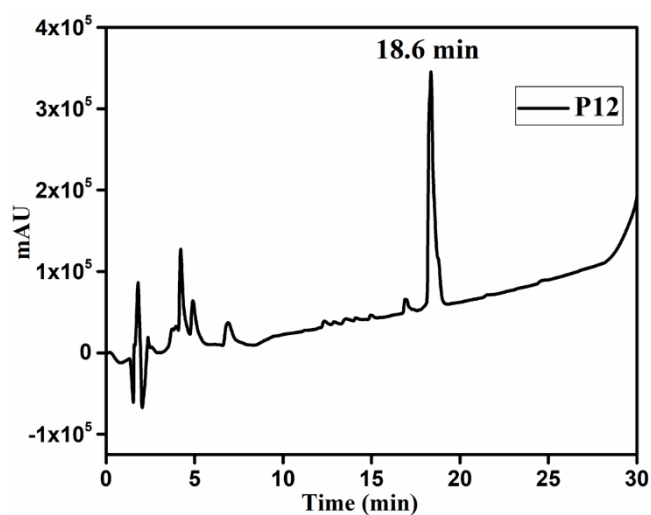
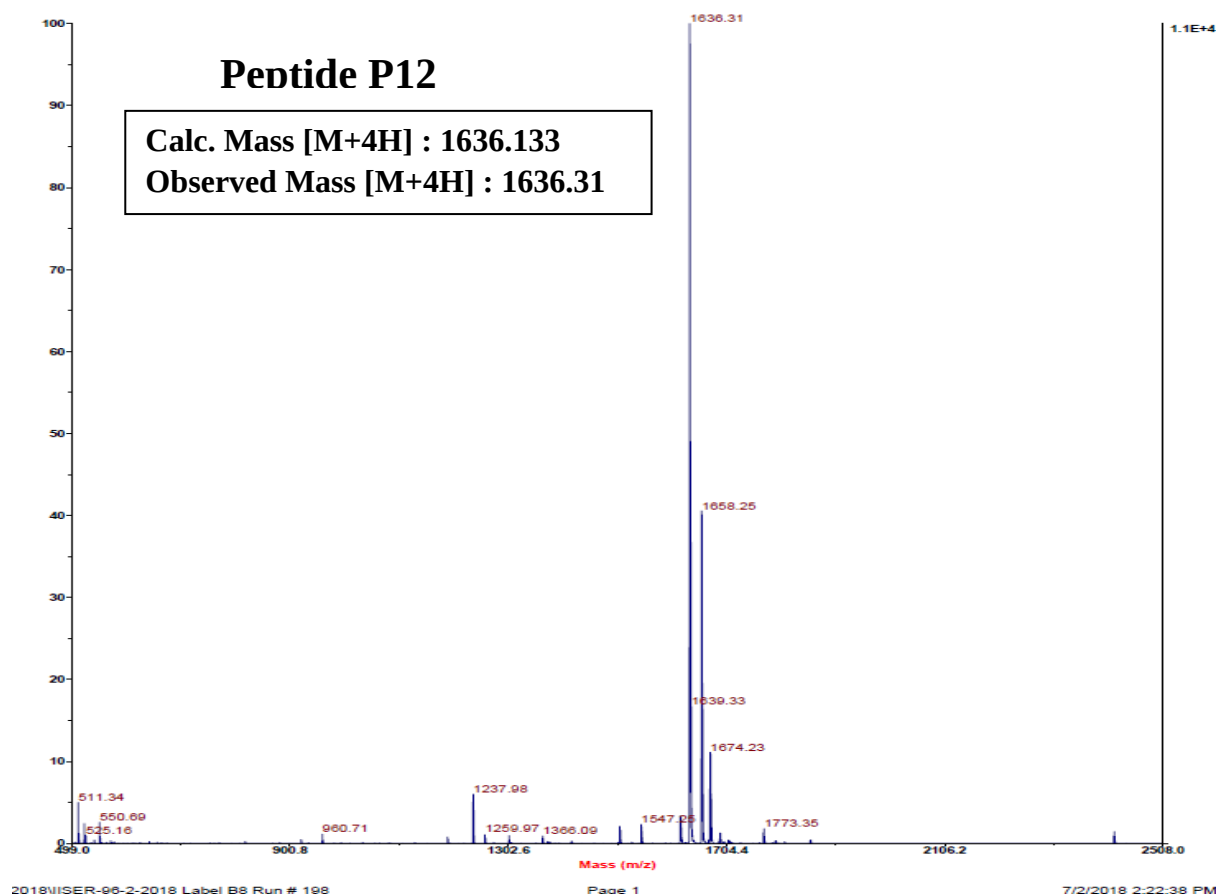


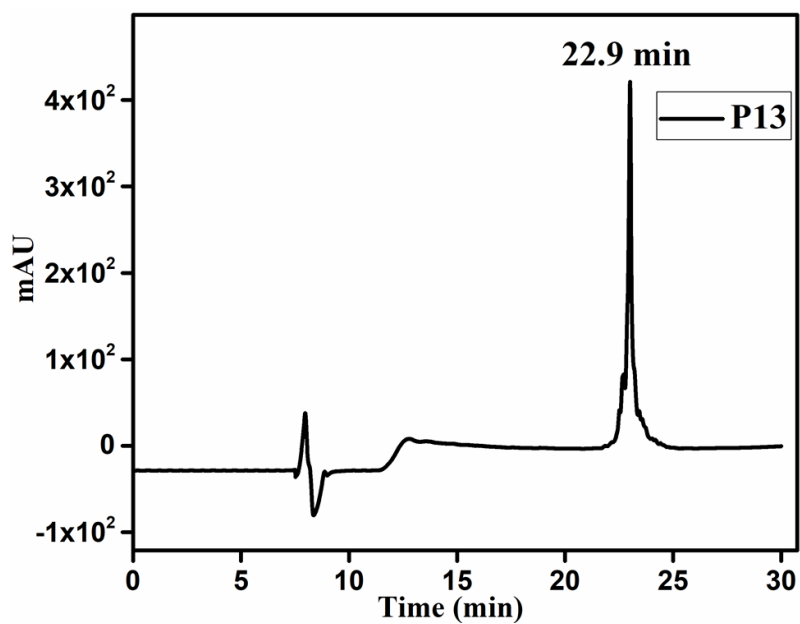
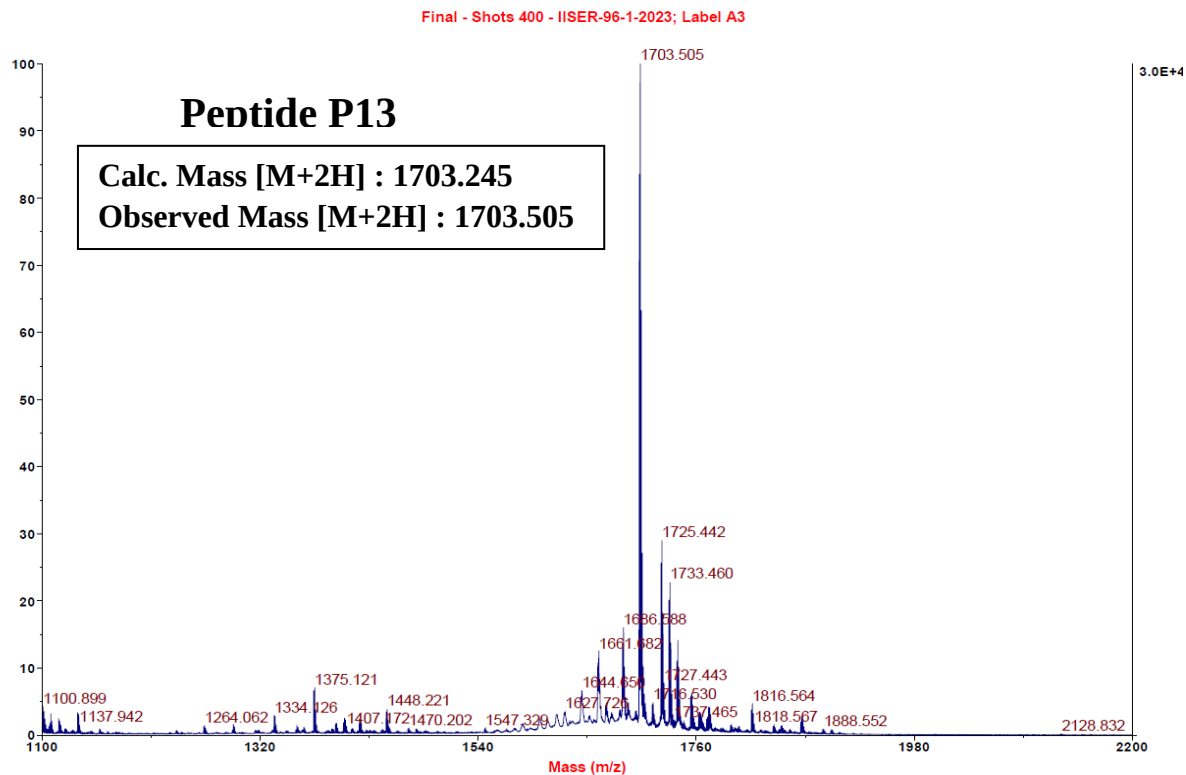
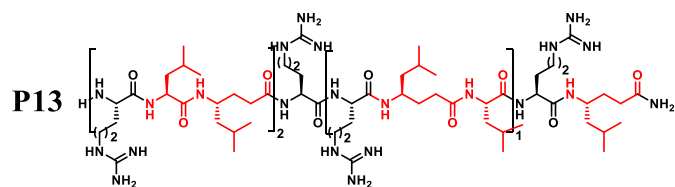
P12



Spectrum Report

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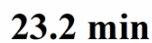




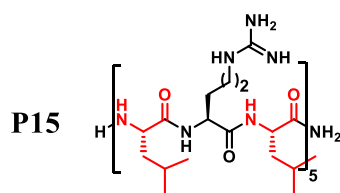
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Observed Mass [M+2H] : 1745.41

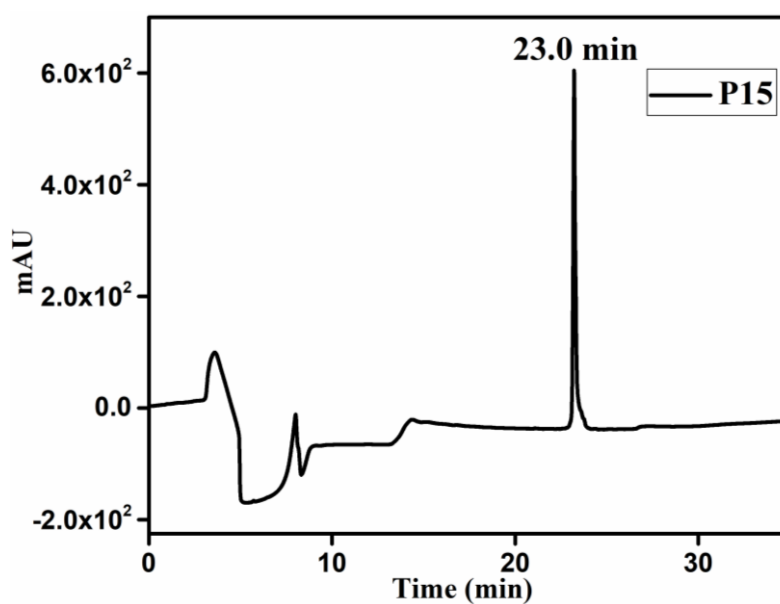
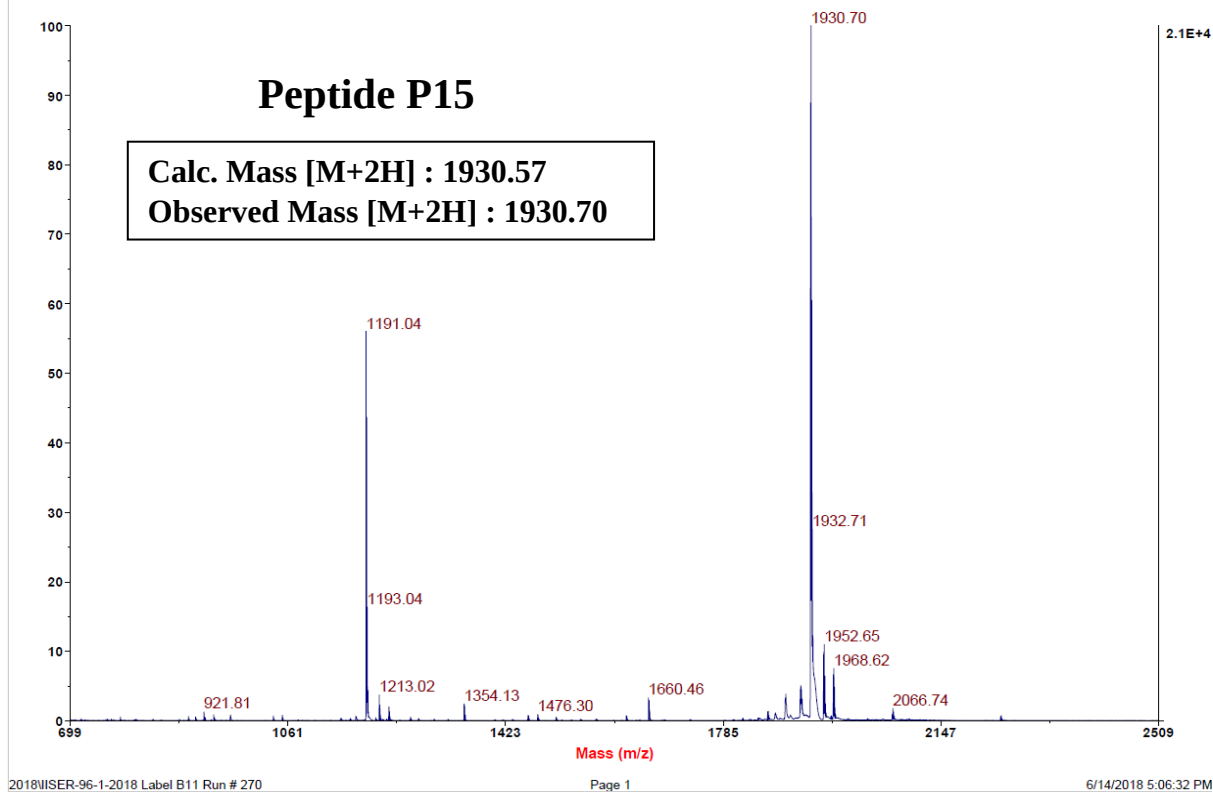


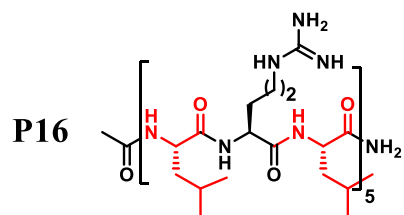
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Spectrum Report

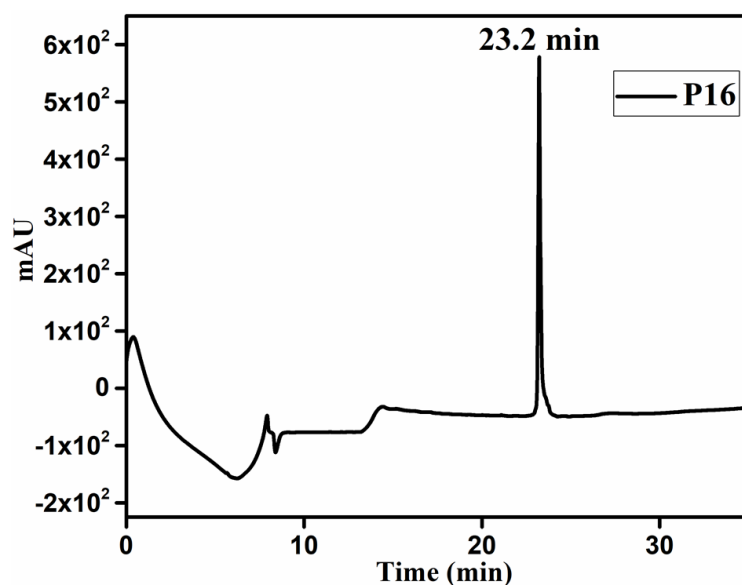
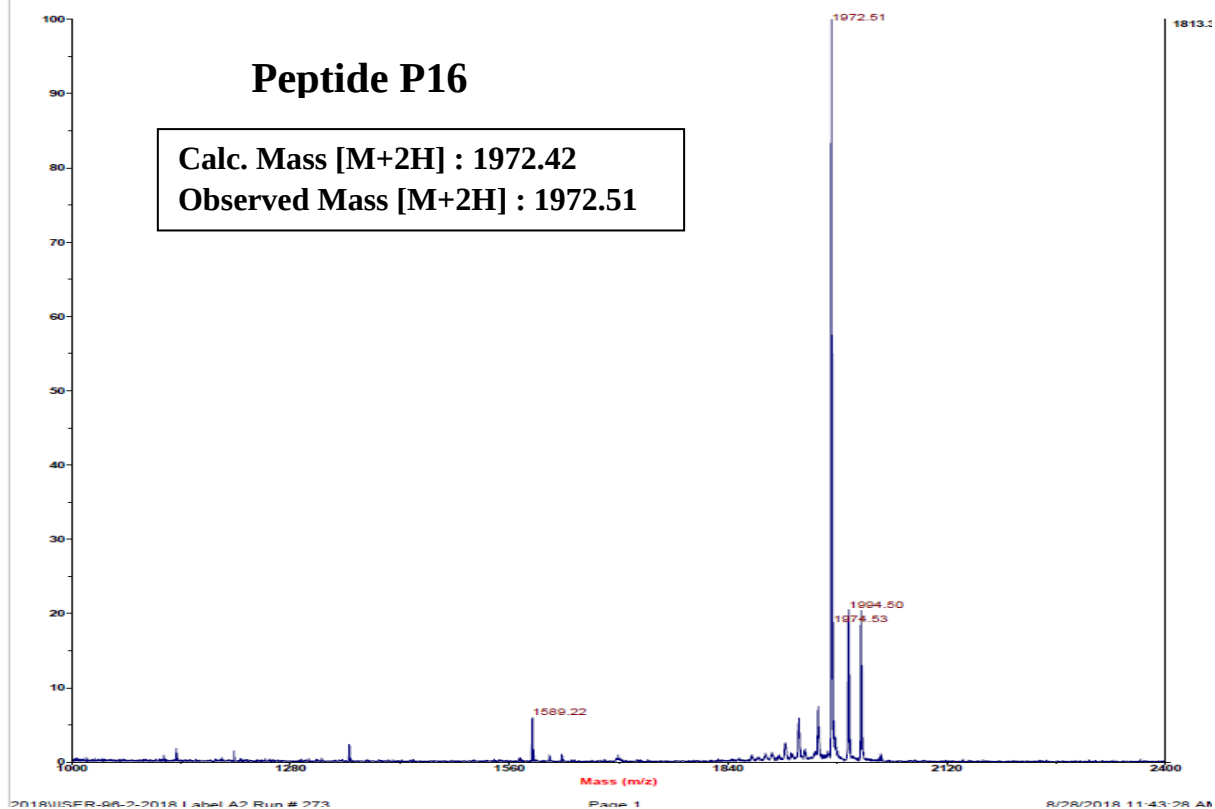
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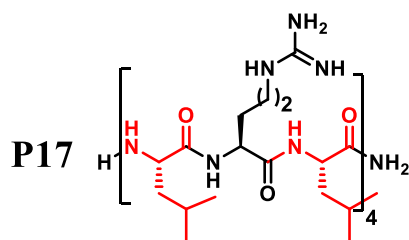




Spectrum Report

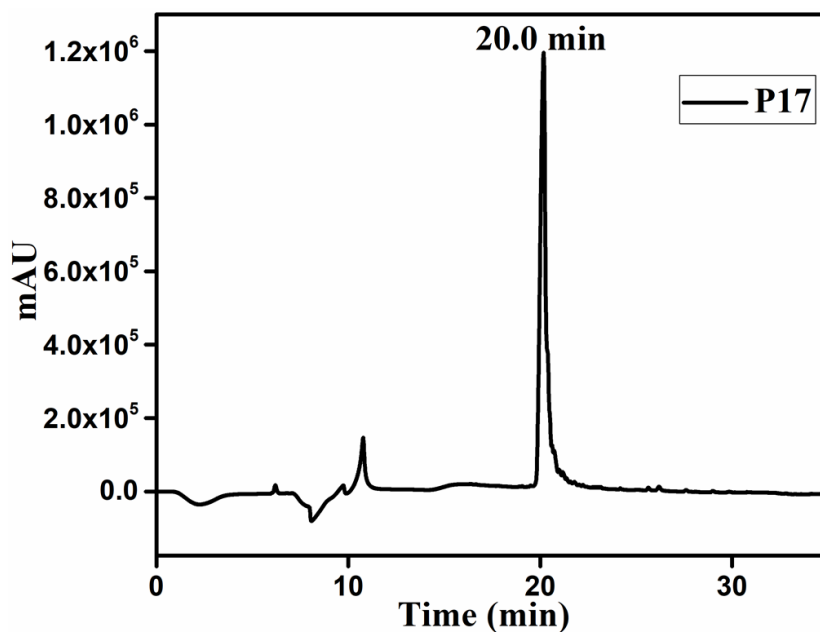
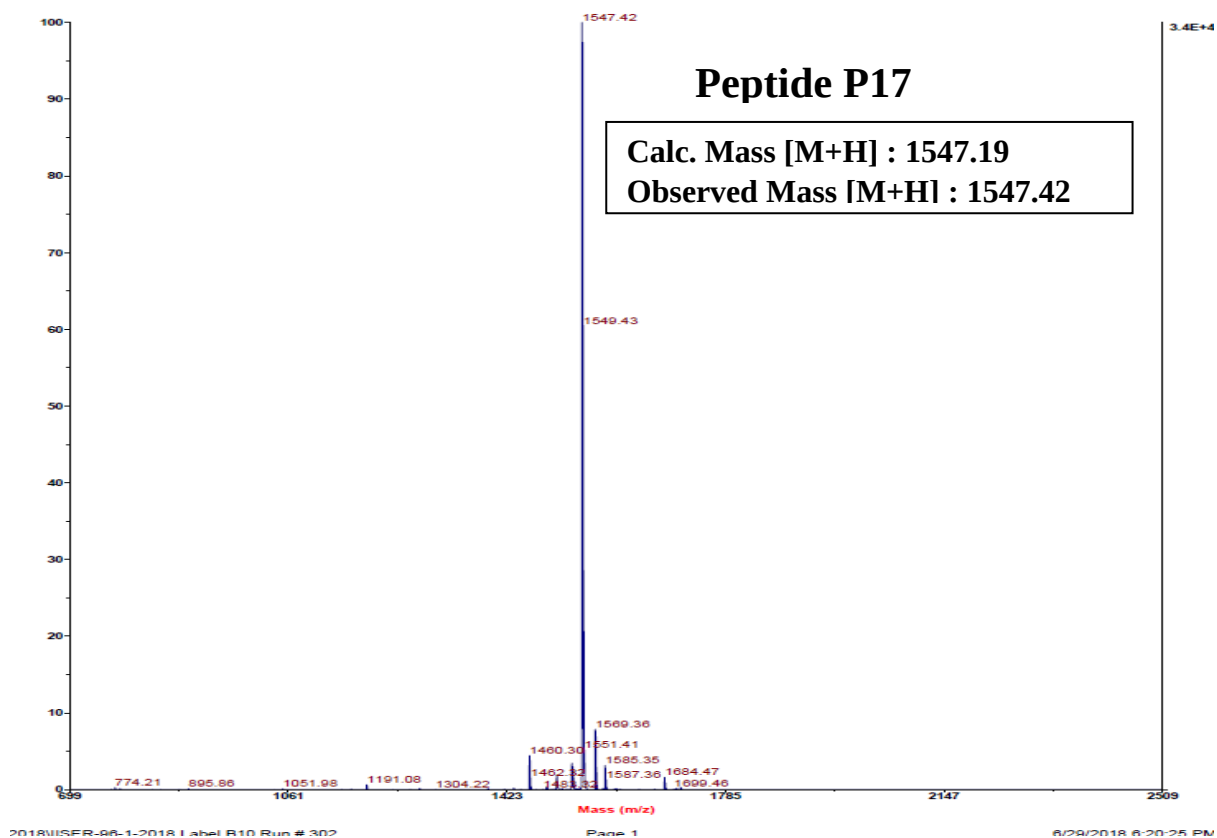
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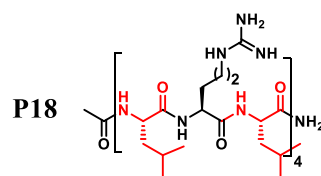




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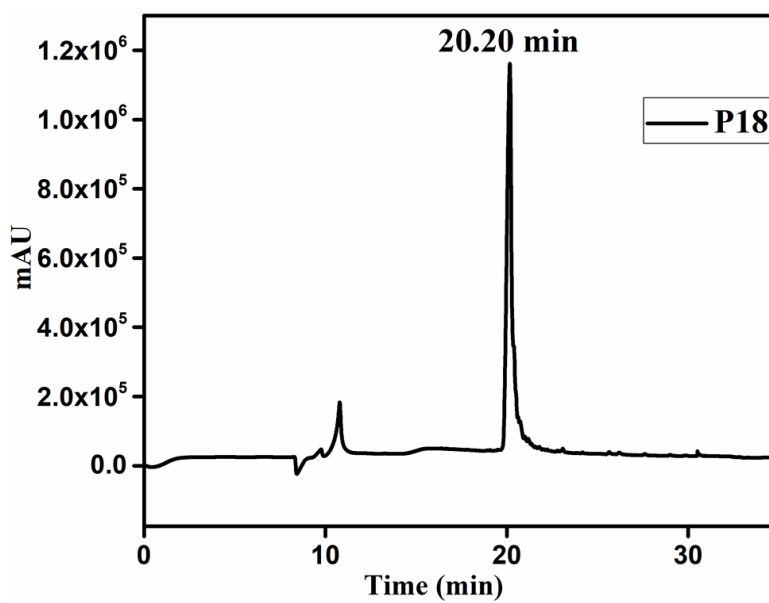
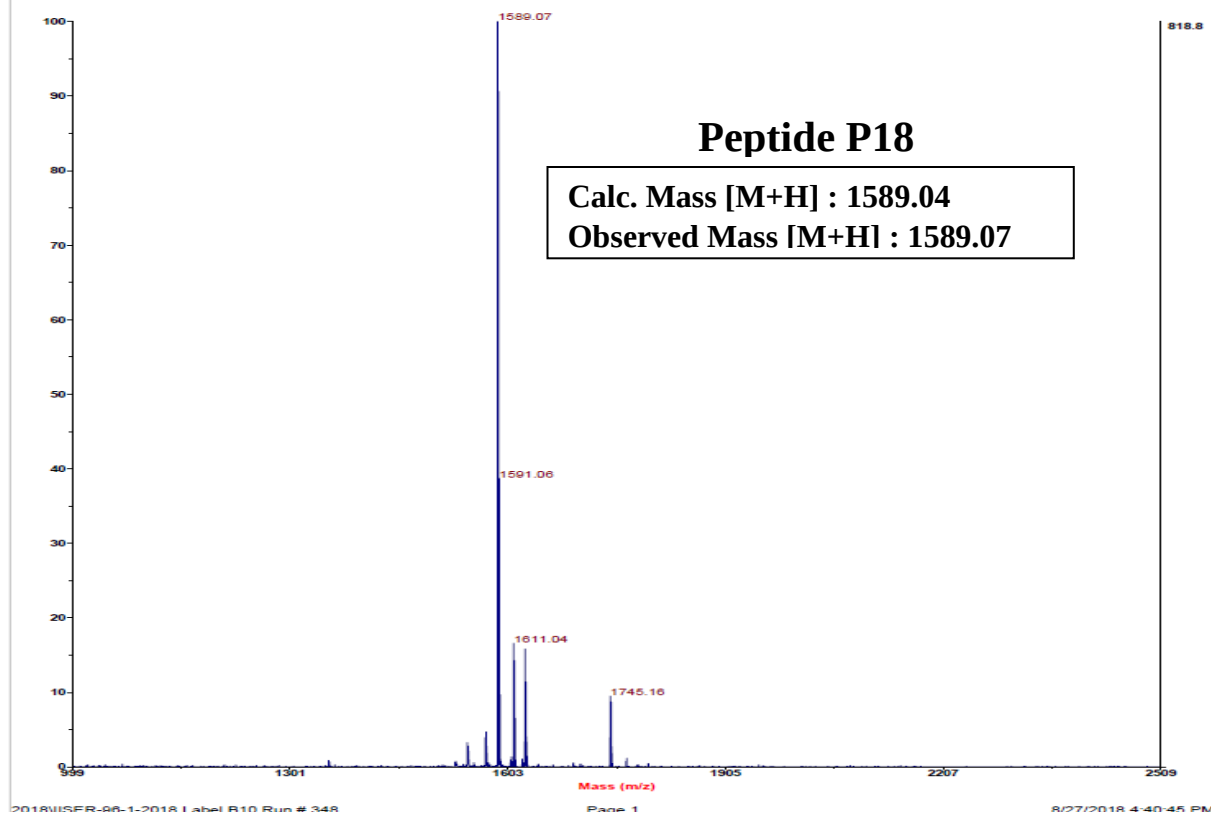
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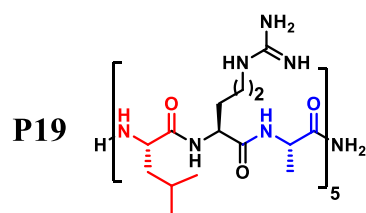




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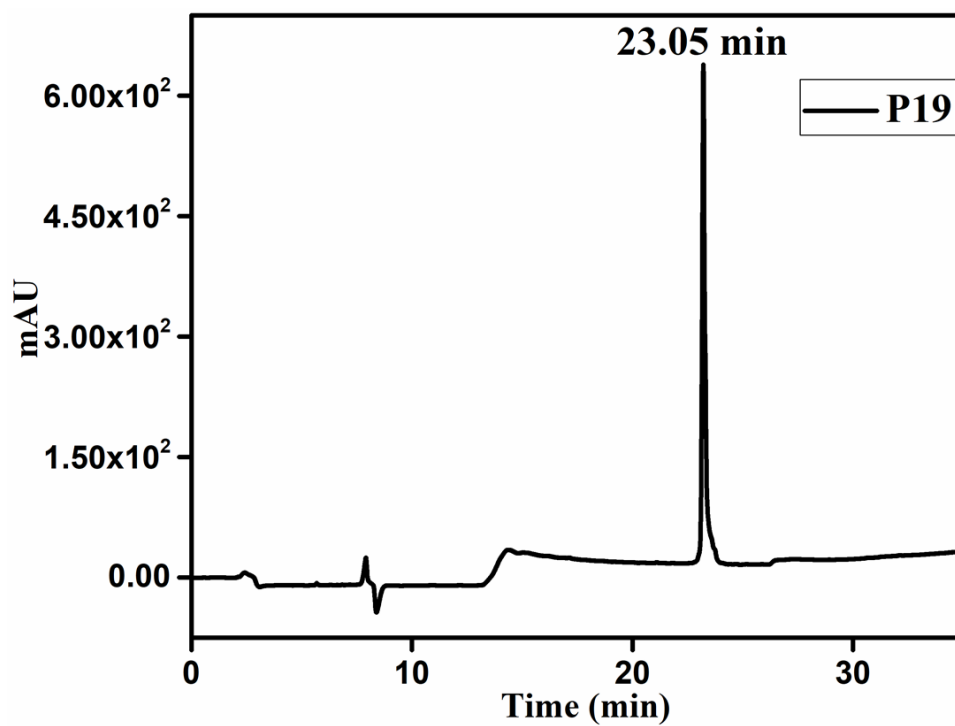
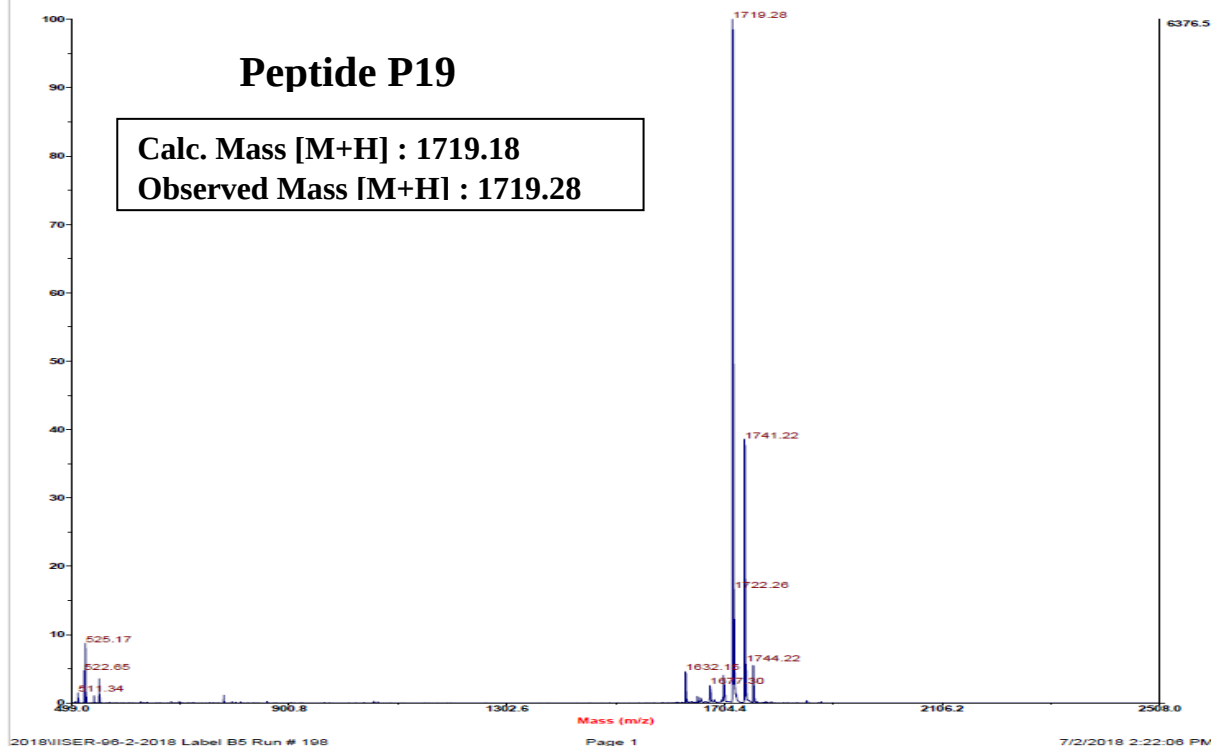
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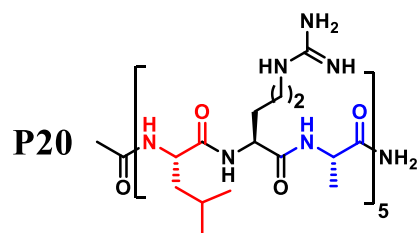




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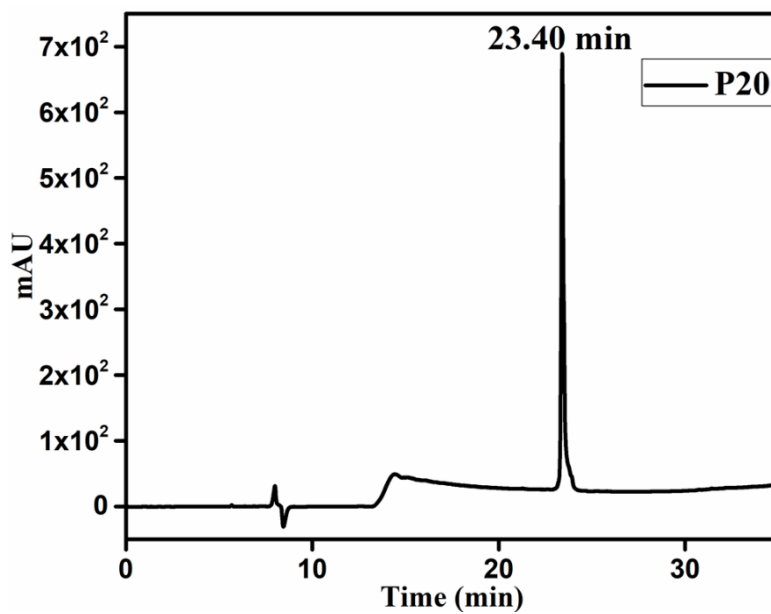
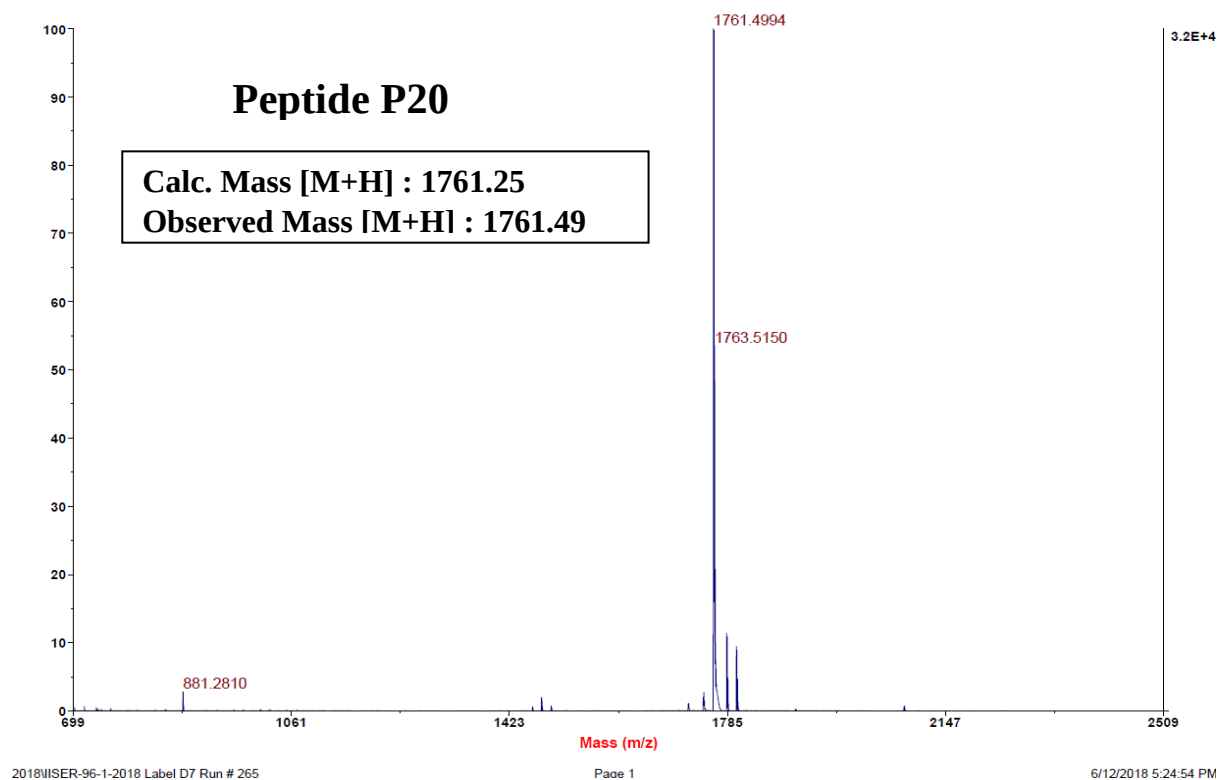
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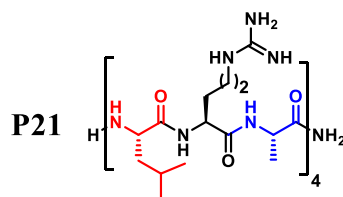




Spectrum Report

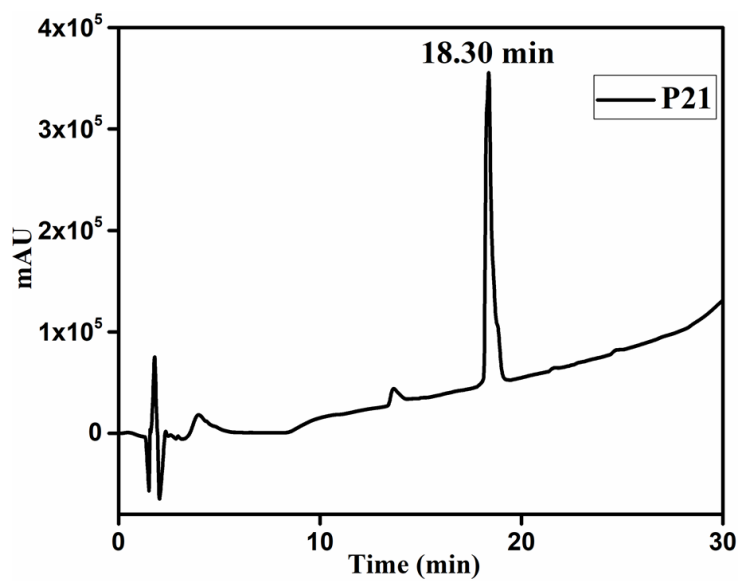
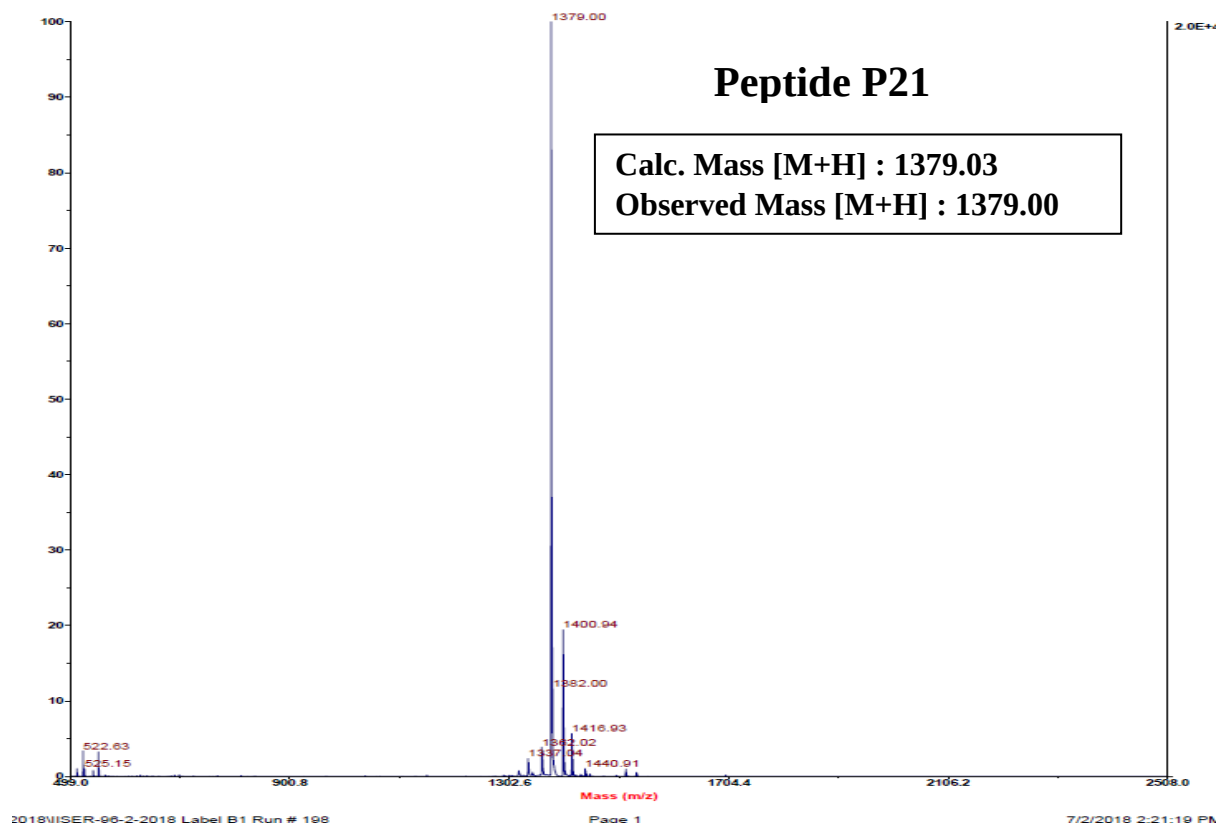
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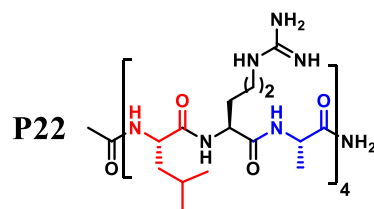




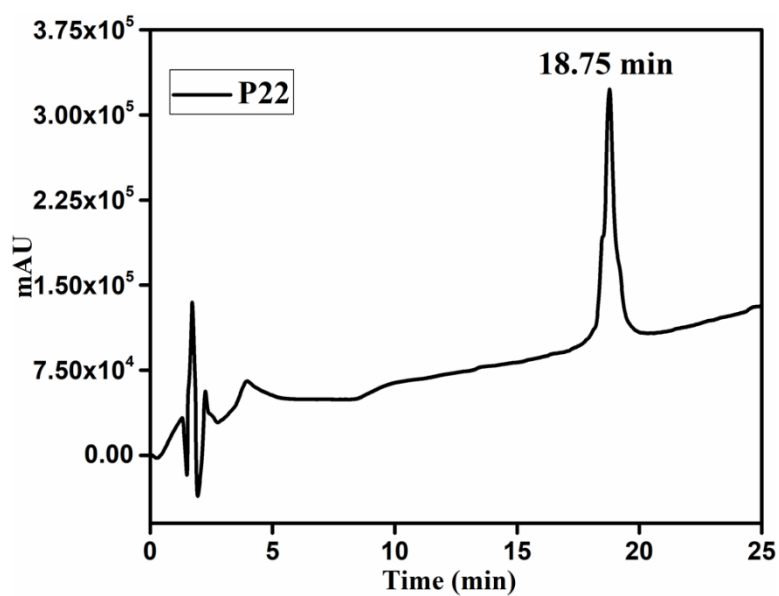
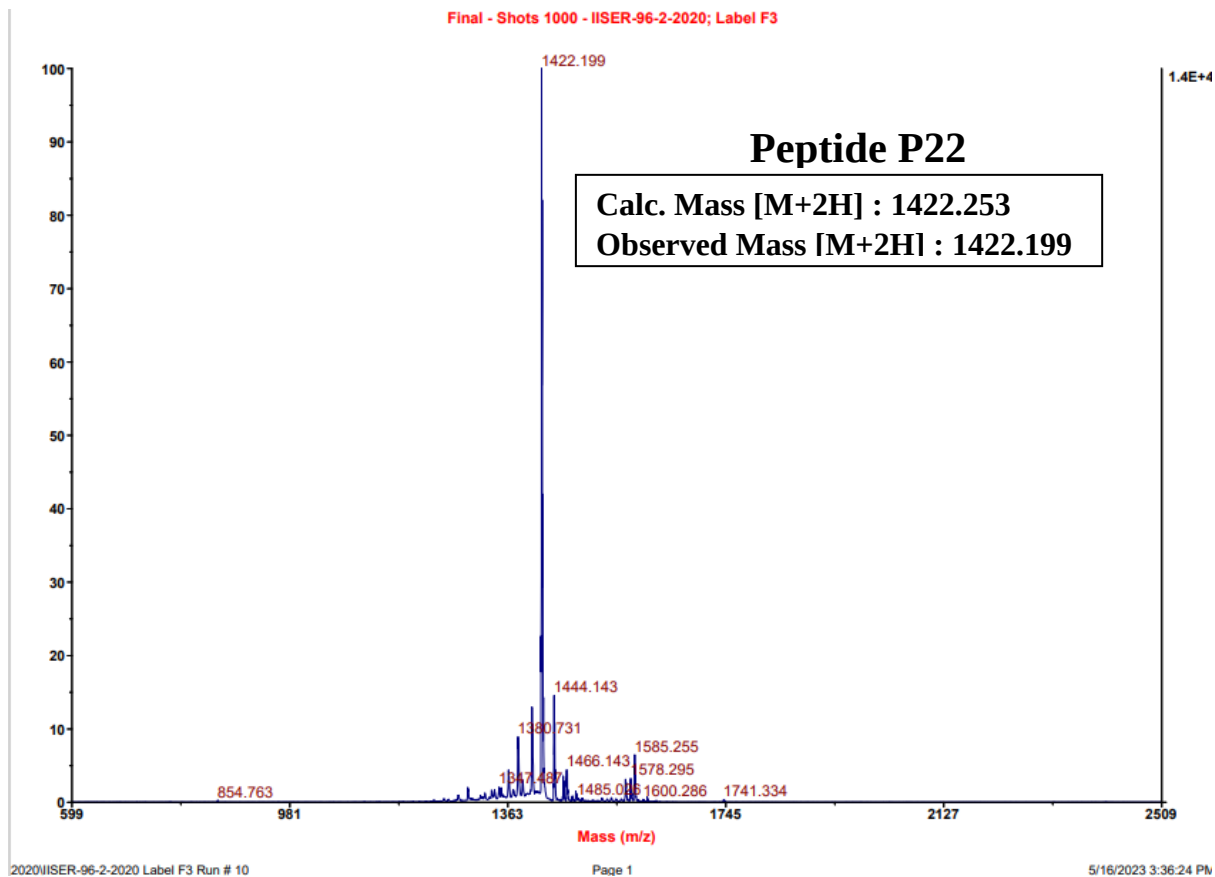
Spectrum Report

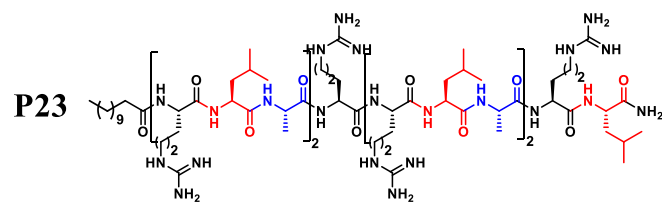
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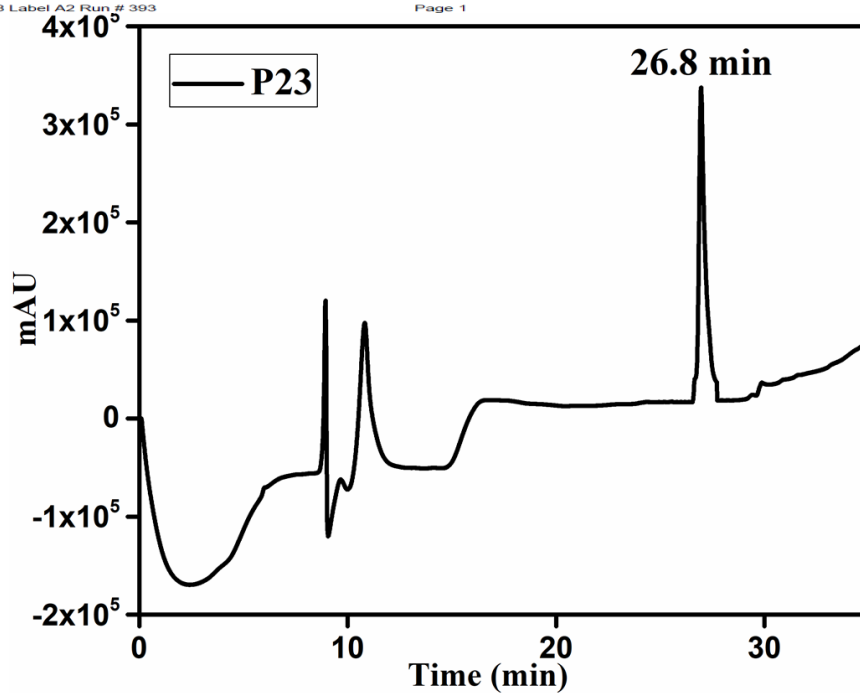
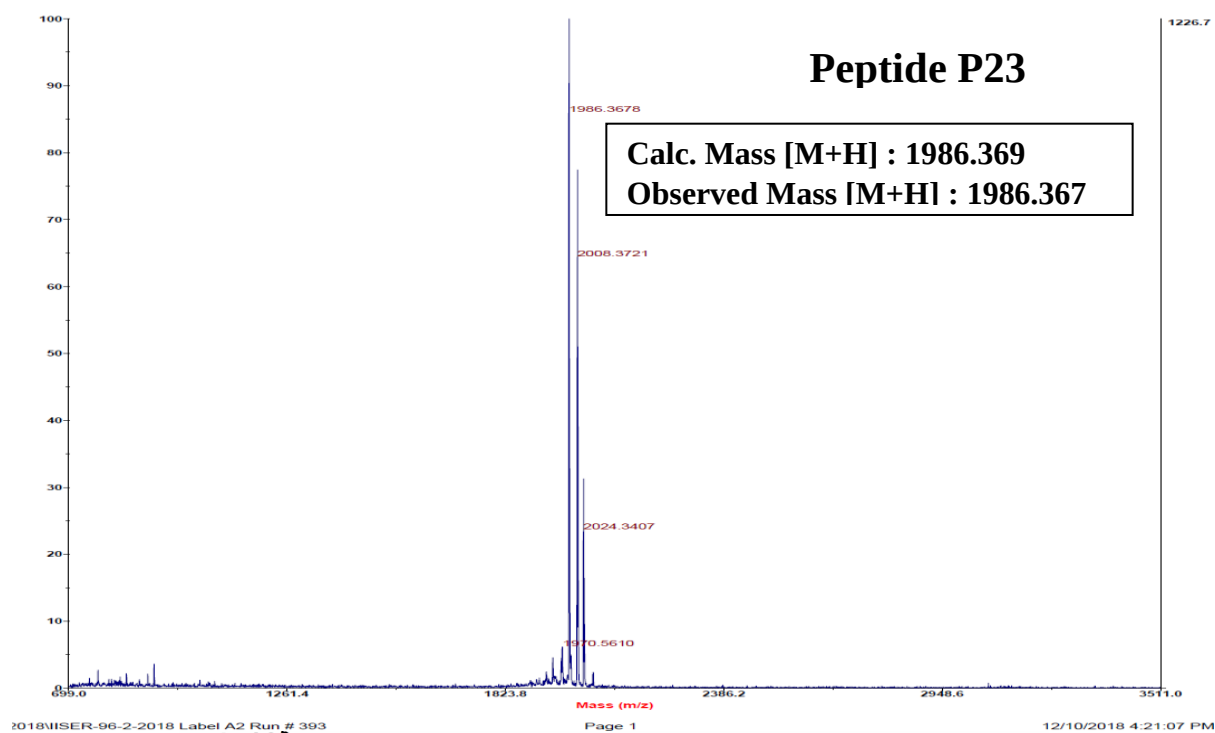
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Spectrum Report

Final - Shots 500 - IISER-96-2-2018; Run #393; Label A2



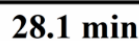


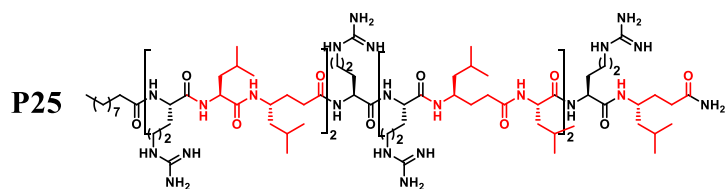
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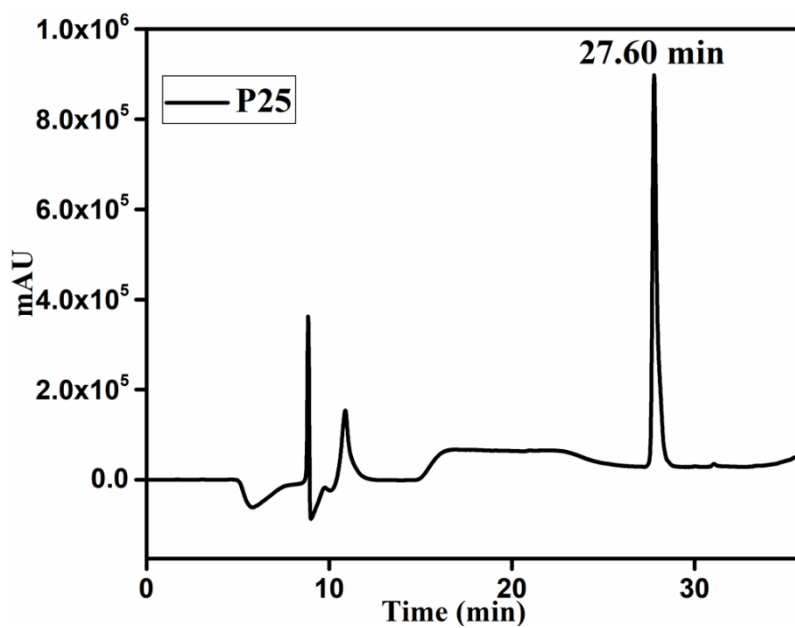
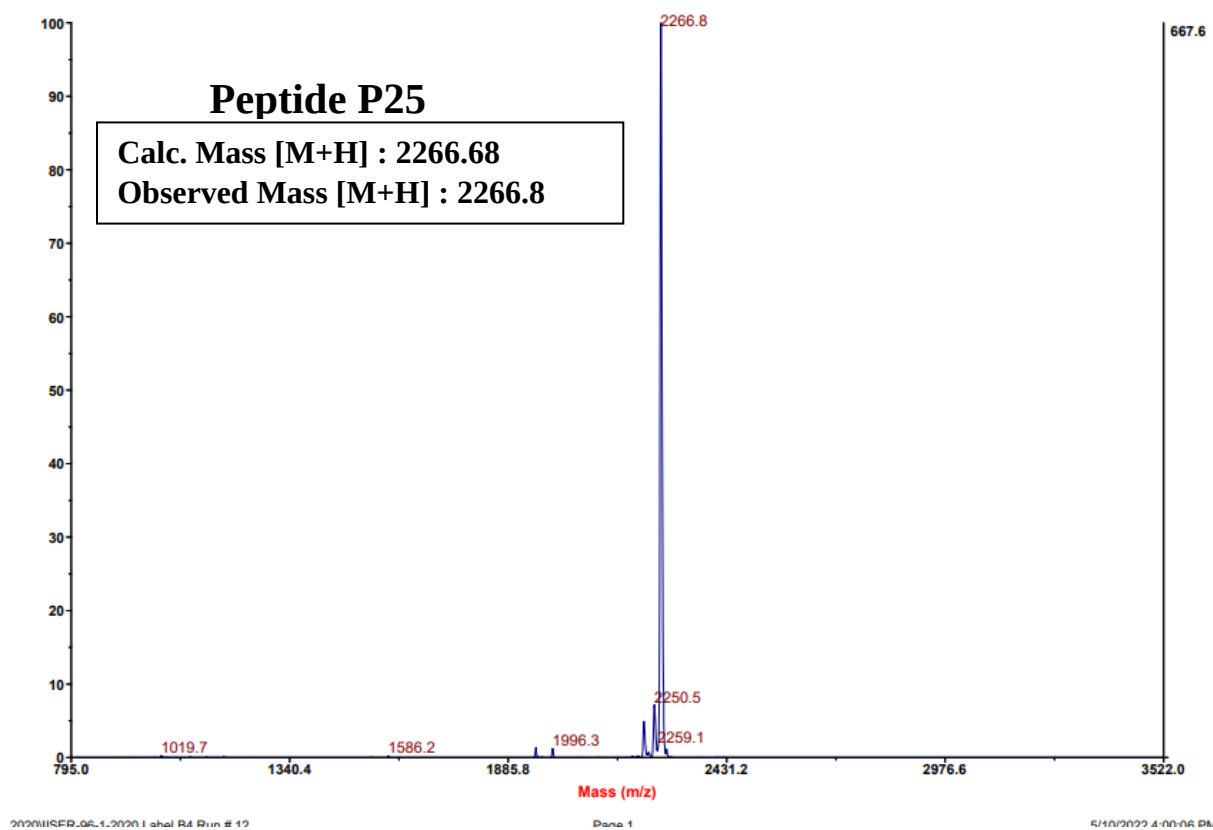
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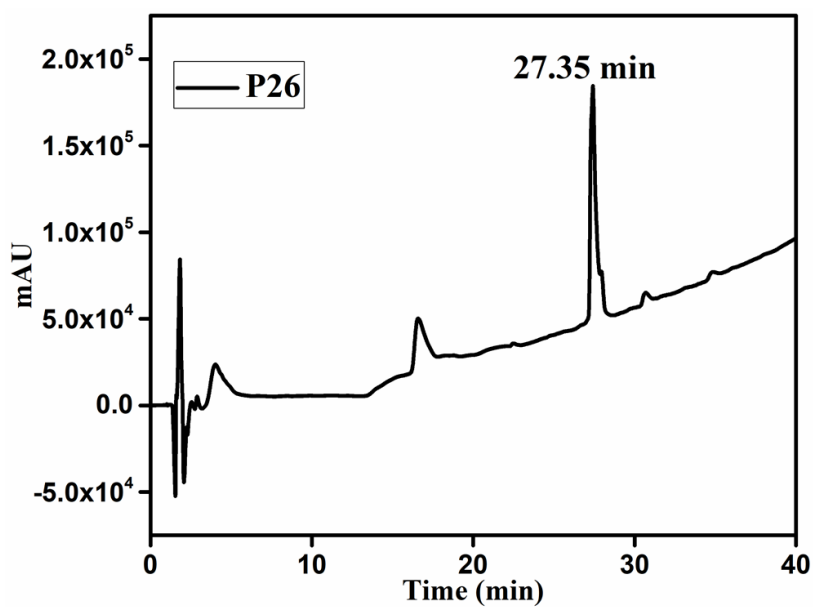
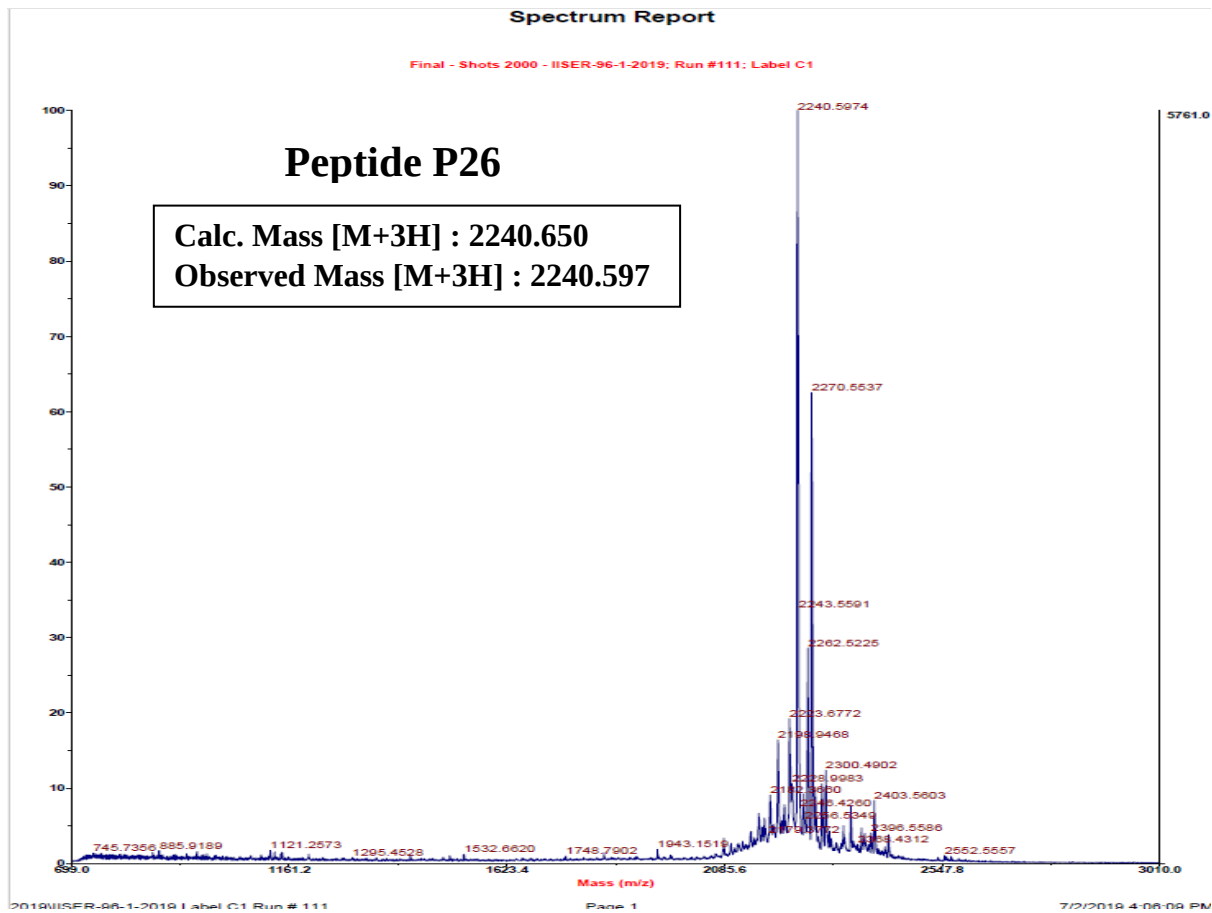
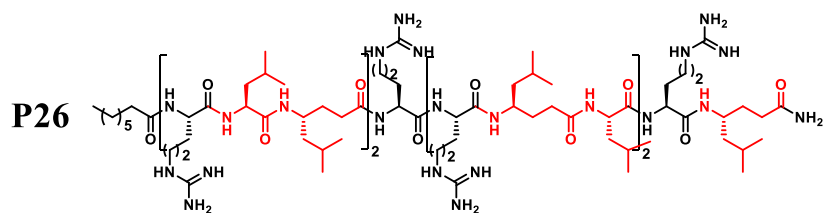
Observed Mass [M+3H] : 2296.491

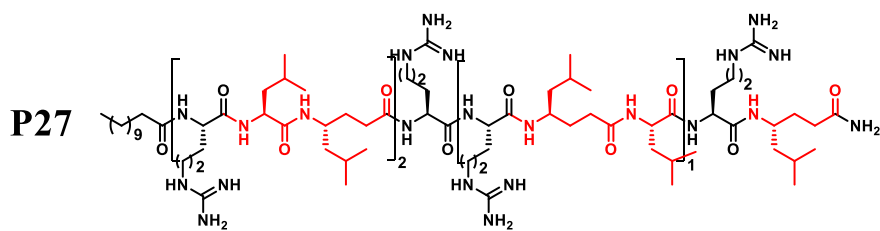




Final - Shots 2000 - IISER-96-1-2020; Label B4

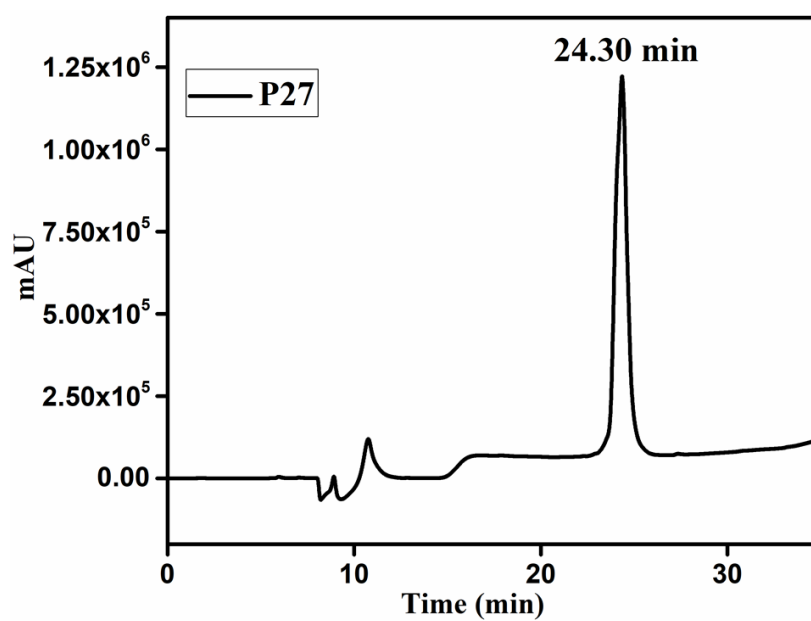
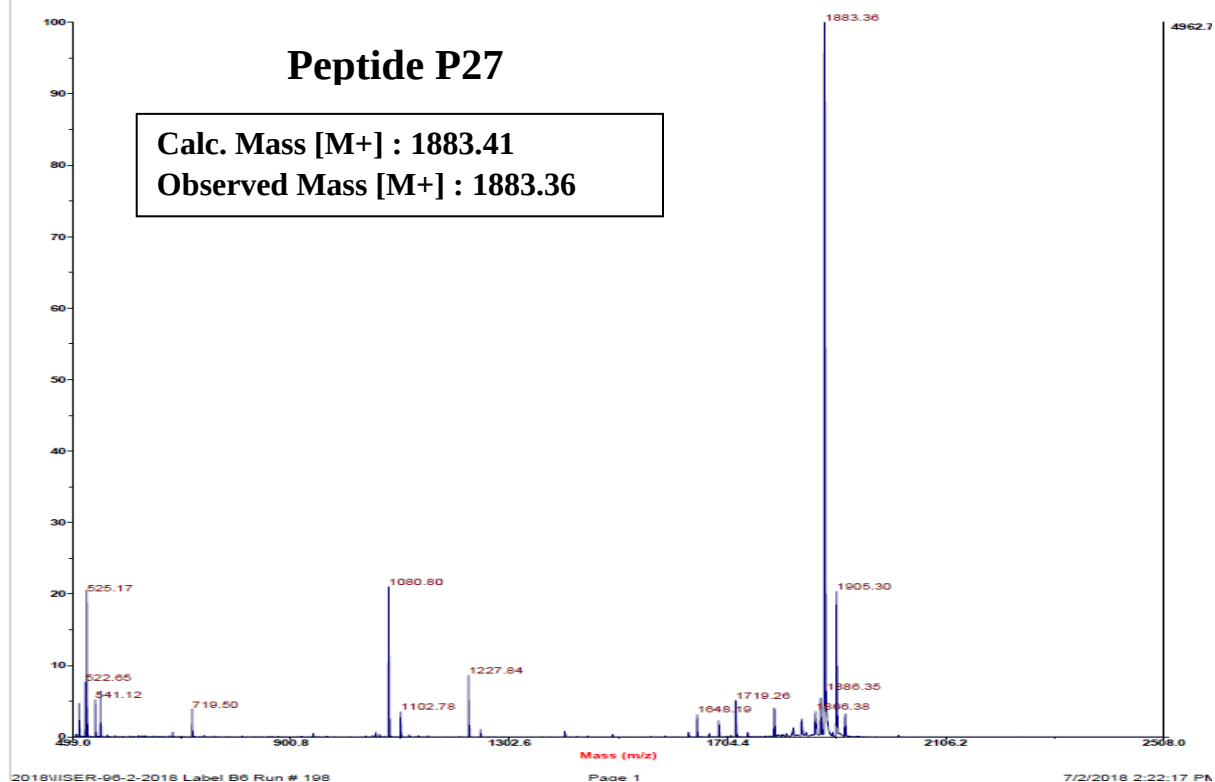


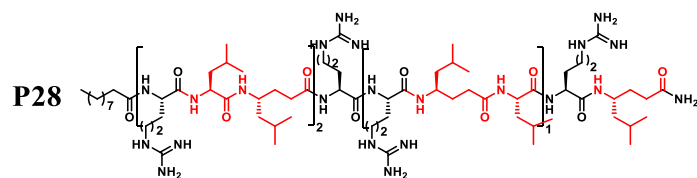




Spectrum Report

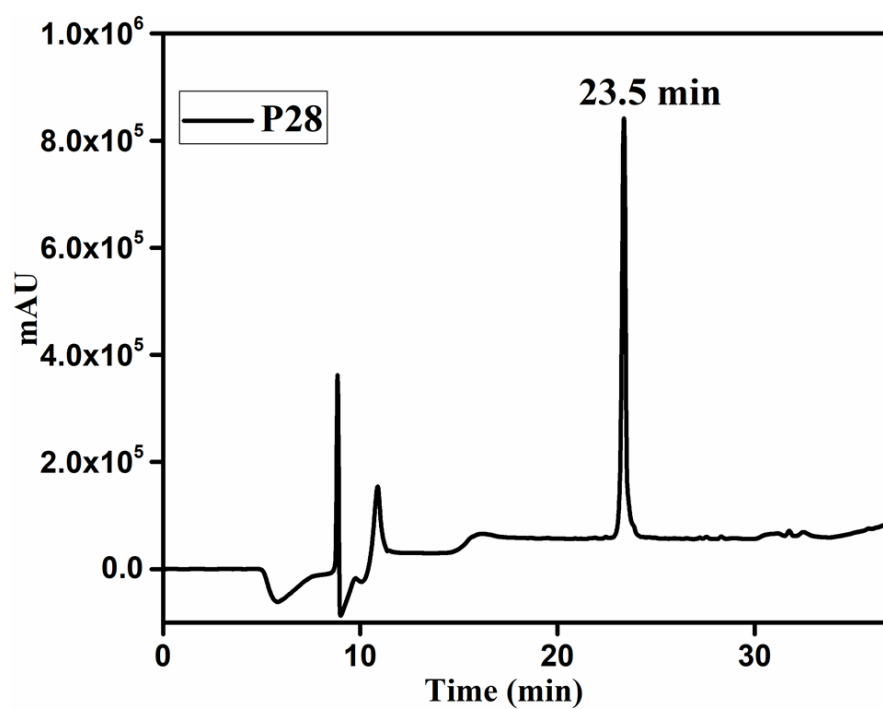
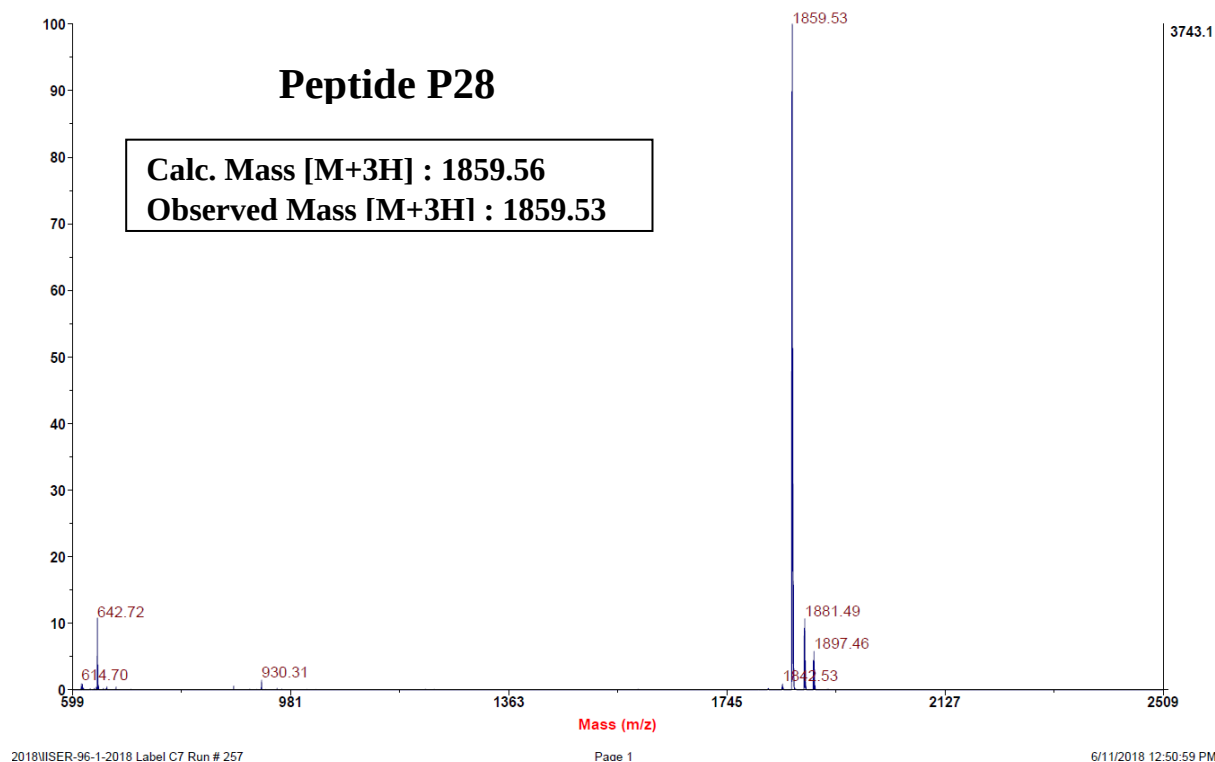
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Spectrum Report

Final - Shots 400 - IISER-96-1-2018; Run #257; Label C7



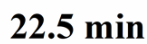


Final - Shots 2000 - IISER-96-2-2019; Run #180; Label A1

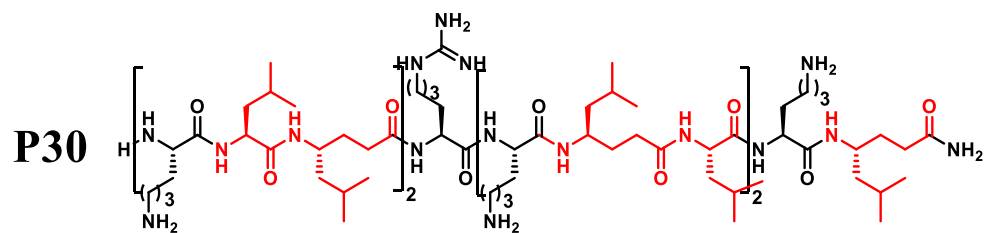


Calc. Mass [M+2H] : 1829.56

Observed Mass [M+2H] :1829.92

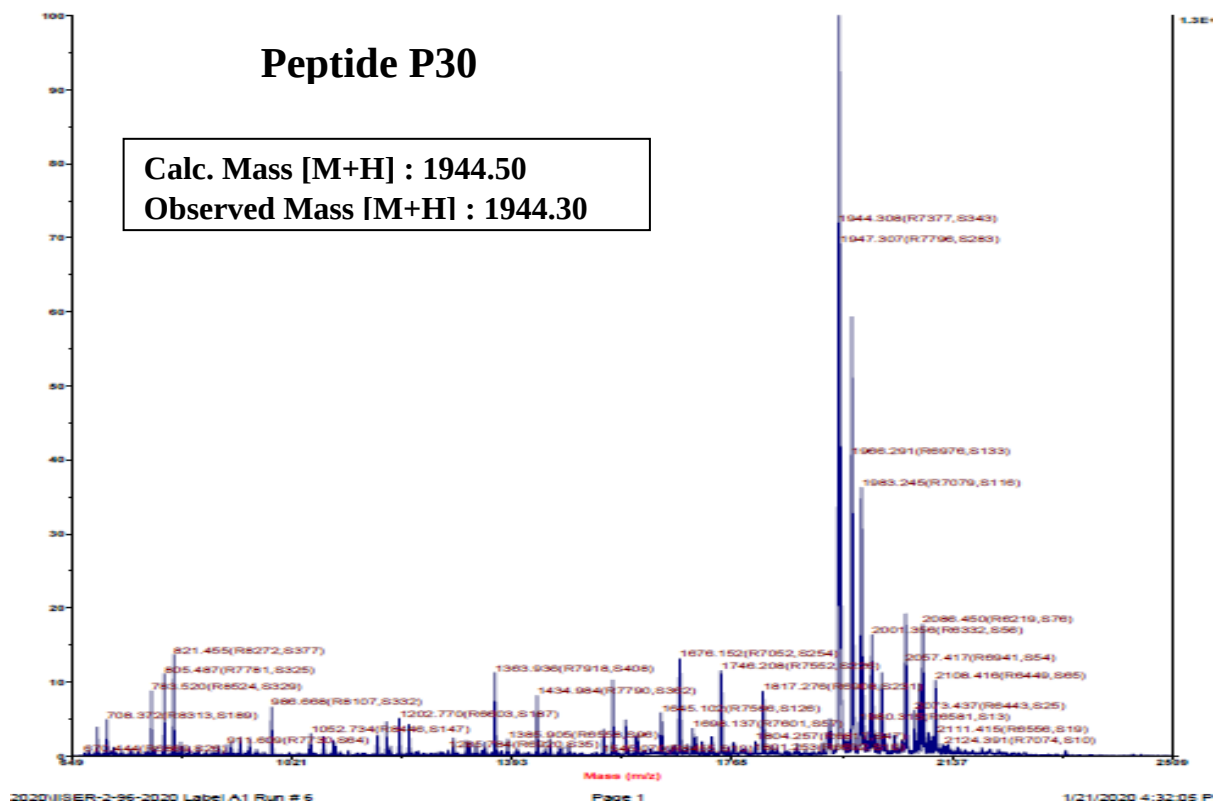


— P29



Spectrum Report

Final - Shots 2009 - SSER-2-96-2020; Label A1

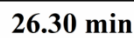




Final - Shots 600 - IISER-06-2-2018; Run #364; Label A2



Observed Mass [M+2H] : 1987.44



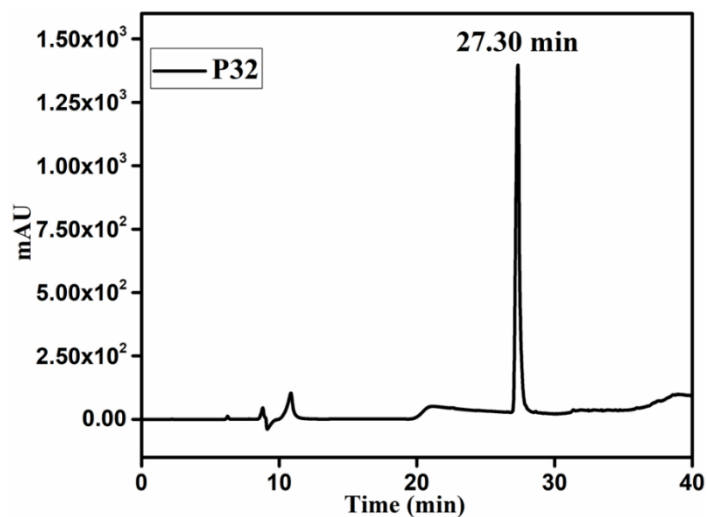


Final - Shots 500 - Chem Dept 96; Label C1



Calc. Mass [M+Na] : 2354.54

Observed Mass [M+Na] = 2354.65



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Chapter 3A

Cationic Amphiphilic Peptide Chaperone Rescues A β ₄₂ Aggregation and Cytotoxicity

3A.1. Introduction

The aggregation of intrinsically disordered polypeptides and proteins into ordered intractable β -amyloid fibrils is a characteristic feature in many neurodegenerative disorders.¹⁻³ Alzheimer's disease is the most pervasive neurodegenerative disease.⁴⁻⁵ It accounts for nearly 60-80% of cases of dementia. According to the World Alzheimer's report more than 75 million people will be suffering from this disease by 2030 and 135 million by 2050.⁶ It will be causing a huge social and economic burden on society. Alzheimer's disease is characterized by the aberrant accumulation of $A\beta$ peptides plaques outside neurons in the brain.⁷ The β -amyloid peptides are the fragments of transmembrane amyloid precursor protein (APP) obtained after the proteolysis by the β - and γ -secretases inside the transmembrane in neuronal cells.⁸ Among many fragments of APP, peptides $A\beta_{40}$ and $A\beta_{42}$ are the most pathologically important species.⁹ Studies suggested that $A\beta_{40}$ is the most abundant among the fragments and $A\beta_{42}$ is more prone to aggregation and more toxic shown in Figure 1.^{10,11} Currently, there is no treatment available to cure Alzheimer's disease, however, present medications temporarily improve or slow the progression of the disease. Being it is a progressive disorder, the effect of these drugs wears off with time. One of the attractive strategies in Alzheimer's treatment is preventing the $A\beta$ -peptides from clumping into plaques or the removal of the plaques of $A\beta$ -peptides from the brain.

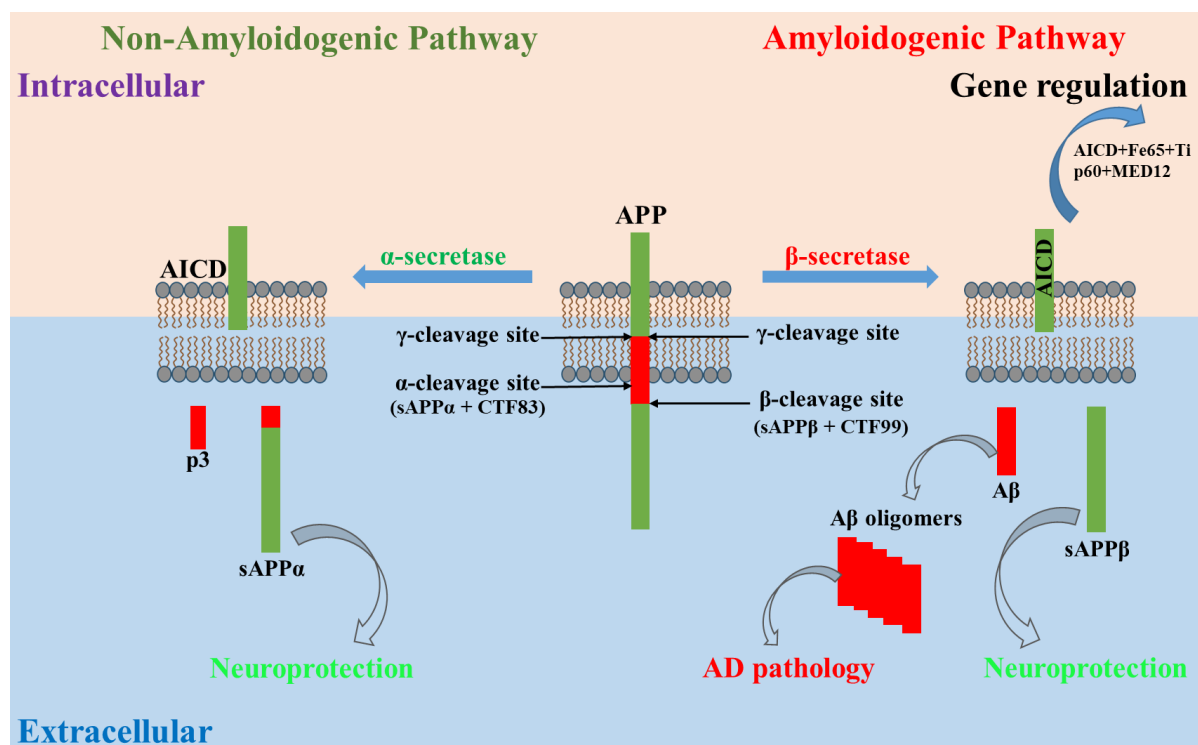


Figure 1: Schematic representation for the pathway of amyloid fibrillation.

A panoply of molecules including synthetic peptides derived from the amyloid core region,¹²⁻¹⁵ small molecules,¹⁶⁻¹⁹ antibodies,²⁰⁻²² nanobodies,²³ affibodies,²⁴ antimicrobial peptides,²⁵⁻²⁷ cyclic peptides,²⁸⁻³⁰ β -hairpins,^{31,32} β -sheet mimetics,^{33,34} peptide-polymer conjugates,³⁵ cell permeable peptides,³⁶ foldamers,³⁷⁻⁴¹ molecular chaperones,⁴² N-methylated peptides⁴³ have been investigated as aggregation inhibitors of A β peptides shown in Figure 2. Previously, we reported the inhibition of amyloid aggregation by a designed β -hairpin composed of cationic residues.³¹ In this chapter, we are reporting the prevention of aggregation of A β ₄₂, disruption of the matured A β ₄₂ peptide plaques into nontoxic species and protection of cells from the toxicity of A β ₄₂ by a designed 15-residue cationic amphiphilic peptide. At the same time, shorter cationic 8- and 12-residue peptides showed a moderate inhibitory effect on the A β ₄₂ aggregation. Further, no inhibitory effect on A β ₄₂ amyloid aggregation was observed after replacing Lys residues with Ala residues in the 15-residue peptide.

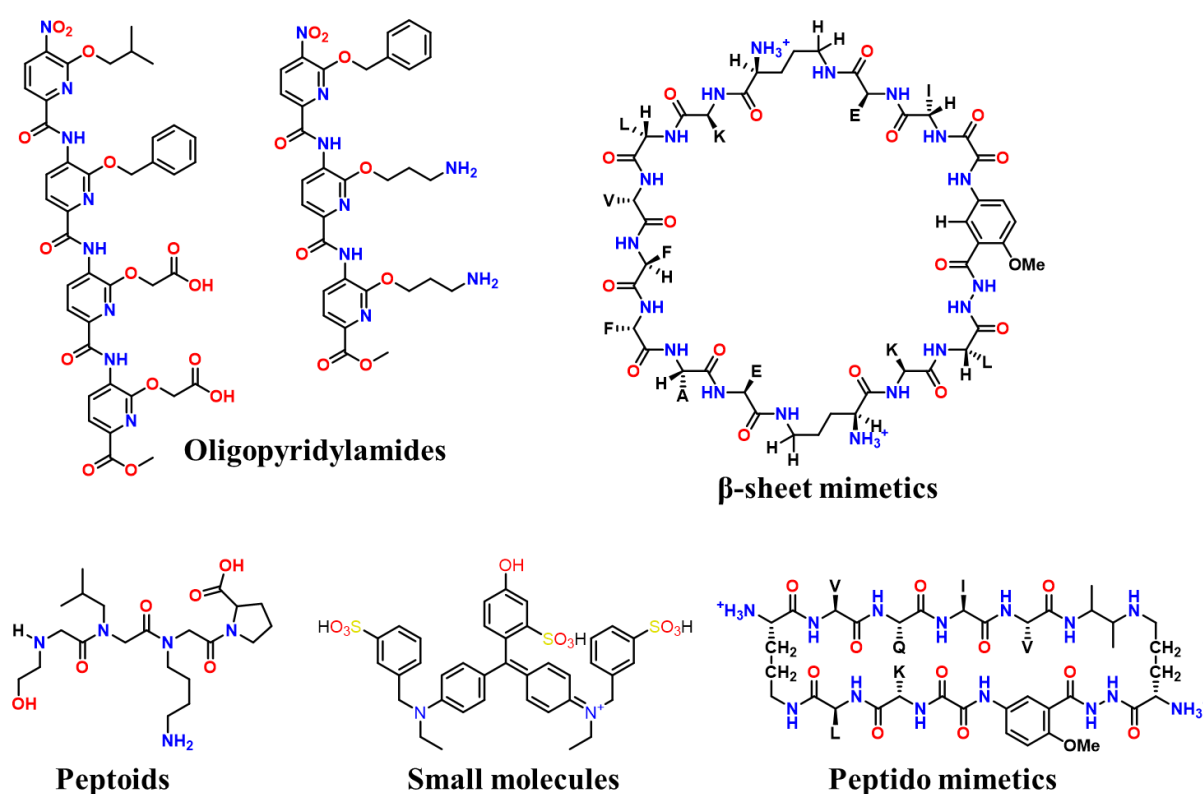


Figure 2: Reported Amyloidogenic Aggregation Inhibitors for Alzheimer's disease.

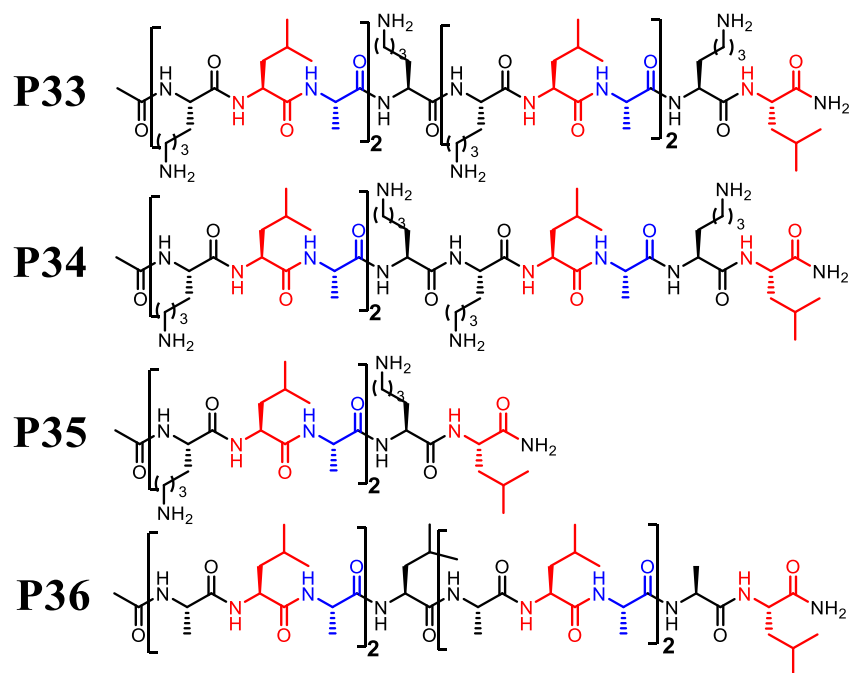
3A.2. Aim and Rationale of the Present Work

We have been interested in the design of antimicrobial cationic amphiphilic peptides. As mentioned above, In our previous reported work we showed the inhibition of amyloid aggregation by a designed β -hairpin composed of cationic residues.³¹ In continuation we sought to check the prevention of aggregation of $A\beta_{42}$, disruption of the matured $A\beta_{42}$ peptide plaques into nontoxic species and protection of cells from the toxicity of $A\beta_{42}$ by a designed 15-residue cationic amphiphilic peptide and also from the shorter cationic 8- and 12-residue peptides along with neutral peptide.

3A.3. Results and Discussion

3A.3.1. Design and Repurposing Amphiphilic Cationic Peptides

We have been interested in the design of antimicrobial cationic amphiphilic peptides. We designed and synthesized peptide **P33** to examine the antibacterial activity. The sequence of the peptide is shown in Scheme 1. The analysis revealed that **P33** displayed a very weak antimicrobial activity against the bacterial strains, *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *K. pneumoniae* BAA 1705, *A. baumannii* BAA 1605, and *P. aeruginosa* ATCC 27853 shown in the Table 1. As the peptide displayed a weak antimicrobial activity, we sought to examine whether or not this peptide can be repurposed as a possible $A\beta_{42}$ aggregation inhibitor. In addition, truncated versions of **P33**, peptides **P34** and **P35**, and **P36** with complete replacement of Lys residues with Ala were designed (Scheme 1) and all peptides were purified by RP-HPLC, pure fractions of peptide were collected by monitoring UV-Vis at 254 nm and 220 nm. Further, peptides were characterized by MALDI-TOF/TOF. After purification peptides were lyophilized to get the dried powder.



Scheme 1. Sequences of peptides.

Table 1: Antimicrobial Activity of the peptide **P33**.

Bacteria	<i>E.coli</i> ATCC 25922	<i>S.aureus</i> ATCC 29213	<i>K.pneumoniae</i> BAA 1705	<i>A.baumannii</i> BAA 1605	<i>Paeruginosa</i> ATCC 27853
P33	>64	>64	>64	>64	>64
Levofloxacin	0.03	0.125	64	8	0.5

The monomeric form of A β ₄₂ peptide adopts a random coil in an aqueous solution, however, upon incubation, it transforms into β -sheet conformation and eventually leads to amyloid fibrils and plaques.^{44,45} Peptides **P33**, **P34** and **P35** possessed the net charges +6, +5 and +3, respectively at pH 7.0. In comparison to the peptides, A β ₄₂ has a net charge of -2.7 at neutral pH. The net -ve charge of A β ₄₂ indicates that it can attract and bind to the cationic peptides.

3A.3.2. Solution conformation of peptide P33

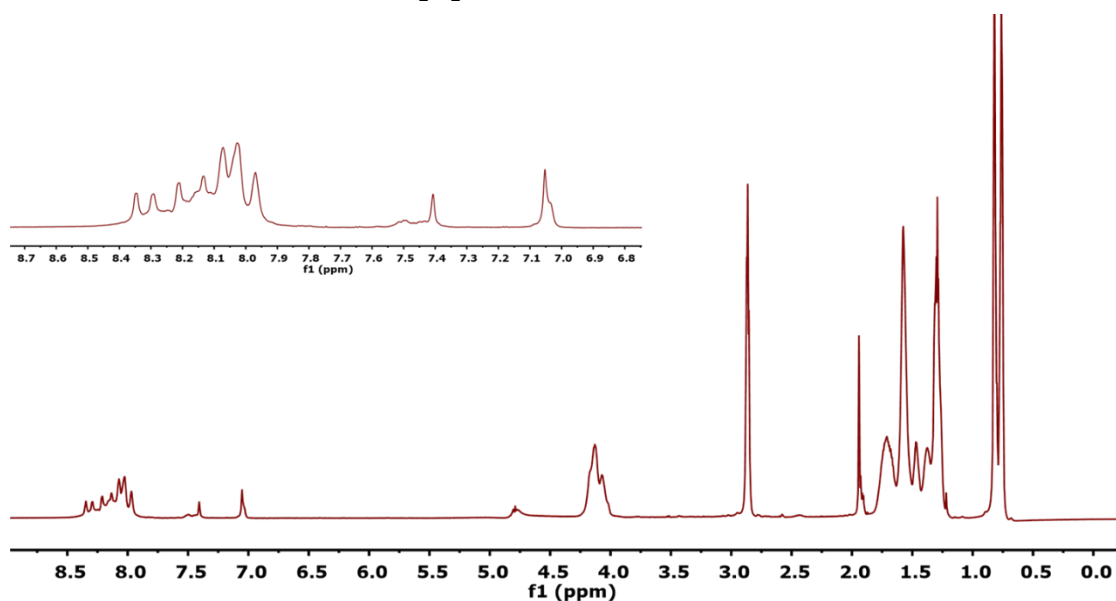


Figure 3: 800 MHz ¹H NMR of peptide **P33** in PBS (pH 7.4) at 288K.

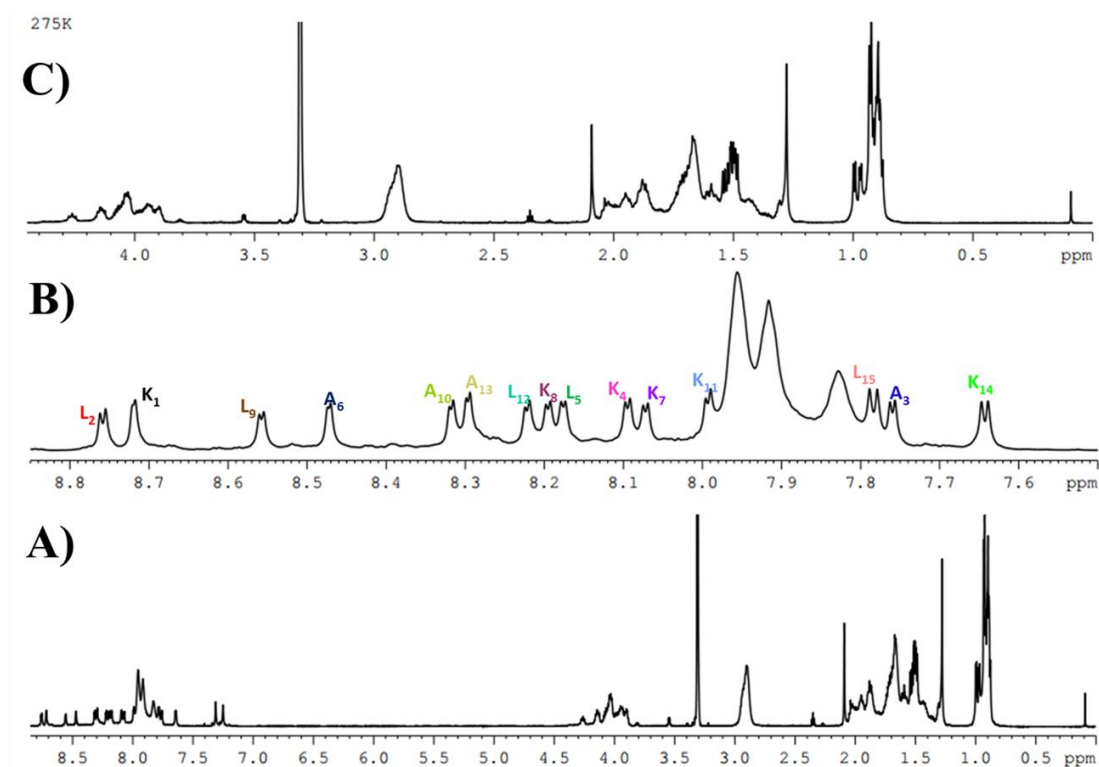


Figure 4: 800 MHz ¹H NMR of peptide **P33** in CD₃OH at 275K. A) Bottom trace shows the full spectrum of the ¹H NMR of peptide **P33**. B) Middle trace shows the amide expanded region of the peptide **P33**. C) Top trace shows the aliphatic region of the peptide **P33**.

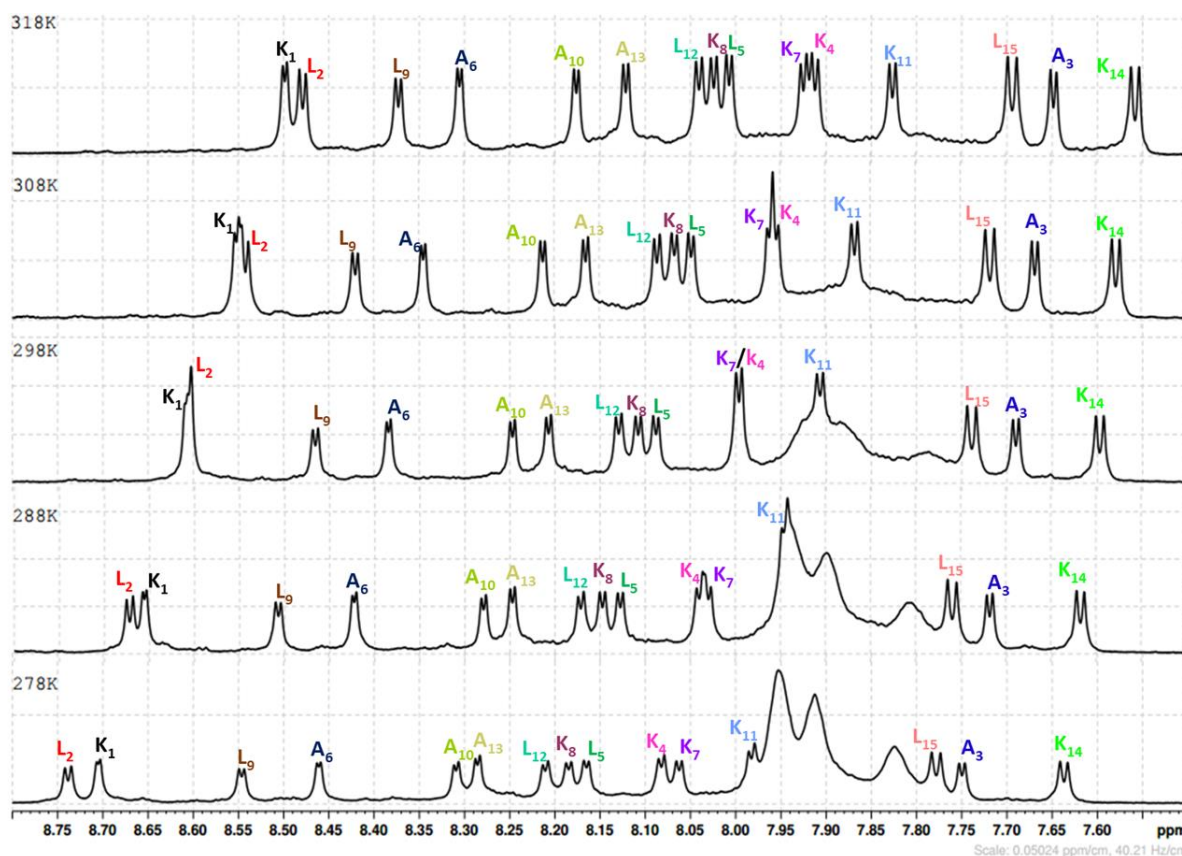


Figure 5: Temperature dependent ^1H NMR spectra of peptide **P33**. Change in chemical shifts of all amide protons with increasing temperature from 278K to 318K. Spectra were recorded in 800 MHz spectrometer in CD_3OH .

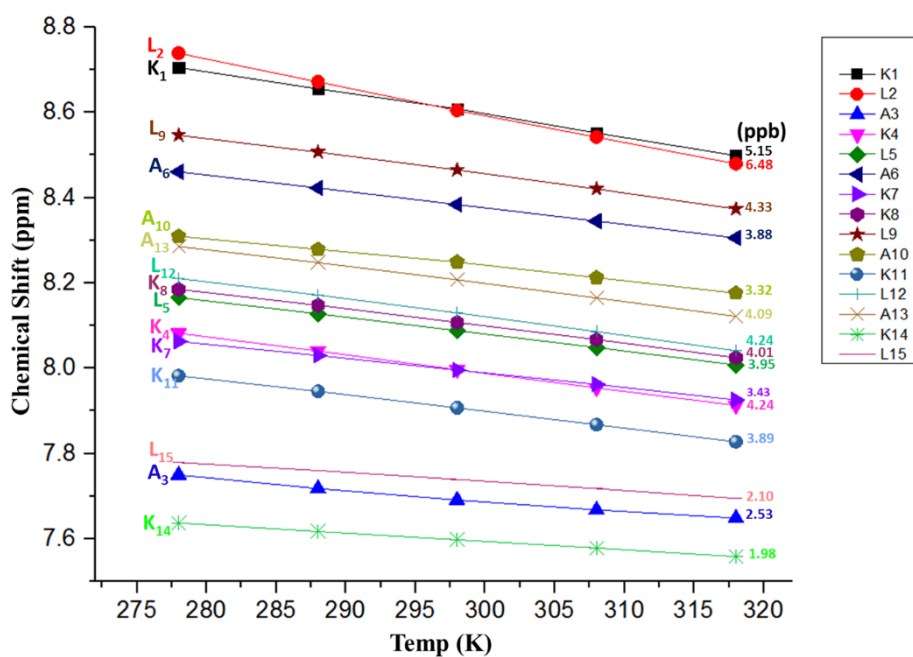


Figure 6: Temperature dependence of NH chemical shifts of the peptide **P33** with increasing temperature from 278K to 318K. Spectra were recorded in 800 MHz spectrometer in CD_3OH .

Table 2: Tabulation of chemical shifts along with $J_{\text{NH(Hz)}}$ scalar couplings and amide temperature coefficients ($d\delta/dT$) for peptide **P33**.

Residue	NH	H α	H β	H γ	H δ	H ϵ	NH $_3^+$	$J_{\text{NH(Hz)}}$	$\Delta\delta/\Delta T$ (ppb)
K ₁	8.72	3.90	1.46	1.58	1.71	1.86/1.81	2.94	2.92	5.15
L ₂	8.76	4.15	1.61	1.75	0.99/0.92			5.50	6.48
A ₃	7.76	4.07	1.49					5.01	2.53
K ₄	8.09	3.91	1.41	1.66	1.89	1.96	2.91	4.90	4.24
L ₅	8.18	4.06	1.73/1.42	1.87	0.97/0.93			4.30	3.95
A ₆	8.47	3.98	1.54					3.30	3.88
K ₇	8.07	3.97	1.47	1.68	1.79/1.89	2.03	2.91	4.64	3.43
K ₈	8.19	3.93	1.42	1.48	1.71/1.67	2.03	2.91	4.53	4.01
L ₉	8.56	4.04	1.89/1.51	1.96	0.89			4.52	4.33
A ₁₀	8.32	4.02	1.52					3.95	3.32
K ₁₁	7.99	3.95	1.43	1.69	1.95	2.02	2.90	5.10	3.89
L ₁₂	8.22	4.03	1.59	1.90	0.98			4.45	4.24
A ₁₃	8.31	4.06	1.51					3.95	4.09
K ₁₄	7.64	4.14	1.56	1.67	1.96	1.99	2.92	6.59	1.98
L ₁₅	7.78	4.27	1.60	1.88	0.93/0.89			7.60	2.10

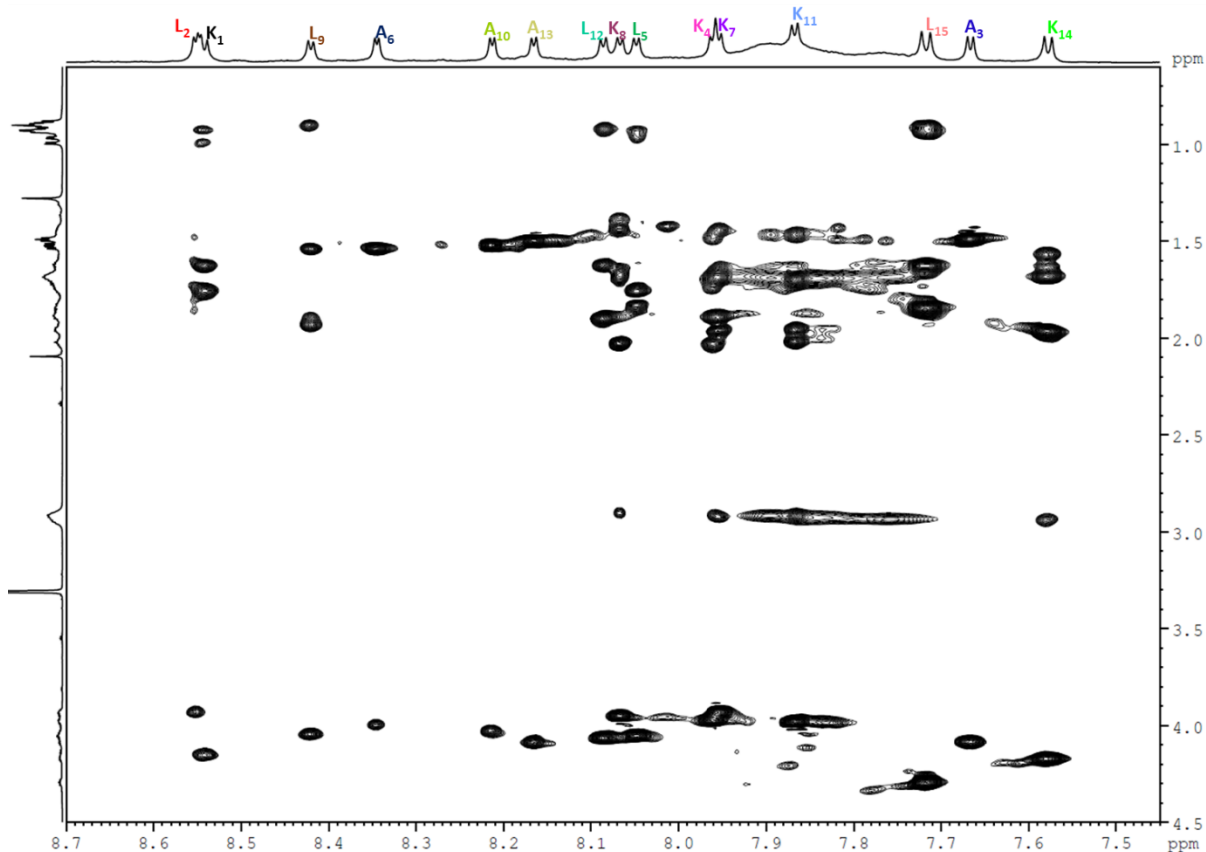


Figure 7: TOCSY spectra of peptide **P33** at 275K. Spectra were recorded in 800 MHz spectrometer in CD₃OH.

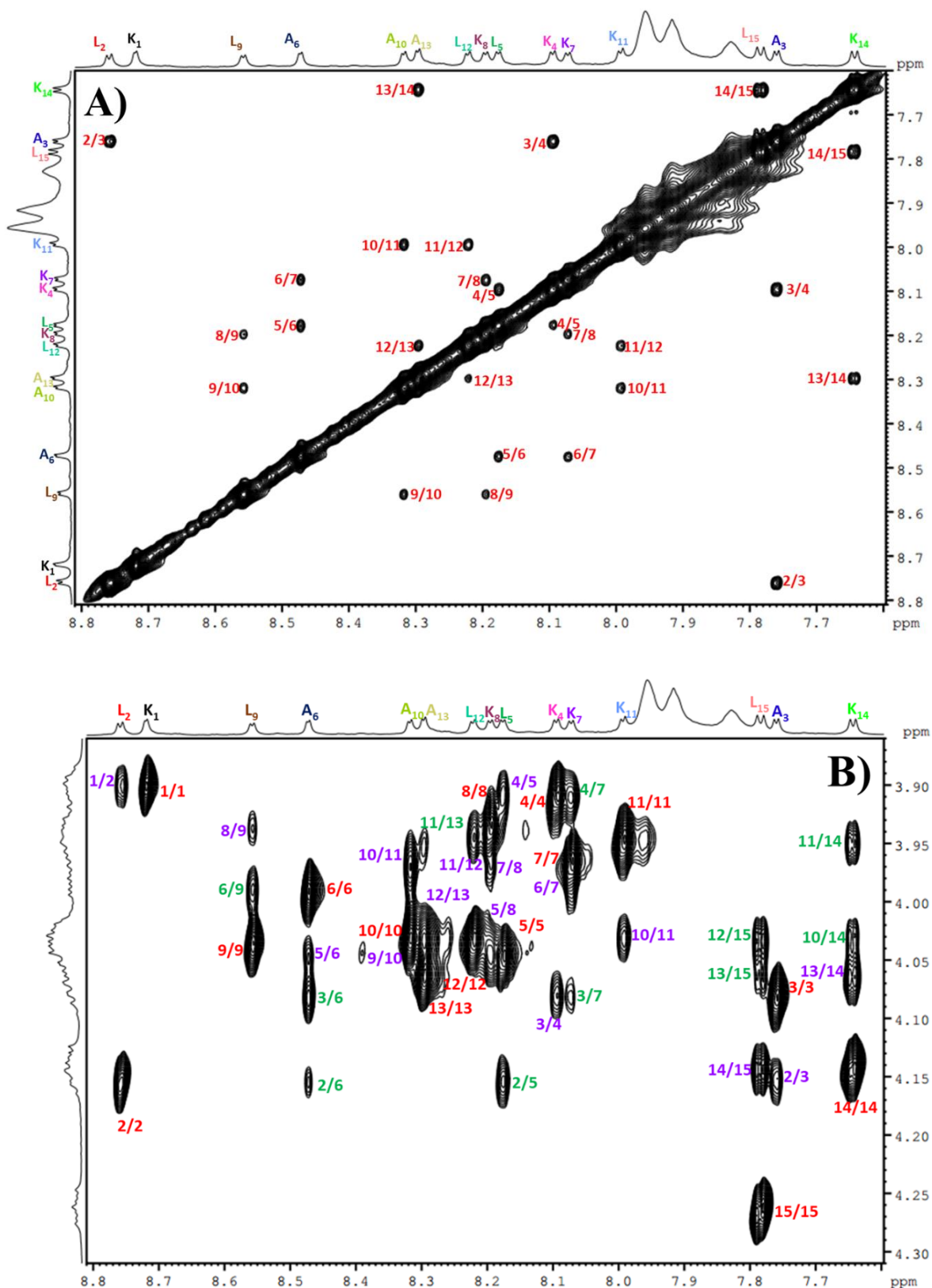


Figure 8: ROESY spectra of peptide **P33** A) Amide region, B) Amide-Aliphatic region. Most of these NOEs were considered in making energy minimized model of the peptide **P33** molecule. Spectra were recorded in 800 MHz spectrometer in CD₃OH.

We used 2D NMR (TOCSY and ROESY) analysis on peptide **P33** to comprehend the solution conformation. While Peptide **P33** produced poor quality ^1H NMR in aqueous PBS solution, CD_3OH (Methanol- d_3) produced a better spectrum. Figures 3 and 4 show, respectively, the ^1H NMR spectra of peptide **P33** in aqueous PBS buffer and in CD_3OH . Figures 5 and 6 demonstrate, respectively, the temperature-dependent ^1H NMR and NH chemical shifts of the peptide **P33** with increasing temperature from 278K to 318K. Table 2 lists the proton's chemical shifts. Figure 7 displays the peptide **P33** partial TOCSY spectrum after being fully assigned. Cross peaks were abundant in CD_3OH 2D ROESY spectra. Figure 8 displays the partial NOESY spectrum for $\text{NH} \leftrightarrow \text{NH}$ and $\text{C}\alpha\text{H} \leftrightarrow \text{NH}$ NOEs. Strong d_{NN} and $d_{\alpha\text{N}}(i, i+1)$ NOEs indicate a helical fold in **P33** clearly. The sequential $\text{NH}(i) \leftrightarrow \text{NH}(i+1)$ and $\text{C}\alpha\text{H}(i) \leftrightarrow \text{NH}(i+1)$ NOEs seen in the ROESY spectrum. These clear NOEs constraints were used to create energy-minimized NMR structures. Figure 9A depicts the superimposition of ten energy-minimized structures. Peptide **P33** assumed a helical shape in solution, as expected. Figure 9B displays the top-view of the side-chain projection of the peptide **P33**.

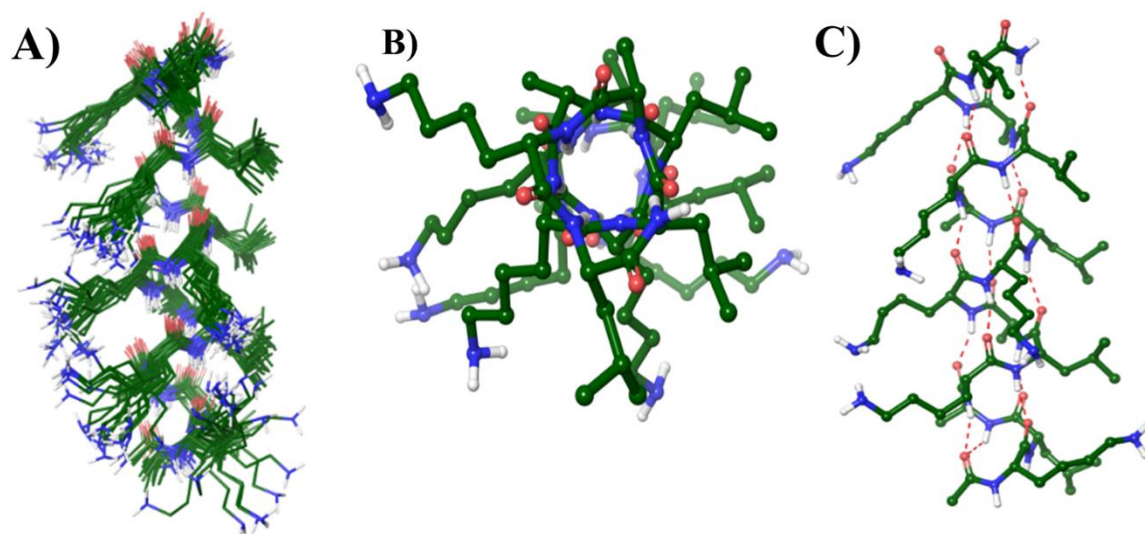


Figure 9: NMR 3D model of the peptide **P33** deduced from the NOEs in 2D NMR. A) 20-structure superposed. B) C-terminal facing. C) Energy minimized structure.

3A.3.3. Effect of cationic peptides on $\text{A}\beta_{42}$ amyloid aggregation

We have examined the effect of cationic peptide **P33** on $\text{A}\beta_{42}$ aggregation using the Thioflavin T (ThT)-fluorescence based assay.^{46,47} The free-aggregated soluble form of $\text{A}\beta_{42}$ was prepared using the reported procedure.³¹ The cationic peptide **P33** was incubated at different concentrations starting from 0.5 to 10 equivalents with a constant 25 μM of soluble $\text{A}\beta_{42}$. The

aggregation of A β ₄₂ in the absence and presence of an inhibitor was quantified using the maximum ThT fluorescence intensity (I_{max}) against the time. The effect of **P33** on the aggregation of soluble A β ₄₂ is shown in Figure 10A. In the absence of an aggregation inhibitor peptide, A β ₄₂ reaches maximum fluorescence intensity with a t_{50} value of 9.2 hours.

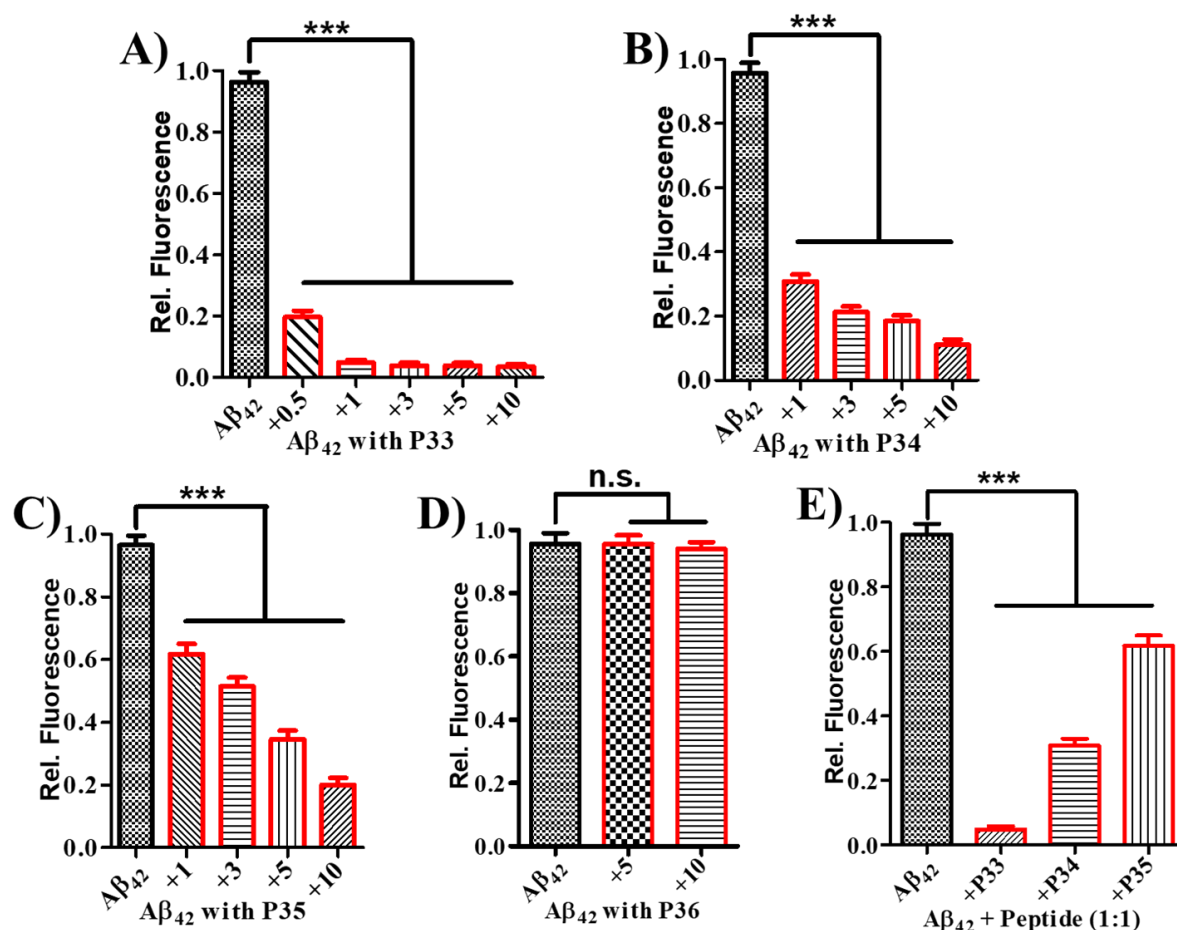


Figure 10: Kinetic study of the fibrillation of the A β ₄₂ in the absence and presence of peptides **P33-P36**. The time dependent aggregation of A β ₄₂ in the absence and presence of varying concentrations of peptides was monitored using ThT fluorescence. The concentrations of A β ₄₂ and ThT were 25 μ M and 20 μ M, respectively used in all experiments. The concentrations of peptides were ranging from 12.5 μ M, 25 μ M, 75 μ M, 125 μ M to 250 μ M. The ThT mediated fluorescence based time dependent A β ₄₂ aggregation kinetic profile in the presence of A) **P33** B) **P34** C) **P35** and D) **P36**. E) The comparative aggregation inhibitory profiles of **P33** to **P35** at 1:1 concentrations (25 μ M each) with A β ₄₂. The GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA) was used to analyse the data using one-way ANOVA and the *post hoc* Tukey's test for manifold comparisons ($p < 0.05$), n.s. = no significance.

In comparison with A β ₄₂ alone, at 0.5 equivalent of **P33** with respect to A β ₄₂ concentration (**P33**: A β ₄₂; 0.5:1), the fluorescence intensity was reduced to 20%. Instructively, the ThT fluorescence intensity was diminished to <~3% at 1:1 concentrations of **P33** and A β ₄₂. These results indicate that at 1:1 concentrations, **P33** effectively prevents the aggregation of A β ₄₂. The effective inhibition of the A β ₄₂ aggregation was also observed at 1:3, 1:5 and 1:10 concentrations of A β ₄₂ and **P33**, respectively.

Based on the exciting results from **P33**, we further synthesized peptides **P34** and **P35**, the shorter analogues of **P33**, and examined their effects on the aggregation of A β ₄₂. Different concentrations of **P34** and **P35** were incubated with A β ₄₂ and examined the ThT fluorescence. Both peptides inhibit the A β ₄₂ aggregation in a concentration dependent manner. Results are shown in Figure 10B and 10C respectively. In comparison to **P33**, the dodecamer **P34** and octamer **P35** required higher concentrations to inhibit the A β ₄₂ aggregation. At a 1:1 concentration of **P34** and A β ₄₂, we still get a good fluorescence intensity of ThT and it reduces with further increasing intensity of ThT and it reduces with further increasing concentration of **P34**. In continuation, the octapeptide **P35** with net charge +3 also inhibits the aggregation in concentration dependent manner similar to **P34**, however, it requires higher concentrations to completely inhibit the A β ₄₂ aggregation. Further, to understand the importance of cationic residues in the inhibition of A β ₄₂ aggregation, we synthesized peptide **P36** by replacing Lys residues with Ala residues. Peptide **P36** with zero net charges was examined for its effect on the A β ₄₂ aggregation under identical conditions. The peptide did not inhibit A β ₄₂ aggregation (Figure 10D), however, showed a delay in the initial association in a concentration independent manner (Figure 22). The comparative profile of the aggregation inhibition activity of peptides **P33-P35** at 1:1 concentration with A β ₄₂ is shown in Figure 10E. The kinetic parameters of ThT assay are tabulated in Table 3. The aggregation rate constant, half-time ($t_{1/2}$), and lag phase were extracted from the ThT assay^{48,49}. At 1:1 molar ratios of A β ₄₂ and **P33**, complete prevention of aggregation of A β ₄₂ was observed. In the absence of the peptide inhibitor, the aggregation of A β ₄₂ showed the $t_{1/2}$ 9.34 $\pm$ 0.25 h and the lag time 6.35 $\pm$ 0.32h. At 1: 0.5 molar ratio of A β ₄₂ and **P33**, the $t_{1/2}$ and lag time reduced to 6.09 $\pm$ 0.39h and 1.8 $\pm$ 0.69 h respectively. The reduction in the $t_{1/2}$ and lag phase suggested the onset prevention of aggregation of A β ₄₂. Similar results were also observed in the case of **P34** and **P35**. At 1:1 molar ratios of **P34** and A β ₄₂, we observed 9.59 $\pm$ 0.37 h of $t_{1/2}$ and 0.75 $\pm$ 0.46 h of lag time. In the case of **P35**, at a 1:1 molar ratio with A β ₄₂, the $t_{1/2}$ and lag time were reduced to 8.22 $\pm$ 0.30h and 2.14 $\pm$ 0.32 h respectively.

These results suggested the peptides prevented aggregation by interfering with the inception of A β ₄₂ aggregation. The ThT fluorescence based amyloid aggregation kinetic analysis clearly suggested that the **P33** is a better A β ₄₂ amyloid aggregation inhibitor compared to **P34**, **P35** and **P36**.

Table 3: ThT assay Kinetic Parameters [t_{50} (h), Lag time (h), k_{app} (h^{-1})] for the aggregation effect on A β ₄₂ in the presence of Peptides.

Sample	t_{50} (h)	Lag time (h)	k_{app} (h^{-1})
A β ₄₂ alone (25 μ M)	9.34533 $\pm$ 0.25	6.3586 $\pm$ 0.32	0.66991 $\pm$ 0.016
A β ₄₂ + P33 (1:0.5)	6.09333 $\pm$ 0.39	1.86553 $\pm$ 0.69	0.47505 $\pm$ 0.036
A β ₄₂ + P34 (1:1)	9.59 $\pm$ 0.37	0.75207 $\pm$ 0.46	0.22631 $\pm$ 0.002
A β ₄₂ + P35 (1:1)	8.22667 $\pm$ 0.30	2.141 $\pm$ 0.36	0.32866 $\pm$ 0.003
A β ₄₂ + P36 (1:5)	18.32 $\pm$ 0.40	7.5234 $\pm$ 0.54	0.18527 $\pm$ 0.002

In order to understand whether or not **P33** can inhibit the aggregation at lower concentrations, we incubated A β ₄₂ with 0.1 and 0.3 equivalents of **P33**. The ThT fluorescence intensity was found to be 40% and 30% for 0.1 and 0.3 concentrations of **P33**, respectively. Even at these lower concentrations, we observe a drastic diminution in the ThT fluorescence intensity indicating that **P33** inhibits the A β -aggregation even at very low concentrations. The results are shown in Figure 11A. Moreover, fluorescence intensity decreases with increasing peptide concentration, suggesting that the peptide inhibits the amyloid aggregation in a concentration dependant manner and almost complete inhibition was observed at 1:1 concentrations of the peptide and A β ₄₂.

As **P33** prevents the aggregation of soluble A β ₄₂ into fibrils, we further sought to investigate whether or not **P33** can disrupt the aggregated species of A β ₄₂ into soluble forms using the ThT-based fluorescence assay. The soluble A β ₄₂ was incubated along with ThT at 37 °C. Peptide **P33** was added to the incubated samples of A β ₄₂ after 16 hours and 24 hours, continued the incubation and monitored the ThT fluorescent intensity. The concentration of **P33** was equivalent to the concentration of A β ₄₂ (1:1). Results are shown in Figure 11B. Instructively, both experiments revealed a gradual diminution of the ThT fluorescence intensity with time. The control experiment without the peptide showed no decrease in the fluorescence intensity of ThT was observed. These results suggest that the peptide disrupted the fibrils of A β ₄₂ in the

solution. Disruption of the amyloid fibrils is one of the most crucial aspects in the design of inhibitors for Alzheimer's diseases and similar protein aggregation mediated disorders. However, many inhibitors failed to disrupt the aggregated fibrils of A β ₄₂. Strikingly, these results suggested that the peptide **P33** not only prevents the aggregation of soluble A β ₄₂ into fibrils and also disrupts the matured amyloid fibrils into soluble precursors.

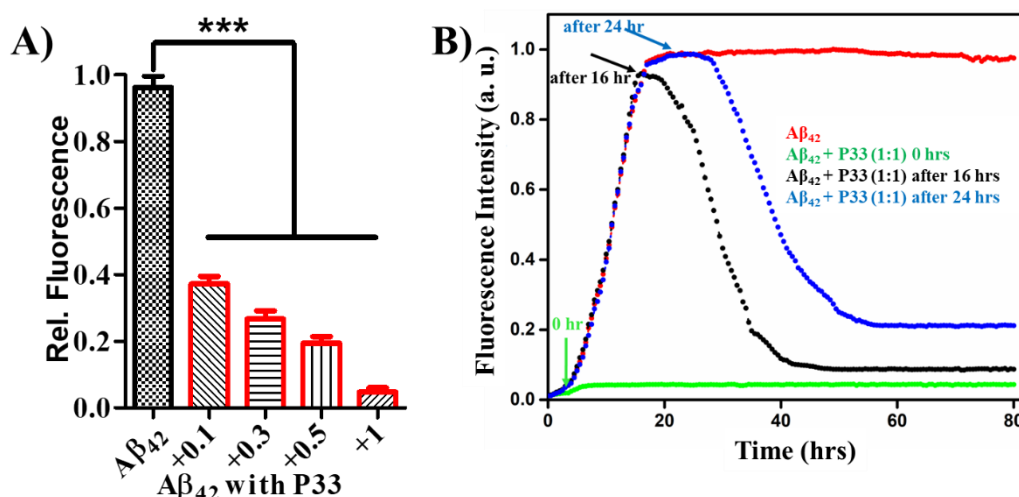


Figure 11: Kinetic study of the fibrillation of the A β ₄₂ with **P33**. A) The normalized kinetic profile of the self-assembly of 25 μ M of A β ₄₂ in the absence and presence of **P33** with stoichiometric ratios (2.5 μ M, 7.5 μ M, 12.5 μ M, and 25 μ M). The GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA) was used to analyse the data using one-way ANOVA and the *post hoc* Tukey's test for manifold comparisons ($p < 0.05$), n.s. = no significance. B) Kinetic study of the fibrillation of the A β ₄₂ with peptide **P33** with equimolar concentration (25 μ M) at different time points. (i) Incubation of A β ₄₂ alone (red line), (ii) addition of **P33** to the solution of A β ₄₂ at 0 hour of incubation (green line), (iii) addition of **P33** after 16 hours of A β ₄₂ incubation (black line) and (iv) addition of **P33** to the solution of A β ₄₂ after 24 hours of incubation (blue line). Buffer Conditions: 10 mM Sodium Phosphate, 150 mM NaCl, 1mM EDTA and pH 7.4. ThT concentration was 20 μ M.

3A.3.4. Dynamic Light Scattering (DLS) Study

The disruption of A β ₄₂ fibrils by **P33** in ThT experiments encouraged us to perform dynamic light scattering (DLS) experiments, which measure the size of the aggregated particles in the solution. We have examined the aggregation of A β ₄₂ using a DLS experiment after incubation of 24 hours in the absence and presence of **P33**. The DLS results are shown in Figure 12A. At

0 hour of incubation, A β ₄₂ displayed small sized aggregated particles. After incubation for 24 hours, the A β ₄₂ displayed aggregated particles around 800 nm in size. In the presence of **P33**, at equal concentration, showing the particle size around 100 nm. Instructively, peptide alone also displayed aggregated particle size around 80 nm. From these experiments, we observed a clear difference in the aggregation profile of A β ₄₂ in the absence and the presence of **P33**. The analysis of the DLS experiments further supported that the peptide prevents the aggregation of A β ₄₂ in the solution.

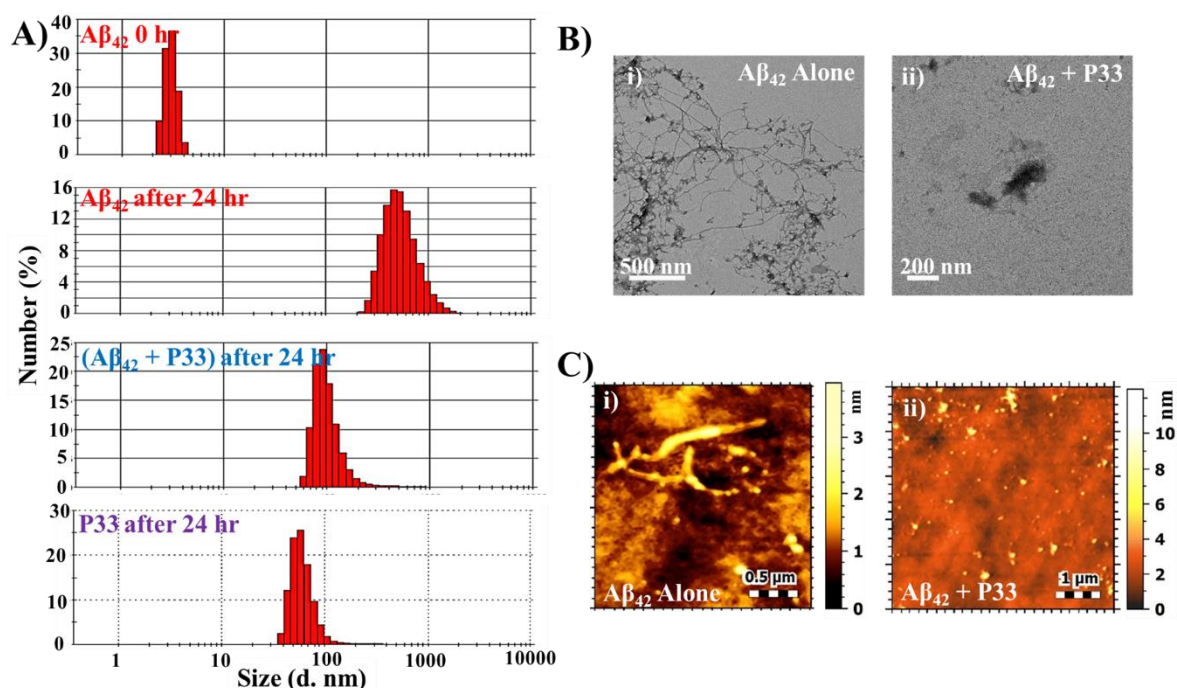


Figure 12: Effect of **P33** on the self-assembly of A β ₄₂. A) Dynamic Light Scattering (DLS) study of the 10 μ M A β ₄₂ in the absence and presence of **P33** after 24 hours incubation at 37 $^{\circ}$ C. The frequency distribution was plotted against the diameter of A β ₄₂ aggregation. B) HR-TEM images A β ₄₂ (10 μ M) after incubation at 37 $^{\circ}$ C for about 48 hours i) in the absence of **P33**, ii) in the presence of **P33**. C) AFM images A β ₄₂ (10 μ M) after incubation at 37 $^{\circ}$ C for about 48 hours i) in the absence of **P33**, ii) in the presence of 10 μ M of **P33**.

3A.3.5. Transmission Electron Microscopy (HR-TEM) and Atomic Force Microscopy (AFM) Analysis

Inspired by the disruption of the A β ₄₂ aggregation in solution and also from the confocal experiments, we have examined the morphology of A β ₄₂ in the absence and presence of peptide **P33** using HR-TEM and AFM. Before subjecting to the HR-TEM experiments, soluble A β ₄₂ was incubated at 37 $^{\circ}$ C in the absence and presence of peptide **P33** at 1:1 concentration. The

TEM images are shown in Figure 12B. In the absence of **P33**, A β ₄₂ displayed an entangled network of self-assembled fibrils. Fascinatingly, in the presence of **P33**, no fibrillar network of A β ₄₂ was observed. These results further support the fact that the peptide **P33** prevents the aggregation of A β ₄₂. Similar results were also observed in AFM analysis. The fibrils of A β ₄₂ observed in the absence of peptide disappeared in the presence of **P33**. The AFM images are shown in Figure 12C.

3A.3.6. Confocal Microscopy Analysis

In continuation, we subjected the A β ₄₂ samples with ThT to the confocal microscopy analysis in the absence and presence of peptide **P33**. The confocal images are shown in the Figure 13. The analysis of the confocal images revealed the A β ₄₂ fibrils with green fluorescence in the absence of the peptide. No fibrils were observed in the presence of **P33**. These observations further support the results obtained in the ThT mediated kinetic and DLS experiments in solution.

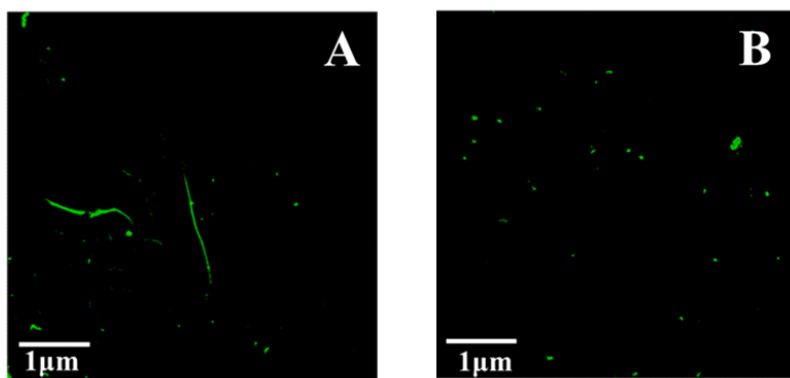


Figure 13: Effect of peptide **P33** on the kinetics of the self-assembly of A β ₄₂ with confocal images. Self-assembly of the 10 μ M A β ₄₂ was incubated at 37 °C up to 48 hours A) In the absence of peptide **P33**. B) in the presence of peptide **P33**. Buffer Conditions: 10 mM Sodium Phosphate, 150 mM NaCl, 1mM EDTA and pH 7.4.

3A.3.7. Enzyme-Linked ImmunoSorbent Assay (ELISA)

We further carried out an ELISA assay to understand the A β ₄₂ aggregation kinetics in the absence and presence of inhibitor **P33** using a commercially available ELISA kit. The solution of A β ₄₂ in PBS buffer was incubated with increasing concentrations of peptide **P33** for 24 hours before starting the ELISA experiment. The samples of A β ₄₂ in the absence and presence of **P33** were incubated in a 96 well microplate pre-coated with A β oligomer-specific monoclonal antibody. The bound oligomers of A β ₄₂ were detected by the biotin conjugated detection

antibody followed by the binding of streptavidin conjugated-HRP to biotin. TMB substrate was used to develop the colour. The absorbance of the samples was measured at 450 nm. The results are shown in Figure 14A. Strikingly, the wells containing A β ₄₂ alone showed increased absorbance at 450 nm compared to the sample wells containing A β ₄₂ along with **P33** and peptide alone. Even at 1:0.5 equivalent concentration of A β ₄₂ and **P33**, respectively showed a substantial decrease in the absorbance value, suggesting that **P33** prevent the aggregation of A β ₄₂ which cannot be recognized by the antibody. Similar results were also observed for 1, 3, 5 and 10 equivalents of **P33** along with A β ₄₂. The results observed in the ELISA experiments were consistent with the ThT, DLS, confocal microscopy and TEM analysis.

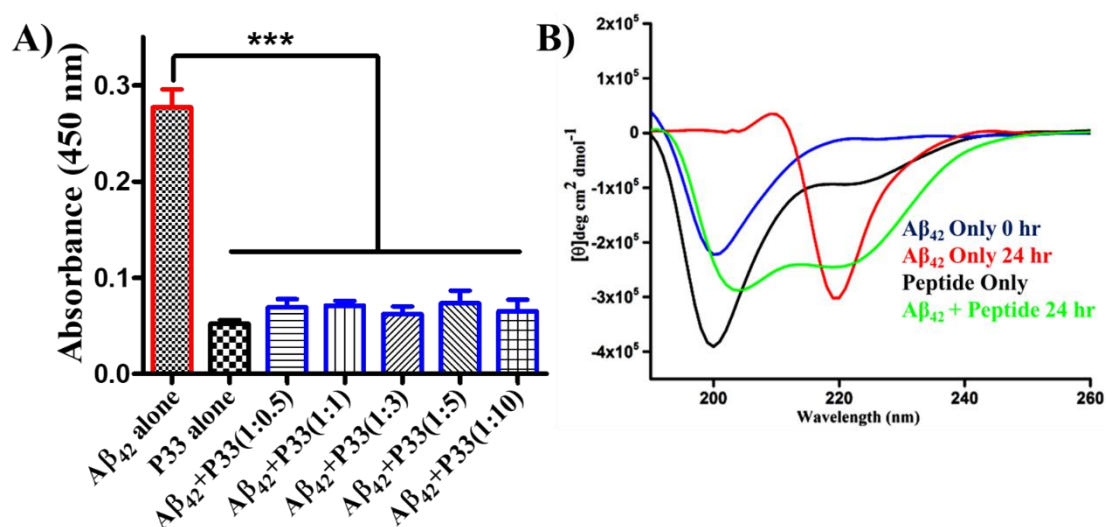


Figure 14: A) Detection of aggregated A β ₄₂ fibrils using ELISA in the absence and presence of **P33**. A substantial decrease in the absorbance was observed in the presence of the peptide at the concentration of 0.5 equivalent and above. The peptide also showed a weak absorbance at under identical conditions. The GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA) was used to analyze the data using one-way ANOVA and the *post hoc* Tukey's test for manifold comparisons ($p < 0.05$). B) The CD analysis of A β ₄₂ samples in the absence and presence of peptide **P33**. The soluble A β ₄₂ (50 μ M) at 0 hour of incubation (blue line), after 24 hours of incubation (red line), in the presence of equimolar concentration **P33** (50 μ M) after 24 hours of incubation (green line) and the peptide **P33** alone (black line) are shown.

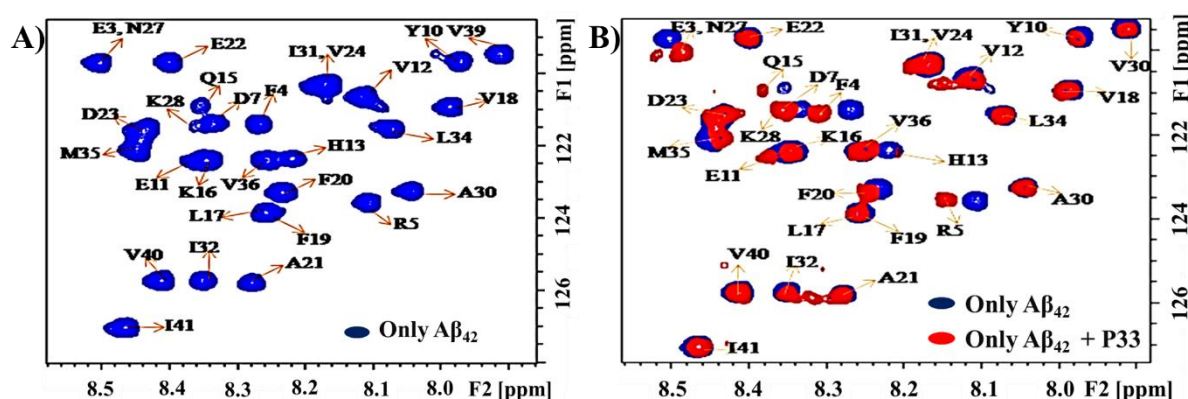
3A.3.8. Circular Dichroism Analysis

The hindsight of the prevention of aggregation of A β ₄₂ and the disruption of aggregated A β ₄₂ fibrils by **P33** inspired us to subject them to CD analysis to understand their molecular

conformations. The secondary structure contents of the peptide and A β ₄₂ can be measured using the quantitative CD analysis. The results are shown in Figure 14B. The A β ₄₂ at 0 hour of incubation without peptide in PBS showed no regular secondary structures. Similarly, **P33** also showed no regular secondary structure signature in the PBS buffer. The CD spectrum of A β ₄₂ after 24 hours of incubation at room temperature showed the CD minima at around 220 nm, suggesting the transformation of the random coil into a β -sheet type structure, while the peptide showed no change in its CD pattern even after 24 hours incubation in the PBS buffer. Instructively, the CD spectrum of 1:1 combination of **P33** and A β ₄₂ after incubating 24 hours showed the CD spectrum corresponding to a helical secondary structure with CD minima at 205 nm and 221 nm. However, we did not observe CD maxima around 200 nm in both β -sheet type and helical type spectra. Based on these results, we speculate that **P33** transformed the random coil and/or β -sheet structure of A β ₄₂ into helix type structure. This type of transformation of β -sheet into helix type structure of A β ₄₂ has also been observed with other aggregation preventing inhibitors.³⁶

3A.3.9. NMR analysis

Far UV-CD analysis suggested that there is a conformational rearrangement in A β ₄₂ upon incubation with **P33** at a 1:1 ratio. To further probe the structural reorganization within A β ₄₂ upon interaction with **P33**, we subjected them to the ¹H-¹⁵N HSQC NMR on an 800 MHz spectrometer attached with a cryo probe. This experiment was performed using ¹⁵N labelled A β ₄₂ at 283 K in 10 mM sodium phosphate buffer, pH 7.4. The amide NH region of HSQC of A β ₄₂ is shown in Figure 15A. All cross peaks were identified with the help of previously assigned spectra from the literature.^{38,39,50} Further, the same HSQC experiment was performed on a sample containing a 1:1 mixture of A β ₄₂ and peptide **P33**. We observed changes in the positions of cross-peaks of A β ₄₂ in the presence of **P33**. To understand the change in the



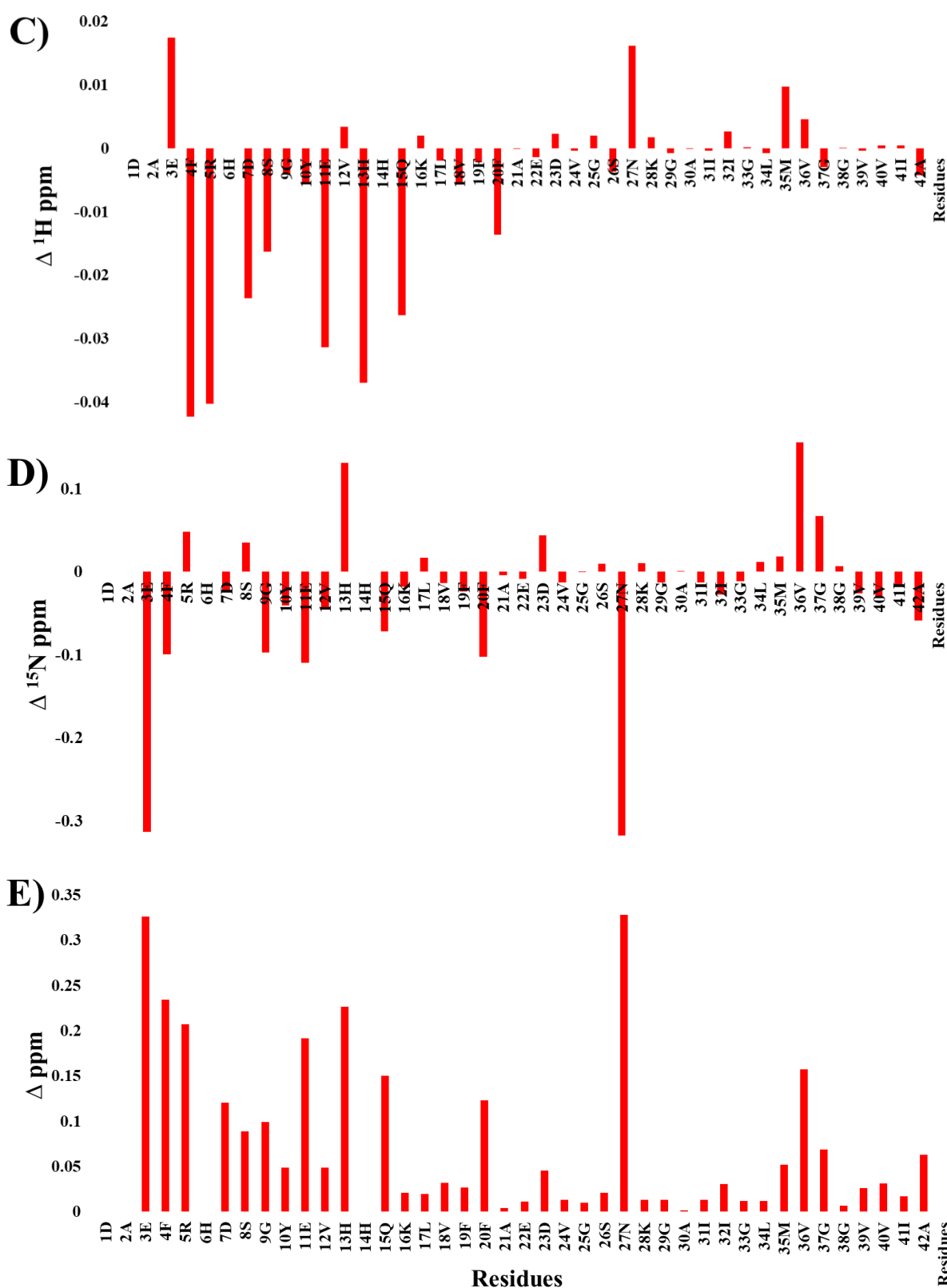


Figure 15: The 2D ^1H - ^{15}N HSQC NMR spectra of A) ^{15}N -isotopically labelled $\text{A}\beta_{42}$ (40 μM) in the absence of **P33**. B) Overlay of 2D ^1H - ^{15}N HSQC NMR spectra of ^{15}N -isotopically labelled $\text{A}\beta_{42}$ (40 μM) in the absence of **P33** (Blue contours) and in the presence of 80 μM **P33** (Red contours). Each peak represents the correlation between

the nitrogen and the amide proton of individual amino acid residues of A β ₄₂. C) and D) The changes in the chemical shift of ¹H and ¹⁵N resonance of A β ₄₂ (40 μ M) induced by **P33** (80 μ M) at a stoichiometric ratio of 1:2 (A β ₄₂: **P33**), respectively. E) Chemical Shift Perturbation study by ¹H-¹⁵N HSQC NMR spectra. A plot of change in peak position of all residues in ¹⁵N-isotopically labelled A β ₄₂ (40 μ M) after addition of peptide **P33** (80 μ M). The HSQC NMR experiments were conducted on an 800 MHz spectrometer attached with a cryo probe at 283 K in 10 mM sodium phosphate buffer, pH 7.4.

position of the cross peaks, we overlay the amide NH region of HSQC spectra of A β ₄₂ in the absence and presence of peptide **P33** which is shown in Figure 15B. The 2D NMR analysis suggested the changes in the chemical shift of ¹H resonance and a clear chemical shift perturbation in many of the residues of A β ₄₂, however, a dominant effect was observed particularly at the N-terminal residues 3E, 4F, 5R, 7D, 11E and 13H. In addition, the perturbation was also observed for the residues 15Q, 20F, 27N and 36V (Figure 15C and 15D, respectively). Except for 5R, no strong perturbation was observed for other cationic residues in the sequence. Based on these results, we speculated that the peptide may bind to the N-terminal region and induces conformational reorganization in A β ₄₂. Among the six negatively charged residues, three residues are strongly involved in the interaction with **P33**.

As already mentioned, In order to understand the structure of the **P33** in solution, we recorded the ¹H NMR in an aqueous solution consisting of 5% D₂O. The peptide displayed a poorly dispersed spectrum in an aqueous solution suggesting a lack of a defined secondary structure in an aqueous solution. However, in contrast, the peptide displayed a well dispersed ¹H NMR spectrum in CD₃OH. The peptide was subjected to the 2D-NMR (TOCSY and ROSEY) in CD₃OH and the structure was calculated using unambiguous NOEs. The peptide adopted a helical conformation in CD₃OH. Further, the CD analysis of the peptide also suggested that the helical conformation in MeOH, however, showed a spectrum similar to random coil type in an aqueous solution. The results suggested that the peptide adopted a helical structure in methanol, however, displayed no defined secondary structure in an aqueous solution.

3A.3.10. Cell toxicity

The soluble aggregates of A β ₄₂ are known to be toxic to neuronal cells. As **P33** has shown to prevent and disrupt the aggregation of A β ₄₂, we examined the toxicity of A β ₄₂ in the presence of **P33** using the MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) reduction assay on SH-SY5Y human neuroblastoma cell lines. Along with A β ₄₂, the toxicity

of **P33** was also examined. The cytotoxicity can be measured based on the ability of the live cells to convert MTT into insoluble formazan crystals. Consequently, the quantity of formazan produced in the assay is proportional to the number of live cells. We used a 10 μ M concentration of A β ₄₂ as a negative control and without A β ₄₂ as a positive control. Different concentrations of **P33** ranging from 1 μ M to 30 μ M were incubated at 37 °C with neuronal cells along with 10 μ M of A β ₄₂. Results are shown in Figure 16. Peptide **P33** displayed no toxicity to the cells up to 30 μ M concentration (Figure 16A). The soluble A β ₄₂ exhibited toxicity to the neuronal cells. Strikingly, with increasing concentrations of **P33**, A β ₄₂ showed reduced toxicity to the neuronal cells. No toxicity of A β ₄₂ was observed at a 1:1 ratio of peptide and A β ₄₂ (Figure 16C). These fascinating results indicate that **P33** rescued the neuronal cells from the toxicity of A β ₄₂. These results suggested that **P33** inhibited the toxicity of 10 μ M A β ₄₂ with an IC₅₀ value of 1.25 μ M (Figure 16D). Peptides **P34** and **P35** are also non-toxic to the cells at 30 μ M concentrations. The comparison of the activity of **P35**, **P34** and **P33** at 1:1 concentrations with A β ₄₂ is shown in Figure 16B. In comparison, **P33** showed more notable activity in rescuing the cells from the toxicity of A β ₄₂ than the other two peptides. In addition to the cytotoxicity experiments, we have also examined haemolytic activity of **P33**. The red blood cells were treated with different concentrations of **P33**. The peptide displayed no haemolytic activity tested up to 250 μ M concentration. These results suggested that **P33** is non-toxic to the neuronal cells and also non-haemolytic. Overall, these results suggested that the **P33** prevents the aggregation of A β ₄₂ by stabilizing its helical conformation and this conformation is non-toxic to the neuronal cells. These results strongly argued that **P33** is a potent candidate for the therapeutic intervention of Alzheimer's disease.

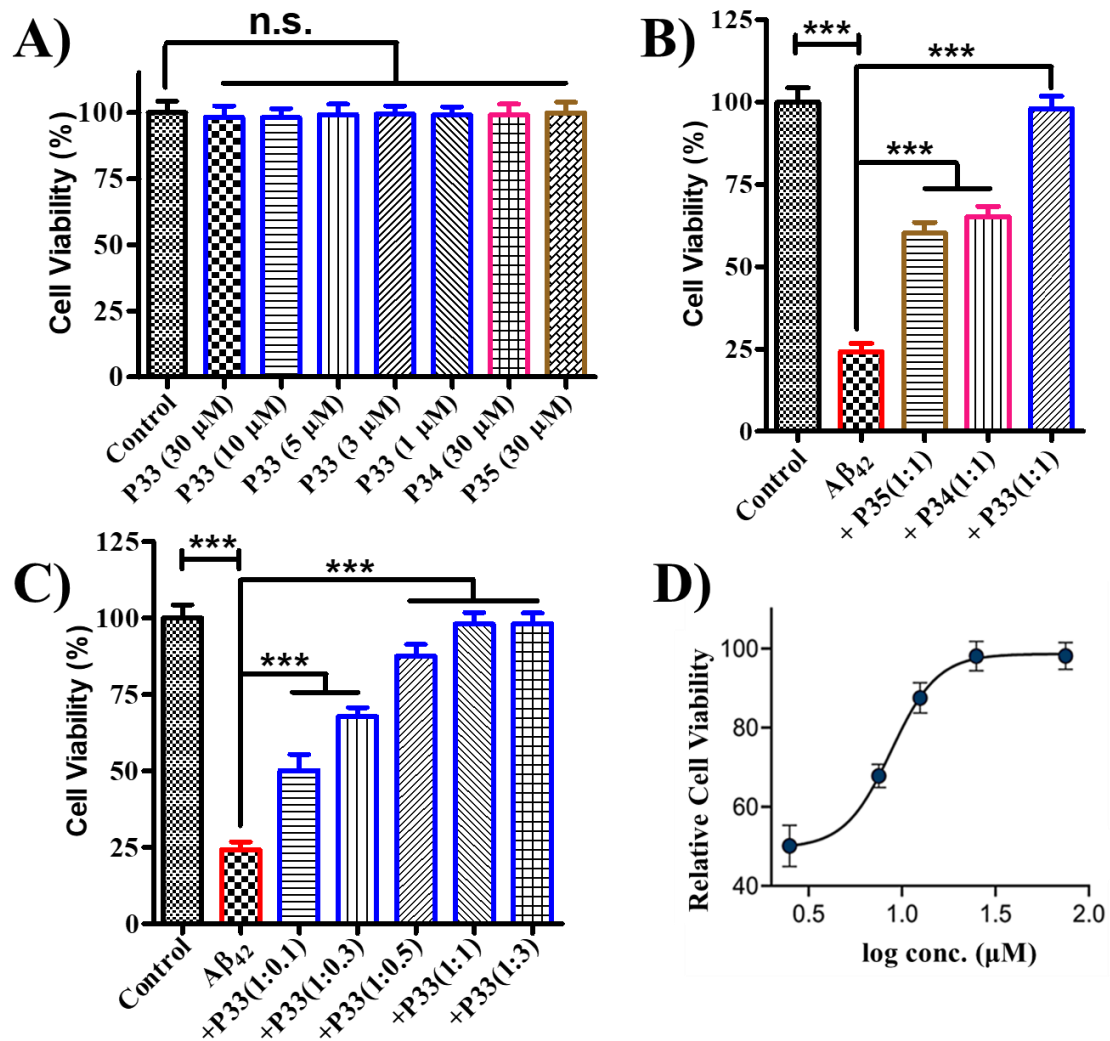


Figure 16: Cell Viability of SHSY5Y cells with Aβ₄₂ (10 μM) in the absence and presence of peptides (P33, P34 and P35). The cells were incubated at 37 °C up to 24 hours. A) Treatment of cells at different concentrations peptides alone. B) Treatment of cells with Aβ₄₂ in the absence and presence (1:1) of peptides P33, P34 and P35. The cell viability was compared to the control cells without any peptide or Aβ₄₂. C) Survival of cells with increasing concentrations of P33 (1 μM, 2 μM, 3 μM, 5 μM, 10 μM and 30 μM) at constant 10 μM of Aβ₄₂ and D) Dose dependent effect of P33 on Aβ₄₂ induced toxicity in SHSY5Y cells. The GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA) was used to analyze the data using one-way ANOVA and the *post hoc* Tukey's test for manifold comparisons ($p < 0.05$), n.s. = no significance.

There is a large body of evidence emerging from recent studies suggesting that the cationic peptides including host defence peptides inhibit the aggregation of β -amyloid peptides.²⁴⁻²⁶ However, the toxicity of these peptides hinders their further progress as inhibitors for Alzheimer's disease. The results reported here suggested that peptide **P33** displayed no toxicity to the cells up to 30 μ M and also showed non-haemolytic activity up to 250 μ M concentration (tested). Further **P33** showed no antibacterial activity against *E. coli* ATCC 25922, *S. aureus* ATCC 29213 *K. pneumoniae* BAA 1705, *A. baumannii* BAA 1605, and *P. aeruginosa* ATCC 27853 up to 64 μ M (tested).

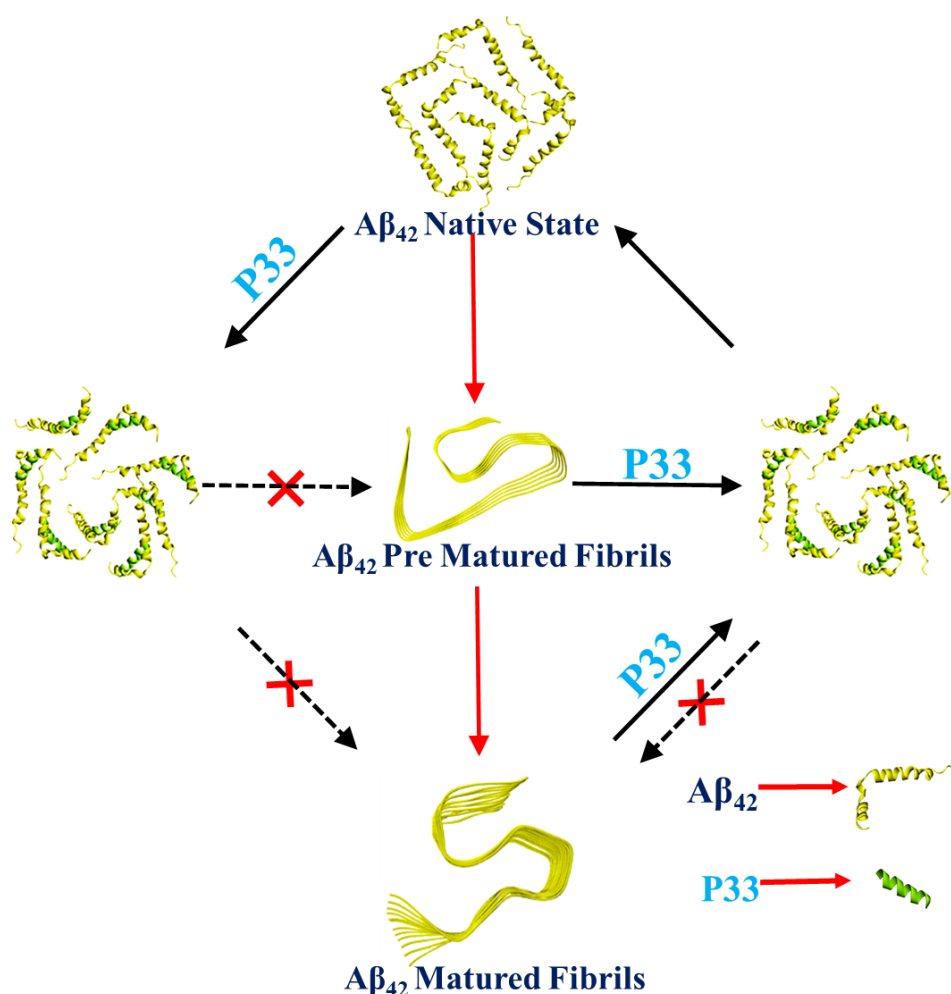


Figure 17: A cartoon representation of the mechanism of the interaction of **P33** with A β ₄₂.

The absence of the elongation phase in the ThT fluorescence kinetic assay, potent suppression of A β ₄₂ aggregation by the inhibitor peptide in solution by DLS experiment, absence of the fibrils of A β ₄₂ in the solution by confocal images, nonappearance of A β ₄₂ fibrils in TEM and AFM, absence of aggregation prone β -sheet structure in the CD analysis and structural re-

organization $A\beta_{42}$ in the presence of **P33** suggested **P33** trapping the $A\beta_{42}$ in non-toxic monomer form or appropriating the oligomers that are nontoxic to the cells. The cell toxicity experiments indeed revealed a reduced toxicity of $A\beta_{42}$ in the presence of equimolar concentration of **P33**. Based on these results, we propose that the peptide **P33** prevents the aggregation of soluble $A\beta_{42}$ and also disintegrates the aggregated $A\beta_{42}$ fibrils into soluble nontoxic precursors. We speculate that upon interacting **P33** induces a conformational change in the $A\beta_{42}$ and this new conformation is not prone to aggregation and also non-toxic to the cells. A cartoon representation of the mechanism of the interaction of **P33** with $A\beta_{42}$ is shown in Figure 17.

3A.4. Conclusion

In conclusion, we report that a 15-residue cationic amphiphilic peptide not only prevents the aggregation of soluble $A\beta_{42}$ into fibrils but also disintegrate the matured fibrils into soluble precursors. The peptide also rescue the neuronal cells by alleviating the toxicity of $A\beta_{42}$. Inspiringly, the results suggested that peptide is non-toxic and also non-haemolytic in nature. The shorter versions of peptide also prevent the aggregation of soluble $A\beta_{42}$, however at higher concentrations. The experimental evidence suggested that upon interaction, the peptide transforms a random coil of soluble $A\beta_{42}$ and the beta-sheet structure of aggregated or matured $A\beta_{42}$ into helical type conformations. We speculated that the peptide traps this helical type conformation which is not toxic to the neuronal cells. Overall, the peptide transformed aggregated fibrils of $A\beta_{42}$ into soluble precursors and rescues the cells from the toxicity of $A\beta_{42}$. These results strongly argue that **P33** may serve as one of the potent therapeutic candidates for Alzheimer's disease.

3A.5. Experimental Section

3A.5.1. Materials and Method

All reagents and chemicals used were purchased from commercial suppliers with good purity grade and used without any further purification. The flat bottom polystyrene 96- well plates and Human Aggregated Beta Amyloid ELISA Kit were purchased from Invitrogen Bio services. Rink Amide Resin, all N-Fmoc protected amino acids and $A\beta_{42}$ peptide (>95% purity) were purchased from GL Biochem, China. ^{15}N -Labeled $A\beta_{42}$ was procured from r-Peptide (Bogart, GA) and used directly in the NMR experiments. Sodium phosphate buffer was prepared using 10 mM sodium phosphate with 150 mM NaCl and 1 mM EDTA and pH was

adjusted to 7.4 by adding either dil. HCl or NaOH. All dry solvents, reagents, Thioflavin T, and antibiotics were obtained from Sigma-Aldrich. The bacterial media were purchased from Becton-Dickinson (Franklin Lakes, USA). RPMI and FBS were purchased from Lonza (Lonza, USA). The bacterial panel including *E. coli* ATCC 25922, *K. pneumoniae* BAA-1705, *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853 *A. baumannii* BAA-1605, and *Enterococcus* sp was procured from Biodefense and Emerging Infections.

3A.5.2. Solid Phase Peptide Synthesis and HPLC Purification

Peptides **P33-P36** were synthesized through a manual solid phase peptide synthesis on Rink amide resin at 0.2 mmol scale using standard Fmoc- conditions. The combination of HBTU and HOBT was used as coupling reagents, DIPEA was used as an activator base, NMP was used as a coupling solvent and 20% piperidine in DMF was used for the Fmoc- group deprotection. To release the peptides from the solid support, the resin was stirred for about 3 hours in a 10 mL cocktail containing trifluoroacetic acid (TFA)/ triisopropylsilane/water/phenol (90:5:5:5). The resin was filtered. The filtrate containing crude peptides was evaporated under reduced pressure and the gummy residue was triturated with cold diethyl ether (50 mL). The precipitate was separated through centrifugation. The crude peptides were again dissolved in 10 mL of ACN/H₂O and purified on a C₁₈ column using reversed-phase HPLC. Acetonitrile and water gradient system containing 0.1% of TFA at a 2 ml flow rate was used to purify the peptides. The mass of the purified peptides was confirmed by MALDI-TOF/TOF analysis. The lyophilized pure peptides were used for further studies.

3A.5.3. Antimicrobial Activity

The antimicrobial activity of **P33** was performed according to the reported procedure.⁵¹ Briefly, the cultures of bacterial strains were inoculated in TSB and OD₆₀₀ was measured, followed by dilution to ~10⁶ cfu/mL. The stock solution of **P33** (10 mg/mL) was prepared in DMSO and tested against the various bacteria from 64 mg/L to 0.5 mg/L in a two-fold serial dilution with 2.5 µL of each concentration added to the well of a 96-well microtiter plate. In continuation, 97.5 µL of bacterial suspension was added to each well containing **P33**. The drug Levofloxacin has used as a positive control and the buffer solution was used as a negative control. The microtiter plates containing bacterial strain and **P33** were incubated at 37 °C for 18-24 hours following which MIC was determined. Experiments were performed in triplicate using duplicate samples.

3A.5.4. β -Amyloid Disruption Study

3A.5.4.1 Preparation of the Amyloid $\text{A}\beta_{42}$

The $\text{A}\beta_{42}$ (2mg) was dissolved in 2 ml of 1,1,1,3,3,3- hexafluoroisopropanol (HFIP) and then kept for occasional vortex at room temperature for 1 hour to maintain in a monomeric state. After this, it was aliquoted into small fractions (0.5 mg/ample) and HFIP was evaporated from the sample by keeping sample vials overnight in a laminar flow hood. The sample vials containing $\text{A}\beta_{42}$ were then lyophilized overnight and stored at $-80\text{ }^{\circ}\text{C}$ until further. Before the usage, the lyophilized $\text{A}\beta_{42}$ was dissolved in DMSO to make the stock 2 mM stock solutions of monomeric $\text{A}\beta_{42}$ in DMSO. The $\text{A}\beta_{42}$ aggregates were prepared by the addition of 10 mM sodium phosphate buffer to the stock solution of $\text{A}\beta_{42}$ in DMSO. The final volume of the $\text{A}\beta_{42}$ solution was maintained at 100 μL and incubated at $37\text{ }^{\circ}\text{C}$. The aggregation kinetics of the $\text{A}\beta_{42}$ in the presence of appropriate concentrations of peptides were studied in 10 mM sodium phosphate buffer (pH 7.4) and the final volume was maintained at 100 μL . The concentration of $\text{A}\beta_{42}$ was kept at a constant 25 μM in all experiments.

3A.5.4.2 Thioflavin T (ThT) Fluorescence-Based Kinetic Assay

The aggregation kinetics of $\text{A}\beta_{42}$ were studied using a ThT-fluorescence assay. The measurements of ThT- fluorescence were carried out on flat bottom black polystyrene nonbinding 96-well plates. The samples were excited at 440 nm and fluorescence emission was monitored at 482 nm. The stock solution of $\text{A}\beta_{42}$ (0.55 mM) was prepared in DMSO and it will be appropriately diluted with sodium phosphate buffer before starting the aggregation kinetic experiments. The stock solution of ThT (200 μM) in 10 mM sodium phosphate buffer (pH 7.4) was prepared. The ratio for ThT to $\text{A}\beta_{42}$ was maintained at 20:25 μM , respectively. The control assay was performed by the addition of 4.5 μL of $\text{A}\beta_{42}$ to the 10 μL of the ThT in sodium phosphate buffer and the volume was adjusted to 100 μL using the buffer. For the inhibition kinetics assay, different concentrations of peptides were added to the wells containing 4.5 μL of $\text{A}\beta_{42}$ and 10 μL of the ThT. The final concentration of the peptides in the wells was maintained at 250, 125, 75, 25, 12.5, 7.5, and 2.5 μM . The solution containing $\text{A}\beta_{42}$, ThT and peptides was incubated at $37\text{ }^{\circ}\text{C}$ and subjected to the fluorescence measurements for a period of 48 hours with orbital shaking for 120 s prior to each reading. Measurements were performed in three independent experiments. Kinetic graphs were plotted using Origin (version 9.1). Kinetic Parameters t_{50} (h), Lag time (h), k_{app} (h^{-1}) were calculated from the ThT assays.^{48,49}

3A.5.4.3. Circular Dichroism Spectroscopy

Circular dichroism spectroscopy analysis was carried out using the Jasco J-815 spectropolarimeter. For CD experiments, the stock solution of A β ₄₂ (0.55 mM in 10mM NaOH) was diluted to 50 μ M in sodium phosphate buffer. The sodium phosphate buffer without peptides and A β ₄₂ was used as a blank. The 50 μ M concentrations of both A β ₄₂ and **P33** were used for CD measurements. The CD spectra of **P33** alone, A β ₄₂ alone and the mixture containing both **P33** and A β ₄₂ at 0 hour and 24 hours of incubation at 37 °C were recorded in triplicate.

3A.5.4.4. High Resolution Transmission Electron Microscopy (HR-TEM) Analysis

A β ₄₂ (100 μ M) was incubated (i) alone and (ii) in the presence of peptide with an equimolar ratio for about 48 hours in sodium phosphate buffer. The stock solutions were prepared as mentioned above. Aliquots of all the samples were diluted 10 times with water. From each sample, 1 μ L of the aliquots were drop casted onto glow discharged individual carbon coated 200 mesh Cu grids (Ted Pella Inc., Redding, CA). Further, the grids were negatively stained with 2% uranyl acetate (Polysciences, Inc, Warrington, PA) in double distilled water and air dried. Stained grids were examined under Bruker High-Resolution Transmission Electron Microscope to take the final images.

3A.5.4.5. AFM Analysis

A β ₄₂ (100 μ M) was incubated (i) alone and (ii) in the presence of peptide with an equimolar ratio for 48 hours. The stock solutions were prepared as mentioned above. The aliquots of all the samples were diluted 10 times with water. From each sample, 1 μ L of the aliquots were drop casted on a cleaned silicon wafer plate (SiO₂/Si substrate), dried at room temperature and finally kept in the desiccator for 10 min before taking the images. Freshly prepared solutions were used in all the experiments. The drop casted samples on silicon wafer plates were examined under Keysight's AFM instrument 5500 to take the final images.

3A.5.4.6. DLS Study

The solution of A β ₄₂ (100 μ M) in 10 mM sodium phosphate buffer (pH 7.4) was prepared as mentioned above and incubated (i) alone and (ii) in the presence of peptide with an equimolar ratio for about 24 hours. The stock solutions were prepared as mentioned above. Aliquots of all the samples were diluted 10 times with water. The size distribution was plotted against the diameter of A β ₄₂ self-assembly.

3A.5.4.7. Laser Scanning Confocal Microscopy study

A β ₄₂ (100 μ M) was incubated (i) alone and (ii) in the presence of peptide with an equimolar ratio for 48 hours. The stock solutions were prepared as mentioned above. The aliquots of all the samples were diluted 10 times with water. 10 μ L of the Thioflavin T was added to each sample as a fluorescent indicator. From each sample, 5 μ L of the aliquots were drop casted on a cleaned glass slide and covered with a thin cover slip then imaged using OLYMPUS ZX81 laser scanning confocal microscopy by inverting the sample (λ_{ex} : 440 nm, λ_{em} : 482 nm).

3A.5.4.8. Cell Viability Assay

SHSY5Y-human neuroblastoma cells were cultured in DMEM (Dulbecco's modified essential medium)/F12 (Sigma, USA) medium in humidified 5% (v/v) CO₂/air at 37 °C in 10% (v/v) (fetal bovine serum) FBS (Invitrogen, USA) and 100 U/mL penicillin. After reaching ~95% confluence, cells were split by using 0.25% trypsin-EDTA (Sigma) in to fractions to carry out the experiment. We were seeded 10⁴ cells in each well in 96-well plates and incubated at 37 °C up to 24 hours.

The MTT experiments (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were performed on human neuroblastoma cell line SH-SY5Y as reported earlier.³¹ Briefly, after 24 hours incubation of cells in a 96-well plate at 37 °C, the medium was replaced with the solution containing 10 μ M A β ₄₂ aggregates diluted in DMEM/F12 medium. In order to understand the effect of **P33** on the cytotoxicity of A β ₄₂ aggregates, 10 μ M A β ₄₂ of was mixed and 1, 3, 5, 10, and 30 μ M concentrations of **P33**, 10 μ M **P34** and 10 μ M **P35** and incubated over a period of 12 hours at 37 °C prior to treatment of cells. The cells were treated with the mixture of A β ₄₂ containing different concentrations of **P33**, **P34** and **P35** and the plates were incubated at 37 °C in humidified 5% (v/v) CO₂/air. The MTT reduction assay was performed after 24 hours of treatment of the peptides and A β ₄₂. After the addition of MTT (0.5 mg/mL), the cells were incubated at 37 °C for about 4 hours in a CO₂ incubator. Further, the supernatant was removed from the each well and DMSO (200 μ L) was added to solubilize the dye formazan. The plates were again incubated in a humidified CO₂ incubator for about 15–20 min. The absorbance was measured at 570 nm using the multi-mode microplate reader. The cell viability was compared to the cells without peptides and A β ₄₂. The A β ₄₂ without peptides was used as a positive control. Similarly, the cytotoxicity of peptides were also examined without A β ₄₂. Each data point represents an average of three independent triplet-well trials.

3A.5.4.9. ¹H-¹⁵N HSQC NMR Spectroscopy

The experiments of ¹H-¹⁵N HSQC NMR were performed on an 800 MHz Bruker instrument. The monomeric ¹⁵N labelled Aβ₄₂ was prepared as described in the above experiments. The NMR experiments were performed by maintaining the solution ratio of 9:1 (H₂O:D₂O) with 20 mM NaPi (pH 7.4). In each experiment, ¹⁵N- Aβ₄₂ sample was prepared freshly to maintain Aβ₄₂ in the monomeric native form to avoid amyloid formation. The stock solution of **P33** (10 mM) was prepared in Mili Q H₂O. ¹H-¹⁵N HSQC NMR experiments were performed using fresh sample of 40 μM ¹⁵N-Aβ₄₂ with 20 mM NaPi buffer having pH 7.4 in the absence and presence of 2 equivalents of **P33** at 283 K with a cryoprobe (Aβ₄₂:peptide **P33**; 1:2). All cross peaks were identified with the help of previously assigned spectra from the literature.^{39,50} The addition of peptide **P33** to the ¹⁵N- Aβ₄₂ sample was less than 2% dilution and the dilution factor was corrected while calculating the perturbation in the chemical shifts of ¹H-¹⁵N resonances. The NMR spectra were processed and analyzed by using TOPSPIN software. Chemical shift perturbations of all the residues (¹H-¹⁵N) were determined by using the equation $\Delta_{\text{ppm}} = [(5X\Delta^1\text{H})^2 + (\Delta^{15}\text{N})^2]^{1/2}$.

3A.5.4.10. ELISA Assay

Human Aggregated Beta Amyloid ELISA Kit was procured from Invitrogen Bioservices. The ELISA assay was performed according to the manufacture's protocol. Briefly, to the N-terminus human Aβ-specific monoclonal antibody-coated wells, 100 μL of the Aβ₄₂ samples in the absence and presence of **P33** were added and incubated for about 2 hours at room temperature. Each sample was prepared by incubating the 0.60 nM of Aβ₄₂ in the absence and presence of peptide **P33** by varying the peptide concentration from 0.5 to 10 equivalent ratio at 37 °C up to 24 hours. The samples were diluted by 1:2 in diluent buffer (contains phosphate buffer saline with bovine protein as a carrier and 0.5% Proclin™ biocide as a preservative) before being added to the 96-well plate. After 2 hours from the addition of the Aβ₄₂ samples, the wells were washed four times with Tris-based wash buffer containing 0.05% Tween-20 pH 7.4 (PBST) followed by the addition of 100 μL of Hu Aggregated Aβ Biotin Conjugate solution into each well. The samples were incubated for about 1 hour at room temperature. After washing the wells four times with PBST, the wells were treated with 100 μL of Streptavidin-HRP (horseradish peroxidase-labelled streptavidin) and incubated for 30 min at room temperature. After washing the wells with PBST, the solution of tetramethylbenzidine (TMB) was added and incubated for 30 about min at room temperature in the dark. The reaction was

stopped by adding 100 μ L of stop solution (0.16 M sulfuric acid) to each well. The absorbance of each well at 450 nm was recorded using multi-mode microplate reader. Experiments were performed in triplicate and averaged.

3A.5.5. NMR of Peptide P33

The NMR experiments of **P33** were performed on 800 MHz spectrometer CD₃OH solvent as reported earlier. The TOCSY was used to assign the residues and ROESY spectrum was used to assign NOEs. The TOPSPIN version 2.1 software was used to analyse the NMR data. High resolution 1D recording was used to measure the Scalar coupling (*J*) values. Amide proton temperature portions ($d\delta/dT$) were measured by recording 1D trial at definite intervals of 10 degrees (K) in the temperature range of 278-318K.

Modelling

Based on the unambiguous NOEs, the energy minimized structure was calculated using Discovery Studio version 3.5 software.

3A.5.6. Statistical Analysis

The GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA, USA) was used to carry out the statistical analysis. One-way ANOVA with Tukey's *post hoc* multiple comparisons test was used to analyze comparisons between three or more groups. *P* values < 0.05 were considered significant.

3A.6. ThT assay

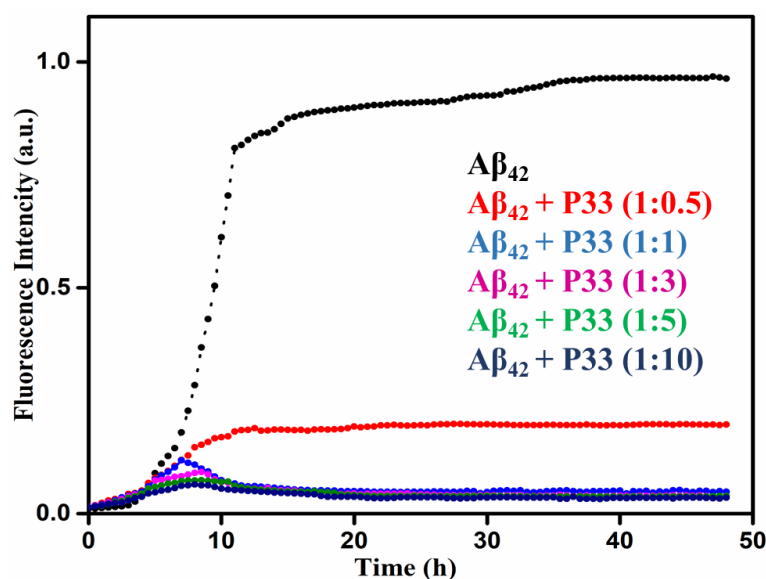


Figure 18: Kinetic study of the fibrillation of the $A\beta_{42}$ with **P33**. The normalized kinetic profile of the self-assembly of 25 μM of $A\beta_{42}$ in the absence and presence of peptide **P33** by varying the concentration (12.5 μM , 25 μM , 75 μM , 125 μM and 250 μM). Buffer Conditions: 10 mM Sodium Phosphate, 150 mM NaCl, 1mM EDTA and pH 7.4. ThT concentration was 20 μM .

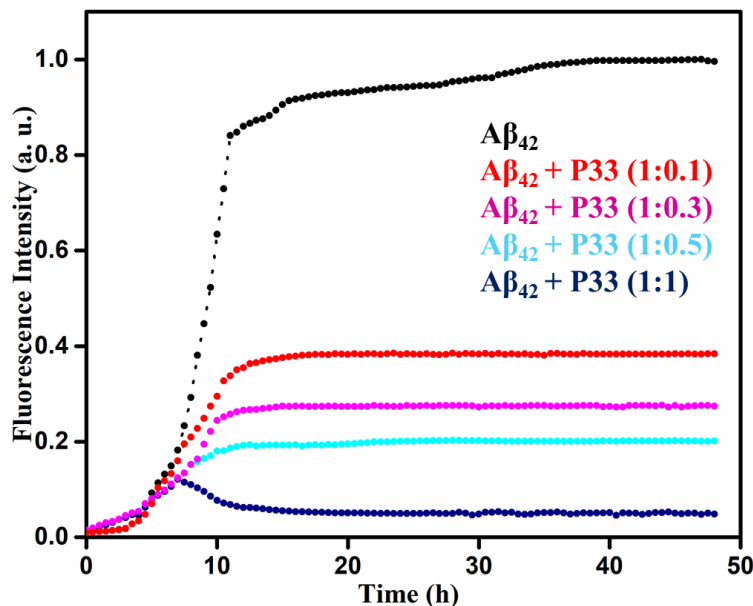


Figure 19: Kinetic study of the fibrillation of the $A\beta_{42}$ with peptide **P33**. The normalized kinetic profile of the self-assembly of 25 μM of $A\beta_{42}$ in the absence and presence of peptide **P33** with stoichiometric ratios (2.5 μM , 7.5 μM , 12.5 μM , and 25 μM). Buffer Conditions: 10 mM Sodium Phosphate, 150 mM NaCl, 1mM EDTA and pH 7.4. ThT concentration was 20 μM .

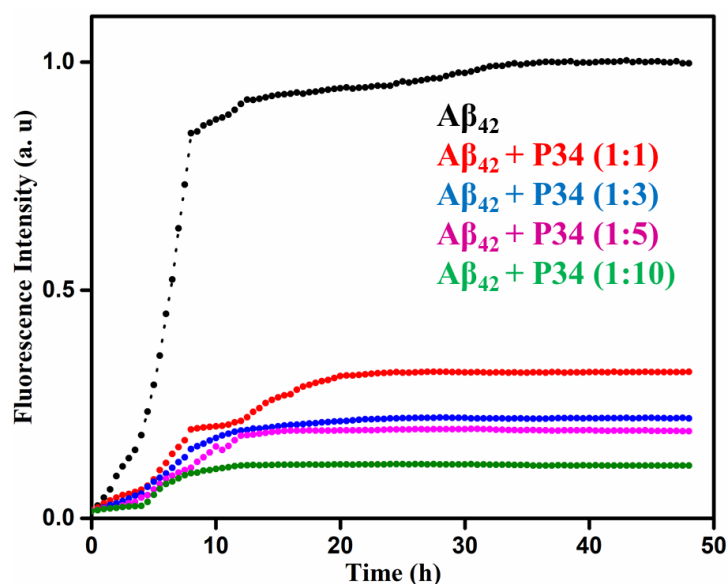


Figure 20: Kinetic study of the fibrillation of the $A\beta_{42}$ with peptide **P34**. The normalized kinetic profile of the self-assembly of 25 μM of $A\beta_{42}$ in the absence and presence of peptide **P34** with stoichiometric ratios (25 μM , 75 μM , 125 μM , and 250 μM). Buffer Conditions: 10 mM Sodium Phosphate, 150 mM NaCl, 1mM EDTA and pH 7.4. ThT concentration was 20 μM .

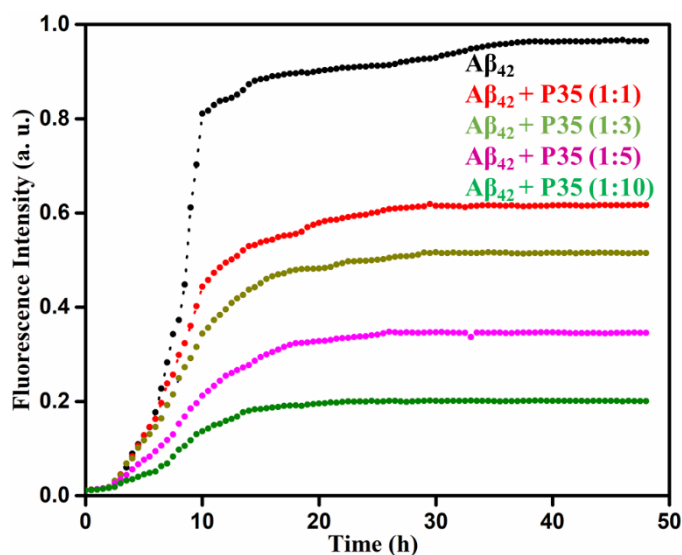


Figure 21: Kinetic study of the fibrillation of the $A\beta_{42}$ with peptide **P35**. The normalized kinetic profile of the self-assembly of 25 μM of $A\beta_{42}$ in the absence and presence of peptide **P35** with stoichiometric ratios (25 μM , 75 μM , 125 μM , and 250 μM). Buffer Conditions: 10 mM Sodium Phosphate, 150 mM NaCl, 1mM EDTA and pH 7.4. ThT concentration was 20 μM .

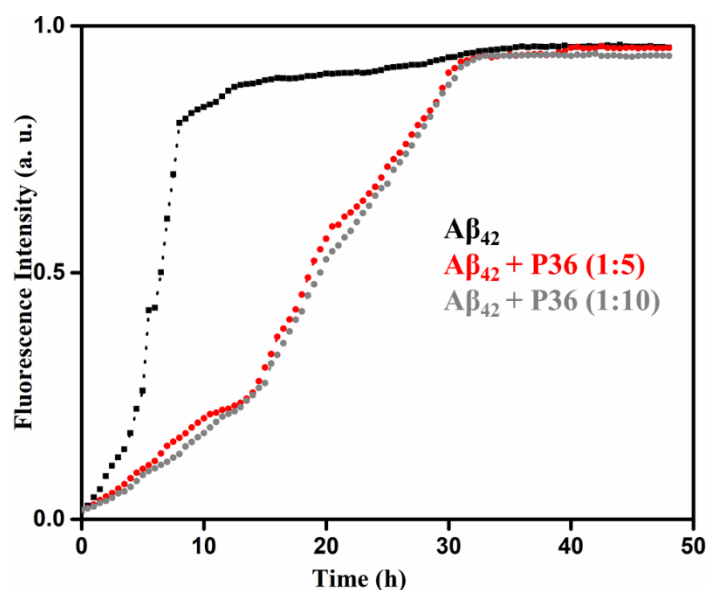


Figure 22: Kinetic study of the fibrillation of the $A\beta_{42}$ with peptide **P36**. The normalized kinetic profile of the self-assembly of 25 μM of $A\beta_{42}$ in the absence and presence of peptide **P36** with stoichiometric ratios (125 μM , and 250 μM). Buffer Conditions: 10 mM Sodium Phosphate, 150 mM NaCl, 1mM EDTA and pH 7.4. ThT concentration was 20 μM .

3A.7. References

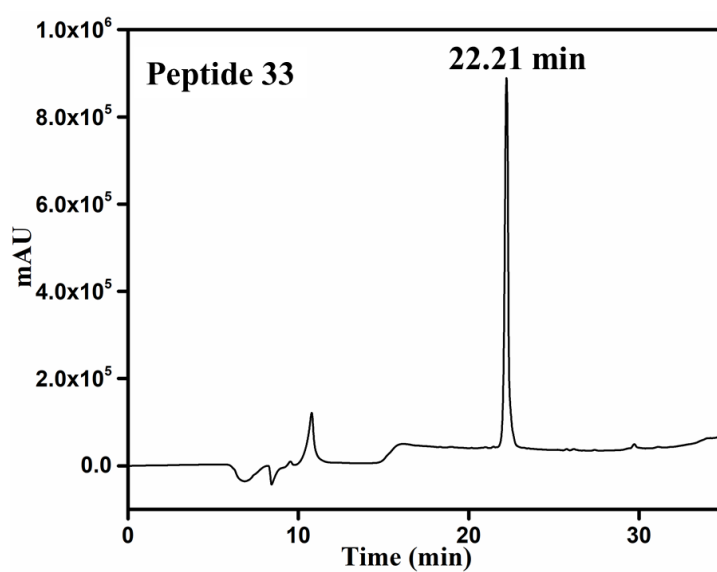
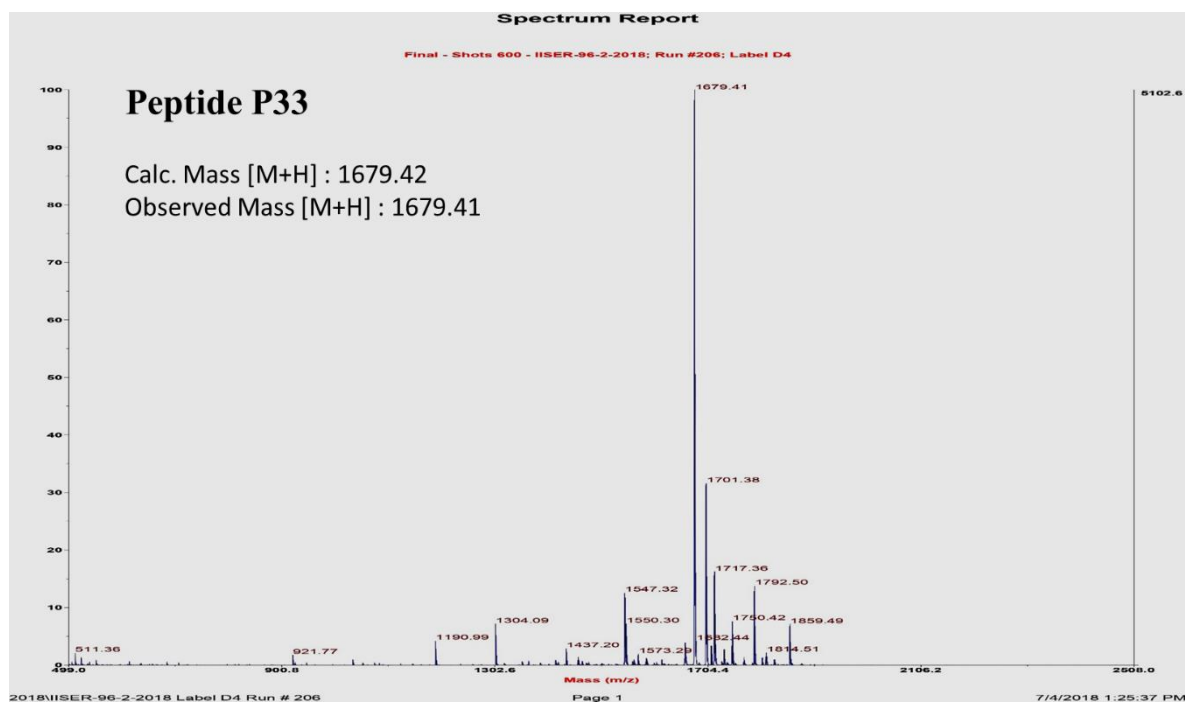
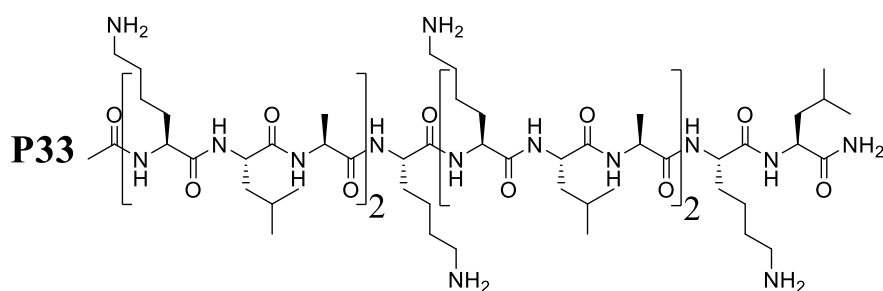
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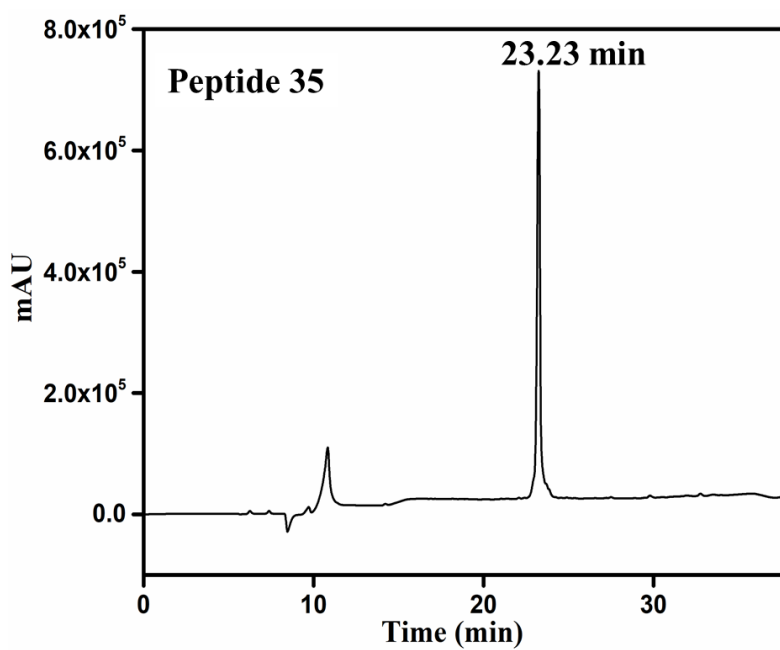
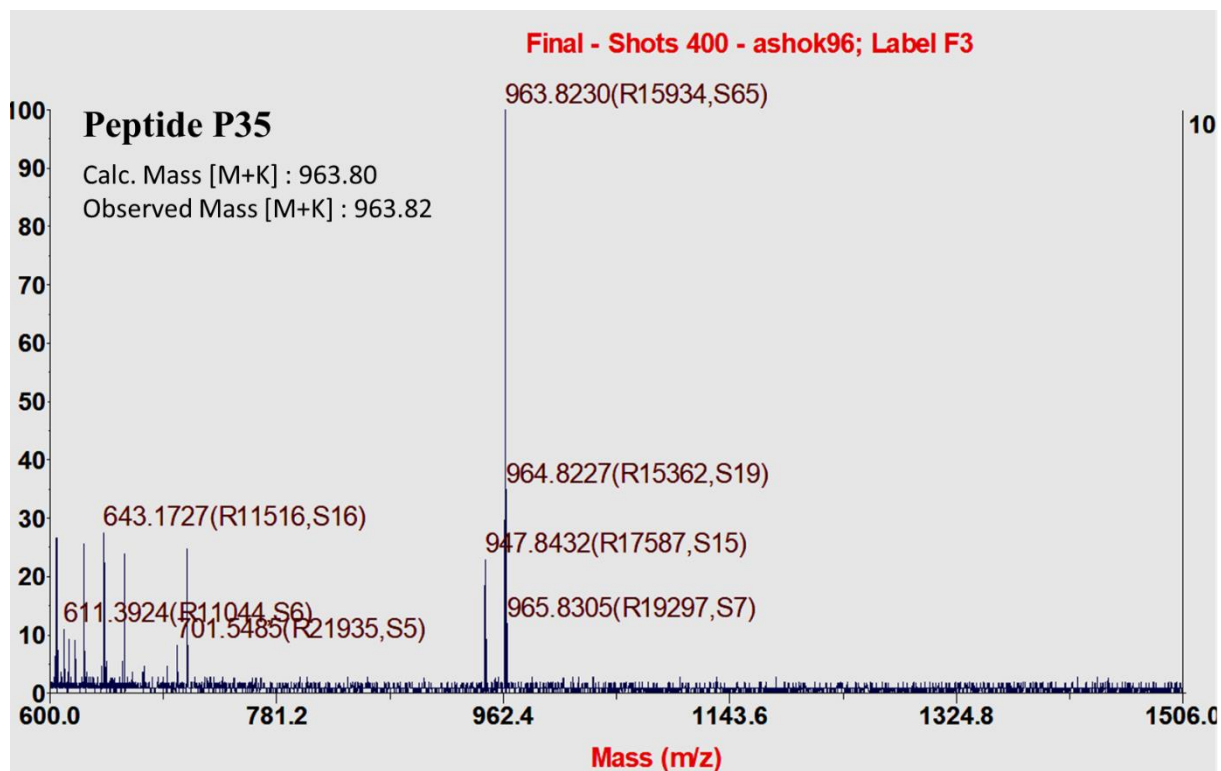
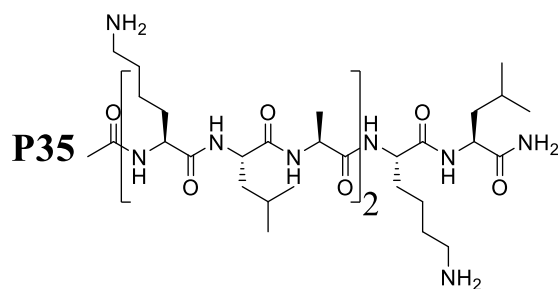
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3A.7. Appendix IIIA: Mass Spectra and RP-HPLC traces of Peptides P33-P36







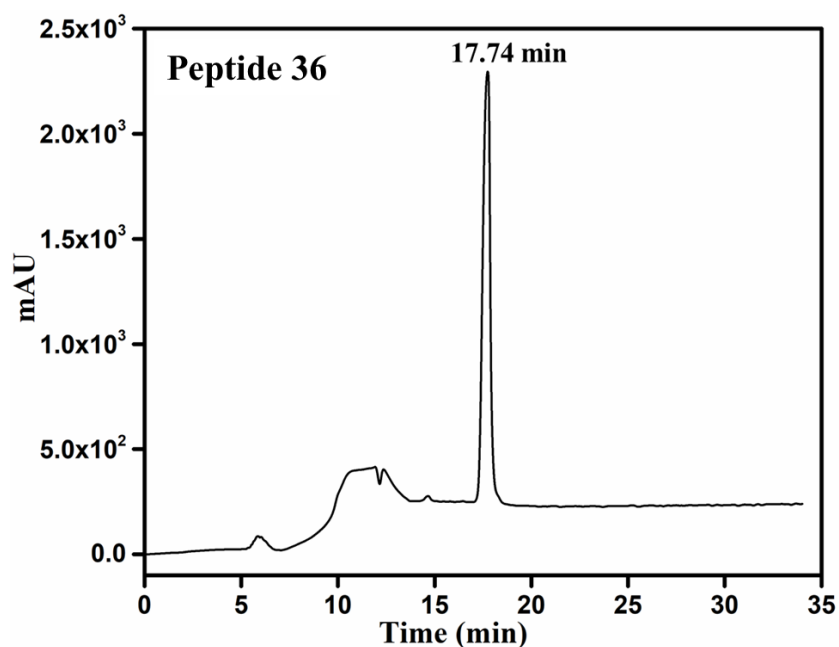
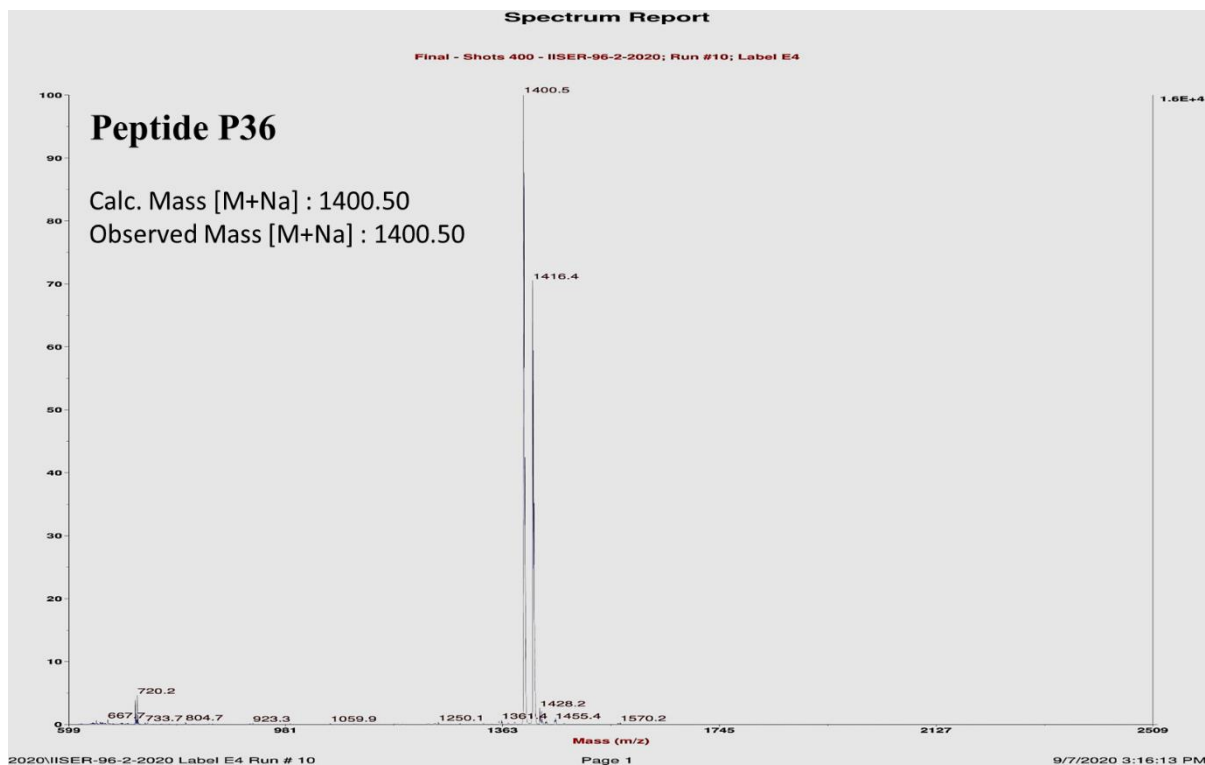
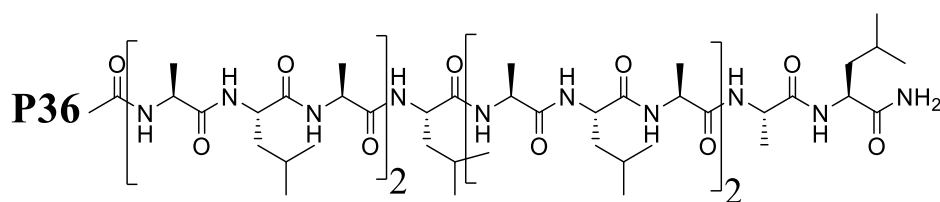


Figure 23: Mass spectra and Reverse phase HPLC profile of peptides **P33-P36** recorded on analytical C₁₈ column (5 μ m, 4.6 x 250 mm column) at 220 nm using ACN/H₂O (**P33-P35**) and MeOH/H₂O (**P36**) gradient system at a flow rate of 2 mL/Min.

Chapter 3B

Proteolytically Stable $\alpha\alpha\gamma$ -Hybrid Peptides Inhibits the Aggregation and Cytotoxicity of $A\beta_{42}$

3B.1 Introduction

Alzheimer's disease (AD) is a progressive neurological disorder that slowly abolishes memory and thinking skills.¹⁻² It is the most common cause of dementia and accounts for nearly 60-80% of cases of dementia. A recent assessment by the world health organization revealed that more than 55 million people are currently suffering from AD and it is expected that more than 135 million people will be suffering from this disease by 2050.³ A large number of AD cases will have a huge social and economic impact not only the people suffering from the AD, but also on their entire families and the society at large. At the moment, there are no approved small molecule drugs available for the treatment of AD and the large and continuous accumulation of AD numbers demands a boost in the drug discovery programmes.

According to the amyloid hypothesis, the key pathogenic event in AD is the aberrant accumulation of the amyloid precursor protein fragments ($A\beta$) in the brain and this pathogenic event triggers the tau pathology, neurodegeneration, decline in cognitive skills and eventually leads to death.⁴⁻⁷ It is believed that the accumulation of $A\beta$ begins 15-20 years before the appearance of clinical symptoms. The hypothesis suggested that there should be a balance between the $A\beta$ production and clearance. The abrupt cleavage of amyloid precursor protein by β - and γ -secretases leads to the $A\beta$ -peptides.⁸ Among these $A\beta$ -oligomers, $A\beta_{1-40}$ ($A\beta_{40}$) is the most abundant and $A\beta_{1-42}$ ($A\beta_{42}$) is highly aggregation prone and also toxic. The amyloid hypothesis also provides therapeutic strategies either inhibiting $A\beta$ -production or clearance of $A\beta$ from the brain. As there is no much success in designing the inhibitors for β -secretase and γ -secretase which are primarily responsible for the $A\beta$ -production, the clearance of $A\beta$ from the brain has attracted much attention.^{9,10} In this context, a variety of organic small molecules,¹¹ synthetic peptides,¹²⁻²⁴ antimicrobial peptides²⁵⁻²⁸ and cell-permeable peptides,²⁹ affibodies,³⁰ β -sheet mimetics,³¹ peptide-polymer conjugates,³² foldamers from aromatic templates³³⁻³⁵ and molecular chaperones³⁶ have been designed and investigated as $A\beta$ aggregation inhibitors. Further, tris-pyrrolidine peptidomimetic (Figure 1) mediated vesicles have also been designed to rescue $A\beta_{42}$ from aggregation and cytotoxicity.³⁷ Recent FDA approval of the humanized IgG1 monoclonal antibody lecanemab (Figure 1) for the treatment of early stages of Alzheimer's disease has boosted the research in inhibiting $A\beta$ aggregation.³⁸ The monoclonal antibody lecanemab bind and dissolves the $A\beta$ plaques in the brine.³⁹ The lecanemab also provides a possible druggable pathway for the design of small molecule therapeutics. Here we are reporting the design, synthesis, and inhibition of soluble amyloid $A\beta_{42}$ -aggregation, the

disintegration of A β ₄₂ fibrils into soluble forms, recusing the neuronal cells from the toxicity of A β ₄₂ and proteolytic stability of $\alpha\alpha\gamma$ -hybrid peptides. We further compared the activity of $\alpha\alpha\gamma$ -hybrid peptide along with the α -peptide counterpart.

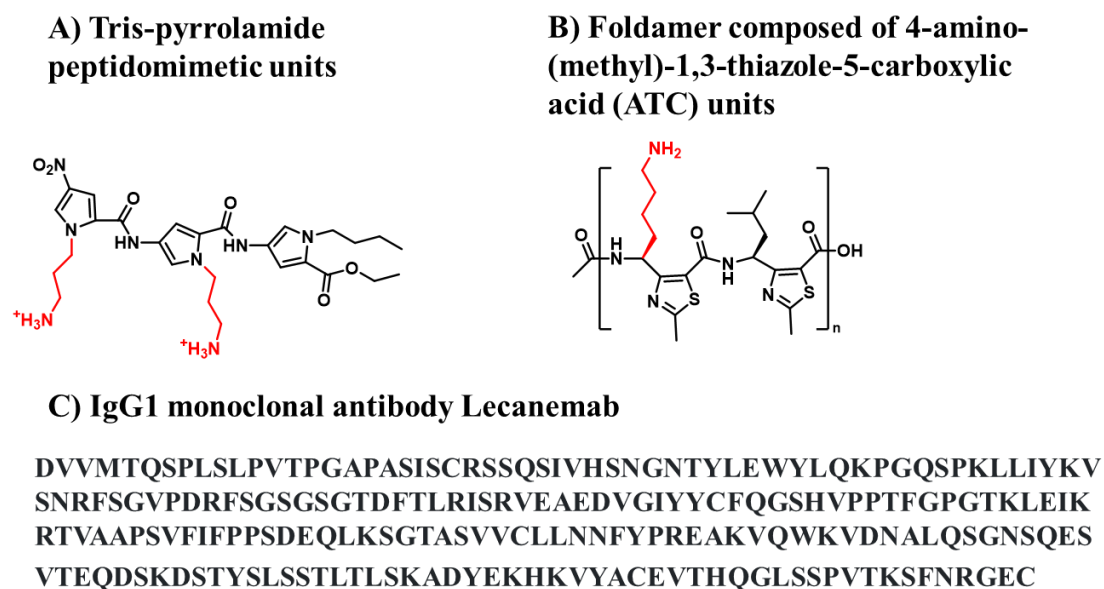


Figure 1: Reported Amyloidogenic Aggregation Inhibitors (A and B) and C) FDA approved antibody for Alzheimer's disease.

3B.2. Aim and Rationale of the Present Work

We have been interested in designing A β aggregation inhibitors using short peptides shown in Figure 2.^{40,41} Recently, we showed the inhibition of A β ₄₂ aggregation by cationic amphiphilic α -peptides (Figure 2).⁴¹ We have been working in the area of foldamers consisting of α,γ -hybrid peptides,⁴²⁻⁴⁴ and demonstrated that hybrid peptides composed of alternating 1:1 α and γ -amino acid adopt 12-helical conformation,⁴⁵ while 2:1 α -and γ -amino acids ($\alpha\alpha\gamma$ -hybrid peptides) adopt 10/12-helical conformation with different side-chain orientations shown in Figure 3.⁴⁶ In the case of $\alpha\alpha\gamma$ -hybrid peptide 10/12-helices, the amino acid side-chains are projected at three side of the helix. Through $\alpha\alpha\gamma$ -hybrid peptides, the side-chains of the all cationic residues can be projected at one side of the helix. As the folding pattern of $\alpha\alpha\gamma$ -hybrid peptide is unique and also requires lesser γ -amino acid residues, we sought to investigate whether or not the $\alpha\alpha\gamma$ -hybrid peptides can inhibit the A β ₄₂ aggregation. Inspired by the results of the cationic amphiphilic peptides, we sought to investigate whether the $\alpha\alpha\gamma$ -hybrid peptides foldamers can inhibit the aggregation of A β ₄₂. The advantage of the $\alpha\alpha\gamma$ -hybrid peptides is that they have better proteolytic stability compared to their α -peptide counterparts.

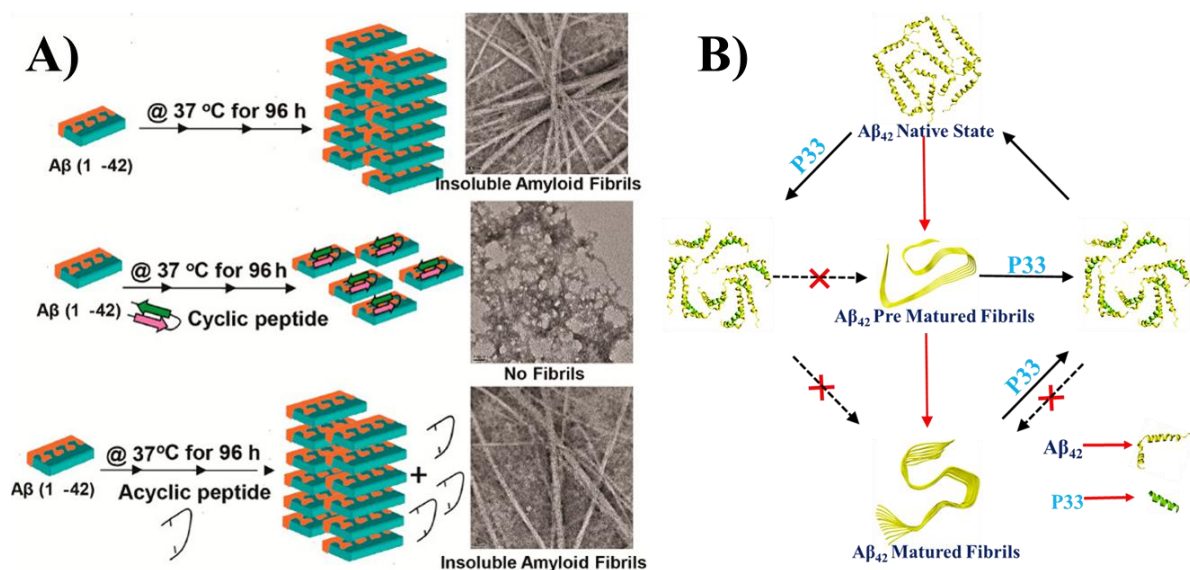


Figure 2: Inhibition of A β_{42} aggregation by cationic amphiphilic α -peptides.

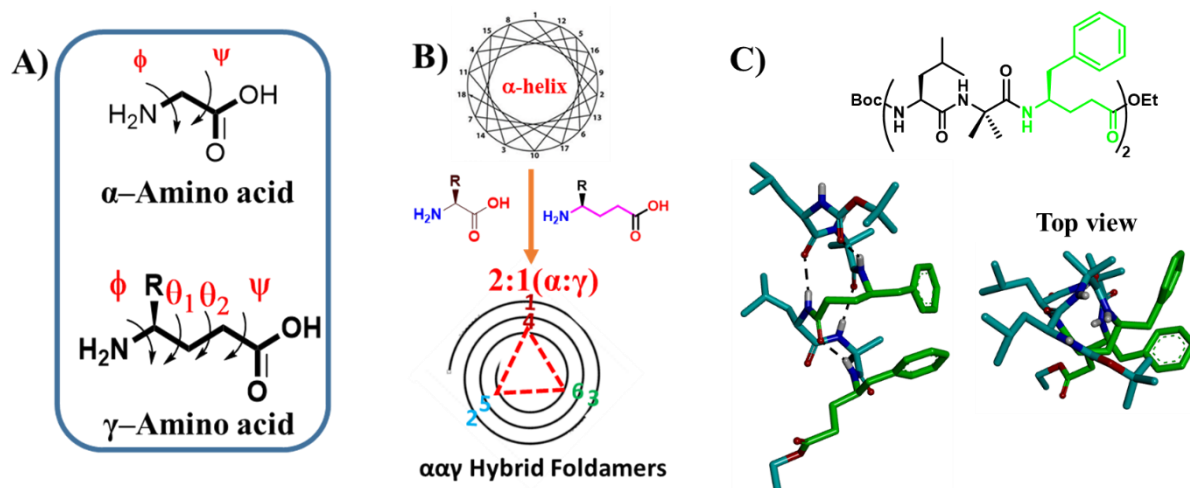


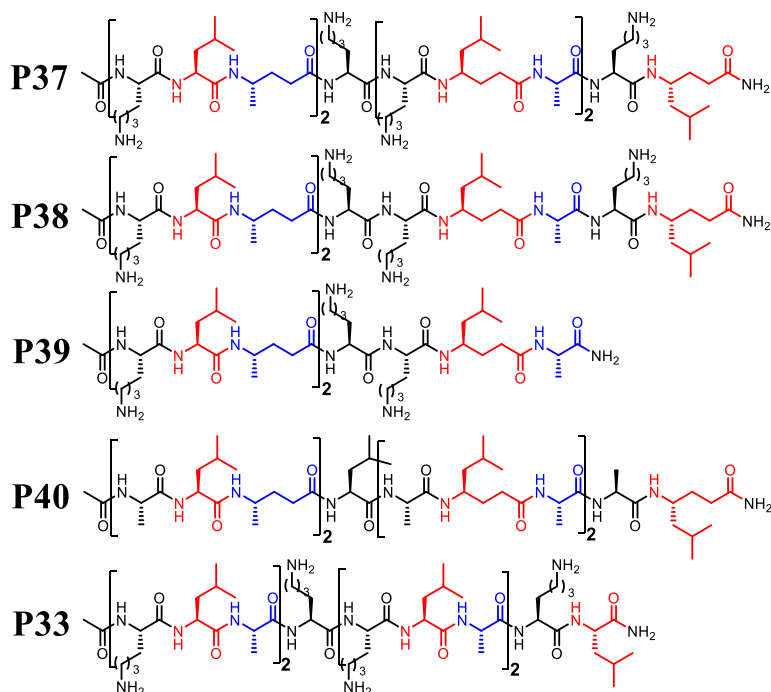
Figure 3: A) Structure of the monomeric building blocks of α - and γ^4 -amino acid. B) Helical wheel diagram depicting side chain projection in α/γ^4 -hybrid peptides. γ^4 -amino acids are shown in green. C) Crystal structure of 10/12-helix from $\alpha\alpha\gamma$ -hybrid peptide [Boc-(Leu-Aib- γ -Phe) $_2$ -OEt].

3B.3. Results and Discussion

3B.3.1. Design of $\alpha\alpha\gamma$ -hybrid peptides

To understand whether or not the $\alpha\alpha\gamma$ -hybrid peptide foldamers can inhibit the A β_{42} aggregations, we designed four peptides **P37-P40** along with the active α -peptide **P33** as a control. The sequences of these $\alpha\alpha\gamma$ -hybrid peptides and the control α -peptide are shown in Scheme 1. The dodecapeptide **P38** and decapeptide **P39** are truncated versions of **P37**. Peptide

P40 is a hydrophobic peptide and all Lys residues were replaced by Ala residues. The γ -amino acids were synthesized as reported earlier.⁴⁴ All peptides were synthesized using solid phase peptide synthesis on Rink Amide resin and purified by RP-HPLC. The purified peptides were authenticated by the mass spectrometry analysis.



Scheme 1: Peptide sequences.

3B.3.2. Inhibition of aggregation of soluble A β ₄₂ by $\alpha\gamma$ -hybrid peptides

The aggregation kinetics of A β ₄₂ was studied using a thioflavin T (ThT) fluorescence assay.⁴⁷ In the absence of any inhibitor peptides, we observed enhancement in the fluorescence of ThT after addition to the solution of soluble A β ₄₂. The ThT fluorescence signal saturates after complete aggregation of soluble A β ₄₂. After observing the marked enhancement in the fluorescence of ThT upon incubation with soluble A β ₄₂, we sought to examine the effect of $\alpha\gamma$ -hybrid peptides **P37-P40** along with the control peptide **P33** on the aggregation of soluble A β ₄₂. We incubated the soluble A β ₄₂ and peptides **P37**, **P38** and **P39** at 1:1, 1:3, 1:5 and 1:10 (A β ₄₂: peptides) concentrations along with ThT to understand the inhibition of aggregation. The ThT fluorescence in the absence and the presence of peptides are shown in Figure 4. In contrast to the ThT fluorescence signal in the absence and presence of peptides at 1:1 and higher concentrations of peptides with A β ₄₂ showed reduced or very minimal fluorescence emission. In comparison, at 1:1 equimolar concentrations of **P37** and A β ₄₂ showed a very weak

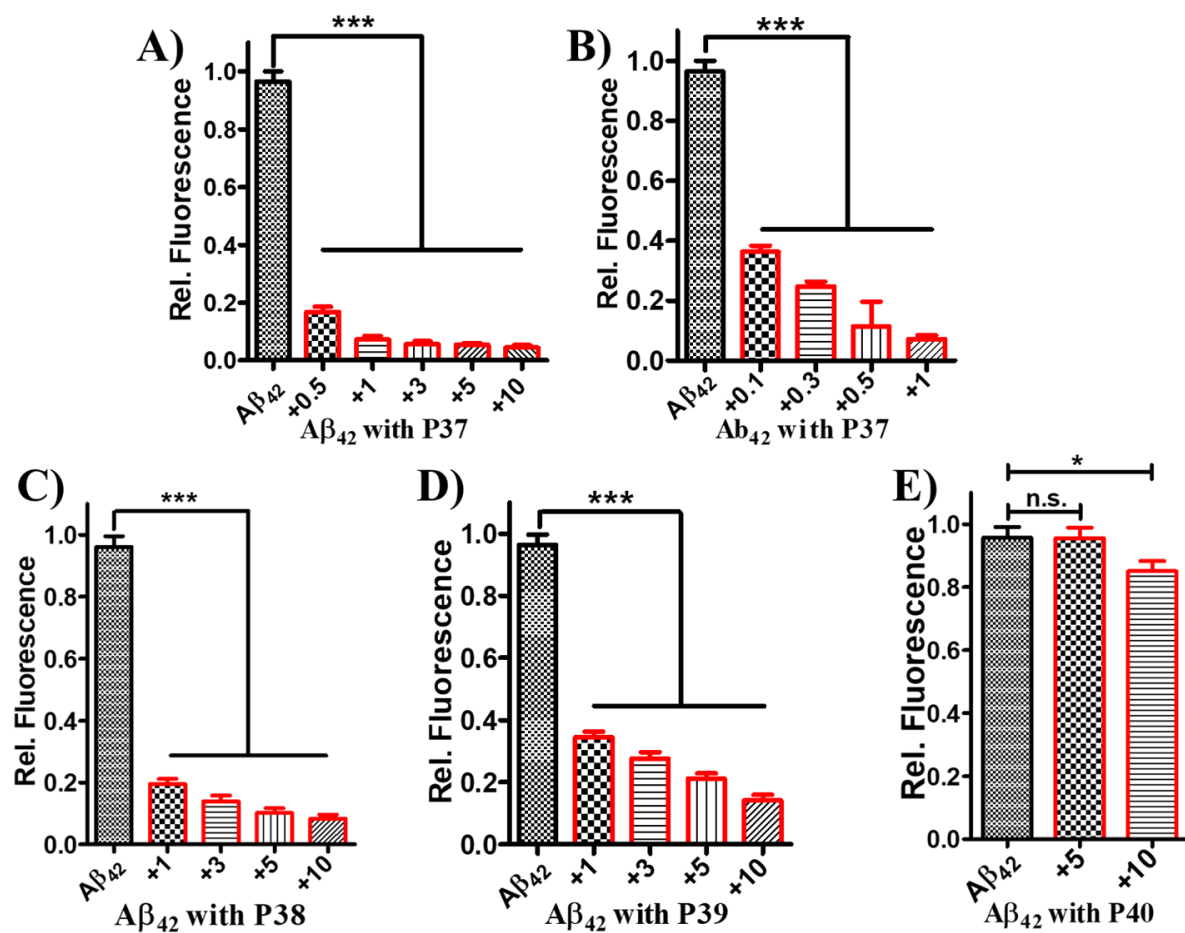


Figure 4: Effect of $\alpha\alpha\gamma$ -hybrid peptide foldamers **P37-P40** on the fibrillation of the Aβ₄₂ (25 μM). The Fibrillation of the Aβ₄₂ alone and in the presence of peptides in different concentrations by varying the time was monitored by measuring the ThT fluorescence. The peptide concentration varied from 2.5 μM, 7.5 μM, 12.5 μM, 25 μM, 75 μM, 125 μM and 250 μM. The time dependent ThT based kinetics of the Aβ₄₂ fibrillation in the presence of A) **P37**, B) **P37** with stoichiometric ratios, C) **P38**, D) **P39** E) **P40**. The 20 μM ThT concentration was used in all the experiments. The GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA) was used to analyse the data using one-way ANOVA and the *post hoc* Tukey's test for manifold comparisons ($p < 0.05$), n.s. = no significance.

ThT fluorescence emission (~5 %) is shown in Figure 1A. At 1:1 concentrations **P38** and **P39** with Aβ₄₂ showed 20% and 38% emission of ThT fluorescence compared to 100% emission in the absence of peptides. At increasing concentrations of both **P38** and **P39** (1:3, 1:5 and 1:10, Aβ₄₂: peptide) showed effective inhibition of Aβ₄₂ aggregation is shown in Figure 4C and 4D. Peptide **P40** exhibits no effect on the Aβ₄₂ aggregation even at higher concentrations is shown in Figure 4E. Based on ThT fluorescence kinetic analysis, the aggregation inhibition activity

of hybrid peptides was found to be **P37>P38>P39>P40**. Inspired by the very weak emission of ThT fluorescence at 1:1 equimolar concentrations of A β ₄₂ and **P37**, we further examined the aggregation inhibition activity of **P37** at lower concentrations. At 1:0.1 equivalent concentrations of A β ₄₂ and **P37**, it showed only 40% fluorescent emission of ThT. At 1:0.3 and 1:0.5 equivalent concentrations, we observed 25% and 10% fluorescent emission. At 1:1 concentrations we observed <5% fluorescent signal. Even at 0.1 equivalent of **P37** with respect to A β ₄₂ 60% reduction in the ThT fluoresce was observed. At 0.5 equivalent of **P37**, we observed a 90% reduction in the fluorescence signal is shown in Figure 4B. We further compared the activity of hybrid peptide **P37** with previously reported α -peptide **P33** at 1:0.5 and 1:1 equivalent concentrations with respect to A β ₄₂. Results are shown in Figure 5A. The hybrid peptide **P37** showed the same inhibition activity as compared to the α -peptide **P33**.

The kinetic parameters of ThT assay are tabulated in Table 1. The aggregation rate constant, half-time ($t_{1/2}$), and lag phase were extracted from the ThT assay.^{53,54} At 1:1 molar ratios of A β ₄₂ and **P37**, complete prevention of aggregation of A β ₄₂ was observed. In the absence of the peptide inhibitor, the aggregation of A β ₄₂ showed the $t_{1/2}$ 8.83 $\pm$ 0.15 h and the lag time 5.75 $\pm$ 0.19 h. At 1: 0.5 molar ratio of A β ₄₂ and **P37**, the $t_{1/2}$ and lag time reduced to 5.42 $\pm$ 0.40 h and 2.12 $\pm$ 0.35 h respectively. The reduction in the $t_{1/2}$ and lag phase suggested the onset prevention of aggregation of A β ₄₂. Similar results were also observed in the case of **P38** and **P39**. At 1:1 molar ratios of **P38** and A β ₄₂, we observed 5.92 $\pm$ 0.32 h of $t_{1/2}$ and 2.29 $\pm$ 0.39 h of lag time. In the case of **P39**, at a 1:1 molar ratio with A β ₄₂, the $t_{1/2}$ and lag time were reduced to 8.70 $\pm$ 0.29 h and 1.64 $\pm$ 0.11 h respectively. These results suggested the peptides prevented aggregation by interfering with the inception of A β ₄₂ aggregation. The ThT fluorescence based amyloid aggregation kinetic analysis clearly suggested that the **P37** is a better A β ₄₂ amyloid aggregation inhibitor compared to **P38**, **P39** and **P40**.

Table 1: ThT assay Kinetic Parameters [t_{50} (h), Lag time (h), k_{app} (h^{-1})] for the aggregation effect on A β ₄₂ in the presence of Peptides.

Sample	t_{50} (h)	Lag time (h)	k_{app} (h^{-1})
A β ₄₂ alone (25 μ M)	8.83 $\pm$ 0.15	5.7582 $\pm$ 0.19	0.6517 $\pm$ 0.009
A β ₄₂ + P37 (1:0.5)	5.42 $\pm$ 0.40	2.129 $\pm$ 0.35	0.6071 $\pm$ 0.008
A β ₄₂ + P38 (1:1)	5.92 $\pm$ 0.32	2.2958 $\pm$ 0.39	0.5520 $\pm$ 0.011
A β ₄₂ + P39 (1:1)	8.70 $\pm$ 0.29	1.6425 $\pm$ 0.11	0.2834 $\pm$ 0.009
A β ₄₂ + P40 (1:5)	13.29 $\pm$ 0.33	4.8209 $\pm$ 0.49	0.2361 $\pm$ 0.004

3B.3.3. Disruption of aggregated A β ₄₂ fibrils by $\alpha\gamma$ -hybrid peptides

Inspired by the inhibition of the aggregation of soluble A β ₄₂ by the hybrid peptides, we sought to investigate the effect of $\alpha\gamma$ -hybrid peptides on the aggregated fibrils of A β ₄₂. To understand the effect of hybrid peptides on the aggregated forms of A β ₄₂, we incubated A β ₄₂ along with ThT, and after complete saturation of ThT fluorescence (approximately 24 h) equimolar concentration of **P37** was added to the solution of A β ₄₂. We observed a steady decrease in the ThT fluorescence with time. In a control experiment, the buffer was added without A β ₄₂ and peptide. We observed no change in the ThT fluorescence. In another control experiment, incubation of soluble A β ₄₂, ThT and **P37** showed a weak fluorescent emission signal. The results are shown in Figure 5B. These results suggested that **P37** not only inhibits the aggregation of A β ₄₂, but also disintegrates the A β ₄₂ fibrils into smaller entities.

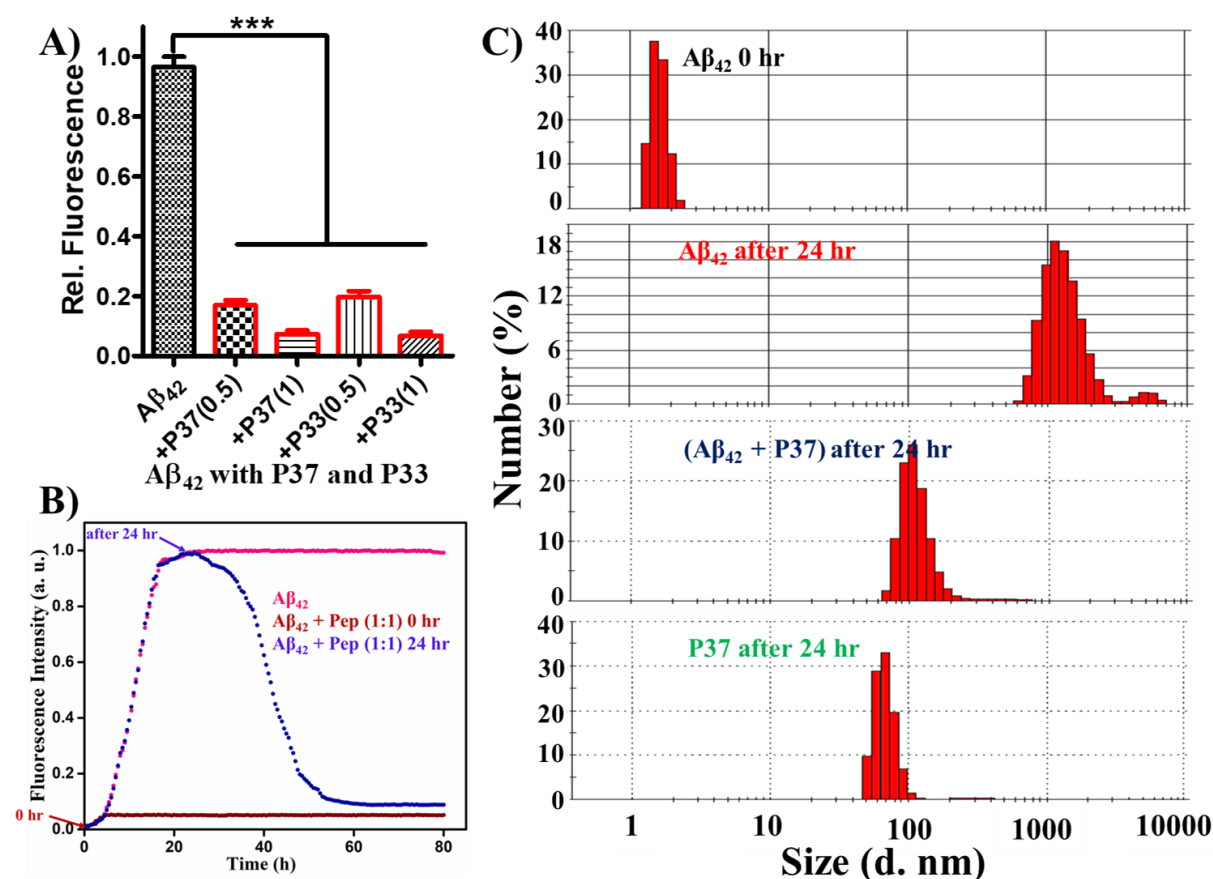


Figure 5: Effect of **P37** on the fibrillation of A β ₄₂. A) Comparison of the effect of **P37** and **P33** on the kinetics of the A β ₄₂ fibrillation and the GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA) was used to analyse the data using one-way ANOVA and the *post hoc* Tukey's test for manifold comparisons ($p < 0.05$). B) The fibrillation of the A β ₄₂ alone and in the presence of peptide **P37** (25 μ M) at different intervals of time. i) A β ₄₂ alone (pink line), ii) **P37**

to the 0 h incubated A β ₄₂ solution (brown line), iii) **P37** to the 24 h incubated A β ₄₂ solution (blue line). C) DLS study for size distribution of A β ₄₂ (10 μ M) in the presence and absence of Peptide **P37** at 37 °C.

3B.3.4. Dynamic light scattering studies

We invoked a dynamic light scattering (DLS) experiment to understand the effect of **P37** on the aggregation of soluble A β ₄₂. DLS experiments help us to understand the size of self-assembled macromolecules in solution and useful tools to investigate the aggregation of A β ₄₂ in the absence and presence of inhibitor peptide **P37**. At 0 h of incubation, soluble A β ₄₂ showed 3-4 nm-sized particles in solution. The size of the A β ₄₂ assembled particles increases to an average diameter of 1100 nm size after 24 h of incubation. In the presence of **P37** at equimolar concentrations, showed particle size with an average diameter of approximately 100 nm. Further, **P37** alone is self-assembled into 80 nm-sized particles. The results are shown in Figure 5C. The DLS experiments suggested that **P37** indeed inhibits the aggregation of A β ₄₂ into larger particles. At equal concentrations, both **P37** and A β ₄₂ aggregate into smaller particles. The DLS experimental results are in accordance with the ThT fluorescence kinetic analysis.

3B.3.5. HR-TEM analysis

To understand the morphology of A β ₄₂ in the absence and the presence **P37**, we used HR-TEM analysis. The aggregation of A β ₄₂ recorded after 24h incubation in the absence and presence of **P37**. The HR-TEM images are shown in Figure 6. In the absence of **P37**, entangled networks of A β ₄₂ fibrils were observed. No fibrils of A β ₄₂ were observed in the presence of **P37**. HR-TEM analysis suggested that **P37** prevents the self-aggregation of A β ₄₂.

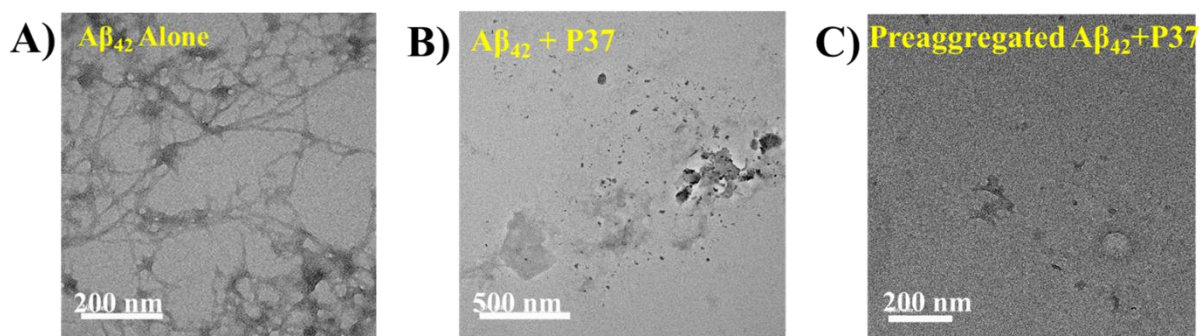


Figure 6: HR-TEM images of 10 μ M A β ₄₂ incubated up to 48 h at 37 °C A) in the absence of **P37** and B) in the presence of **P37**. C) Addition of **P37** after 24 h of the incubated A β ₄₂. No fibrils of A β ₄₂ was observed in the presence of **P37**.

3B.3.5. AFM Analysis

In addition to the HR-TEM analysis, the atomic force microscopy (AFM) was also invoked to understand the effect of **P37** on A β ₄₂ aggregation. The solution of A β ₄₂ (10 μ M) was incubated for 48 hours in the presence and the absence **P37** at an equimolar concentration and subjected to the AFM analysis. The fibrils of A β ₄₂ was observed after 48 hours of incubation in the absence of **P37**. In the presence of **P37**, no fibrils of A β ₄₂ observed. The AFM images of A β ₄₂ in the absence and presence of inhibitor peptide were shown in Figure 7A and Figure 7B, respectively. The AFM analysis suggested that **P37** effectively controls the aggregation property of A β ₄₂.

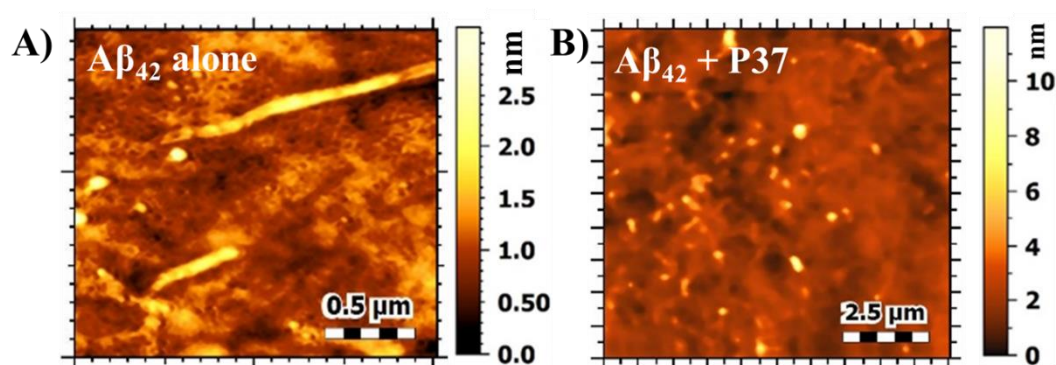


Figure 7: Effect of peptide **P37** on the kinetics of the self-assembly of A β ₄₂ with AFM images. Self-assembly of the 10 μ M A β ₄₂ was incubated at 37 °C up to 48 h. A) In the absence of peptide **P37**. B) In the presence of peptide **P37**. Buffer Conditions: 10 mM Sodium Phosphate, 150 mM NaCl, 1mM EDTA and pH 7.4.

3B.3.6. ELISA analysis

In order to test, whether the antibody can recognize the **P37** disrupted A β ₄₂ assemblies, we performed ELISA analysis on a commercially available ELISA kit coated with an antibody that recognizes the aggregated assemblies of A β ₄₂. The soluble A β ₄₂ was incubated in the absence and the presence of **P37** for 24 h. ELISA was performed after 24h of incubation as suggested by the supplier protocols. The results are shown in Figure 8. Results suggested that the antibody recognized the A β ₄₂ fibrils in the absence of **P37**, however in the presence of the **P37** antibody did not recognize the A β ₄₂. These results further supported that the peptide prevents the A β ₄₂ aggregations.

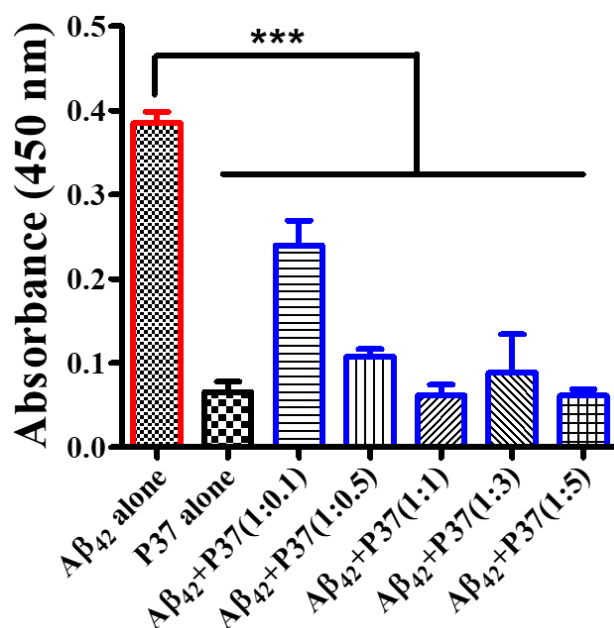


Figure 8: Mitigation of Aβ₄₂ aggregation was confirmed by ELISA assay using the monoclonal antibody specific for the N-terminus of human Aβ. Aβ₄₂ samples were incubated at 37 °C up to 24 h in the absence and presence of peptide **P37** with different concentrations. Data was plotted after subtracting from the chromogen blank. Error bars shown here represents an average of three independent triplicate values and the GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA) was used to analyse the data using one-way ANOVA and the *post hoc* Tukey's test for manifold comparisons ($p < 0.05$).

3B.3.7. Conformational Analysis by Circular Dichroism

Encouraged by the aggregation inhibition activity of **P37**, we sought to investigate its influence on the conformations of Aβ₄₂ using circular dichroism. The CD spectra of soluble Aβ₄₂ and **P37** were shown in Figure 9. The soluble Aβ₄₂ showed a random coil structure. Similarly, hybrid peptides showed no proper secondary structure. After 24h incubation, Aβ₄₂ transformed into β-sheet type structure in the absence of **P37**. Interestingly, incubation of 1:1 equivalent concentrations of Aβ₄₂ and **P37**, displayed a new CD spectrum resembling the helix structure. It is remarkable to notice that individually both Aβ₄₂ and **P37** showed different CD signature, however, upon mixing 1:1 concentration, they showed helix-type CD spectrum. The CD analysis revealed that **P37** induces a conformational reorganization in Aβ₄₂ and possibly transformed it into a helix-type structure.

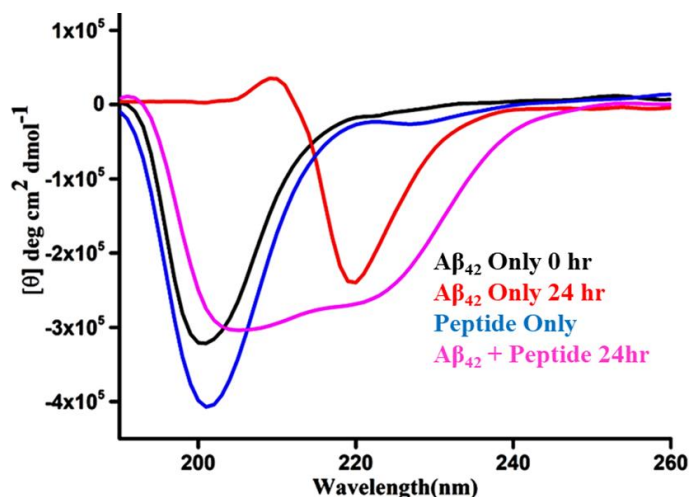
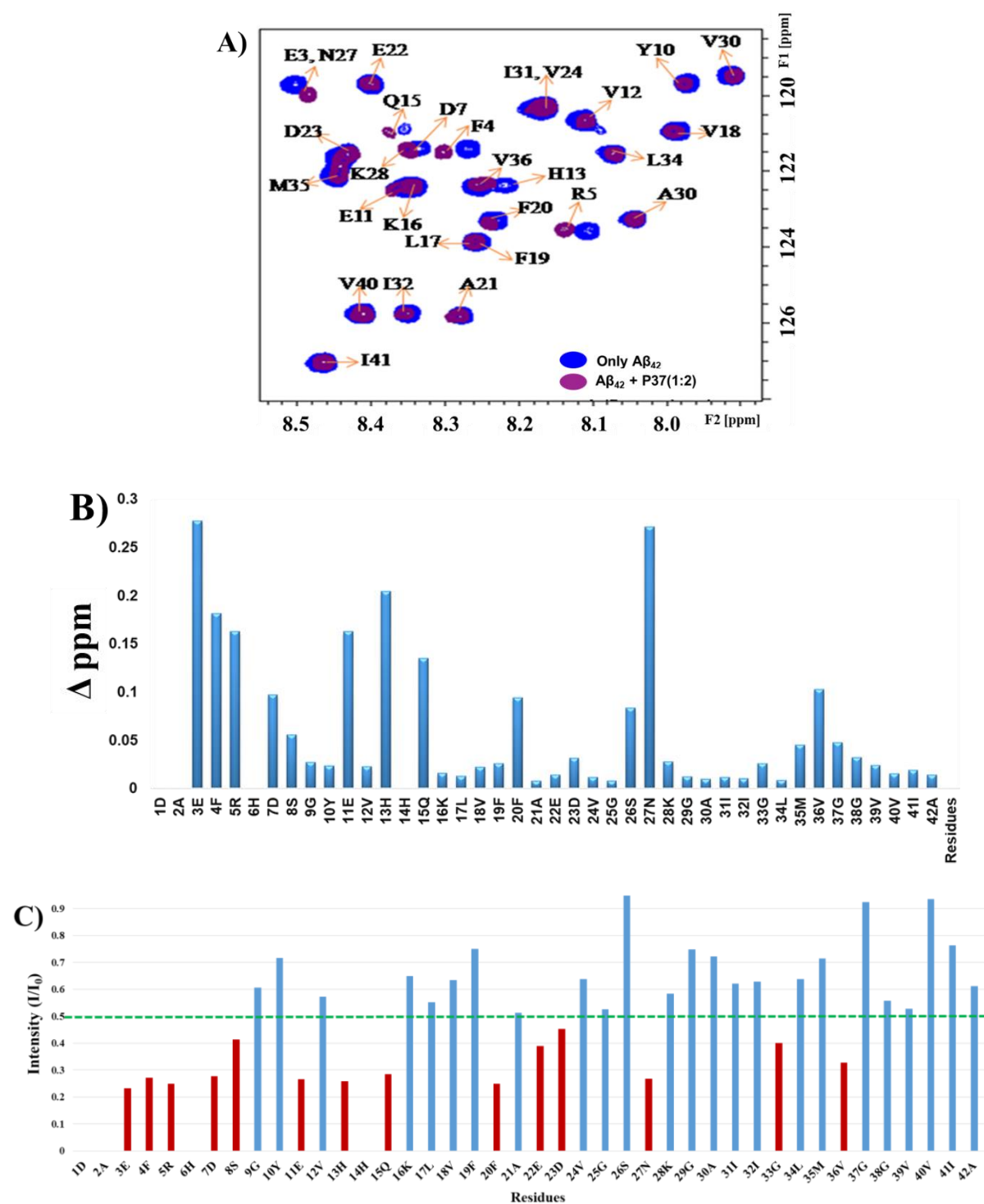


Figure 9: The CD spectra of A β_{42} (50 μ M) in the presence and absence of peptide **P37**. A β_{42} without incubation (black line), with 24 h incubation (red line), and 24 h incubation of peptide **P37** alone (blue line) and also A β_{42} with Peptide **P37** (50 μ M) with 24 h incubation (pink line).

3B.3.8. 2-Dimentional NMR analysis

In order to understand the interaction of **P37** with A β_{42} , we invoked heteronuclear single quantum correlation (HSQC) experiments using N¹⁵ labelled A β_{42} in the absence and presence of **P37**. The overly of the partial HSQC spectra of A β_{42} in the absence and the presence of **P37** is shown in Figure 10A. The inspection of HSQC revealed a large perturbation in the amide NH chemical shifts of A β_{42} in the presence of **P37**. Mostly the N-terminal residues 3E, 4F, 5R, 7D, 11E, 13H, 15Q, 20F and 27N showed a measurable change in their chemical shifts after the addition of **P37** is shown in Figure 10B. Additionally, we observed perturbation in amide NH of C-terminal residue 36V. Though out of six anionic residues in A β_{42} , three residues are strongly interacting with the **P37**, the threshold of cationic and hydrophobicity is required for the inhibition of A β_{42} aggregation. In addition to the change in the chemical shifts, we showed the decreased intensity (I/I_0) by measuring the cross-peak intensity of the labelled A β_{42} alone from the HSQC spectrum was measured (I_0), as well as the intensity of the same cross-peak from the spectrum of labelled A β_{42} in the presence of unlabelled peptide **P37** (I). The results were studied by constructing the bar diagram shown in Figure 10C. We also measured the change in the peak volume of A β_{42} residues upon interaction with **P37** by subtracting the cross-peak volume from the HSQC spectra of the labelled A β_{42} alone (V_0) with the volume of the same cross-peak from the spectrum of the labelled A β_{42} in the presence of unlabelled peptide **P37** (V) and the results was analyzed by plotting the bar diagram shown in Figure 10D. The

eight residues 3E, 4F, 5R, 7D, 11E, 12V, 13H and 15Q at the N-terminus, 20F from the central hydrophobic core, two residues 23D, 27N from the central polar region and 36V from the C-terminal hydrophobic region showed decreased intensity and change in the volume upon interaction with the **P37**. The analysis of HSQC experiments suggested that peptide induces a conformational change in A β_{42} after interacting with it. The results of NMR analysis support the results observed in the circular dichroism experiments.



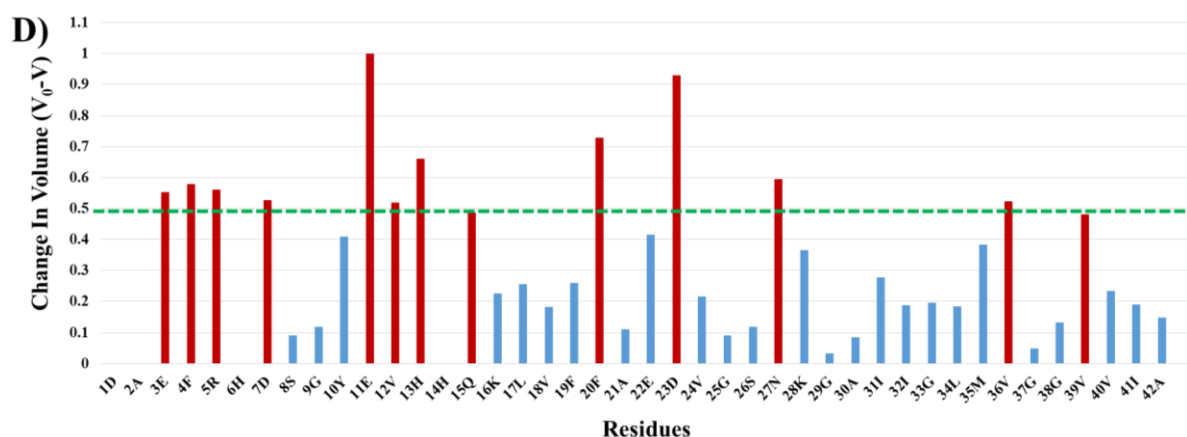


Figure 10: Effect of Peptide **P37** on the conformational structure of A β ₄₂. A) Superimpose of ¹⁵N-A β ₄₂ (40 μ M) ¹H-¹⁵N HSQC NMR spectra without peptide **P37** (blue colour) and with peptide **P37** with 1:2 stoichiometric ratio of (A β ₄₂ : **P37**) (purple colour). B) The chemical shift perturbation of ¹⁵N-A β ₄₂ induced from the Peptide **P37** with 1:2 stoichiometric ratio of (A β ₄₂ : Peptide **P37**). C) The reduced intensity (I/I₀) of ¹⁵N-A β ₄₂ induced from the Peptide **P37** with 1:2 stoichiometric ratio of (A β ₄₂ : Peptide **P37**). The residues which are highlighted by brown colour have more interaction with peptide. D) The Change in peak volume of ¹⁵N-A β ₄₂ induced from the Peptide **P37** with 1:2 stoichiometric ratio of (A β ₄₂ : Peptide **P37**). Change In Volume $\Delta V = (V_0 - V)$, the residues which are highlighted by brown colour having more interaction with peptide. The NMR experiments were carried out in sodium phosphate buffer, pH 7.4 at 283 K using cryo probe attached 800 MHz spectrometer.

3B.3.9. Cytotoxicity Analysis

Encouraged by the structural reorganization of A β ₄₂ from the hybrid peptide, we sought to investigate the cytotoxicity using the MTT (3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) of **P37** treated A β ₄₂, soluble A β ₄₂ without the inhibitor and **P37** alone against the SHSY5Y human neuroblastoma cell lines. The cells without A β ₄₂ were used as a positive control. To understand the toxicity of A β ₄₂ on the neuronal cells, we incubated the cells with 10 μ M A β ₄₂. To check whether **P37** has any impact on the toxicity of A β ₄₂, cells were incubated with the combination of 10 μ M A β ₄₂ along with increasing concentrations of hybrid peptide **P37** from 1 μ M to 30 μ M at 37 °C. To check the toxicity of hybrid peptides, we incubated the cells with increasing concentrations of **P37** starting from 1 μ M to 30 μ M. In addition, To check the toxicity of **P38** and **P39**, cells were incubated with the 30 μ M concentration of peptides **P38** and **P39**. After 24 h of incubation, the cells treated with A β ₄₂

alone showed only 25% survivability of cells compared to the cells without A β_{42} , suggesting the toxicity of A β_{42} to the cells. Further, Cells treated with only peptides (up to 30 μ M) displayed no toxicity to the cells. Results are shown in Figure 11A. Remarkable results were observed from the cells treated with a combination of A β_{42} and **P37**. With increasing concentrations of hybrid peptide, A β_{42} displayed reduced toxicity to the neuronal cells. No toxicity of A β_{42} was observed at 0.5 or above an equivalent concentration of **P37** with respect to A β_{42} . Results are shown in Figure 11C. To understand the effect of all three hybrid peptides **P37-P39**, we incubated the cells with 1:1 concentrations of peptides with A β_{42} . The cytotoxicity results are shown in Figure 11B. We observed no effect of A β_{42} at 1:1 concentration with **P37**. In comparison to other peptides, **P37** showed better activity. These results suggested that hybrid peptide **P37** rescued the neuroblastoma cells from the A β_{42} toxicity (10 μ M) with an IC₅₀ value of 2.5 μ M (Figure 11D).

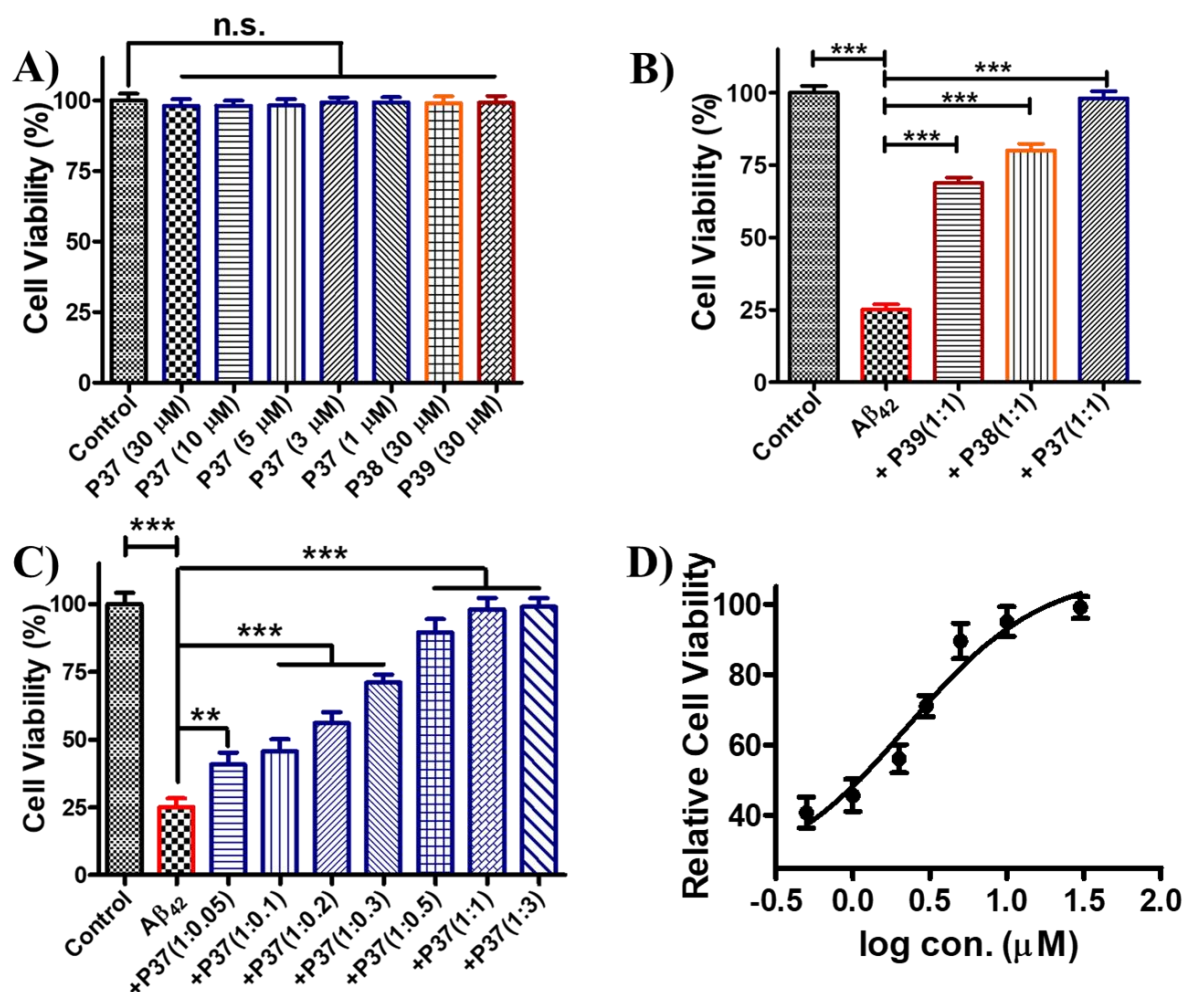


Figure 11: Survival of neuroblastoma (SHSY5Y) cells with A β ₄₂ in the presence and absence of Peptides **P37-P39** by incubating the cells at 37 °C for about 24 h. A) Cells were treated with Peptides alone (**P37-P39**) at different concentrations. B) Cells were treated with A β ₄₂ with an equimolar ratio (1:1) in the presence and absence of peptide **P37-P39**. The results of the cell viability was compared the cells without the treatment of A β ₄₂ and peptides. C) Toxicity of A β ₄₂ (10 μ M) to SHSY5Y cells in the absence and presence of peptide **P37** with increasing the concentrations (0.5 μ M, 1 μ M, 2 μ M, 3 μ M, 5 μ M, 10 μ M and 30 μ M). D) Effect of Peptide **P37** on the toxicity induced by A β ₄₂ (10 μ M) in SHSY5Y cells by Dose-dependent. The GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA) was used to analyze the data using one-way ANOVA and the *post hoc* Tukey's test for manifold comparisons ($p < 0.05$), n.s. = no significance.

3B.3.10. Proteolytic stability

As $\alpha\gamma$ -hybrid peptide shows remarkable activity against the aggregation of A β ₄₂ and also saves the cells from the toxicity of A β ₄₂, Further, we examined its proteolytic stability using protease trypsin. The α -peptide **P33** was used as a control. The stability of the peptides against trypsin with time was monitored using RP-HPLC analysis. The pure peptides **P37** and **P33** were incubated with trypsin in an Tris-buffer and examined the stability with time by passing through a C18-column using reverse-phase HPLC. The time-dependent HPLC traces of both peptides are shown in Figure 12. Within 15 min of incubation, we observed a reduction in the size of the **P33** peak and the appearance of other peaks. At 120 min, we observed an almost complete reduction in the size of the **P33** peak. By 12 h, we observed almost complete vanishing of the **P33** peak. In sharp contrast, no reduction in the size of the **P37** peak with time was observed, In addition no other peaks of **P37** were observed even after 24 h incubation. The $\alpha\gamma$ -peptide was found to be stable against the protease trypsin, these hybrid peptides may serve as potential therapeutic agents.

The $\alpha\gamma$ -hybrid peptide displayed a distinctive path in arresting the activity of A β ₄₂. The ThT-based fluorescence analysis revealed that the peptide inhibits the aggregation of soluble A β ₄₂ and also disintegrates the aggregated fibrils of A β ₄₂ into smaller assemblies. This was further supported by the DLS analysis. In addition, a clear difference was observed in the aggregation of A β ₄₂ in the absence and the presence of **P37**. Further, the CD and 2D NMR HSQC experiments suggested that peptide induces a conformational reorganization upon interacting with A β ₄₂. The **P37** induced new conformation of A β ₄₂ has shown less tendency to form larger

fibrils and also showed no toxicity to the neuronal cells. More importantly, **P37** disintegrates the aggregated fibrils of A β ₄₂ into nontoxic smaller entities.

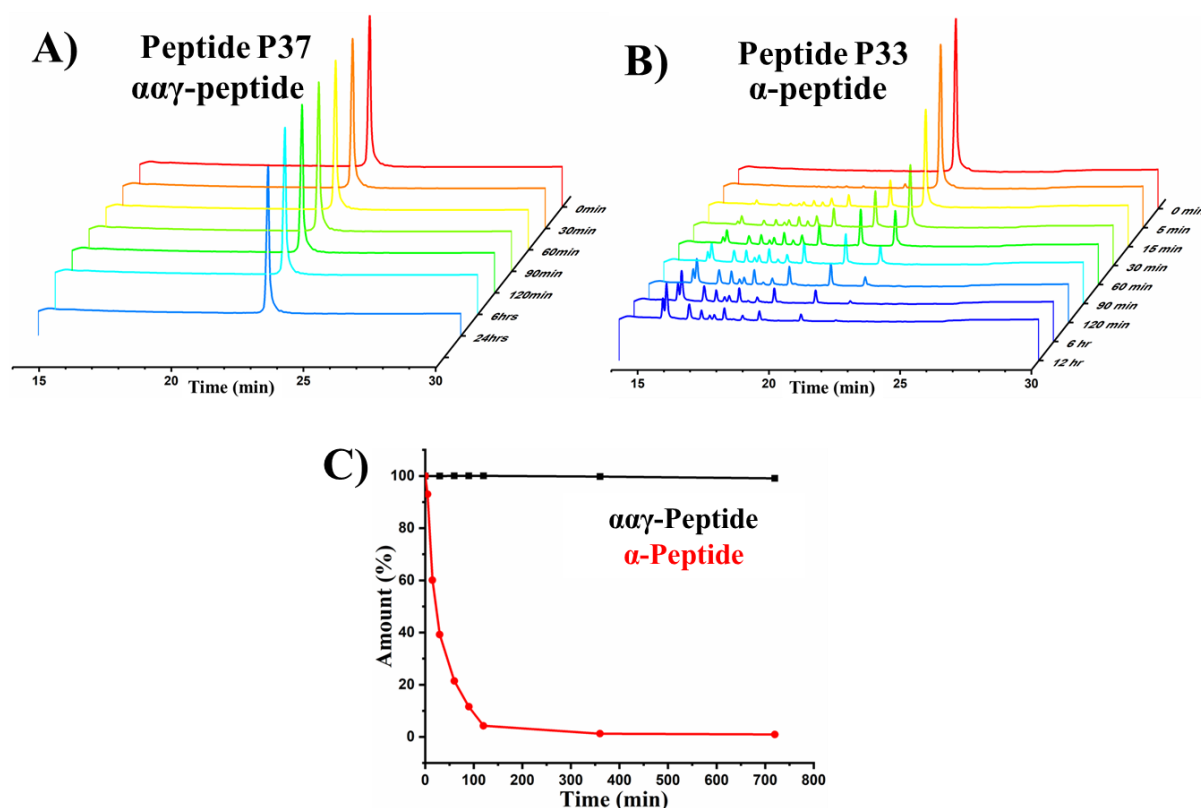


Figure 12: Proteolysis of A) Peptide **P37** and B) Control Peptide **P33** by treating with trypsin. C) Time dependent tryptic digestion of **P37** and **P33**. Peptide **P37** is stable even after 12 h.

3B.4. Conclusions

Here we demonstrated the inhibition of aggregation of soluble A β ₄₂ by designing 10-mer, 12-mer and 15-mer $\alpha\alpha\gamma$ -hybrid peptides. Among the three, the 15-mer hybrid peptide showed better aggregation inhibition activity compared to other deca- and dodeca- peptides. Further, the 15-mer peptide disintegrated the aggregated assemblies of A β ₄₂ into smaller entities. At equimolar concentration with A β ₄₂, the hybrid peptide saved the cells from the toxicity of A β ₄₂. The cytotoxicity experiments also suggested that hybrid peptides are nontoxic to the cells. In contrast to the α -peptide counterpart, the $\alpha\alpha\gamma$ -hybrid peptide showed stability against the protease trypsin. The CD and NMR analysis revealed that upon interaction the peptide induces a conformational change in A β ₄₂, and this conformation is less prone to self-aggregation and also nontoxic to the cells. Overall, the proteolytically stable, non-cytotoxic $\alpha\alpha\gamma$ -hybrid peptide showed that the inhibition of aggregation of soluble A β ₄₂, disintegrate the fibrils of A β ₄₂, and

also attenuate the toxicity of A β ₄₂. The results reported here can be explored to design new therapeutics for the Alzheimer's disease.

3B.5. Experimental Section

3B.5.1. Materials and Method

All reagents, fluorescent-dyes, enzymes and chemicals used were purchased from commercial suppliers with good purity grade and used without any further purification. The flat bottom polystyrene 96-well plates and Human Aggregated Beta Amyloid ELISA Kit were purchased from Invitrogen Bio services. Rink Amide Resin, all N-Fmoc protected amino acids and A β ₄₂ peptide (>95% purity) were purchased from GL Biochem, China. ¹⁵N-Labeled A β ₄₂ was procured from r-Peptide (Bogart, GA) and used directly in the NMR experiments. Tris-buffer was prepared using 10 mM Tris-base, 150 mM NaCl and 0.1 mM EDTA and pH was adjusted to 8.2 by adding either dil. HCl or NaOH. Sodium phosphate buffer was prepared using 10 mM sodium phosphate with 150 mM NaCl and 1 mM EDTA and pH was adjusted to 7.4 by adding either dil. HCl or NaOH. All dry solvents, reagents, Trypsin, Thioflavin T, Rhodamine-B and antibiotics were obtained from Sigma-Aldrich.

3B.5.2. Solid Phase Peptide Synthesis and HPLC Purification

The N-acetylated peptides **P37-P40** were synthesized through a manual solid phase peptide synthesis on Rink amide resin at 0.2 mmol scale using standard Fmoc- conditions. The combination of HBTU and HOBT was used as coupling reagents, DIPEA was used as an activator base, NMP was used as a coupling solvent and 20% piperidine in DMF was used for the Fmoc- group deprotection. To release the peptides from the solid support, the resin was stirred for about 3 h in a 10 mL cocktail containing trifluoroacetic acid (TFA)/triisopropylsilane/water/phenol (90:5:5:5). The resin was filtered. The filtrate containing crude peptides was evaporated under reduced pressure and the gummy residue was triturated with cold diethyl ether (50 mL). The precipitate was separated through centrifugation. The crude peptides were again dissolved in 10 mL of ACN/H₂O and purified on a C₁₈ column using reversed-phase HPLC. Acetonitrile and water gradient system containing 0.1% of TFA at a 2 ml flow rate was used to purify the peptides. The mass of the purified peptides was confirmed by MALDI-TOF/TOF analysis. The lyophilised pure peptides were used for further studies.

3B.5.3. β -Amyloid Disruption Study

3B.5.3.1. Preparation of the Amyloid Beta₄₂

The A β ₄₂ (2mg) was dissolved in 2 ml of 1,1,1,3,3,3- hexafluoroisopropanol (HFIP) and then kept for occasional vortex at room temperature for 1 h to maintain in a monomeric state. After this, it was aliquoted into small fractions (0.5 mg/ample) and HFIP was evaporated from the sample by keeping sample vials overnight in a laminar flow hood. The sample vials containing A β ₄₂ were then lyophilized overnight and stored at -80 °C until further use. Before the usage, the lyophilized A β ₄₂ was dissolved in DMSO to make the 2 mM stock solutions of monomeric A β ₄₂ in DMSO. The A β ₄₂ aggregates were prepared by the addition of sodium phosphate buffer (10 mM) to the stock solution of A β ₄₂ in DMSO. The final volume of the A β ₄₂ solution was maintained at 100 μ L and incubated at 37 °C. The fibrillation of the A β ₄₂ with the appropriate concentrations of peptides were studied in sodium phosphate buffer (10 mM, pH 7.4) and the final volume was maintained at 100 μ L. The concentration of A β ₄₂ was kept at a constant 25 μ M in all experiments.

3B.5.3.2. Thioflavin T (ThT) Fluorescence-Based Kinetic Assay

The aggregation kinetics of A β ₄₂ were studied using a ThT assay. The measurements of ThT based fluorescence were carried out on flat bottom black polystyrene nonbinding 96-well plates. The samples were excited at 440 nm and fluorescence emission was monitored at 482 nm. The stock solution of A β ₄₂ (0.55 mM) was prepared in DMSO and it will be appropriately diluted with sodium phosphate buffer before starting the aggregation kinetic experiments. The stock solution of ThT (200 μ M) in 10 mM sodium phosphate buffer (pH 7.4) was prepared. The ratio for ThT to A β ₄₂ was maintained at 20:25 μ M, respectively. The control assay was performed by the addition of A β ₄₂ (4.5 μ L) to the ThT (10 μ L) in sodium phosphate buffer (10 mM) and the volume was adjusted to 100 μ L using the buffer. For the inhibition kinetics assay, different concentrations of peptides were incubated with the wells containing A β ₄₂ (4.5 μ L) and ThT (10 μ L). The final concentration of the peptides in the wells was maintained at 250, 125, 75, 25, 12.5, 7.5, and 2.5 μ M. The solution containing A β ₄₂, ThT and peptides was incubated for about 48 h at 37 °C and subjected to the fluorescence measurements with orbital shaking for 120 s before taking the each reading. Measurements were performed in three independent experiments. Kinetic graphs were plotted using Origin (version 9.1). Kinetic Parameters t_{50} (h), Lag time (h), k_{app} (h^{-1}) were calculated from the ThT assays.^{48,49}

3B.5.3.3. Circular Dichroism Spectroscopy

Circular dichroism spectroscopy analysis was carried out using the Jasco J-815 spectropolarimeter. For CD experiments, the stock solution of A β ₄₂ (0.55 mM in 10mM NaOH) was further diluted to 50 μ M in sodium phosphate buffer (10 mM). The sodium phosphate buffer without peptides and A β ₄₂ was used as a blank. The 50 μ M concentrations of both A β ₄₂ and **P37** were used for CD measurements. The CD spectra of **P37** alone, A β ₄₂ alone and the mixture containing both **P37** and A β ₄₂ at 0 h and 24 h of incubation were recorded in triplicate at 37 °C.

3B.5.3.4. High Resolution Transmission Electron Microscopy (HR-TEM) Analysis

100 μ M A β ₄₂ was incubated (i) alone and (ii) in the presence of peptide with an equimolar ratio for about 48 h in sodium phosphate buffer. The stock solutions were prepared as mentioned above. Aliquots of all the samples were diluted 10 times with water. From each sample, 1 μ L of the aliquots were drop casted onto glow discharged individual carbon coated 200 mesh Cu grids (Ted Pella Inc., Redding, CA). Further, the grids were negatively stained with 2% uranyl acetate (Polysciences, Inc, Warrington, PA) in double distilled water and air dried. Stained grids were examined under Bruker High-Resolution Transmission Electron Microscope to take the final images.

3B.5.3.5. DLS Study

The solution of A β ₄₂ (100 μ M) in 10 mM sodium phosphate buffer (pH 7.4) was prepared as mentioned above and incubated (i) alone and (ii) in the presence of peptide with an equimolar ratio for about 24 h. The stock solutions were prepared as mentioned above. Aliquots of all the samples were diluted 10 times with water. The diameter of the self-assembly of A β ₄₂ was plotted against the size distribution.

3B.5.3.6. AFM Analysis

The solution of A β ₄₂ (100 μ M) was incubated (i) alone and (ii) in the presence of peptide with an equimolar ratio for 48 h. The stock solutions were prepared as mentioned above. The aliquots of all the samples were diluted 10 times with water. From each sample, 1 μ L of the aliquots were drop casted on a cleaned silicon wafer plate (SiO₂/Si substrate), dried at room temperature and finally kept in the desiccator for 10 min before taking the images. Freshly prepared solutions were used in all the experiments. The drop casted samples on silicon wafer plates were examined under Keysight's AFM instrument 5500 to take the final images.

3B.5.3.7. Cell Viability Assay

SHSY5Y-human neuroblastoma cells were cultured in DMEM (Dulbecco's modified essential medium)/F12 (Sigma, USA) medium in humidified 5% (v/v) CO₂/air at 37 °C in 10% (v/v) FBS (fetal bovine serum) (Invitrogen, USA) and 100 U/mL penicillin. After reaching ~95% confluence, cells were split by using 0.25% trypsin-EDTA (Sigma) into fractions to carry out the experiment. We seeded 10⁴ cells in each well in 96-well plates and incubated at 37 °C up to 24 h.

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) experiments were performed on SH-SY5Y cell line as reported earlier.^{40,41} Briefly, after 24 h incubation of cells at 37 °C in a 96-well plate, the medium was removed and further cells were incubated with the solution containing 10 µM Aβ₄₂ aggregates diluted in DMEM/F12 medium. To check the effect of **P37** on the cytotoxicity of Aβ₄₂ aggregates, 10 µM Aβ₄₂ was mixed with 1, 3, 5, 10, and 30 µM concentrations of **P37**, 10 µM **P38** and 10 µM **P39** and incubated at 37 °C up to 12 h prior to treatment of cells. The cells were treated with the mixture of Aβ₄₂ containing different concentrations of peptides **P37-P39** and the plates were incubated at 37 °C in humidified 5% (v/v) CO₂/air. The MTT reduction assay was performed after 24 h of treatment of the peptides and Aβ₄₂. After the addition of MTT (0.5 mg/mL), the cells were incubated at 37 °C for about 4 h in a CO₂ incubator. Further, the supernatant was removed from each well and DMSO (200 µL) was added to solubilize the dye formazan. The plates were again incubated in a humidified CO₂ incubator for about 15–20 min. The absorbance was measured at 570 nm using the multi-mode microplate reader. The cell viability was compared to the cells without peptides and Aβ₄₂. The Aβ₄₂ without peptides was used as a positive control. Similarly, the cytotoxicity of peptides was also examined without Aβ₄₂. Each data point represents an average of three independent triplet-well trials.

3B.5.3.8. ¹H-¹⁵N HSQC NMR Spectroscopy

The experiments of ¹H-¹⁵N HSQC NMR were performed on an 800 MHz Bruker instrument. The monomeric ¹⁵N labelled Aβ₄₂ was prepared as described in the above experiments. The NMR experiments were performed by maintaining the solution ratio of 9:1 (H₂O:D₂O) with 20 mM NaPi (pH 7.4). In each experiment, ¹⁵N- Aβ₄₂ sample was prepared freshly to maintain Aβ₄₂ in the monomeric native form to avoid amyloid formation. The stock solution of **P37** (10 mM) was prepared in Milli Q H₂O. The fresh sample of ¹⁵N-Aβ₄₂ (40 µM) ¹H-¹⁵N HSQC NMR experiments were performed using NaPi buffer (20 mM) having pH 7.4 in the absence

and presence of 2 equivalents of **P37** at 283K with a cryoprobe ($A\beta_{42}$:peptide **P37**; 1:2). Previously assigned 1H - ^{15}N HSQC NMR spectra of ^{15}N - $A\beta_{42}$ from the literature were used to identify all cross peaks.^{41,50,51} The addition of peptide **P37** to the ^{15}N - $A\beta_{42}$ sample was less than 2% dilution and the dilution factor was corrected while calculating the perturbation in the chemical shifts of 1H - ^{15}N resonances. The NMR spectra were processed and analyzed by using TOPSIN software. Chemical shift perturbations of all the residues (1H - ^{15}N) were determined by using the equation $\Delta_{ppm} = [(5X\Delta^1H)^2 + (\Delta^{15}N)^2]^{1/2}$. The decreased intensity (I/I_0) was determined by measuring the cross-peak intensity of the labelled $A\beta_{42}$ alone from the HSQC spectrum was measured (I_0), as well as the intensity of the same cross-peak from the spectrum of labelled $A\beta_{42}$ in the presence of unlabelled peptide **P37** (I). Change in the peak volume of the amino acid residues were determined by subtracting the cross-peak volume from the HSQC spectra of the labelled $A\beta_{42}$ alone (V_0) with the volume of the same cross-peak from the spectrum of the labelled $A\beta_{42}$ in the presence of unlabelled peptide **P37** (V), ($\Delta V = V_0 - V$).

3B.5.3.9. ELISA Assay

Human Aggregated Beta Amyloid ELISA Kit was procured from Invitrogen Bioservices. The ELISA assay was performed according to the manufacture's protocol. Briefly, to the N-terminus human $A\beta$ -specific monoclonal antibody-coated wells, 100 μ L of the $A\beta_{42}$ samples alone and in the presence of **P37** were added and further, incubated for about 2 h at room temperature. Each sample was prepared by incubating the 0.60 nM of $A\beta_{42}$ alone and in the presence of peptide **P37** by varying the peptide concentration from 0.5 to 10 equivalent ratio at 37 °C up to 24 h. The samples were diluted by 1:2 in diluent buffer (contains phosphate buffer saline with bovine protein as a carrier and 0.5% Proclin™ biocide as a preservative) before being added to the 96-well plate. After 2 h from the addition of the $A\beta_{42}$ samples, the wells were washed four times with Tris-based wash buffer containing 0.05% Tween-20 pH 7.4 (PBST) followed by the addition of 100 μ L of Hu Aggregated $A\beta$ Biotin Conjugate solution into each well. The samples were incubated for about 1 hour at room temperature. After washing the wells four times with PBST, the wells were treated with 100 μ L of Streptavidin-HRP (horseradish peroxidase-labelled streptavidin) and incubated for about 30 min at room temperature. After washing the wells with PBST, the solution of tetramethylbenzidine (TMB) was added and incubated for about 30 min at room temperature in the dark. The reaction was stopped by adding 100 μ L of stop solution (0.16 M sulfuric acid) to each well. The absorbance of each well at 450 nm was recorded using multi-mode microplate reader. Experiments were performed in triplicate and averaged.

3B.5.4. Proteolytic Resistance of Peptide P37 to the Trypsin hydrolysis

1 mg of each Peptide was dissolved in 1 mL of Tris-buffer (50 mM, pH 8.2). Here we used 0.05% trypsin in EDTA solution which we procured from sigma to determine the proteolytic resistance of peptides. In this study to check the stability of peptide, we took 200 μ L of peptide solution from the main stock solution (1 mg/mL) which we prepared earlier, to this 2 μ L of 0.05% trypsin in EDTA were added and incubated at 37 $^{\circ}$ C. Further 25 μ L aliquots of incubated solution were taken at different time intervals [0, 30, 60, 90, 120, 360 and 1440 min, respectively] to this 25 μ L of Tris-buffer (50 mM) was added for further dilution then stability of the peptide against trypsin hydrolysis was analysed through reverse phase HPLC with ACN/H₂O as a mobile phase.⁵²

3B.5.5. Statistical Analysis

The GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA, USA) was used to carry out the statistical analysis. One-way ANOVA with Tukey's *post hoc* multiple comparisons test was used to analyse comparisons between three or more groups. *P* values < 0.05 were considered significant.

3B.6. ThT assay

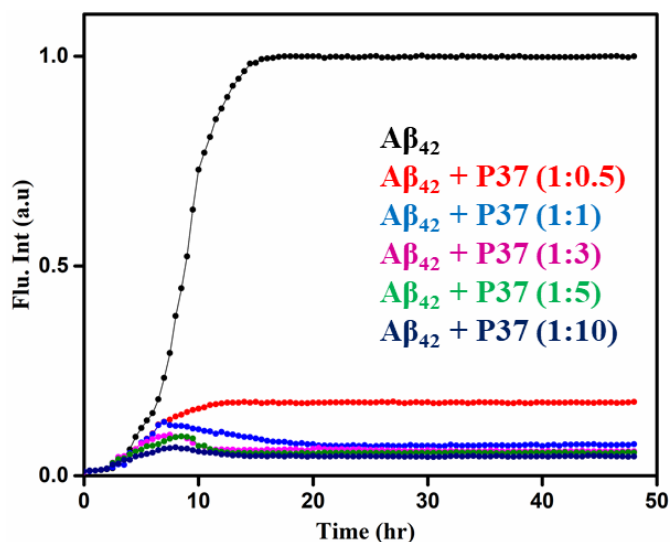


Figure 13: Kinetic study of the fibrillation of the A β_{42} with **P37**. The normalized kinetic profile of the self-assembly of 25 μ M of A β_{42} in the absence and presence of peptide **P37** by varying the concentration (12.5 μ M, 25 μ M, 75 μ M, 125 μ M and 250 μ M). Buffer Conditions: 10 mM Sodium Phosphate, 150 mM NaCl, 1mM EDTA and pH 7.4. ThT concentration was 20 μ M.

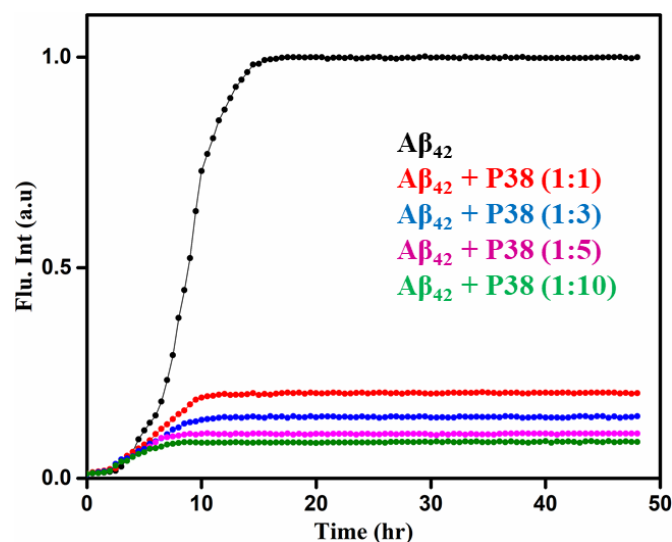


Figure 14: Kinetic study of the fibrillation of the $A\beta_{42}$ with peptide **P38**. The normalized kinetic profile of the self-assembly of 25 μM of $A\beta_{42}$ in the absence and presence of peptide **P38** by varying the concentration (25 μM , 75 μM , 125 μM , and 250 μM). Buffer Conditions: 10 mM Sodium Phosphate, 150 mM NaCl, 1mM EDTA and pH 7.4. ThT concentration was 20 μM .

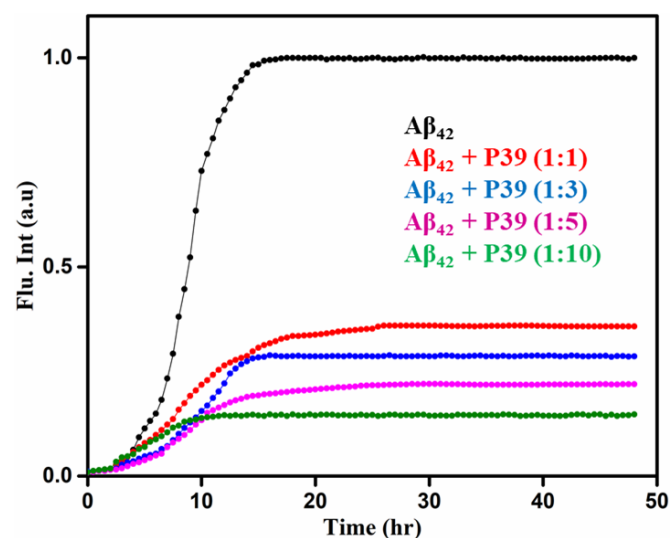


Figure 15: Kinetic study of the fibrillation of the $A\beta_{42}$ with peptide **P39**. The normalized kinetic profile of the self-assembly of 25 μM of $A\beta_{42}$ in the absence and presence of peptide **P39** by varying the concentration (25 μM , 75 μM , 125 μM , and 250 μM). Buffer Conditions: 10 mM Sodium Phosphate, 150 mM NaCl, 1mM EDTA and pH 7.4. ThT concentration was 20 μM .

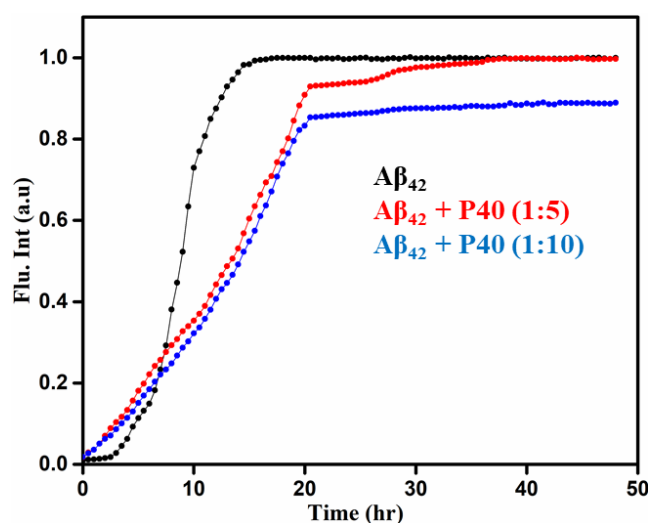


Figure 16: Kinetic study of the fibrillation of the A β_{42} with peptide **P40**. The normalized kinetic profile of the self-assembly of 25 μ M of A β_{42} in the absence and presence of peptide **P40** by varying the concentration (125 μ M, and 250 μ M). Buffer Conditions: 10 mM Sodium Phosphate, 150 mM NaCl, 1mM EDTA and pH 7.4. ThT concentration was 20 μ M.

3B.7. References

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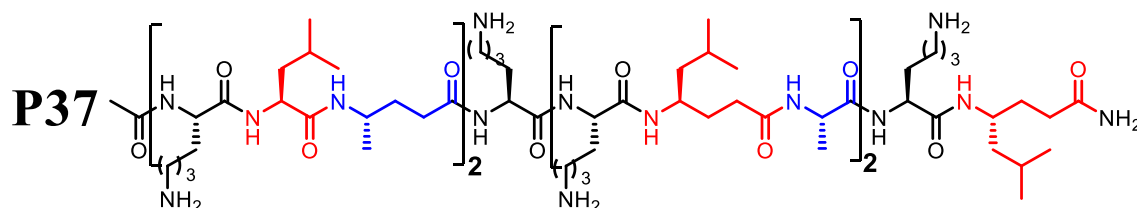
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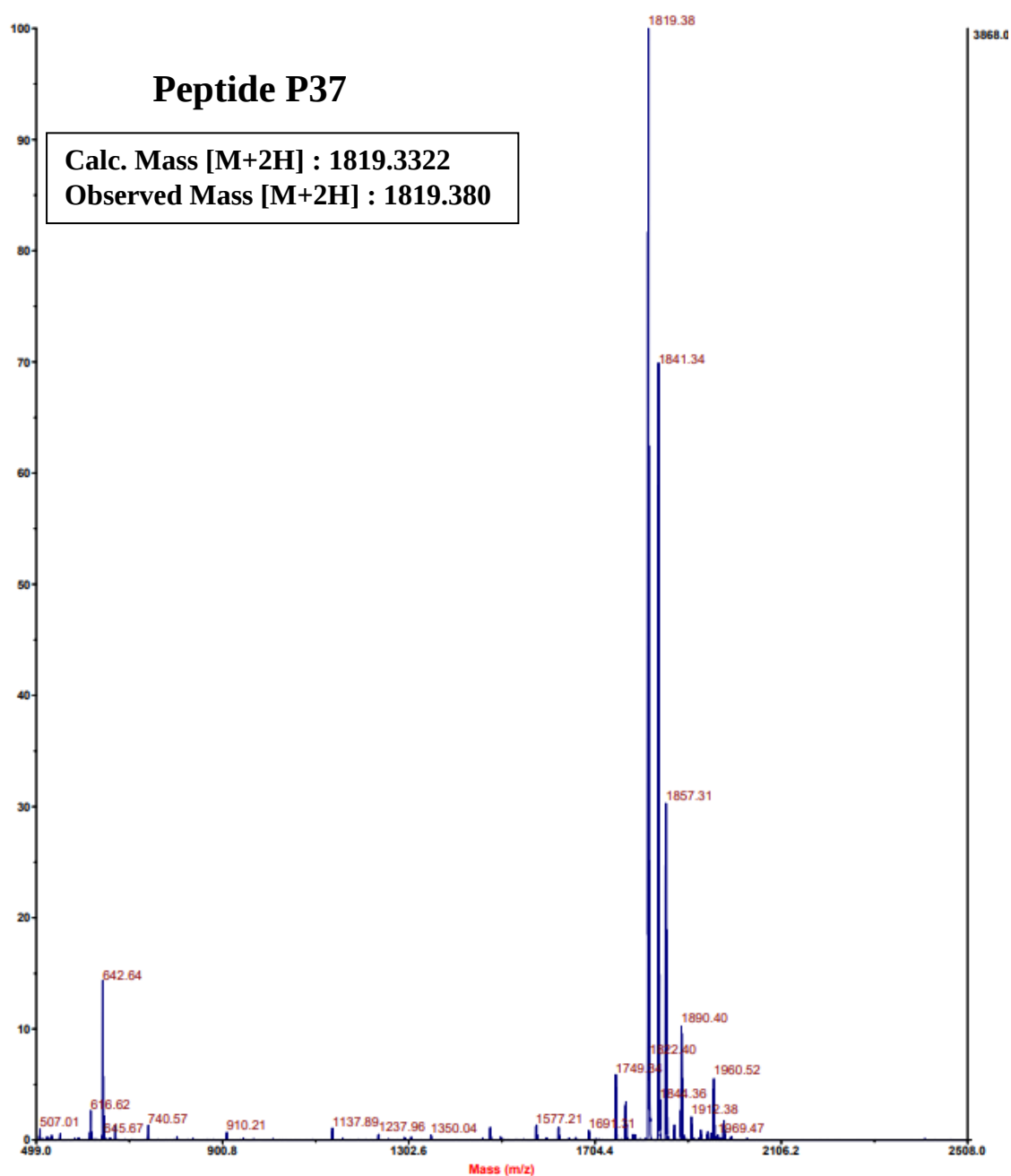
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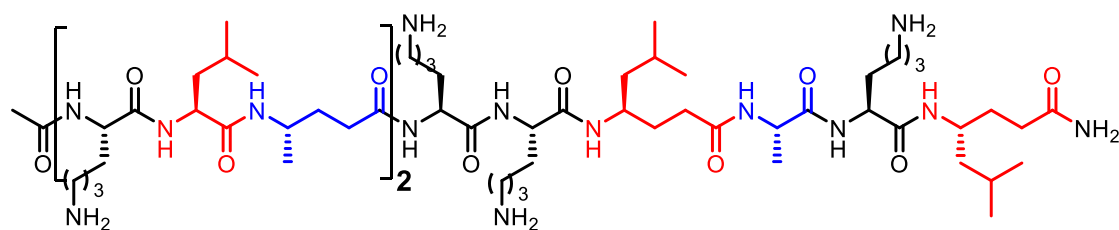
3B.8.1. Mass spectra of peptides



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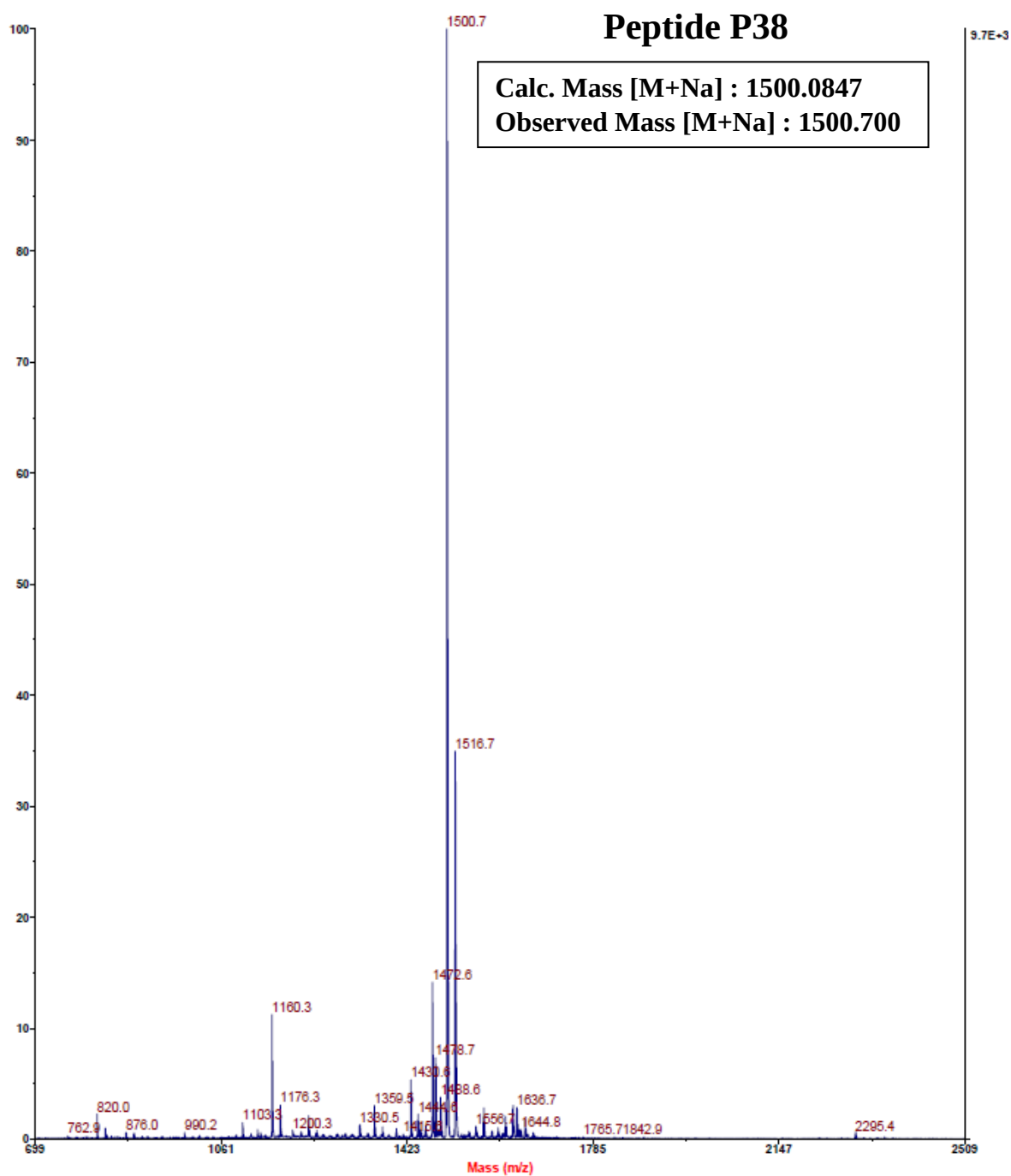


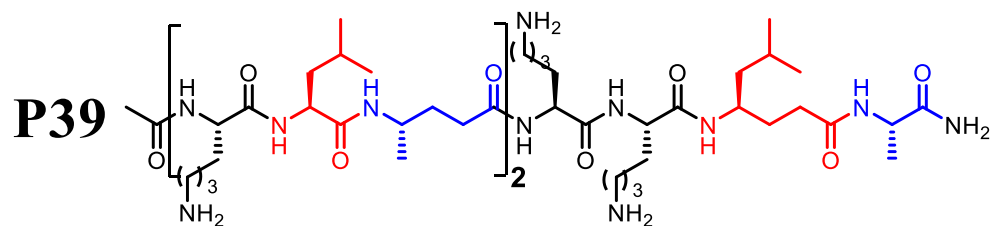
P38



Spectrum Report

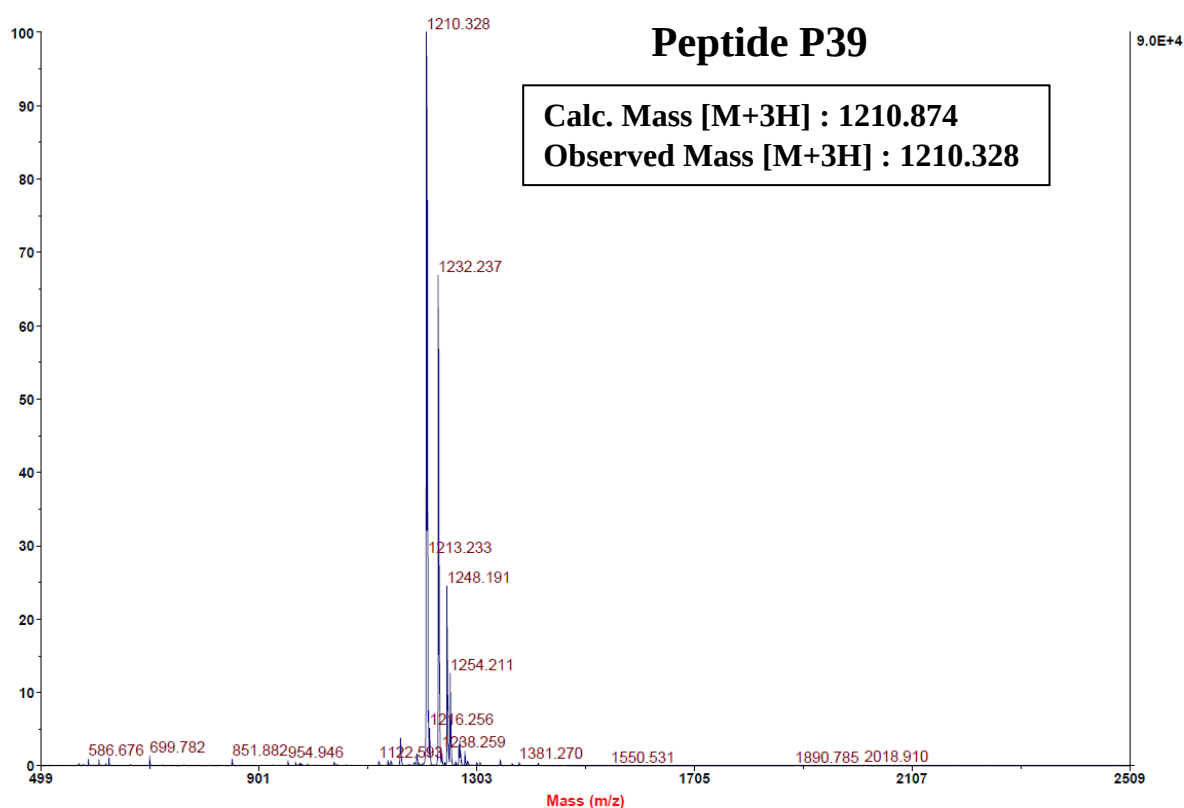
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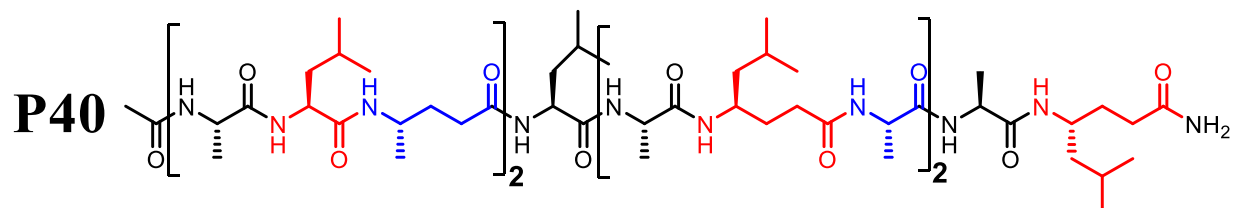




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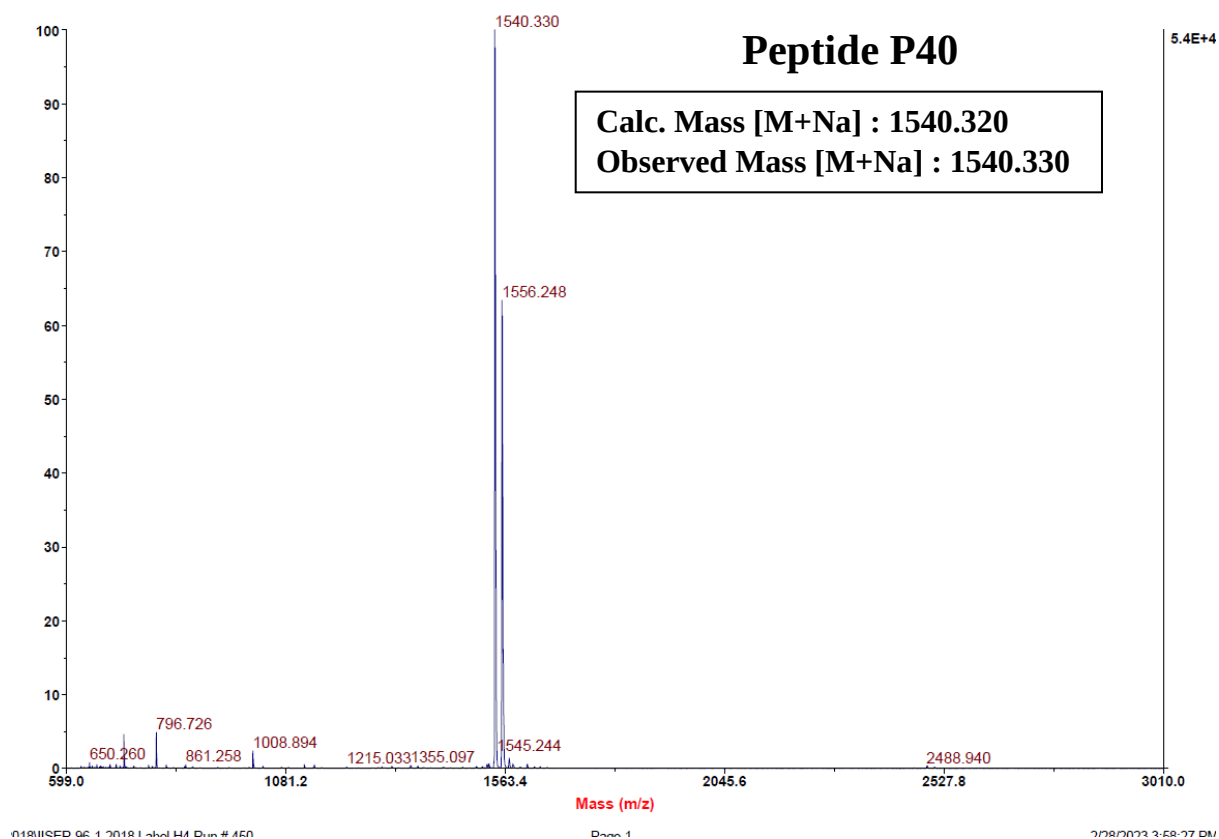
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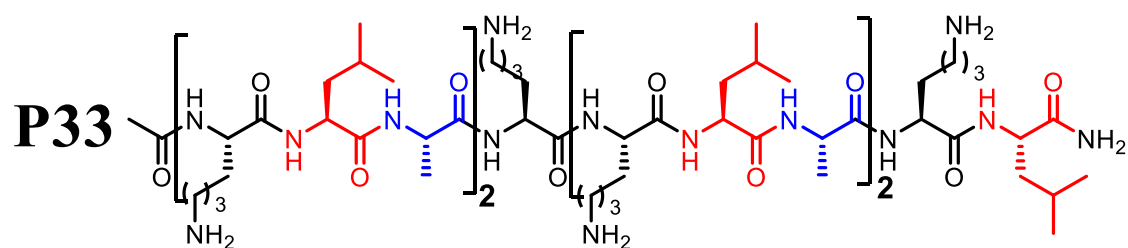




Spectrum Report

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Spectrum Report

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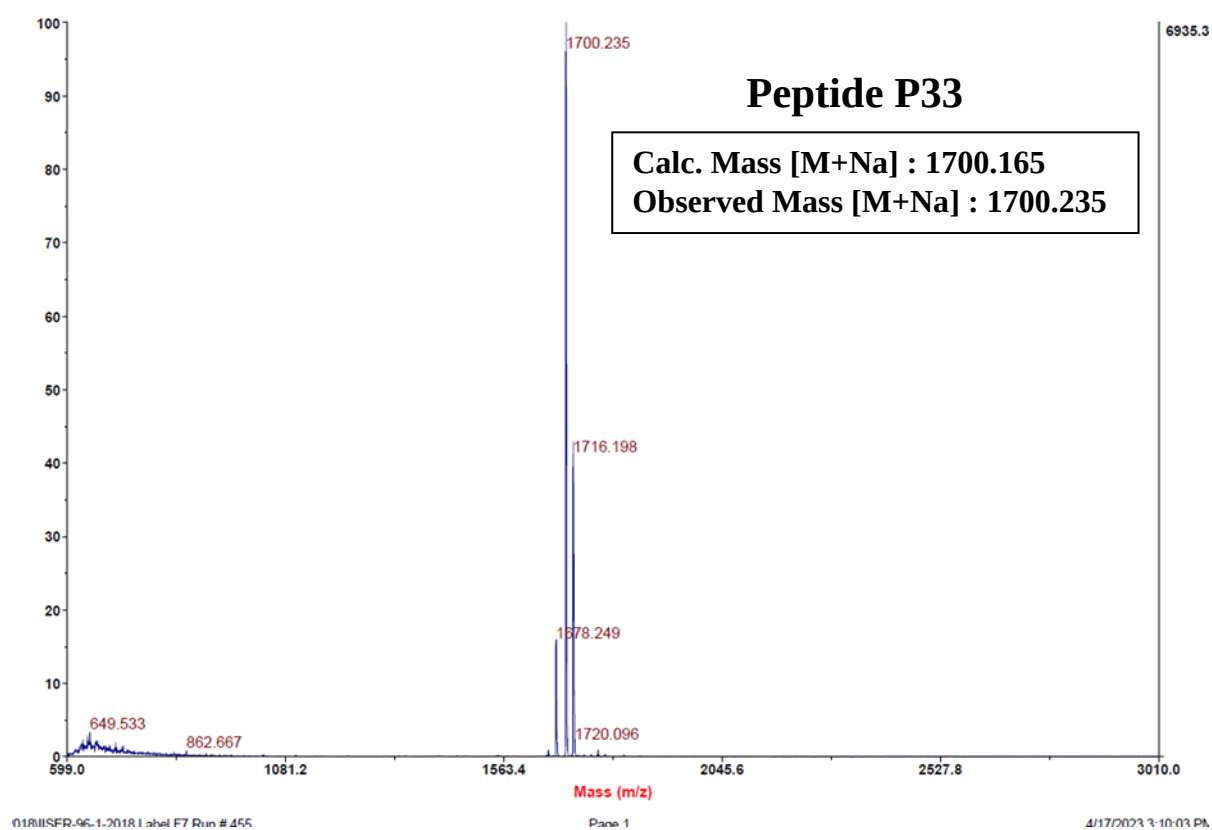
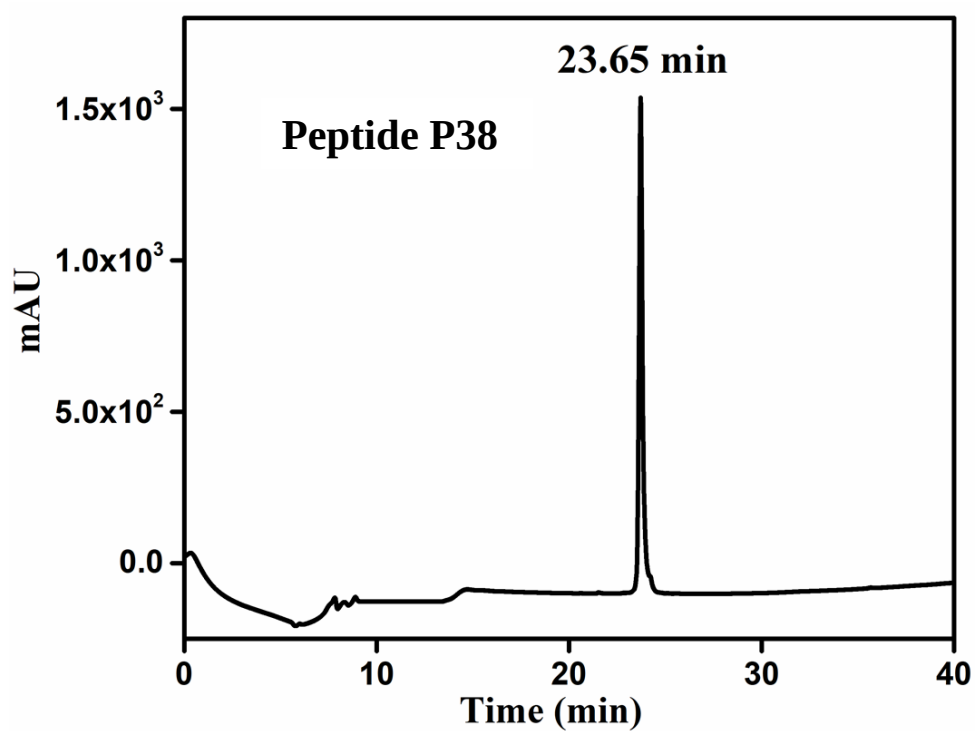
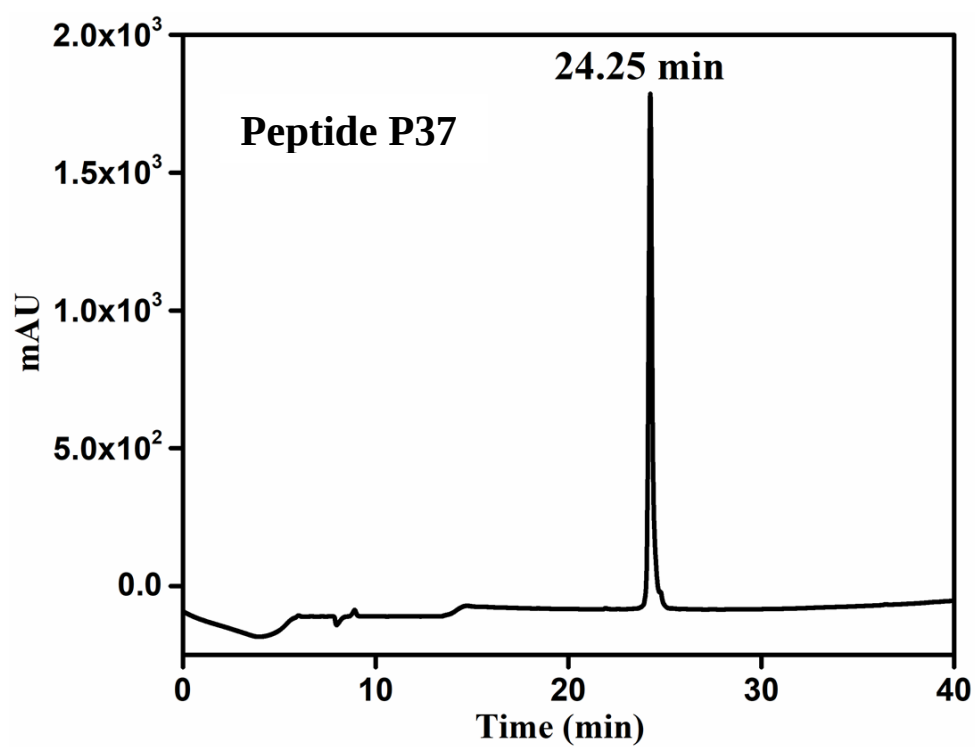
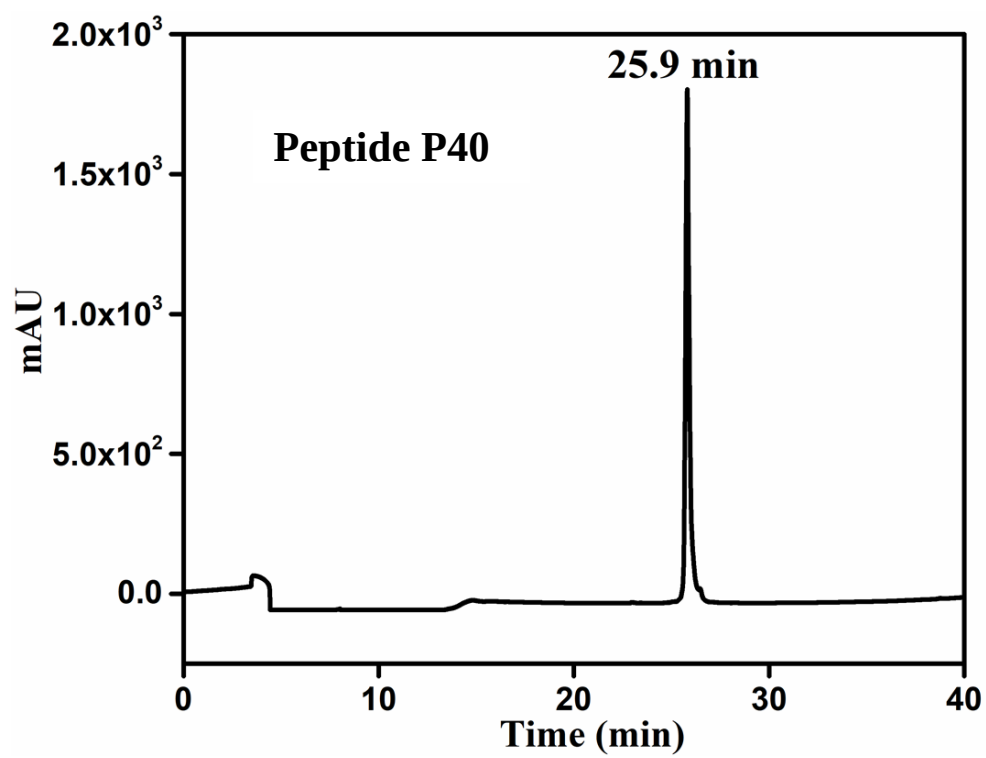
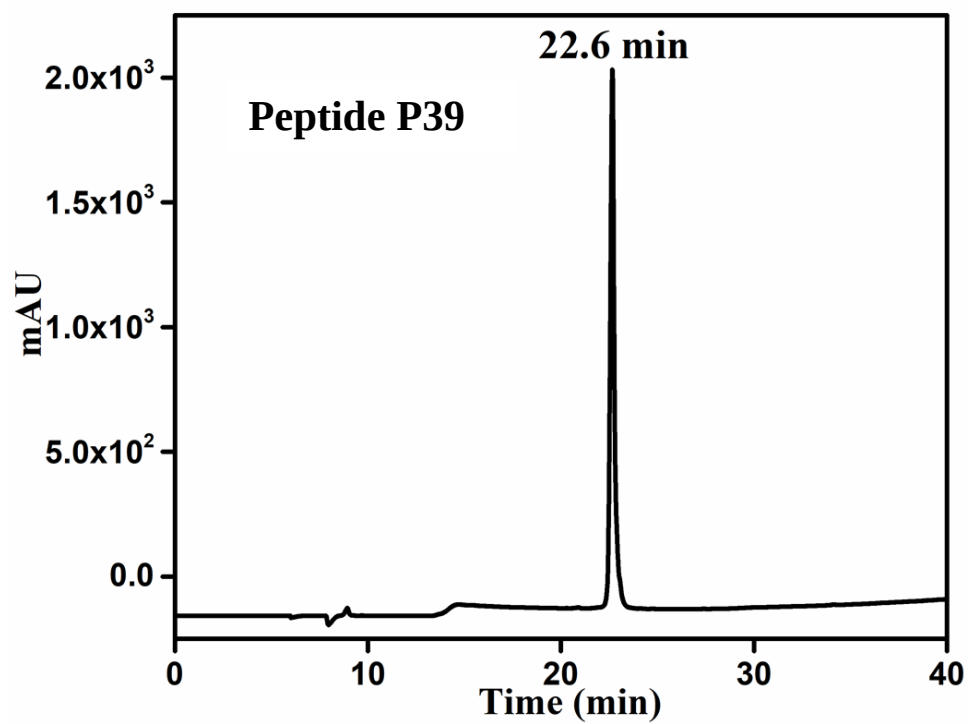


Figure 17: Mass spectra of peptides.

3B.8.2. HPLC profiles of peptides





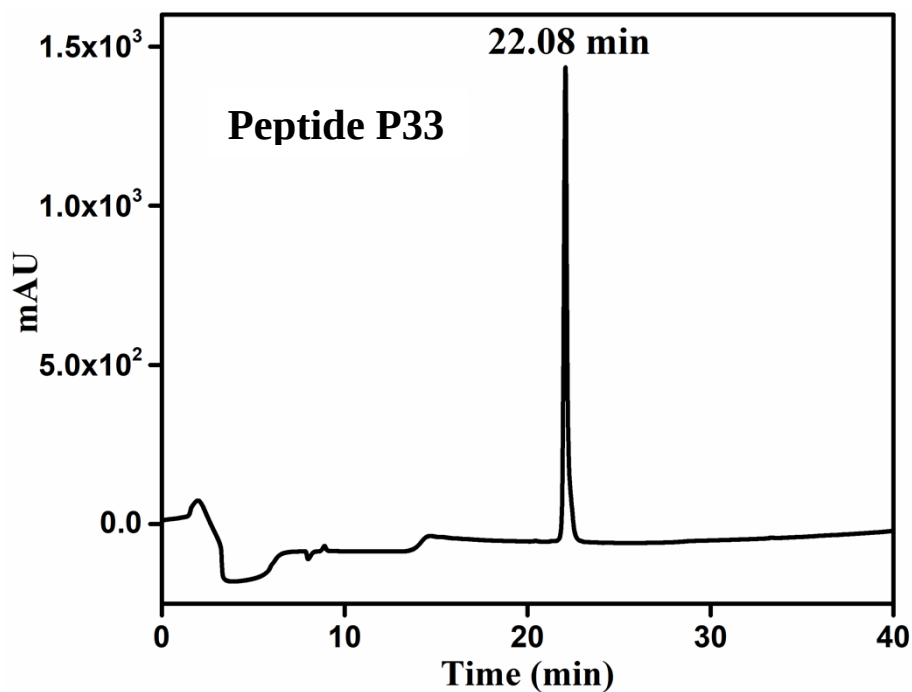


Figure 18: HPLC profile of Peptides in reverse phase was recorded on analytical C₁₈ column (5 μ m, 4.6 x 250 mm column) by using ACN/H₂O (**P37**, **P38**, **P39**, and **P33**) and MeOH/H₂O (**P40**) gradient system at 220 nm with a flow rate of 2 mL/min.

Chapter 4

Foldamer Nanotubes Mediated Label-Free Detection of Protein-Small Molecule Interactions

4.1. Introduction

Self-assembly of biomolecules is ubiquitous in nature. Over the years, extensive efforts have been made to understand the self-assembly of proteins and other biomolecules outside the cellular environment.¹ Peptides and proteins have been shown to adopt a variety of self-assembled architectures shown in Figure 1.^{2,3} The self-assembled peptide materials have been gaining momentum due to their wide range of applications including cargo delivery,⁴ energy storage⁵ and piezoelectric properties.^{6,7} Among the various self-assembled architectures of peptides, peptide nanotubes (PNTs) have been recognized as one of the most attractive one-dimensional nanostructures.⁸ The peptide nanotube assemblies have shown diverse applications such as nanoreactors,⁹ sensors,¹⁰ light-harvesting systems,¹¹ artificial photosynthesis,¹² drug delivery¹³ and antimicrobials.¹⁴ In their pioneering work, Gazit and co-workers showed the self-assembly of Phe-Phe dipeptide into distinct nanotubes and demonstrated the potential optoelectronic applications of the peptide nanotubes.¹⁵ Ghadri and co-workers demonstrated the antimicrobial and antiviral properties of self-assembled cyclic peptide nanotubes.¹⁶ Further, Lynn and colleagues demonstrated the nanotubular assembly of amyloid β -peptides.¹⁷ The peptide nanotubes have also been derived from surfactant-like peptides,¹⁸ polyglutamine rich peptides,¹⁹ and coiled-coil peptides²⁰ and proteins with β -helix.²¹

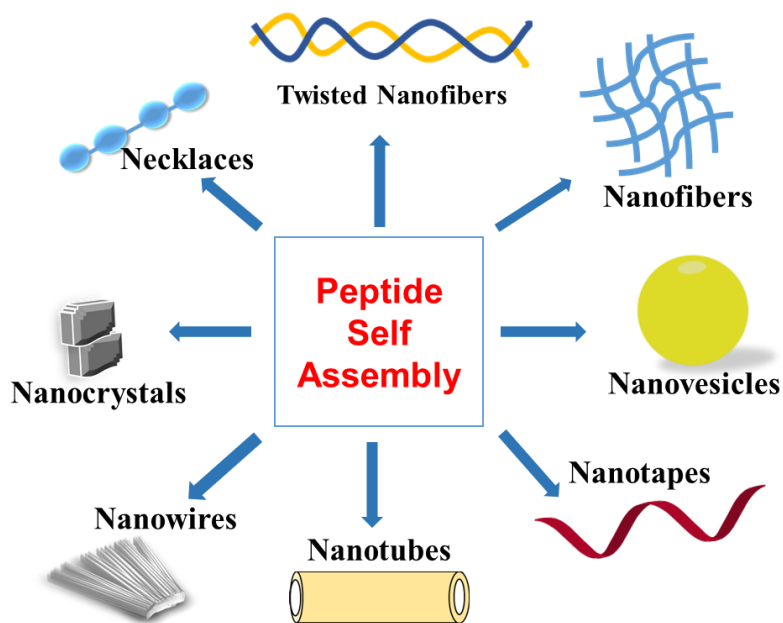


Figure 1: Various self-assembled nanoarchitecture made of peptides.

Foldamers are structural mimetics of peptides and proteins constructed from non-natural amino acid building blocks.²² Among the various types of foldamers, the oligomers constructed from β -amino acids, γ -amino acids and mixed sequences containing $(\alpha\beta)_n$,²³ $(\alpha\gamma)_n$,²⁴ and $(\alpha\delta)_n$ ²⁵ have attracted considerable attention due to their structural similarities with the natural peptides and proteins. In addition, foldamers have also displayed a variety of self-assembled architectures such as fibrils, vesicles and microtubes.²⁶ We have been interested in understanding the conformations and self-assembly of hybrid peptides containing 1:1 alternating α - and γ -amino acids.^{24d,24g} Previously, we reported peptide nanotubes derived from the α,γ -hybrid peptide 12-helix and their utility in casting the silver nanowire from silver ions shown in Figure 2.²⁷

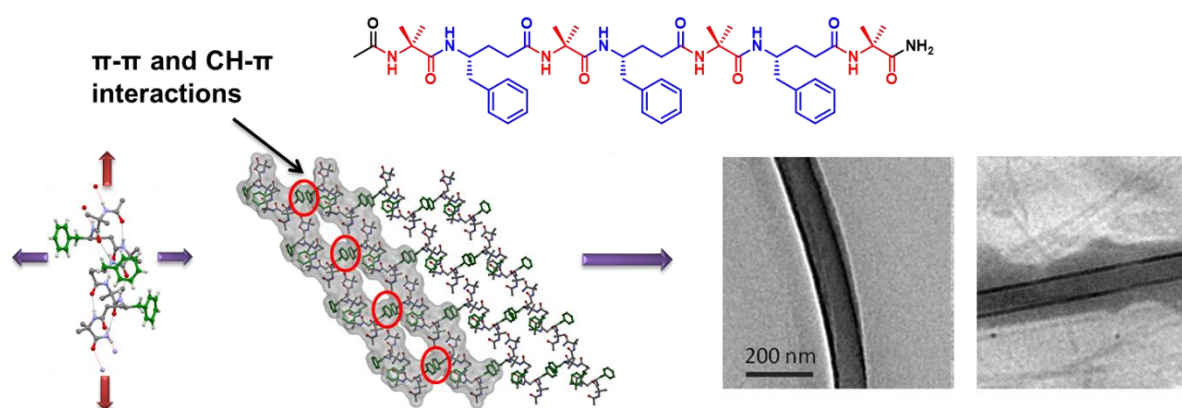


Figure 2: Nanotube self-assembly from α,γ -hybrid peptide foldamers.²⁷

4.2. Aim and Rationale of the Present Work

Since we are working on the various foldamers from different hybrid peptides, we sought to investigate the conformations of α,γ -hybrid peptides containing Cys residues, which can dimerize the hybrid peptide foldamers and also provide a useful handle to conjugate different functional groups on the surface of the self-assembled foldamers architectures. Herein, we are reporting the single crystal conformation of α,γ -hybrid peptide 12-helix dimer, self-assembly of helical peptides as nanotubes, exploration of the peptide nanotube as tools to detect the streptavidin-biotin interactions using impedance spectroscopy, the schematic representation is shown in Figure 3.

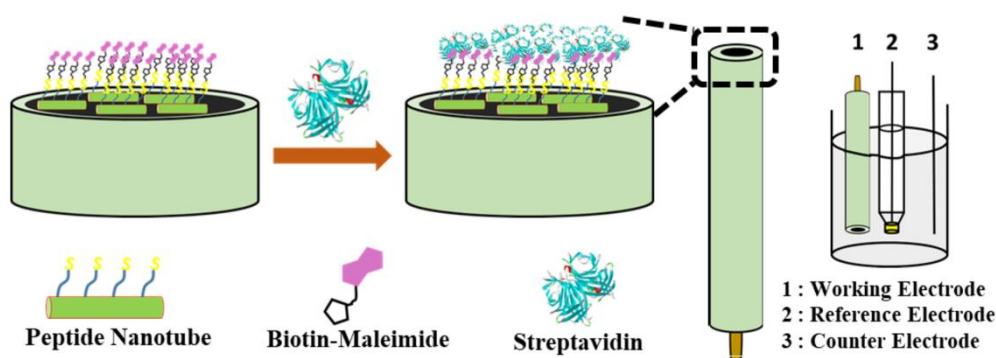
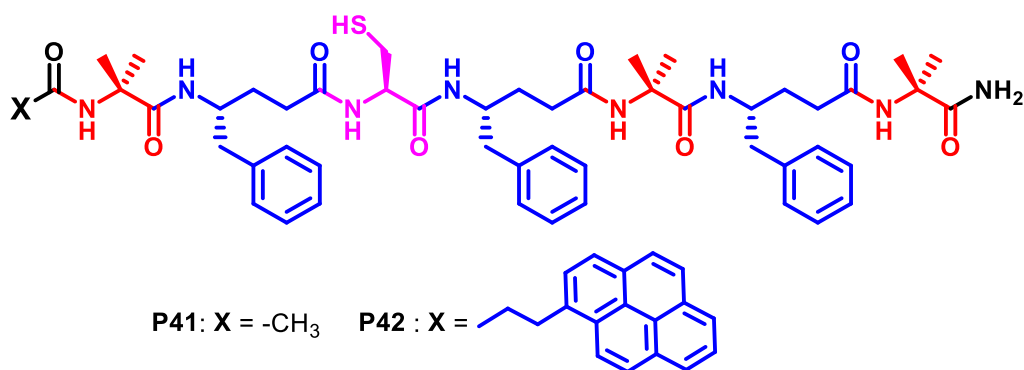


Figure 3: Schematic representation of the peptide nanotube based sensors to detect the protein-small molecule interactions.

4.3. Results and Discussion

4.3.1. Design and Synthesis

In order to understand the conformation of α,γ -hybrid peptides consisting of Cys residue, we have synthesized peptide **P41** and **P42**. The sequence of the peptide is shown in Scheme 1. The Cys residue was incorporated at the middle of the sequence. The peptide was synthesized through solid-phase synthesis. The γ^4 Phe was synthesized using the Wittig reaction as reported earlier. The peptide was purified by RP-HPLC, pure fractions of peptide were collected by monitoring UV-Vis absorption at 254 nm and 220 nm. Further, peptides were characterized by MALDI-TOF/TOF. After purification peptides were lyophilised to get the dried powder.



Scheme 1: Sequence of the α,γ -hybrid peptide helices.

4.3.2. Conformational (Single Crystal) Analysis of Peptides

In order to understand the conformation in single crystals, we subjected peptide to crystallization in aqueous methanol and Chloroform/Trifluoroethanol (CHCl₃/TFE). The peptide solution in CHCl₃/TFE gave single crystals after slow evaporation of the solvents. The X-ray diffracted single crystal structure of **P41** is shown in Figure 4. The peptide adopted helical conformation in single crystals and dimerized through a disulphide bridge. The helix in the dimer, is stabilized by six continuous intramolecular hydrogen bonds between the residues 1 and 4 ($i \rightarrow i+3$). The peptide adopted 12-helix conformation in single crystals. The γ -Phe adopted *gauche*, *gauche* conformation along N-C γ -C β -C α and C γ -C β -C α -C(O) and accommodated into the 12-helix.

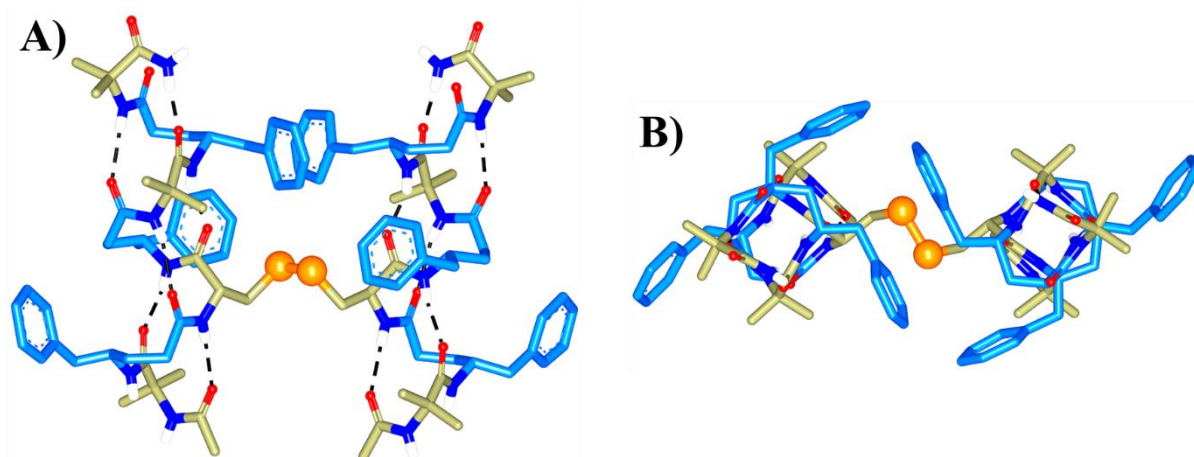


Figure 4: A) X-ray diffracted single crystal structure of **P41**. B) Top view of the helix dimer.

Table 1: Torsion angles [°] for **P41**.

Peptide Unit	ϕ [°]	θ_1 [°]	θ_2 [°]	ψ [°]
Aib 1	-52	-	-	-40
γ^4 Phe 2	-130	44	65	-124
Cys 3	-67	-	-	-28
γ^4 Phe 4	-118	57	56	-116
Aib 5	-53	-	-	-42
γ^4 Phe 6	-130	57	55	-115
Aib 7	-54	-	-	-43

Table 2: Intramolecular Hydrogen bonds for **P41** [Å and °].

D-H...A	d(D-H) [Å]	d(H...A) [Å]	d(D...A) [Å]	$\angle$ (DHA) [°]
N(4)-H(4)...O(2)	0.860	2.068	2.894	161
N(6)-H(6)...O(4)	0.860	1.961	2.768	156
N(7)-H(7)...O(5)	0.860	2.187	3.042	173
N(8)-H(8)...O(6)	0.861	1.828	2.645	158

The helical dimer in the peptide **P41** adopted by the α,γ -hybrid 12-helix, is similar to helical dimers observed in protein structures. The torsion angles and H-bond parameters are shown in the Table 1 and Table 2 respectively. From the crystal structure it is evident that the peptide **P41** get packed in columnar hexagonal packing where the aromatic Phe-residues get additional noncovalent interaction with 3.71 Å for intermolecular π - π stacking interaction, 3.55 Å for intramolecular π - π stacking interaction and 3.02 Å for intramolecular CH- π interaction is shown in Figure 5. Inspired by the 12-helical structure of **P41**, we sought to investigate its supramolecular architectures. We speculated **P41** may adopt ordered architectures in an aqueous solution due to the presence of aromatic amino acids in the sequence.

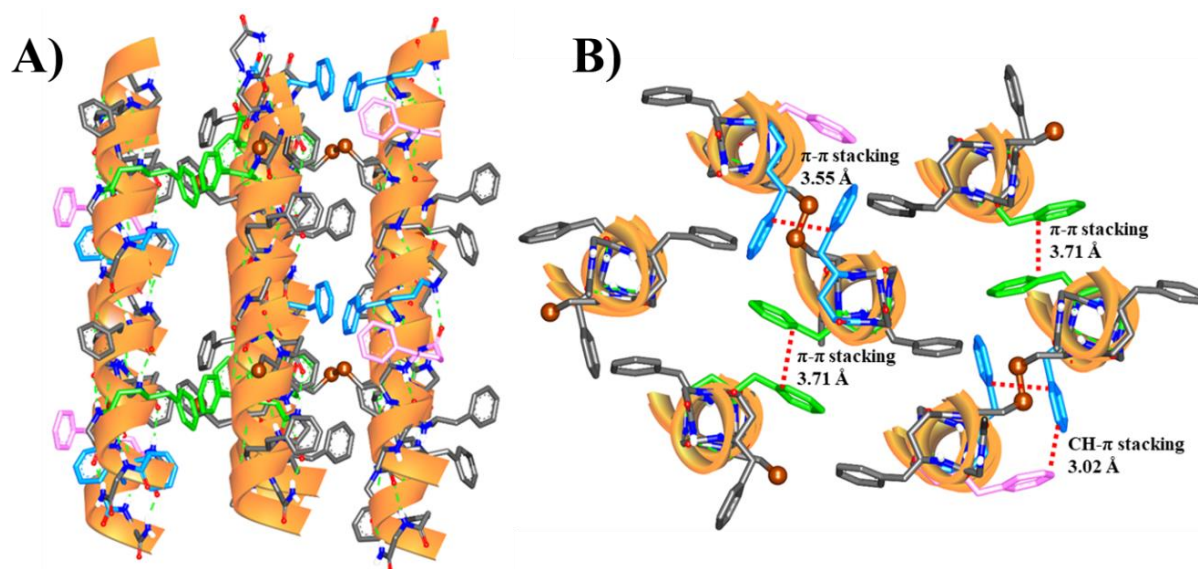


Figure 5: A) Crystal structure of **P41** depicting the noncovalent interactions. B) Top view of helical structure (except γ^4 Phe all other amino acids side chains are omitted for clarity).³¹

4.3.3. Self-Assembly of Peptides

In order to understand its supramolecular architecture, the solution of **P41** in THF (1mg/1 mL) was further diluted with water and examined. At 1:1.5 combination of THF: H₂O after incubation of the sample for about 24 h. The supramolecular assembly of the peptide was examined using FE-SEM analysis. After 24 h of incubation, the aqueous peptide solution was vortexed for about 1 min and centrifuged. The filtrate was subjected to the FE-SEM analysis and their images are shown in Figure 6. Instructively, the peptide displayed elongated discrete nanotubes. Better nanotubes were obtained after 3 days of incubation of peptide solution.

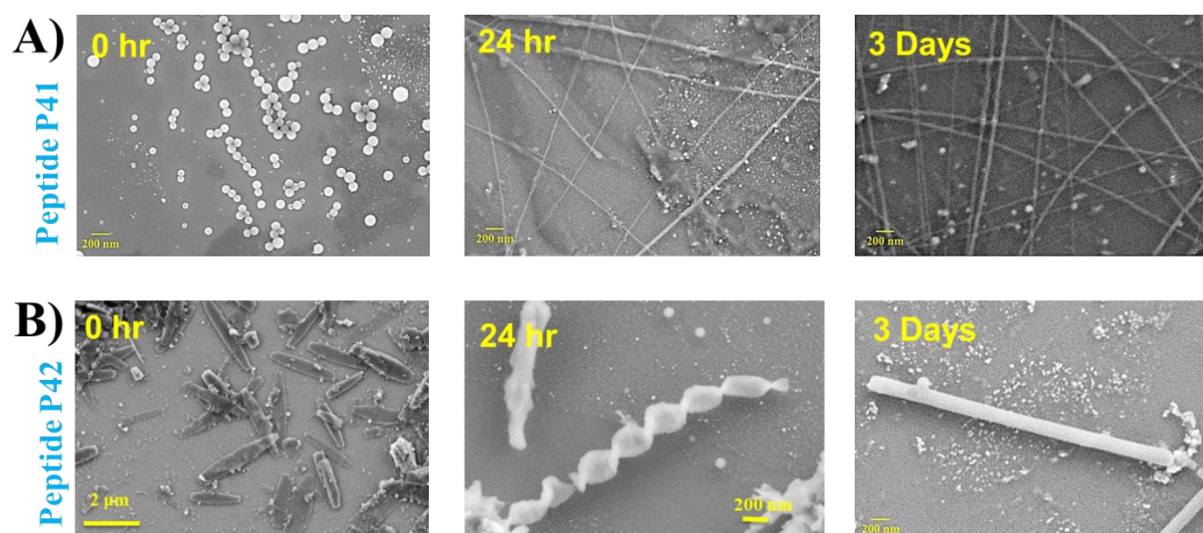


Figure 6: A) FE-SEM images of peptide **P41**. B) FE-SEM images of peptide **P42**. FE-SEM images were recorded after 3 days of incubation of peptides in THF: H₂O (1:1.5) solution.

Inspired by the self-assembly of the peptide **P41** into elongated nanotubes, we synthesized the peptide **P42** with pyrene moiety at the N-terminus. The sequence of the peptide is shown in Scheme 1. As aromatic groups assist the peptide to self-assemble into nanotubes, we speculated that the incorporation of pyrene will further augment the ordered aggregation in an aqueous solution. The FE-SEM images of **P42** are shown in Figure 6. Immediately after dissolving in a solution containing THF: H₂O (1:1.5) mixture **P42** displayed flat plate-like assemblies, after 24 h of incubation it showed twisted helical assemblies. Strikingly, after 3 days of incubation, peptide **P42** self-assembled into elongated discrete nanotubes, the schematic representation of the supramolecular assembly of **P42** with different time intervals is shown in Figure 7. The HR-TEM images further supported the formation of elongated discrete nanotubes of **P42** shown in Figure 8.

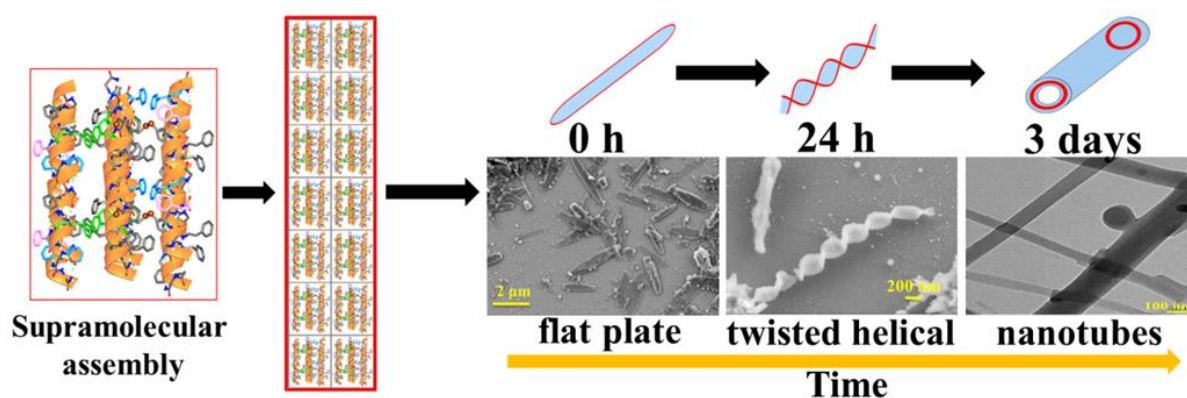


Figure 7: Non-covalent interactions mediated supramolecular assembly of **P42**. Formation of nanotubes with time is depicted.

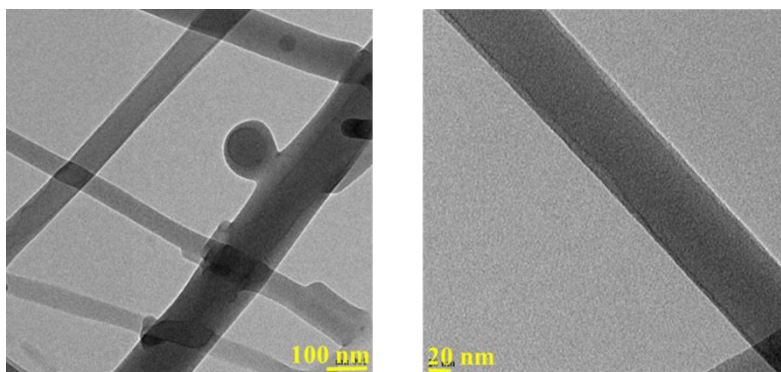


Figure 8: HR-TEM images of peptide **P42**. HR-TEM images were recorded after 3 days of incubation of peptides in THF: H₂O (1:1.5) solution.

As pyrene has been serving as a reporter molecule to understand the conformations of peptides and proteins,²⁸ we sought to explore the pyrene fluorescence emission to understand the morphology of peptide self-assemblies in solution. The fluorescence spectra of **P42** at various concentrations are shown in Figure 9A. The pyrene excimer was observed at higher concentrations of **P42**. Further, we subjected the incubated solution of **P42** to fluorescence microscopy imaging. The solution was excited at 342 nm and emission was monitored at 395 nm. The fluorescent images of **P42** self-assembly in solution are shown in Figure 9B. Fascinatingly, we observed elongated discrete blue fluorescent nanotubes of **P42** in solution. These results suggested that peptide retained the same morphology both in solution and on the surface. The mass spectral analysis of the incubated sample showed the mass of the peptide with a free SH group suggesting that the nanotubes consist of free SH groups. The fluorescent images of **P42** nanotubes after conjugation with N-(5-Fluoresceinyl) maleimide in solution are shown in Appendix. Along with the colour froth, coloured nanotubes are also seen in the solution.

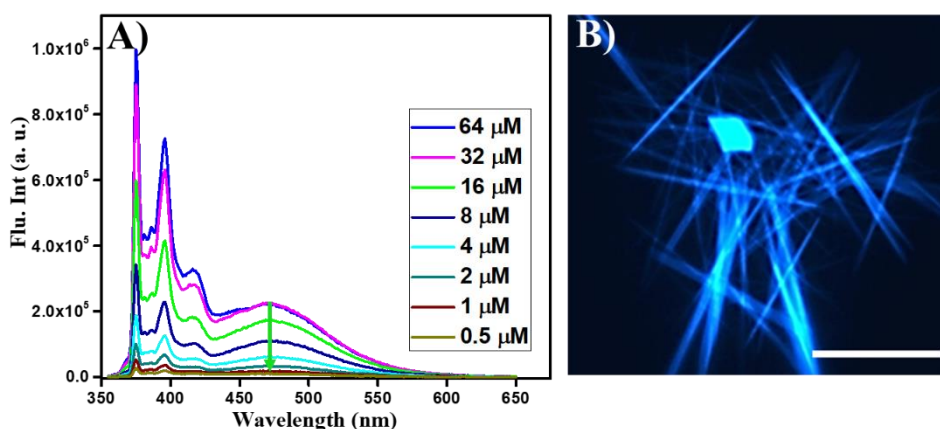
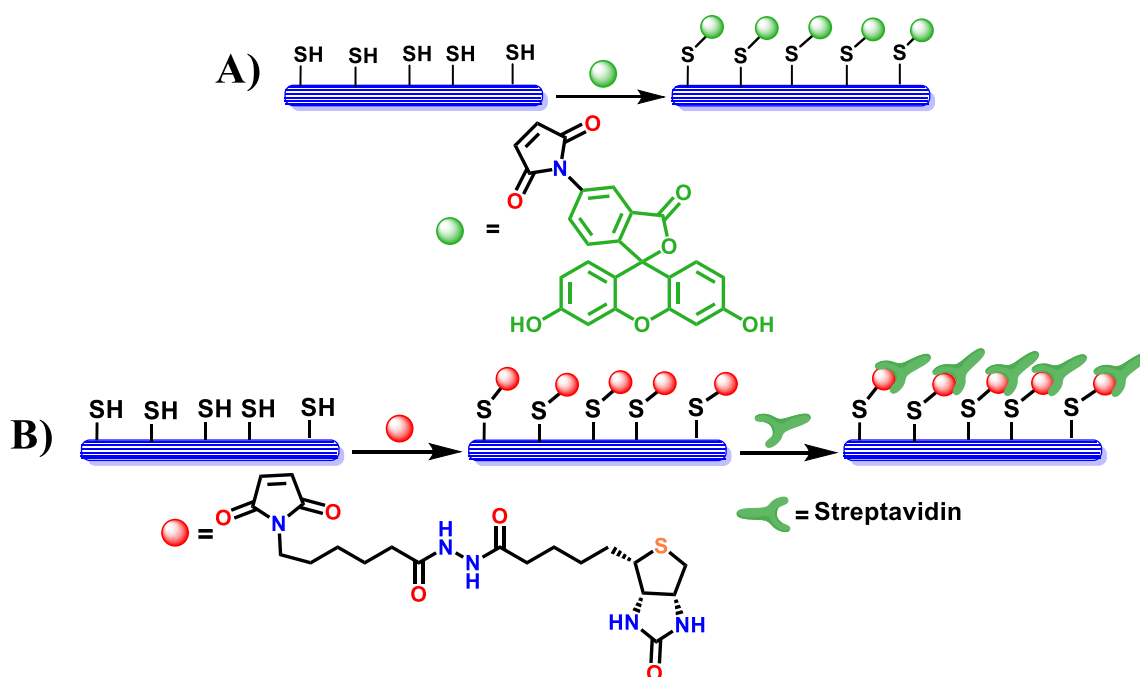


Figure 9: A) Fluorescence spectra of peptide **P42** in CHCl₃. The pyrene excimer peak was observed at 475 nm. B) Fluorescence microscope images of elongated fluorescent nanotubes

of hybrid peptide **P42** in solution. The solution was excited at 342 nm (λ_{ex} : 342 nm) and images were recorded at 395 nm (λ_{em} : 395 nm) and the scale bar was 200 μm .

As the free SH group can serve as a useful handle to functionalize through conjugate addition, we treated the incubated solution of **P42** nanotubes with N-(5-Fluoresceinyl) maleimide. The schematic representation of the reaction is shown in Scheme 2. We anticipated the same methodology can be explored to conjugate small molecules to **P42** and they can be recognized as respective proteins and antibodies through impedance spectroscopy. Encouraged by the conjugation reaction on **P42** nanotubes, we sought to explore whether the same technology can be used to understand the small molecule-protein interactions on nanotubes through impedance spectroscopy. As a proof of concept, we sought to investigate the Biotin-streptavidin interactions on **P42** nanotubes using impedance spectroscopy. The aqueous solution of biotin maleimide was added to the solution of pre-incubated, self-assembled **P42** in water and THF and incubated for another 3 days at room temperature. The schematic representation of the reaction is shown in Scheme 2. The biotin conjugation to the **P42** was confirmed by the mass spectral analysis (Appendix).



Scheme 2: A) Conjugation of nanotubes of **P42** with N-(5-Fluoresceinyl) maleimide. B) Conjugation of nanofibers of **P42** with biotin maleimide and the interaction of conjugated biotin with streptavidin.

In order to understand any changes in the morphology of **P42** assembly after biotin conjugation, we drop casted the incubated solution on TEM grids and subjected it to HR-TEM analysis. The image of biotin-conjugated **P42** nanotubes is shown in Figure 10. Instructively, there is no change in the morphology of the **P42** self-assembly after biotin conjugation and also further confirmed through confocal microscope fluorescent images of the **P42** nanotubes with N-(5-Fluoresceinyl) maleimide in solution shown in Figure 10. Encouraged by these results, we added an aqueous solution of streptavidin to the solution of biotin-conjugated **P42** and incubated for about 4 h at room temperature. The solution was drop casted on TEM grids and subjected to HR-TEM analysis and the image is shown in the Figure 10. We observed no change in the morphology of **P42** nanotubes even after the biotin-streptavidin complexation. As there is no change in the morphology of the nanotubes after biotin and streptavidin interactions, encouraged to investigate these interactions on **P42** nanotubes using nanoscale capacitance and impedance spectroscopy. Matsui and colleagues previously showed the detection of virus particles using non-covalently immobilized antibodies on peptide nanotubes derived from bola-amphiphilic peptide monomers.¹⁰ The electrochemical impedance and capacitance measurements have also been used previously to detect cells.^{29,30} However, it has not been used to detect small molecule-protein interactions.

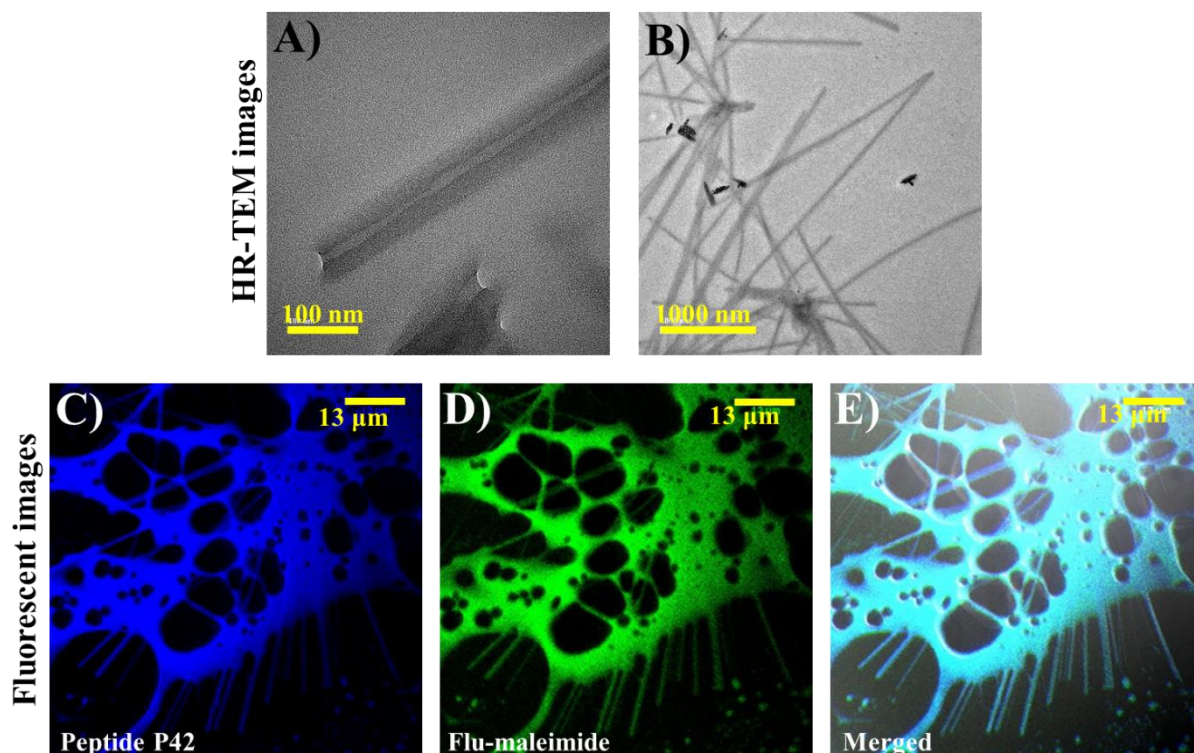


Figure 10: A) HR-TEM images of biotin attached **P42** nanotubes, and B) HR-TEM images of biotin-streptavidin attached **P42** nanotubes. HR-TEM images were recorded after 3 days of incubation of peptides in THF: H₂O (1:1.5) solution. Fluorescent images of the **P42** nanotubes with N-(5-Fluoresceinyl) maleimide in solution. C) Image of **P42** nanotubes in solution excited at 342 nm D) Images of **P42** nanofibers in solution excited at 494 nm and E) Merged images of A) and B), (λ_{ex} : 494 nm, λ_{em} : 519 nm).

4.3.4. Impedance Study: Protein-Small molecule interactions

To understand whether or not the helical foldamer nanotubes can be used to detect the small molecule-protein interactions, the solution of biotin-conjugated **P42** nanotubes was drop casted on a polished glassy carbon electrode. The electrochemical impedance spectra were recorded by the addition of increasing concentrations of streptavidin to the electrolyte. The Nyquist plot was acquired at the open circuit voltage by superimposing an AC amplitude of 10 mV (peak-to-peak) in the frequency range from 1 MHz to 10 kHz. The Nyquist Plot with the nanotube sensor is shown in Figure 11A. There is a clear change in the impedance spectra with increasing concentration of streptavidin in the electrolyte. Before drop casting the biotin-conjugated **P42** nanotubes, the solution was centrifuged to remove the excess biotin-maleimide. Peptide **P42** nanotubes without biotin conjugation were used as a control. No change in the impedance spectra was observed even after increasing concentrations of streptavidin in the control experiment. Further to understand the specificity of protein interaction with the small molecule and also to see any change in the impedance, we replaced streptavidin with bovine serum albumin (BSA) and measured the impedance. No change in the impedance spectra was observed even with increasing concentrations of BSA in the electrolyte. The results are shown in Figure 12A.

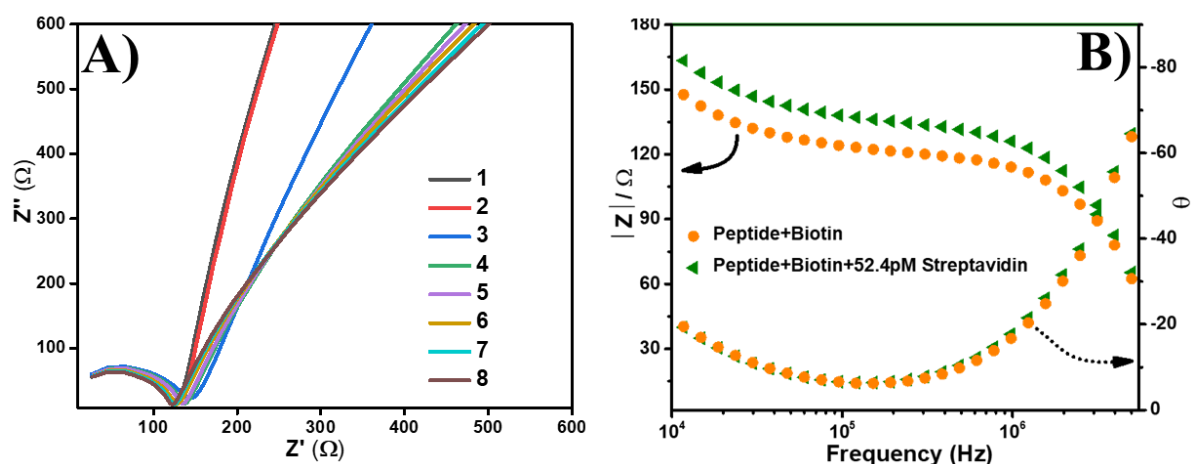


Figure 11: Electrochemical Impedance Spectra of specificity of protein interaction with biotin-conjugated **P42** Nanotubes. Nyquist plot A) peptide **P42** with biotin by varying the streptavidin concentration (1: **P42** only, 2: **P42**+52.4 pM streptavidin, 3: **P42**+biotin, 4: **P42**+biotin+10.48 pM streptavidin, 5: **P42**+biotin+20.96 pM streptavidin, 6: **P42**+biotin+31.44 pM streptavidin, 7: **P42**+biotin+41.92 pM Streptavidin, 8: **P42**+biotin+52.4 pM streptavidin). Bode plot B) biotin conjugated Peptide **P42** with the addition of streptavidin.

Further, the Bode plots demonstrating the magnitude of impedance versus the phase angle for the biotin-conjugated nanotubes sensor, and the nanotubes with biotin are shown in Figure 11B. In addition, the Bode plot of biotin-conjugated nanotubes with control protein BSA is shown in Figure 12B. These results demonstrate that enhancement in the impedance values is due to the specific interactions of biotin on the **P42** nanotubes and the streptavidin. To understand whether or not the **P42** nanotubes are important for the interaction, we follow the same protocol, but without peptide. The solution of biotin maleimide was centrifuged similarly to the solution of biotin-conjugated **P42**, the solution was decanted, the residue was re-suspended with the same buffer solution, and drop casted on the electrode. Increasing concentrations of streptavidin were added to the electrolyte and measured the impedance. The Nyquist Plot and Bode plot demonstrate no change in the impedance spectra even at high concentrations of streptavidin, as shown in Figure 12C and 12D. These experiments clearly suggest that peptide nanotubes play an important role in biotin-streptavidin interactions. In addition, the phase angle was approaching -90° at the medium to higher frequencies, suggesting that the system displayed a characteristic behaviour of a capacitor. The recognition of biotin-streptavidin on the nanotube increased the impedance which leads to a decrease in capacitance as a result of the accumulation of low-permittivity materials on the working electrodes.

To assess whether or not these peptide nanotube sensors can be used as a platform to measure the protein-small molecule interactions, we have plotted the changes in capacitance against the increasing concentrations of streptavidin. The results are shown in Figure 13. The values of capacitance were obtained for every concentration of streptavidin by fitting the data to the circuit shown in the inset of Figure 13. The changes in the capacitance were detectable even with 20-40 pM concentrations of streptavidin. Therefore, these peptide nanotube sensors mediate the detection of small molecule-protein interactions in picomolar concentrations. The streptavidin detection limit of 20 pM observed in this method is lower or comparable to the fluorescence based detection methods reported in the literature.³²⁻³⁵ Overall, these results

demonstrated that small-molecule protein interaction can be detected in at very low concentrations without any labelling.

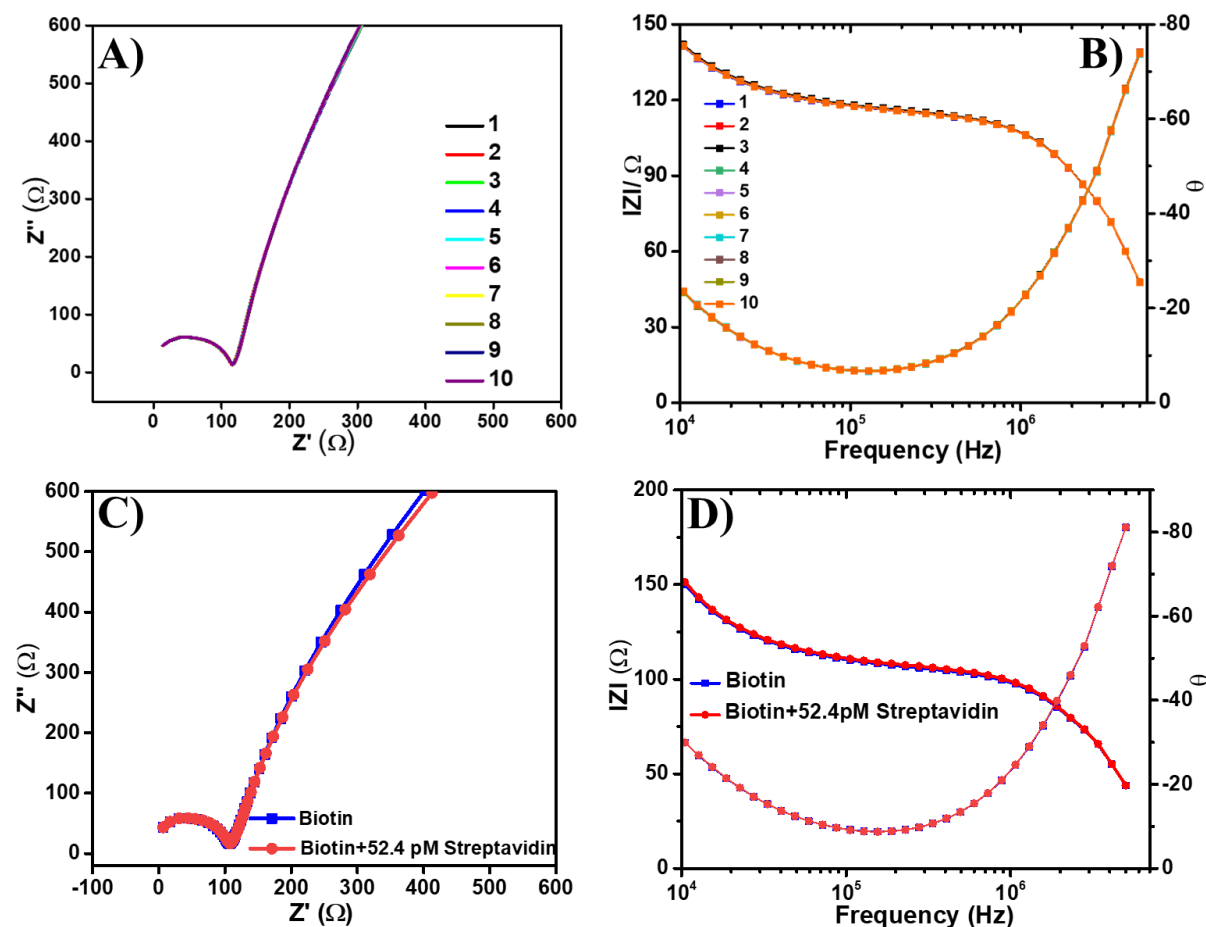


Figure 12: Impedance Spectra of specificity of protein interaction with biotin conjugated **P42** Nanotubes. Peptide **P42** with biotin by varying the BSA concentration (1: **P42**+Biotin, 2: **P42**+Biotin+0.53 nM BSA, 3: **P42**+Biotin+1.06 nM BSA, 4: **P42**+Biotin+1.59 nM BSA, 5: **P42**+Biotin+2.12 nM BSA, 6: **P42**+Biotin+2.65 nM BSA, 7: **P42**+Biotin+3.18 nM BSA, 8: **P42**+Biotin+3.71 nM BSA, 9: **P42**+Biotin+4.24 nM BSA, 10: **P42**+Biotin+4.77 nM BSA), A) Nyquist plot, B) Bode Plot. Only biotin with streptavidin in the absence of Peptide **P42**. C) Nyquist plot, D) Bode Plot.

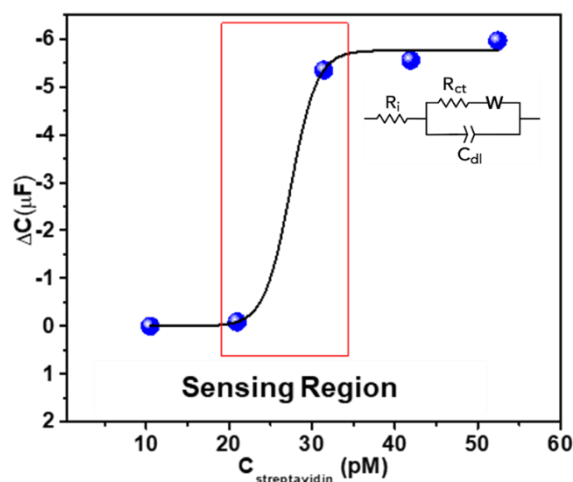


Figure 13: Protein-small molecule interaction. Correlation between the concentration of streptavidin in the solution and changes in interfacial capacitance. Data were fitted to the circuit shown in the inset. R_s is the Solution resistance, R_{ct} is the Charge transfer resistance, C_{dl} is the Double layer capacitance.

4.4. Conclusion

In conclusion, we have demonstrated exploration of the small molecule conjugated nanotubes of the self-assembled α,γ -hybrid foldamers as sensors for the label-free detection of small molecule-protein interactions using impedance spectroscopy and associated capacitance. The X-ray diffracted single crystal analysis revealed the α,γ -hybrid peptides containing Cys residue adopted helical conformations in a single crystal. The inter-helical side-chain aromatic interactions and the head-to-tail intermolecular H-bonds promote these helical foldamers to assemble them into a nanotubular architecture. The free SH of the Cys provides the handle for the small molecule conjugation. More importantly, even after the conjugation with a small molecule and also after the binding of the protein, peptides retain the nanotubular architectures. As detected by fluorescence microscopy, these nanotubes are stable in aqueous buffer conditions, enabling them to serve as valuable platforms for the label-free detection of biomolecular interactions. Using impedance spectroscopy, we detected the interactions of biotin and streptavidin in picomolar concentrations by monitoring the alterations in the interfacial capacitance. We anticipate that the same strategy can be explored to detect and understand various small molecule protein interactions.

4.5. Experimental Section

4.5.1. Materials and Method

The amino acid, reagents and proteins which are used in this work, Phenylalanine, 2-Aminoisobutyric acid (Aib), Cysteine, Pyrene butyric Acid, Pd/C (10%), Streptavidin, N-(5-Fluoresceinyl) maleimide, Biotin-Maleimide and BSA protein were purchased from Sigma-Aldrich and N-hydroxysuccinimide, NaBH₄, Oxone, DCC, HOBt, Ethyl bromoacetate, PPh₃, 2-Iodobenzoic acid was purchased from Spectrochem. THF was distilled over sodium before use. Gamma amino acids were prepared from the reported protocol, and all those reactions were monitored by analytical thin-layer chromatography and purified by normal Column chromatography with Merck silica gel (120-200 mesh). All monomers are protected with Fmoc-OSu to make peptides by SPPS. Protein concentration was determined spectroscopically at 280 nm by UV spectrometer. The Matrix Assisted LASER Desorption Ionization mass spectrometer (MALDI-TOF/TOF, Applied Biosciences) was used to obtain accurate mass. Data for X-ray structure determination were obtained from Bruker APEX II DUO diffractometer using Mo-K α (λ = 0.71073 Å) graphite monochromatic radiation.

4.5.2. Peptide Synthesis

The peptides (**P41** and **P42**) were synthesized on a solid support Rink amide resin (0.2 mmol) by manual synthesis method. All peptide coupling reactions were done with NMP as a solvent, HOBt/HBTU as coupling reagents and DIPEA as an activator base. Deprotection of the Fmoc was carried out by a solution of 20% piperidine in DMF. Capping after the first coupling was done with acetic anhydride/pyridine/DMF in the ratio of (2:1:1) before Fmoc deprotection. Cleavage of the final Peptide from the resin was done by treatment of the resin with a cocktail of trifluoroacetic acid (TFA)/water/ triisopropylsilane/phenol (90:5:5:5) for 3 h, at the end the resin was filtered. The filtrate containing crude peptides was evaporated under reduced pressure and the gummy residue was precipitated with cold diethyl ether (50 mL). The precipitate was separated through centrifugation. The crude peptides were again dissolved in 10 mL of MeOH/H₂O and purified on a C₁₈ column using reversed-phase HPLC. Methanol and water gradient system applied was from 95% A to 95% B (A: 95% water and B: 95% methanol) at a 2 ml flow rate was used to purify the peptides. The mass of the purified peptides was confirmed by MALDI-TOF/TOF analysis. The lyophilised pure peptides were used for further studies.

4.5.3. FE-SEM Study

To check the self-assembly of the peptides, Peptides were dissolved in THF (1 mg/mL) as a main stock solution, then the stock solution was diluted with distilled H₂O (THF/H₂O = 400/600 microliter). After diluting with H₂O, samples were kept for incubation at room temperature. The aqueous peptide solution was vortexed for about 1 min and centrifuged. The filtrate was finally drop casted (3 μ L) on the previously cleaned silicon vapours plate (SiO₂/Si substrate) with different time intervals (0 h, after 24 h, after 3 days), dried at room temperature and finally kept in the desiccator for 10 mins before taking the images. For all the experiments freshly prepared solutions have been made. Samples were examined under the FE-SEM ZEISS ULTRA PLUS instrument to take the final images.

4.5.4. HR-TEM Study

HR-TEM Samples were prepared in a similar way of FE-SEM analysis and samples were drop casted (4 μ L) on a carbon-coated copper grid and dried at room temperature and imaged it. Further, we checked the Nanotube flexibility with the addition of Biotin-Maleimide and Streptavidin protein. Sample preparation was the same as earlier, here we took the Biotin-maleimide in the aqueous part of the self-assembled solution for the peptide **P42** and then kept it for 3 days incubation at room temperature. After that, Streptavidin protein was added to the solution, the sample was kept for 4 h incubation. Before drop casting on the glass slide, we centrifuged the sample to remove the excess biotin-maleimide and streptavidin. The self-assembly was resuspended with the same solvent combination ratio. Samples were drop casted on TEM grids to check the stability of the peptide nanotubes. Drop casted samples were examined under Bruker High-Resolution Transmission Electron Microscope to take the final images.

4.5.5. Excimer Study

To check the excimer character in the pyrene, peptide **P42** was dissolved with the solvent CHCl₃ by varying the concentration (0.5 to 64 micromolar), this experiment was carried out using FluoroMax-4 HORIBA fluorimeter (λ_{ex} : 342 nm, λ_{em} : 395 nm and Excimer Peak: 475 nm).

4.5.6. Laser Scanning Confocal Microscopy

Nanotube solution (Peptide **P42**) was drop casted on a previously cleaned glass slide and covered with a thin cover slip then imaged using OLYMPUS ZX81 laser scanning confocal microscopy by inverting the sample (λ_{ex} : 342 nm, λ_{em} : 395 nm).

To check the bioconjugation of the cysteine of the peptide **P42** with Biotin-Maleimide, here we choose the *N*-(5-Fluoresceinyl) maleimide (λ_{ex} : 494 nm, λ_{em} : 519 nm). Nanotube preparation was done like the previously mentioned FE-SEM sample preparation. The aqueous solution of *N*-(5-Fluoresceinyl) maleimide was added to the solution of pre-incubated, self-assembled **P42** in water and THF then kept for 3 days incubation at room temperature. The sample was drop casted on a previously cleaned glass slide and covered with a thin cover slip then imaged by inverting the sample.

4.5.7. DLS Study

The size distribution of the peptide Nanotube in THF/H₂O solution was done by DLS (Dynamic Light Scattering) experiment using a 90° scattering angle. The sample preparation was done by following same as the HR-TEM samples. Here we took the blank with THF/H₂O in the same ratio of the prepared sample. This experiment was carried out by the instrument Zeta Sizer Nanoseries ZS90.

4.5.8. Impedance Experimental Study

Electrochemical impedance spectroscopy was carried out in the three-electrode setup with peptide modified glassy carbon electrode as the working electrode, Ag/AgCl (3.5 M KCl) as the reference electrode and platinum mesh as a counter electrode. Before each experiment, the glassy carbon electrode was cleaned manually as well as electrochemical polishing. The analysis was carried out at the open circuit voltage with 10 mV AC amplitude from 1 MHz to 10 kHz. The glassy carbon electrode was modified by drop-casting a known amount of the incubated self-assembled peptide nanotube and dried it in the atmosphere before the electrochemical analysis. The impedance spectra were taken at different concentrations of protein and were fitted with Randle's circuit in Z-sim software to find the capacitance. The change in the capacitance values was calculated and plotted against the concentration of protein to get the sensing region. This experiment was carried out by the instrument Biologic VMP-300 electrochemical workstation.

4.5.9. Crystal Information of Peptide P41 (CCDC No: 2233515)

Crystals of **P41** were grown by slow evaporation from a solvent composition of Trifluoroethanol/CHCl₃ (TFE/CHCl₃) with 1:1 ratio. A single crystal, Needle in shape (0.70 X 0.35 X 0.25 mm³), was mounted on a loop. The X-ray data were collected at 100 K on a Bruker AXS SMART APEX CCD diffractometer using Mo-K α radiation (λ =0.71073 Å), ω -scans (2θ =56.00°) for a total number of 162013 independent reflections. Space group *P* 21 21 2; *a*=26.978(5), *b*=15.573(3), *c*=16.434(3) Å; α =90, β =90, γ =90, *V*=6904(2), Orthorhombic C₁₀₀H₁₃₈N₁₆O₁₆S₂; *Z*=2 for chemical formula C₁₀₀H₁₃₈N₁₆O₁₆S₂; ρ_{calcd} = 1.001 gcm⁻³; μ =0.103 mm⁻¹; *F*(000)=2212. The structure was obtained by direct methods using SHELXS-97. All non-hydrogen atoms were refined anisotropically. The hydrogen atoms were fixed geometrically in the idealized position and refined in the final cycle of refinement as riding over the atoms to which they are bonded. The final *R* value was 0.1910 (*wR*²=0.5071) for 17074 observed reflections ($F_0 \geq 4\sigma(|F_0|)$); *S*=1.119.

4.6. References

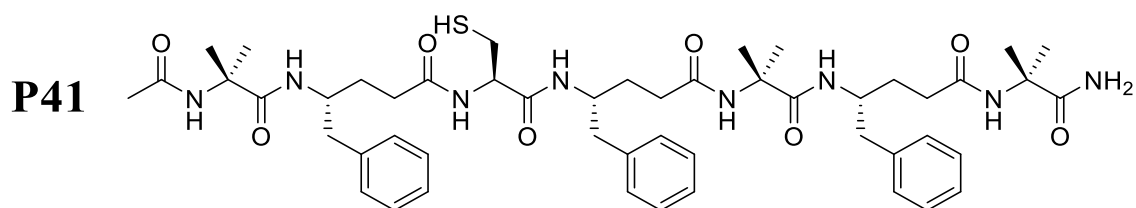
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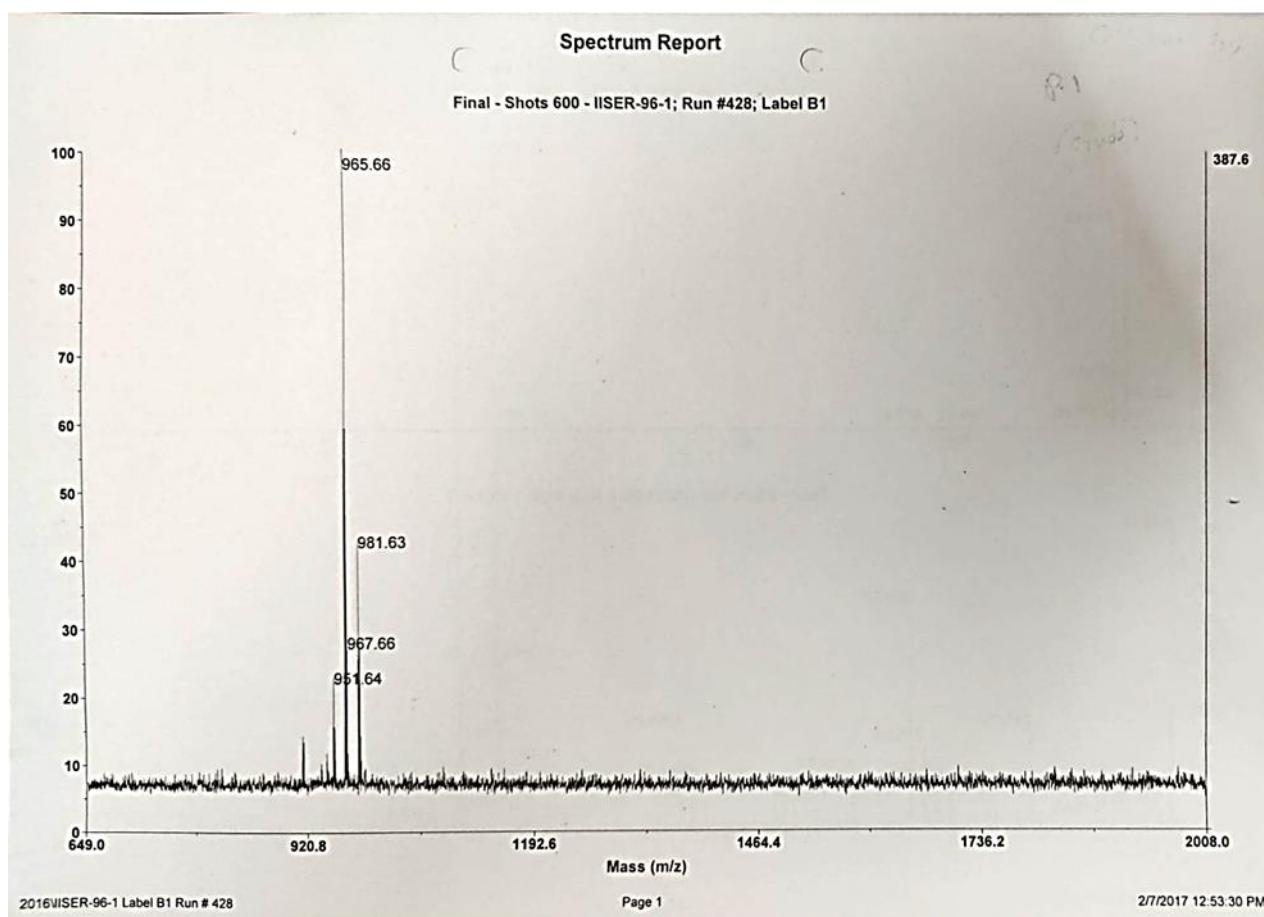
31. Deposition number 2233515 (for **P1**) contains the supplementary crystallographic data for this paper. These data are provided free of charge by the joint Cambridge Crystallographic Data Centre and Fachinformationszentrum Karlsruhe Access Structures service.
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4.7. Appendix IV: Mass Spectra of the Peptide P41-P42 and Peptide P42 with Biotin-Maleimide

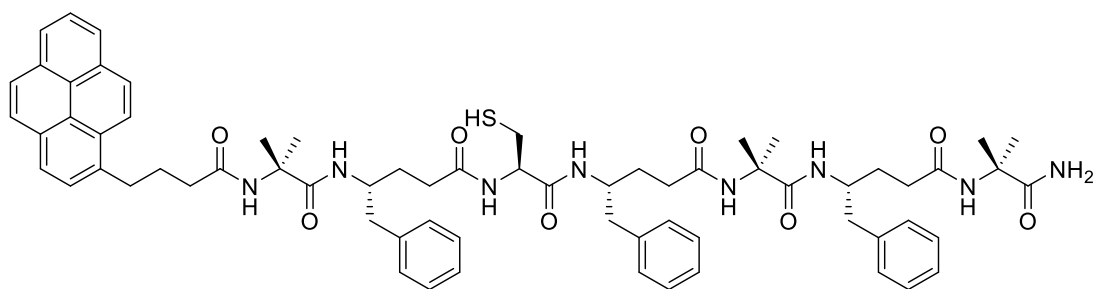


Calculated Mass for $C_{50}H_{70}N_8O_8S$ $[M+Na]^+$: 965.50

Observed Mass for $C_{50}H_{70}N_8O_8S$ $[M+Na]^+$: 965.66

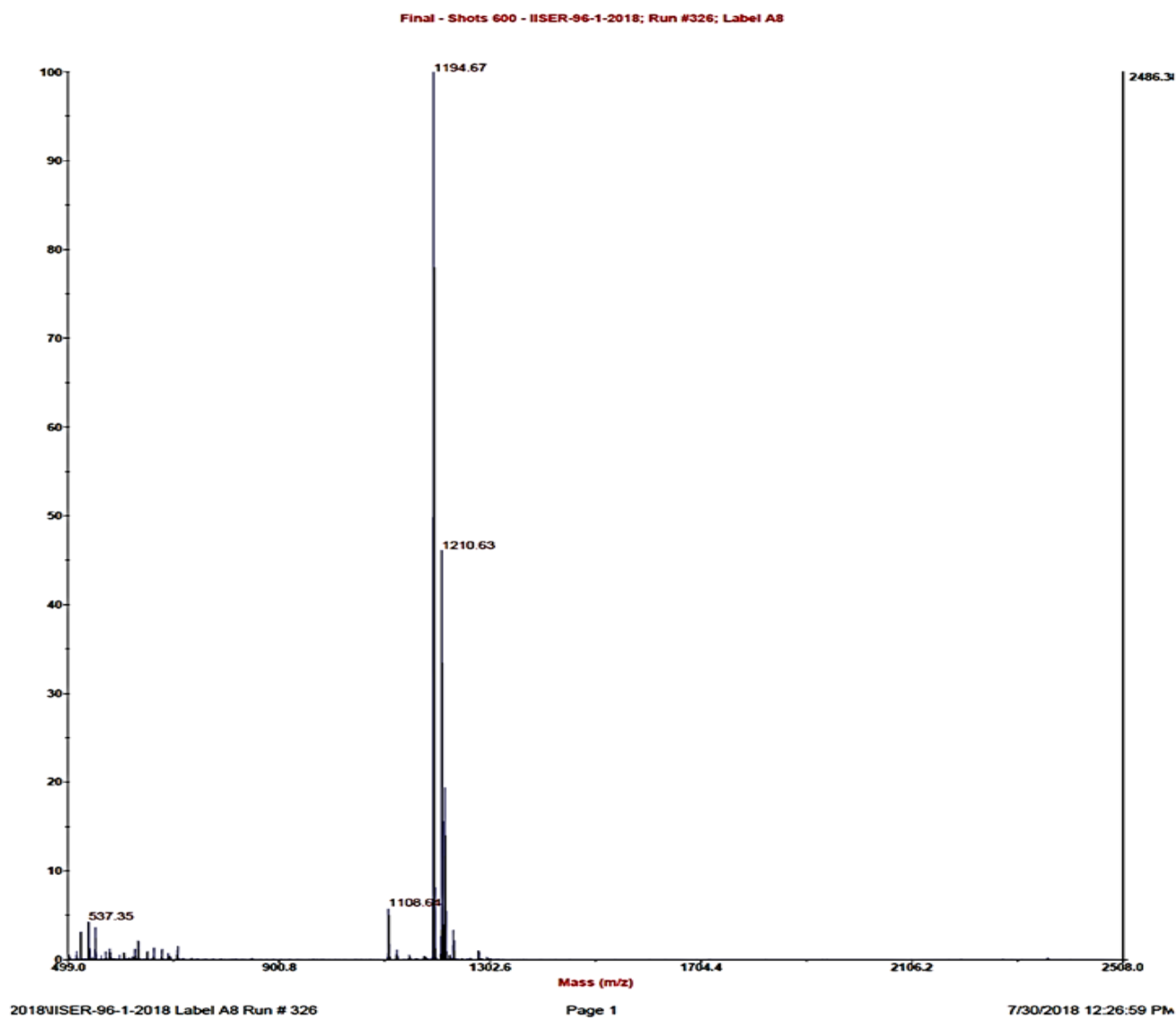


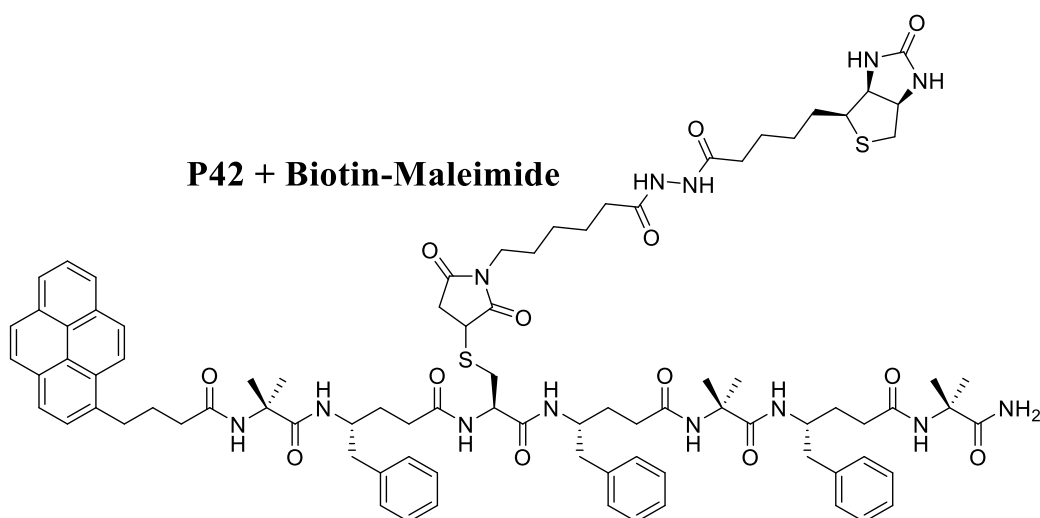
P42



Calculated Mass For $C_{68}H_{82}N_8O_8S$ $[M+Na]^+$: 1194.60

Observed Mass For $C_{68}H_{82}N_8O_8S$ $[M+Na]^+$: 1194.67





Calculated Mass For $\text{C}_{88}\text{H}_{111}\text{N}_{13}\text{O}_{13}\text{S}_2$ $[\text{M}+\text{Na}]^+$: 1646.05

Observed Mass For $\text{C}_{88}\text{H}_{111}\text{N}_{13}\text{O}_{13}\text{S}_2$ $[\text{M}+\text{Na}]^+$: 1646.60

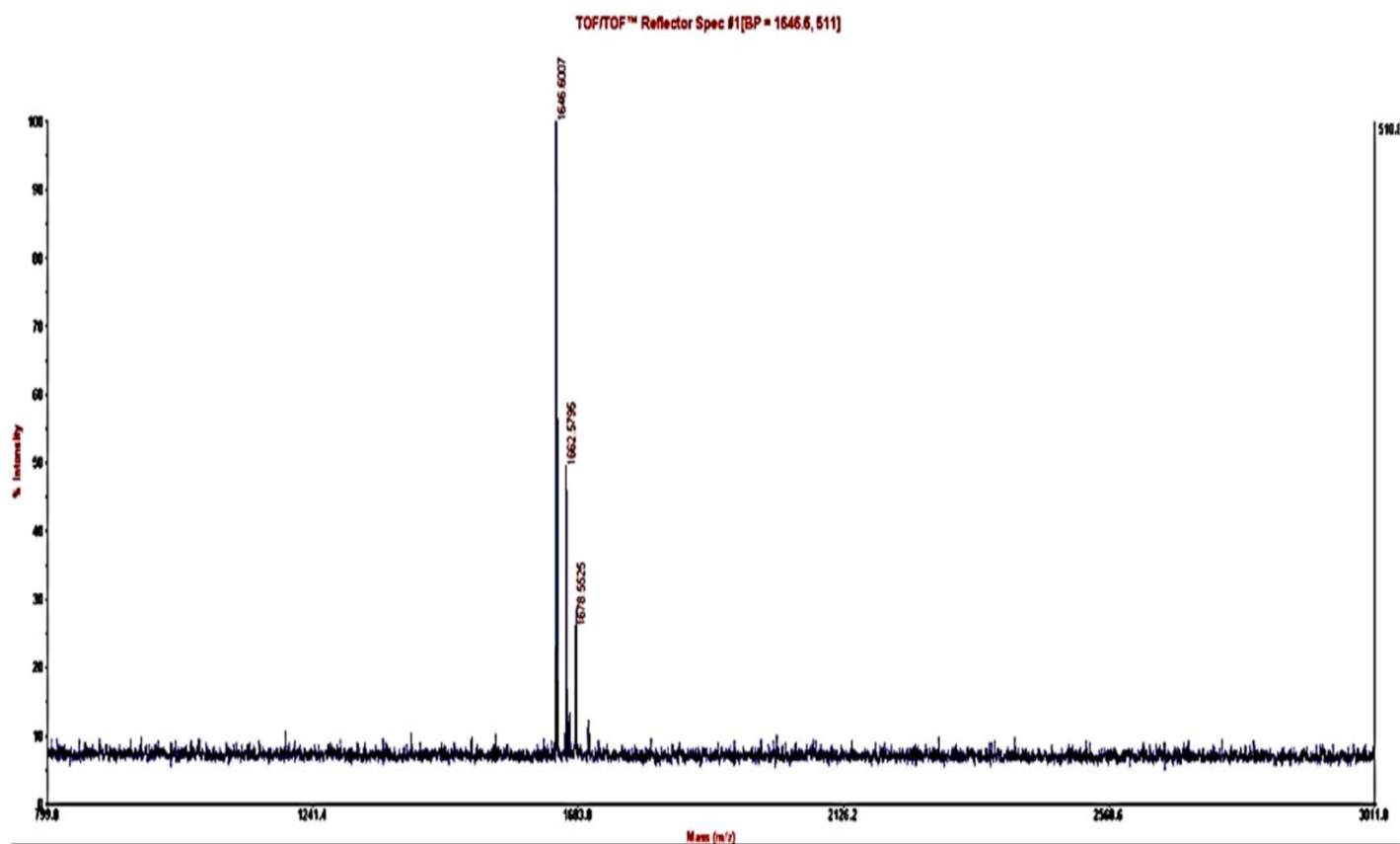


Figure 14. Mass spectra of the Peptides.

Chapter 5

Designing β -Double Helix Structures from α,γ -Hybrid Foldamers

5.1. Introduction

Mimicking the folded structures of biomolecules using artificial building blocks that possess diverse H-bonding capabilities is an area of research that has garnered significant interest.¹ This pursuit has been eloquently explored to design mimetics of protein secondary structures, employing oligomers of β -, γ -, and higher homologue amino acids.² In comparison to α -peptides and homooligomers of β - and γ -amino acids, both theoretical and experimental investigations have revealed a diverse range of helical structures with different H-bond pseudocycles present in the heterooligomers of $(\alpha\beta)_n$,³ $(\alpha\gamma)_n$,⁴ and $(\beta\gamma)_n$ ⁵ sequences. The incorporation of more than one type of amino acid in these heterooligomers has significantly expanded the repertoire of folded helical structures. The heterooligomers of $(\alpha\beta)_n$ -sequences have demonstrated the adoption of various helical structures, including 11-, 14/15-, and 11/9-helices. Similarly, the $(\alpha\gamma)_n$ sequences have exhibited the ability to form 12- and 12/10-helical structures. In contrast to the 14-helical structures observed in $(\gamma)_n$ sequences,⁶ our recent work has demonstrated the formation of β -double helical structures from oligomers of 4,4-dialkyl α,β -unsaturated γ -amino acids (commonly known as (*E*)-vinyllogous amino acids).⁷ Furthermore, we demonstrated that oligomers of (*E*)- α,β -unsaturated γ -amino acids without 4,4-dialkyl substitution adopt β -sheet type structures.⁸ Previously, Clardy, Schreiber, and their colleagues also demonstrated β -sheet type structures from oligomers of (*E*)-vinyllogous amino acids.⁹ Through computational studies, Hofmann and colleagues explored the various types of helical structures available to homooligomers of (*E*)- and (*Z*)-vinyllogous amino acids.¹⁰ In addition, α,β -unsaturated γ -amino acids have been investigated for their potential to design a wide array of folded structures.¹¹ In contrast to the prevalent protein secondary structures such as α -helix and β -sheets, β -double helices are rare in proteins. However, these unique β -double helix structures have been observed in certain natural peptide sequences such as gramicidins A-D and feglymycin.^{12,13} And also In nucleic acid, DNA,¹⁴ two nucleotide strands join together through regular H-bonding between nucleotide bases to form a double-helical secondary structure, which is a frequent and significant structural motif. The natural double helical structures are shown in Figure 1.

Numerous attempts have been made over the years in the literature to design β -double helix structures using polypeptides made up of alternate L- and D- α amino acids.¹⁵ Beneditti and colleagues used polypeptides with 1:1 alternate L- and D- α amino acids to study the double helix formation, which was influenced by the double helix structure in gramicidin A.^{15a} It has been demonstrated that aromatic oligoamide foldamers^{16,17} and synthetic polymers¹⁸ adopt

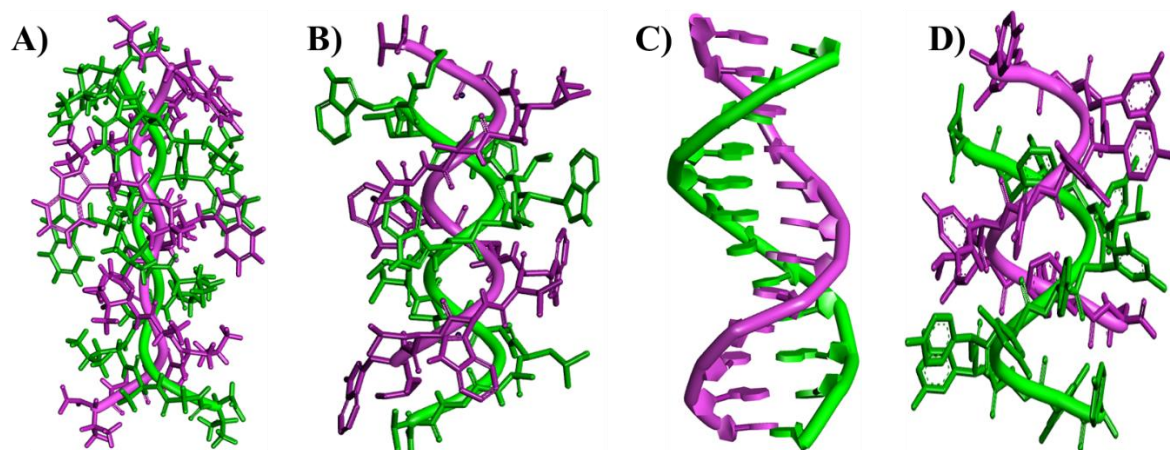


Figure 1: Occurrences of double helices in nature (A) Left-handed parallel double helical structure of gramicidin A (PDB: 1MIC). (B) Right-handed antiparallel double helical structure of gramicidin A (PDB: 1AV2). (C) Double-helical DNA structure (PDB: 1BNA). (D) Feglymycin, a right-handed antiparallel double helical structure (PDB: 1W7R).

double helical conformations in contrast to peptides. Lehn and his colleagues first described double-stranded helical metal complexes in their fundamental work, in which two linear oligodonor ligands wrap around two or more metal centers to design a double helix.¹⁹ Iverson and colleagues described the double helical structures that resulted from the alternating aromatic stacking in water of electron deficient 1,4,5,8-naphthalene-tetracarboxylic diimide (NDI) and electron rich 1,5-dialkoxy-naphthalene(DAN).²⁰ Additionally, Yashima and colleagues showed that the double helices formed from the meta-terphenyl derivatives are stabilised by amidinium-carboxylate salt bridges.²¹ Lehn and Huc also described the homodimeric double helical structures made from aromatic oligoamides that were supported by H-bonds between the β -strands and π - π staking.^{16a} As shown in the aromatic oligoamides, organic templates, L, D-peptides, and (*E*)-vinyllogous $\gamma^{4,4}$ -amino acid homooligomer, representative examples of synthetic double helical structures are illustrated in Figure 2. In this Chapter, we are demonstrating the antiparallel double helix conformations from a new class of α,γ -hybrid peptides formed by the combination of 1:1 L- α amino acid and (*E*)-vinyllogous $\gamma^{4,4}$ -amino acid homooligomer. Though natural and synthetic parallel and antiparallel double helix are reported are made by either L and D- α polypeptides or aromatic oligomide or through salt-bridge interaction, there is no examples in the literature showing the chiral antiparallel double helix from the L- α amino acid except feglymycin.

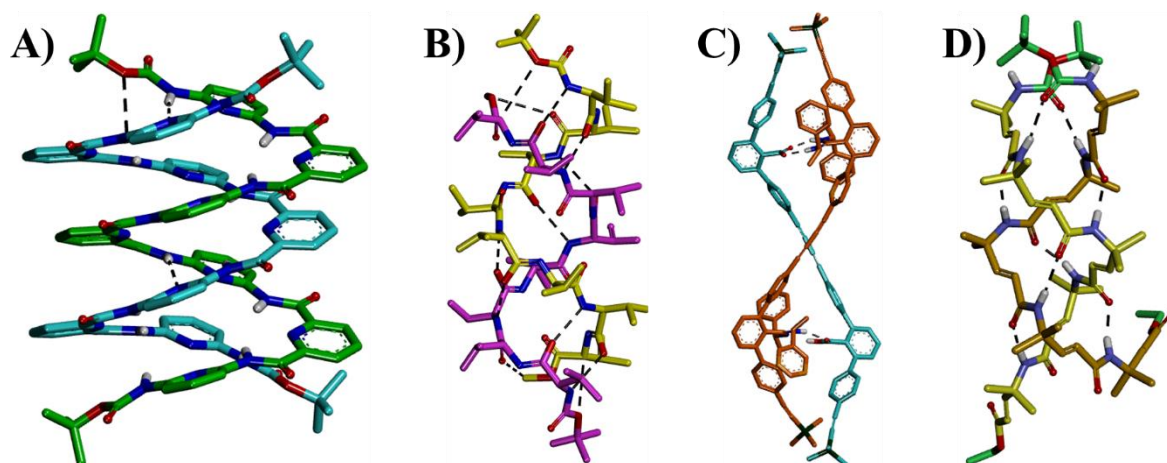


Figure 2: Artificial structures with double helices. A) Aromatic oligomides,^{16a} B) Polypeptide of L, D, valine,^{15a} C) Meta-terphenyl derivatives via salt bridge interaction,^{21a} D) (*E*)-vinyllogous $\gamma^{4,4}$ -amino acid homooligomer.⁷

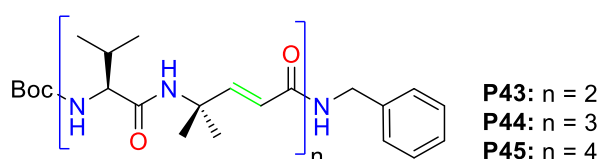
5.2. Aim and Rationale of the Present Work

Inspired by the structural diversity observed in the $(\alpha\gamma)_n$ -hybrid peptides and the β -double helix from the γ -amino acid oligomers, we sought to investigate the types of structures available to the $\alpha\gamma$ -hybrid sequences consisting of 1:1 alternating α -Val and (*E*)- α,β -unsaturated 4,4-dimethyl γ -amino acids. Here, we are reporting a novel β -double helix structure from the octapeptide consisting of alternating α - and γ -amino acids in solution and the stability of the double helix. In contrast to the hexapeptide, the octapeptide spontaneously adopted a β -double helix structure in solution.

5.3. Results and Discussions

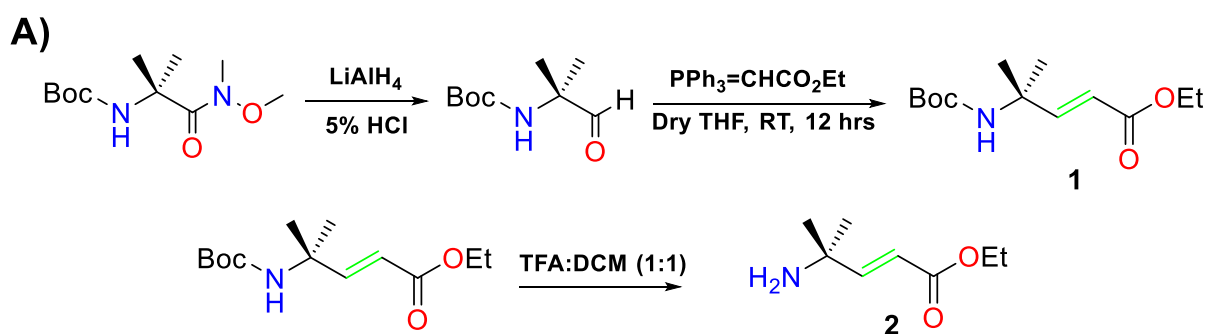
5.3.1. Design and synthesis

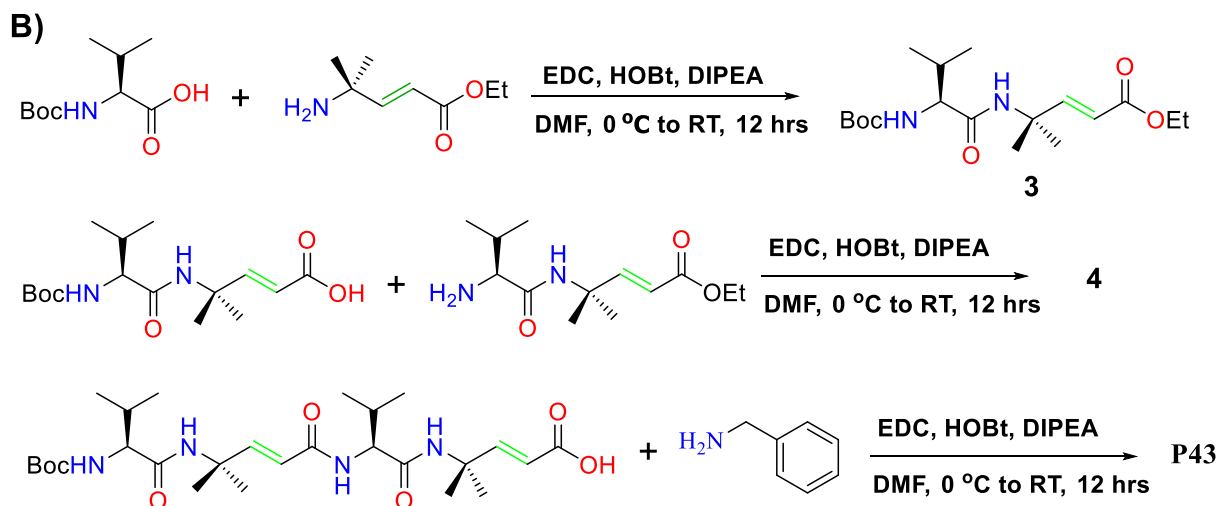
To investigate the structural properties of α,γ -hybrid peptides consisting of a 1:1 alternating pattern of α -Val and 4,4-dimethyl- γ -amino acids, we designed tetrapeptide, hexapeptide and octapeptide sequences, as depicted in Scheme 1. All peptides were synthesized in the solution phase using a fragment coupling strategy. The Boc-(*E*)- α,β -unsaturated 4,4-dimethyl- γ -amino acid (**1**) was synthesised using the procedures described earlier.²² The N terminal was protected using a *tert*-butoxycarbonyl (Boc) group, while the C terminus was protected using an ethyl



Scheme 1: The α,γ -hybrid peptides composed of α -Val and (*E*)- α,β -unsaturated 4,4-dimethyl- γ -amino acids.

ester group. For the N- and C-termini, protecting groups were eliminated using trifluoroacetic acid and saponification, respectively. N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBt) were used to perform couplings. By using a condensation approach that included Boc-Val-OH and H₂N-d γ Aic-OEt (**2**), DIPEA as an activator base, DMF as the solvent, and EDC/HOBt as coupling reagents, the dipeptides were synthesised. After being purified using silica gel (120-200 mesh), the resulting dipeptides (**3**) were used in other applications. Boc-Val-d γ Aic-COOH and NH₂-Val-d γ Aic-OEt were used in a 2+2 condensation approach to synthesize the tetrapeptide Boc-Val-d γ Aic-Val-d γ Aic-OEt (**4**). The N-terminal tetrapeptide acid Boc-Val-d γ Aic-Val-d γ Aic-OH and benzylamine (Bn) were used in a [4 + 1] condensation to yield the peptide Boc-Val-d γ Aic-Val-d γ Aic-Bn (**P43**). The N-terminal dipeptide acid Boc-Val-d γ Aic-OH and H₂N-Val-d γ Aic-Val-d γ Aic-Bn were used in a [2 + 4] condensation that yielded the hexapeptide Boc-Val-d γ Aic-Val-d γ Aic-Val-d γ Aic-Bn (**P44**). The N-terminal tetrapeptide acid Boc-Val-d γ Aic-Val-d γ Aic-COOH and H₂N-Val-d γ Aic-Val-d γ Aic-Bn were used in a [4 + 4] condensation to yield the octapeptide Boc-Val-d γ Aic-Val-d γ Aic-Val-d γ Aic-Val-d γ Aic-Bn (**P45**). Finally, using a MeOH/H₂O gradient system and reversed-phase HPLC, all peptides were purified and their identities were confirmed through mass spectral analysis.





Scheme 2: A schematic illustration of the synthetic process for A) Building block of a monomer and B) Peptide **P43**. Peptide **P44** and **P45** were both synthesised using a similar method.

5.3.2. Circular Dichroism (CD) analysis of peptides

To understand the conformations of **P43**, **P44** and **P45**, we conducted circular dichroism (CD) analysis on these peptides in CHCl_3 . The peptide **P43** showed CD spectra with CD minima observed at 246 nm, on the other hand hexapeptide (**P44**) and octapeptide (**P45**) displayed similar CD spectra with CD maxima observed at around 254 and 252 nm, respectively (Figure 3). The CD spectra displayed by peptides **P44** and **P45** were different from peptide **P43** and also different from those of β -sheet peptides composed of complete α,β -unsaturated γ -amino acids. Typically, β -sheet structures consisting of L-amino acids exhibit CD minima, not maxima. The CD analysis suggests that **P44** and **P45** adopted different conformations in solution compared to β -sheet structures.

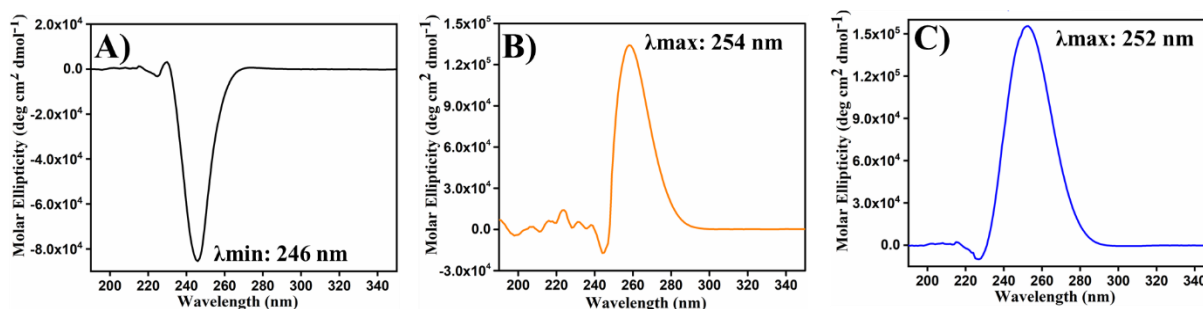


Figure 3: Circular dichroism spectrum of (A) tetrapeptide (B) hexapeptide and (C) octapeptide consisting of α -Val and (E)- α,γ -unsaturated 4,4-dimethyl- γ -amino acids in CHCl_3 .

5.3.3. Single crystal X-ray analysis of peptides P43

Crystal structures are very important in understanding the helical parameters such as residue – per-turn, rise-per-turn, helical radius and the projection of amino acids side-chains in the newly designed peptides. Keeping this in mind, we attempted to grow the X-ray quality crystals from all these peptides in a various solvent combinations. We got the single crystal only for the peptide **P43** obtained from the slow evaporation of MeOH/H₂O solution, for other peptides (**P44** and **P45**) we did not get the single crystals even after several trials with various solvent combinations. The X-ray structure of peptide **P43** is shown in Figure 4. This structure suggested that in peptide **P43** there is no intra molecular hydrogen bonds and peptide backbone is taking almost planar structure. The backbone torsion angles of the peptide **P43** are tabulated in the Table 1. Since we did not get the single crystals for the peptide **P44** and **P45** even after several trials with various solvent combinations, we thought to investigate their structure by analysing their 2D-NMR experiments.

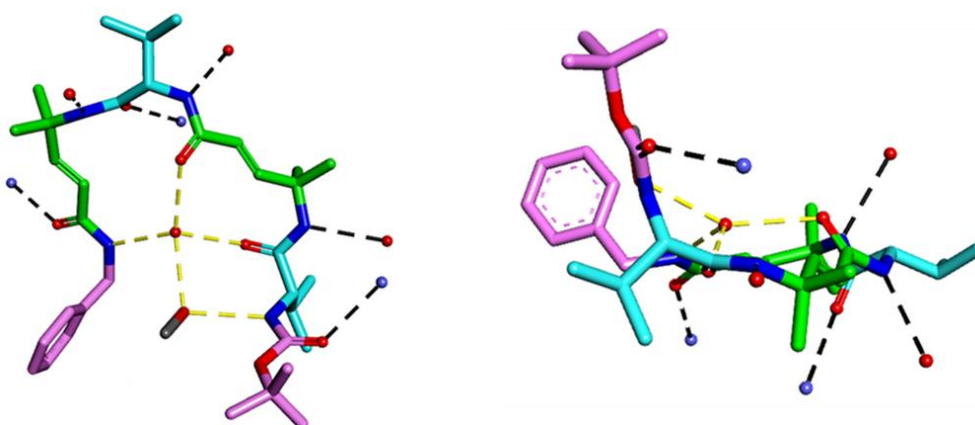


Figure 4: X-ray structures of peptide **P43** in MeOH/H₂O.

Table 1: Backbone torsion angles (in degree) of peptide **P43**.

Peptide Unit	Φ [°]	θ_1 [°]	θ_2 [°]	Ψ [°]
Val (1)	-88.60	-	-	-127.54
dγAic (2)	45.68	-155.21	-178.24	171.65
Val (3)	-71.92	-	-	143.78
dγAic (4)	81.56	-0.04	177.46	-179.95

5.3.4: Solution conformation of peptide **P44** and **P45**

To further investigate the conformations of these peptides, we subjected them to 2D NMR analysis. In order to get well-resolved ^1H NMR, we subjected **P44** and **P45** for temperature-dependent NMR in the solvent CDCl_3 . Both peptides are highly soluble in chloroform, indicating the possibility of ordered structures of peptides in solution. The temperature-dependent partial ^1H NMR spectra are shown in Figure 5. The ^1H NMR analysis revealed that **P44** exhibited a poorly dispersed spectrum in chloroform at room temperature. As the temperature decreased from 313K to 233K, we observed a more dispersed ^1H NMR spectrum at 263K and lower temperatures. However, the analysis indicates that there may be more than one conformation of **P44** in solution. In comparison, the temperature-dependent ^1H NMR of **P45** displayed a well-dispersed spectrum at 283K and at lower temperatures.

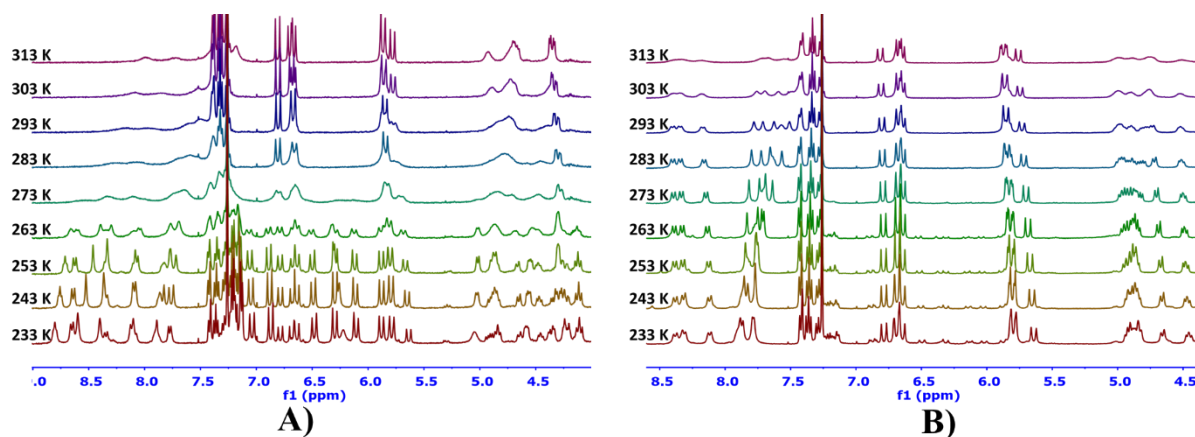


Figure 5: Temperature dependent ^1H NMR of A) **P44** and B) **P45** in CDCl_3 .

As peptide **P45** displayed well dispersed ^1H NMR, we chose 278K to record 2D-NMR. We utilized TOCSY to identify the amino acids (Figure 6), and their positions were further assigned using ROESY analysis.

Upon conducting the preliminary inspection of the 2D ROESY spectrum for the peptide **P45**, we made an intriguing observation of several unusual long-range Nuclear Overhauser Effects (NOEs), particularly between the Boc- and Benzyl groups. This intriguing finding suggested the possible formation of a dimer of the peptide **P45** in an antiparallel manner. Motivated by these long-range NOEs, we conducted a preliminary replica exchange molecular dynamics (REMD) simulation involving two peptide **P45** molecules.

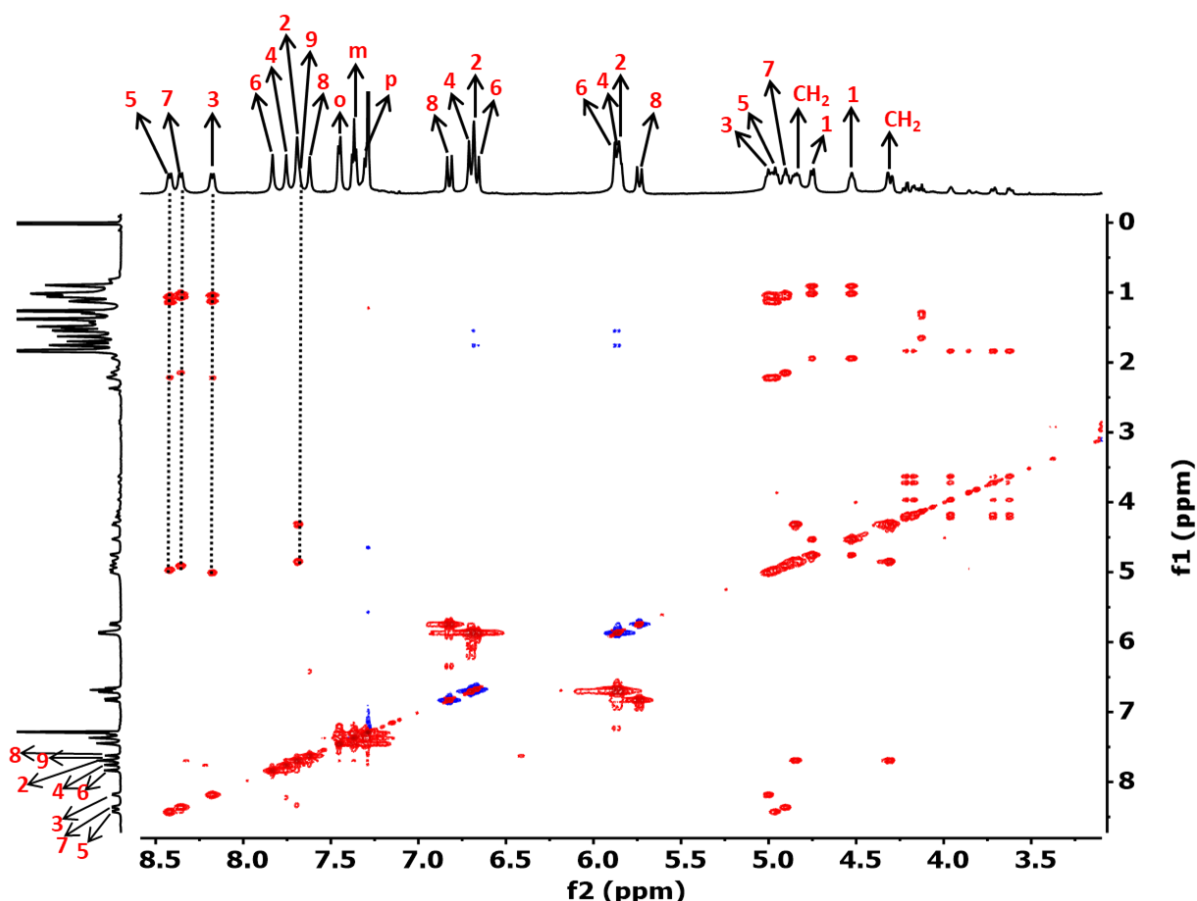


Figure 6: TOCSY spectrum of peptide **P45** (5 mM) in CDCl₃ at 278K.

The REMD simulation results indicated that the peptide **P45** adopted an antiparallel β -double helix conformation as its minimum energy structure. This finding strongly supported the NOEs pattern observed in the ROESY analysis, providing valuable insight into the spatial arrangement and interactions within the peptide.

Based on the model double helix structure obtained from the REMD simulation for the peptide **P45**, we categorized the observed Nuclear Overhauser Effects (NOEs) as either intramolecular (within the same peptide molecule) or intermolecular (between different peptide molecules in the dimer). Notably, we observed very strong NOE between 1NH $\leftrightarrow$ 7CaH, indicating strong interactions within the dimers. As the peptide consisting of four α,β -unsaturated γ -amino acids in the sequence, we rule out the possibility of close proximity between 1NH and 7CaH (in a single molecule). The long-range intermolecular NOEs between NH $\leftrightarrow$ NH and NH $\leftrightarrow$ C β H=C α H are presented in Figure 7A, while the NOEs involving NH $\leftrightarrow$ C α H and NH $\leftrightarrow$ C γ H (γ -residues) are illustrated in Figure 7B. Additionally, Figure 8A and 8B showcase the NOEs between CH $\leftrightarrow$ CH double protons and C α H $\leftrightarrow$ C α H, respectively. These distinct

NOE patterns offer valuable insights into the spatial arrangement and interactions within the β -double helix structure of the peptide.

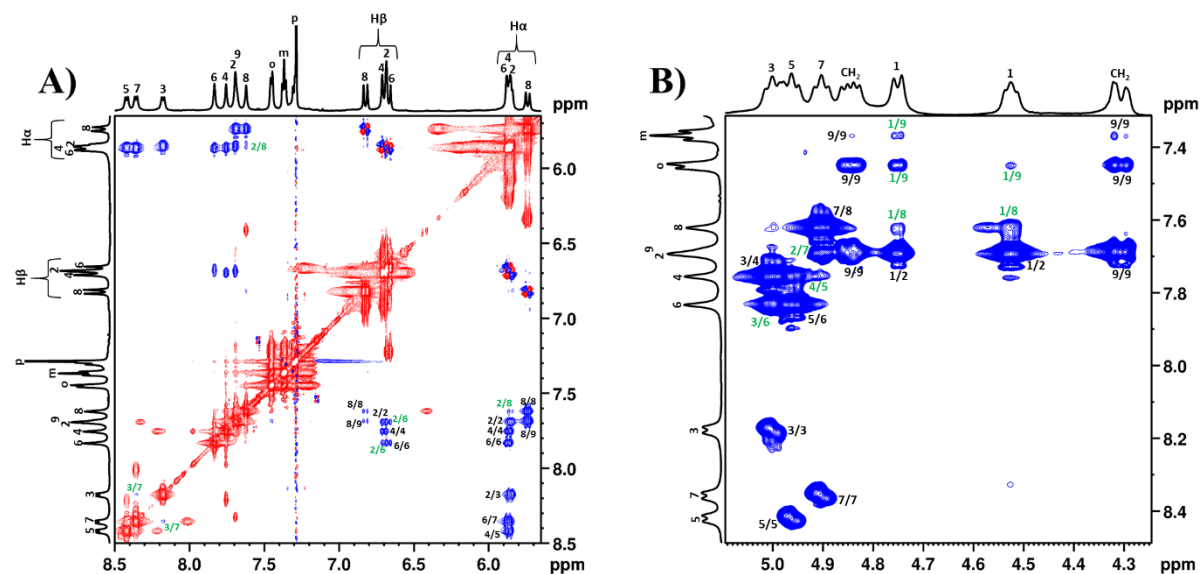


Figure 7: A) Partial ROESY spectrum of peptide **P45** depicting the inter-strand long range NOEs. (A) NH $\leftrightarrow$ NH, NH $\leftrightarrow$ C β H (γ -residues) and NH $\leftrightarrow$ C α H (γ -residues) (B) NH $\leftrightarrow$ C α H (α -Val) C β H, C α H (γ -residues) $\leftrightarrow$ C α H (α -Val), -CH₂-Ph. (**Intra** and **inter**-molecular NOEs are marked with **black** and **green** labels respectively).

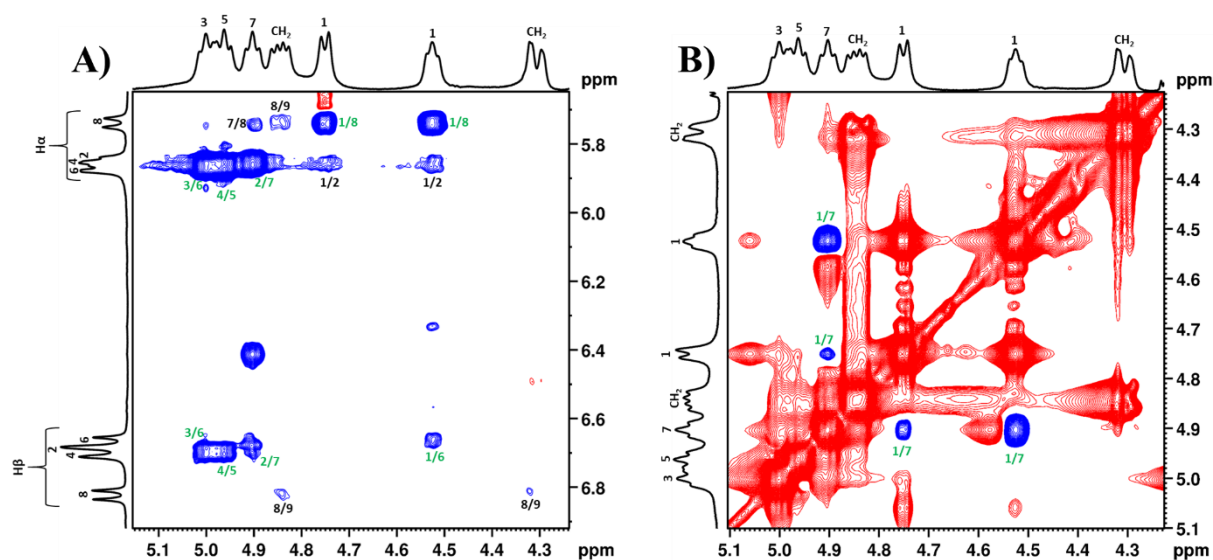


Figure 8: Partial ROESY spectrum of peptide **P45** depicting the long range inter-strand NOEs. (A) NH $\leftrightarrow$ H α (γ -residues) and H α $\leftrightarrow$ H β (γ -residues), (B) NH $\leftrightarrow$ H α (α -residue) and H α $\leftrightarrow$ H α (α -residue) (**Intra** and **inter**-molecular NOEs are marked with **black** and **green** labels respectively).

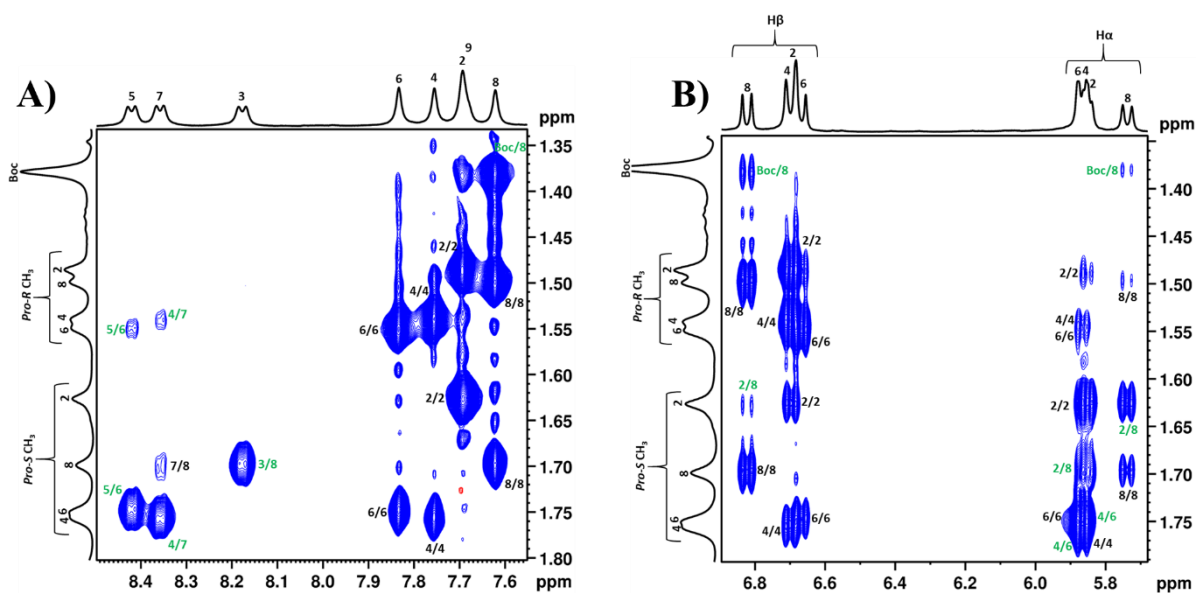


Figure 9: Partial ROESY spectrum of **P45** (5 mM) in CDCl₃ showing A) NH $\leftrightarrow$ *pro-R* or *S* CH₃ NOEs. B) H α or H β $\leftrightarrow$ *pro-R* or *S* CH₃ and Boc $\leftrightarrow$ H α or H β NOEs. (**Intra** and **inter**-molecular NOEs are marked with **black** and **green** labels respectively).

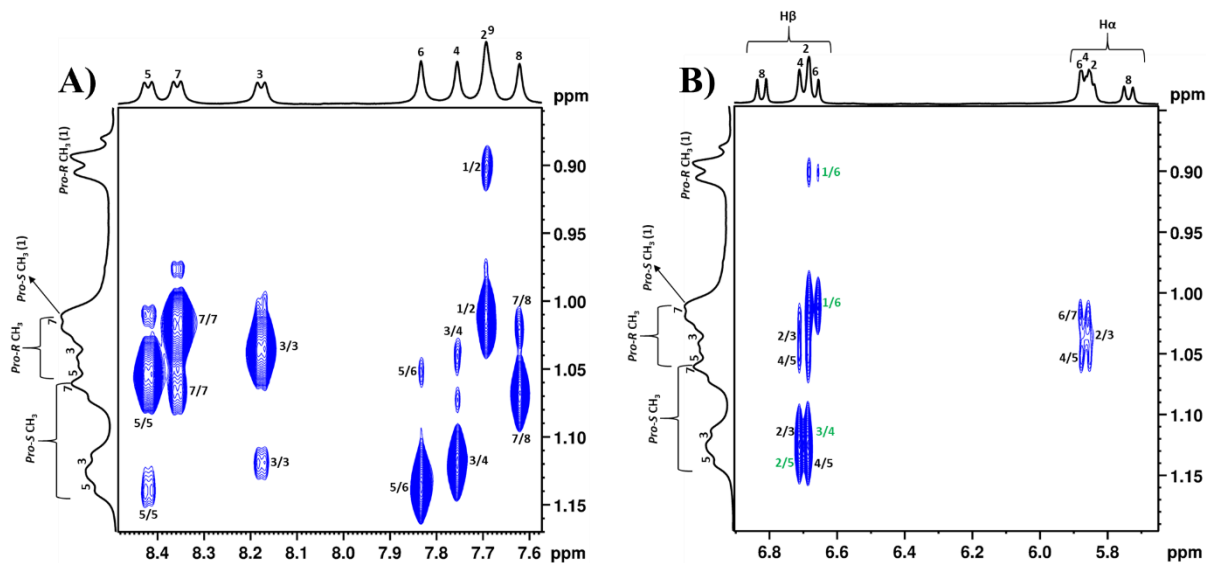


Figure 10: Partial ROESY spectrum of **P45** (5 mM) in CDCl₃ showing A) NH $\leftrightarrow$ *pro-R* or *S* CH₃ NOEs. B) H α or H β $\leftrightarrow$ *pro-R* or *S* CH₃ NOEs. (**Intra** and **inter**-molecular NOEs are marked with **black** and **green** labels respectively).

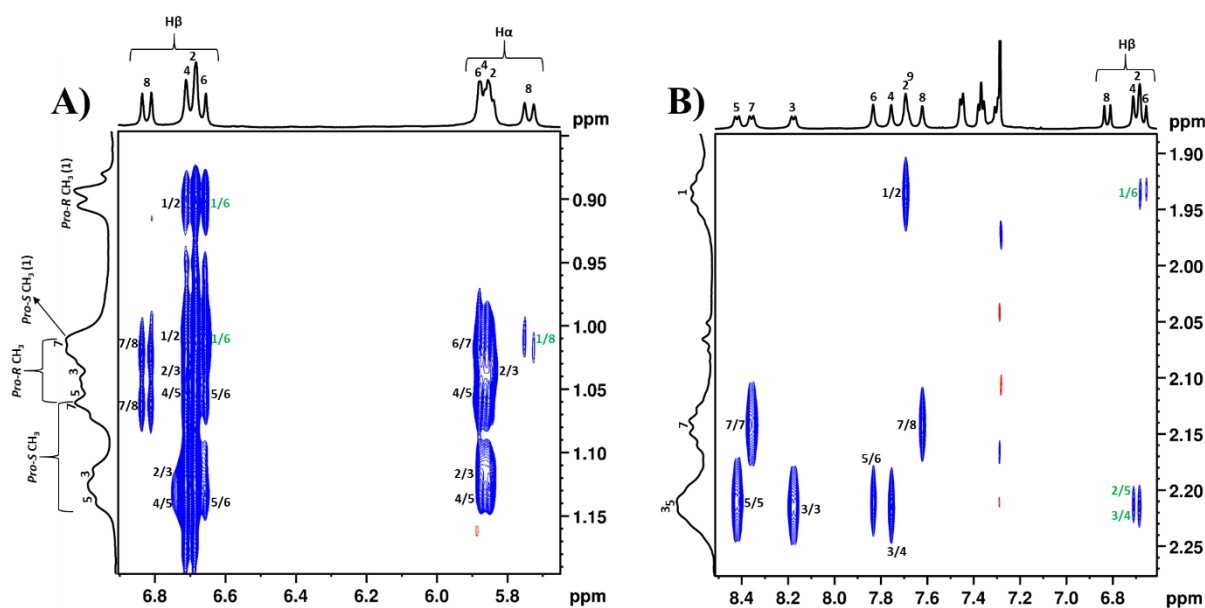


Figure 11: Partial ROESY spectrum of **P45** (5 mM) in CDCl_3 showing A) $\text{H}\alpha \leftrightarrow \text{pro-R or S CH}_3$ and $\text{pro-R or S CH}_3 \leftrightarrow \text{H}\alpha$ or $\text{H}\beta$ NOEs. B) $\text{H}\beta \leftrightarrow \text{NH}$ and $\text{H}\beta \leftrightarrow \text{H}\beta$ NOEs. (**Intra** and **inter**-molecular NOEs are marked with **black** and **green** labels respectively).

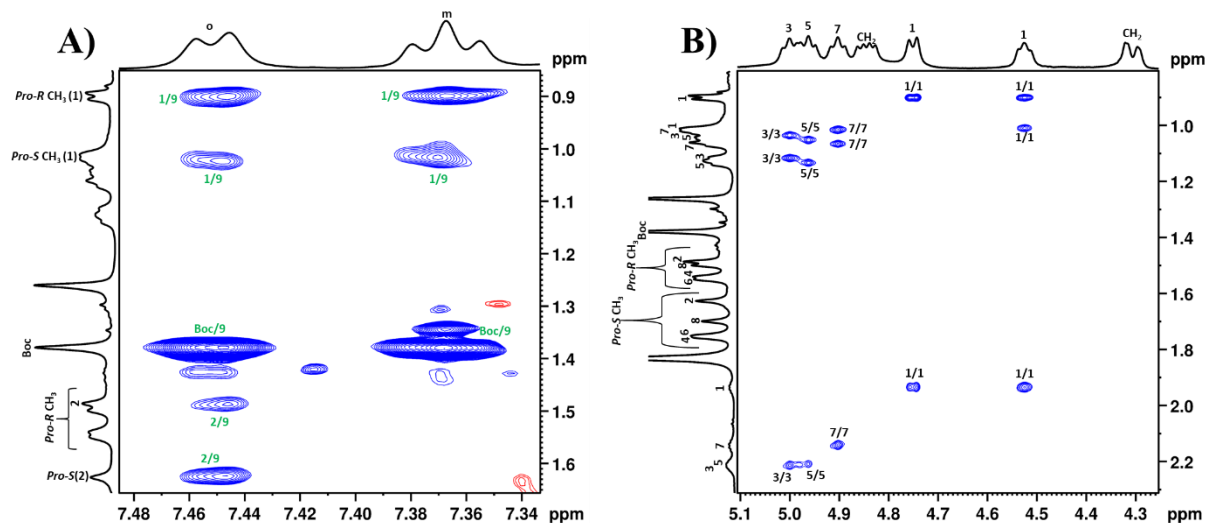


Figure 12: Partial ROESY spectrum of **P45** (5 mM) in CDCl_3 showing A) $\text{pro-R or S CH}_3 \leftrightarrow \text{Ho or Hm}$ and $\text{Boc} \leftrightarrow \text{Ho or Hm}$ NOEs. B) $\text{H}\alpha \leftrightarrow \text{pro-R or S CH}_3$, $\text{NH} \leftrightarrow \text{pro-R CH}_3$, $\text{H}\alpha \leftrightarrow \text{H}\beta$ and $\text{NH} \leftrightarrow \text{H}\beta$ NOEs. (**Intra** and **inter**-molecular NOEs are marked with **black** and **green** labels respectively).

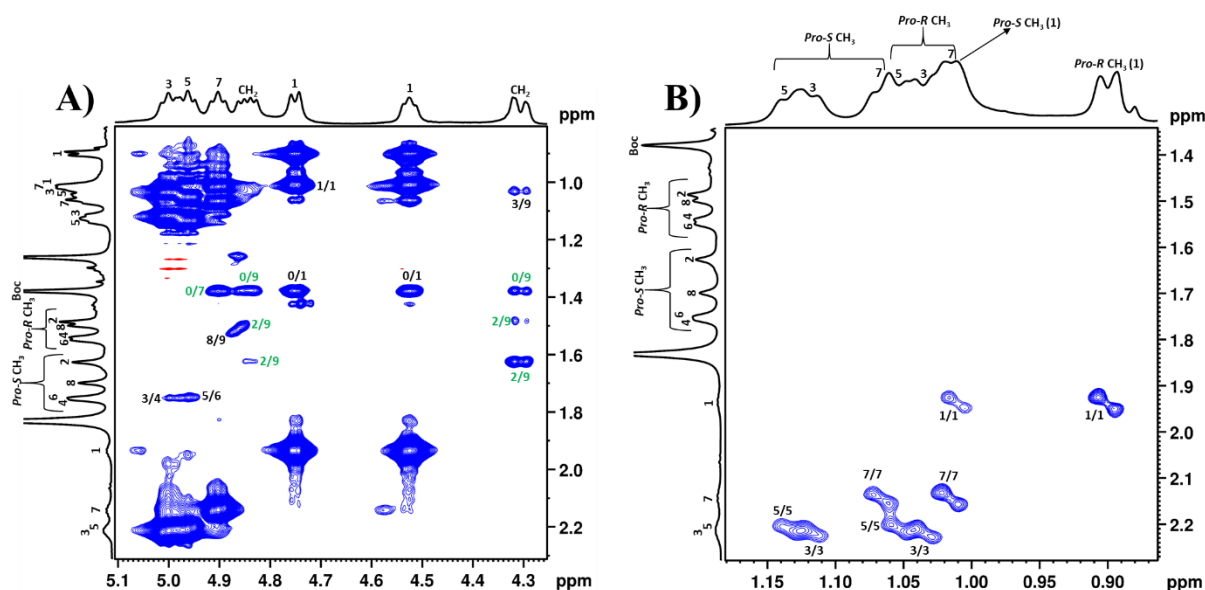


Figure 13: Partial ROESY spectrum of **P45** (5 mM) in CDCl₃ showing A) Boc $\leftrightarrow$ H α , Boc $\leftrightarrow$ NH, Boc $\leftrightarrow$ CH₂, NH or H α $\leftrightarrow$ pro-S CH₃, and pro-R or S CH₃ $\leftrightarrow$ CH₂ NOEs. B) H β $\leftrightarrow$ pro-R or S CH₃ NOEs. (**Intra** and **inter**-molecular NOEs are marked with **black** and **green** labels respectively).

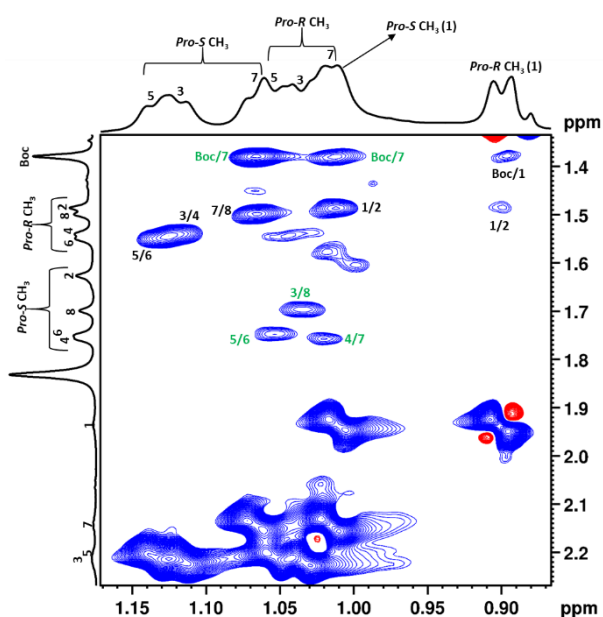


Figure 14: Partial ROESY spectrum of **P45** (5 mM) in CDCl₃ showing Boc $\leftrightarrow$ pro-R or S CH₃, pro-R or S CH₃ $\leftrightarrow$ pro-R CH₃ and pro-R CH₃ $\leftrightarrow$ pro-S CH₃ NOEs. (**Intra** and **inter**-molecular NOEs are marked with **black** and **green** labels respectively).

In addition to the $1\text{NH} \leftrightarrow 7\text{C}\alpha\text{H}$, We observed several long-range inter-strand strong NOEs, between $\text{Boc} \leftrightarrow 8\text{NH}$, $2\text{C}\gamma\text{H}_3 \leftrightarrow 8\text{C}\beta\text{H}$, $4\text{C}\alpha\text{H} \leftrightarrow 8\text{C}\gamma\text{H}_3$, and $\text{Boc} \leftrightarrow \text{CH}$ (m-benzyl group). Additionally, we observed medium NOEs between $2\text{NH} \leftrightarrow 7\text{C}\alpha\text{H}$, $3\text{C}\alpha\text{H} \leftrightarrow 6\text{NH}$, $4\text{NH} \leftrightarrow 5\text{C}\alpha\text{H}$, $3\text{NH} \leftrightarrow 8\text{C}\gamma\text{H}_3$, $4\text{C}\gamma\text{H}_3 \leftrightarrow 7\text{NH}$, $5\text{NH} \leftrightarrow 6\text{C}\gamma\text{H}_3$, and $\text{Boc} \leftrightarrow 8\text{C}\alpha\text{H}$, as well as weak NOEs between $2\text{C}\beta\text{H} \leftrightarrow 5\text{C}\beta\text{H}$, $2\text{C}\beta\text{H} \leftrightarrow 6\text{NH}$, $2\text{C}\alpha\text{H} \leftrightarrow 8\text{NH}$, $6\text{C}\beta\text{H} \leftrightarrow 2\text{NH}$, $1\text{C}\alpha\text{H} \leftrightarrow 8\text{NH}$, $1\text{NH} \leftrightarrow \text{CH}$ (o-benzyl group), $1\text{C}\alpha\text{H} \leftrightarrow 8\text{C}\alpha\text{H}$, $2\text{C}\alpha\text{H} \leftrightarrow 7\text{C}\alpha\text{H}$, $1\text{NH} \leftrightarrow 7\text{C}\alpha\text{H}$, $2\text{C}\beta\text{H} \leftrightarrow 5\text{C}\beta\text{H}$, $3\text{NH} \leftrightarrow 7\text{NH}$, $2\text{C}\beta\text{H} \leftrightarrow 6\text{NH}$, and $3\text{C}\beta\text{H} \leftrightarrow 4\text{C}\beta\text{H}$. Furthermore, several very weak NOEs were also observed. Detailed information regarding all the intra and inter-strand NOEs can be found in the Table 1 and 2.

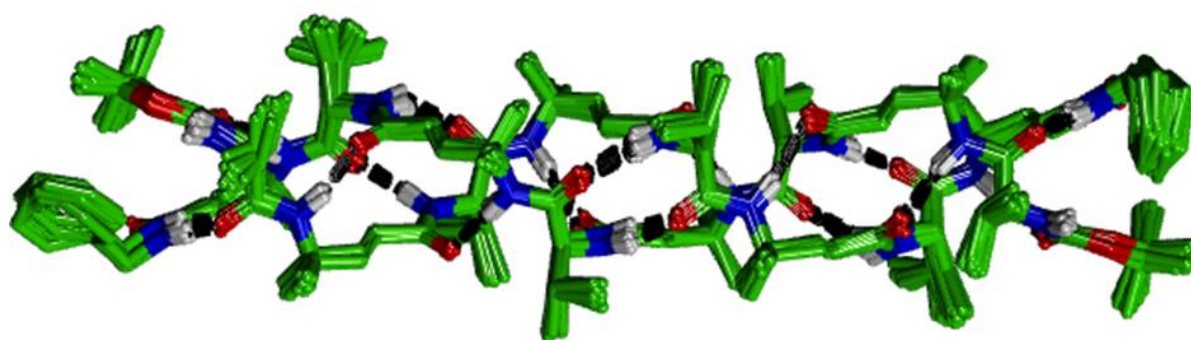
Table 1: Intra-molecular NOEs used in structure calculation and their classification.

$\text{H}\beta/\text{Pro-R CH}_3$ (4/4), $\text{H}\alpha/\text{NH}$ (3/4), $\text{H}\alpha/\text{Pro-S CH}_3$ (2/2), $\text{H}\beta/\text{Pro-R CH}_3$ (3/3), $\text{H}\alpha/\text{NH}$ (1/2), $\text{H}\beta/\text{Pro-S CH}_3$ (3/3), $\text{H}\alpha/\text{NH}$ (8/9), $\text{H}\alpha/\text{Pro-S CH}_3$ (4/4), $\text{H}\beta/\text{Pro-S CH}_3$ (8/8), $\text{H}\alpha/\text{Pro-R CH}_3$ (1/1), $\text{H}\alpha/\text{H}\beta$ (1/1), $\text{H}\beta/\text{Pro-R CH}_3$ (2/2), $\text{NH}/\text{Pro-R CH}_3$ (1/1), $\text{NH}/\text{H}\alpha$ (8/8), $\text{H}\alpha/\text{Pro-S CH}_3$ (6/6), $\text{H}\beta/\text{Pro-R CH}_3$ (5/5), $\text{H}\beta/\text{Pro-S CH}_3$ (5/5), $\text{H}\beta/\text{Pro-R CH}_3$ (7/7), $\text{NH}/\text{H}\beta$ (7/7), $\text{H}\beta/\text{Pro-S CH}_3$ (7/7), $\text{H}\alpha/\text{NH}$ (4/5), $\text{H}\alpha/\text{NH}$ (6/7), $\text{H}\beta/\text{Pro-R CH}_3$ (6/6), $\text{H}\beta/\text{Pro-S CH}_3$ (1/1), $\text{NH}/\text{H}\beta$ (3/3), $\text{H}\beta/\text{Pro-R CH}_3$ (8/8), $\text{H}\alpha/\text{Pro-S CH}_3$ (3/3), $\text{H}\alpha/\text{NH}$ (7/8), $\text{H}\alpha/\text{NH}$ (2/3), $\text{H}\beta/\text{Pro-R CH}_3$ (1/1), $\text{H}\alpha/\text{Pro-S CH}_3$ (7/7), $\text{H}\alpha/\text{Pro-R CH}_3$ (7/7), $\text{H}\alpha/\text{H}\beta$ (7/7), $\text{H}\alpha/\text{Pro-S CH}_3$ (5/5), $\text{H}\alpha/\text{Pro-R CH}_3$ (5/5), $\text{H}\alpha/\text{H}\beta$ (5/5), $\text{NH}/\text{H}\beta$ (5/5), $\text{H}\alpha/\text{NH}$ (5/6), $\text{H}\alpha/\text{Pro-R CH}_3$ (3/3), $\text{H}\alpha/\text{H}\beta$ (3/3), $\text{H}\alpha/\text{Pro-S CH}_3$ (1/1), $\text{NH}/\text{H}\beta$ (1/1)	Very Strong
$\text{H}\beta/\text{NH}$ (3/4), $\text{H}\beta/\text{NH}$ (7/8), $\text{NH}/\text{H}\alpha$ (2/2), $\text{NH}/\text{Pro-R CH}_3$ (5/5), $\text{NH}/\text{Pro-R CH}_3$ (7/7), $\text{NH}/\text{Pro-S CH}_3$ (2/2), $\text{NH}/\text{Pro-R CH}_3$ (4/4), $\text{NH}/\text{Pro-R CH}_3$ (2/2), $\text{NH}/\text{Pro-R CH}_3$ (6/6), $\text{H}\beta/\text{Pro-S CH}_3$ (6/6), $\text{H}\beta/\text{Pro-S CH}_3$ (4/4), $\text{NH}/\text{Pro-R CH}_3$ (8/8), $\text{NH}/\text{H}\alpha$ (4/4), $\text{NH}/\text{Pro-R CH}_3$ (3/3), $\text{H}\beta/\text{Pro-S CH}_3$ (2/2), $\text{H}\beta/\text{NH}$ (1/2), $\text{NH}/\text{H}\alpha$ (7/7), $\text{NH}/\text{H}\alpha$ (6/6), NH/NH (1/2), $\text{NH}/\text{Pro-S CH}_3$ (1/1)	Strong
$\text{NH}/\text{H}\beta$ (2/2), $\text{Pro-S CH}_3/\text{Pro-R CH}_3$ (5/6), $\text{Pro-S CH}_3/\text{Pro-R CH}_3$ (3/4), $\text{NH}/\text{H}\beta$ (4/4), $\text{H}\beta/\text{NH}$ (5/6), $\text{NH}/\text{H}\beta$ (6/6), $\text{H}\beta/\text{Pro-S CH}_3$ (2/3), $\text{Pro-S CH}_3/\text{NH}$ (7/8), $\text{Pro-S CH}_3/\text{NH}$ (5/6), $\text{Pro-S CH}_3/\text{NH}$ (3/4), $\text{Pro-S CH}_3/\text{NH}$ (1/2), $\text{H}\alpha/\text{Pro-S CH}_3$ (8/8), $\text{H}\alpha/\text{Pro-R CH}_3$ (8/8), $\text{H}\alpha/\text{Pro-R CH}_3$ (6/6), $\text{NH}/\text{H}\alpha$ (3/3), $\text{H}\alpha/\text{Pro-R CH}_3$ (2/2), $\text{NH}/\text{H}\alpha$ (5/5), $\text{Pro-S CH}_3/\text{Pro-R CH}_3$ (7/8), $\text{H}\beta/\text{Pro-S CH}_3$ (4/5), $\text{NH}/\text{Pro-S CH}_3$ (8/8), $\text{Pro-S CH}_3/\text{Pro-R CH}_3$ (1/2), $\text{H}\alpha/\text{Pro-R CH}_3$ (4/4)	Medium
$\text{Pro-R CH}_3/\text{NH}$ (7/8), $\text{Pro-R CH}_3/\text{NH}$ (1/2), $\text{H}\beta/\text{NH}$ (8/9), $\text{H}\beta/\text{Pro-R CH}_3$ (4/5), $\text{NH}/\text{Pro-S CH}_3$ (6/6), $\text{NH}/\text{Pro-S CH}_3$ (4/4), $\text{NH}/\text{Pro-S CH}_3$ (3/3), $\text{NH}/\text{Pro-S CH}_3$ (5/5), $\text{NH}/\text{H}\beta$ (8/8), $\text{NH}/\text{Pro-S CH}_3$ (7/7), $\text{H}\beta/\text{Pro-R CH}_3$ (2/3), $\text{H}\alpha/\text{Pro-R CH}_3$ (6/7), $\text{H}\alpha/\text{Pro-R CH}_3$ (4/5), $\text{H}\alpha/\text{Pro-R CH}_3$ (2/3)	Weak
$\text{Pro-R CH}_3/\text{NH}$ (5/6), $\text{NH}/\text{Pro-S CH}_3$ (7/8), $\text{H}\alpha/\text{H}\alpha$ (7/8), $\text{Pro-R CH}_3/\text{NH}$ (3/4), $\text{H}\alpha/\text{Pro-S CH}_3$ (3/4), $\text{H}\alpha/\text{Pro-S CH}_3$ (5/6), $\text{Pro-S CH}_3/\text{H}\beta$ (5/6), $\text{Pro-R CH}_3/\text{H}\beta$ (5/6), $\text{Pro-R CH}_3/\text{H}\beta$ (7/8), $\text{Pro-R CH}_3/\text{Pro-R CH}_3$ (1/2), $\text{H}\alpha/\text{Pro-S CH}_3$ (4/5), $\text{Pro-S CH}_3/\text{H}\beta$ (7/8), $\text{Pro-S CH}_3/\text{H}\beta$ (1/2), $\text{Pro-R CH}_3/\text{H}\beta$ (1/2), $\text{H}\alpha/\text{Pro-S CH}_3$ (2/3)	Very Weak

Table 2: Inter-molecular NOEs used in structure calculation and their classification.

H α /H α (1/7)	Very Strong
<i>Pro-S</i> CH ₃ /H α (2/8), <i>Pro-S</i> CH ₃ /H α (4/6), H α / <i>Pro-S</i> CH ₃ (4/6), H α / <i>Pro-S</i> CH ₃ (2/8)	Strong
<i>Pro-R</i> CH ₃ / <i>Pro-S</i> CH ₃ (5/6), <i>Pro-S</i> CH ₃ / <i>Pro-R</i> CH ₃ (4/7), <i>Pro-R</i> CH ₃ / <i>Pro-S</i> CH ₃ (3/8), NH/ <i>Pro-S</i> CH ₃ (3/8), <i>Pro-S</i> CH ₃ /H β (2/8), <i>Pro-S</i> CH ₃ /H β (1/6), H β / <i>Pro-S</i> CH ₃ (2/5), <i>Pro-S</i> CH ₃ /H β (3/4), NH/H α (2/7), H α /NH (3/6), NH/H α (4/5), NH/ <i>Pro-S</i> CH ₃ (5/6), <i>Pro-S</i> CH ₃ /NH (4/7)	Medium
<i>Pro-R</i> CH ₃ /H β (1/6), H β /H β (2/5), H β /H β (3/4), H β /H β (1/6), <i>Pro-R</i> CH ₃ /Hm (1/9), H α /H α (4/5), NH/H α (1/8), H α /H α (1/8), <i>Pro-S</i> CH ₃ /Ho (2/9), <i>Pro-R</i> CH ₃ /Ho (1/9), H α /H α (2/7), H α /H α (3/6), NH/H(o) (1/9), H α /NH (1/8), NH/H α (1/7), H β /NH (6/2), NH/NH (3/7), H α /NH (2/8), H β /NH (2/6)	Weak
<i>Pro-S</i> CH ₃ /H α (1/8), NH/H(m) (1/9), H α /H(o) (1/9), <i>Pro-S</i> CH ₃ /Ho (1/9), <i>Pro-S</i> CH ₃ /Hm (1/9), H α /H β (1/6), H α /H(m) (1/9), NH/ <i>Pro-R</i> CH ₃ (5/6), <i>Pro-R</i> CH ₃ /NH (4/7), H β /H α (2/7), H α /H β (3/6), NH/NH (1/8), H β /H α (4/5), <i>Pro-R</i> CH ₃ /Ho (2/9)	Very Weak

All the observed NOEs were utilized for the calculation of the structure of **P45**. The solution structure calculation was performed using a simulated annealing protocol in a vacuum with DESMOND (Version- 6.8.137) and the OPLS 2005 force field. A low-energy antiparallel double helix structure was selected based on a preliminary REMD simulation. The upper limit distance restraints used for H...H NOEs were 2.5 Å for very strong, 3 Å for strong, 3.5 Å for medium, 4.5 Å for weak, and 5.5 Å for very weak NOEs. In cases where NOEs involved one CH₃, C...H upper limit distance restraints were set at 3 Å for very strong, 3.5 Å for strong, 4.5 Å for medium, 5.5 Å for weak, and 6.5 Å for very weak NOEs. For NOEs involving two CH₃ groups, C...C upper limit distance restraints were chosen at 5 Å for medium and 7 Å for very weak NOEs. All lower limits for NOE distance restraints were set at 1.8 Å, and a force constant of 1 Kcalmol⁻¹ was used for all the restraints. The final temperature was maintained at 300K.

**Figure 15:** Twenty energy minimized structures of **P45** are shown. The peptide **P45** adopted anti-parallel double helix conformation.

The structural analysis of the twenty minimum energy structures derived from the calculation revealed that the peptides **P45** adopted an anti-parallel β -double helix conformation. Moreover,

our analysis suggested that two independent **P45** molecules intertwined in a manner reminiscent of DNA double helix, forming an intriguing structural arrangement. Figure 15 showcases these twenty minimum energy structures.

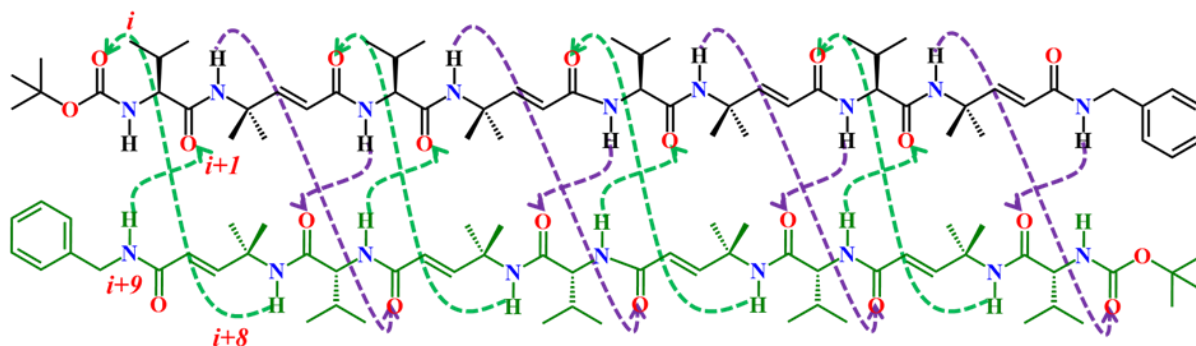


Figure 16: The H-bonding pattern observed in the anti-parallel β -double helix of **P45**.

The side chains of Val and Aib residues in **P45** were observed to project outwards from the double helix conformation. The β -double helix structure is stabilized by sixteen inter-strand hydrogen bonds. Careful analysis revealed that all amide NH and CO groups were involved in these inter-strand hydrogen bonds. The specific hydrogen bonding pattern observed in **P45** is illustrated in Figure 16. Notably, the Boc-NH is not involved in the inter-strand H-bond in the dimer. This conclusion was further supported by temperature-dependent ^1H NMR experiments conducted on **P45**. Only the Boc-amide NH showed the very less change in the chemical shift upon increasing temperature, while other amide NH groups displayed change in the chemical shift with varying temperatures. Concentration-dependent ^1H NMR experiments also confirmed the intertwined nature of the **P45** structure, as no significant changes in amide NH chemical shifts were observed with increasing peptide concentration (Figure 17).

These experimental results collectively offer strong support to the notion of **P45** adopting an anti-parallel β -double helix structure, which is in contrast to the parallel β -double helix observed in the oligomers of 4,4-dimethyl- γ -amino acids. Instead, **P45** structure resembles that of the natural peptide gramicidin A. Furthermore, the hydrogen bonding pattern in **P45** differs from that observed in the γ -peptide double-helix. Overall, these findings provide valuable insights into the unique structural characteristics of **P45** and highlight its potential significance in the realm of foldamers.

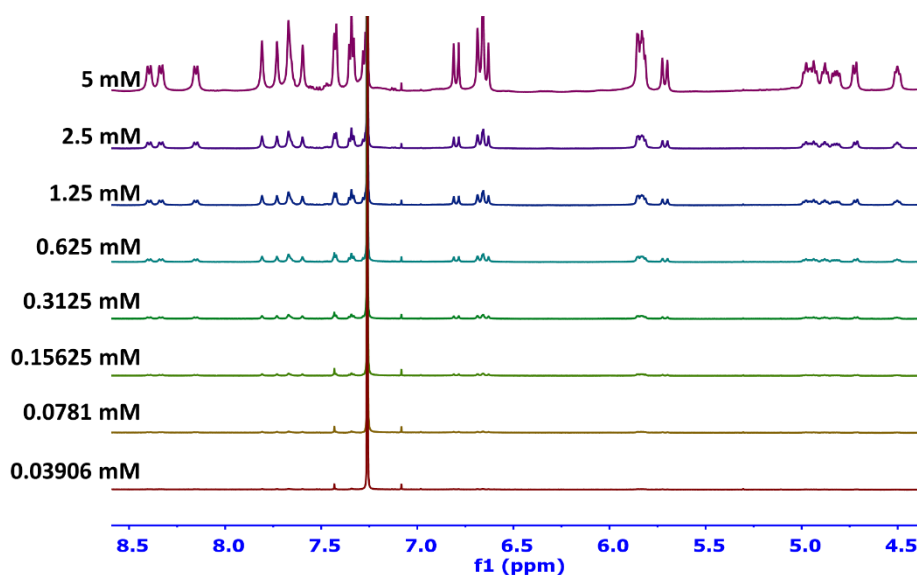


Figure 17: Partial ^1H NMR spectra of peptide **P45** in CDCl_3 at different concentration.

5.3.5. Circular Dichroism Spectroscopy

Inspired by the intriguing double-stranded β -helix structure of **P45**, we embarked on investigating its stability through temperature-dependent CD analysis with the temperature range spanning from 288K to 378K in 1, 1, 2, 2-tetrachloroethane shown in Figure 18. As the temperature increased, we observed a gradual and continuous decrease in the CD maxima at 252 nm. This observation strongly suggested the unfolding of the peptide structure with rising temperatures. However, even at the highest temperature of 378K, the presence of CD maxima indicated that the peptide still partially retained its double helix conformation. This intriguing finding indicates that despite the increase in temperature, the peptide retained some degree of its original double helix structure.

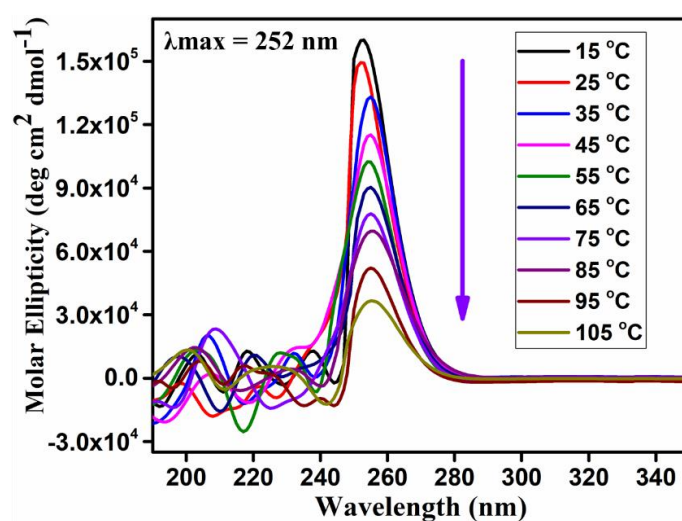


Figure 18: Temperature dependent CD spectra of the Peptide **P45** in tetrachloroethane.

The average torsion angles of the **P45** double helix are provided in Table 3. All valine residues adopted extended conformations. Regarding the (*E*)- α,β -unsaturated 4,4-dimethyl γ -amino acid residue, it displayed distinct torsion angles compared to those observed in its continuous oligomers. The average torsion values for ϕ and θ_1 were found to be 166 ± 23 and θ_1 varied from -22 ± 19 to -87 ± 22 . In contrast to Val, the ϕ values in the unsaturated amino acids are positive. Additionally, there was considerable variation in the θ_1 values. Both θ_2 and ψ showed the values for fully extended conformation.

Table 3: Average torsion angles of peptide **P45** derived from the energy minimized superimposed β -double helix structures.

Peptide Unit	Φ [$^\circ$]	θ_1 [$^\circ$]	θ_2 [$^\circ$]	Ψ [$^\circ$]
Val (1)	-71 ± 19			157 ± 11
d γ Aic (2)	177 ± 16	-54 ± 23	174 ± 9	172 ± 20
Val (3)	-128 ± 12			155 ± 15
d γ Aic (4)	159 ± 16	-44 ± 23	176 ± 10	171 ± 26
Val (5)	-103 ± 20			150 ± 10
d γ Aic (6)	171 ± 17	-87 ± 22	174 ± 10	-173 ± 18
Val (7)	-101 ± 23			158 ± 12
d γ Aic (8)	156 ± 15	-22 ± 19	179 ± 9	169 ± 23

In comparison, the average torsion angles of the β -double helix derived from alternating α_L and α_D -residues (ϕ_L , ψ_L , ϕ_D , ψ_D) were found to be -123 ± 15 , $+137\pm30$, -121 ± 30 , and $+135\pm20$, respectively. A comparison of torsion values between the two double helices revealed that they displayed similar sign patterns, but different numerical values. In both cases, L- α -residues displayed negative and positive signs for ϕ_L and ψ_L , respectively. The sign of the ϕ_D and ψ_D torsion angles of D- α -residue were consistent with the positive and negative signs of ϕ and θ_1 values of (*E*)- α,β -unsaturated γ -amino acids.

These torsion angle analyses provide valuable insights into the conformational characteristics of the **P45** double helix and its comparison with other β -double helices derived from α_L and α_D amino acid residues, shedding light on the structural diversity on the α,γ -hybrid peptide systems.

5.4. Conclusions

In conclusion, our study successfully demonstrated the formation of an anti-parallel double-stranded β -helix structure from 1:1 alternating α , γ -hybrid octapeptide composed of Val and (*E*)- α,β -unsaturated 4,4-dimethyl γ -amino acids. The β -double helix structure is stabilized by a network of sixteen inter-strand hydrogen bonds. Unlike the conventional 12-helix or β -sheet structures, the α,γ -hybrid peptide adopted a novel double-stranded β -helix conformation in solution. The presence of (*E*)- α,β -unsaturated γ -amino acids was found to be crucial for the formation and stability of the double helix structure. Notably, our structural analysis revealed that both the double-helix systems of L, D-peptides, and the α,γ -hybrid peptide shared a common sign pattern for the ϕ and ψ torsion angles. It is worth mentioning that although the hexapeptide exhibited an identical CD spectrum, the ^1H NMR analysis indicated the presence of multiple conformations, suggesting the requirement of eight residues to achieve a stable structure. The discovery of this novel β -double helix structure presents exciting opportunities for incorporating different amino acid side chains, potentially leading to the development of biologically active mimetics with applications akin to gramicidin A-D and feglymicin. Overall, our findings provide valuable insights into the structural versatility of α,γ -hybrid peptides, offering promising prospects for further research and the development of functional peptide mimetics.

5.5. Experimental Section

5.5.1. Materials and Method

Oxone, 2-Aminoisobutyric acid (Aib), NaBH_4 , N-hydroxysuccinimide, HOBt, EDC.HCl, Triphenyl Phosphine (PPh_3), Ethyl bromoacetate, EtOAc, THF, 2-Iodobenzoic acid were procured from different commercial sources. Prior to usage, THF was distilled over sodium. Column chromatography was carried out on silica gel with a mesh size of 120–200. Analytical thin layer chromatography was used for monitoring reactions. UV illumination and KMnO_4 or ninhydrin stain were used to visualize. Spectrometers operating at 400 or 600 MHz were used for all NMR experiments. Chemical shifts are expressed in parts per million (ppm, δ) relative to residual solvent (CHCl_3 , δ 7.26 for ^1H) or tetramethylsilane (δ 0.00). Unless otherwise stated. Singlet (s), doublet (d), triplet (t), doublet of doublets (dd) and multiplet (m) are the several types of ^1H NMR splitting patterns. In Hertz (Hz), coupling constants (*J*) are reported. Accurate mass was obtained using the Matrix Assisted LASER Desorption Ionisation mass spectrometer (MALDI-TOF/TOF).

5.5.2. Synthesis of peptides

The (*E*)- α,β -unsaturated 4,4-dimethyl- γ -amino acid (**1**) was synthesised using the procedures described earlier.²² We used traditional solution-phase strategies for making peptides. The N terminal was protected using a tert-butyloxycarbonyl (Boc) group, while the C terminus was protected using an ethyl ester group. For the N- and C-termini, protecting groups were eliminated using trifluoroacetic acid and saponification, respectively. N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBt) were used to perform couplings. By using a condensation approach that included Boc-Val-OH and H₂N-d γ Aic-OEt (**2**), DIPEA as an activator base, DMF as the solvent, and EDC/HOBt as coupling reagents, the dipeptides were synthesised. After being purified using silica gel (120-200 mesh), the resulting dipeptides (**3**) were used in other applications. Boc-Val-d γ Aic-COOH and NH₂-Val-d γ Aic-OEt were used in a 2+2 condensation approach to synthesise the tetrapeptide Boc-Val-d γ Aic-Val-d γ Aic-OEt (**4**). The N-terminal tetrapeptide acid Boc-Val-d γ Aic-Val-d γ Aic-OH and benzylamine (Bn) were used in a [4 + 1] condensation to yield the peptide Boc-Val-d γ Aic-Val-d γ Aic-Bn (**P43**). The N-terminal dipeptide acid Boc-Val-d γ Aic-COOH and H₂N-Val-d γ Aic-Val-d γ Aic-Bn were used in a [2 + 4] condensation that yielded the hexapeptide Boc-Val-d γ Aic-Val-d γ Aic-Val-d γ Aic-Bn (**P44**). The N-terminal tetrapeptide acid Boc-Val-d γ Aic-Val-d γ Aic-OH and H₂N-Val-d γ Aic-Val-d γ Aic-Bn were used in a [4 + 4] condensation to yield the octapeptide Boc-Val-d γ Aic-Val-d γ Aic-Val-d γ Aic-Val-d γ Aic-Bn (**P45**). Finally, using a MeOH/H₂O gradient system and reversed-phase HPLC, all peptides were purified and their identities were confirmed through mass spectral analysis.

5.5.3. Crystal Structure Information of Peptide P43 (CCDC No: 2289279)

Crystals of **P43** were grown by slow evaporation from a solvent composition of MeOH/H₂O. A single crystal, Needle in shape (0.65 X 0.40 X 0.30 mm³), was mounted on a loop. The X-ray data were collected at 150 K on a Bruker APEX(II) DUO CCD diffractometer using Mo-K α radiation (λ =0.71073 Å), ω -scans (2θ =56.618°) for a total number of 41348 independent reflections. Space group *P*1; a=9.6687(6), b=13.4613(8), c=16.9982(11) Å; α =111.655(2), β =104.433(2), γ =91.113(2), V=1976(2), Triclinic C₇₁H₁₁₆N₁₀O₁₆; Z=1 for chemical formula C₇₁H₁₁₆N₁₀O₁₆; ρ_{calcd} = 1.148 gcm⁻³; μ =0.081 mm⁻¹; F(000)=740 The structure was obtained by direct methods using SHELXS-97. All non-hydrogen atoms were refined anisotropically. The hydrogen atoms were fixed geometrically in the idealized position and refined in the final cycle of refinement as riding over the atoms to which they are bonded. The final R value was 0.0750 (wR_2 =0.2079) for 19666 observed reflections ($F_0 \geq 4\sigma(|F_0|)$); S=1.017.

5.5.4. NMR spectroscopy

All NMR spectroscopy experiments were conducted with spectrometers operating at 400 or 600 MHz. ROESY and TOCSY analyses were used to determine resonance assignments. Using the time-proportional phase incrementation (TPPI) technique, all 2D data were gathered in phase sensitive mode. In the t₂ and t₁ dimensions, sets of 1024 and 512 data points, respectively, were used. A total of 32 and 72 transients, respectively, were gathered for the TOCSY and ROESY studies. In all dimensions, a spectral width of 6007 Hz was applied. To acquire ROESY spectra, spin-lock periods of 200 and 250 ms were used. Finally, zero filling was done to provide a 2 KV1 K data set. Prior to processing, a shifted square-sine-bell window was used.

5.5.4.1. Details of Structure calculation

Structure calculation was done using a simulated annealing protocol in vacuum using DESMOND (Version- 6.8.137) with OPLS 2005 force field and NOE restraints. A low-energy antiparallel double helix structure was selected from a preliminary REMD simulation and kept in an orthorhombic simulation cell of dimensions 32.64 Å * 32.54 Å * 56.04 Å. H...H upper limit distance restraints used for very strong, strong, medium, weak, and very weak NOEs are 2.5 Å, 3 Å, 3.5 Å, 4.5 Å, and 5.5 Å, respectively. In the case of NOEs involving one CH₃, C...H upper limit distance restraints used are 3 Å, 3.5 Å, 4.5 Å, 5.5 Å, and 6.5 Å for very strong, strong, medium, weak, and very weak NOEs respectively. In the case of NOEs involving two CH₃ groups, C...C upper limit distance restraints used are 5 Å and 7 Å for medium and very weak NOEs, respectively. All the lower limits for NOE distance restraints were chosen to be 1.8 Å. 1 Kcalmol⁻¹ force constant was used for all the restraints. Before the production run simulation, a default NVT relaxation was done as implemented in DESMOND. NVT ensemble with Langevin thermostat was used for the production run simulation. A RESPA integrator was used in which, for all the bonded interactions, near non-bonded interactions, and far non-bonded interactions, a time step of 1 fs was used. A cut-off of 9 Å was used for short-range electrostatic interactions. A smooth particle mesh Ewald method was used for treating long-range electrostatic interactions. Simulated annealing was done in 7 stages. The first stage consists of simulation for 30 ps at 10K. In the second stage, the temperature was linearly increased to 300K till 200 ps. In the third stage, the temperature was linearly increased to 700K till 400 ps. In the fourth stage, the temperature was linearly increased to 1000K till 600 ps. In the fifth stage, the temperature was linearly increased to 1500K till 1000 ps. In the sixth stage, the temperature was linearly decreased to 300K till 2000 ps. In the

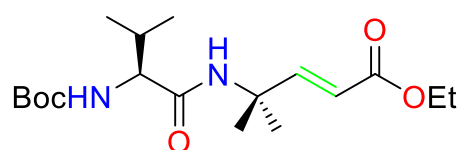
seventh stage, the temperature was maintained at 300K up to 2400 ps. Twenty minimum energy structures were extracted from the trajectory between 2000 ps and 2400 ps and superimposed.

5.5.5. Circular Dichroism (CD) Spectroscopy

A JASCO J-815 spectropolarimeter with a Peltier temperature controller was used to record CD spectra. Peptides (**P43-P45**) were dissolved in CHCl_3 while maintaining a 1 mM peptide concentration in order to measure CD spectra at 20 °C. Peptide **P45** temperature-dependent studies were carried out by dissolving the peptide in tetrachloroethane (TCE) at intervals of 10 °C from 15 to 105 °C.

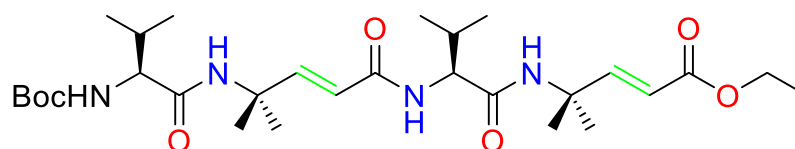
5.6. NMR details of compounds and Peptides

5.6.1. Compound 3: Boc-Val-d γ Aic-OEt



^1H NMR (400 MHz, Chloroform-*d*) δ 6.98 (d, J = 15.9 Hz, 1H), 6.08 (s, 1H), 5.83 (d, J = 15.9 Hz, 1H), 5.02 (d, J = 8 Hz, 1H), 4.17 (q, J = 8 Hz, 2H), 3.74 (dd, J = 8.7, 6.7 Hz, 1H), 2.12 – 2.02 (m, 1H), 1.47 (s, 3H), 1.44 (s, 9H), 1.43 (s, 3H), 1.27 (t, J = 8 Hz, 3H), 0.95 (d, J = 6.7 Hz, 3H), 0.92 (d, J = 7.0 Hz, 3H), MALDI TOF/TOF- m/z calcd. for $\text{C}_{18}\text{H}_{32}\text{N}_2\text{O}_5$ $[\text{M}+\text{Na}]^+$: 379.23, obsvd: 379.39

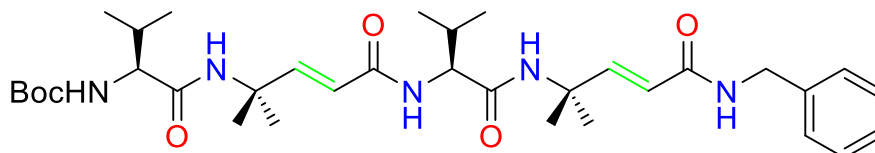
5.6.2. Compound 4: Boc-(Val-d γ Aic) $_2$ -OEt



^1H NMR (400 MHz, Chloroform-*d*) δ 7.02 (d, J = 15.9 Hz, 1H), 6.93 (d, J = 5.92 Hz, 1H), 6.83 (d, J = 15.5 Hz, 1H), 6.63 (s, 1H), 6.55 (s, 1H), 5.87 (d, J = 15.5 Hz, 1H), 5.81 (d, J = 15.9 Hz, 1H), 5.21 (d, J = 9.1 Hz, 1H), 4.30 (t, J = 8.0 Hz, 1H), 4.16 (q, J = 7.2 Hz, 2H), 3.95 (t, J

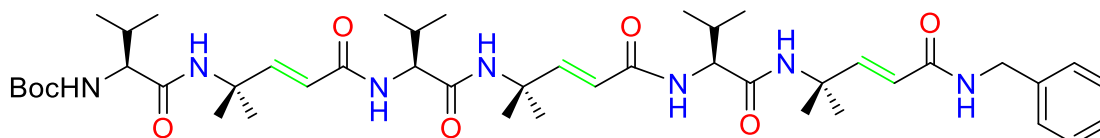
= 6.5 Hz, 1H), 2.11 – 2.00 (m, 2H), 1.48 (s, 3H), 1.44 – 1.42 (m, 18H), 1.26 (t, $J = 7.2$ Hz, 3H), 0.98 – 0.92 (m, 9H), 0.89 (d, $J = 6.8$ Hz, 3H), MALDI TOF/TOF- m/z calcd. for $C_{29}H_{50}N_4O_7$ $[M+Na]^+$: 589.36, obsvd: 589.57

5.6.3. Peptide P43: Boc-(Val-d γ Aic)₂-Bn



1H NMR (400 MHz, Chloroform- d) δ 7.32 – 7.28 (m, 4H), 7.28 – 7.26 (m, 1H), 7.23 (dt, $J = 6.9, 3.0$ Hz, 1H), 7.19 (d, $J = 7.2$ Hz, 1H), 6.95 (s, 1H), 6.82 (d, $J = 15.6$ Hz, 1H), 6.82 (s, 1H), 6.75 (d, $J = 15.6$ Hz, 1H), 5.87 (d, $J = 15.6$ Hz, 1H), 5.83 (d, $J = 15.6$ Hz, 1H), 5.04 (d, $J = 9.0$ Hz, 1H), 4.54 (dd, $J = 14.8, 6.3$ Hz, 1H), 4.45 – 4.32 (m, 2H), 3.99 (t, $J = 7.6$, 1H), 2.09 (m, 1H), 1.96 (m, 1H), 1.53 (s, 3H), 1.47 (s, 3H), 1.41 (s, 9H), 1.38 (s, 3H), 1.37 (s, 3H), 0.97 – 0.91 (m, 9H), 0.86 (d, $J = 6.8$ Hz, 3H), MALDI TOF/TOF- m/z calcd. for $C_{34}H_{53}N_5O_6$ $[M+Na]^+$: 650.39, obsvd: 650.63

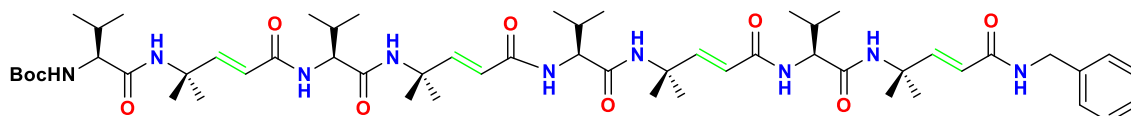
5.6.4. Peptide P44: Boc-(Val-d γ Aic)₃-Bn



1H NMR [400 MHz, Chloroform- d , 243K, (1:0.6, major : minor)] δ 8.76 (t, $J = 6.1$ Hz, 1H, major), 8.64 (d, $J = 9.8$ Hz, 1H, major), 8.52 (s, 1H, major), 8.36 (s, 1H, major), 8.33 (s, 1H, minor), 8.10 (s, 1H, minor), 8.08 (s, 1H, major), 7.86 (m, 1H, minor), 7.83 (s, 1H, minor), 7.78 (s, 1H, minor), 7.74 (s, 1H, minor), 7.42 (d, $J = 7.5$ Hz, 1H, minor), 7.36 (t, $J = 7.4$ Hz, 1H, minor), 7.29 (d, $J = 7.2$ Hz, 1H, minor), 7.24 – 7.16 (m, 3H, major), 7.16 – 7.12 (m, 2H, major), 7.05 (d, $J = 15.8$ Hz, 1H, major), 6.88 (d, $J = 15.6$ Hz, 1H, major), 6.79 (d, $J = 15.6$ Hz, 1H, minor), 6.67 (d, $J = 15.7$ Hz, 1H, major), 6.63 (d, $J = 15.7$ Hz, 1H, minor), 6.49 (d, $J = 15.5$ Hz, 1H, major), 6.30 (d, $J = 15.5$ Hz, 1H, major), 6.26 (s, 1H, minor), 6.11 (d, $J = 15.4$ Hz, 1H, major), 5.88 (d, $J = 15.7$ Hz, 1H, major), 5.80 (d, $J = 15.4$ Hz, 1H, minor), 5.65 (d, $J = 15.6$ Hz, 1H, minor), 5.03 (d, $J = 8.2$ Hz, 1H, major), 4.88 (m, 2H), 4.65 (d, $J = 9.8$ Hz, 1H), 4.56

(dd, $J = 10.2, 4.9$ Hz, 1H), 4.47 (t, $J = 8.6$ Hz, 1H), 4.39 – 4.19 (m, 3H), 4.12 (t, $J = 10.1$ Hz, 1H), 3.69 (t, $J = 9.3$ Hz, 1H), 2.18 – 1.94 (m, 3H), 1.91 – 1.79 (m, 2H), 1.75 – 1.41 (m, 20H), 1.37 (s, 14H), 1.33 (s, 5H), 1.24 (s, 2H), 1.13 – 1.02 (m, 5H), 1.00 – 0.91 (m, 9H), 0.88 – 0.79 (m, 11H), 0.62 (d, $J = 6.6$ Hz, 2H), MALDI TOF/TOF- m/z calcd. for $C_{45}H_{71}N_7O_8 [M+Na]^+$: 860.53, obsvd: 860.81

5.6.5. Peptide P45: Boc-(Val-d γ Aic) $_4$ -Bn



1H NMR (400 MHz, Chloroform- d , 283K) δ 8.40 (d, $J = 10.1$ Hz, 1H), 8.34 (d, $J = 9.8$ Hz, 1H), 8.16 (d, $J = 10.1$ Hz, 1H), 7.80 (s, 1H), 7.72 (s, 1H), 7.66 (s, 1H), 7.64 (t, $J = 6.32$ Hz, 1H), 7.57 (s, 1H), 7.43 (d, $J = 7.5$ Hz, 2H), 7.34 (t, $J = 7.4$ Hz, 2H), 7.28 (d, $J = 7.4$ Hz, 1H), 6.80 (d, $J = 15.6$ Hz, 1H), 6.67 (d, $J = 15.0$, 2H), 6.64 (d, $J = 15.16$, 1H), 5.85 (d, $J = 15.24$, 1H), 5.84 (d, $J = 15.32$, 1H), 5.82 (d, $J = 15.44$, 1H), 5.72 (d, $J = 15.6$ Hz, 1H), 4.99 (t, $J = 9.0$ Hz, 1H), 4.95 (t, $J = 8.9$ Hz, 1H), 4.89 (t, $J = 8.8$ Hz, 1H), 4.82 (dd, $J = 14.7, 7.2$ Hz, 1H), 4.72 (d, $J = 9.7$ Hz, 1H), 4.51 (t, $J = 8.2$ Hz, 1H), 4.29 (dd, $J = 14.3, 4.0$ Hz, 1H), 2.24 – 2.15 (m, 2H), 2.15 – 2.08 (m, 1H), 1.97 – 1.88 (m, 1H), 1.73 (s, 6H), 1.68 (s, 3H), 1.61 (s, 3H), 1.52 (s, 3H), 1.51 (s, 3H), 1.47 (s, 3H), 1.46 (s, 3H), 1.36 (s, 9H), 1.11 (d, $J = 7.0$ Hz, 3H), 1.09 (d, $J = 7.0$ Hz, 3H), 1.06 – 0.97 (m, 15H), 0.88 (d, $J = 6.4$ Hz, 3H), MALDI TOF/TOF- m/z calcd. for $C_{56}H_{89}N_9O_{10} [M+Na]^+$: 1070.67, obsvd: 1070.97

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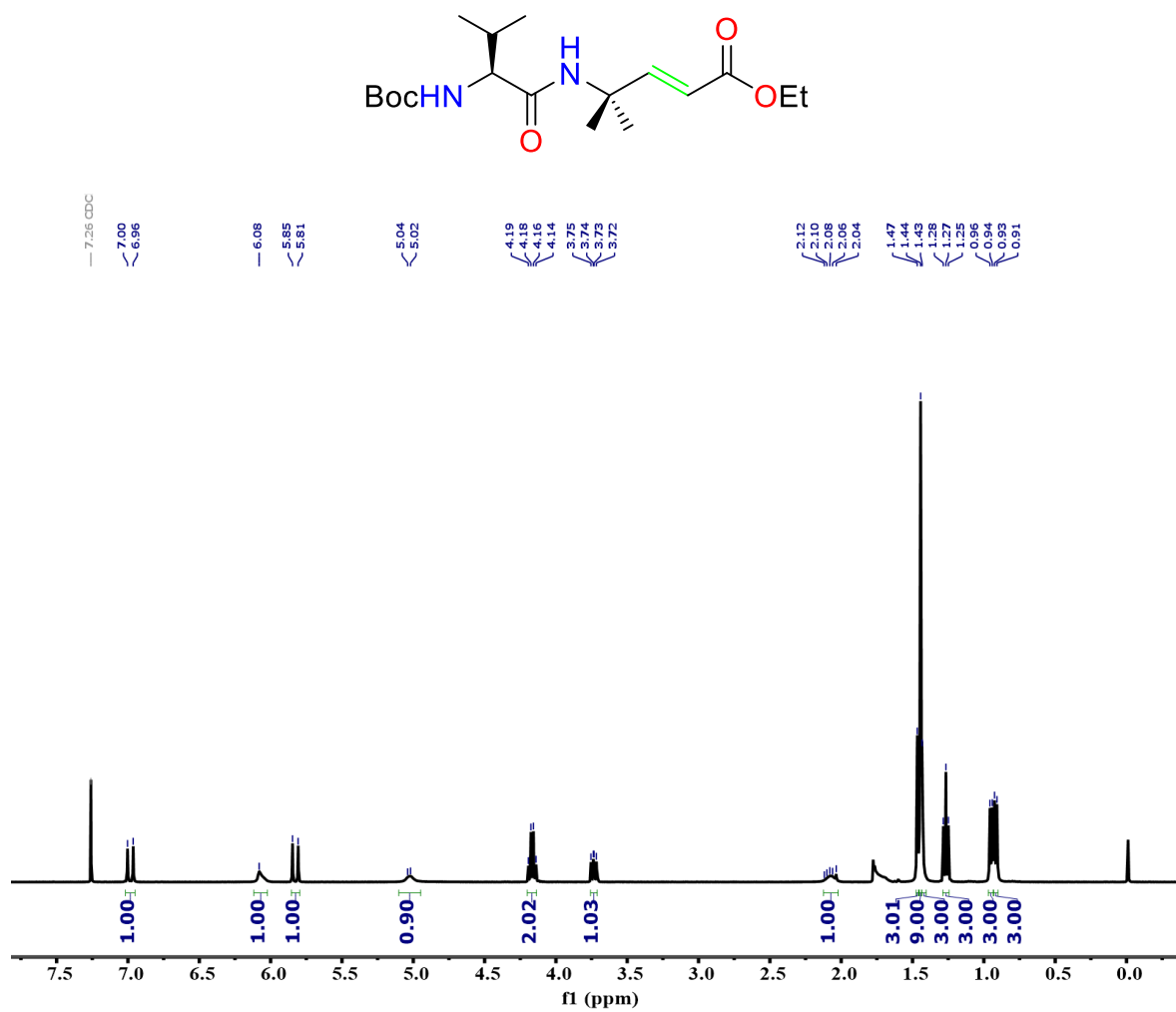
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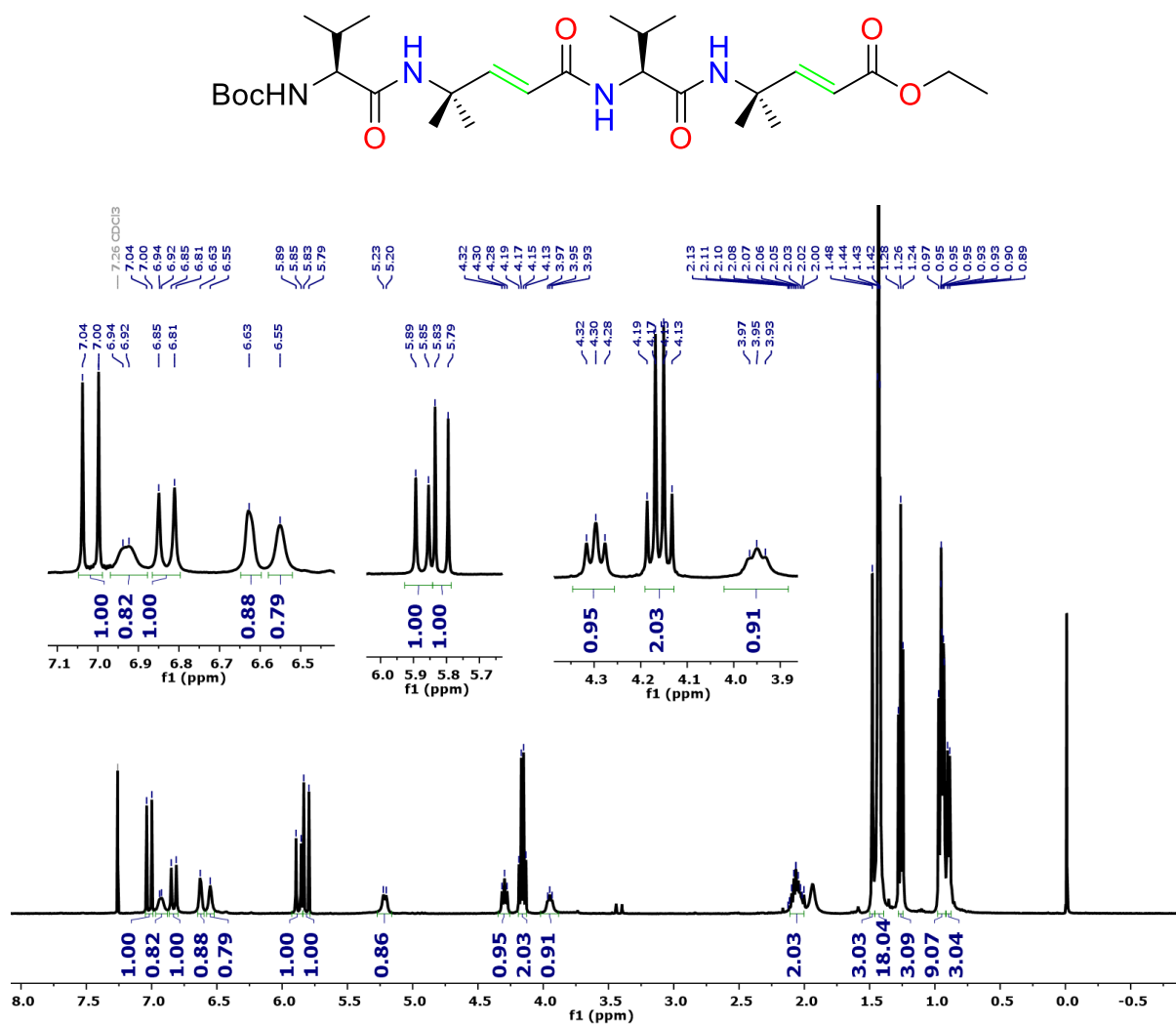
5.8. Appendix V: ^1H NMR Spectra and Mass Spectra for the Compounds and Peptides

5.8.1. ^1H NMR spectra

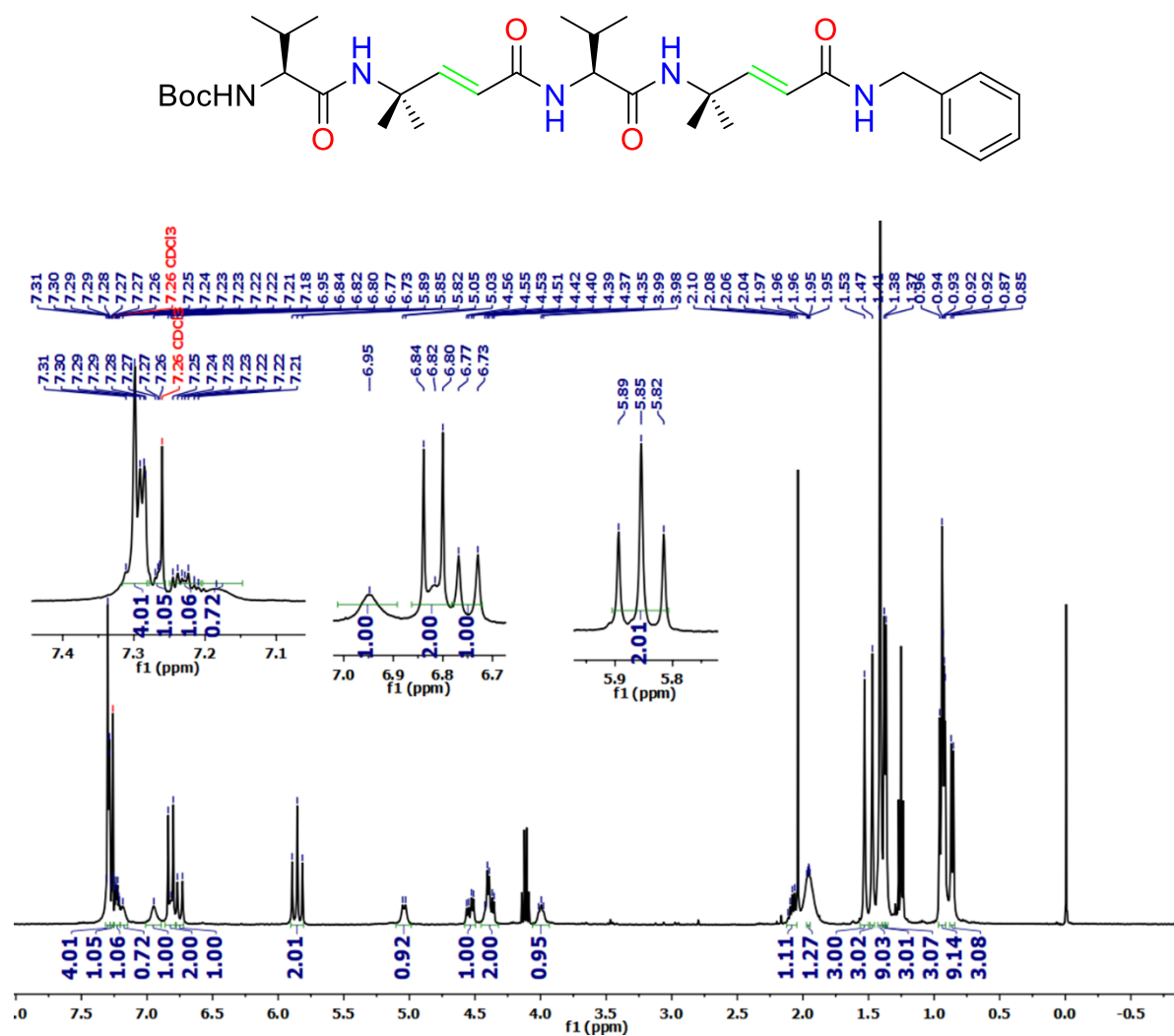
5.8.1.1. ^1H NMR spectra of Compound 3: Boc-Val-d γ Aic-OEt



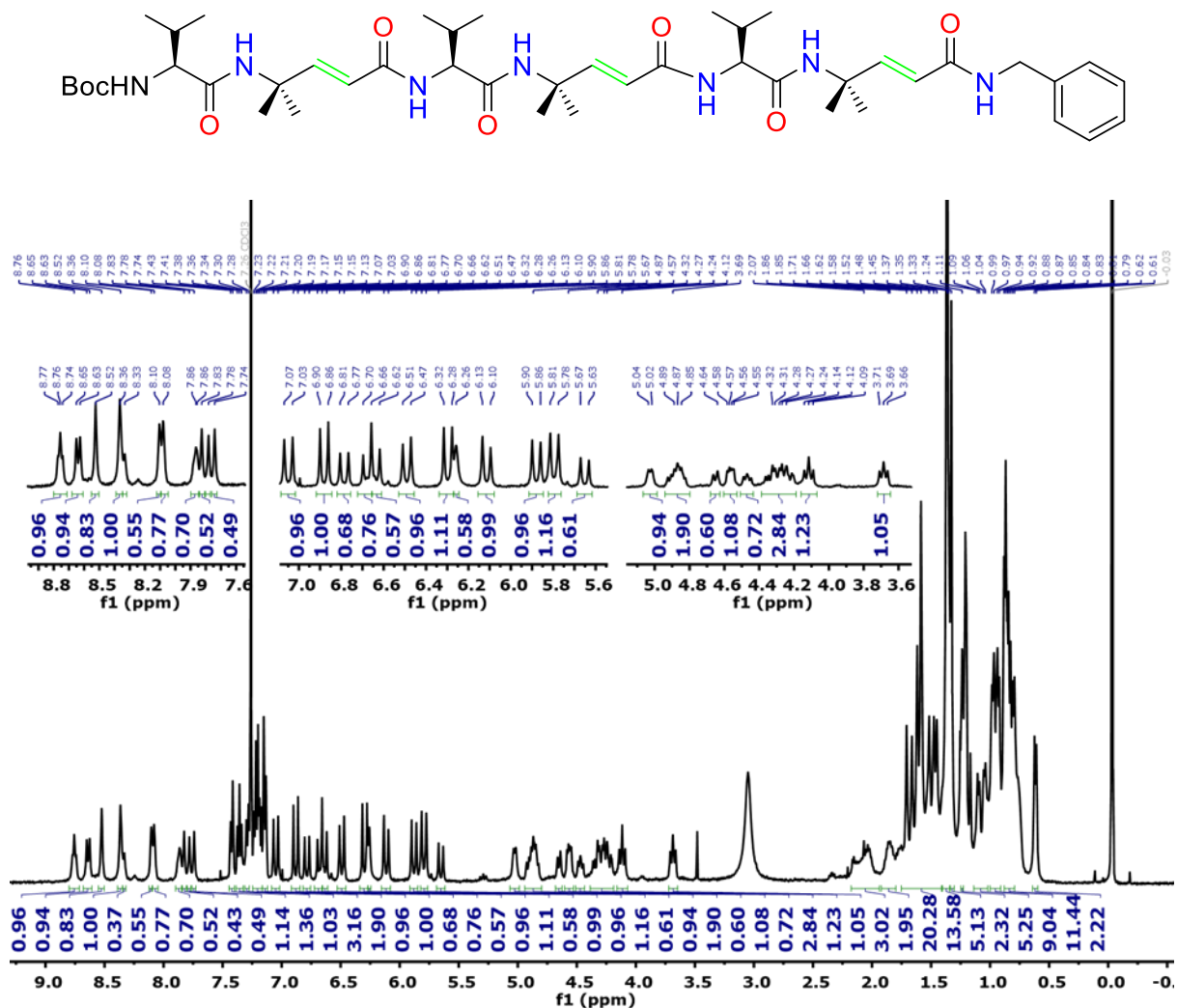
5.8.1.2. ^1H NMR spectra of Compound 4: Boc-(Val-d γ Aic) $_2$ -OEt



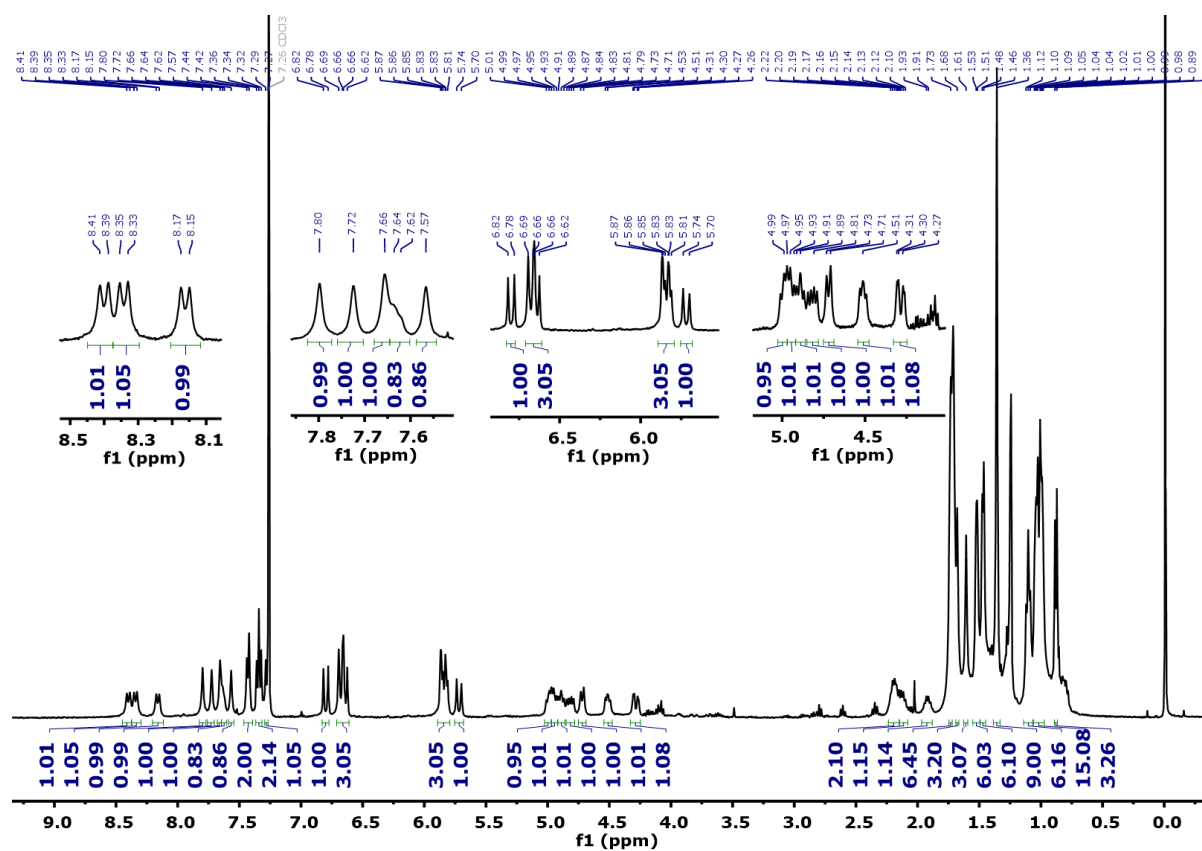
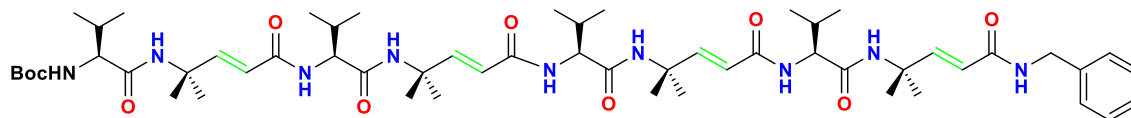
5.8.1.3. ^1H NMR spectra of Peptide P43: Boc-(Val-d γ Aic) $_2$ -Bn



5.8.1.4. ^1H NMR spectra of Peptide P44: Boc-(Val-d γ Aic) $_3$ -Bn

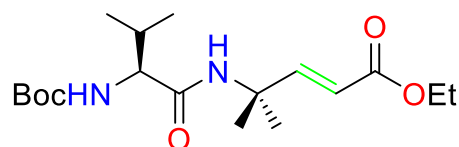


5.8.1.5. ¹H NMR spectra of Peptide P45: Boc-(Val-d₅Aic)₄-Bn



5.8.2. Mass Spectra of Compounds and Peptides

5.8.2.1. Mass Spectra of Compound 3: Boc-Val-dγAic-OEt

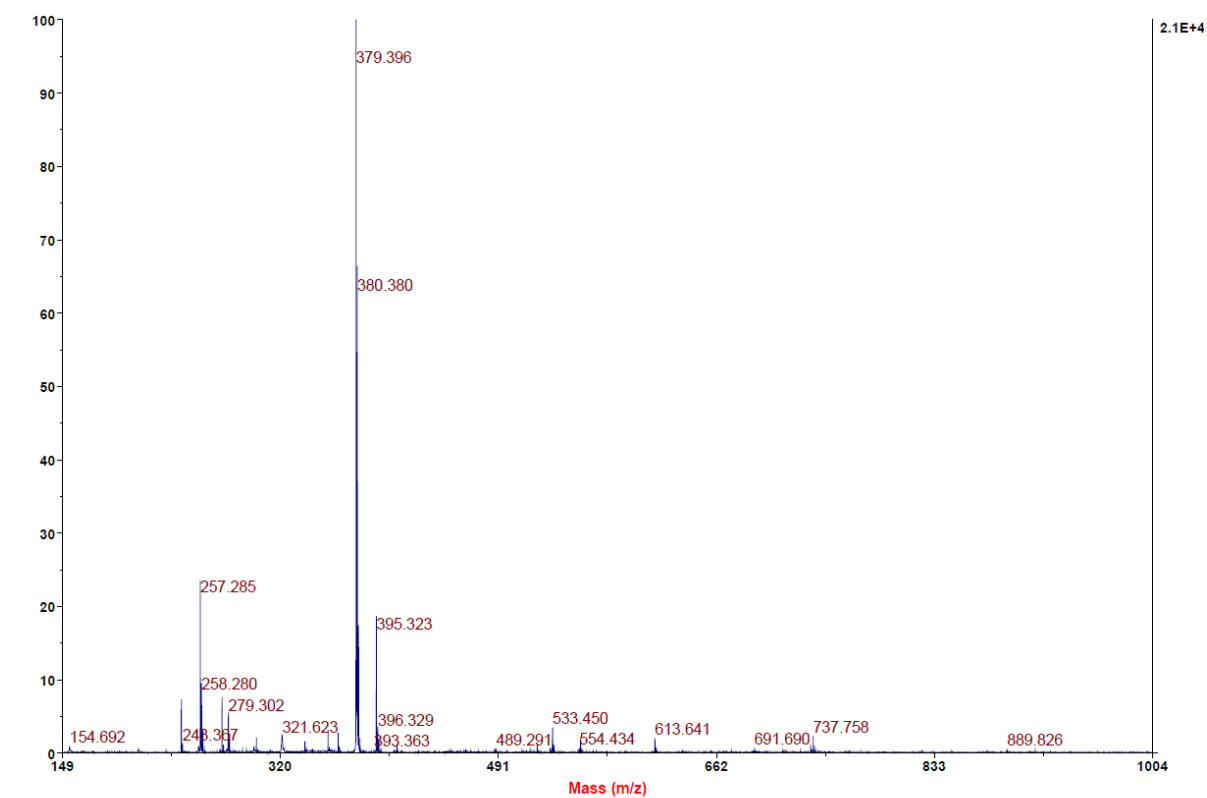


Calculated Mass for $C_{18}H_{32}N_2O_5$ $[M+Na]^+$: 379.23

Observed Mass for $C_{18}H_{32}N_2O_5$ $[M+Na]^+$: 379.39

Spectrum Report

Final - Shots 200 - IISER-96-2-2023; Label D1

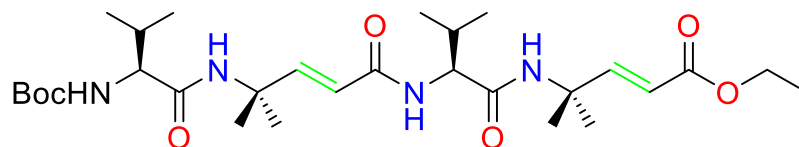


SER-2023\IISER-96-2-2023 Label D1 Run # 22

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5.8.2.2. Mass Spectra of Compound 4: Boc-(Val-d γ Aic)₂-OEt

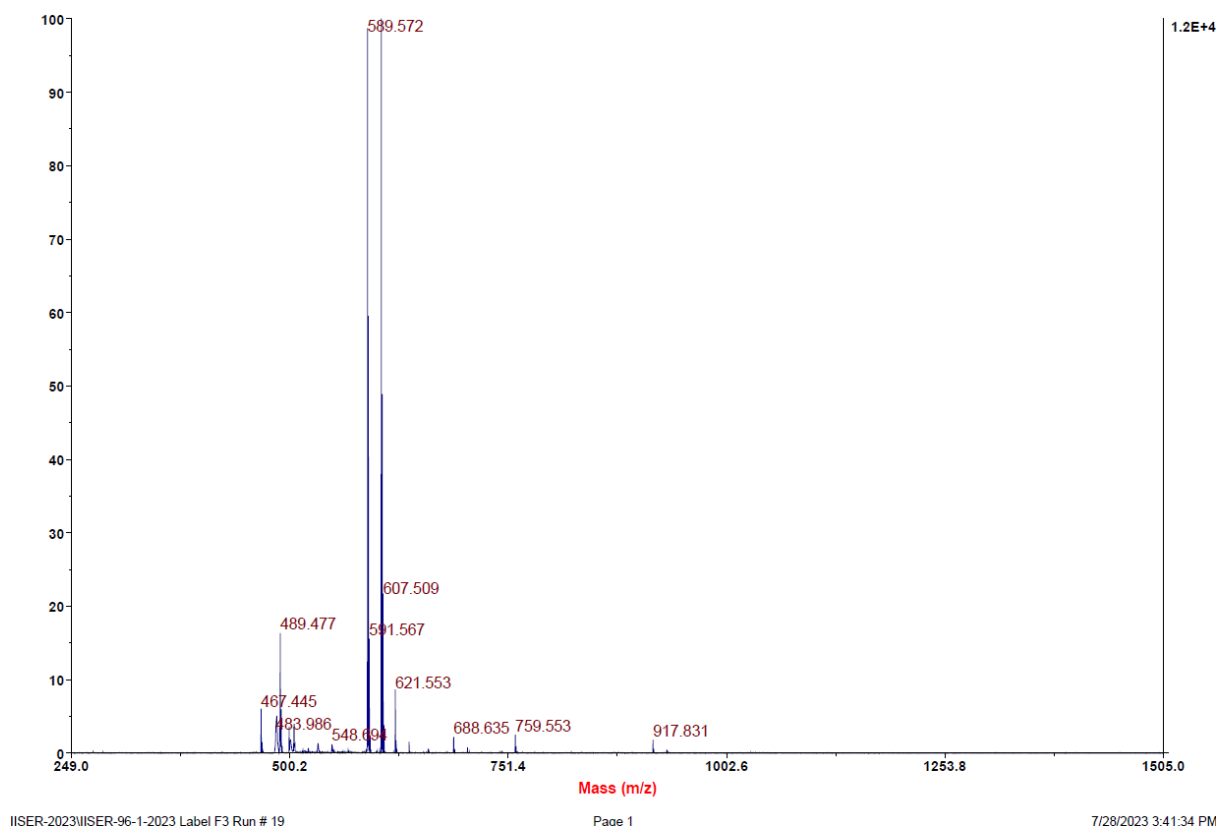


Calculated Mass for C₂₉H₅₀N₄O₇ [M+Na]⁺ : 589.36

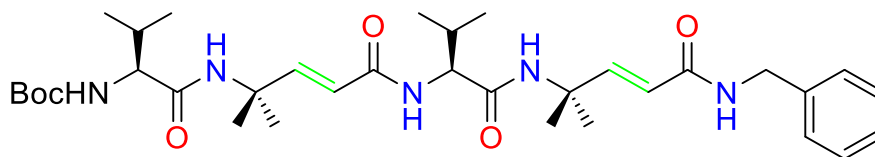
Observed Mass for C₂₉H₅₀N₄O₇ [M+Na]⁺ : 589.57

Spectrum Report

Final - Shots 200 - IISER-96-1-2023; Label F3



5.8.2.3. Mass Spectra of Peptide P43: Boc-(Val-d γ Aic)₂-Bn

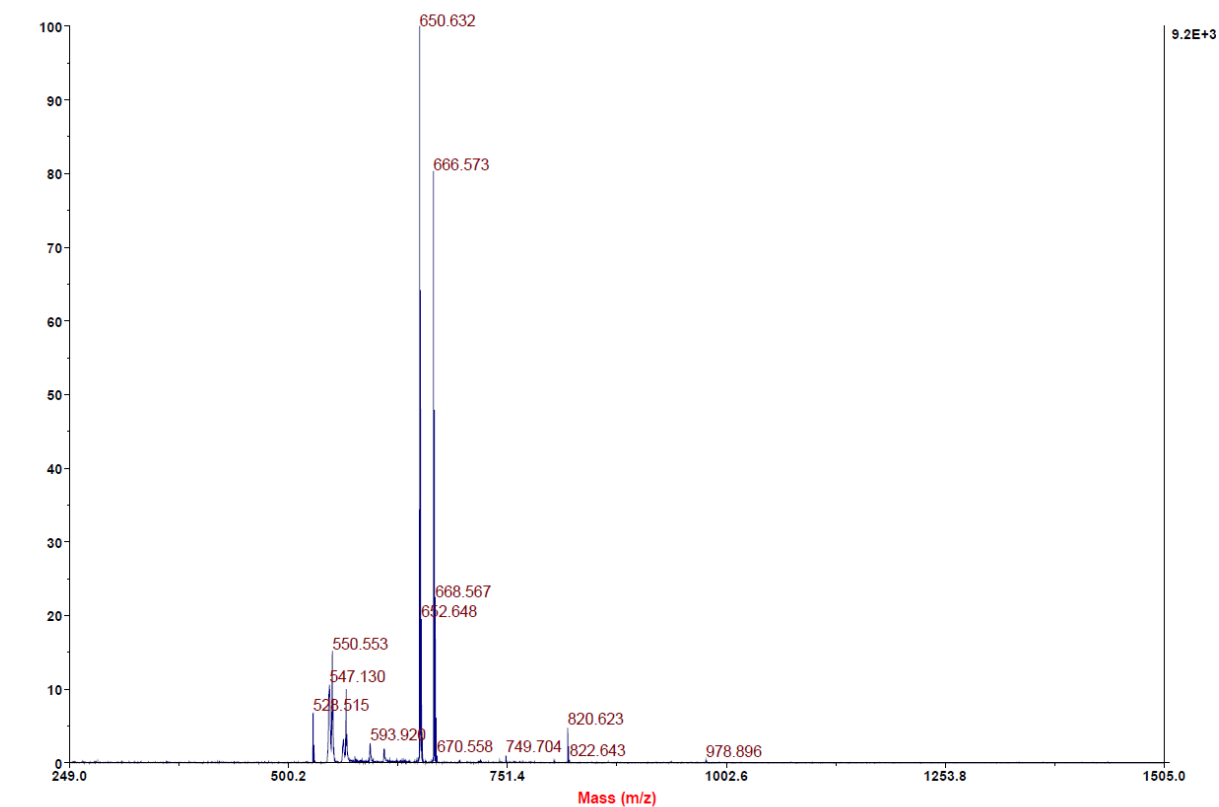


Calculated Mass For C₃₄H₅₃N₅O₆ [M+Na]⁺ : 650.39

Observed Mass For C₃₄H₅₃N₅O₆ [M+Na]⁺ : 650.63

Spectrum Report

Final - Shots 200 - IISER-96-1-2023; Label F5

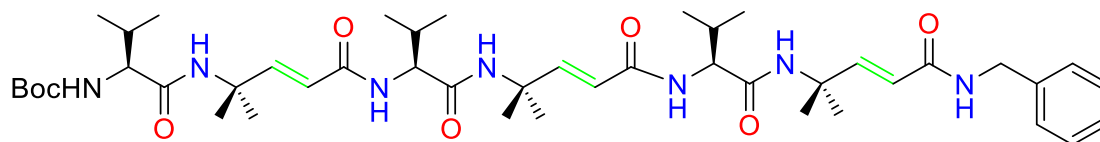


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5.8.2.4. Mass Spectra of the Peptide P44: Boc-(Val-dγAic)₃-Bn

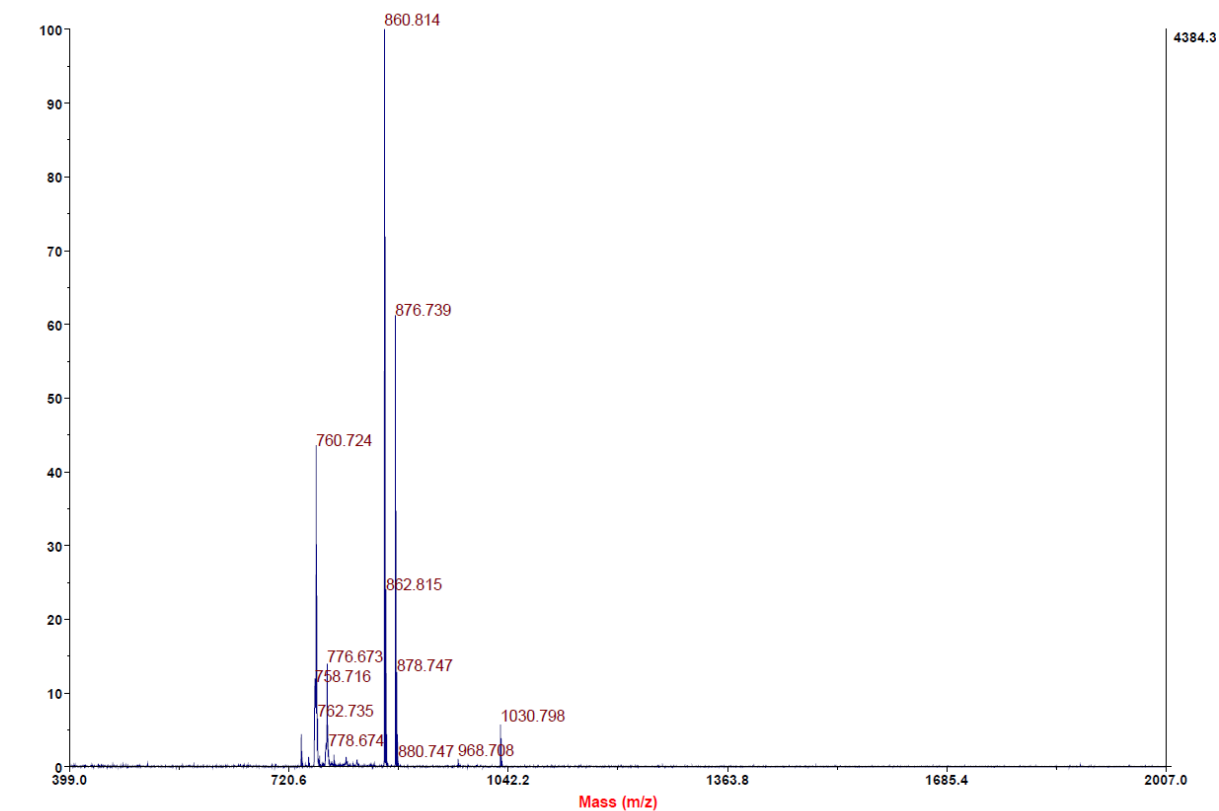


Calculated Mass For C₄₅H₇₁N₇O₈ [M+Na]⁺ : 860.53

Observed Mass For C₄₅H₇₁N₇O₈ [M+Na]⁺ : 860.81

Spectrum Report

Final - Shots 200 - IISER-96-1-2023; Label F9

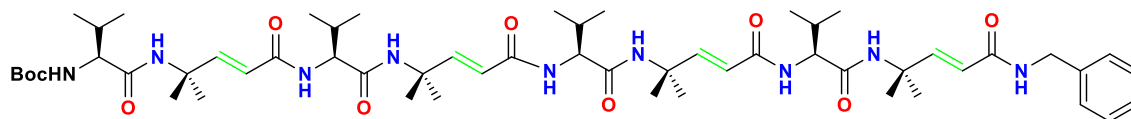


IISER-2023/IISER-96-1-2023 Label F9 Run # 19

Page 1

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5.8.2.5. Mass Spectra of the Peptide P45: Boc-(Val-dγAic)₄-Bn

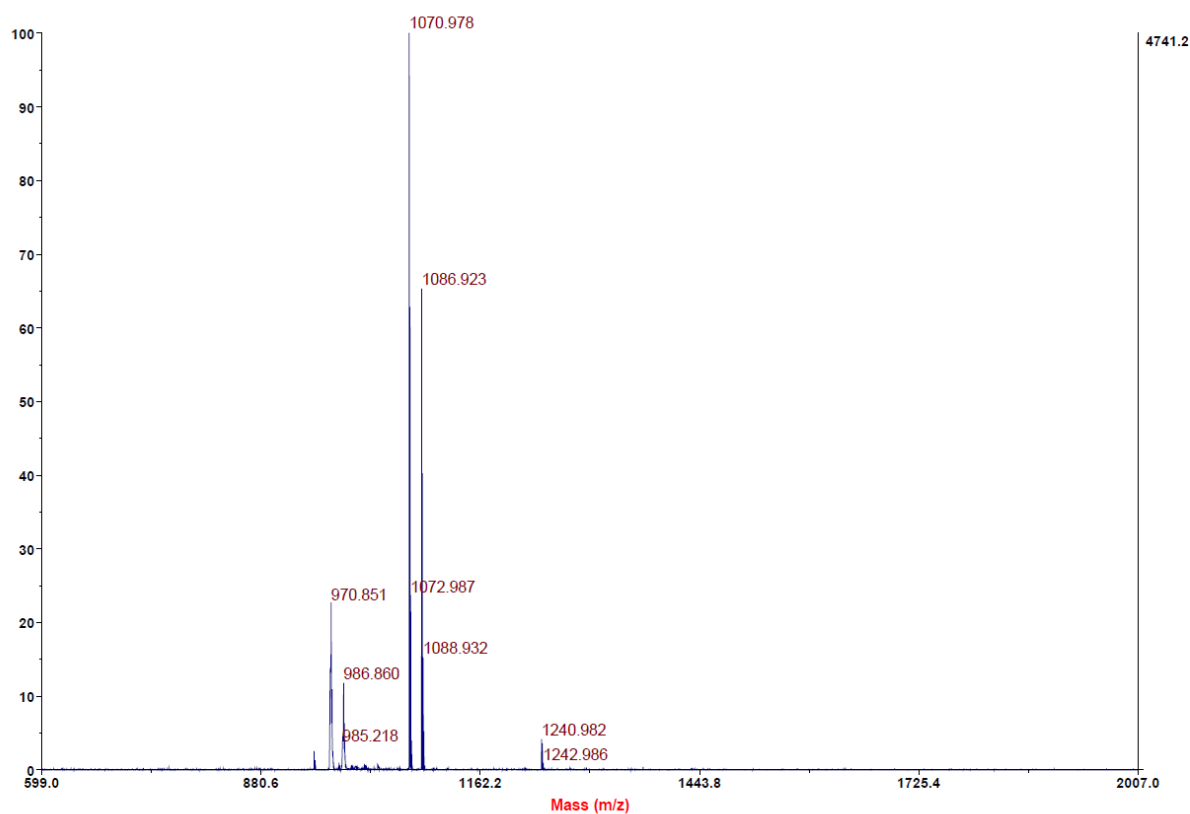


Calculated Mass For C₅₆H₈₉N₉O₁₀ [M+Na]⁺ : 1070.67

Observed Mass For C₅₆H₈₉N₉O₁₀ [M+Na]⁺ : 1070.97

Spectrum Report

Final - Shots 200 - IISER-96-1-2023; Label F10



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A cationic amphiphilic peptide chaperone rescues A β ₄₂ aggregation and cytotoxicity†

DRGKoppalu R. Puneeth Kumar,^a Rahi M. Reja,^a Dillip K. Senapati,^b Manjeet Singh,^a Sachin A. Nalawade,^a Gijo George,^b Grace Kaul,^{cd} Abdul Akhir,^c Sidharth Chopra,^{cd} Srinivasarao Raghothama,^{†b} and Hosahudya N. Gopi,^{†*a}

⊕ Author affiliations

Abstract

Directing A β ₄₂ to adopt a conformation that is free from aggregation and cell toxicity is an attractive and viable strategy to design therapeutics for Alzheimer's disease. Over the years, extensive efforts have been made to disrupt the aggregation of A β ₄₂ using various types of inhibitors but with limited success. Herein, we report the inhibition of aggregation of A β ₄₂ and disintegration of matured fibrils of A β ₄₂ into smaller assemblies by a 15-mer cationic

✕

A cationic amphiphilic peptide chaperone rescues A β ₄₂ aggregation and cytotoxicity

D. R. Puneeth Kumar, R. M. Reja, D. K. Senapati, M. Singh, S. A. Nalawade, G. George, G. Kaul, A. Akhir, S. Chopra, S. Raghothama and H. N. Gopi, *RSC Med. Chem.*, 2023, **14**, 332
DOI: 10.1039/D2MD00414C

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Inhibition of β -Amyloid Aggregation through a Designed β -Hairpin Peptide

Author: Anjali Jha, Mothukuri Ganesh Kumar, Hosahudya N. Gopi, et al

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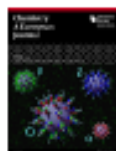
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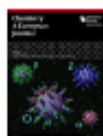
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