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TAILORING SHORT SELF-ASSEMBLING PEPTIDES
INTO BIOCOMPATIBLE GELATORS

A thesis submitted for the partial fulfillment of the degree of

Doctor of Philosophy

BY
DEBIKA DATTA

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***Dedicated to
My Family***

My circle of strength and love...



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DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING

DECLARATION

The research work presented in this thesis is an original work carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, for the degree of the Doctor of Philosophy. Research work was executed under the guidance of my supervisors Dr. Nitin Chaudhary and Dr. Vibin Ramakrishnan, and no work has been submitted, in part or whole, for any other degree to any other University/Institute. As per the standards of reporting research findings, due acknowledgements have been made wherever the work described is based on the findings of other researchers.

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Certificate

This is to certify that the work incorporated in the thesis entitled “Tailoring Short Self-Assembling Peptides into Biocompatible Gelators” submitted by Ms. Debika Datta, for the award of the degree of Doctor of Philosophy is an authentic record of the research work carried out under our supervision and guidance in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India. The work is original and has not been submitted in part or full for any other degree at any other University/Institute.

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Table of Contents

List of Abbreviations	vii
List of Figures	ix
List of Tables	xv
Synopsis	xvii
List of Publications	xxi
1 Introduction and Research Objectives	1
1.1 A Brief Background On Molecular Self-Assembly	3
1.2 Nucleic-Acid Self-Assembly	5
1.3 Protein And Peptide Self-Assembly	6
1.3.1 Amyloid Self-Assembly	10
1.3.2 Cyclic peptides	12
1.3.3 Peptide amphiphiles.....	13
1.3.4 Turn containing peptides	17
1.3.5 Aromatic-moiety containing peptides and amino acids.....	18
1.3.6 Applications of self-assembling peptides	20
1.4 Research Design and Objectives	22
1.5 References	23
2 Self-assembly of β-turn motif-connected tandem repeats of Aβ_{16-22} and its aromatic analogs	37
2.1 Introduction	39
2.2 Materials And Methods	41
2.2.1 Materials	41
2.2.2 Peptide synthesis and characterization	41
2.2.3 Peptide aggregation	42
2.2.4 Circular dichroism (CD) spectroscopy	42
2.2.5 Fourier transform infrared (FTIR) spectroscopy.....	43
2.2.6 Molecular dynamics [MD] simulations	43

2.2.7 Thioflavin T (ThT) fluorescence assay	44
2.2.8 Transmission electron microscopy (TEM)	44
2.3 Results And Discussion	44
2.3.1 CD spectroscopy	45
2.3.2 FTIR spectroscopy	48
2.3.3 MD simulations	49
2.3.4 ThT fluorescence spectroscopy	62
2.3.5 Transmission electron microscopy	63
2.4 Conclusions	65
2.5 References	66
3 Hydrogel formation by the Aβ₁₆₋₂₂ aromatic analog, Aβ₁₆₋₂₂(Phe₂₀Tyr)...	71
3.1 Introduction	73
3.2 Materials And Methods	74
3.2.1 Materials	74
3.2.2 Peptide synthesis and characterization	74
3.2.3 Self-assembly/hydrogelation	74
3.2.4 CD spectroscopy	75
3.2.5 FTIR spectroscopy	75
3.2.6 Raman Spectroscopy	75
3.2.7 AFM imaging	75
3.2.8 TEM imaging	76
3.2.9 ThT fluorescence spectroscopy	76
3.2.10 Microrheology	76
3.2.11 Doxorubicin release assay	77
3.2.12 Rhodamine B release assay	77
3.2.13 Cell seeding and cultivation	77
3.2.14 Cell viability assay	78
3.2.15 Insulin gene expression assay	78
3.3 Results and Discussion	79
3.3.1 CD spectroscopy	81
3.3.2 FTIR spectroscopy	81
3.3.3 Raman Spectroscopy	82

3.3.4 AFM and TEM imaging	83
3.3.5 ThT fluorescence spectroscopy	84
3.3.6 Microrheology	84
3.3.7 Doxorubicin and rhodamine B release assay	87
3.3.8 Cell culture, growth and insulin expression	88
3.4 Conclusions	90
3.5 References	91

4 Lipid hydrogels from β -turn motif-connected tandem repeats of A β ₁₆₋₂₂ and its aromatic analogs

4.1 Introduction	95
4.2 Materials And Methods	95
4.2.1 Materials	95
4.2.2 Peptides	95
4.2.3 Self-assembly/hydrogelation	95
4.2.4 CD spectroscopy	96
4.2.5 FTIR spectroscopy	96
4.2.6 AFM imaging	96
4.2.7 TEM imaging.....	96
4.2.8 ThT fluorescence spectroscopy	96
4.2.9 Mechano-responsive behaviour of A β FF-NG and A β FY-NG as the representative gels	96
4.2.10 Microrheology	96
4.2.11 Doxorubicin release assay	97
4.2.12 Cell seeding and cultivation	97
4.2.13 Cell viability assay	98
4.2.14 Insulin gene expression assay	98
4.3 Results and Discussion	98
4.3.1 CD and FTIR spectroscopy	100
4.3.2 AFM and TEM imaging	102
4.3.3 ThT fluorescence spectroscopy	103
4.3.4 Mechano-responsive behaviour of A β FF-NG and A β FY-NG gels	104
4.3.5 Microrheology	105
4.3.6 Doxorubicin release assay	106

4.3.7 Cell culture, growth and insulin expression	107
4.4 Conclusions	109
4.5 References	111
5 Investigation into the β-turn-supporting motifs using proton NMR ...	115
5.1 Introduction	117
5.2 Materials And Methods	117
5.2.1 Materials	117
5.2.2 Peptides	118
5.2.3 Nuclear Magnetic Resonance (NMR) Spectroscopy	118
5.3 Results and Discussion	119
5.3.1 A β FF and its turn analogs	119
5.3.2 A β FY and its turn analogs	122
5.3.3 A β YY and turn analogs	125
5.3.4 A β WW and turn analogs	127
5.4 Conclusions	157
5.5 References	157
6 Water-alcohol bigels from fatty-acylated dipeptides	161
6.1 Introduction	163
6.2 Materials And Methods	165
6.2.1 Materials	165
6.2.2 Peptide synthesis and characterization	165
6.2.3 Self-assembly	165
6.2.4 TEM imaging	166
6.2.5 Scanning Electron Microscopy (SEM) imaging	166
6.2.6 FTIR spectroscopy	166
6.2.7 Microrheology	166
6.2.8 Rhodamine B release assay	167
6.2.9 Docetaxel release assay	167
6.3 Results and Discussion	167
6.3.1 Electron microscopy	170
6.3.2 FTIR spectroscopy	172

6.3.3 Microrheology	175
6.3.4 Rhodamine B release assay	178
6.3.5 Docetaxel release	179
6.4 Conclusions	180
6.5 References	182
7 Conclusions and Future Directions.....	185
7.1 Conclusions and Future Directions	187





List of Abbreviations

Aβ	β -amyloid
AFM	Atomic force microscopy
ATR	Attenuated total reflection
BHK-21	Baby hamster kidney cells
Boc	Tert-butoxycarbonyl
Calcein AM	Calcein acetoxymethyl ester
CD	Circular dichroism
CSI	Chemical shift index
DIPEA	N,N-diisopropylethylamine
DMEM	Dulbecco's modified Eagle's medium
DMF	Dimethylformamide
DLS	Dynamic light scattering
DLS-μR	DLS-microrheology
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
Fmoc	Fluorenylmethyloxycarbonyl
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSER	Generalized Stokes-Einstein relation
FTIR	Fourier-transform infrared spectroscopy
HBTU	N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-yl)uronium Hexafluorophosphate
HCCA	α -Cyano-4-hydroxycinnamic acid
HEK-293	Human embryonic kidney cells
HFIP	1,1,1,3,3,3-Hexafluoro-2-propanol
HOBt	1-hydroxybenzotriazole hydrate
HPLC	High performance liquid chromatography
IAPP	Human islet amyloid polypeptide
IMR-32	Human neuroblastoma cells
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
MD	Molecular dynamics
MSD	Mean squared displacement
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy
PA	Peptide amphiphile
PBS	Phosphate-buffered saline
RIN-5F	Rat pancreatic cells
RPMI 1640	Roswell Park Memorial Institute 1640 Medium
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
ThT	Thioflavin T
TFE	Trifluoroethanol
TOCSY	Total Correlation Spectroscopy



List of Figures

1.1	A diagrammatic representation depicting self-assembly of soap molecules forming the bubbles (top) and phospholipid molecules forming bilayer and micelles (bottom).	4
1.2	A diagrammatic representation of DNA self-assembly.	6
1.3	A diagrammatic representation of peptide self-assembled structures and their potential applications.	18
1.4	A diagrammatic representation of the various applications of peptide-based hydrogels.	22
2.1	CD spectra of 24 h-old peptide solutions in 20% HFIP.	46
2.2	ATR-FTIR spectra of the dried films from 24 h-old peptide solutions in 20% HFIP.	48
2.3	MD simulation of A β FY-NG.	50
2.4	MD simulation of A β FY-pG.	51
2.5	MD simulation of A β YY-NG.	52
2.6	MD simulation of A β YY-pG.	53
2.7	MD simulation of A β FF-NG.	55
2.8	MD simulation of A β FF-pG.	56
2.9	MD simulation of A β WW-NG.	57
2.10	MD simulation of A β WW-pG.	58
2.11	MD simulation of A β FF-Up.	59
2.12	MD simulation of A β FY-Up.	60
2.13	MD simulation of A β YY-Up.	61
2.14	MD simulation of A β WW-Up.	62
2.15	ThT fluorescence intensity at 482 nm for 24 h-old samples in 20% HFIP.	63
2.16	TEM analyses of 72 h-old peptide samples in 20% HFIP.	65
3.1	Gelation by peptides as assessed by tube inversion.	80
3.2	Gelation caused by 5 mM A β FY at pH 2 and 4 as assessed by inverting the tube.	80
3.3	Characterization of self-assembled A β FF structures and A β FY gel.	81
3.4	Raman scattering caused by the sample obtained from A β FY gel.	83
3.5	Self-assembled fibrillar structures formed by A β FF and A β FY as observed using AFM and TEM.	84
3.6	ThT fluorescence spectra for the self-assembled peptides.	84

List of Figures

3.7	A diagrammatic representation of how DLS-microrheology works.	85
3.8	DLS-microrheology characterization.	85
3.9	Plots of MSD against time for the 5 mM A β FY gel at different temperatures starting from 25 °C.	87
3.10	Release of doxorubicin and rhodamine B from A β FY gel.	88
3.11	Brightfield and calcein fluorescence images of RIN-5F, HEK-293, and BHK-21 cells grown for 24 h in A β FY gel.	89
3.12	Human neuroblastoma cell line, IMR-32 grown for 24 h in A β FY gel.	89
3.13	Expression of GAPDH and insulin 1.	90
4.1	Images of the inverted tubes showing self-supporting gels.	99
4.2	Hydrogelation by A β FF-NG and A β FY-NG at acidic pH.	100
4.3	Far-UV CD spectra recorded for A β FF-NG, A β FY-NG, A β FF-pG, A β FY-pG, A β FF-Up, and A β FY-Up.	101
4.4	ATR-FTIR spectra of A β FF-NG, A β FF-pG, A β FF-Up and A β FY-NG, A β FY-pG and A β FY-Up.	101
4.5	AFM images of the self-assembled structures that underlie hydrogel formation.	102
4.6	TEM images of A β FF-NG, A β FF-pG, A β FF-Up, A β FY-NG, A β FY-pG, and A β FY-Up gel samples.	103
4.7	ThT fluorescence spectra recorded for the sheared hydrogels; A β FF-turn-based peptides, and A β FY-turn-based peptides.	103
4.8	Mechano-responsive behaviour of the A β FF-NG and A β FY-NG gels.	104
4.9	DLS-microrheology data recorded for hydrogels.	105
4.10	Doxorubicin release kinetics from A β FF-NG, A β FF-pG, A β FF-Up, and A β FY-NG gels.	106
4.11	Doxorubicin release kinetics from A β FF-NG gel recorded after washing, and entrapped drug in the gel.	107
4.12	Fluorescence images of the calcein AM treated HEK-293 and RIN-5F cells grown for 24 h in peptide hydrogels.	108
4.13	Agarose gel electrophoretic analysis of GAPDH and insulin-1 mRNA amplification from RIN-5F cells grown for 24 h in A β FF-NG and A β FY-NG hydrogels.	109
5.1	The pulse programs used for recording the water-suppressed 1D proton NMR, and the 2D TOCSY spectra.	119
5.2A	The aliphatic region of the proton NMR spectra of A β FF and its turn-connected repeats in DMSO-d ₆ .	120
5.2B	The C α region of the proton NMR spectra of A β FF and its turn-connected repeats in DMSO-d ₆ .	121

List of Figures

5.2C	The aromatic region of the proton NMR spectra of AβFF and its turn-connected repeats in DMSO-d ₆ .	121
5.2D	The amide region of the proton NMR spectra of AβFF and its turn-connected repeats in DMSO-d ₆ .	122
5.3A	The aliphatic region of the proton NMR spectra of AβFY and its turn-connected repeats in DMSO-d ₆ .	123
5.3B	The C _α region of the proton NMR spectra of AβFY and its turn-connected repeats in DMSO-d ₆ .	123
5.3C	The aromatic region of the proton NMR spectra of AβFY and its turn-connected repeats in DMSO-d ₆ .	124
5.3D	The amide region of the proton NMR spectra of AβFY and its turn-connected repeats in DMSO-d ₆ .	124
5.4A	The aliphatic region of the proton NMR spectra of AβYY and its turn-connected repeats in DMSO-d ₆ .	125
5.4B	The C _α region of the proton NMR spectra of AβYY and its turn-connected repeats in DMSO-d ₆ .	126
5.4C	The aromatic region of the proton NMR spectra of AβYY and its turn-connected repeats in DMSO-d ₆ .	126
5.4D	The amide region of the proton NMR spectra of AβYY and its turn-connected repeats in DMSO-d ₆ .	127
5.5A	The aliphatic region of the proton NMR spectra of AβYY and its turn-connected repeats in DMSO-d ₆ .	128
5.5B	The C _α region of the proton NMR spectra of AβYY and its turn-connected repeats in DMSO-d ₆ .	128
5.5C	The aromatic region of the proton NMR spectra of AβYY and its turn-connected repeats in DMSO-d ₆ .	129
5.5D	The amide region of the proton NMR spectra of AβYY and its turn-connected repeats in DMSO-d ₆ .	129
5.6A	The aliphatic region of the 1D proton NMR spectra of AβFF in water and DMSO.	130
5.6B	The aromatic and amide region of the 1D proton NMR spectra of AβFF in water and DMSO.	131
5.6C	The aliphatic region of the 1D proton NMR spectra of AβFY in water and DMSO.	131
5.6D	The aromatic and amide region of the 1D proton NMR spectra of AβFY in water and DMSO.	132
5.6E	The aliphatic region of the 1D proton NMR spectra of AβYY in water and DMSO.	132
5.6F	The aromatic and amide region of the 1D proton NMR spectra of AβYY in water and DMSO.	133

List of Figures

5.6G	The aliphatic region of the ¹ D proton NMR spectra of AβWW in water and DMSO.	133
5.6H	The aromatic and amide region of the ¹ D proton NMR spectra of AβWW in water and DMSO.	134
5.7A	¹ H- ¹ H TOCSY spectra of AβFF recorded in DMSO-d ₆ .	135
5.7B	¹ H- ¹ H TOCSY spectra of AβFF-NG recorded in DMSO-d ₆ .	136
5.7C	¹ H- ¹ H TOCSY spectra of AβFF-pG recorded in DMSO-d ₆ .	137
5.7D	¹ H- ¹ H TOCSY spectra of AβFF-Up recorded in DMSO-d ₆ .	138
5.8A	¹ H- ¹ H TOCSY spectra of AβFY recorded in DMSO-d ₆ .	139
5.8B	¹ H- ¹ H TOCSY spectra of AβFY-NG recorded in DMSO-d ₆ .	140
5.8C	¹ H- ¹ H TOCSY spectra of AβFY-pG recorded in DMSO-d ₆ .	141
5.8D	¹ H- ¹ H TOCSY spectra of AβFY-Up recorded in DMSO-d ₆ .	142
5.9A	¹ H- ¹ H TOCSY spectra of AβYY recorded in DMSO-d ₆ .	143
5.9B	¹ H- ¹ H TOCSY spectra of AβYY-NG recorded in DMSO-d ₆ .	144
5.9C	¹ H- ¹ H TOCSY spectra of AβYY-Up recorded in DMSO-d ₆ .	145
5.9D	¹ H- ¹ H TOCSY spectra of AβYY-pG recorded in DMSO-d ₆ .	146
5.10A	¹ H- ¹ H TOCSY spectra of AβWW recorded in DMSO-d ₆ .	147
5.10B	¹ H- ¹ H TOCSY spectra of AβWW-NG recorded in DMSO-d ₆ .	148
5.10C	¹ H- ¹ H TOCSY spectra of AβWW-pG recorded in DMSO-d ₆ .	149
5.10D	¹ H- ¹ H TOCSY spectra of AβWW-Up recorded in DMSO-d ₆ .	150
5.11	The NOE cross-peaks between the H _N of Gly with that of Lys observed for AβFF-pG and AβYY-pG.	155
6.1	The fatty-acylated peptides employed in this study.	164
6.2	Images of the inverted tubes containing 24 h-old 20 mg/ml peptides in methanol/water; Pa-VV, Ma-VV, Pa-VL, Ma-VL, Ma-IV, Pa-LV, Ma-IL, and Pa-II.	169
6.3	The tube inversion test for gelation of 50% ethanol/water; Pa-VV, Pa-VL, Pa-LV, Pa-II, Ma-VL, Ma-VV, Ma-IV, and Ma-IL.	170
6.4	TEM images of the 24 h-old fatty-acylated dipeptide samples (20 mg/ml) in 50% methanol/water.	171
6.5	SEM images of 50% ethanol/water bigels.	171
6.6	ATR-FTIR spectra recorded for the fatty-acylated peptide samples.	173
6.7	MSD data for Pa-VV, Pa-VL, Pa-LV, Pa-II and Ma-VV, Ma-VL, Ma-IV, Ma-IL gels in 50% methanol/water.	176

List of Figures

6.8	The G' and G'' data, as determined from the MSD data using generalized Stokes-Einstein relation; Pa-VV, Pa-VL, Pa-II, Pa-LV, Ma-VV, Ma-VL, Ma-IV, and Ma-IL in 50% methanol/water.	176
6.9	MSD data of Pa-VV, Pa-VL, Pa-LV, Pa-II, and Ma-VV, Ma-VL, Ma-IV, Ma-IL 50% ethanol/water bigels.	177
6.10	The G' (black squares) and G'' (red circles) data, as determined from the MSD data using generalized Stokes-Einstein relation; Pa-VV, Pa-VL, Pa-II, Pa-LV, Ma-VV, Ma-VL, Ma-IV, and Ma-IL 50% ethanol/water bigels.	178
6.11	Rhodamine B release from Pa-VV, Pa-VL, Pa-LV, Pa-II, Ma-VV, Pa-VL, Ma-IV, and Ma-IL 50% methanol/water bigels.	179
6.12	Fraction docetaxel release from Ma-IV 50% ethanol/water bigel.	179
6.13	Schematic models of probable self-assembling modes of the fatty-acylated dipeptides.	181





List of Tables

1.1	Some of the important literature about self-assembly of the peptides, amino acids, and their analogs.	7
1.2	Some of the peptide amphiphiles sequences and their self-assembly.	16
2.1	Dihedral angles of central residues in β -turns.	40
2.2	List of the peptides studied: their ID, sequence, calculated monoisotopic mass, and the m/z values obtained in MALDI mass spectrometry.	45
3.1	Sequences of the primers used for amplifying GAPDH and insulin-1 cDNA.	79
5.1	The random coil chemical shifts of $H_{C\alpha}$ and H_N for the amino acids used in this study.	151
5.2	The chemical shifts of the $H_{C\alpha}$ and H_N obtained from the TOCSY assignment.	152
5.3	The $H_{C\alpha}$ and H_N chemical shifts of the amino acids in the turn-supporting dipeptide motifs.	154
6.1	List of the fatty-acylated peptides employed in this study; their sequence, ID, calculated monoisotopic mass, and the m/z values obtained in MALDI mass spectrometry.	168
6.2	FTIR data of gel-forming fatty-acylated peptides in ethanol-water.	175



Synopsis

Molecular self-assembly is pervasive in living systems. The assembly of viral particles, assembly of lipids to form the biological membranes, the formation of double helical DNA through hydrogen bonding between individual strands, structural proteins, and the disease-associated or functional amyloids are some of the examples of self-assembly in living systems. Non-biological examples of molecular self-assembly include the formation of molecular crystals and assembly of amphiphilic molecules into monolayers, micelles, and vesicles.

The bottom-up approach of designing smart and functional materials has been of interest in the past few decades. A variety of molecules have been reported to self-assemble into ordered superstructures. Among them, peptides stand out as the molecule of choice for the several advantages they have to offer over other molecules *viz.* biocompatibility, stability, chemical versatility, ease of synthesis, possibilities of chemical modifications, incorporation of desired stereoisomers, to mention a few. A variety of superstructures have been obtained through the self-assembly of peptides, peptidomimetics, and peptide amphiphiles. A number of such self-assembled molecules can fix a large amount of solvent molecules resulting in gels. Gels, an important class of soft matter, have gained considerable attention. Hydrogels obtained from biocompatible molecules such as amino acids, amino acid derivatives, and peptides have found applications in medicine. They have been used as drug delivery vehicles, scaffolds in tissue engineering, and biosensors. The gels (organogels, hydrogels, and bigels) have been reported to possess interesting optical, electrical, mechanical, and catalytic properties. Peptides, therefore, happen to be an interesting class of self-assembling molecules and their potential as gelating materials have been realized in the past decade.

In this study, I explored the self-assembly and the hydrogelating propensity of the A β_{16-22} peptide, its aromatic analogs, as well as their β -turn motif-connected repeats. Despite their potential in diverse applications, high production cost limit their commercialization. Keeping this in mind, another set of peptides wherein the peptidic chain contained rather inexpensive amino acids *viz.* Val, Leu, and Ile coupled with fatty acids, was designed, synthesized, and their gelation potential investigated.

The scientific content of this thesis is organized in the seven chapters.

Chapter 1: Introduction and research objectives

The chapter gives a brief introduction to molecular self-assembly. It provides an overview of some eminent work carried out on nucleic acid, peptide, protein, and amino acid self-assembly. The chapter largely focuses on the self-assembly of amyloidogenic and designed peptides. A section of the chapter deals with the peptide amphiphiles followed by potential applications of the peptide-based superstructures.

Chapter 2: Self-assembly of β -turn motif-connected tandem repeats of $A\beta_{16-22}$ and its aromatic analogs

Self-assembly of short amyloidogenic peptides has been important in understanding the various interactions that drive the process as well as the molecular architecture of the assembled structures. The short amyloidogenic-stretch of β -amyloid, $A\beta_{16-22}$ (Ac-KLVFFAE-am), is a useful model peptide to study the aspects of β -amyloid fibril formation. Herein, the self-assembly was investigated by incorporating β -turn-inducing motifs, in the Ac-KLVXZAE-am chains, where X and Z are the aromatic amino acids, Phe, Tyr, or Trp. In this chapter, the self-assembly of the control (turn-lacking) peptides Ac-KLVFFAE-am, Ac-KLVFYAE-am, Ac-KLVYYAE-am, and Ac-KLVWWAE-am as well as the peptide repeats separated by turn-inducing dipeptide motifs, Asn-Gly, D Pro-Gly, and Aib- D Pro are discussed. The peptides harboring β -turn-inducing motifs aggregate rapidly, cause substantial enhancements in thioflavin T (ThT) fluorescence compared to the controls, the β -turn motif-lacking peptides. The morphology of the fibrils strongly depends on the type of β -turn. Ac-KLVFYAE-am repeats separated by Aib- D Pro and D Pro-Gly have the highest aggregation propensity among all the peptides studied. The chapter also discusses the theoretical results obtained from molecular dynamics simulation of the turn-containing peptides, giving insights into the type of turn formed. The study indicates that β -turn motifs can be exploited to modulate and control the aggregation propensity of the peptides and the morphology of the aggregates.

Chapter 3: Hydrogel formation by the $A\beta_{16-22}$ aromatic analog, $A\beta_{16-22}$ (Phe₂₀Tyr)

The results from the first chapter led to a follow-up investigation into the amyloidogenic propensity of the short 7-residue stretch, $A\beta_{16-22}$ (Ac-KLVFFAE-

am), and its aromatic analogue A β FY (Ac-KLVFYAE-am) at higher concentrations. The results showed that the aromatic analogue A β FY forms self-supporting soft gels at concentrations ≥ 2 mM, even though the end-capped parent peptide does not form hydrogel up to 20 mM (1.8% w/w) concentration in 10% HFIP. This chapter delves into the characterization and possible applications of the hydrogel. The hydrogel made up of amyloid-like fibers possess distinct elastic properties. The gel is shown to support the growth of rat pancreatic cells (RIN-5F), human embryonic kidney cells (HEK-293), baby hamster kidney cells (BHK-21), and human neuroblastoma cells (IMR-32). The results include the doxorubicin release assay showing its potential as a drug-delivery vehicle, the thermal response of the gels, as well as the gelation at the acidic pH. The other two aromatic analogs Ac-KLVYYAE-am, and Ac-KLVWWAE-am did not cause gelation at a 2 mM concentration under identical condition.

Chapter 4: Lipid hydrogels from β -turn motif-connected tandem repeats of A β_{16-22} and its aromatic analogs

Inspired by the insights gained from the results detailed in the chapters 2 and 3, an attempt was made to look into the hydrogelation propensity of the A β_{16-22} repeats, connected through β -turn-supporting motifs specifically the ones having Phe-Phe or Phe-Tyr aromatic cassette in their sequence. This chapter discusses the results wherein the A β_{16-22} repeats connected through Asn-Gly, Aib-^DPro, and ^DPro-Gly form transparent gels at concentration ≥ 2 mM in 10% HFIP. The repeats of the aromatic analog A β FY also resulted in similar hydrogels. These gels could trap the anticancer drug, doxorubicin and displayed its steady release in water. In addition, the gels supported the growth of mammalian cell lines, HEK-293 and RIN-5F. The gels turned out to be mechano-responsive, wherein the shear-thinned gels re-gel upon standing. The results support that the turn-inducing motifs can have marked influence on the hydrogelating propensity of the self-assembling peptides.

Chapter 5: Investigation into the β -turn-supporting motifs using proton NMR

The results described in chapters 2 and 4 showed that the β -turn supporting motif-connected tandem repeats of A β_{16-22} and its aromatic analogs display distinct self-assembling propensities. The results prompted me to examine the β -turn region in the peptides using NMR spectroscopy. The peptides,

unfortunately, were poorly soluble in water and CD₃OH. The NMR spectra, therefore, were recorded in DMSO-d₆. Assignments were made from the TOCSY spectra. Large spectral overlap alongside aggregation, however, made it difficult to carry out sequential assignment from the NOESY spectra. Individual amino acids or pairs of amino acids such as AA, KK, LL, VV, Aromatic-Aromatic, EE, on the other hand, could be unambiguously identified. The data, there, was interpreted using the chemical shift indices and indicated turn formation.

Chapter 6: Water-alcohol bigels from fatty-acylated dipeptides

Short fatty-acylated peptides with polar amino acids have been reported to self-assemble and cause gelation. Amino acids with non-polar aliphatic side-chains are cheap resource material as side-chains need not be protected during synthesis. In addition to that, the high hydrophobicity of the peptides facilitates ready purification through solvent extraction. In this chapter, the results of investigations into the self-assembly of short amphipathic peptides having two amino acids coupled to a fatty-acylated chain in the alcohol-water mixture have been discussed. The peptide backbone and C-terminal carboxylate group happen to be the only polar moieties in the peptides. The dipeptides made up of amino acids from the three hydrophobic aliphatic amino acids (Ile, Leu, and Val) coupled with one of the three fatty acids *viz.* the lauric acid, the myristic acid, and the palmitic acid were investigated. This resulted in a total of 27 fatty-acylated peptides. Out of the 27 fatty-acylated peptides, 8 resulted in distinct water-alcohol gels. The 2 wt% gels can entrap the hydrophobic anticancer drug, docetaxel causing sustained release for 3 days in PBS. Such bigels can find applications in the delivery of drugs that have low solubility in water and therefore tend to precipitate in the hydrogels.

Chapter 7: Conclusions and Future Directions

This chapter discusses the highlights of the findings described in chapters 2-6 and the future possibilities.

List of Publications

Journal Articles

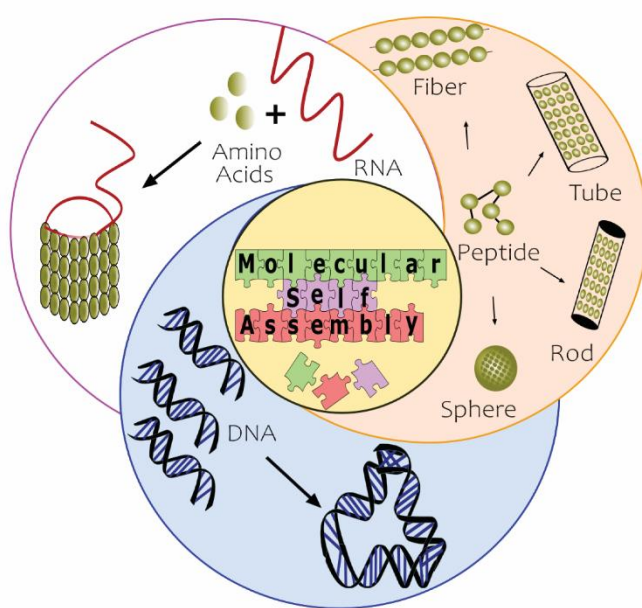
- (1) **Debika Datta**, Ramakrishnan Nagaraj*, Nitin Chaudhary*. Water-alcohol bigels from fatty-acylated dipeptides. The Journal of Physical Chemistry B. 2020; 124(3), 577-588, DOI:10.1021/acs.jpcb.9b10002.
- (2) **Debika Datta**, Vishnu Kumar, Sachin Kumar, Ramakrishnan Nagaraj*, Nitin Chaudhary*. Lipid hydrogels from β -turn motif-connected tandem repeats of $A\beta_{16-22}$. Soft Matter. 2019; 15(24), 4827-4835, DOI: 10.1039/c9sm00343f.
- (3) **Debika Datta**, Vishnu Kumar, Sachin Kumar, Ramakrishnan Nagaraj, Nitin Chaudhary*. Hydrogel formation by an aromatic analog of β -amyloid fragment, $A\beta_{16-22}$ – a scaffold for 3-D cell culture. ACS Omega. 2019; 4(1), 620-627, DOI: 10.1021/acsomega.8b02771.
- (4) **Debika Datta**, Adicherla Harikrishna, Ramakrishnan Nagaraj*, Nitin Chaudhary*. Self-assembly of β -turn motif-connected tandem repeats of $A\beta_{16-22}$ and its aromatic analogs. Peptide Science. 2019; 111(3), 2475-8817, DOI: 10.1002/pep2.24099.

Conference Proceedings

- (1) **Debika Datta**, Richa Arya and Nitin Chaudhary*. Investigations into the role of amphiphilicity in $A\beta_{16-22}$ self-assembly, Journal of Proteins and Proteomics, 2014; 5(3), SPECIAL ISSUE Conference.



INTRODUCTION AND RESEARCH OBJECTIVES





1.1 A BRIEF BACKGROUND ON MOLECULAR SELF-ASSEMBLY

Nature has always amazed us with its simple, beautiful creation, ever wondered how the water droplets turn into amazing ice crystals or snowflakes under two different conditions? Well, the process underlying this phenomenon is known as self-assembly. Any physical system has the natural propensity to exchange energy with its surroundings to attain a low energy state. To bring the constituent particles to a stable configuration, they assume structures with reduced free energy state. This spontaneous, self-assembly process happens without any external intervention. In the language of a chemist or biologist, the term molecular self-assembly is often referred to as the process of spontaneous arrangement of molecules into ordered structures through various non-covalent interactions. From the assembly of the soap molecules in a soap bubble to the assembly of four haemoglobin subunits to form a functional tetrameric protein, there are numerous examples of self-assembly in nature [1, 2]. Inspired by these examples, chemists and material scientists have designed a variety of molecules that self-assemble into ordered superstructures [3-7].

The prevalent forces that mediate the self-assembly process include electrostatic interactions (ionic bonds), stacking (aromatic) interactions, van der Waal's forces, hydrophobic interactions, hydrogen bonding (intra and intermolecular), and metal-chelation [8, 9]. While all these non-covalent interactions are rather weak in isolation, when combined together, they play the decisive role in the conformation a molecule can take and the subsequent self-assembly [10-13]. Through these interactions, small building blocks associate to produce large superstructures with distinct physical and chemical properties. This approach of assembly has been commonly called the "bottom-up" technique for designing and fabrication of nano- and microstructures [14].

Self-assembly of molecules is central to life. In biological systems, simpler building blocks associate themselves in solution through molecular recognition and self-assembly to give rise to rather complex structures. The self-assembly of lipid molecules in water to give rise to a lipid bilayer is perhaps one of the most

Chapter 1

well-known examples of self-assembly in biology. The amphipathic nature of the phospholipids causes the hydrophobic tail to be shielded from the water inside the hydrophobic core, whereas the hydrophilic polar head group is exposed to the aqueous environment [15, 16]. Other examples include assembly of nucleic acids through H-bonding and stacking interactions between the nitrogenous bases, the self-assembly of single protein/glycoprotein in the S-layers in bacterial cell surface (all archaea and many bacteria) [17], spontaneous formation of template-assisted virus shell (like tobacco mosaic virus) [18], the folding of polypeptide chain to form the tertiary or quaternary protein structure [19], silk fibroin protein fibrillar assembly [20, 21], and amyloid fibrils [22-25].

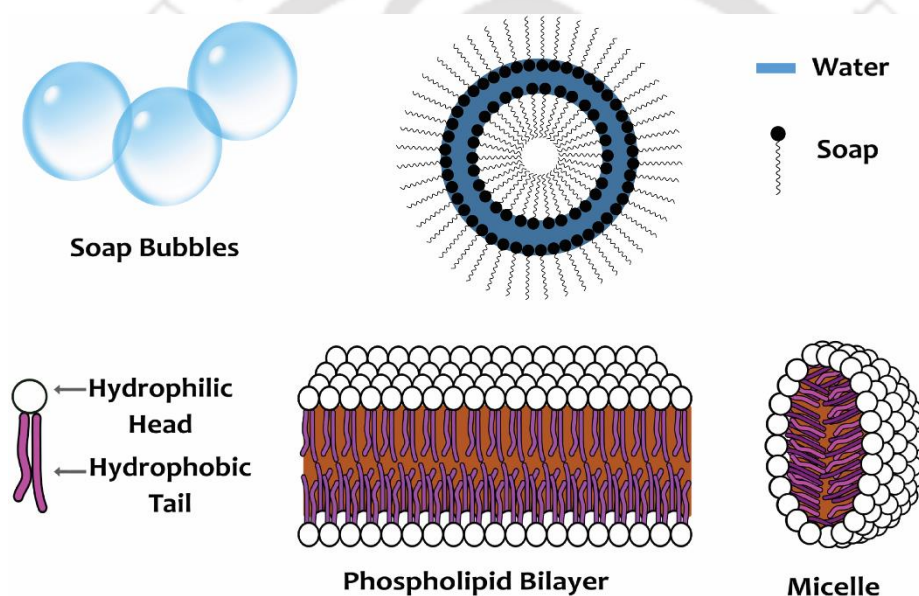


Figure 1.1 A diagrammatic representation depicting self-assembly of soap molecules forming the bubbles (top) and phospholipid molecules forming bilayer and micelles (bottom).

Molecular self-assembly has shown promise to be a powerful tool in the fabrication of sophisticated nanomaterials. Self-assembly of molecules in biological systems is highly regulated and carried out with very high fidelity. This is necessary to ensure proper function and avoid deleterious effects. Designing the molecules that can self-assemble in a controlled way and can respond to stimuli in a predicted manner is an emerging area in material science. Inspired by the tight control of assembly and precise emerging properties of the biological

self-assembly, researchers have looked towards the molecules of biological origin to design such self-assembling molecules. Researchers have utilized molecules that include, but are not limited to, lipids, nucleic acid complexes, amino acids, peptides, proteins, and peptidomimetics [15, 26-32]. Some of the examples are discussed in the subsequent sections.

1.2 NUCLEIC-ACID SELF-ASSEMBLY

The double-helical DNA happens to be one of the most well-known cases of biomolecular recognition and self-assembly. The two strands recognize the complementary nucleotide sequence on each other and self-assemble to fold into a double-helical structure. The exceptional stability to the DNA structure, conferred by the aromatic stacking interactions and H-bonding, made the molecule to be considered as a potential nanomaterial from the late 90s. An additional H-bond between guanosine and cytosine compared to those between adenine and thymine provides high thermostability to the DNA molecules. Similarly, in the case of RNA (ribonucleic acid), multiple single-stranded structures with intramolecular interactions lead to the formation of complex 3D structures.

One of the pioneering works that demonstrated the potential of DNA as a nanomaterial was carried out by Nadrian C. Seeman and coworkers in 1998. They designed the DNA molecules that self-assemble to produce 2-dimensional DNA crystals. The assembly was driven by intermolecular interactions between the base pairs of the designed DNA molecules having “sticky ends” [30]. Subsequent studies showed the formation of more complex 3D structures from DNA templates [33-36]. Design of programmable DNA nanotubes by Rothemund and coworkers expanded the possibilities of nucleic acids as building blocks for nanomaterials [37]. The rather straightforward synthesis of oligonucleotides using chemical methods has enabled nucleic acid strands to be tagged with other materials like nanoparticles, small molecules, or proteins. The excellent molecular recognition of the complementary sequences by the DNA overhangs has been exploited in developing conducting silver nanowires between the

Chapter 1

electrodes that have been tagged with oligonucleotides of defined sequences [38]. Braun and coworkers further exploited DNA molecular recognition properties to precisely localize the carbon nanotubes to build carbon nanotube field-effect transistor [39]. In another exciting piece of work, Yan and coworkers designed 4 x 4 DNA tile that self-assembles into nanoribbons and highly ordered two-dimensional nanogrids [40]. Subsequent deposition of streptavidin on the DNA microarrays made up of biotinylated nucleotides resulted in protein microarrays. The nanoribbons also acted as a scaffold for depositing metal to generate conducting nanowires [40]. Turberfield and coworkers developed a tweezer-like DNA structure; the opening and closing of which could be fuelled by supplying single-stranded DNA molecules. [41]. An excellent review by Feldkamp and Niemeyer discusses the various designed DNA hybrid conjugates giving rise to programmed supramolecular DNA nanostructures [42].

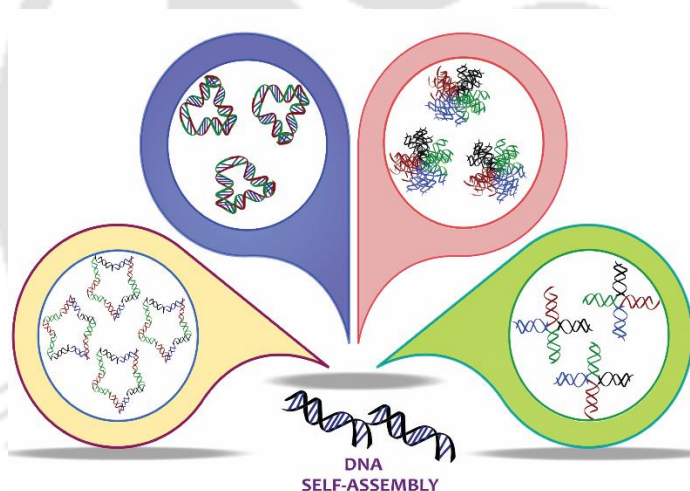


Figure 1.2 A diagrammatic representation of DNA self-assembly.

1.3 PROTEIN AND PEPTIDE SELF-ASSEMBLY

The basic building block of proteins and peptides are the amino acids. The presence of the 20 natural amino acids along with the unnatural amino acids offers numerous possible combinations of sequences with intriguing physicochemical properties. The chemical space of a polymer made up of 'X' different monomers is given by X^N , where N is the length of the polymer. Thus, the larger the number of monomers available, the more diverse the polymers are.

For example, for a tetrapeptide made up of naturally-occurring 20 amino acids, there are 20^4 i.e. 160,000 different sequences possible. An oligonucleotide of the same length, on the other hand, would have only 4^4 i.e. 256 different sequences. Considering the possibility of including the D-enantiomers expands the diversity of a tetrapeptide to 40^4 i.e. 1,560,000, a number four orders of magnitude larger than that for an oligonucleotide. Due to their inherent chemical versatility, ease of synthesis, physical and chemical stability, well-established principles of their folding, the peptides stand out as an excellent choice of starting material. Due to their properties such as biocompatibility and biodegradability, peptides turn out to be ideal for making nanomaterials for biomedical applications.

Peptide and amino acid based materials have shown interesting electrical, optical, mechanical, and biological properties. Some of the exciting reports on self-assembled structures are listed and discussed in table 1.1.

Table 1.1 Some of the important literature about self-assembly of the peptides, amino acids, and their analogs.	
Reference	Work done
Amyloidogenic peptides	
Rudolf Virchow	Found that corpora amylacea in ependyme (the thin lining of ventricular system of brain) and choroid plexus (an area in the brain that produces cerebrospinal fluid) stained violet with iodine. He strongly believed that the stained material was starch and named it “amyloid.
Friedreich and Kekulé, 1859 [43]	Showed that protein was the main substance present in the amyloid-rich segments isolated from spleen.
Cohen and Calkins, 1959 [44]	Observed elongated fibrils in the electron micrographs of various amyloid disease tissue.
Eanes and Glenner, 1968 [45]	The first report on the structural organization of proteins/peptides in amyloid fibrils (X-ray fiber diffraction). The first proof of the cross- β structure of amyloid fibrils.
Petkova et al., 2002 [46]	Structural model of $A\beta_{1-40}$ from solid-state NMR.
Török et al., 2002 [47]	Structural features of amyloid fibrils of Alzheimer's $A\beta$ peptide using Electron paramagnetic resonance (EPR) spectroscopy.
Lu et al., 2003 [48]	Formation of highly-ordered nanotubes by the amyloidogenic fragment of β -amyloid, $A\beta_{16-22}$ at acidic pH.
Scheibel et al., 2003 [49]	Conducting nanowires from amyloid fiber yeast Sup35p self-assembly.
Jayasinghe et al., 2004 [50]	Structural feature identification of fibrillar islet amyloid polypeptide using EPR.
Nelson et al., 2005 [51]	The structure of the cross- β spine of a seven residue amyloidogenic fragment of Sup35. The first atomic-resolution structure of an amyloid fibril.
Heise et al., 2005 [52]	Structure and dynamics of full-length α -synuclein fibrils by solid-state NMR.

Chapter 1

Smith et al., 2006 [53]	Studied mechanical properties of insulin fibrils. The fibrils were found to have strength comparable to that of steel and mechanical stiffness similar to that of silk.
del Mercato et al., 2007 [54]	Intrinsically conducting and fluorescent amyloid-like fibrils of poly (VGGLG).
Krysmann et al., 2008 [55]	Hydrogel from an amyloidogenic fragment of β -amyloid, KLVFF.
Bhak et al., 2010 [56]	Accumulation of curly protein fibrils of α -synuclein produced amyloid hydrogel.
Tayeb-Fligelman et al., 2017 [57]	The crystal structure of 22-residue phenol-soluble modulin α_3 (PSM α_3) peptide secreted by <i>Staphylococcus aureus</i> forms amphipathic α -helices that are folded to stack perpendicular to the fibril axis into sheets revealing a “cross- α ” amyloid-like architecture.
Guerrero-Ferreira et al., 2018 [58]	Structure of α -synuclein fibrils using Cryo-EM.
Peptide nanotubes	
Ghadiri et al., 1993 [59]	Formation of nanotubes from cyclic syndiotactic peptides. Extensive H-bonding network stabilizes the self-assembled structures.
Ghadiri et al., 1994 [26]	Artificial ion-channels using nanotubes made up of cyclic syndiotactic peptides having membrane-interacting properties.
Reches and Gazit, 2003 [60]	Nanotubes from diphenylalanine. Casting wires using nanotubes as the molds.
Reches and Gazit, 2004 [61]	Nanosphere formation from diphenylglycine derivatives.
Horne et al., 2005 [62]	Charge transfer through naphthalenetetracarboxylic acid diimide (NDI) side chain in cyclic peptide self-assembly.
Carny et al., 2006 [63]	Coaxial wires using diphenylalanine nanotubes.
Görbitz, 2006 [64]	Structure of the diphenylalanine nanotubes.
Liang et al., 2008 [65]	Amyloid fibrils as the scaffold for light harvesting.
Kholin et al., 2010 [66]	Reported strong shear piezoelectric activity in the diphenylalanine nanotubes.
Yan et al., 2011 [67]	Hexagonal crystalline microtubes through higher order organization of the diphenylalanine nanotubes. Show linear birefringence and optical waveguiding.
Tarabout et al., 2011 [68]	Modulation of the peptide tube diameters by chemical modifications of the aromatic residues in close contacts.
Non-amyloid peptides-based fibers, ribbons, tapes, and hydrogels	
Aggeli et al., 1997 [69]	Short oligopeptides form polymeric β -sheet nanotapes in water and methanol.
Niece et al., 2003 [70]	Nanofibers from peptide-amphiphile molecules. Assembly through electrostatic attraction and hydrophobic interaction.
Ozbas et al., 2004 [71]	β -hairpin peptide self-assembles to form hydrogel triggered by the presence of salt.
Yokoi et al., 2005 [72]	Self-complementary peptide RADA16 forms nanofibers and hydrogel.
Schneider et al., 2002 [73] Kretsinger et al., 2005 [74]	MAX1 peptide folds into β -hairpin conformation and thereby forms the hydrogel.
Letchford and Burt., 2006 [75]	Formation of micelles, nanospheres, nanocapsules, and polymerosomes from amphiphilic copolymers of polyethyleneglycol derivatives.

Mahler et al., 2006 [76]	Aromatic Phenylalanine dipeptides forming hydrogels.
Zhang et al., 2003 [77] Smith et al., 2008 [78]	Hydrogel formation from Fmoc-diphenylalanine and other Fmoc-dipeptides.
Yang et al., 2007 [79]	Intracellular formation of nanofibers (catalyzed by enzymes) that leads to hydrogelation and cell death.
Hirst et al., 2008 [80]	Supramolecular gel-based nanostructures find application as biomaterials, regenerative medicine, hydrogels, metallogels, optical technologies, etc.
Lim et al., 2009 [81]	A cyclic RGD-coated peptide self-assembles to form nanoribbons structure.
Rajagopal et al., 2009 [82]	MAX1 peptide variant [MAX1(K15E)] undergoes thermally triggered hydrogelation at physiological buffer conditions.
Cui et al., 2009 [83]	A peptide amphiphiles consisting of 4 amino acids and an alkyl tail self-assembles to form giant peptide nanobelts.
Ma et al., 2010 [84]	Nanofibers and hydrogel formation from glycine-rich pentapeptide derivatives.
Huang et al., 2011 [85]	Photoresponsive hydrogels from azobenzene substituted short peptides.
Fletcher et al., 2011 [86]	A pH-responsive reversible hydrogel formed from a 21 residue α -helical peptide.
Castelletto et al., 2011 [87]	A peptide amphiphiles (C16-KTTKS) forms tapes, twisted ribbons, and fibrils on the addition of the oppositely charged anionic surfactant.
Ghosh et al., 2013 [88]	Derivatives of diphenylalanine conjugates form fibers.
Xie et al., 2015 [89]	Helical nanoribbons from Fmoc-tripeptides.
Amino acid-based structures	
Gortner et al., 1921 [90]	One of the first examples of an amino acid based hydrogel: the dibenzoyl cysteine formed a hydrogel.
Vegniers et al., 1995 [91]	First report of a Fmoc dipeptide hydrogelator (Fmoc-Leu-Asp).
Yang et al., 2004 [92]	Hydrogel formation by N α -Fmoc-Tyr and N ϵ -Fmoc-Lys.
Toledano et al., 2006 [93]	Reversed hydrolysis by proteases formed potent peptide hydrogelators, from Fmoc-tripeptides.
Jayawarna et al., 2006 [94]	Fmoc dipeptides form hydrogels capable of supporting 3D cell culture.
Yang et al., 2007 [95]	A β -amino acid derivative forms supramolecular hydrogel, <i>in vivo</i> and <i>in vitro</i> , triggered by phosphatase enzyme.
Gao et al., 2009 [96]	Enzymatic dephosphorylation triggered the self-assembly and hydrogelation of a C-terminal methyl ester derivative of Fmoc-Tyr.
Liang et al., 2009 [97]	Controlled drug release from D-amino acid dipeptide hydrogels.
Sutton et al., 2009 [98]	Controlled dye-release from Fmoc-amino acid hydrogels.
Ryan et al., 2010 [99]	Compared to Fmoc-Tyr that forms hydrogels in 30 minutes with a rigidity, G' of ~ 1000 Pa, Fmoc-Phe(F ₅) forms optically transparent hydrogels within 5 minutes with a G' of ~ 3000 Pa. Perfluorination of the phenyl side chain results in a significant electronic perturbation of the phenyl side chain (the side chain is much more electron deficient than that of Tyr) and an increase in hydrophobicity.

Chapter 1

Koley et al., 2011 [100]	Formation of nanorods, nanofibrils, nanotubes, and nanovesicles from single hydrophobic amino acid (Phe, Trp, Tyr and Leu) based molecules.
Cao et al., 2012 [101]	Controlled self-assembly from achiral amino acid derivatives to form chiral twisted nanostructures.
Irwansyah et al., 2015 [102]	Antimicrobial activity against gram-positive bacteria, from coassembly of Fmoc-Phe and Fmoc-Leu hydrogels.

1.3.1 Amyloid Self-Assembly

Amyloid fibrils constitute a particularly important class of self-assembling proteins and peptides. Amyloid fibrils are long, fibrous aggregates that are composed of long β -sheets and are characterized by a meridional reflection at 4.7-4.8 Å and an equatorial reflection at ~ 10 Å. Amyloid fibrils are mostly known as the aggregates of misfolded proteins that lead to various neurodegenerative disease conditions like Alzheimer's, Parkinson's, type II diabetes, Huntington's disease, etc. [103]. However, studies in the past decade have identified various amyloidogenic proteins that are native functional forms in various living forms, including humans. The disease-unrelated amyloids have been found in aerial hyphae of filamentous bacteria, bacterial biofilm formation, memory consolidation in *Drosophila*, as well as in melanin synthesis in humans [23, 104-106]. The growing evidence suggesting non-toxicity of amyloid fibrils eased their way for being used as functional materials in technological applications.

Amyloid fibrils have drawn the attention from the biological, chemistry, and material science community alike owing to their impressive material properties. Amyloid fibers could have unusually high mechanical properties; insulin fibrils, for example, show mechanical strength comparable to that of steel, stiffness similar to that of silk [53]. Owing to their stability and high aspect ratio (can go up to hundreds of micrometres), amyloid fibrils have been utilized to make nanowires by metal deposition [49, 60]. Furthermore, the fibers could be intrinsically conducting, as reported by del Mercato and coworkers [54]. It is always advantageous to have short peptide sequences as monomers for self-assembly. Short peptides are not only economical and easy to synthesize but also self-assemble through a limited set of interactions, thereby providing better control over self-assembly. Görbitz and Gazit showed the formation of

nanotubes from the dipeptide diphenylalanine, which is the core recognition motif of the β -amyloid protein [60, 64]. Diphenylalanine nanotubes have been used as molds for casting metal nanowires [60].

Furthermore, through metal deposition on the surface of the tubes, coaxial wires have been generated [63]. These applications highlight the potential uses of the hollow nanostructures in molecular electronics. Phe-Phe dipeptide motif is one of the most widely studied self-assembling peptide sequences. Subtle changes in the assembly conditions have resulted in rods and fibers with interesting properties like thermal stability, mechanical stability, piezoelectricity, field sensitivity, and as biosensors [66, 107-113].

Several short (4-10 residues) peptide sequences have been shown to form ordered amyloid-like fibrils with characteristic structural properties can serve as model systems for understanding the self-assembly of amyloid fibrils [114-118]. The 16-22 residue stretch of β -amyloid ($A\beta_{16-22}$: Ac-KLVFFAE-am) has been known to assemble into fibers, nanotubes, and annular rings under different conditions [48, 119-121]. Like Phe-Phe, highly ordered self-assembled nanotubes were obtained from the $A\beta_{16-22}$ fragment at acidic pH [48]. Krysmann et al., showed the formation of hydrogels from an amyloidogenic fragment of β -amyloid, KLVFF [55]. The aromatic cassette Phe-Phe is believed to be critical in modulating self-assembly [122-125]. Nagaraj and coworkers investigated various aromatic analogs of $A\beta_{16-22}$, substituting Phe with Tyr and Trp residues and obtained highly amyloidogenic variants [124]. In another remarkable study, Buchanan et al., investigated the mechanism of IAPP (human islet amyloid polypeptide) associated with type II diabetes, using spectroscopic methods [126]. Kapurniotu and coworkers identified short amyloidogenic stretches from human IAPP. The stretches IAPP₂₃₋₃₇ (FGAIL), IAPP₂₂₋₂₇ (NFGAIL), and IAPP₂₀₋₂₇ (SNNFGAIL), and IAPP₂₀₋₂₉ (SNNFGAILSS) are reported to form characteristic amyloid-like fibrils *in vitro* [115]. Johansson and coworkers have reported typical amyloid-like fibrils from *de novo* designed tetrapeptides viz. KFFE and KVVE; the charge complementary is reported to play a critical role in the assembly [116].

Chapter 1

Goux and coworkers identified short amyloidogenic stretches (≤ 6 residues) from the tau protein [117].

Research in past 20 years has shed light on the possible toxic protein species in amyloid diseases; it turns out that the toxicity is mostly restricted to prefibrillar aggregates and fibrils are the innocent bystanders [127-129]. This feature could make amyloid fibrils potential candidates as tissue engineering and drug delivery vehicles. The recognition principles involving the key use of aromatic interactions in driving the self-assembly of amyloid fibrils could thus be used in the design and fabrication of nanomaterials.

Assembly of designed peptide-based molecules

The understanding of principles of peptide chemistry and folding has enabled the researchers to *de novo* design the peptide sequences that can fold and assemble predictably. The conformational space has been expanded by incorporating the D-amino acids. Peptide self-assembly is rather sensitive to self-assembly conditions. The terminal structures and their emergent properties strongly depend on the solvent, pH, temperature, ionic strength, agitation, and incubation period. Some of the principal classes of the *de novo* designed peptides are discussed below.

1.3.2 Cyclic peptides

A seminal work by Ghadiri and coworkers in 1993 gave the first peptide nanotubes from syndiotactic cyclic peptides [59]. The unique design principle involved incorporation of amino acids with alternate $C\alpha$ stereochemistry (alternate L and D-amino acids). In addition, the peptides were cyclized, thereby putting large scale conformational constraints. The peptide molecules stack up, giving rise to hollow nanotubular structures. The stacked ring subunits are stabilized by the intermolecular H-bonding to form a β -sheet conformation [27]. Artificial transmembrane ion channels were subsequently derived through self-assembly of similar cyclic peptides [26]. Self-assembly of the syndiotactic cyclic peptides tagged with 1,4,5,8-naphthalenetetracarboxylic acid diimide (NDI)

resulted in NDI excimer formation [62]. This study shows that self-assembling cyclic syndiotactic peptides could be used to develop nanomaterials with interesting electronic and optical properties. A tryptophan-rich cationic peptide designed on the same principles has been shown to self-assemble into tubular structures that disrupt bacterial membranes, thereby acting as an antimicrobial agent. [130]. The Lanreotide, a synthetic analog of somatostatin, is used as growth hormone inhibitor. This cyclic octapeptide has been shown to form tubular structures in aqueous solution [131]. The nanotubes have been reported to have viral capsid-like dimension. An alternating pattern of aromatic and aliphatic amino acid residues resulted in antiparallel β -sheet conformation in the peptide building blocks. Furthermore, the self-assembly process for several cyclic peptides formed from 3-5 repeats of dipeptides having hydrophobic and charged amino acid residues have also been investigated [132]. The self-assembly of several cyclic syndiotactic peptides has been described in an excellent review by Perrier and coworkers [133].

1.3.3 Peptide amphiphiles

A peptide amphiphile (PA) is a peptide or peptide conjugate having distinct polar and non-polar regions. An amphiphilic peptide can be made by attaching a stretch of non-polar amino acid residues (forming the hydrophobic tail) to polar or charged amino acids (forming the hydrophilic head). Besides, the amphipathicity could be conferred through folding and self-assembly. The peptides may lack distinct hydrophilic and hydrophobic regions in their primary structure but can fold/assemble to attain an amphipathic character [72, 86, 87].

Lipopeptides

An important class of PAs is a peptide chain conjugated to a fatty acid/lipid/surfactant [134-139]. Surfactin, a cyclic lipopeptide of the *Bacillus subtilis* origin, is an example of naturally-occurring lipopeptides [140]. The generalized structure of lipopeptides includes an acyl carbon chain linked to one of the peptide termini. Stupp and coworkers made extensive contribution to this class of self-assembling molecules by investigating several such peptide

Chapter 1

amphiphiles. They synthesized several amphiphilic molecules by attaching a long hydrophobic alkyl tail with a hydrophilic peptide chain head, mimicking a phospholipid molecule [83, 134, 141-143]. One such example is that of formation of giant nanobelts of length up to 0.1 mm formed from the self-assembly of $C_{16}H_{31}OVEVE$ [83]. The headgroup of this lipopeptide had alternating hydrophilic (E) and hydrophobic residues (V). Another interesting work is that on the formation of nanofibres from self-assembled branched peptide amphiphiles. The PAs had palmitoyl chain coupled to the peptide sequence containing RGDS as cell adhesion epitope [142]. Other lipopeptides like $C_{10}H_{19}OA_4G_3S(PO_4)RGD$ and $C_{16}H_{31}OC_4G_3S(PO_4)RGD$ have been shown to self-assemble into nanofibers at low pH and disassemble fully with increasing pH, thus making them pH-responsive [134]. Greenfield et al. showed that the addition of salts in PAs resulted in nanofibrillar gels having stronger rheological properties. The calcium-mediated ionic bridges in $CaCl_2-C_{16}-V_3A_3E_3$ lipopeptide resulted in gels with stronger intra- and inter-fiber crosslinks; thereby enabling them withstand higher strains than the HCl-PA lipopeptide gel [143]. Deng et al., coupled a 7-residue peptide stretch from the β -amyloid ($A\beta_{11-17}$) to dodecanoic acid and found that the otherwise non-assembling $A\beta_{11-17}$ sequence, when synthesized as $C_{12}-A\beta_{11-17}$, was able to self-assemble into nanofibres and nanobelts at pH 3 and 10, respectively [144]. The lipopeptides have shown to form a variety of interesting superstructures. The peptide $C_{16}H_{31}-WA_4KA_4KA_4KA$ was found to self-assemble into spherical micelles with diameters around 10 nm, that gradually transformed into worm-like micelles on prolonged incubation [145].

Interestingly, fatty acids mixed with di- and multi-amines have been investigated and shown to form distinct hydrogels [146]. This, however, is not an example of a peptide amphiphile but co-assembly of polar molecules with non-polar ones. On a similar line, hydrogels were obtained from sodium salts of fatty acids mixing with oligolysines [135].

Peptide-only amphiphiles

As the naturally-occurring amino acid pool contains both hydrophilic and hydrophobic amino acids, the amphiphilic character can be imparted to the peptides, without the need of an alkyl or a lipidic moiety. A number of such peptides have been investigated. One of the earliest works with such peptide amphiphiles was carried out by Walton and coworkers. They reported the self-assembly of (SG)_n and (AG)_n peptide amphiphiles into fibrillar β -sheet structures [147, 148]. Zhang and coworkers studied several lipid-like peptides like A₆D, V₆D, V₆D₂, that formed the self-assembled structures like nanotubes, micelles, and vesicles [149, 150].

In a similar study, several glycine-rich peptides behaved like “surfactant-like” molecules, thereby having the potential to stabilize membrane proteins [151]. The length of the hydrophobic tail has also been shown to modulate self-assembly in peptide amphiphiles. One such example is A_nK (where n= 3, 6, 9) showed aggregates of different size and shape on increasing the length of the hydrophobic residues [152, 153]. Electron microscopy imaging showed the formation of sheets, fibrillar morphology (worm-like micelles), and nanorods formation for A₃K, A₆K, and A₉K, respectively. Zhang and coworkers further investigated the self-complementary of the ionic peptides consisting of aspartic or glutamic acid residues (at N-terminus) and arginine or lysine residues (at C-terminus) to drive the self-assembly [154]. Rich and coworkers studied the sequence Ac-(AEAEAKAK)₂-NH₂, which resulted in β -sheet fibres of 10-20 nm in diameter [155, 156]. Replacing the Glu with Arg, and the Lys with Asp (RADA16 peptide) resulted in hydrogels that find application in tissue engineering [72, 157]. Congo red birefringence proved that the fibrillar morphology is similar to that of the amyloid fibrils [158].

The peptide EFK16-II (FEFEFKFKFEFEFKFK) has been shown to form a β -sheet structure [159]. To investigate the effect of changing the size and sequence of the non-polar residues, Mueller and coworkers studied peptide self-assembly by introducing residues like Val, Leu, Ile, Gly in the place of Phe in the sequence

Chapter 1

(FEFEFKFK)₂. The size and sequence of the non-polar residues caused variations in the β -sheet secondary structure of the peptides that affected the self-assembly [160].

Bolaamphiphiles

A family of bolaamphiphile peptides was investigated by Matusi and coworkers, wherein the assembly resulted in tubular structures [161, 162]. The histidine-rich sequences were able to immobilize Au and Cu nanocrystals on the nanotube surface. Another study by Banerjee et al. shows phenylalanine-based bolaamphiphile with an oligomethylene group to be a potent metallo-hydrogelator [163]. The hydrogel can adsorb various toxic dyes from waste-water and also act as a carrier for vitamin B12.

The literature available on the work of peptide amphiphiles is vast. Some of the excellent reports provide essential insights into the assembly [10, 134, 136, 138, 164, 165]. Some of the superstructures derived from PAs are listed below in table 1.2.

Table 1.2 Some of the peptide amphiphiles sequences and their self-assembly.		
Sequence	Self-assembled structures	References
Ac-G _m D _n -OH	Nanotubes, nanovesicles	[151]
Ac-GAVILRR-NH ₂	Nanodonuts, spherical micelles	[166]
Ac-I ₃ K-NH ₂	Nanotubes	[167]
K _m L _n	Nanofibers	[168, 169]
NH ₂ -X ₅ -H ₄ R ₈ -CONH ₂ (X = I, W, F)	Nanoparticles	[170]
A ₁₂ H ₅ K _{10 or 15}	Core shell nanoparticles	[171]
C ₁₆ H ₃₁ -A ₄ K ₄	Nanofibers	[172]
C ₁₆ H ₃₁ -V ₃ A ₃ E ₃	Nanofibrillar gels	[143, 173]
C ₁₆ H ₃₁ -LSQETFSDLWKLLPEN	Rod-like micelles	[174]
Cholesterol-G ₃ R ₆ YGRKKRRQRRR	Nanoparticles	[175]
C ₁₂ H ₂₃ -EVHHQKL	Nanofibrils	[144]
Ac-QQRQQQQEQQ-NH ₂	Twisted ribbon, nanotapes	[29, 176, 177]
Ac-QQRFQWQFEQQ-NH ₂	helical β -sheet tapes	[29, 177, 178]
Palmitoyl-GV ₃ A ₃ E ₃	Fibrils	[179]
VEVE	Nanobelt	[83, 180]
VVEE	Cylindrical Nanofibers	[83]
R-GRKKRRQRRRPPQGSGG-FKFEFKFEFKFE	Nanoribbon	[181]
C ₁₂ -GAGAGAGY	Nanofibers	[182]
C ₁₆ -KTTKS and C ₁₆ -ETTES (mixed)	Nanotapes	[183]

H ₂ N-VKVKVKVK-V ^D PPT-KVKVKVKV-NH ₂	Fibrils	[71, 73, 184]
Fmoc-RGD	Fibrillar hydrogel	[185]
COOH-GE ₃ L ₃ -COC ₅ H ₁₀ -(urea oligomer), COOH-GE ₃ L ₃ -COC ₅ H ₁₀ -(urea oligomer), COOH-E ₃ L ₃ -COC ₅ H ₁₀ -(urea oligomer)	Nanofibrils	[186]
C ₁₂ -EVHHQKL	Amyloid fibrillisation	[187]
C ₁₆ -(G) ₃ (S) ₂ PHSRN(SG) ₅ RGDSP	Nanofibrils	[188]

1.3.4 Turn containing peptides

Reverse turns happen to be an important structural motif in proteins. Tight turns are often involved in sudden reversal of the peptide backbone. A β -hairpin is one of the most common outcomes of such a chain reversal. As a large majority of peptide self-assembled structures are composed of β -sheets, a β -hairpin could be an interesting monomeric unit to obtain β -sheet rich superstructures. One of the most pioneering works on β -hairpin self-assembly has been carried out by Schneider and coworkers [71, 73, 184, 189]. A series of short peptides, named MAX peptides, were designed wherein the dipeptide ^DPro-^LPro was incorporated in the middle of the sequence. As ^DPro-^LPro is a well-known type-II' β -turn-inducing motif, the designed peptides folded into β -hairpin structures and self-assembled to form hydrogels. The MAX₁ peptide (H₂N-VKVKVKVK-V^DPPT-KVKVKVKVNH₂) folds into a β -hairpin conformation in an aqueous solution buffered at pH 9 and self-assembles to form hydrogels. Lowering the pH below the lysine side-chain pK_a causes inter-strand electrostatic repulsion and thereby unfolding the hairpin conformation [73]. An investigation into the folding transition of two other analogs called the MAX₂ (H₂N-VKVKVKVK-V^DPPT-KVKTKVKVNH₂) and MAX₃ (H₂N-VKVKVKTK-V^DPPT-KVKTKVKVNH₂) showed MAX₃ to undergo thermally reversible self-assembly leading to reversible hydrogels [189].

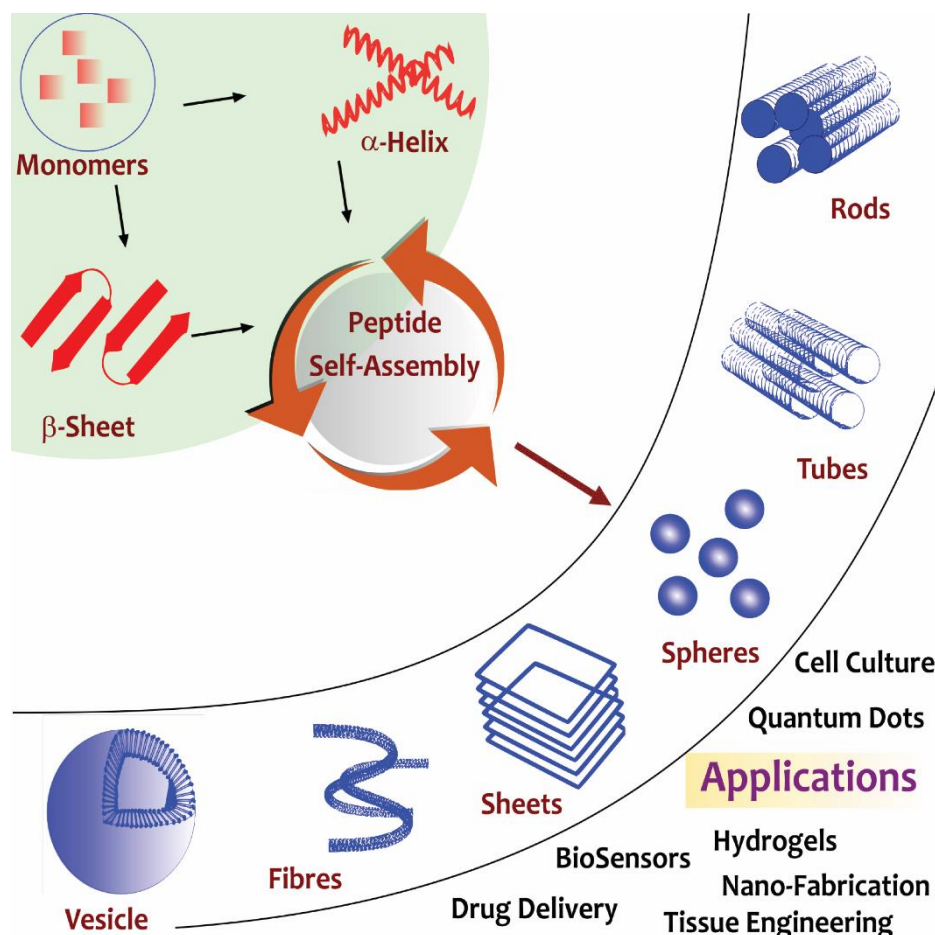


Figure 1.3 A diagrammatic representation of peptide self-assembled structures and their potential applications.

1.3.5 Aromatic-moiety containing peptides and amino acids

Small aromatic peptides have been shown to form well-ordered nanostructures. The Phe-Phe aromatic cassette present in the $A\beta_{16-22}$ is believed to play a critical role in its self-assembly [64]. Görbitz and Gazit subsequently showed that the dipeptide Phe-Phe, by itself, can self-assemble into highly ordered nanotubes. Diphenylglycine, an analog wherein C_β is not there, and the phenyl rings are directly attached to the α -carbon, self-assembled into nanospheres in aqueous solution [61]. End capping with bulky aromatic groups like Fmoc (9-fluorenylmethoxycarbonyl) in peptides has been shown to promote self-assembly through aromatic π - π stacking interactions. The earliest example of an aromatic self-assembling peptide was Fmoc-LD, which was reported to be a potent thermoreversible hydrogelator by Janmey and coworkers, in 1995 [91]. Xu

and coworkers were the first to report the self-assembly by an Fmoc-protected amino acid (Fmoc-Tyr-phosphate), which also showed enzyme responsiveness [190, 191]. Ulijn and Gazit's groups carried out investigations on Fmoc-FF independently and were able to obtain hydrogels at physiological pH [94, 192]. In another study, Das and coworkers designed and developed a series of Fmoc-amino acid/peptide-functionalized cationic amphiphiles to test their gelation efficacy. Replacing the N-terminal Fmoc group with Boc (tert-butoxycarbonyl) moiety rendered most of the cationic dipeptides/amino acids non-gelating. The study thus showed the presence of planar aromatic ring to be critical for the gelation efficiency of the cationic amphiphiles [193]. Apart from Fmoc, various other aromatic moieties such as Boc, phenyl, naphthalene, azobenzene, and pyrene derivatives have been attached to the N-terminus of the peptides to drive self-assembly through aromatic interactions [194-196]. Peptides with an N-terminal naphthyl moiety have been shown to form structures like nanofibers and hydrogels that were found to have various applications as in inhibition of growth of glioblastoma cells, induce cell death, as potent anti-cancer agents, and inhibition of bacterial growth [79, 197-200]. Peptide derivatives having a perfluorinated phenyl moiety at the N-terminus have been shown to form hydrogels when compared to their non-fluorinated analogs [201]. The assembly is believed to be directed by the quadrupole-quadrupole interactions between aromatic amino acid side chains and the perfluorinated N-terminal group. Another noteworthy example is the incorporation of an azobenzene moiety in a peptide sequence. Azobenzene is a photoresponsive molecule that can switch between cis and trans configurations by absorbing light of different excitation wavelengths. It is thus possible to obtain photoresponsive peptide hydrogels (having the potential to switch between gel-sol) by suitably incorporating azobenzene moiety in the peptide [202]. Various nucleotide bases like adenine, thymine, guanine, cytosine has been coupled with peptides to obtain hydrogels [203, 204]

In addition to peptides, several small Fmoc protected amino acids have also been investigated for their self-assembling propensity. Fmoc-Phe and Fmoc-Tyr, for

Chapter 1

example, were found to be potent hydrogelators [98]. The effect of covalent disulphide linkage along with aromatic interactions, has also been explored. Fmoc-CF-OMe, for example, self assembles to form a hydrogel in a reducing environment [205]. Oxidation of the cysteine residues resulted in the collapse of the gel. An investigation on varying the C-terminus of the different Fmoc-phenylalanine derivatives revealed that COOH promotes gelation, COOMe promotes precipitation, and CONH₂ results in solutions generally [206]. An extensive review by Fleming and Ulijn on aromatic self-assembling amino acids and peptides explores various design possibilities [207].

1.3.6 Applications of self-assembling peptides

The applications of the self-assembled peptide nanostructures are manifold. Owing to their versatility and biocompatibility, peptide-based superstructures have been used as drug delivery vehicles, tissue engineering matrices, scaffolds for catalysis, and casting nanowires [49, 208-210]. Designed amyloid fibrils have been reported to possess intrinsic conductivity [54]. *De novo* designed peptides, short peptides from amyloidogenic peptides and proteins, and Fmoc-protected amyloidogenic peptides have been found to form hydrogels which find applications in drug delivery and regenerative medicine [211, 212]. The ability of the hydrogel network to trap and release small molecules makes the peptide hydrogels promising candidates as drug delivery vehicles. Peptide nanotubes have found applications as ion channels [26], antimicrobial agents [130], cast for nanowires and coaxial wires [60, 63], channels for fluid flow. Furthermore, peptide nanostructures have been employed as the scaffolds to provide physical support for cell culture and growth. This proved their potential to be used in tissue engineering [213-216]. Fiber-forming peptides have been tagged with molecules to obtain self-assembled structures decorated with the tagged molecules. The tag could also be tethered to the preformed structures. Such derivatization has resulted in superstructures with biological, catalytic, optical, electrical, and electronic properties [7, 217-224]. Besides, the self-assembled peptide nanotubes have been utilized as supports for enzymes or in certain cases to improve electrochemical parameters in an electrode [225, 226]. This shows the

application of peptide nanostructures as biosensors. Peptides synthesized with strategic design have been used in bionanomaterials like 3D cell culture, gene delivery, as well as membrane stabilization [227-230]. Short designed self-assembling peptide amphiphiles behaved like surfactants and have been found to stabilize bovine rhodopsin in the presence of lipid molecules [228]. Several peptide-based hydrogels have been reported to have functions like antibacterial activity, removal of dyes from wastewater, as well as exhibit semiconducting and photoswitching behaviour [231-233]. While above are only a few examples, applications of peptide-based self-assembled structures are numerous.

The various properties and applications of the self-assembled structures obtained from the nucleic acid, proteins, amino acids, peptides and peptidomimetics highlight the importance and possibilities of the bottom-up approach of fabricating smart, functional materials. In fact, the monomers need not restrict to the peptides, amino acids, or other biomolecules but could be any molecule appropriately designed to allow the required interactions for self-assembly. However, because of the inherent advantages like biocompatibility and biodegradability that they offer, the peptide and amino-acids have an edge as the monomers over other molecules as far as biomedical applications are concerned. Peptide and peptide analogs have thus been taken up as the monomers to obtain physical gels.

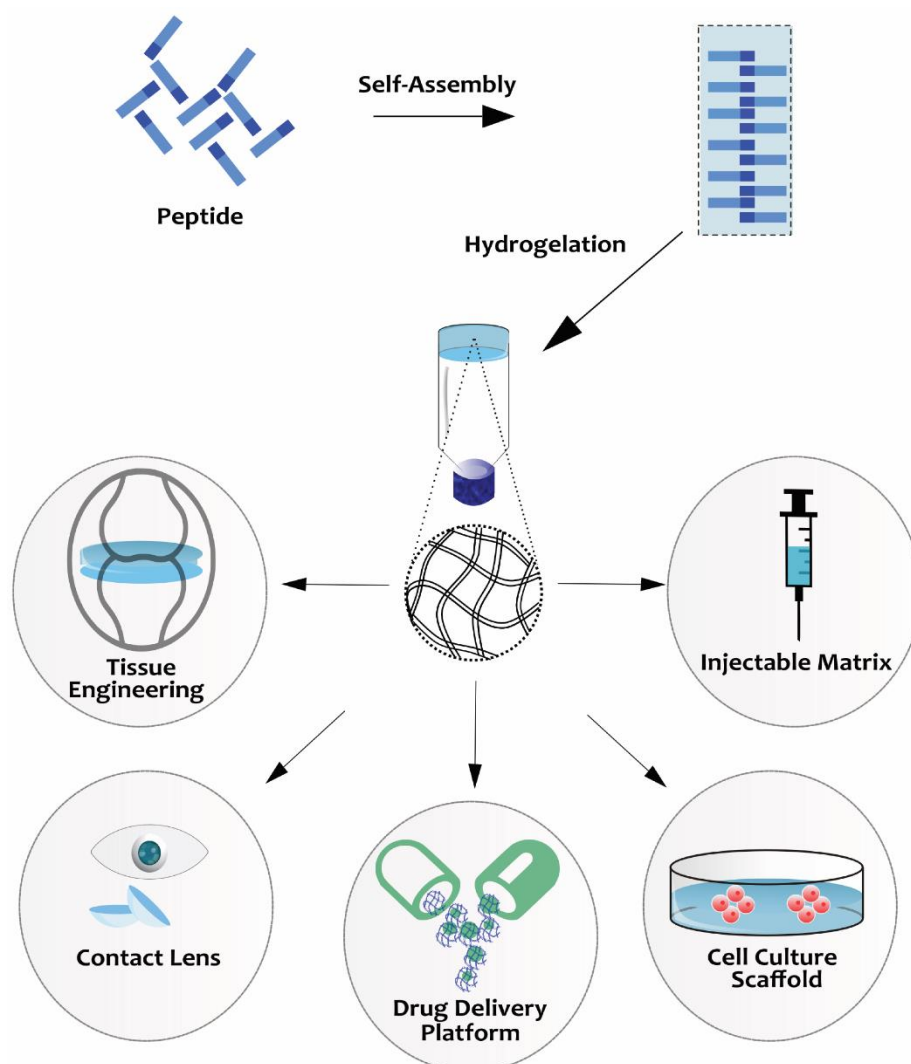


Figure 1.4 A diagrammatic representation of the various applications of peptide-based hydrogels.

1.4 RESEARCH DESIGN AND OBJECTIVES

Getting inspired by the ordered fibrillary structures formed by the amyloidogenic peptides, a model peptide was chosen which is known to self-assemble with an antiparallel β -sheet architecture. The idea of choosing such a peptide was to investigate the role the β -hairpin structure plays on amyloidogenic propensity and the subsequent potential for gelation. $A\beta_{16-22}$ happens to be one of the most widely studied amyloidogenic sequences that self-assemble to form in-register antiparallel β -sheets. Therefore, tandem repeats of $A\beta_{16-22}$ (KLVFFAE) connected via dipeptide motifs that are well-known to support/induce type I' or II' β -turn were designed, synthesized and explored. $A\beta_{16-22}$ offers another advantage;

several aromatic analogs of the peptide have been investigated. This also allowed certain selected aromatic analogs in the context of β -hairpin formation to be investigated.

Furthermore, a series of 27 peptidomimetics sequences were designed, which were short, cheap, and easy to synthesize. In this set of sequences, dipeptides consisting of Val, Leu, and Ile were coupled with fatty acids and their assembly in the alcohol-water mixture was investigated.

The following objectives were undertaken in this thesis:

Objectives

1. Designing the molecules, their synthesis, characterization, and purification.
2. Self-assembly of the peptides, their characterization, and mechanism of assembly.
3. Computer modelling and simulation.
4. Designing analogs to selectively vary the physicochemical properties and their effects on the emergent properties of the self-assembled structures.

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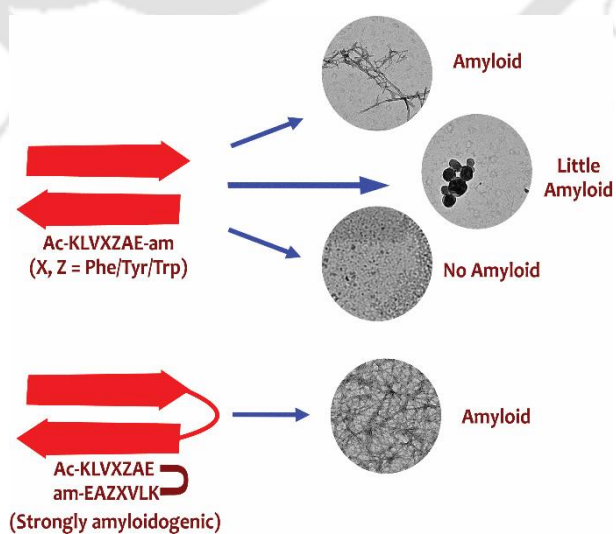
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**SELF-ASSEMBLY OF β -TURN MOTIF-CONNECTED TANDEM
REPEATS OF A β ₁₆₋₂₂ AND ITS AROMATIC ANALOGS**





2.1 INTRODUCTION

The β -sheet rich fibrous aggregates of amyloid proteins and peptides were initially identified as toxic deposits associated with various neurodegenerative diseases. However, research work has unveiled several disease-unrelated amyloids as in bacteria, *Drosophila*, and even in humans [1-4]. Amyloid fibrils are formed by peptides and proteins that differ from each other in their sequence, amino acid composition, and length. On the molecular level, however, they possess striking similarity with interdigitated β -sheets. Many amyloidogenic proteins need to denature, at least partially, to yield amyloid aggregates and aggregation kinetics could be characterized by a lag phase. Short amyloidogenic peptides, on the other hand, could aggregate rather rapidly as they would be largely unordered in the monomeric state and structural perturbation is not required as in the case of folded proteins. The rate of the assembly would depend on the intrinsic amyloidogenic propensity and stability of the oligomeric nucleus. To be able to modulate the aggregation propensity of amyloid peptides thus stands as an interesting research proposition. Several short amyloidogenic peptides self-assemble through the antiparallel β -sheet formation. Such peptides happen to be interesting candidates as the two-strands could be brought together by connecting them through a tight turn. This, was hypothesized, to increase their aggregation propensity. As β -turns are often found in proteins connecting two antiparallel strands of β -sheets [5], they were chosen for introducing the tight turns. The β -turn was identified in 1968 by Venkatachalam [6]. A beta-turn motif involves four amino acid residues, and various types have been defined based on the backbone torsion angles of the second and third residues [7]. The different types of β -turns (4-residue turns) are listed in table 2.1 [8].

Among these, type I and type II β -turns are the most common ones in proteins [9]. Interestingly, however, the mirror image β -turns, type I' and type II' are the preferred turns for β -hairpin structures [10, 11]. Several dipeptide motifs have been reported in the literature that shows a strong preference for a particular type of β -turn. Asn-Gly, for example, prefers to populate a type I' turn [12, 13].

Chapter 2

Introduction of non-natural amino acids, ^DPro and Aib in particular, has resulted in rather tight turns with very strong turn type preference. Aib-^DPro dipeptide motif, for example, is shown to bring about an obligatory type I' turn [14]. ^DPro-Gly and ^DPro-^LPro dipeptide motifs have been reported to preferentially nucleate type II' turns [15, 16]. It was therefore chosen, to connect the self-assembling peptide sequences through dipeptide motifs that could support the type I' and type II' β -turns. Schneider and coworkers designed a 20-residue peptide, termed MAX-1 wherein the dipeptide motif ^DPro-^LPro is introduced in the middle of the sequence to introduce a β -turn. The peptide self-assembles into β -sheet rich fibrils that ultimately cause hydrogelation [17, 18].

Table 2.1 Dihedral angles of central residues in β -turns.				
β -turn type	ϕ_{i+1}	ψ_{i+1}	ϕ_{i+2}	ψ_{i+2}
I	-60	-30	-90	0
I'	60	30	90	0
II	-60	120	80	0
II'	60	-120	-80	0
III	-60	-30	-60	-30
III'	60	30	60	30
IV	Any bend with at least two angles differing by $>40^\circ$ from those given here			
V	-80	80	80	-80
V'	80	-80	-80	80
VIa*	-60	120	-90	0
VIb*	-120	120	-60	0
VII	Kink in chain created by $\psi_2 \approx 180^\circ$, $ \phi_3 < 60^\circ$ OR $ \psi_2 < 60^\circ$, $\psi_3 \approx 180^\circ$			
VIII	-60	-30	-120	120
* The peptide bond between residue $i + 1$ and $i + 2$ is cis and residue $i + 2$ is Pro.				

Thus, the self-assembly of the tandem repeats of A β_{16-22} , an amyloidogenic fragment from the β -amyloid peptide, wherein the two repeats are connected via β -turn supporting dipeptide motifs, was investigated. A β_{16-22} self-assembles into amyloid-like fibrillar aggregates with a characteristic antiparallel organization of β -strands [19]. The aromatic dipeptide stretch 'Phe-Phe' is believed to be critical in its self-assembly [20, 21]. The self-assembly of the tandem A β_{16-22} repeats connected through a type I' turn has been previously investigated [22]. Previous work by Pachahara and Nagaraj identified several highly amyloidogenic variants of A β_{16-22} while exploring the effect of specific substitution of the aromatic amino

acid Phe with Trp and Tyr [23]. The idea was to investigate if the aggregation of short amyloidogenic peptides could be facilitated by connecting the two strands through a tight turn. Through a careful analysis of the aromatic analogs investigated by Pachahara and Nagaraj [23], the self-assembly of A β ₁₆₋₂₂, Ac-KLVFFAE-am (A β FF) and its aromatic analogs, Ac-KLVFYAE-am (A β FY), Ac-KLVYYAE-am (A β YY), and Ac-KLVWWAE-am (A β WW), connected in-tandem through type I' and type II' β -turn-inducing motifs were chosen to be investigated. Asn-Gly and Aib-^DPro were chosen as the type I' β -turn-inducing motifs [16, 24, 25] while ^DPro-Gly was chosen as the type II' turn-inducing motif [15, 26]. Table 2.2 lists the peptide sequences designed and synthesized for this purpose.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids, and *N,N,N',N'*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU) were procured from Novabiochem (Darmstadt, Germany). Rink amide resin, *N,N*-diisopropylethylamine (DIPEA), piperidine, trifluoroacetic acid, ethanedithiol, thioanisole, acetic anhydride, 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), and Thioflavin T (ThT) were from Sigma-Aldrich Chemicals Pvt. Ltd. *N,N*-dimethylformamide, diethyl ether, and *m*-cresol was obtained from Merck, India. 1-hydroxybenzotriazole hydrate (HOBt) was obtained from Sisco Research Laboratory, India. All other reagents were of the highest grade available.

2.2.2 Peptide synthesis and characterization

Peptides were synthesized on solid support using Fmoc chemistry by employing HBTU/HOBt/DIPEA activation strategy. For 14-residue peptides, HATU activation was used for coupling the turn-inducing amino acids (8th and 9th amino acids). On-resin acetylation of the N-terminus was carried out using five equivalents of acetic anhydride and ten equivalents of DIPEA. The peptides were cleaved from the resin using a cocktail mixture containing TFA:*m*-

Chapter 2

cresol:thioanisole:ethanedithiol (20:2:2:1) and precipitated in ice-cold diethyl ether. The crude peptides were dried, dissolved in DMSO, and purified on a reversed-phase C-18 column on a Shimadzu Prominence Modular HPLC instrument (Shimadzu, Kyoto, Japan) using a linear gradient of acetonitrile containing 0.1% TFA (2.25% acetonitrile/min). The purified peptides were characterized by MALDI-TOF (Bruker Autoflex Speed MALDI-TOF-TOF, USA) mass spectrometry.

2.2.3 Peptide aggregation

Peptides were dissolved in HFIP and their concentrations estimated by measuring absorption at 280 nm for peptides containing tyrosine or tryptophan and at 254 nm for peptides lacking these amino acids. Molar absorption coefficients of peptides were calculated by adding the molar absorption coefficients of the aromatic amino acids at corresponding wavelengths i.e Tyr ($\epsilon_{280} = 1280 \text{ M}^{-1}\text{cm}^{-1}$), Trp ($\epsilon_{280} = 5690 \text{ M}^{-1}\text{cm}^{-1}$), and Phe ($\epsilon_{254} = 143 \text{ M}^{-1}\text{cm}^{-1}$). The concentrations of the peptides were adjusted to 1.25 mM in HFIP. Subsequently, the peptides were diluted in water to obtain 250 μM concentration having 20% HFIP, and their aggregation studied.

2.2.4 Circular dichroism (CD) spectroscopy

Far-UV CD spectra were recorded on a Chirascan CD spectropolarimeter (Applied Photophysics, UK) for the 24 h old samples with suitable dilution in 20% HFIP. The spectra were recorded in a 1 mm path length cell with a step size of 0.5 nm, corrected by subtracting the blank spectrum, i.e. the spectrum for 20% HFIP, and smoothed. The data was converted to mean residue ellipticity, $[\theta]_{\text{MRE}}$, using the formula: $[\theta]_{\text{MRE}} = (M_r \times \theta_{\text{obs}})/(100 \times l \times c)$, where M_r is the mean residue weight (peptide molecular weight/number of amino acid residues), θ_{obs} is the observed ellipticity in millidegrees, l is the path length in decimeters, and c is the peptide concentration in mg/ml [27].

2.2.5 Fourier transform infrared (FTIR) spectroscopy

FTIR spectra were recorded on a Bruker Alpha-E spectrometer (Bruker Optik GmbH, Germany) with Eco attenuated total reflection (ATR) single reflection ATR sampling module equipped with ZnSe ATR crystal. Peptides (10 μ l) were spread out and dried as films on ZnSe crystal, and ATR-FTIR spectra were recorded at a resolution of 4 cm^{-1} .

2.2.6 Molecular dynamics [MD] simulations

Peptides in a fully extended conformation ($\phi = \psi = 180^\circ$) were constructed using Ribosome.^[24] The dihedral angles ϕ and ψ for ^DPro , wherever applicable, were fixed to 70° and -45° , respectively. The amino-terminus was acetylated while C-terminus was amidated for each of the peptides. The lysine and glutamate side-chains were electrically charged, rendering the peptide electrically neutral; no counter-ion was added separately. Energy minimization and MD simulation were performed using the Gromacs software package (v 5.0.4) [28] with the GROMOS96 54a7 forcefield [29] at the CDAC-IIT Guwahati Supercomputing facility. The Ribosome-constructed structures were energy minimized in vacuum using the Steepest Descent algorithm. The vacuum energy-minimised peptide structures were solvated with water using simple point charge [30] water model in a cubic box with a distance of 1.5 nm between the peptide and the edge of the box. The system comprising of one peptide molecule and 25,148 water molecules was energy-minimised. A small position restrained dynamics was performed before the final production of the MD run. MD simulation was carried out for 100 ns at 300 K and 1 bar by using leap-frog integration method [31]. Temperature and pressure couplings were done with v-rescale [32] and Berendsen [33] methods, respectively. Long-range electrostatics was calculated with fast, smooth Particle mesh Ewald (PME) method with cubic interpolation and Fourier grid spacing of 0.1 nm [34, 35]. The Verlet cut off-scheme was followed for short-range electrostatics and van der Waals interactions, with the cut-off value set to 1.0 nm for both r_{coulomb} and r_{vdw} . Bond lengths were constrained using the LINCS algorithm [36]. All the simulations were carried out using periodic

Chapter 2

boundary conditions in all the directions. The results were analysed using the built-in Gromacs utilities. Discovery Studio Visualizer was used for visualization [37].

2.2.7 Thioflavin T (ThT) fluorescence assay

ThT fluorescence spectra for the 24 h-old peptide samples in 20% HFIP were recorded on a Jasco FP8500 spectrofluorometer (Jasco, Japan). The peptides were diluted to 20 μ M concentration in 5 mM phosphate buffer, pH 7.0, containing 10 μ M ThT. The samples were excited at 440 nm, and fluorescence emission recorded from 460 to 550 nm. Excitation and emission slit widths were 2.5 and 5 nm, respectively. Spectra of the peptides without ThT were recorded as blanks and subtracted. Fluorescence intensity at 482 nm is reported in the results.

2.2.8 Transmission electron microscopy (TEM)

The peptide samples (72 h-old) were diluted 2.5-fold in deionized water and drop-casted on Formvar/Carbon coated 200-mesh copper grids. After 2 minutes, the solvent was blotted out from the periphery of the grid by Whatman I filter paper. The grids were stained with saturated uranyl acetate negative stain solution, which was blotted out after 30 seconds. The grids were then dried and images recorded on JAM-2100 LaB6 (JEOL, Japan) transmission electron microscope at 120 kV.

2.3 RESULTS AND DISCUSSION

Twelve peptides containing β -turn-inducing motifs between the two peptide repeats of A β FF, A β FY, A β YY, and A β WW (Table 2.2) were synthesized along with 4 control peptides, and their self-assembly investigated. Previously, Pachahara and Nagaraj studied the aggregation of the aromatic analogues in 20% HFIP [23]. The assembly of β -turn analogs was chosen to be studied under the same conditions: the peptides were dissolved in 20% HFIP and analyzed using CD spectroscopy, ATR-FTIR spectroscopy, ThT fluorescence spectroscopy, and TEM imaging.

Table 2.2 List of the peptides studied: their ID, sequence, calculated monoisotopic mass, and the m/z values obtained in MALDI mass spectrometry.

Peptide ID [#]	Sequence [#]	Calculated monoisotopic mass	Observed m/z
AβFF	Ac-KLVFFAE-am	893.49	895.602
AβFY	Ac-KLVFYAE-am	909.48	911.29
AβYY	Ac-KLVYYAE-am	925.48	927.36
AβWW	Ac-KLVWWAE-am	971.51	973.43
AβFF-Up	Ac-KLVFFAE-U ^D P-KLVFFAE-am	1910.06	1911.02
AβFF-pG	Ac-KLVFFAE- ^D PG-KLVFFAE-am	1882.03	1884.29
AβFF-NG	Ac-KLVFFAE-NG-KLVFFAE-am	1899.02	1900.09
AβFY-Up	Ac-KLVFYAE-U ^D P-KLVFYAE-am	1942.05	1944.07
AβFY-pG	Ac-KLVFYAE- ^D PG-KLVFYAE-am	1914.02	1916.27
AβFY-NG	Ac-KLVFYAE-NG-KLVFYAE-am	1931.01	1933.79
AβYY-Up	Ac-KLVYYAE-U ^D P-KLVYYAE-am	1974.04	1975.91
AβYYpG	Ac-KLVYYAE- ^D PG-KLVYYAE-am	1946.01	1947.78
AβYY-NG	Ac-KLVYYAE-NG-KLVYYAE-am	1963.00	1964.68
AβWW-Up	Ac-KLVWWAE-U ^D P-KLVWWAE-am	2066.10	2068.29
AβWW-pG	Ac-KLVWWAE- ^D PG-KLVWWAE-am	2038.07	2040.21
AβWW-NG	Ac-KLVWWAE-NG-KLVWWAE-am	2055.06	2057.02

[#]Letter U in the peptide ID and the sequence represents Aib; D-Proline is indicated by lower case 'p' in the ID and by ^DP in the sequence.

2.3.1 CD spectroscopy

The secondary structures of tandem repeats of AβFF, AβFY, AβYY, and AβWW connected via β-turn-inducing motifs were examined using CD spectroscopy. Peptides dissolved in HFIP (1.25 mM) were diluted in deionized water to obtain 250 μM peptide solutions in 20% HFIP. Far-UV CD spectra were recorded for the 24 h-old peptide samples (Figure 2.1).

Chapter 2

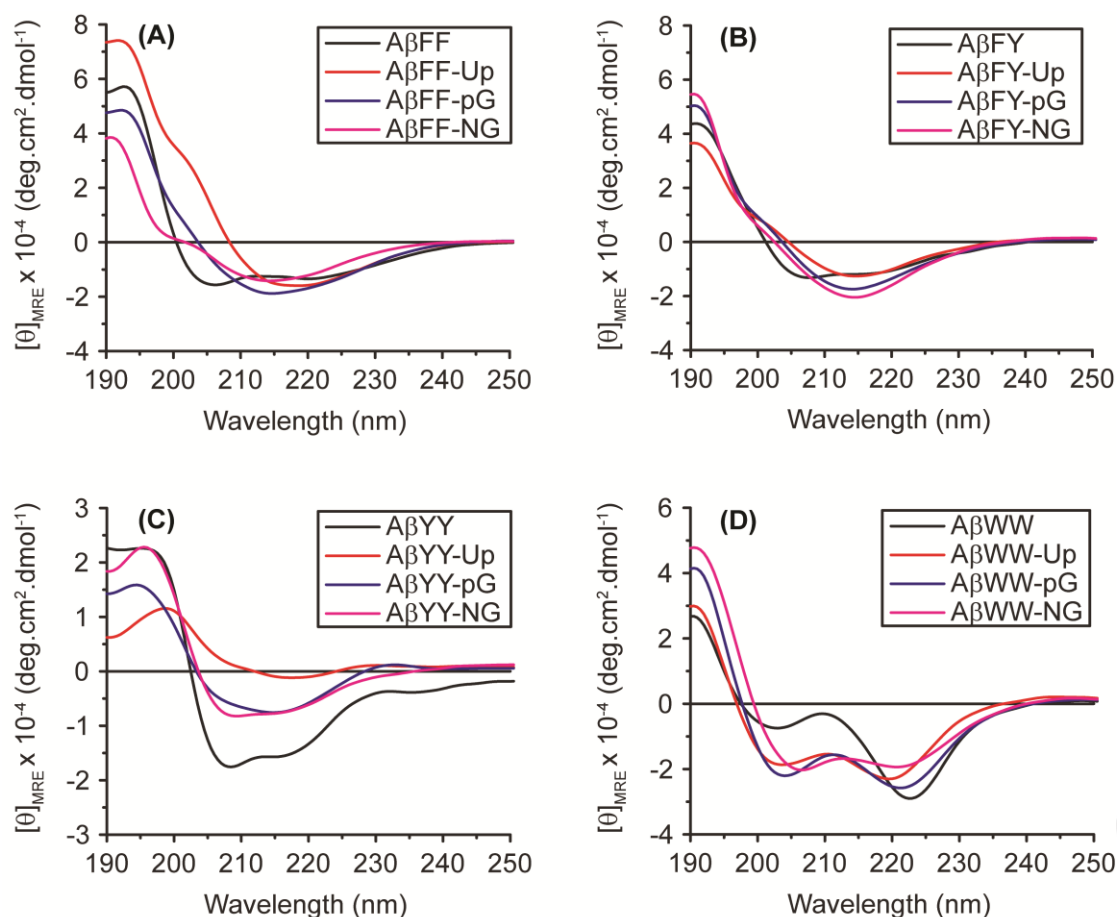


Figure 2.1 CD spectra of 24 h-old peptide solutions in 20% HFIP.

AβFF displays negative bands at 206 and 220 nm with an intense positive band ~193 nm (Panel A). Such helix-like spectra have been obtained in TFE for tri- to hexapeptide methylamides that harbor Asn-Pro-Gly sequence and have been assigned to β-turns [38]. AβFY (panel B) and AβYY (panel C) show spectra similar to that of AβFF, albeit with the long wavelength band blue-shifted to ~216 nm. The spectra suggest β-sheet conformation alongside turns. AβWW, on the other hand, displays an intense negative band ~223 nm, a positive band of comparable intensity ~191 nm, and a weak negative band ~203 nm (panel D). The spectrum is similar to that reported by Pachahara and Nagaraj for AβWW in 20% HFIP [23]. Similar spectra have been reported for indolicidin in SDS micelles and tryptophan-rich self-assembling peptides, and are believed to arise due to the stacking of Trp residues [39, 40]. Tryptophan stacking could take place for AβWW through intermolecular interaction. The Asn-Gly β-turn motif-harboring

peptides, A β FF-NG (panel A) and A β FY-NG (panel B) display broad negative band centered ~214 nm and a shoulder around 202 nm suggesting largely β -sheet conformation. CD spectrum with a weak negative band between 220-230 nm and a positive band ~200 nm arises from β -turns [41]. A positive shoulder ~203 nm could therefore be a contribution from β -turn motif. A β YY-NG displays a spectrum with negative bands around 209 and 216 nm, suggesting β -sheet conformation with an appreciable contribution from turn (panel C). A β WW-NG displays negative bands around 207 and 221 nm, a spectrum characteristic of distinct α -helix (panel D). The data indicate that A β WW-NG folds into a largely α -helical conformation in 20% HFIP, whereas other Asn-Gly motif harboring peptides largely populate β -sheet structure. Fluorinated alcohols are well known to promote α -helical conformation in peptides [42, 43]. Furthermore, Asn-Gly dipeptide motif supports type I' β -turn formation; but is not an obligatory turn-nucleating motif. Aib-^DPro, on the other hand, is an obligatory type I' β -turn inducing motif. A β FF-Up displays a broad negative band around 218 nm and a positive band around 192 nm along with a 203 nm shoulder (panel A). The spectrum is suggestive of β -sheet and β -turn conformations. A β FY-Up spectrum also displays β -sheet and β -turn conformations (panel B). A β YY-Up shows a very weak band around 218 nm and a positive band ~199 nm (panel C). Such spectra have been assigned to turns in designed β -turn peptides [41]. A β WW-Up displays a spectrum with negative bands around 219 and 204 nm. The positive band ~190 nm is of comparable intensity. Spectra similar to these have been assigned to β -turns in literature [44]. Assignment of the secondary structure to the A β WW and its analogs' CD spectra is complicated by aromatic exciton coupling. The A β WW-Up spectrum appears to be influenced by aromatic exciton coupling of tryptophan. The type II' β -turn nucleating motif ^DPro-Gly appears to nucleate β -turn in A β FF-pG (panel A) and A β FY-pG (panel B). Both the peptides display a negative band ~214-215 nm, a positive band around 192 nm with a shoulder around 203 nm. A β YY-pG displays a broad negative band ~215 nm with a hump ~207 nm (panel C). The spectrum is suggestive of β -sheet conformation with turn signature as well. A β WW-pG spectrum (panel D) is qualitatively similar to that

Chapter 2

of A β WW-Up. All the analogs of A β FF and A β FY that harbor β -turn-inducing motifs display distinct β -conformation with a negative band around 214-218 nm.

2.3.2 FTIR spectroscopy

ATR-FTIR spectra were recorded for the 24 h-old peptide samples to investigate the secondary structure in the dried films (Figure 2.2).

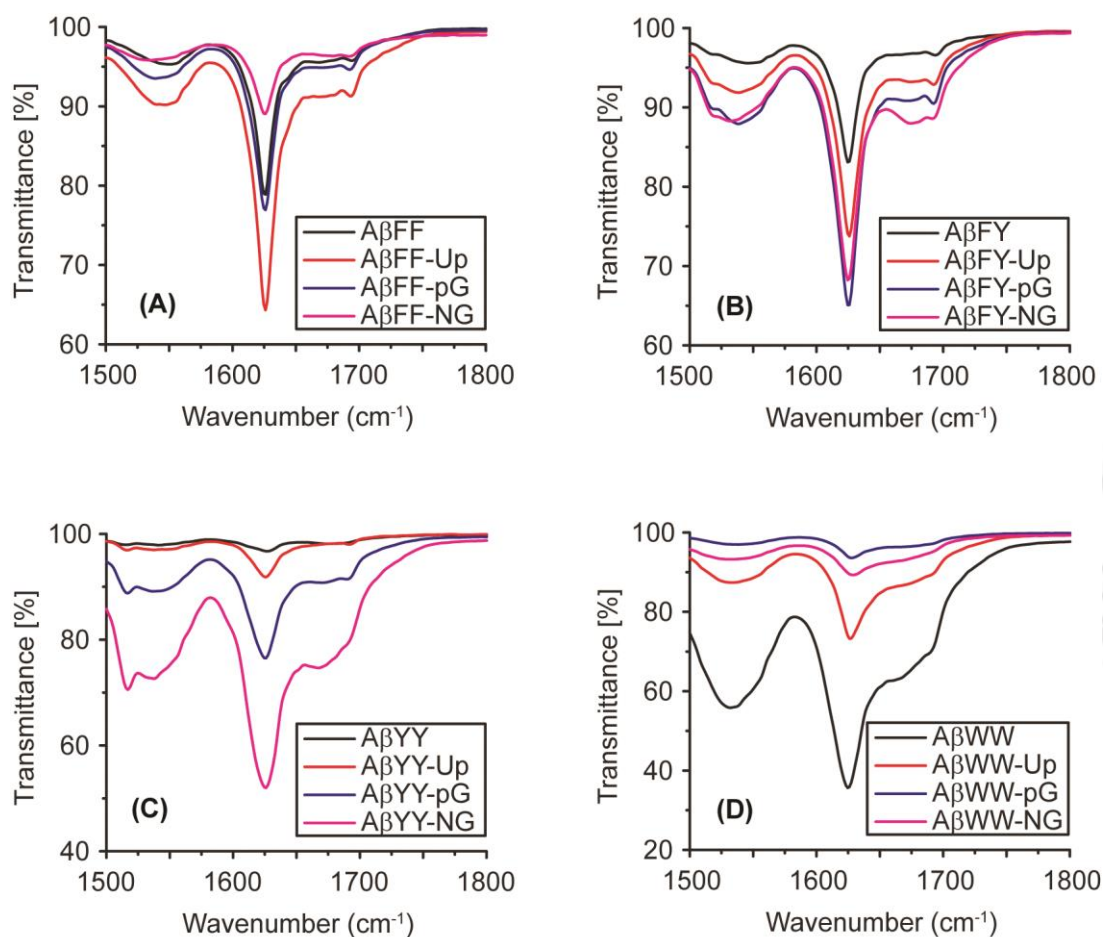


Figure 2.2 ATR-FTIR spectra of the dried films from 24 h-old peptide solutions in 20% HFIP.

The spectra of A β FF, A β FY, A β YY, and their turn containing analogs show intense bands around 1624-1626 cm⁻¹, a characteristic signature of β -sheets. Tryptophan-containing peptides A β WW, A β WW-NG, A β WW-Up, A β WW-pG, though displayed a major band ~1625 cm⁻¹, a shoulder ~1650 cm⁻¹ is observed. The results suggest that the β -sheet conformation of the peptides is retained in the dried films.

2.3.3 MD simulations

MD simulations (100 ns) were carried out in water to gain insights about the type of turn mediated by the Asn-Gly, ^DPro-Gly, and Aib-^DPro dipeptide motifs in the peptides. The trajectory files obtained following MD simulations were analyzed to examine the folding of the peptides. Distance between the α -carbons of terminal amino acid residues and that between the α -carbons of the residues preceding and succeeding the β -turn dipeptide motifs were calculated. The dihedral angles of the β -turn dipeptide motifs gave insights into the type of turn formed. Furthermore, the possibility of H-bonding between C=O of preceding residue and NH of the succeeding residue was investigated for certain representative peptides viz. A β FY-NG, A β FY-pG, A β YY-NG and A β YY-pG.

The data for A β FY-NG is shown in figure 2.3. A β FY-NG simulation was started with an extended peptide structure ($\phi_{\text{all}}=\psi_{\text{all}}=180^\circ$). The peptide folds to take a β -hairpin conformation after ~30 ns; the distance between K1C α and E16C α is ~0.6 nm and shows only small variations (black trace, panel A). The distance between the E7C α and K10C α is also ~0.6 nm, and little variation is observed through the simulation (red trace, panel A), wherein a hydrogen-bond is formed between Glu7 C=O and Lys10 NH (panels D and E), suggesting a β -turn [6, 9]. At 34 ns, the dihedral angles, ϕ and ψ for Asn are -56° and 91° , respectively. The angles show variations around ϕ and ψ values of $\sim -57^\circ$ and $\sim 105^\circ$ for the largest cluster (panel B). Gly, on the other hand, adopts ϕ and ψ values of 56° and 51° after 34 ns. After 34 ns, Gly9 ϕ samples values largely between $40 - 120^\circ$, spread around $+66^\circ$ (panel C). The values of ψ range from $-60 - +60^\circ$ with $\psi = 30^\circ$ as the largest cluster. The dihedral angles of the largest cluster indicate a type II β -turn. At 34 ns, the distance between E7CO and K10NH is ~0.19 nm (panel D) with angle HNO of 19° (panel E), indicating an H-bond. Panels F-K show the snapshots at 20 ns interval supporting hairpin structures.

Chapter 2

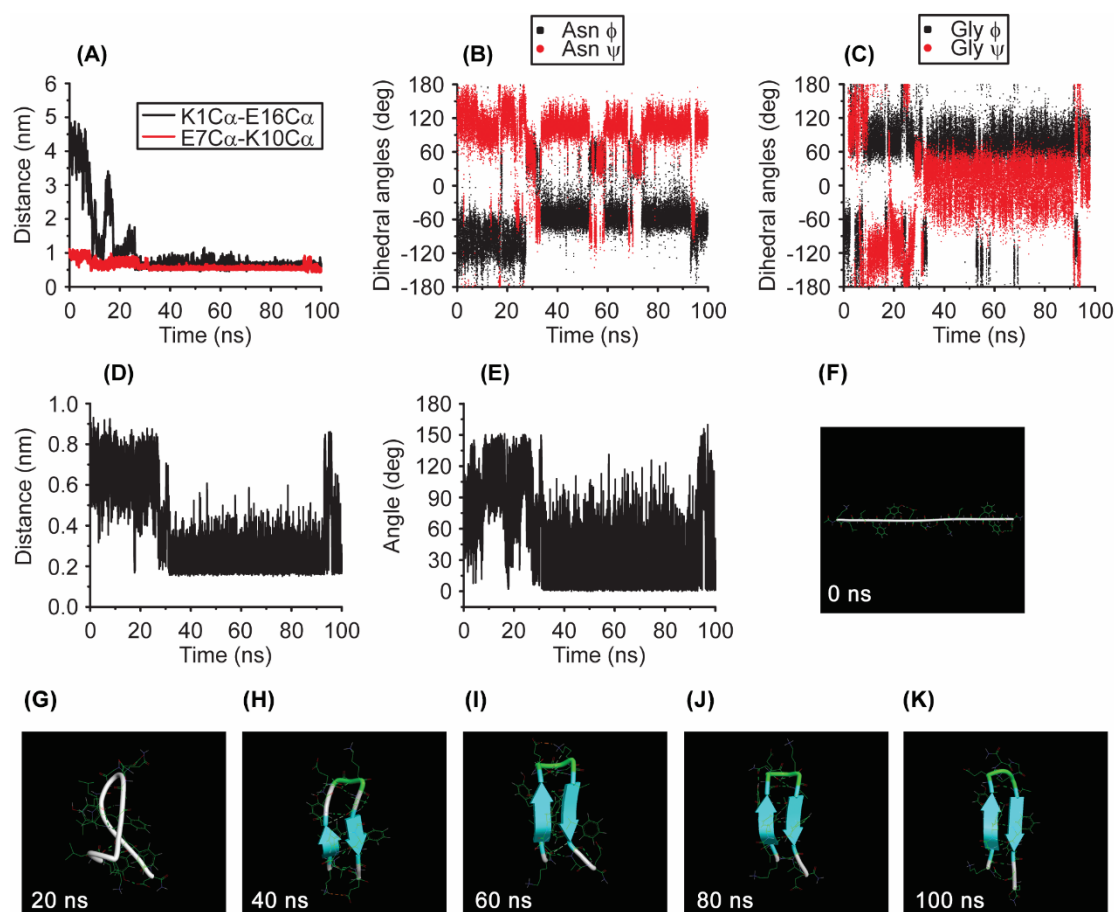


Figure 2.3 MD simulation of AβFY-NG. (A), distance between K1Cα and E16Cα (black) and that between E7Cα and K10Cα (red); (B), dihedral angles, ϕ (black) and ψ (red) of Asn residue; (C), dihedral angles, ϕ (black) and ψ (red) of Gly residue; (D), distance between amide oxygen of Glu7 and amide proton of Lys10; (E), angle HNO, wherein HN belongs to Lys10 amide whereas O belongs to the Glu7 amide; after ~ 32 ns, the distance and the angle are, mostly, within 0.35 nm and 60° , respectively; (F-K), snapshots from the MD trajectory at 20 ns interval.

AβFY-pG simulation was started with extended conformation; ϕ and ψ values for ^DPro , however, were taken 70° and -45° , respectively. The peptide folds into a β -hairpin-like structure around 19 ns (Figure 2.4). The distance between K1Cα and E16Cα as well as that between E7Cα and K10Cα is ~ 0.5 - 0.6 nm and is invariant over time. After 19 ns, the largest cluster takes the ϕ and ψ values for ^DPro spread around $+60^\circ$ and $\sim -110^\circ$, respectively (panel B). Gly9 takes ϕ and ψ values around -120° and 30° , respectively (panel C). The ^DPro and Gly dihedral angles conform to a type II' turn. The hairpin appears to be stabilized by an $i - i+3$ hydrogen-

bond in the β turn region as suggested by the distance between E7CO and K10NH (panel D) and angle HNO (panel E).

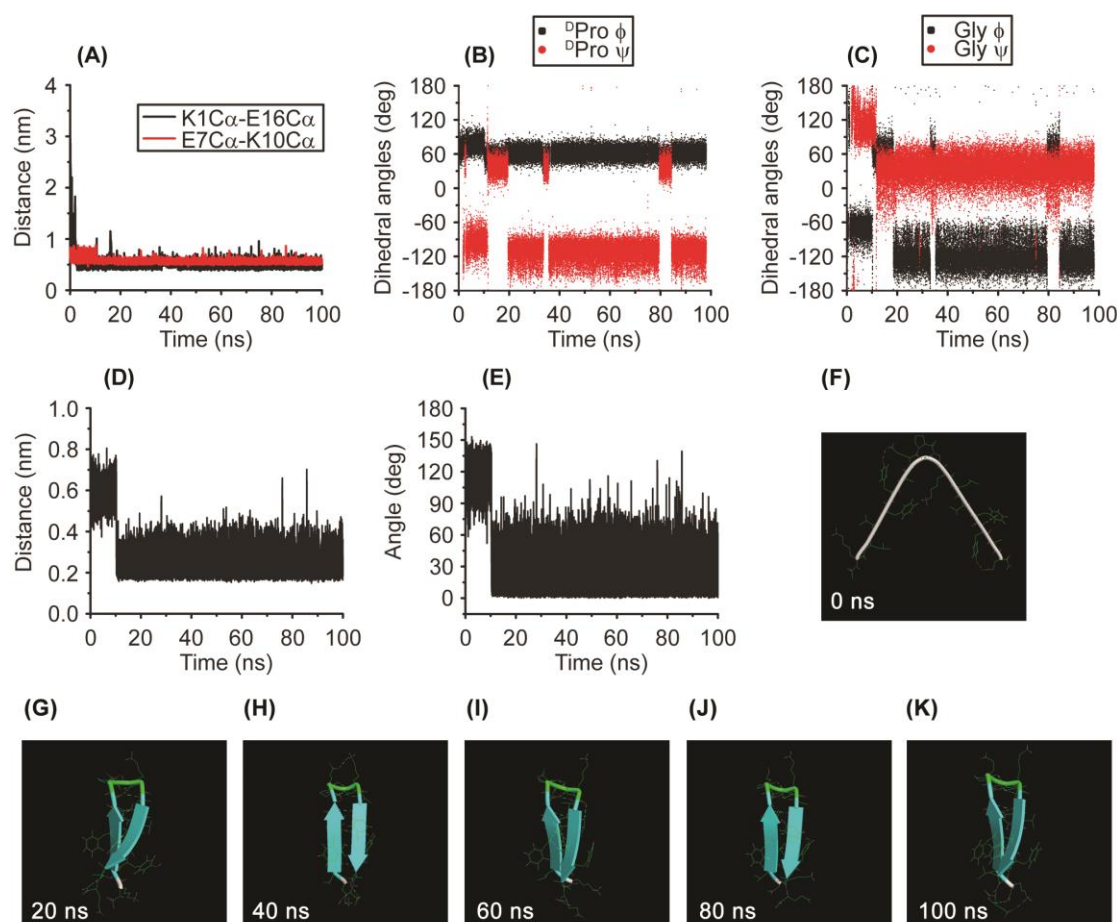


Figure 2.4 MD simulation of A β FY-pG. (A), distance between K1C α and E16C α (black) and that between E7C α and K10C α (red); (B), dihedral angles, ϕ (black) and ψ (red) of D Pro residue; (C), dihedral angles, ϕ (black) and ψ (red) of Gly residue; (D), distance between amide oxygen of Glu7 and amide proton of Lys10; (E), angle HNO, wherein HN belongs to Lys10 amide whereas O belongs to the Glu7 amide; after ~ 11 ns, the distance and the angle are, mostly, within 0.35 nm and 60°, respectively; (F-K), snapshots from the MD trajectory at 20 ns interval.

MD simulation data for A β YY-NG are shown in figure 2.5. The data show a K1C α and E16C α distance of ~ 0.6 - 0.7 nm within 10 ns of simulation (Panel A). However, β -turn is not mediated by residues 7-10, but by residues 8-11. Instead of Asn8 and Gly9, Gly9 and Lys10 happen to be the $i+1$ and $i+2$ turn residues, respectively. This results in a shift in the β -strand registry (Panels F-K). Large fluctuations observed in the distance between K1C α and E16C α could be attributed to the

Chapter 2

flexible N-terminal residue. Around 40 ns, Gly9 takes up ϕ and ψ values spread around $+65^\circ$ and -105° , respectively (Panel B). Lys10 takes ϕ and ψ values around -90° and -30° , respectively (Panel C). The distance between N8C α and L11C α are also within 0.6 nm. The distance between N8CO and L11NH is largely within 0.3 nm (panel D) with angle HNO within 60° (panel E) indicating an H-bond. The presence of H-bond between i^{th} and $(i+3)^{\text{th}}$ residues and the dihedral angles of the $i+1$ and $i+2$ residues indicate a type II' β -turn.

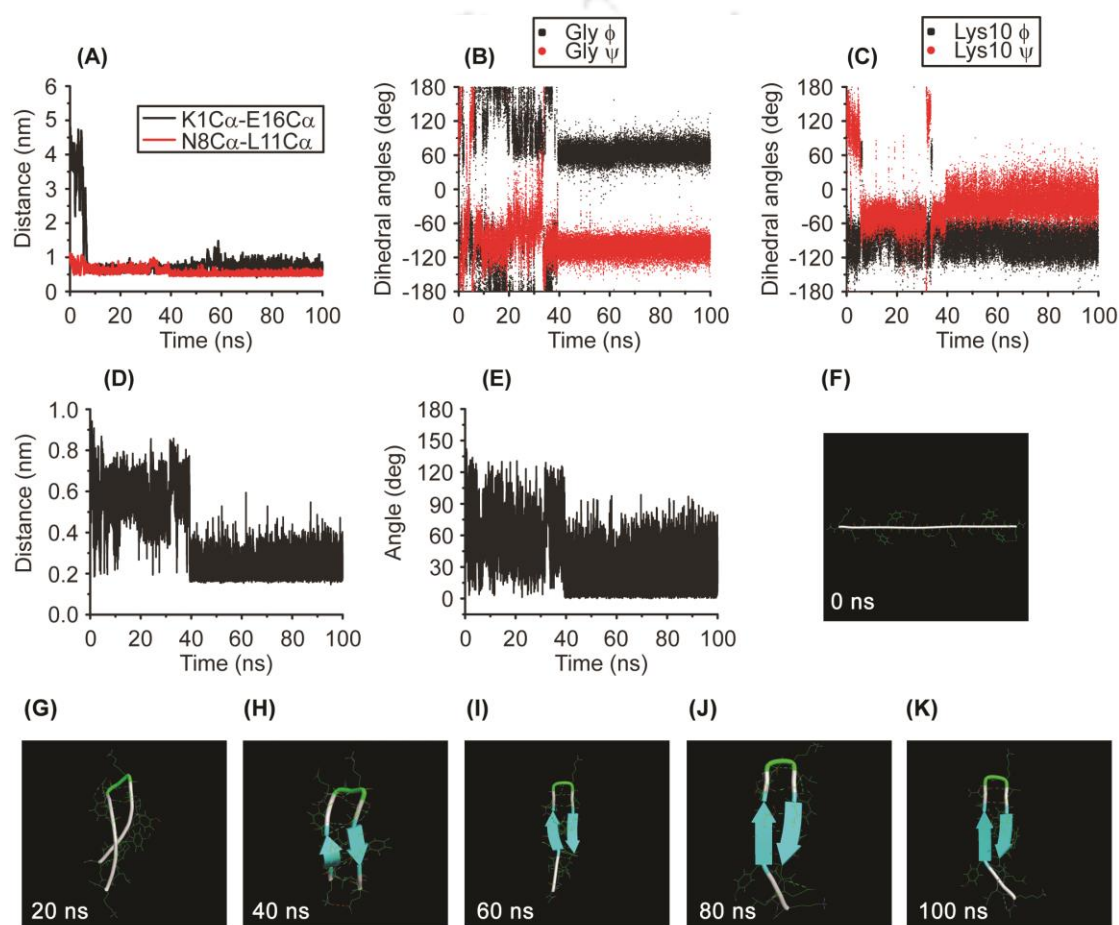


Figure 2.5 MD simulation of AβYY-NG. (A), distance between K1C α and E16C α (black) and that between N8C α and L11C α (red); (B), dihedral angles, ϕ (black) and ψ (red) of Gly residue; (C), dihedral angles, ϕ (black) and ψ (red) of Lys10 residue; (D), distance between backbone amide oxygen of Asn8 and amide proton of Leu11; (E), angle HNO, wherein HN belongs to Leu11 amide whereas O belongs to the Asn8 backbone amide; after 40 ns, the distance and the angle are, mostly, within 0.35 nm and 60° , respectively; (F-K), snapshots from the MD trajectory at 20 ns interval.

A β YY-pG rapidly folds into a β -hairpin structure (Figure 2.6). Around 7 ns, the distance between K1C α and E16C α and that between E7C α and K10C α are within 0.6 ns and are largely invariant throughout the simulation (Panel A). ^DPro8 takes up ϕ and ψ values spread around +60° and -110°, respectively (Panel B). Gly9 takes up ϕ and ψ values spread around -90° and 0°, respectively (Panels C); ψ values largely reside around 30° and -30° suggesting two major clusters around 0°. The data suggest a classic type II' turn mediated by ^DPro8 and Gly9.

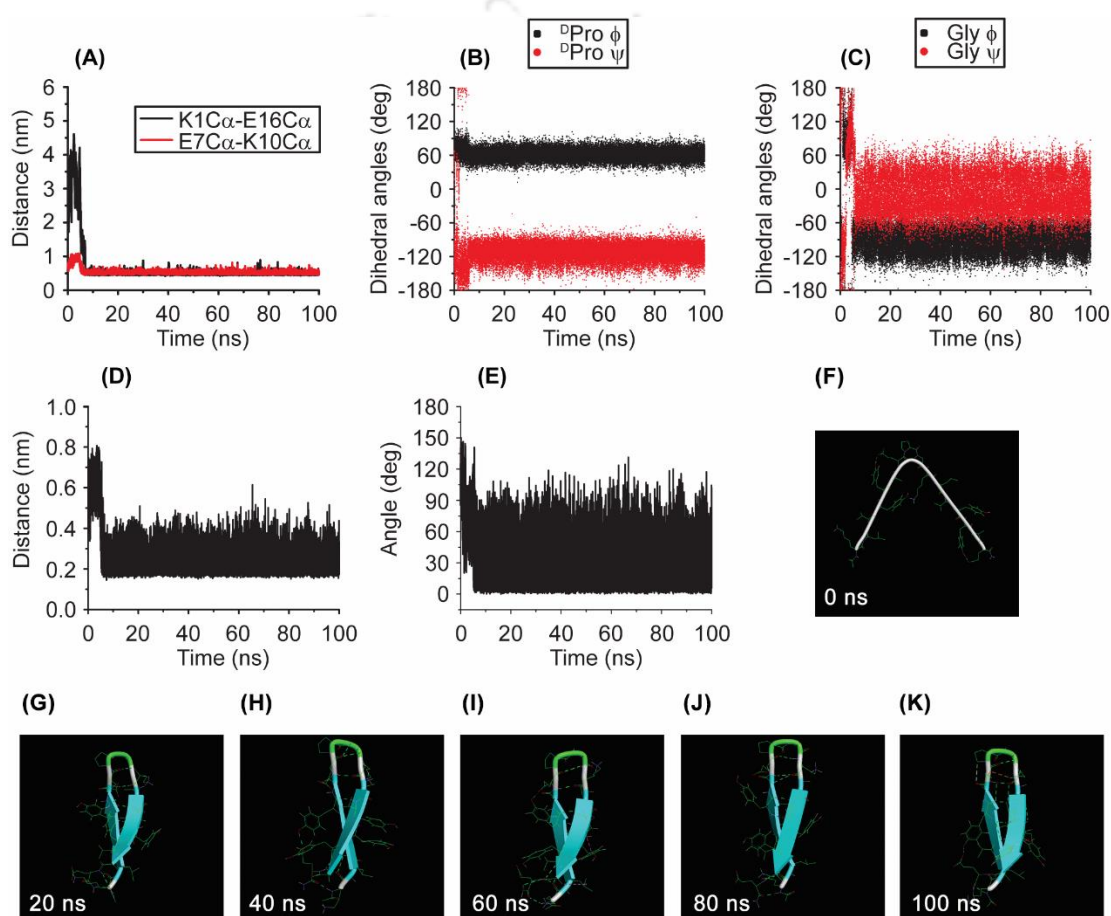


Figure 2.6 MD simulation of A β YY-pG. (A), distance between K1C α and E16C α (black) and that between E7C α and K10C α (red); (B), dihedral angles, ϕ (black) and ψ (red) of ^DPro residue; (C), dihedral angles, ϕ (black) and ψ (red) of Gly residue; (D), distance between amide oxygen of Glu7 and amide proton of Lys10; (E), angle HNO, wherein HN belongs to Lys10 amide whereas O belongs to the Glu7 amide; after ~6 ns, the distance and the angle are, mostly, within 0.35 nm and 60°, respectively; (F-K), snapshots from the MD trajectory at 20 ns interval.

Chapter 2

The hairpin appears to be stabilized by an $i - i+3$ hydrogen-bond in the beta-turn region as suggested by the distance between E7CO and K10NH (panel D) and angle HNO (panel E). Panels F-K show the snapshots at 20 ns interval and confirm the β -hairpin structure.

Snapshots of the MD simulations of A β FF-NG, A β FF-pG, A β WW-NG, A β WW-pG, A β FF-Up, A β FY-Up, A β YY-Up, and A β WW-Up are shown from figures 2.7-2.14, respectively. A β FF-NG folds into a β -hairpin structure. The turn, however, is a non-classical one (Figure 2.7). A β FF-pG takes up a β -hairpin structure mediated by a β -turn within 20 ns (Figure 2.8). D Pro mostly takes up the dihedral angles characteristic of a type II' turn, but Gly shows high conformational flexibility (Panels B and C, Figure 2.8). A β WW-NG coils but a β -hairpin-like conformation is obtained only around 96 ns (Figure 2.9, panel F). The hairpin, however, is not mediated by a β -turn. As suggested by the unstable dihedral angles near the end of the simulation, a 100 ns simulation appears to be insufficient to comment if the hairpin is indeed the kinetically-stable structure. A β WW-pG takes up a β -hairpin structure, but the turn is neither of the classical β -turns (Figure 2.10). A β FF-Up takes type I' β -turn (Figure 2.11). In A β FY-Up, the turn is not only mediated by Aib and D Pro but Lys11 as well, suggesting a γ turn (Figure 2.12). A β YY-Up folds to take a hairpin-like structure (Figure 2.13). The Aib and D Pro dihedral angles take up values those of classical type I' turn in the 10-25 ns region; the chains, however, do not take up a β -conformation. It appears that the bulky Tyr side-chains disrupt the β -sheet structure due to steric clashes; the β -hairpin structure is finally achieved with a shift in the β -strand registry, possibly to ease out the steric clashes. A β WW-Up failed to form any β -hairpin-like structure, possibly due to the steric hindrance caused by bulky indole groups of Trp residues (Figure 2.14).

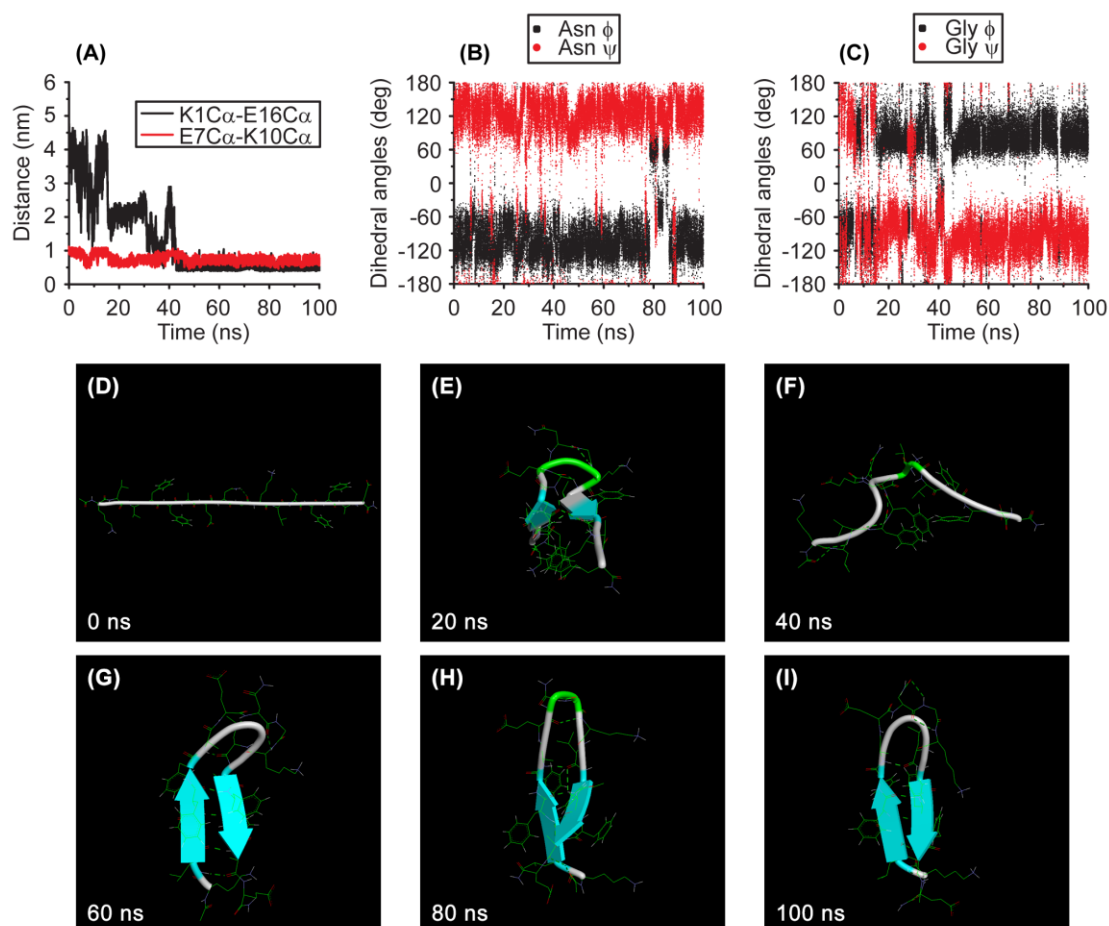


Figure 2.7 MD simulation of AβFF-NG. (A), distance between K1Cα and E16Cα (black) and that between E7Cα and K10Cα (red); (B), dihedral angles, ϕ (black) and ψ (red) of Asn residue; (C), dihedral angles, ϕ (black) and ψ (red) of Gly residue; (D-I), snapshots from the MD trajectory at 20 ns interval.

Chapter 2

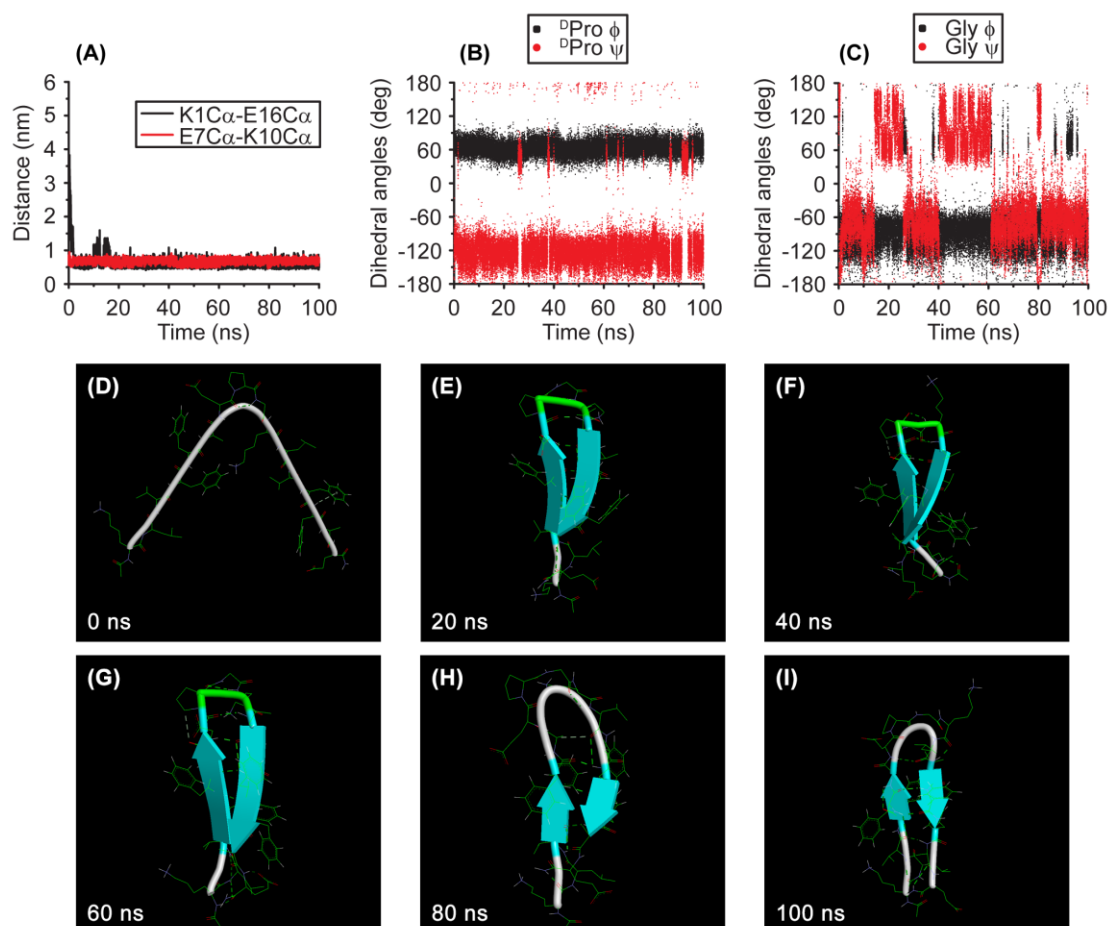


Figure 2.8 MD simulation of AβFF-pG. (A), distance between K1Cα and E16Cα (black) and that between E7Cα and K10Cα (red); (B), dihedral angles, ϕ (black) and ψ (red) of ^DPro residue; (C), dihedral angles, ϕ (black) and ψ (red) of Gly residue; (D-I), snapshots from the MD trajectory at 20 ns interval.

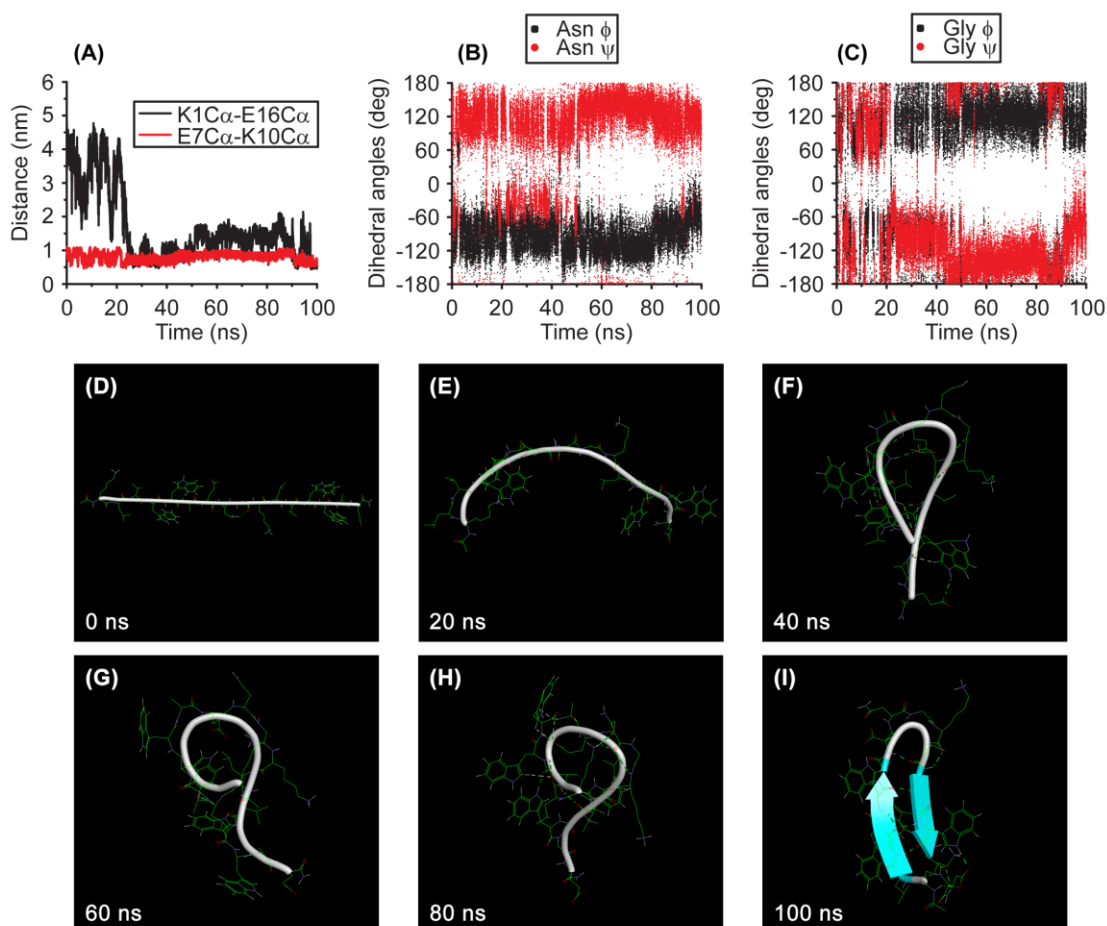


Figure 2.9 MD simulation of AβWW-NG. (A), distance between K1Cα and E16Cα (black) and that between E7Cα and K10Cα (red); (B), dihedral angles, ϕ (black) and ψ (red) of Asn residue; (C), dihedral angles, ϕ (black) and ψ (red) of Gly residue; (D-I), snapshots from the MD trajectory at 20 ns interval.

Chapter 2

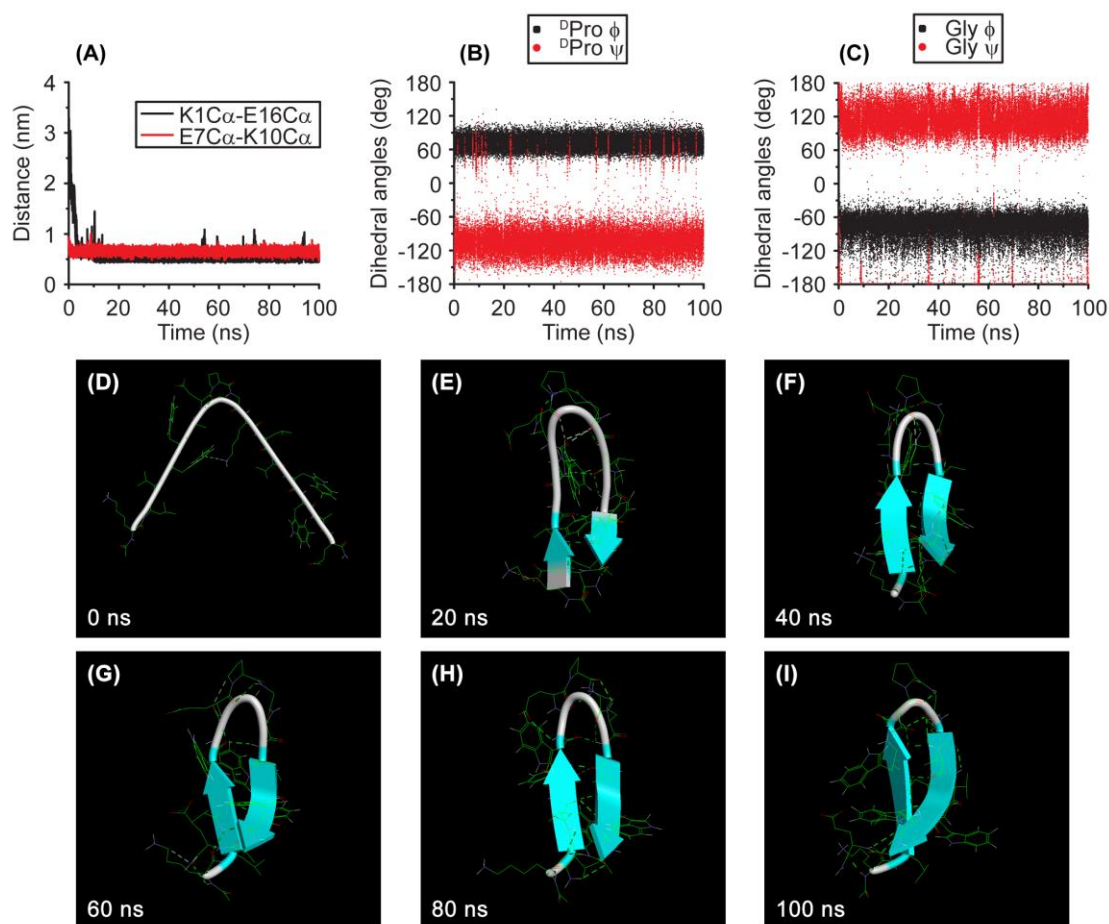


Figure 2.10 MD simulation of AβWW-pG. (A), distance between K1Cα and E16Cα (black) and that between E7Cα and K10Cα (red); (B), dihedral angles, ϕ (black) and ψ (red) of ^DPro residue; (C), dihedral angles, ϕ (black) and ψ (red) of Gly residue; (D-I), snapshots from the MD trajectory at 20 ns interval.

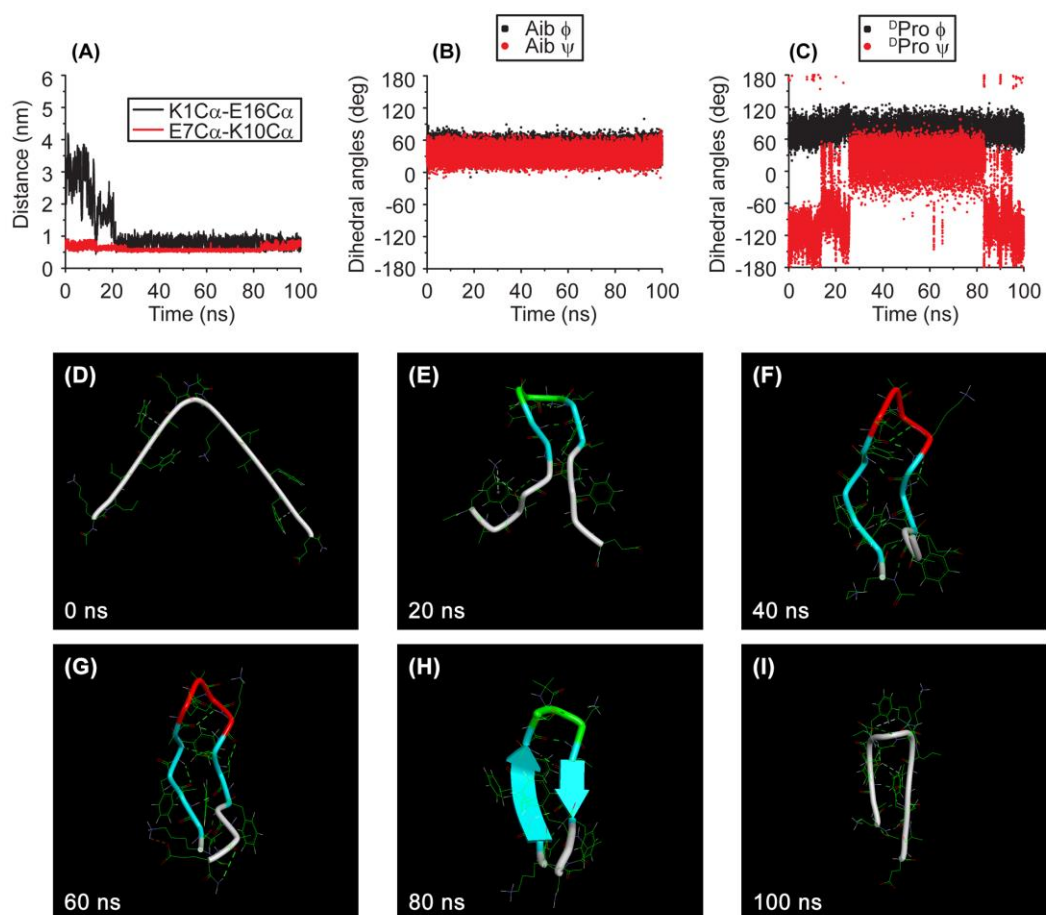


Figure 2.11 MD simulation of AβFF-Up. (A), distance between K1Cα and E16Cα (black) and that between E7Cα and K10Cα (red); (B), dihedral angles, ϕ (black) and ψ (red) of Aib residue; (C), dihedral angles, ϕ (black) and ψ (red) of D Pro residue; (D-I), snapshots from the MD trajectory at 20 ns interval.

Chapter 2

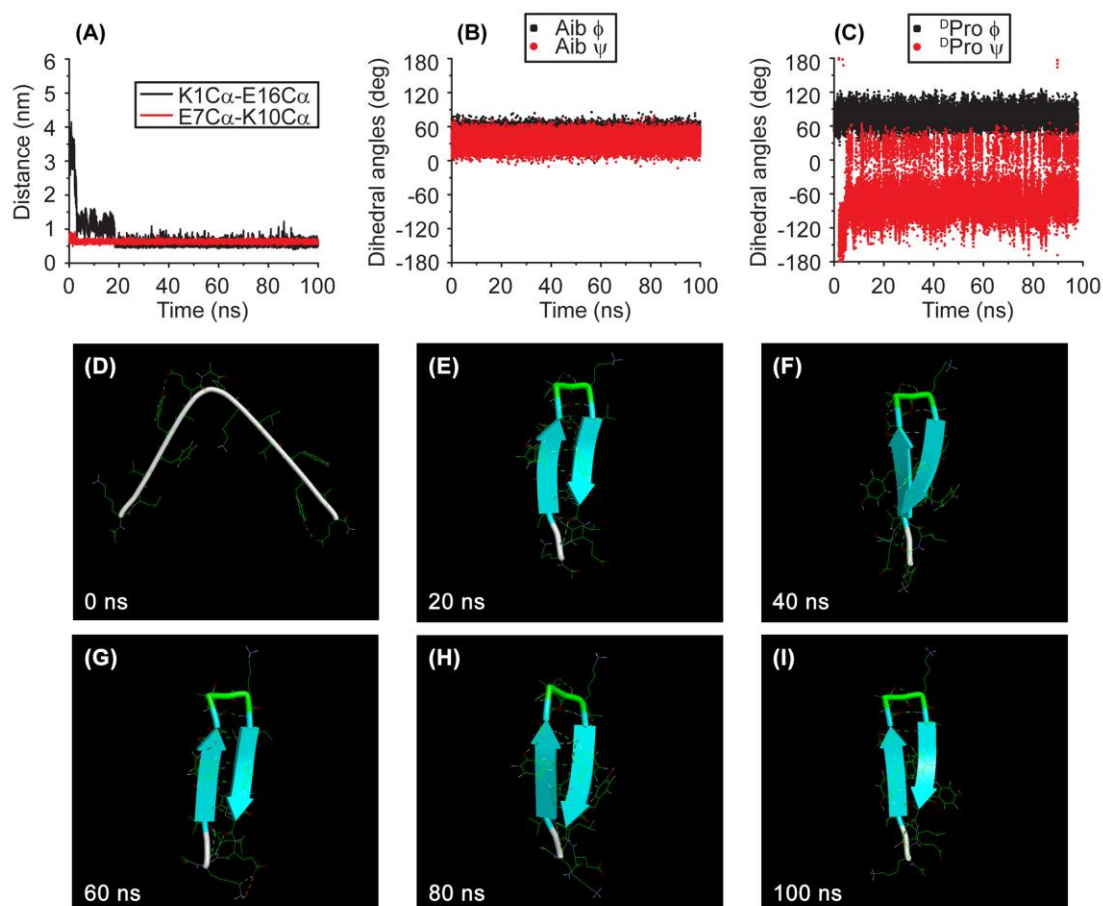


Figure 2.12 MD simulation of AβFY-Up. (A), distance between K1Cα and E16Cα (black) and that between E7Cα and K10Cα (red); (B), dihedral angles, ϕ (black) and ψ (red) of Aib residue; (C), dihedral angles, ϕ (black) and ψ (red) of ^DPro residue; (D-I), snapshots from the MD trajectory at 20 ns interval.

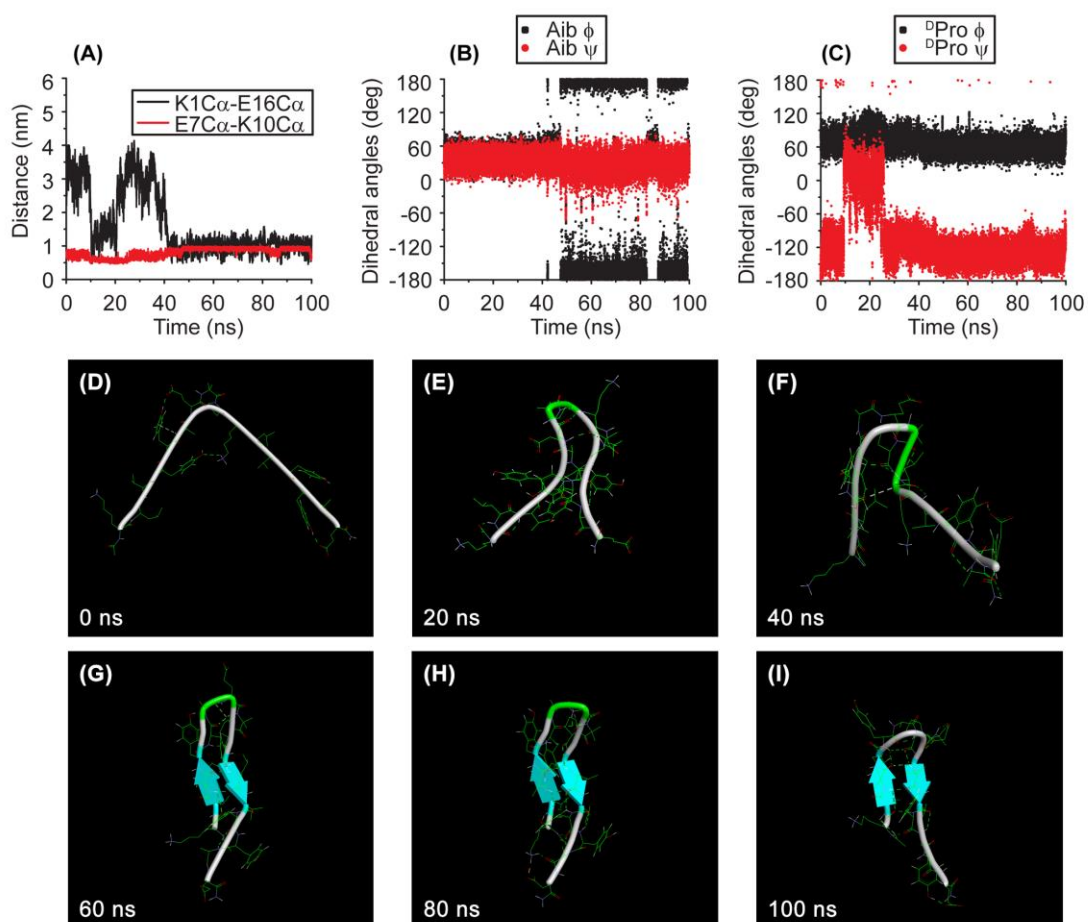


Figure 2.13 MD simulation of AβYY-Up. (A), distance between K1Cα and E16Cα (black) and that between E7Cα and K10Cα (red); (B), dihedral angles, ϕ (black) and ψ (red) of Aib residue; (C), dihedral angles, ϕ (black) and ψ (red) of D Pro residue; (D-I), snapshots from the MD trajectory at 20 ns interval.

Chapter 2

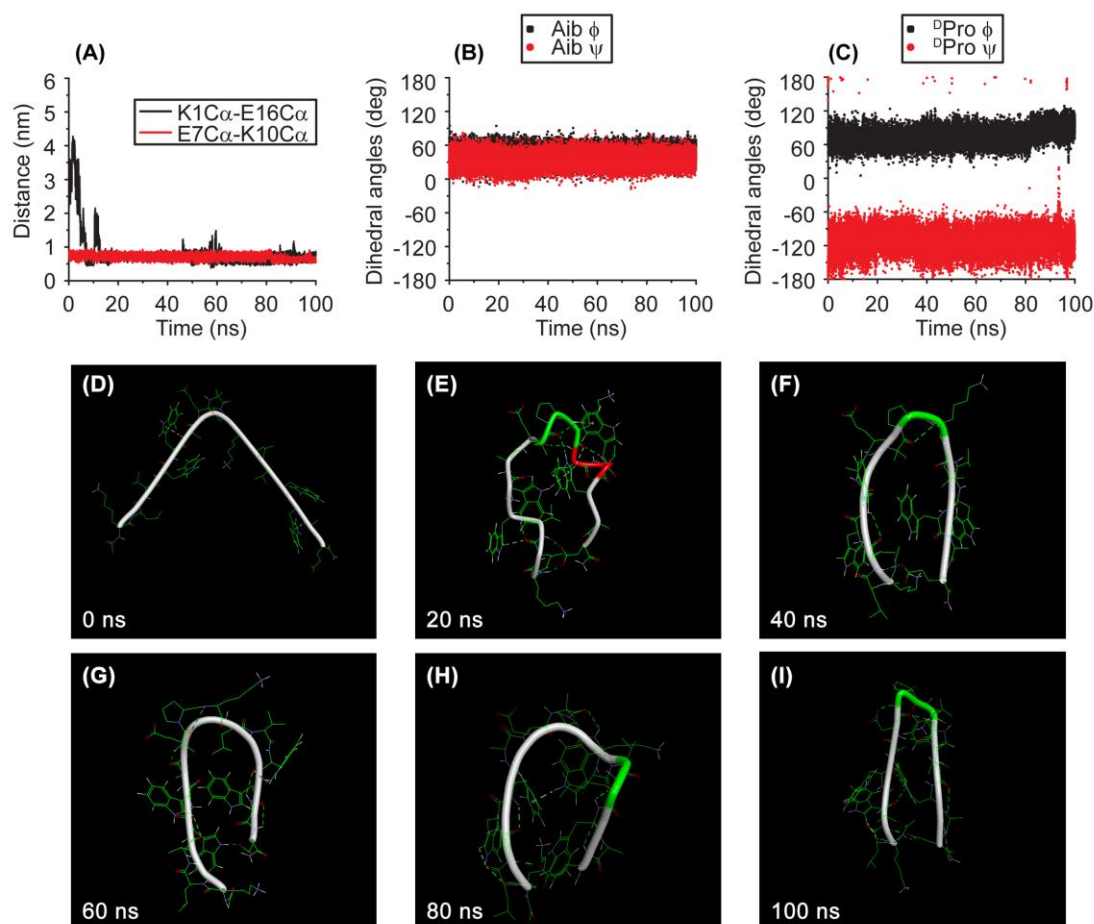


Figure 2.14 MD simulation of AβWW-Up. (A), distance between K1Cα and E16Cα (black) and that between E7Cα and K10Cα (red); (B), dihedral angles, ϕ (black) and ψ (red) of Aib residue; (C), dihedral angles, ϕ (black) and ψ (red) of ^DPro residue; (D-I), snapshots from the MD trajectory at 20 ns interval.

2.3.4 ThT fluorescence spectroscopy

Peptide solutions at 250 μ M in 20% HFIP were incubated at room temperature and analyzed using ThT fluorescence spectroscopy after 24 h incubation. AβFF causes small enhancement ($\sim$ 3-fold) in ThT fluorescence in agreement with the previous reports (Figure 2.15). AβFY caused $\sim$ 4-fold enhancement in ThT fluorescence. The other two peptides, AβYY and AβWW, caused only little enhancement ($<$ 2-fold) in ThT fluorescence. All the turn-containing peptides, on the other hand, cause larger enhancement in ThT fluorescence compared to control peptides. The effect of the turn-inducing motifs, however, is sequence dependent. These results are unsurprising as control peptides have different aggregation properties by themselves [23]. More than 10-fold enhancement in

ThT fluorescence was observed for the A β FF-derived turn-harboring peptides. The A β FY-derived peptides turned out to be highly amyloidogenic; >20-fold enhancement was observed for A β FY-Up and A β FY-NG, whereas ~50-fold enhancement was observed for A β FY-pG. A β YY and A β WW are largely non-amyloidogenic as is suggested by the ThT fluorescence. Turn-harboring A β YY repeats, on the other hand, caused ~4 to 5-fold enhancement in ThT fluorescence. A β WW-derived peptides caused ~10-fold enhancement in ThT fluorescence suggesting their higher self-assembling propensity.

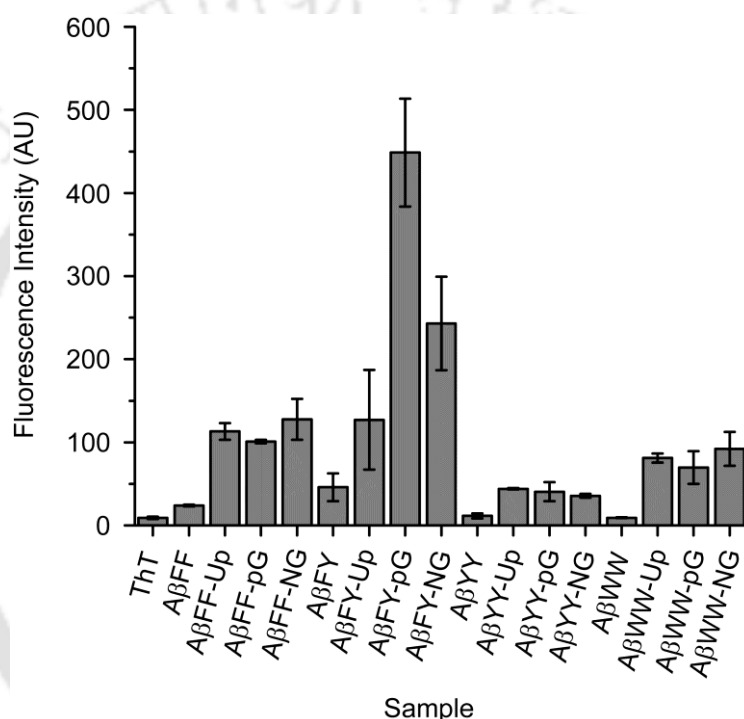


Figure 2.15 ThT fluorescence intensity at 482 nm for 24 h-old samples in 20% HFIP. The 24 h-old samples were diluted in 5 mM phosphate buffer, pH 7.0 and spectra recorded. The error bars represent standard deviation.

2.3.5 Transmission Electron Microscopy

TEM images of the 72 h-old peptide samples are shown in figure 2.16. A β FF, A β FY, and their β -turn analogs show distinct fibrillar structures. The fibril morphology, however, depends on the type of β -turn motif present in the peptide. The control A β FF shows thick, short fibers. A β FF-Up forms long ribbon-like structures, whereas long needle-like aggregates were obtained from A β FF-pG. A β FF-NG shows thin, long fibers; the fibers show lateral assembly and

Chapter 2

entanglement. A β FY and its analogs resulted in long, thin fibrils; A β FY forms thin sub-micrometer fibers as well (inset, panel A β FY). Very extensive networks of fibrils were obtained for all the β -turn motif harboring peptides that corroborates with the large enhancement in ThT fluorescence (Figure 2.16). A β YY does not show any fibrillar aggregates while A β YY-NG showed few fibrous structures suggesting that peptides are sluggish to aggregate. MD simulation of A β YY-NG shows a β -turn mediated by Gly9-Lys10 (Figure 2.5). This renders the two ends of the β -hairpin cationic and could diminish the self-assembly through electrostatic repulsions. A β YY-Up and A β YY-pG, on the other hand, resulted in distinct fibrillar structures. The data suggest that the introduction of rigid β -turn could impart amyloidogenicity to the otherwise non-amyloidogenic peptides. A tight β -hairpin essentially brings two self-assembling motifs (β -strands) in close proximity. Such a structure is a small β -sheet that could nucleate the growth of fibrils by incorporating monomers. A β WW and its analogs resulted in largely spherical clumps. Thin, long fibrils, were also obtained from A β WW-Up. MD simulations of A β WW-NG and A β WW-pG suggest the formation of hairpin-like structures, albeit not mediated by classical β -turns. Such a structure could result in self-assembly through tryptophan-zipper interactions; the structures formed by A β WW turn analogs, in fact, caused large enhancement in ThT fluorescence suggesting the formation of β -sheet rich aggregates.

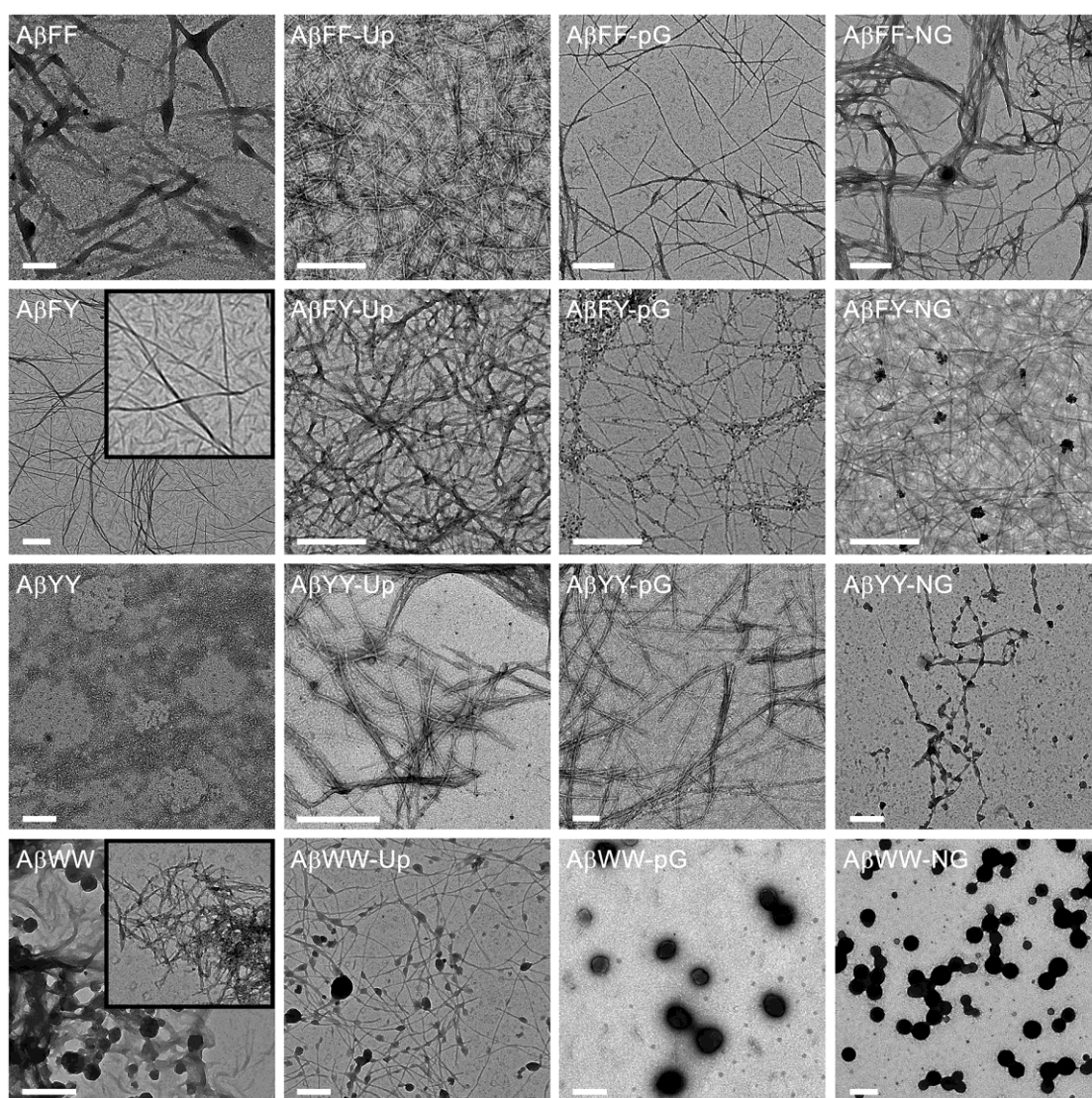


Figure 2.16 TEM analyses of 72 h-old peptide samples in 20% HFIP. The scale bars represent 0.5 μm .

2.4 CONCLUSIONS

Turn-inducing motifs play an important role in protein folding [45]. Thus it is shown in this chapter that the turns can have deciding roles on the aggregation propensity of the amyloidogenic peptides and the properties of the self-assembled structures. Compared to A β FF, that formed short fibers after three days; the β -turn analogs form distinct long fibers that cause large enhancement in ThT fluorescence. A β FY is more amyloidogenic than A β FF, as shown previously [23] and is evident from ThT fluorescence and TEM imaging data in this study. Beta-turn connected A β FY repeats resulted in a very large

Chapter 2

enhancement in ThT fluorescence and resulted in distinct fibrils as revealed by TEM imaging. Among all the β -turn analogs, A β FY-derived peptides turned out to be highly amyloidogenic, forming a distinct fibrillar mesh and causing very large enhancement in ThT fluorescence. Phe₁₉ has been shown to be critical for the assembly of A β ₁₆₋₂₂ in previous studies; substitution of Phe₁₉ with Tyr or Trp severely diminishes its aggregation propensity suggesting its role in cross-strand interactions [20, 21]. Consequently, neither A β YY nor A β WW causes any significant enhancement in ThT fluorescence. Beta-turn analogs of A β YY, however, caused enhancement in ThT fluorescence and resulted in fibrillar structures indicating that a non-amyloidogenic peptide could be rendered amyloidogenic by inducing β -hairpin formation. The role of β -hairpin in amyloid formation has been studied using model polyQ peptides [46, 47]. A model polyQ peptide harboring a ^DPro-Gly motif in the middle of the polyQ region displays faster aggregation kinetics suggesting that β -turn formation could be the rate-determining step in the aggregation [47]. The same study reveals that the polyglutamine core formed by Huntington exon-1 is based on interdigitated hairpin-like structures [47]. These data suggest that hairpin-like structures could be crucial in the assembly of other self-assembling peptides as well. In addition, the site of incorporation of the β -turn inducing motifs in could be exploited to alter the registry of the strands thereby modulating their self-assembly.

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Chapter 2

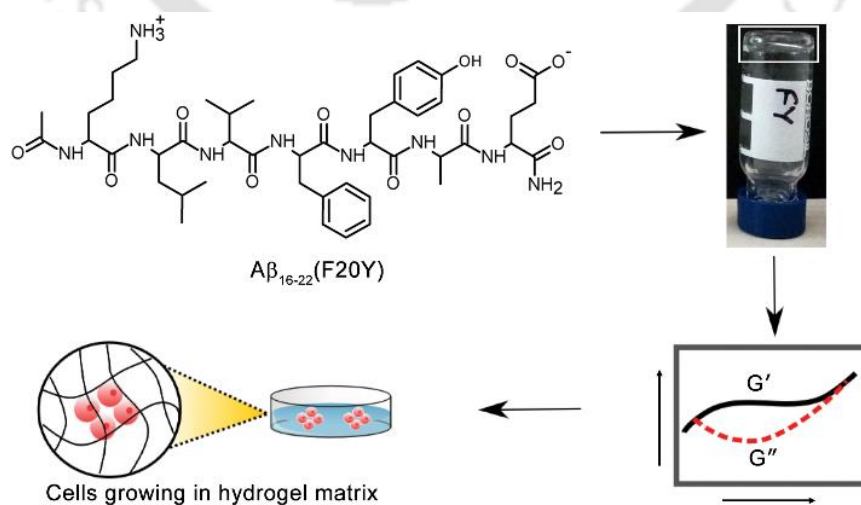
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HYDROGEL FORMATION BY THE $A\beta_{16-22}$ AROMATIC ANALOG, $A\beta_{16-22}$ (Phe20Tyr)





3.1 INTRODUCTION

Gels constitute an important class of soft materials and play an important role in our day-to-day life like toothpaste gels, shaving gels, and shampoos. Gels are characterized by a large amount of solvent, typically $\geq 98\%$ w/w, fixed by a small amount of solid. Supramolecular gels or physical gels are characterized by the self-assembly of gelator molecules through non-covalent interactions. Water-based gels (hydrogels) have gained considerable attention in the past two decades and turn out to be promising materials for various biomedical applications [1-4]. Among different classes of molecules that have been explored for biocompatible hydrogels, peptides stand out as the material of choice as they are biocompatible, easily synthesized using standard methods, and readily hydrolysed by proteases [1, 5, 6]. Peptide-based hydrogels are promising candidates as drug-delivery vehicles and scaffolds for 3-dimensional cell culture [3, 7]. Their potential has quickly been realized, and several peptide-based hydrogels have already been commercialized as media for cell culture [8].

A large majority of peptide-based hydrogels are based on β -sheet architecture [9]. Non- β -sheet peptide hydrogels, however, are also known [10, 11]. The peptide molecules self-assemble into β -sheet rich fibrillar structures, much like amyloid fibrils. The fibrils entangle to make a three-dimensional network trapping water molecules. In fact, hydrogels have been obtained from amyloidogenic proteins [12] and Fmoc-capped amyloidogenic peptides [13]. It is important to mention that a great majority of peptide hydrogelators are rich in aromatic moieties and aromatic stacking interactions are believed to be critical for self-assembly [5, 14]. Incorporation of bulky aromatic moieties such as Fmoc and naphthyl at the amino-terminus of peptides has resulted in a variety of hydrogelators. In fact, the Fmoc-protected amino acids, Fmoc-Tyr and Fmoc-Phe are reported to cause hydrogelation at slightly acidic pH [7]. Interestingly, Fmoc-Tyr forms hydrogels with a storage modulus at least two orders of magnitude larger than that of Fmoc-Phe hydrogels. The reasons underlying different hydrogelation potential are not clear. Packing of bulkier Tyr side chain and possibility of forming hydrogen-bonds with water molecules could be the reason. A β_{16-22} (Ac-

Chapter 3

KLVFFAE-am) harbors a diphenylalanine moiety which is involved in the aromatic interactions in the amyloid fibrils [15]. Therefore an attempt was made to investigate if A β ₁₆₋₂₂ and its aromatic analogs discussed in chapter 2 could form hydrogels.

3.2 MATERIALS AND METHODS

3.2.1 Materials

The materials for peptide synthesis are the same as described in section 2.2.1, chapter 2. Calcein acetoxymethyl ester (Calcein AM) was procured from Sigma-Aldrich Chemicals Pvt. Ltd. Trypan blue was obtained from Sisco Research Laboratory, and rhodamine B were obtained from Merck. Doxorubicin hydrochloride was procured from TCI Chemicals Pvt. Ltd. All the cell lines were obtained from the National Centre for Cell Science (Pune, India). All other reagents were of the highest grade available.

3.2.2 Peptide synthesis and characterization

The synthesis, purification, and characterization of the peptides A β FF, A β FY, A β YY, and A β WW has been described in chapter 2 (section 2.2.2). In fact, the very same peptides were employed in this study.

3.2.3 Self-assembly/hydrogelation

The peptide stock solutions were prepared in HFIP, and their concentrations determined as described in section 2.2.3, chapter 2. The peptides were subsequently diluted in water to obtain the desired peptide concentrations (final HFIP concentration = 10%). The peptides were left undisturbed at room temperature and monitored for gelation by inverting the tube after 12 h of standing. A β FY caused gelation at 2 mM peptide concentrations. None of the other three peptides formed hydrogel up to 10 mM peptide concentration. As A β YY and A β WW failed to cause hydrogelation, they were not investigated any further. The parent peptide, A β FF, however, was included in the study.

Gelation of acidic solutions by A β FY

A β FY stock solution (50 mM) was prepared in HFIP. Water, acidified to pH 2 and 4 using HCl, was added to the peptide solutions (5 mM peptide concentration) and left undisturbed. Both the samples resulted in gels within 4 h of incubation as ascertained by tube-inversion.

3.2.4 CD spectroscopy

Far-UV CD spectra were recorded on a Jasco 1500 spectropolarimeter. The 24 h-old peptide samples (2 mM) were diluted to obtain 35-50 μ M concentration and spectra recorded in 0.1 cm path length cell with a scan rate of 100 nm/min. Each spectrum is an average of 4 scans and corrected by subtracting the solvent spectrum. The data are converted to mean residue ellipticity, as described in section 2.2.4, chapter 2.

3.2.5 FTIR spectroscopy

FTIR spectra were recorded for the 24 h-old 2 mM peptide samples on a Shimadzu IRAffinity-1S Fourier Transform Infrared Spectrophotometer on the attenuated total reflection (ATR) module equipped with a diamond ATR crystal. Peptide samples (10 μ l) were spread out and dried as films on the crystal, and ATR-FTIR spectra recorded at a resolution of 4 cm^{-1} .

3.2.6 Raman spectroscopy

About 20 μ l of the 5 mM A β FY gel sample was deposited on a clean glass slide and air dried. Spectrum was recorded using a 514 nm laser on a Horiba Jobin Vyon Raman spectrometer, (Model LabRam HR).

3.2.7 AFM imaging

24 h-old peptide samples (2 mM concentration in 10% HFIP) were 100-fold diluted and quickly deposited on clean glass slides. Excess solution was blotted out after 2 min using Whatman I filter paper and slides allowed to air-dry. AFM

Chapter 3

images were acquired in non-contact mode using a silicon probe on an Asylum MFP-3D Origin atomic force microscope (Oxford Instruments).

3.2.8 TEM imaging

Peptide samples (2 mM; incubated for 24 h) were diluted 100-fold and 5 μ l volume deposited on carbon-coated 200-mesh copper grids. After 2 minutes, the sample was gently blotted out from the periphery of the grid using a Whatman I filter paper. The grids were stained with saturated uranyl acetate solution, which was blotted out after 30 seconds. Sample images were recorded at 200 kV on JEM-2100F (JEOL, Japan) transmission electron microscope. Widths of the fibrils were determined using ImageJ software. At least 20 fibrils were measured for each of the samples.

3.2.9 ThT fluorescence spectroscopy

ThT fluorescence assay was carried out at 10 μ M ThT concentration in 5 mM phosphate buffer, pH 7.4 on a Jasco FP8500 spectrofluorometer. 24 h-old peptide samples (2 mM) were diluted to 20 μ M concentration and excited at 440 nm, slit width 2.5 nm. Fluorescence emission spectra were recorded from 460-540 nm with a 5 nm emission slit width.

3.2.10 Microrheology

Microrheology measurements were carried out on a Malvern Zetasizer Nano ZS instrument using the 173° non-invasive back-scattering configuration. Carboxylated melamine beads (0.6 μ m) were used as the tracer particle. The concentration of the tracer particles was optimized so as to prevent tracer aggregation as well as the interaction between the sample and the tracer. All the microrheology experiments were carried out at 25 °C in a quartz cuvette having a final tracer volume fraction of 0.025% in the sample. Briefly, the tracer particles were added along with the peptide during the gel formation. Each of the gel was set in the DLS cuvette and left undisturbed for 12 h. The mean squared displacement (MSD) data were recorded for the 12 h-old peptide samples. The elastic (G') and viscous (G'') moduli were determined using the Malvern

Zetasizer instrument software from the mean squared displacement (MSD) data using the generalized Stokes-Einstein equation, as introduced by Mason [16].

Temperature dependent microrheology

MSD for the A β FY gel prepared at 25 °C was recorded. Subsequently, the temperature was increased up to 55 °C with a step size of 10 °C and brought back to 25 °C with the same step size. MSD data recorded at these temperatures were used to determine G' and G'' .

3.2.11 Doxorubicin release assay

Doxorubicin at 0.5 mg/ml concentration was trapped inside the A β FY gel (5 mM peptide concentration) during the gelation process. Briefly, 5 μ l of 50 mM A β FY stock solution in HFIP was added to a quartz cuvette; 45 μ l of 0.55 mg/ml doxorubicin stock solution prepared in water was subsequently added and left undisturbed for 12 h resulting in the gel. Release of the drug was assayed by gently adding 950 μ l water on the doxorubicin-loaded gel and monitoring absorbance at 490 nm as a function of time.

3.2.12 Rhodamine B release assay

Rhodamine B loaded gels (5 mM peptide concentration; 200 μ M rhodamine B) were prepared in a quartz fluorescence cell using method similar to that used for doxorubicin loading. Water (950 μ l) was subsequently added on top of the gel and rhodamine B release monitored by measuring fluorescence emission intensity at 573 nm upon excitation with 545 nm light. The excitation and emission slit widths were 2.5 and 5 nm, respectively.

3.2.13 Cell seeding and cultivation

The human embryonic kidney (HEK-293), baby hamster kidney (BHK-21), rat pancreas (RIN-5F), and human neuroblastoma (IMR-32) cells were cultured in T25 culture flasks containing RPMI 1640 medium (RIN-5F) or DMEM medium (HEK-293, BHK-21, and IMR-32) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (Gibco, Life Technologies). The cells were

Chapter 3

incubated at 37 °C containing 5% CO₂ and were subsequently re-cultured and maintained for further assays. Cells were harvested by trypsinization, stained with trypan blue, and counted using Countess II FL Automated Cell Counter (Thermo Fisher Scientific). AβFY gels (5 mM concentration) were prepared in the wells of a 96-well plate at 37 °C in 5% CO₂. After 12 h of gelation, RPMI or DMEM (2-fold volume) was gently added to the gel and decanted carefully after 60 minutes; this step was repeated once more. Cells (2×10⁵) were then seeded in the 96-well plate and incubated at 37 °C in 5% CO₂.

3.2.14 Cell viability assay

RIN-5F, HEK-293, and BHK-21 cells grown in the gels for 24 h were washed twice with phosphate-buffered saline (PBS, pH 7.4) and incubated with 5 μM calcein AM in plain RPMI/DMEM medium (without FBS) for 30 min at 37 °C. After staining, the cells were washed three times with PBS, visualized under white light, and the images recorded on EVOS FLoid Cell Imaging Station (Thermo Fisher Scientific). Subsequently, fluorescence images were recorded using the green channel. Green fluorescence indicates hydrolysis of the non-fluorescent calcein AM by cellular esterases to fluorescent calcein. RIN-5F cells were assayed for the insulin-1 expression as well.

3.2.15 Insulin gene expression assay

Functional characterization of RIN-5F cells grown in the gel was carried out by assaying for insulin-1 gene expression by PCR. Total RNA was extracted from the RIN-5F cells grown for 24 h using RNeasy mini kit (QIAGEN) according to the manufacturer's protocol. Genomic DNA contamination, if any, was removed by treating with DNase I (NEB, USA) for 30 min at 37 °C. cDNA was synthesized from 500 ng RNA using high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and rat insulin-1 was confirmed by PCR amplification. The sequences of primers used for PCR amplification are shown in Table 3.1. The expected PCR product sizes for insulin-1 and GAPDH are 129 bp and 189 bp, respectively.

Table 3.1 Sequences of the primers used for amplifying GAPDH and insulin-1 cDNA.	
GAPDH	5'-ATGGAGAAGGCTGGGGCTCA-3'
	5'-GTTGTCATGGATGACCTTGGC-3'
Insulin-1	5'-GCACCTTTGTGGTCCTCACCT-3'
	5'-GCCTCCACCCAGCTCCAGTT-3'

3.3 RESULTS AND DISCUSSION

The peptides stock solutions were prepared in HFIP. For gelation, peptides were diluted in water to obtain 2, 5, and 10 mM concentrations such that the final HFIP concentration is 10%. Dilution in water resulted in clear transparent solutions. The peptide solutions thus obtained were left undisturbed at room temperature for 12 h. Formation of the gel was examined by inverting the tubes. A β FF, A β YY, and A β WW failed to cause gelation at all three concentrations, whereas A β FY resulted in self-supporting transparent gels at all three concentrations. As shown in chapter 2 (Figure 2.16), no fibrillar structures were obtained for A β YY in 20% HFIP, suggesting its intrinsically poor amyloidogenicity. A β WW had formed large structures with little fibrillar structures. As both A β YY and A β WW failed to cause gelation as well, the investigation pertaining to hydrogelation was no longer continued with them. The hydrogelation of A β FF and A β FY were also examined at a further higher concentration of 20 mM. Expectedly, A β FY formed hydrogel that was, interestingly, transparent even at 20 mM concentration. A β FF, on the other hand, failed to cause gelation. It can, therefore, be inferred that the A β_{16-22} aromatic mutant, A β FY is a promising hydrogelator wherein gels could be obtained in the concentration range from 2–20 mM (~0.18–1.8% w/w). Figure 3.1 shows the picture of the inverted tube for 2 mM A β FY gel. A β_{16-22} failed to form gels up to 20 mM concentration; tilting the tube caused the sample to flow, indicating largely viscous behaviour.

Chapter 3

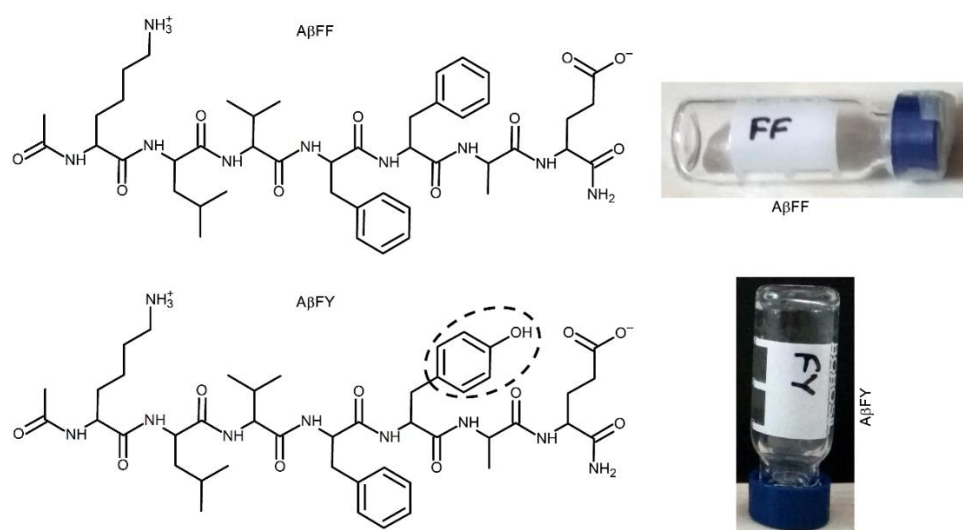


Figure 3.1 Gelation by peptides as assessed by tube inversion. The 12 h-old AβFF (20 mM) flows when the tube is tilted (top) whereas AβFY (2 mM) supports its weight in the inverted tube (bottom).

Gelation of acidic solutions by AβFY

It was a curiosity to see if the AβFY could cause gelation at acidic pH values. It was found that the peptide resulted in self-supporting gels at pH 2 and 4 as well (Figure 3.2). These gels were not further investigated.



Figure 3.2 Gelation caused by 5 mM AβFY at pH 2 and 4 as assessed by inverting the tube. The gel area is marked with white rectangles.

As the peptide AβFY is derived through subtle modification of the amyloidogenic peptide Aβ₁₆₋₂₂, the structural makeup of the self-assembled structures was

investigated using CD spectroscopy, infrared spectroscopy, Raman spectroscopy, ThT fluorescence spectroscopy, non-contact mode AFM, and TEM imaging.

3.3.1 CD spectroscopy

Far-UV CD spectra were recorded to probe the differences, if any, at the secondary structure level. The A β FY spectrum displays a negative band centered $\sim$ 216 nm and a positive band around 191 nm indicating a distinct β -sheet conformation (Figure 3.3, panel A). The CD spectrum of A β FF, on the other hand, displays a negative band around 221 nm and an intense positive band around 199 nm. The spectrum is suggestive of twisted β -sheet [17]. Distinct CD spectra indicate differences at the secondary structure level for the self-assembled peptides.

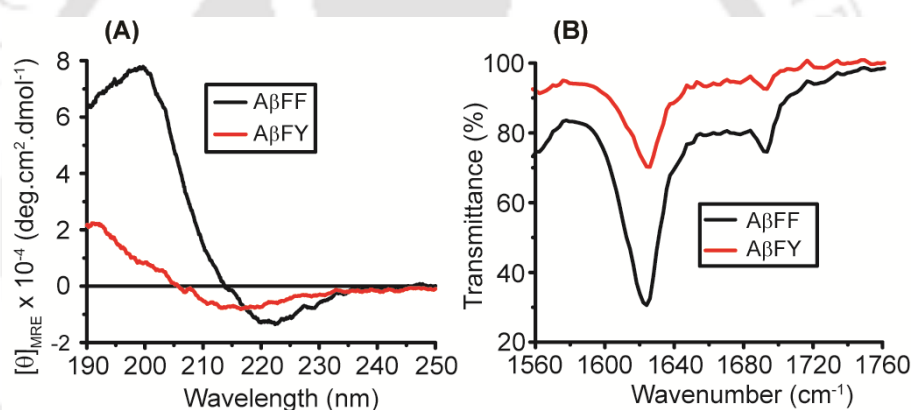


Figure 3.3 Characterization of self-assembled A β FF structures and A β FY gel. CD spectra (panel A), and ATR-FTIR spectra (panel B) for the self-assembled peptides.

3.3.2 FTIR spectroscopy

Both A β FF and A β FY, harbor dipeptide aromatic motifs and clusters of aromatic residues can have a significant contribution to far-UV CD spectra of short peptides [18, 19]. Aromatic residues constitute the core of A β FF fibrils [20]. In fact, intermolecular aromatic interactions are believed to play a critical role in the A β FF self-assembly. Such a cluster of aromatic residues can cause marked perturbation in the far-UV CD spectra. Secondary structure of the self-assembled peptides was, therefore further characterized using ATR-FTIR spectroscopy (Figure 3.3, panel B). Amide I band $\sim$ 1626 cm^{-1} indicates a β -sheet conformation,

Chapter 3

even for peptides rich in aromatic residues [21]. Both A β FF and A β FY showed an amide I band at 1624 cm⁻¹, a signature characteristic of β -sheet rich fibrils [22]. The high-frequency peak often observed for amyloid-like fibrils [22] was observed ~1692 cm⁻¹ for both the peptides. Tyr side-chain is bulkier than that of Phe. In addition to that, Tyr is less hydrophobic than Phe and could be more solvent exposed. These characteristics, alongside the electronic effects of the electron-withdrawing hydroxyl group in the Tyr side chain and the possibility of H-bonding, could result in distinct hydrogelation propensity.

3.3.3 Raman spectroscopy

The involvement of the Tyr side chain hydroxyl group in H-bonding is often characterized using Raman spectroscopy. Tyrosine generates a pair of fairly intense Raman bands around 850 and 830 cm⁻¹ [23]. The ratio of the intensities of these bands (I_{850}/I_{830}) depends on the H-bonding status of the phenolic OH group [23, 24]. An I_{850}/I_{830} ratio of 0.3 suggests the hydroxyl group to be a strong H-bond donor, whereas a ratio of 2.5 suggests it to be a strong H-bond acceptor. An intermediate value suggests the hydroxyl group to be both an H-bond donor and acceptor. The Raman spectra recorded from the A β FY hydrogels resulted in distinct bands centred around 853 and 830 cm⁻¹ (Figure 3.4). The ratio I_{853}/I_{830} turns out to be ~0.98, indicating that the Tyr hydroxyl group acts as both H-bond donor and acceptor in the A β FY hydrogel.

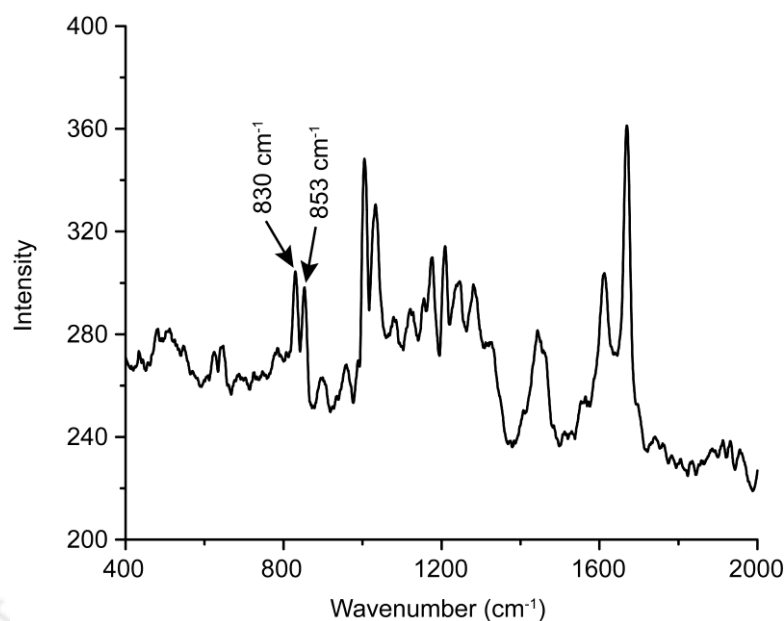


Figure 3.4 Raman scattering caused by the sample obtained from 5 mM A β FY gel. Comparable intensity of the peaks ~ 830 and 853 cm^{-1} , marked by arrows, indicate Tyr side-chain involvement in H-bonding.

3.3.4 AFM and TEM imaging

AFM imaging of the 2 mM peptide samples showed entangled fibrous structures for both the peptides (Figure 3.5, panels A and B). Fibrils formed by the two peptides are very similar. The data show that the substitution of Phe₂₀ by Tyr does not compromise the self-assembling propensity of the β -amyloid-derived peptide. Aggregation of both the peptides resulted in micrometer-long fibers. The TEM analysis, however, showed considerable difference in their width (Figure 3.5, panel C and D). A β_{16-22} fibrils turned out to be 7.6 ± 1.6 nm wide, whereas the average width of A β FY fibrils was found to be 14.3 ± 4.2 nm.

Chapter 3

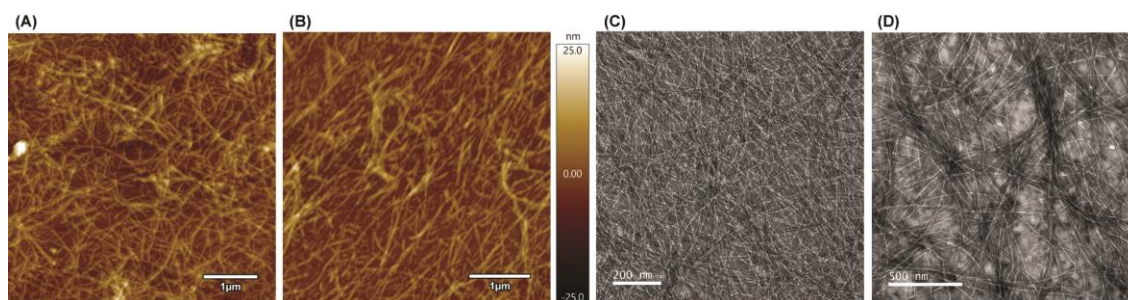


Figure 3.5 Self-assembled fibrillar structures formed by A β FF (panels A and C) and A β FY (panels B and D) as observed using AFM (panels A and B) and TEM (panels C and D).

3.3.5 ThT fluorescence spectroscopy

ThT fluorescence assay carried out in 5 mM phosphate buffer, pH 7.4 shows ~10-fold enhancement in fluorescence intensity in the presence of 20 μ M peptide. Both the peptides caused similar enhancement in fluorescence intensity (Figure 3.6). The data confirms the cross- β nature of the fibrillar structures.

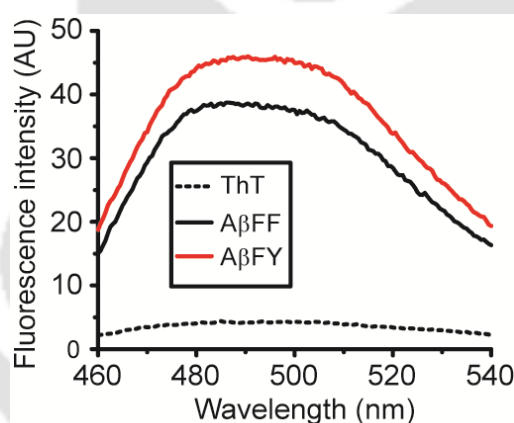


Figure 3.6 ThT fluorescence spectra for the self-assembled peptides.

3.3.6 Microrheology

As A β FY formed transparent gels, the rheological properties could readily be investigated using DLS-microrheology [16, 25]. Polyacrylamide gel (5%) was used as a reference gel material. Diffusion of spherical colloid particles is the principle underlying DLS-microrheology. For a Newtonian fluid, the mean squared displacement (MSD) of the colloid particles increases linearly over time. A non-linear increase in MSD is indicative of a viscoelastic material. MSD can be related to the viscoelastic modulus of the fluid using generalized Stokes-Einstein

relation [26]. Figure 3.8 shows the microrheology data recorded using 600 nm diameter carboxylated melamine beads.

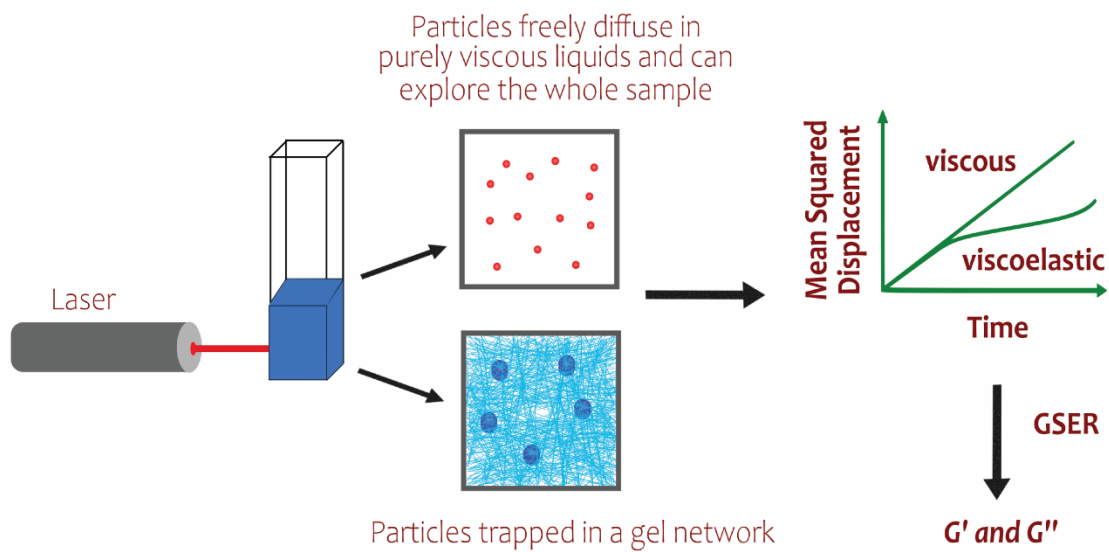


Figure 3.7 A diagrammatic representation of how DLS-microrheology works.

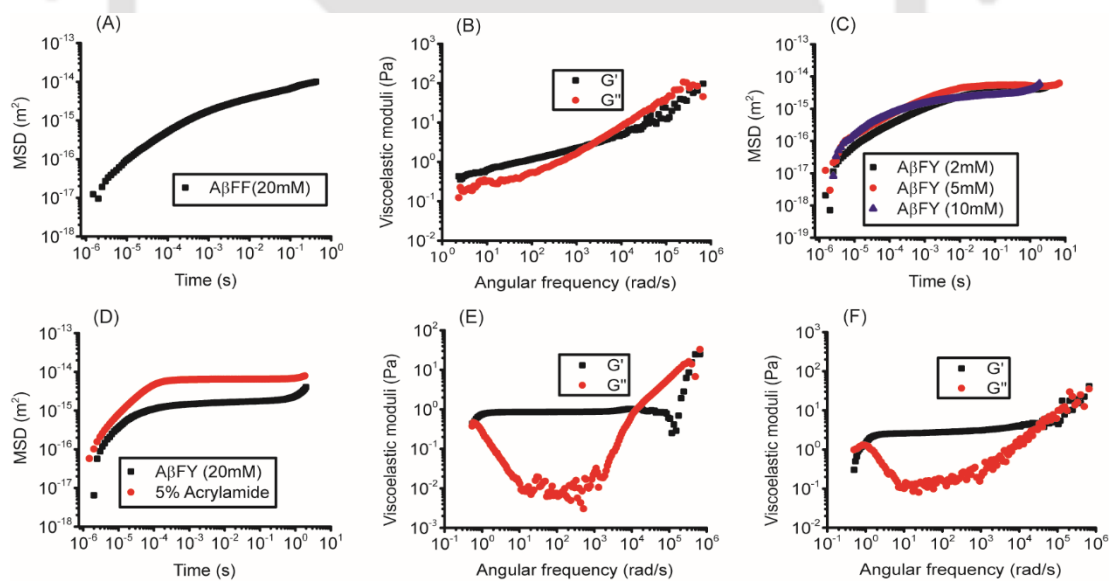


Figure 3.8 DLS-microrheology characterization. Plot of MSD against time for 20 mM AβFF (panel A). Plots of G' and G'' against angular frequency for 20 mM AβFF (panel B). Plots of MSD against time for 2 mM, 5 mM, and 10 mM AβFY gels (panel C). Plots of MSD against time for 20 mM AβFY and 5% acrylamide gels (panel D). Plots of G' and G'' against angular frequency for 5% acrylamide (panel E) and 20 mM AβFY gel (panel F).

Chapter 3

A β FF (20 mM) displays a non-linear MSD plot suggesting a viscoelastic behaviour (Panel A). Plots of storage (G') and loss (G'') moduli against frequency indicate comparable storage and loss moduli across the frequency range (Panel B). Panel C shows the MSD plots of 2, 5, and 10 mM A β FY samples. The traces indicate viscoelastic behaviour at all three concentrations. The peptide at 10 mM concentration exhibits distinct elastic behaviour. The MSD trace of 20 mM A β FY is similar to that of 5% acrylamide gel (panel D). Such MSD traces have been reported in the literature for Fmoc-Tyr hydrogels [27]. Plots of G' and G'' against angular frequency for 5% acrylamide gel (Panel E) and 20 mM A β FY (Panel F) indicate higher storage modulus than loss modulus in the 1 – 10,000 rad/s frequency range. The storage modulus for 5% acrylamide gel is ≥ 10 -fold higher than loss modulus in the 2–3000 rad/s frequency range whereas that for 20 mM A β FY is at least 3-5 times higher in the same frequency range. The storage modulus is at least one order of magnitude higher than loss modulus in the 5–500 rad/s frequency range.

The sensitivity of the gel to the changes in temperature was assessed for the 5 mM A β FY gel by monitoring the changes in microrheological properties at different temperatures (Figure 3.9). Increase in temperature causes subtle changes in MSD, which is also reflected in the viscoelastic moduli wherein G' , and G'' decrease a little. No distinct melting temperature is observed up to 55 °C. Increasing the temperature beyond 55 °C caused considerable solvent evaporation; data beyond 55 °C, therefore could not be recorded. The subsequent decrease in temperature did not cause any appreciable change in MSD and viscoelastic moduli. As viscoelastic moduli for most peptide hydrogels reported in the literature are determined using bulk rheology, comparing them with those of A β FY gel was not attempted. In fact, Frith and coworkers have shown that DLS-microrheological properties of Fmoc-Tyr hydrogel do not reproduce the bulk rheological properties [27].

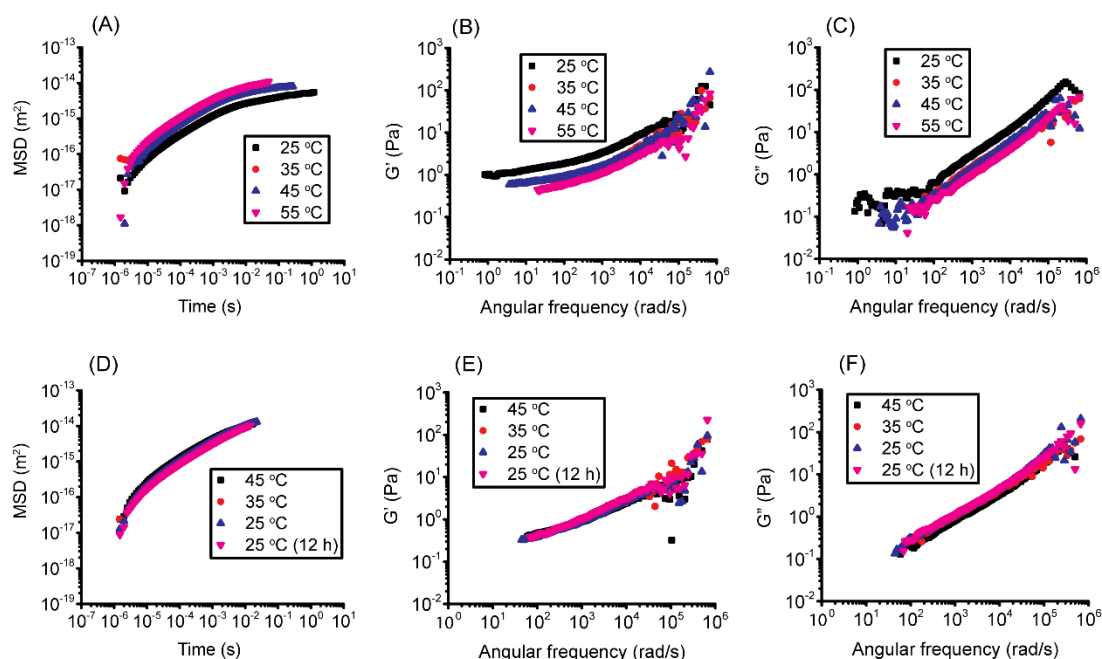


Figure 3.9 Plots of MSD against time for the 5 mM AβFY gel prepared at 25 °C at different temperatures starting from 25 °C (panel A). Panels B and C show the G' and G'' plots against angular frequency at these temperatures, respectively. Panel D shows the plots at different temperatures while decreasing the temperature from 55 °C. The label, 25 °C (12 h) in panels D-F indicate the data recorded for the sample that was incubated at 25 °C for 12 h. Panels E and F show the G' and G'' plots against angular frequency at these temperatures, respectively.

3.3.7 Doxorubicin and rhodamine B release assay

Peptide hydrogels based on the fibrillar network can trap small molecules and hold promise to be used as drug delivery vehicles. The entrapment of anticancer drug, doxorubicin in AβFY gel and subsequent release in water was studied. Doxorubicin was trapped inside the gel during the gelation process itself. The release of doxorubicin was monitored by gently adding water (19-fold volume) on the gel and measuring the absorbance at 490 nm as a function of time. Doxorubicin displayed a slow release (Figure 3.10, panel A); saturation in absorbance, an indication of equilibrium, was achieved around 150 minutes indicating a potential application of the gel as a drug delivery vehicle. Release of fluorescent dye, rhodamine B was also examined by monitoring its fluorescence as a function of time. The dye displayed a rather slow release for three days (Figure 3.10, panel B).

Chapter 3

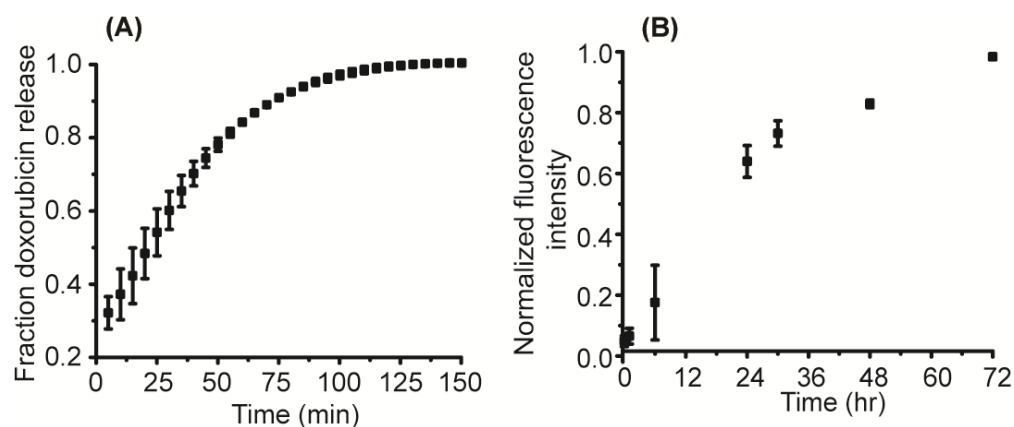


Figure 3.10 Release of doxorubicin (panel A) and rhodamine B (panel B) from 5 mM AβFY gel. Error bars indicate standard deviation for three independent experiments.

3.3.8 Cell culture, growth and insulin expression

Peptide hydrogels find much interest in regenerative medicine as they can act as scaffolds to support cell growth in 3-dimensions. AβFY gelation at 5 mM concentration was tested at 37 °C. The peptide formed a distinct transparent gel. The potential of gel as a 3D cell culture scaffold was investigated using rat pancreatic cell line RIN-5F, human embryonic kidney cells (HEK-293), and baby hamster kidney cells (BHK-21). Gels (5 mM) were prepared in the wells of a 96-well plate. The cells were seeded as described in section 3.2.13 and examined after 24 h using microscopy (Figure 3.11). All the three cells grow in 3-dimensional clumps as has been reported in the literature for 3-dimensional cell cultures [28]. Viability of the cells was assessed through calcein AM uptake. Calcein AM is a cell-permeable non-fluorescent derivative of calcein. Following cellular uptake, the ester bonds are hydrolysed by the intracellular esterases resulting in green fluorescence [29]. All the three cells display green fluorescence upon calcein AM treatment indicating that the cells are alive inside the gel (Panels D-F).

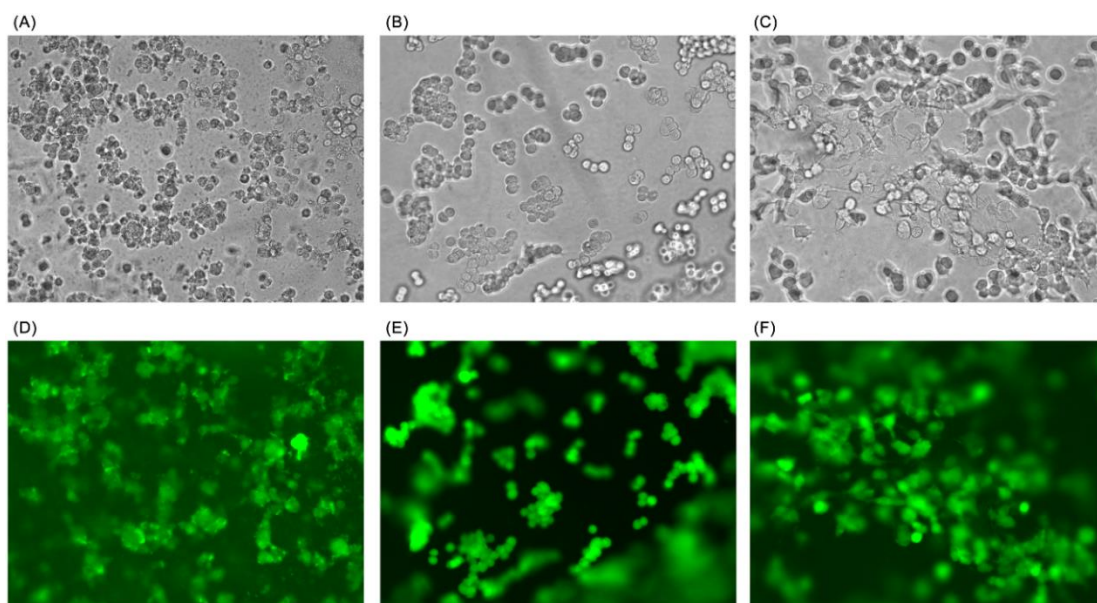


Figure 3.11 Brightfield (top panels) and calcein fluorescence (bottom panels) images of RIN-5F (panels A and D), HEK-293 (panels B and E), and BHK-21 (panels C and F) cells grown for 24 h in A β FY gel.

In addition to RIN-5F, HEK-293, and BHK-21 cells, the gel also supported the growth of human neuroblastoma cell line, IMR-32 (Figure 3.12).

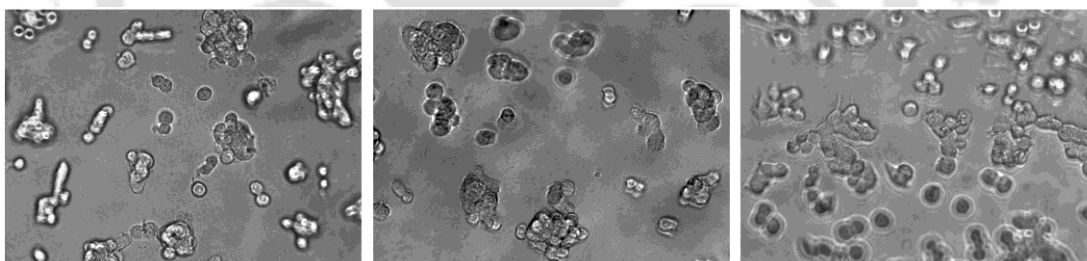


Figure 3.12 Human neuroblastoma cell line, IMR-32 grown for 24 h in 5 mM A β FY gel.

RIN-5F cells were assayed for insulin expression using PCR. The cDNA synthesized from total mRNA was amplified using rat insulin-1 primers and analysed using agarose gel electrophoresis. The cells were found to express insulin-1 suggesting that they are functional while growing in the A β FY gel (Figure 3.13). Amyloid-based hydrogels have the ability to act as matrices for 3-dimensional cell culture and tissue engineering [13, 30]. Clearly, A β FY hydrogel supports the growth and function of RIN-5F cells.

Chapter 3

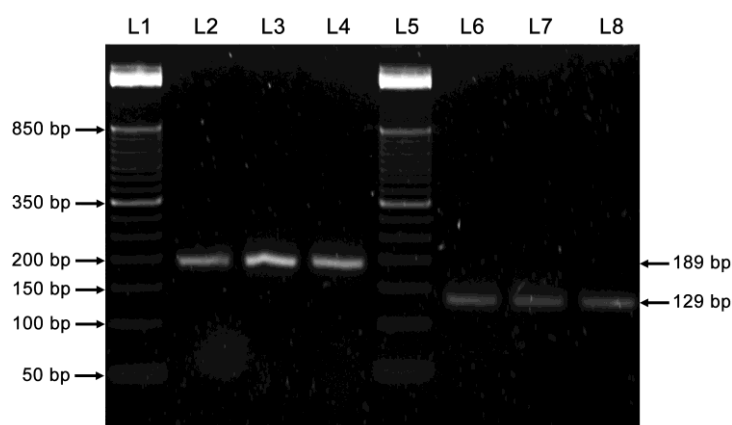


Figure 3.13 Expression of GAPDH (L2-L4) and insulin 1 (L6-L8) from 3 independent samples. L1 and L5 show the bands from the molecular weight marker. L2, L6; L3, L7; and L4, L8 correspond to the three independent samples. The bands ~189 bp in L2-L4 correspond to GAPDH while those ~129 bp in L6-L8 correspond to insulin-1.

3.4 CONCLUSIONS

Physical gels constitute an important class of soft materials. Large molecular weight biopolymers, polysaccharides, in particular, are known for long to form hydrogels. Proteins and peptides have emerged as another important class of hydrogelating biopolymers in the past two decades. Peptides make an interesting class of molecules. Physical and chemical properties that determine the structure, self-assembling propensities and the characteristics of the self-assembled structures can be modulated by suitably modifying the sequence. Most peptide-based hydrogels are characterized by β -sheet rich fibrillar structures that entangle to form a three-dimensional network similar to that of amyloid fibrils. Amyloidogenic proteins and peptides can form hydrogels [12, 31, 32]. This study presents a hydrogelating analog of the well-studied amyloidogenic fragment from β -amyloid, $A\beta_{16-22}$. The analogue, $A\beta_{FY}$, self-assembles to yield a soft gel at a concentration as low as 2 mM (~0.18% w/w) whereas $A\beta_{16-22}$ ($A\beta_{FF}$) fails to form hydrogels even at 10-fold higher concentration. Aromatic amino acids are present in various amyloidogenic peptides and are believed to play a critical role in their self-assembly through aromatic stacking interactions; Phe has received special attention [33]. Even though the fibrils formed by the two peptides are very similar, the CD spectra are

quite distinct. The difference in the secondary structure alongside the H-bonding potential of Tyr could possibly account for A β FY gelation propensity. These data indicate that Tyr could be as promising as Phe in designing small peptide-based hydrogelators. Furthermore, this study shows that the native amyloidogenic peptides hold the promise to be developed into non-toxic hydrogelators through subtle modifications.

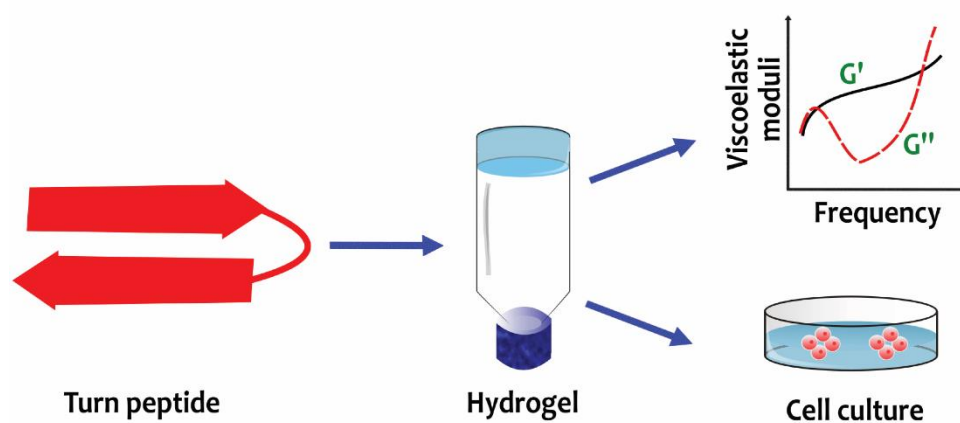
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***LIMPID HYDROGELS FROM β -TURN MOTIF-CONNECTED TANDEM
REPEATS OF $A\beta_{16-22}$ AND ITS AROMATIC ANALOGS***





4.1 INTRODUCTION

The results in chapter 2 established that the tandem repeats of A β_{16-22} and its aromatic analogs, when connected via β -turn-supporting dipeptide motifs viz. Asn-Gly, Aib-^DPro, and ^DPro-Gly turn out to be more amyloidogenic than the parent 7-residue peptides. In addition, A β FY (the Phe²⁰Tyr mutant), and the β -turn motif-harboring repeats of A β FY are, by and large, more amyloidogenic than their A β FF counterparts. Schneider and coworkers designed short peptides that self-assemble, through β -turn mediated hairpin formation, to form responsive hydrogels [1, 2]. Growing evidence suggesting amyloids as the non-toxic functional materials eased their way for technological applications [3-9]. Such functionalization has resulted in fibers with biological, catalytic, optical, electrical, and electronic properties [10-18]. Among the various soft materials, hydrogels happen to be an important class as they find applications in enzyme immobilization, drug delivery, tissue engineering, catalysis, waste treatment [5, 19-34]. Considering these reports and the hydrogels, obtained from A β FY, described in chapter 3, exploring the hydrogelating potential of the β -turn-supporting dipeptide motif-connected repeats of A β_{16-22} and its aromatic analogs described in chapter 2, seemed worthwhile.

4.2 MATERIALS AND METHODS

4.2.1 Materials

The materials used in this chapter are the same as described in section 2.2.1, chapter 2 and section 3.2.1, chapter 3.

4.2.2 Peptides

The peptides employed for the studies described in chapters 2 and 3 were used for the studies carried out for this chapter.

4.2.3 Self-assembly/hydrogelation

The peptide stock solutions were prepared in HFIP, and the concentrations estimated as described in section 2.2.3, chapter 2. For self-assembly and gelation,

Chapter 4

the peptides were diluted using water to obtain the desired peptide concentrations; final HFIP concentration in all the reactions was 10%. Gelation was examined by inverting the tubes after 12 h incubation at room temperature.

Gelation of representative peptides at acidic solutions

A β FF-NG and A β FY-NG were chosen as the representative peptides from the A β FF and A β FY turn-harboring series of peptides, respectively. Stock solutions (50 mM) was prepared in HFIP. Water, acidified to pH 2 and 4 using HCl, was added to the peptide solutions to obtain a 5 mM peptide concentration in 10% HFIP. The samples were left undisturbed for 12 h at room temperature. Gelation was ascertained by tube-inversion.

4.2.4 CD spectroscopy

The 24 h-old peptide samples were diluted in 10% HFIP and spectra recorded as described in section 3.2.4, chapter 3. The data are converted to mean residue ellipticity, as described in section 2.2.4, chapter 2.

4.2.5 FTIR spectroscopy

FTIR spectra were recorded for the 24 h-old samples as described in Chapter 3, section 3.2.5.

4.2.6 AFM imaging

AFM images for the peptide samples (incubated for 24 h) were acquired as described in Chapter 3, section 3.2.7.

4.2.7 TEM imaging

The 24 h-old peptide samples were diluted in deionized water, and samples prepared as described in section 2.2.8, chapter 2. TEM images were recorded as described in, section 3.2.8, chapter 3.

4.2.8 ThT fluorescence spectroscopy

ThT fluorescence assay was carried out for 24 h-old peptide samples exactly as described in section 3.2.9, chapter 3.

4.2.9 Mechano-responsive behaviour of A β FF-NG and A β FY-NG as the representative gels

The 5 mM gels formed by A β FF-NG and A β FY-NG were sheared using a pipette. The sheared gels were transferred to the clean empty vials and left undisturbed to observe if the sheared samples re-gel.

4.2.10 Microrheology

Microrheology measurements were carried out at 25 °C. The tracer particles in water were added to the peptide stock solutions in HFIP to obtain the final peptide concentration of 5 mM and tracer volume fraction of 0.025% and mean squared displacement (MSD) data was obtained as described in Chapter 3, section 3.2.10. The elastic (G') and viscous (G'') moduli were subsequently derived from the MSD data by the inbuilt Malvern Zetasizer instrument software based on the generalized Stokes-Einstein equation as introduced by Mason [35].

4.2.11 Doxorubicin release assay

Doxorubicin was trapped inside the 5 mM peptide gels at 0.5 mg/ml concentration during the gelation process. Water (950 μ l) was gently loaded on top of the drug-loaded gel (50 μ l), and absorbance monitored at 490 nm with respect to time as described in Chapter 3, section 3.2.11.

4.2.12 Cell seeding and cultivation

Rat pancreatic cell line (RIN-5F) and human embryonic kidney cells (HEK-293) were seeded and cultured in T25 culture flasks containing RPMI 1640 and DMEM media, respectively, as described in section 3.2.13, chapter 3. A β FF-NG and A β FY-NG gels (2 mM peptide concentration) were prepared in a 96-well plate at 37 °C in 5% CO₂. RPMI or DMEM media (2-fold volume) was gently added to the gels

Chapter 4

obtained after 12 h incubation and carefully decanted after 60 minutes; this step was repeated once more. Approximately 2×10^5 cells were then seeded on the gel and incubated at 37 °C in 5% CO₂.

4.2.13 Cell viability assay

RIN-5F and HEK-293 cells grown in the gels for 24 h were washed with PBS, treated with 5 μ M calcein AM dye and visualized as described in section 3.2.14, chapter 3.

4.2.14 Insulin gene expression assay

Expression of insulin-1 gene by the RIN-5F cells grown in hydrogels along with GAPDH serving as an internal control was studied as described in section 3.2.15, chapter 3. The same sets of primers were used for PCR amplification (Table 3.1, chapter 3).

4.3 RESULTS AND DISCUSSION

The β -turn-motif harboring peptides turned out to be poorly soluble in water and ethanol. As the peptides dissolved to a very high concentration in HFIP; the self-assembly/hydrogelation was investigated in 10% HFIP. The peptides were dissolved in HFIP; water was subsequently added to achieve the desired peptide concentration such that the final HFIP concentration is 10%.

The tubes were left undisturbed at room temperature and the gelation examined by inverting the tubes after 12 h of incubation (Figure 4.1). All three A β ₁₆₋₂₂ β -turn analogs viz. A β FF-NG, A β FF-pG, and A β FF-Up resulted in self-supporting hydrogels at 2 mM ($\sim$ 0.4 wt%) peptide concentration (Panels A-C). A β FY-NG also resulted in hydrogels at 2 mM peptide concentration (Panel D). A β FY-pG and A β FY-Up, on the other hand, failed to cause gelation at 2 mM concentration; at higher concentration (5 mM, $\sim$ 1 wt%), however, both the peptides resulted in clear gels (Panels E and F). All six peptides resulted in limp hydrogels at 5 mM peptide concentration. A β YY-NG formed gel at 5 mM concentration (Panel G), whereas A β YY-pG (Panel H) and A β YY-Up (Panel I) formed partial gels at 5 mM

concentration. A β WW β -turn motif-harboring peptides failed to cause gelation up to 5 mM concentration. Studies with A β YY and A β WW peptides were not carried out any further.

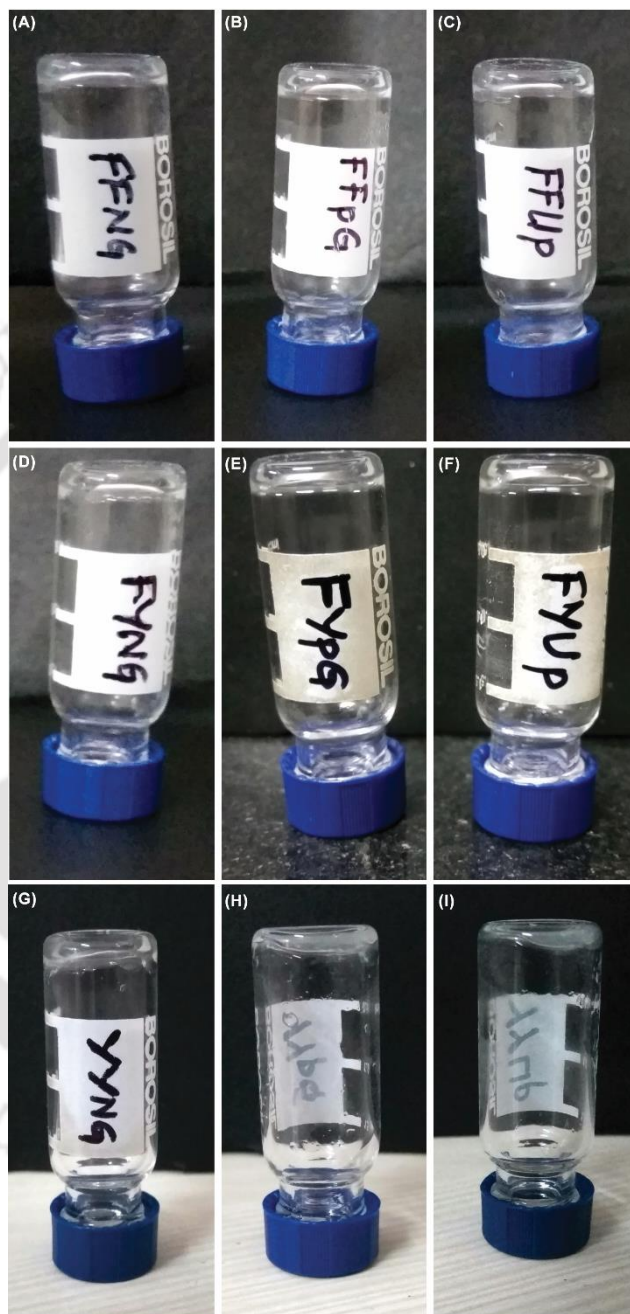


Figure 4.1 Images of the inverted tubes showing self-supporting gels. Panels A, B, C, and D represent the gels formed by A β FF-NG, A β FF-pG, A β FF-Up, and A β FY-NG, respectively at 2 mM peptide concentration. Panels E and F represent the gels formed by A β FY-pG and A β FY-Up, respectively at 5 mM peptide concentration. Panels G, H, and I represent gels formed by A β YY-NG, A β YY-pG (partial gel), and A β YY-Up (partial gel) respectively at 5 mM peptide concentration.

Chapter 4

Gelation of A β FF-NG and A β FY-NG at acidic solutions

The gelation at acidic pH was investigated using two representative peptides A β FF-NG and A β FY-NG at 5 mM peptide concentration. Both the peptides resulted in transparent self-supporting gels at both pH 2 and 4 (Figure 4.2). These gels were not investigated further.

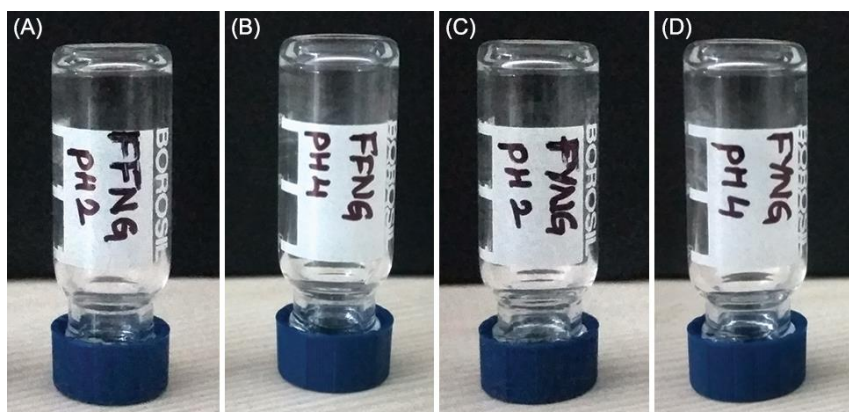


Figure 4.2 Hydrogelation by A β FF-NG and A β FY-NG at acidic pH. A β FF-NG at pH 2 (A) and 4 (B); A β FY-NG at pH 2 (C) and 4 (D).

Structural characterization of the hydrogels was carried out using CD spectroscopy, FTIR spectroscopy, ThT fluorescence spectroscopy, AFM, and TEM.

4.3.1 CD and FTIR spectroscopy

Secondary structure of the peptides in the hydrogels was examined using far-UV CD spectroscopy (Figure 4.3). The peptides display a negative band centered around 215–218 nm with a positive band near 190 nm suggesting distinct β -sheet conformation. Far-UV CD spectra can have a significant contribution from the aromatic side-chains if the peptide's aromatic content is high or the peptide folds to form aromatic clusters [36, 37]. As the peptides are designed to fold into β -hairpins that places aromatic residues in the β -sheet core, the secondary structure was further characterized using ATR-FTIR spectroscopy of the dried peptide films (Figure 4.4). Amide I band given by proteins/peptides largely arises from carbonyl stretching vibration. The transition is sensitive to the backbone conformation, thereby allowing secondary structure determination. Amide I

band for all 6 peptides appeared around 1624 cm^{-1} supporting β -sheet conformation.

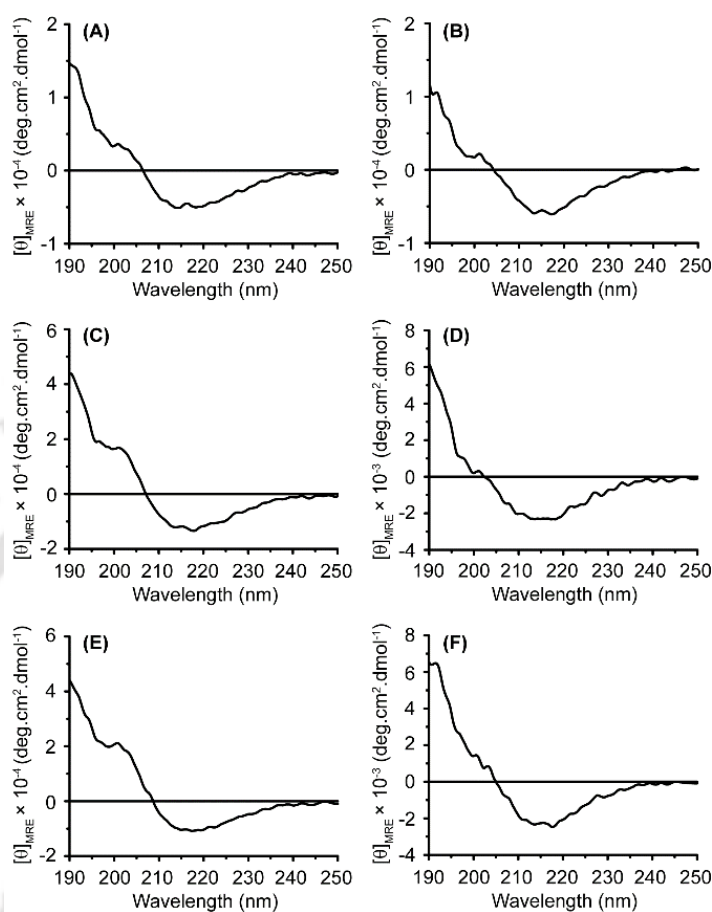


Figure 4.3 Far-UV CD spectra recorded for A β FF-NG (A), A β FY-NG (B), A β FF-pG (C), A β FY-pG (D), A β FF-Up (E), and A β FY-Up (F).

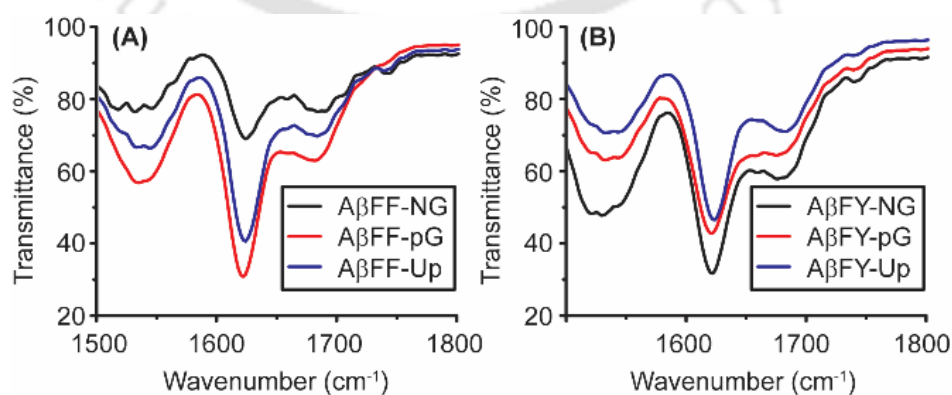


Figure 4.4 ATR-FTIR spectra of A β FF-NG, A β FF-pG, A β FF-Up (A) and A β FY-NG, A β FY-pG, and A β FY-Up (B).

Chapter 4

4.3.2 AFM and TEM imaging

Figure 4.5 shows the morphology of the self-assembled peptide structures as determined using AFM. The peptides, predictably, formed amyloid-like fibrous aggregates. Networks of such fibrillar structures are known to cause hydrogelation by fixing water molecules.

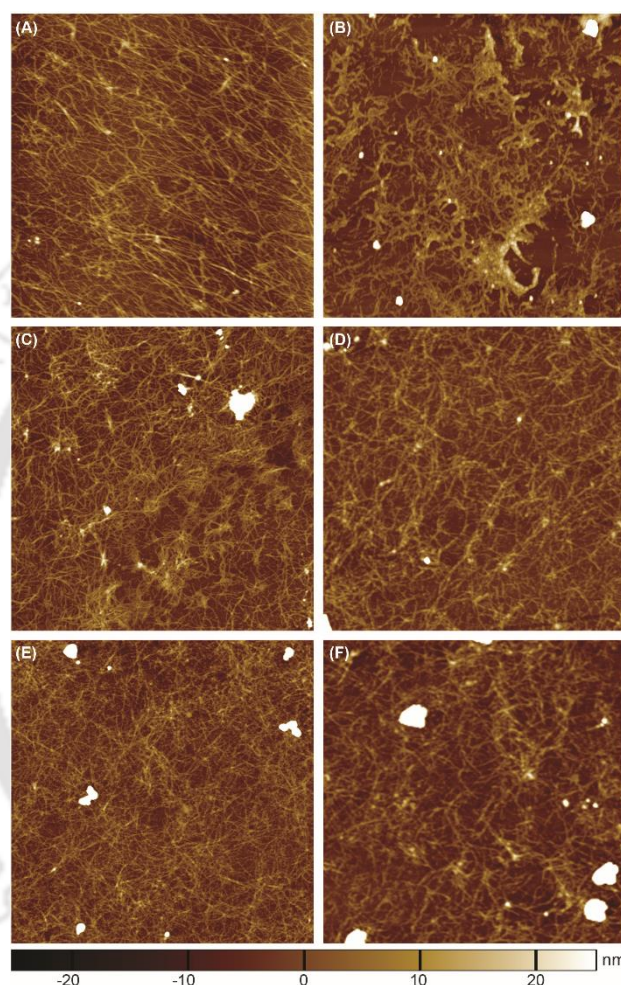


Figure 4.5 AFM images of the self-assembled structures that underlie hydrogel formation. A β FF-NG (A), A β FF-pG (B), A β FF-Up (C), A β FY-NG (D), A β FY-pG (E), and A β FY-Up (F). Each panel represents 10 μ m $\times$ 10 μ m scan area.

The fibrous structures were characterized by TEM as well (Figure 4.6). It is interesting to note that A β_{16-22} , though amyloidogenic by itself, failed to cause hydrogelation (Chapter 3), repeats connected via β -turn-supporting motifs turned out to be promising hydrogelators.

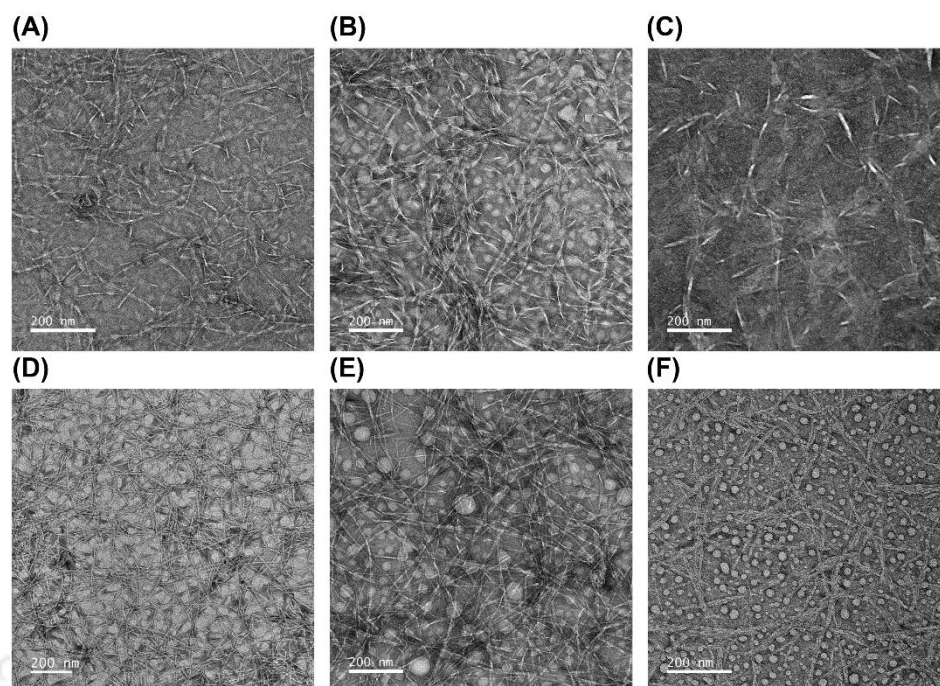


Figure 4.6 TEM images of A β FF-NG (A), A β FF-pG (B), A β FF-Up (C), A β FY-NG (D), A β FY-pG (E), and A β FY-Up (F) gel samples.

4.3.3 ThT fluorescence spectroscopy

As the gels are made up of amyloid-like fibrils, they were characterized by monitoring the fluorescence of ThT, a benzothiazole dye that exhibits higher quantum yield when bound to amyloid fibrils. The hydrogel fibrils caused very large enhancement in ThT fluorescence (Figure 4.7).

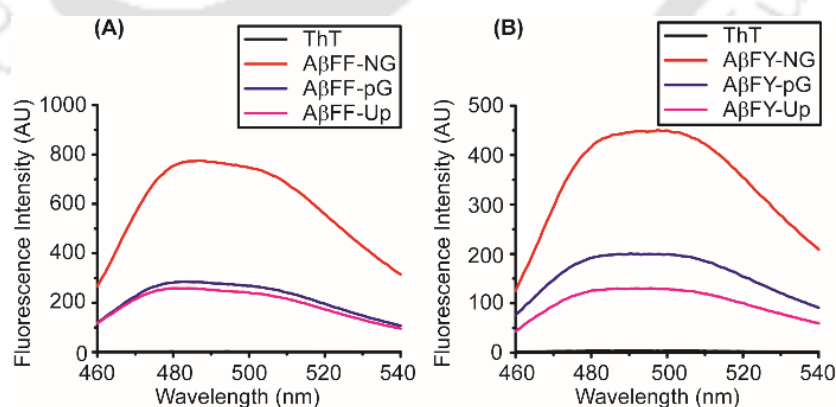


Figure 4.7 ThT fluorescence spectra recorded for the sheared hydrogels; A β FF-turn-based peptides (A), and A β FY-turn-based peptides (B).

Chapter 4

The A β FF-turn-based peptides, by and large, caused higher enhancement compared to the A β FY-turn-based peptides. In addition, the peptides harboring Asn-Gly as the turn-supporting motif caused at least 2-fold higher enhancement compared to the peptides harboring ^DPro-Gly and Aib-^DPro as the turn-supporting motifs. Asn-Gly mediates a flexible turn compared to the rather tight turns mediated by ^DPro-Gly and Aib-^DPro. A flexible turn would provide conformational flexibility to the two strands; such flexibility might be important for optimal packing of the intra- and inter-sheet side chains.

4.3.4 Mechano-responsive behaviour of A β FF-NG and A β FY-NG gels

The mechano-responsive behaviour was studied using A β FF-NG and A β FY-NG as the representative gels [38]. The gels exhibited shear-thinning when pulled out using a pipette. Overnight standing of the sheared samples resulted in gels suggesting reversibility (Figure 4.8).

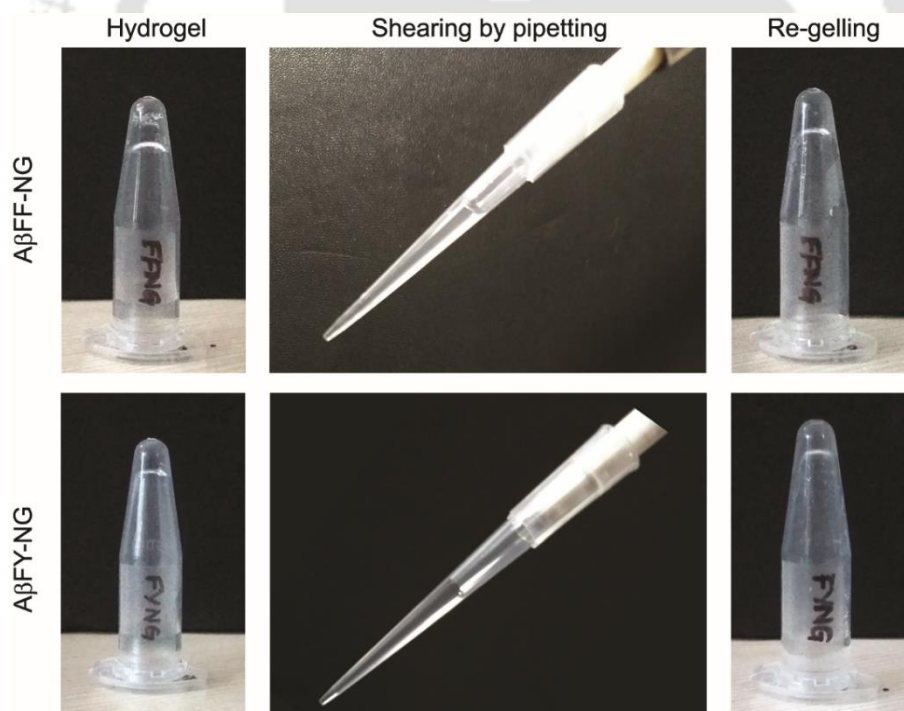


Figure 4.8 Mechano-responsive behaviour of the 5 mM A β FF-NG and A β FY-NG gels. The shear-thinned samples form gels upon 12 h standing.

4.3.5 Microrheology

The viscoelastic properties of the 5 mM gels were examined by DLS-microrheology measurements using 600 nm carboxy-melamine tracer particles. The MSD traces obtained for the peptide hydrogels are shown in figure 4.9 (panels A and E). The tracer particles display non-linear displacement indicating distinct viscoelastic properties. The complex shear modulus, $G^*(\omega) = G'(\omega) + iG''(\omega)$ is obtained from the MSD data using the generalized Stokes-Einstein relation. It is important to note that DLS-microrheology makes it possible to investigate the rheological properties at very high frequencies; frequencies higher than a few hundred Hz are otherwise inaccessible using bulk rheology [39].

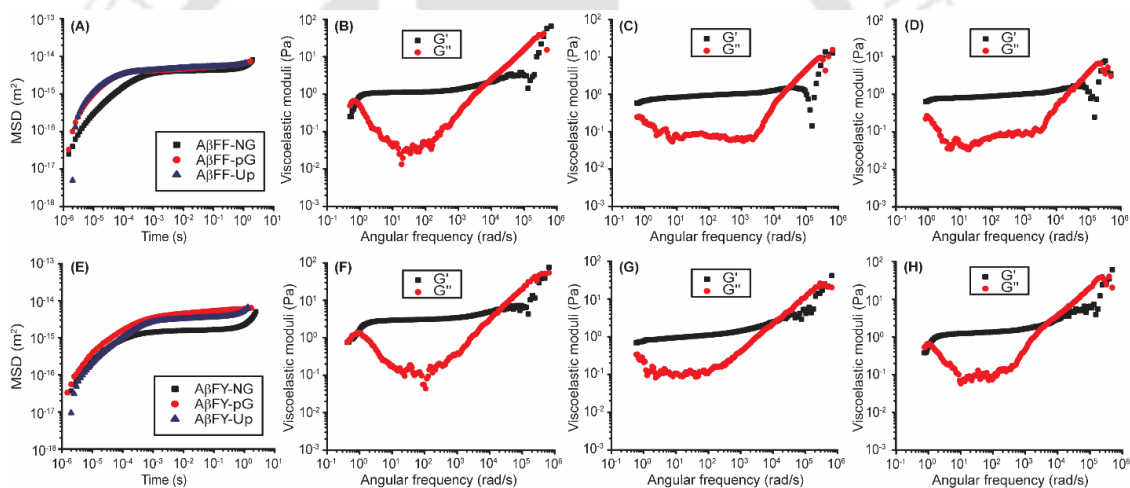


Figure 4.9 DLS-microrheology data recorded for 5 mM hydrogels. Panels A and E show the MSD data for the AβFF and AβFY-turn-based peptides, respectively. The G' (storage modulus, black squares) and G'' (loss modulus, red circles) data, as determined from the MSD data using the generalized Stokes-Einstein relation; AβFF-NG (B), AβFF-pG (C), AβFF-Up (D), AβFY-NG (F), AβFY-pG (G), and AβFY-Up (H).

The storage and loss moduli obtained from MSD data are shown in panels B-D and F-H. All six peptides display distinct elastic properties; storage moduli are higher than the loss moduli in at least 2–2000 rad/s angular frequency range. AβFF-pG, AβFF-Up, AβFY-NG, and AβFY-pG display storage moduli higher than loss moduli in the 1–10⁴ rad/s range.

Chapter 4

4.3.6 Doxorubicin release assay

Peptide-based hydrogels are promising drug-delivery vehicles [34]. The gels can trap the drug molecules that can be released at the target site in a sustained manner. In order to investigate if the gels obtained from the β -turn motif-harboring amyloidogenic peptides can trap and show the sustained release of the drug, the release of the anticancer drug, doxorubicin from the hydrogels was examined. Figure 4.10 shows the release of doxorubicin (trapped at 0.5 mM concentration during gelation) from 5 mM A β FF-NG (panel A), A β FF-pG (panel B), A β FF-Up (panel C) and A β FY-NG (panel D) gels. All four gels show the sustained release of doxorubicin for around 2.5 hours.

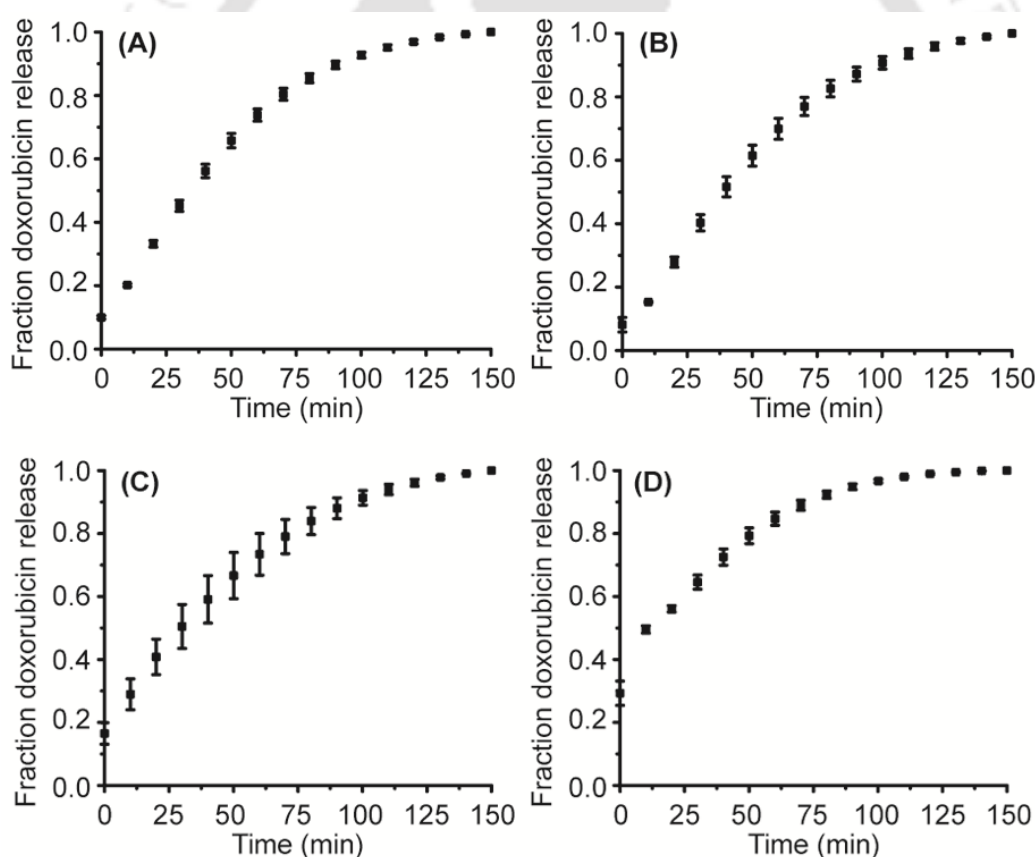


Figure 4.10 Doxorubicin release kinetics from A β FF-NG (A), A β FF-pG (B), A β FF-Up (C), and A β FY-NG (D) gels as determined by monitoring the absorbance at 490 nm.

Using A β FF-NG (5 mM peptide concentration) as the representative peptide, the gelation was studied in the presence of higher doxorubicin concentration (5

mM). The peptide gelation was unaffected by the high doxorubicin concentration and doxorubicin up to at least 5 mM concentration could be trapped by the gel.

In order to verify the drug entrapment, the experiment for drug release was repeated, after washing the A β FF-NG gel four times with water (each washing with a volume equal to the gel volume). The washings were performed within 1 minute, and doxorubicin release kinetics recorded (Figure 4.11, panel A). The release kinetics was very similar to that from the previously shown unwashed gel. In fact, no doxorubicin could be detected in the water used for washing the gel by the naked eye. The entrapment of the drug in the gel was also evident from the picture of gel (Figure 4.11, panel B).

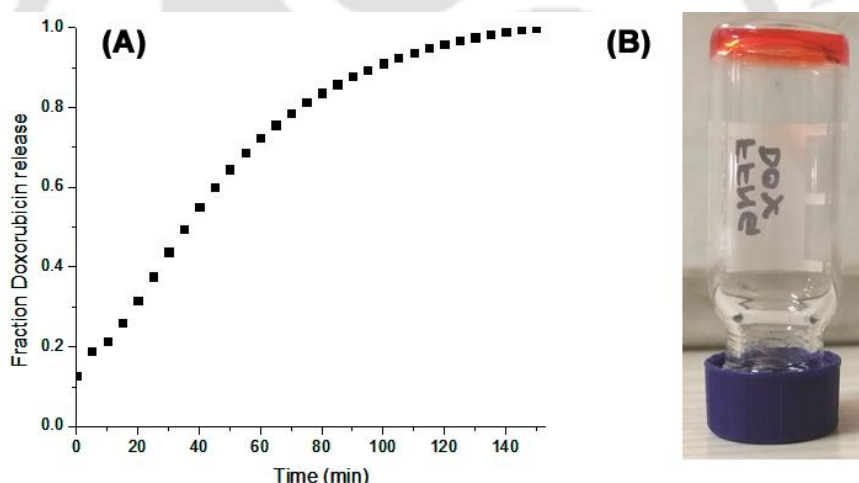


Figure 4.11 Doxorubicin release kinetics from A β FF-NG gel recorded after washing (A), and entrapped drug in the gel (B).

4.3.7 Cell culture, growth, and insulin expression

Supporting cell-growth in 3-dimensions is yet another biomedical application of peptide-based hydrogels. In fact, many peptide-based hydrogels have been commercialized for 3-D cell cultures. The potential of the hydrogels described in this study to support the growth and function of mammalian cells was also investigated. Peptides harboring Asn-Gly β -turn motif, *i.e.* A β FF-NG and A β FY-NG, were chosen as the representative peptides for cell culture studies. Human

Chapter 4

embryonic kidney cells, HEK-293 and rat pancreatic cells, RIN-5F were seeded on the 2 mM peptide gels prepared in 96-well plate as described in the section 4.2.12. The cells grow in the hydrogel matrix as ascertained by the uptake of calcein AM. Ester hydrolysis caused by the cellular esterases results in green fluorescence indicating cell viability. Both HEK-293 and RIN-5F cells grow in clumps, and yield green fluorescence upon calcein AM treatment (Figure 4.12).

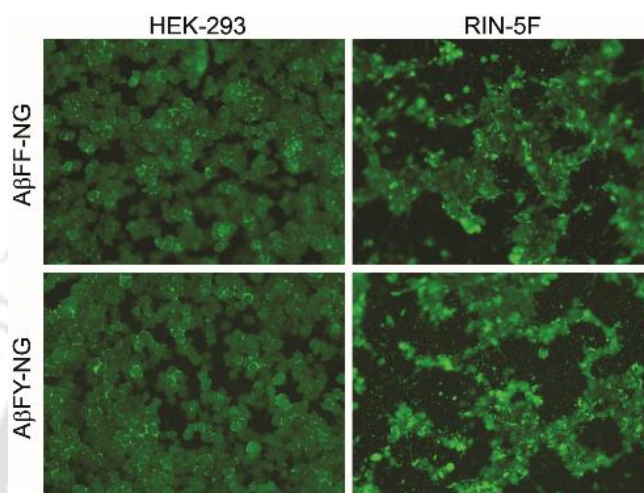


Figure 4.12 Fluorescence images of the calcein AM treated HEK-293 (left panels) and RIN-5F (right panels) cells grown for 24 h in peptide hydrogels.

As RIN-5F is a pancreatic cell line, expression of insulin-1 gene by the cells grown in the gels was examined. Total mRNA isolated from the RIN-5F cells grown in the hydrogels for 24 h was converted to cDNA and amplified by PCR using insulin-1 and GAPDH primers. The cells growing in the gels were found to be expressing the housekeeping gene GAPDH as well as insulin-1 (Figure 4.13). These data indicate that the peptide hydrogels reported here can serve as a scaffold for 3-D cell culture.

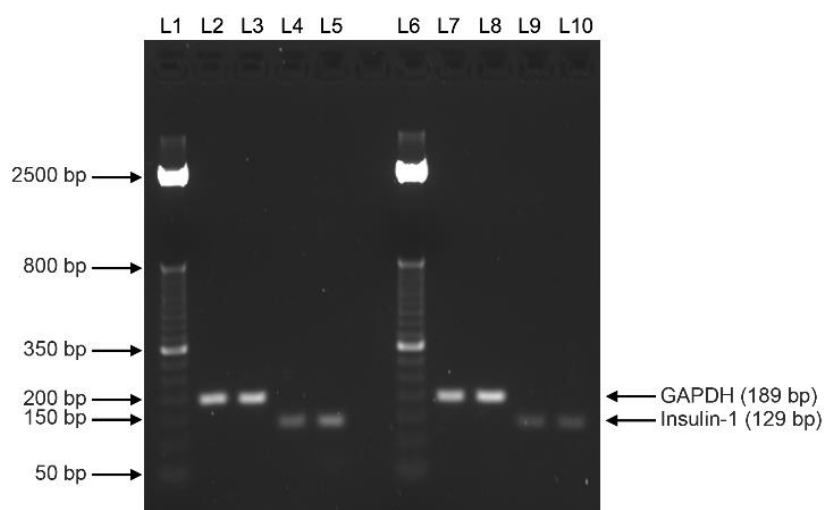


Figure 4.13 Agarose gel electrophoretic analysis of GAPDH and insulin-1 mRNA amplification from RIN-5F cells grown for 24 h in A β FF-NG (lanes 2-5) and A β FY-NG (lanes 7-10) hydrogels. Lanes 2, 3, 7, and 8 correspond to GAPDH PCR amplified product while lanes 4, 5, 9, and 10 correspond to insulin-1 amplification product. Lanes 1 and 6 represent size markers.

Schneider and coworkers have designed a series of short peptides, named MAX peptides that harbor a $^D\text{Pro}^L\text{Pro}$ dipeptide motif the middle of the peptide sequence [1, 2, 19]. $^D\text{Pro}^L\text{Pro}$ is a well-established type-II' β -turn-inducing dipeptide motif. The designed peptides fold into the β -hairpin structures mediated by the predicted type-II' β -turn triggering self-assembly and consequently hydrogelation. Our results presented in this study show that β -turn can have a marked influence on the hydrogelating propensity of the peptides. The otherwise, non-hydrogelating peptide A β_{16-22} becomes a potent hydrogelator when the two strands are connected via a turn-supporting motif. Results with the A β FY-based peptides indicate the preference of Asn-Gly as the β -turn motif over Aib- ^DPro and ^DPro -Gly. It would, therefore, be interesting to see the effects of β -turn-inducing motifs other than $^D\text{Pro}^L\text{Pro}$ for the MAX series of peptides as well.

4.4 CONCLUSIONS

In this chapter, an attempt has been made to explore the hydrogelating propensity of the tandem repeats of A β_{16-22} (A β FF) and its aromatic analogs A β FY, A β YY, and A β WW when connected by β -turn-supporting motifs. Aib-

Chapter 4

^DPro and ^DPro-Gly are well-established dipeptide motifs that nucleate tight type I' and type II' β -turns, respectively. Asn-Gly, on the other hand, supports type I' β -turn but is not an obligatory type I' turn forming motif. Irrespective of the type of turn-inducing motif, the A β FF repeats resulted in limp hydrogels at concentrations as low as 2 mM. The structures underlying hydrogelation were found to be amyloid-like fibrils. Even though CD, FTIR, and AFM suggested no marked difference between the fibrils formed by A β FF-NG, A β FF-pG, and A β FFUp; ThT fluorescence was at least two-fold higher for A β FF-NG fibrils compared to A β FF-pG and A β FF-Up. As described in chapter 3, the A β FY peptide forms transparent hydrogels at concentration ≥ 2 mM. When the A β FY repeats are connected via Asn-Gly motif, the peptide resulted in hydrogels at 2 mM concentration. Interestingly, however, repeats connected via ^DPro-Gly and Aib-^DPro motifs failed to cause hydrogelation under these conditions. The peptides resulted in hydrogels at 5 mM concentration. As is the case with the A β FF β -turn peptides, the Asn-Gly connected A β FY caused at least 2-fold higher enhancement in ThT fluorescence compared to the repeats connected via ^DPro-Gly and Aib-^DPro motifs. An Asn-Gly dipeptide motif provides higher conformational flexibility in the turn region compared to ^DPro-Gly and Aib-^DPro. The MD simulations of the β -turn supporting motif-harboring peptides have been discussed in section 2.3.3, chapter 2. Even though a 100 ns simulation may not be sufficient for sampling the entire conformational space of a peptide, hairpin-like were obtained for all the A β FF and A β FY series of peptides within 100 ns. Such rapid folding can be attributed, alongside the turn inducing motifs, to the self-assembling propensity of the strands. Interestingly, however, the major cluster for A β FY-NG was a type II turn instead of type I'. A β FF-NG, A β FF-pG does fold into a hairpin structure, but the hairpin is not mediated by a classical β -turn.

The data presented in this chapter show that the turn-supporting motifs can play deciding role on the emergent properties of self-assembled structures; the otherwise non-hydrogelating peptide A β_{16-22} becomes a potent hydrogelator when the two strands are connected via a turn-supporting motif. Results with

the A β FY-based peptides indicate a preference of Asn-Gly as the β -turn motif over Aib-^DPro and ^DPro-Gly for gelation. A β FY-NG causes gelation at 2 mM peptide concentration, whereas A β FY-pG and A β FY-Up fail to cause gelation at this concentration. It appears that the bulky Tyr side chain might be difficult to pack, thereby affecting assembly and consequently gelation at lower concentrations. This is further supported by the fact that A β FY-NG displays higher G''/G' ratio than that displayed by A β FF-NG, suggesting that A β FF-NG makes a gel with higher elasticity.

To conclude, the β -turn motif-connected repeats of A β FF and A β FY form transparent hydrogels. These gels are mechano-responsive, i.e. display shear-thinning upon mechanical shearing and re-gel upon standing. Like various other peptide-based hydrogels reported in the literature, these hydrogels show continual drug release and also act as scaffolds for mammalian cell culture.

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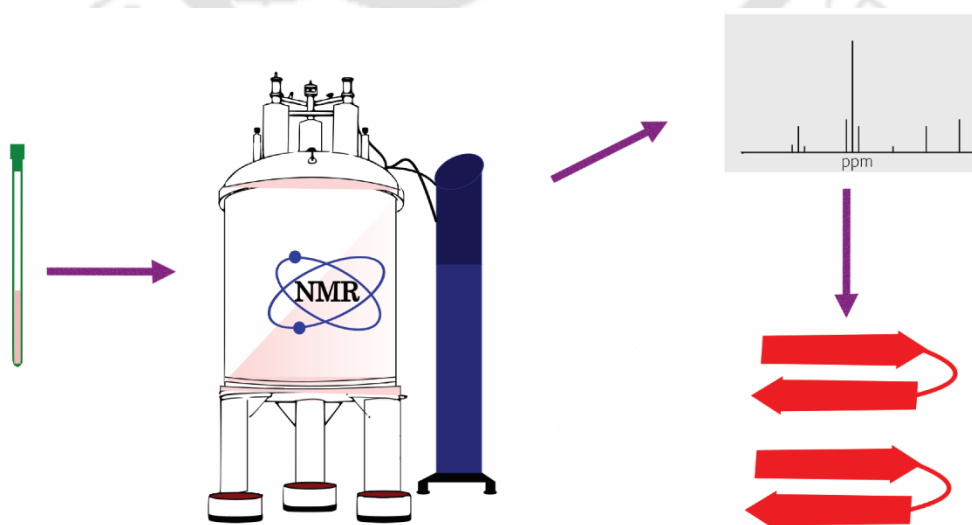
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**INVESTIGATION INTO THE β -TURN-SUPPORTING MOTIFS USING
PROTON NMR**





5.1 INTRODUCTION

The results described in chapter 2, and 4 show that the β -turn supporting motif-connected tandem repeats of $A\beta_{16-22}$ and its aromatic analogs display self-assembling propensities very different from the parent peptides. When dipeptide sequences such as NG, Up, and pG, that have the propensities to mediate β -turn, were introduced to obtain $A\beta FF(NG/pG/Up)A\beta FF$ peptides as well as those of $A\beta FY$, $A\beta YY$ and $A\beta WW$, the ability to form fibrils was evident. The turn analogs are, by and large, more amyloidogenic than the parent peptides. Besides, the turn-supporting motifs rendered the peptides hydrogelating. The folding of the turn-harboring peptides in water using MD simulations was investigated, the results of which have been described in chapter 2.

Short peptides, judiciously engineered, have the ability to form β -hairpin structures in solutions, details of which have emerged from NMR studies [1-7]. The conformation of β -hairpin-forming peptides has been examined in organic solvents such as $CHCl_3$, MeOH, DMSO, and in aqueous medium [1-12]. It emerges from these studies that the dipeptide sequences viz. Asn-Gly, D Pro-Gly and Aib- D Pro favour β -turns and β -hairpin structures. Though short peptides do not generally adopt folded conformations in DMSO solutions, β -turns and β -hairpin structures have been observed in DMSO too [1-7]. In an effort to examine whether the $A\beta_{16-22}$ -derived peptides adopt folded conformation, we have carried out NMR investigation in DMSO and aqueous media. The turn motif-harboring peptides were insoluble in solvents such as water, $CDCl_3$, and CD_3OH at concentrations required to record NMR spectra. The control peptides, however, were soluble in water and $1D$ proton NMR spectra for these peptides were recorded in water as well to compare with those recorded in DMSO.

5.2 MATERIALS AND METHODS

5.2.1 Materials

DMSO- d_6 and D_2O were purchased from Sigma-Aldrich Chemicals Pvt. Ltd. Deionized water was used for all the experiments.

Chapter 5

5.2.2 Peptides

The peptides employed for the studies described in chapters 2 and 3 were used for the studies carried out for this chapter. All the peptides were fully characterized by HPLC and mass spectrometry, as described in Chapter 2. They were homogenous with a minimum 95% purity.

5.2.3 Nuclear Magnetic Resonance (NMR) Spectroscopy

All NMR experiments were recorded on a Bruker ASCEND 600 MHz NMR at the Central Instruments Facility, IIT Guwahati using the TCI600S3 cryoprobe. ^1H spectra were recorded for the control peptides A β FF, A β FY, A β YY, and A β WW in 2% D_2O . As the β -turn containing peptides did not dissolve in D_2O , the spectra could not be recorded in water. NMR samples were prepared by dissolving 1 mg peptide in 600 μl of 2% D_2O (for native control peptides) or DMSO-d_6 (for all the peptides). ^1H NMR spectra were recorded using *zgesgp* pulse program (water suppression using excitation sculpting with gradients) (Figure 5.1A) [13]. The sample was locked with the solvent deuterium signal, followed by tuning and match of the probehead. The shimming was optimized and the conventional ^1H spectra recorded. The frequency of the water signal to be suppressed was set for recording the water-suppressed ^1H spectra. Each ^1H spectrum was recorded with 16 scans. The ^1H - ^1H homonuclear TOCSY experiment was recorded for the turn-containing samples in DMSO-d_6 using the *mlevesgp* pulse program (homonuclear Hartman-Hahn transfer using MLEV17 sequence for mixing and using two power levels for excitation and spinlock) (Figure 5.1B) [13, 14]. The spectra were recorded in phase-sensitive modes using the States-TPPI method with 2048 and 256 data points collected in F_2 and F_1 dimensions for all β -turn motif-containing peptides except for A β FF-pG where points collected in F_2 and F_1 were 2048 and 512, respectively. The mixing time for each experiment was 80 ms. NOESY spectra were recorded using *noesyegpph* (2D homonuclear correlation via dipolar coupling) pulse program in phase-sensitive mode with a 350 ms mixing time. Data points collected in F_2 , and F_1 dimensions were 4096

and 256, respectively. All the spectra were processed using the Topspin v4.0.7 (academic license) software.

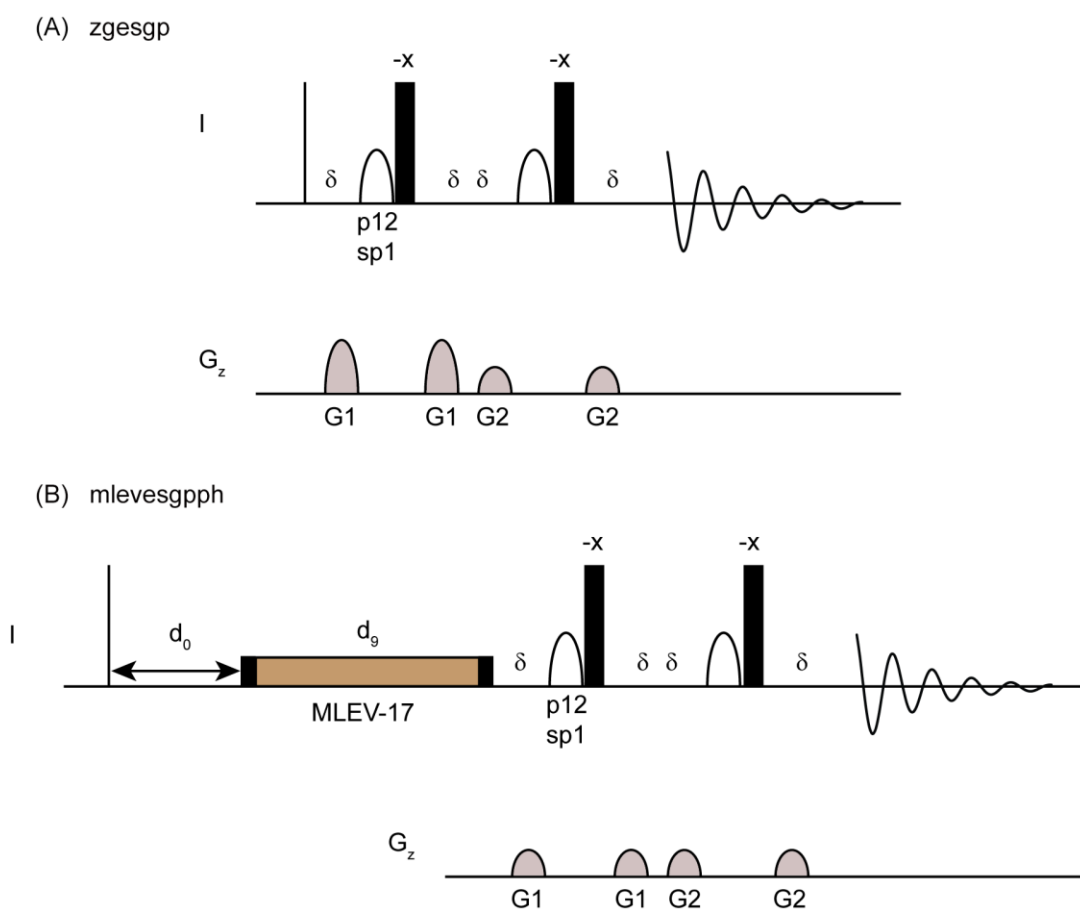


Figure 5.1 The pulse programs used for recording the water-suppressed 1D proton NMR (panel A) and the 2D TOCSY spectra (panel B).

5.3 RESULTS AND DISCUSSION

The 1D proton NMR spectra recorded in DMSO- d_6 for the control 7-residue peptides and their turn motif-connected tandem repeats are shown in figures 5.2 – 5.5. The spectra have been expanded to identify the differences in the aliphatic, C_α , aromatic, and the amide regions.

5.3.1 A β FF and its turn analogs

The spectra in figure 5.2A show that the methyl resonances of Leu and Val are dispersed in the turn peptides as compared to A β FF. This is more pronounced in the A β FF-pG and A β FF-Up containing peptides. The two Ala methyl resonances ~ 1.2 ppm are also resolved. Dispersion of the C_α resonances is observed (Figure

Chapter 5

5.2B). The aromatic protons of Phe show complexity as compared to A β FF (Figure 5.2C). Dispersion is also observed for the H_N protons (Figure 5.2D). Although dispersed, the amide protons in A β FF-Up peptide are relatively broader and less resolved, suggesting aggregation. These observations suggest that the turn peptides are not unordered and populate folded conformations.

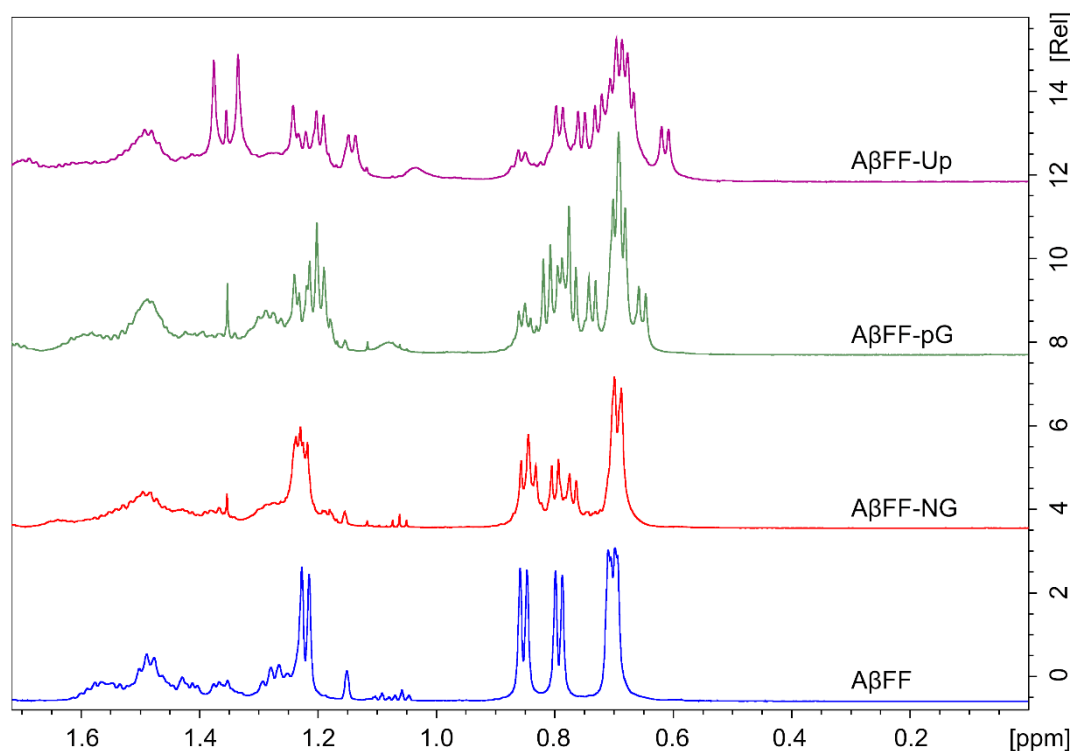


Figure 5.2A The aliphatic region of the proton NMR spectra of A β FF and its turn-connected repeats in DMSO- d_6 .

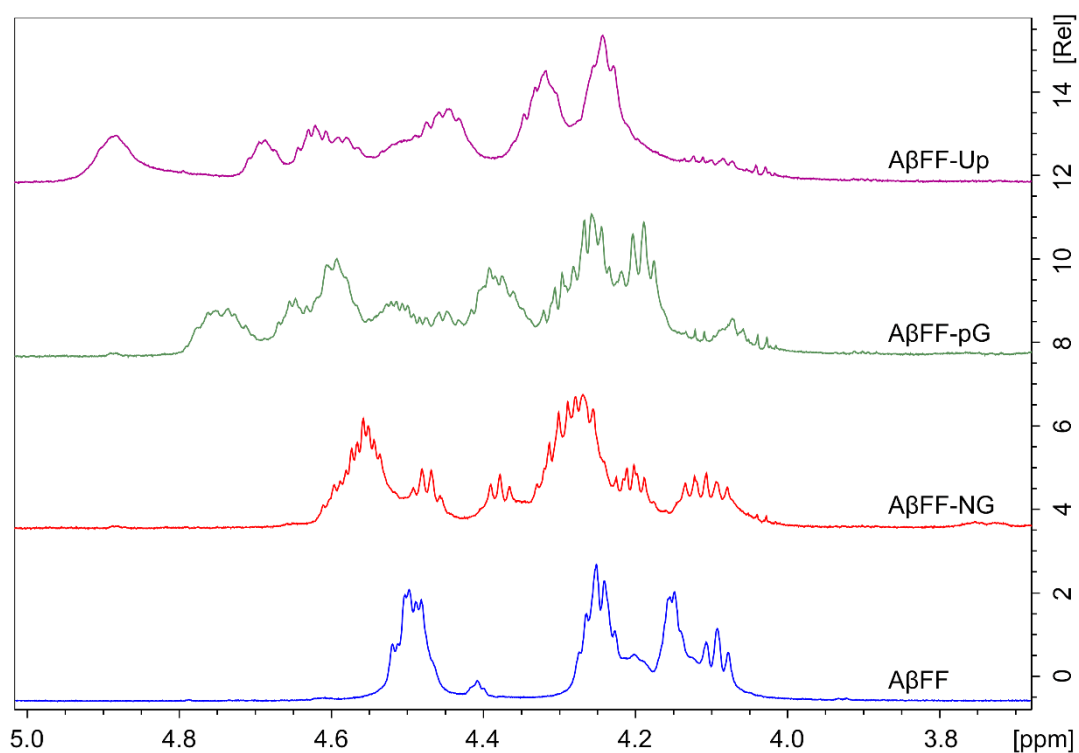


Figure 5.2B The C_{α} region of the proton NMR spectra of A β FF and its turn-connected repeats in DMSO- d_6 .

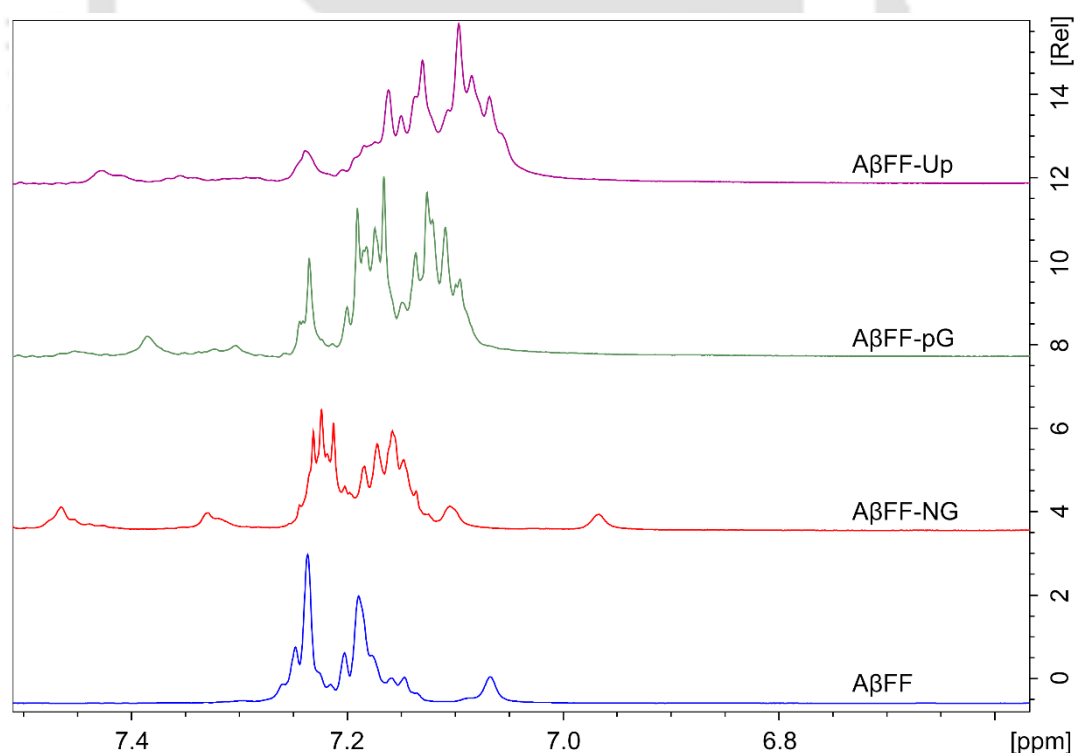


Figure 5.2C The aromatic region of the proton NMR spectra of A β FF and its turn-connected repeats in DMSO- d_6 .

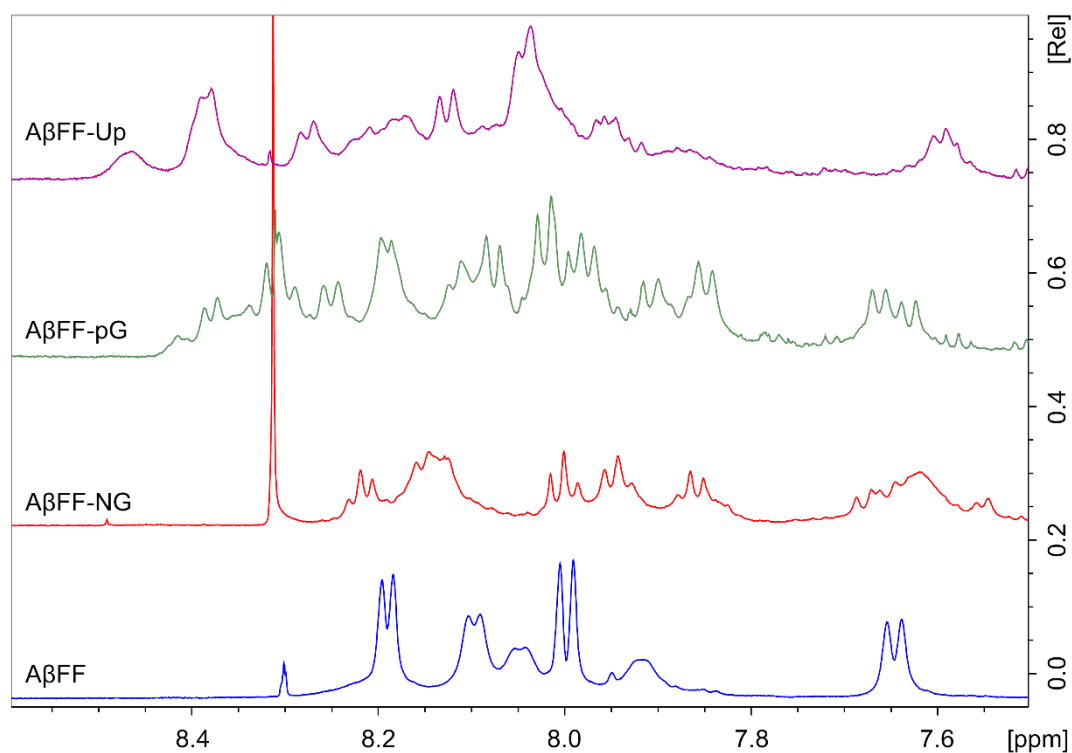


Figure 5.2D The amide region of the proton NMR spectra of A β FF and its turn-connected repeats in DMSO- d_6 .

5.3.2 A β FY and its turn analogs

The 1D spectra shown in figures 5.3 A-D suggest that the turn analogs of A β FY have populations in folded conformations. As with A β FF-Up analog, the A β FY-Up analog also tends to aggregate.

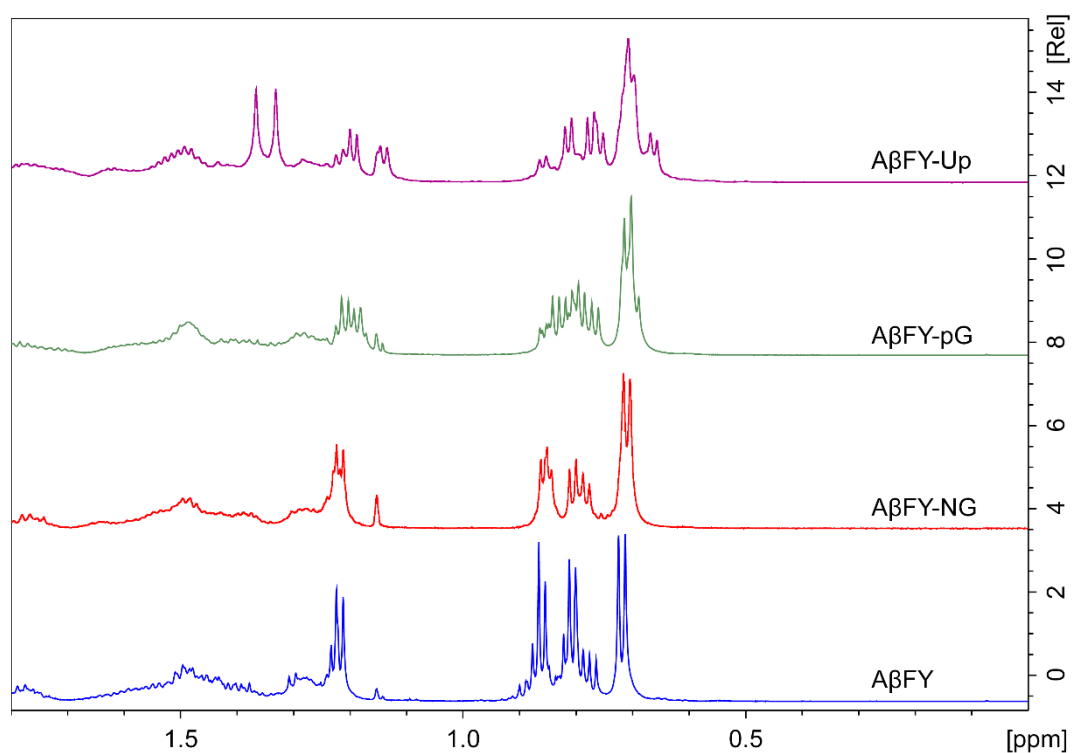


Figure 5.3A The aliphatic region of the proton NMR spectra of A β FY and its turn-connected repeats in DMSO- d_6 .

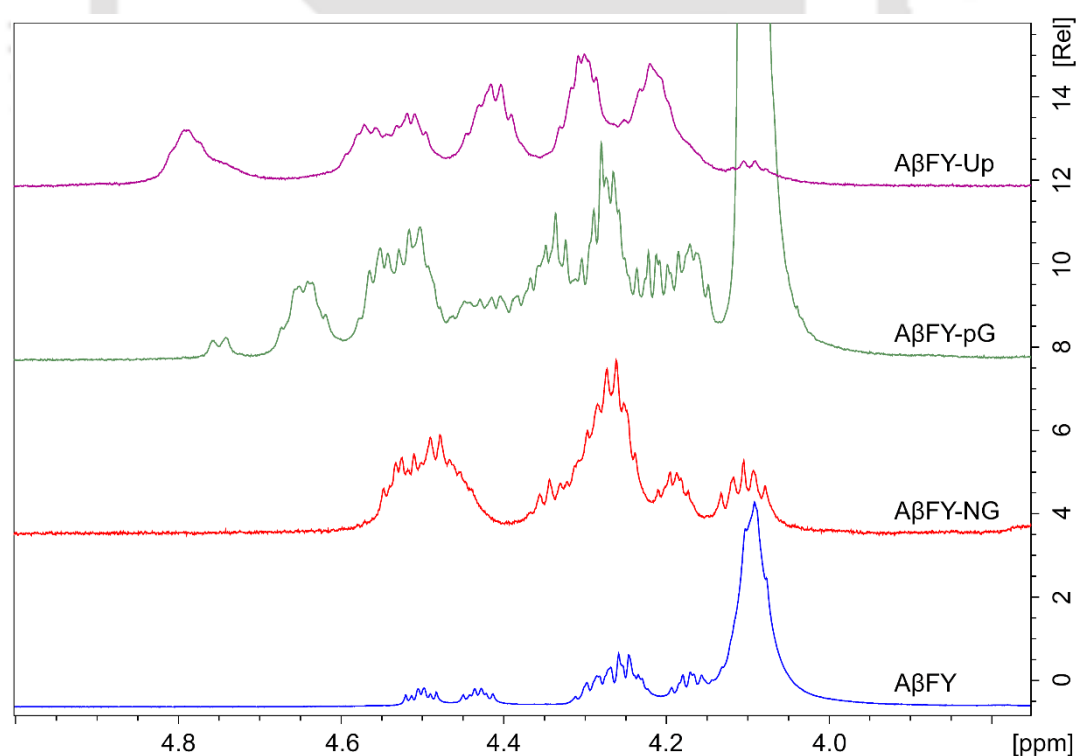


Figure 5.3B The C_α region of the proton NMR spectra of A β FY and its turn-connected repeats in DMSO- d_6 .

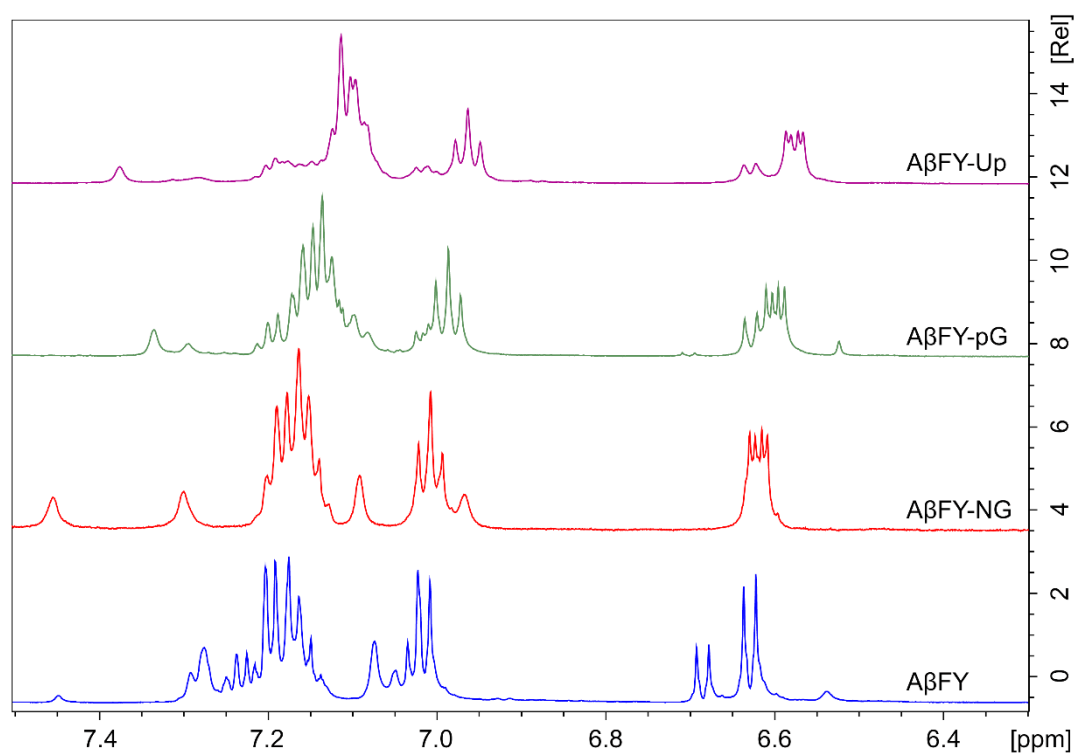


Figure 5.3C The aromatic region of the proton NMR spectra of AβFY and its turn-connected repeats in DMSO-d₆.

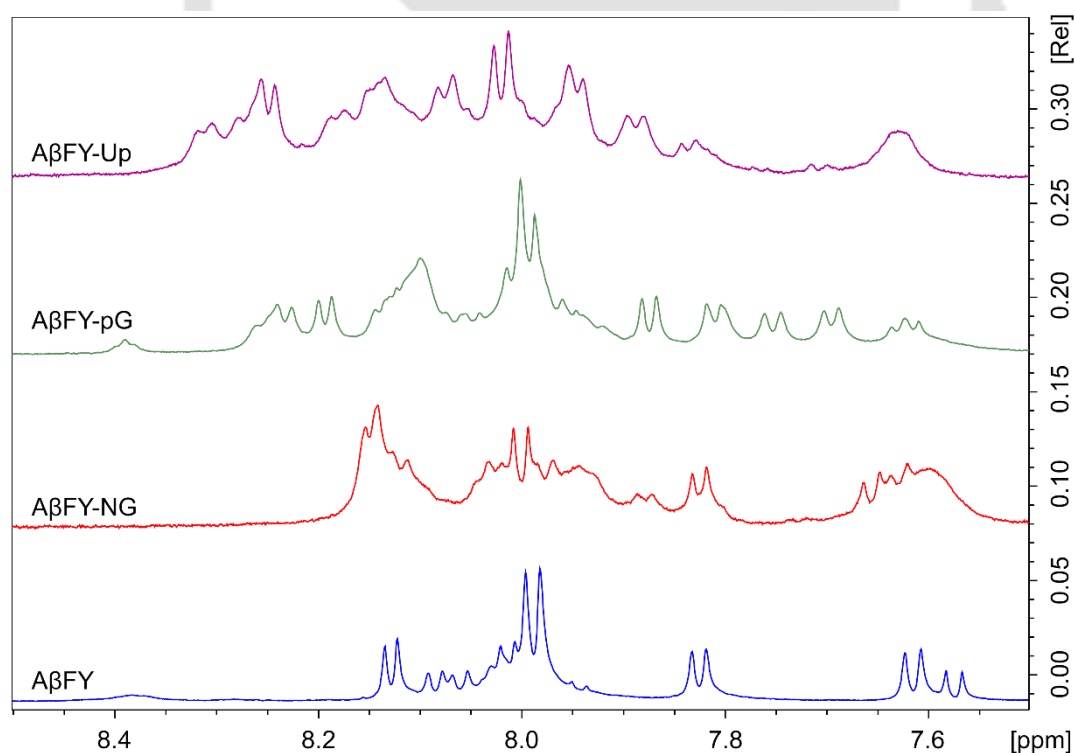


Figure 5.3D The amide region of the proton NMR spectra of AβFY and its turn-connected repeats in DMSO-d₆.

5.3.3 A β YY and turn analogs

In the case of the A β YY peptides (Figures 5.4 A-D), the resonances in the aliphatic, as well as the aromatic regions, are relatively sharp. Only the amide protons are less resolved though dispersed. The multiple peaks observed for the aromatic protons in the A β YY-pG and A β YY-Up analogs as compared to the A β YY and the A β YY-NG analog suggest differences in the orientations of the aromatic rings in the A β YY-pG and A β YY-Up due to multiple conformations existing on the NMR time scale.

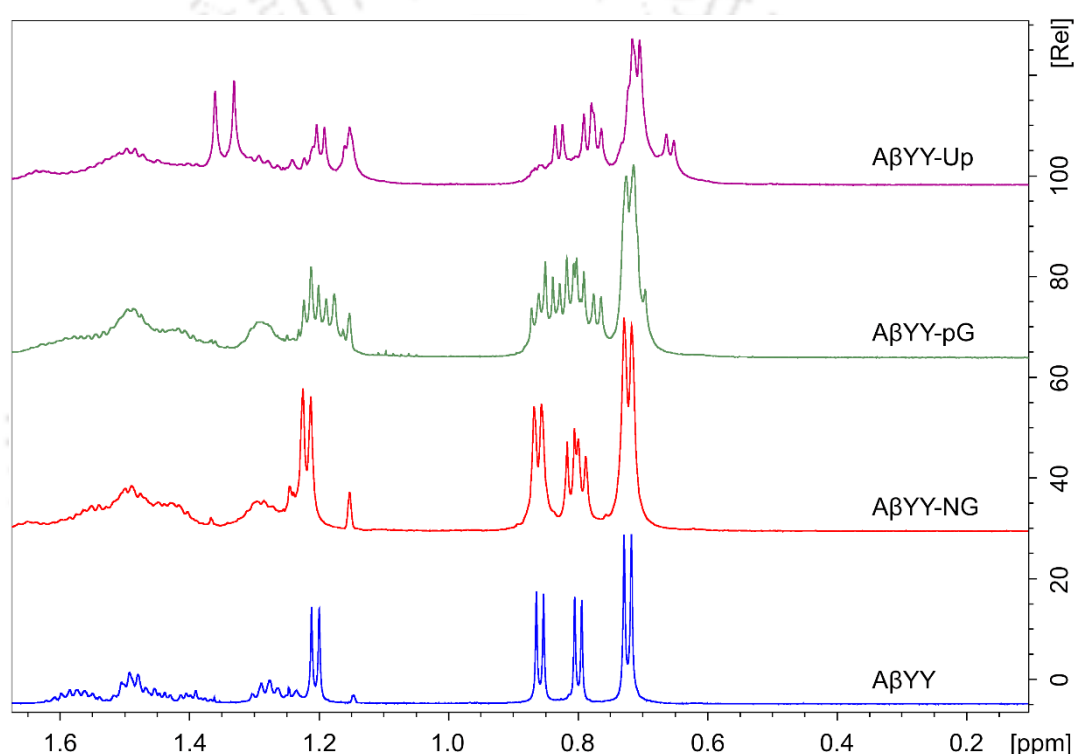


Figure 5.4A The aliphatic region of the proton NMR spectra of A β YY and its turn-connected repeats in DMSO- d_6 .

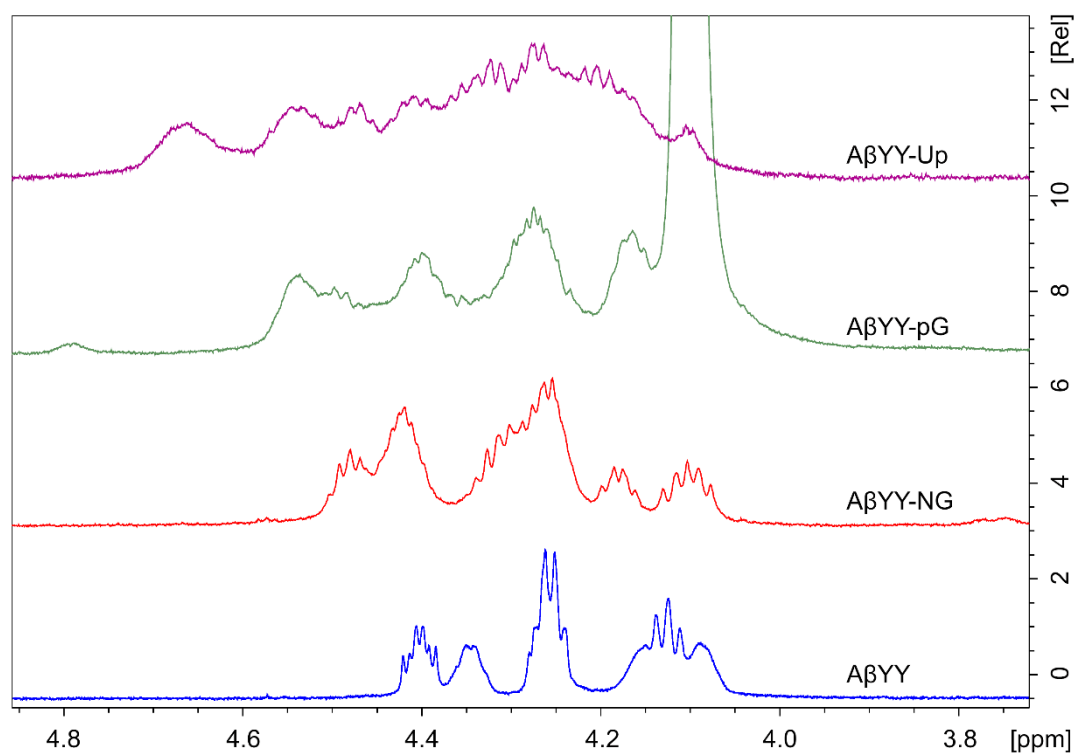


Figure 5.4B The C_{α} region of the proton NMR spectra of $A\beta YY$ and its turn-connected repeats in $DMSO-d_6$.

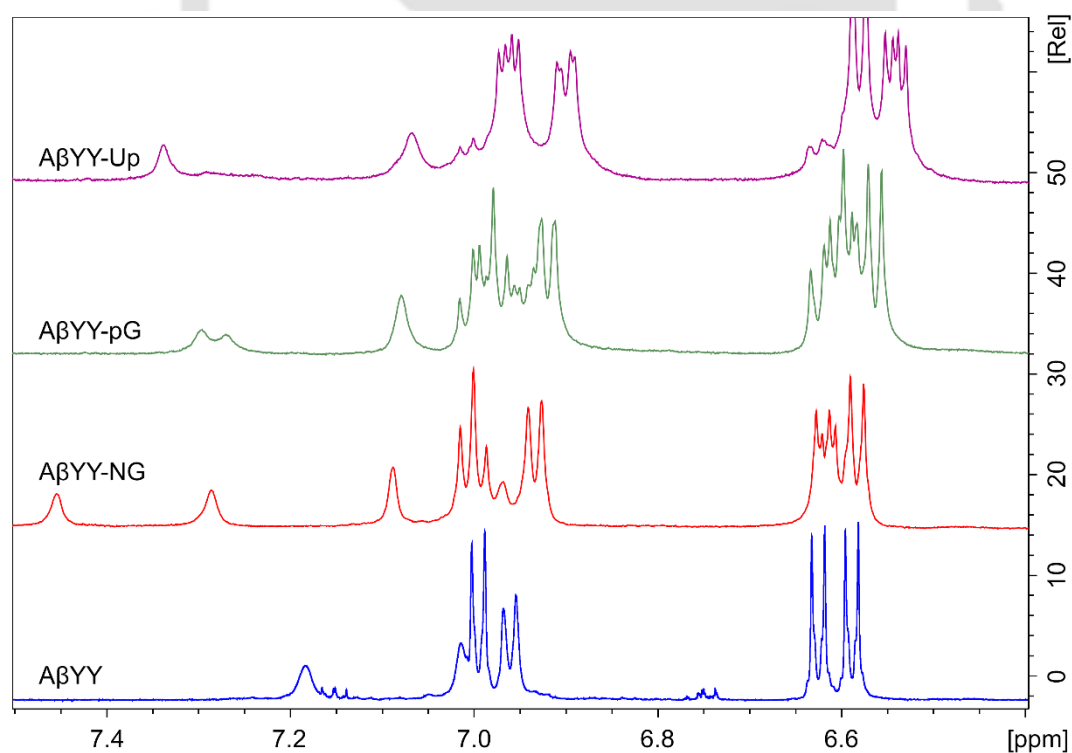


Figure 5.4C The aromatic region of the proton NMR spectra of $A\beta YY$ and its turn-connected repeats in $DMSO-d_6$.

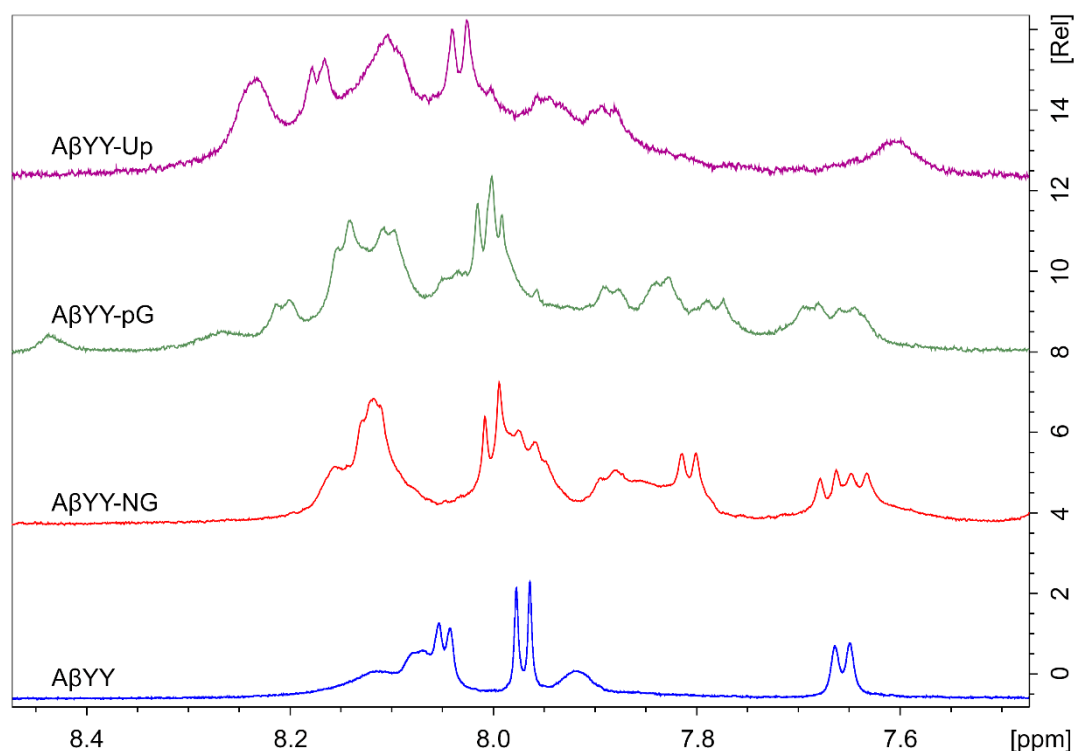


Figure 5.4D The amide region of the proton NMR spectra of A β YY and its turn-connected repeats in DMSO- d_6 .

5.3.4 A β WW and turn analogs

In the NMR spectra of these peptides (Figures 5.5 A-D), complex pattern of protons in all the regions as well as broadening in A β WW-pG and A β WW-Up peptides indicate aggregation and population of multiple conformations. The amide protons are dispersed in the turn analogs arguing for folded conformations.

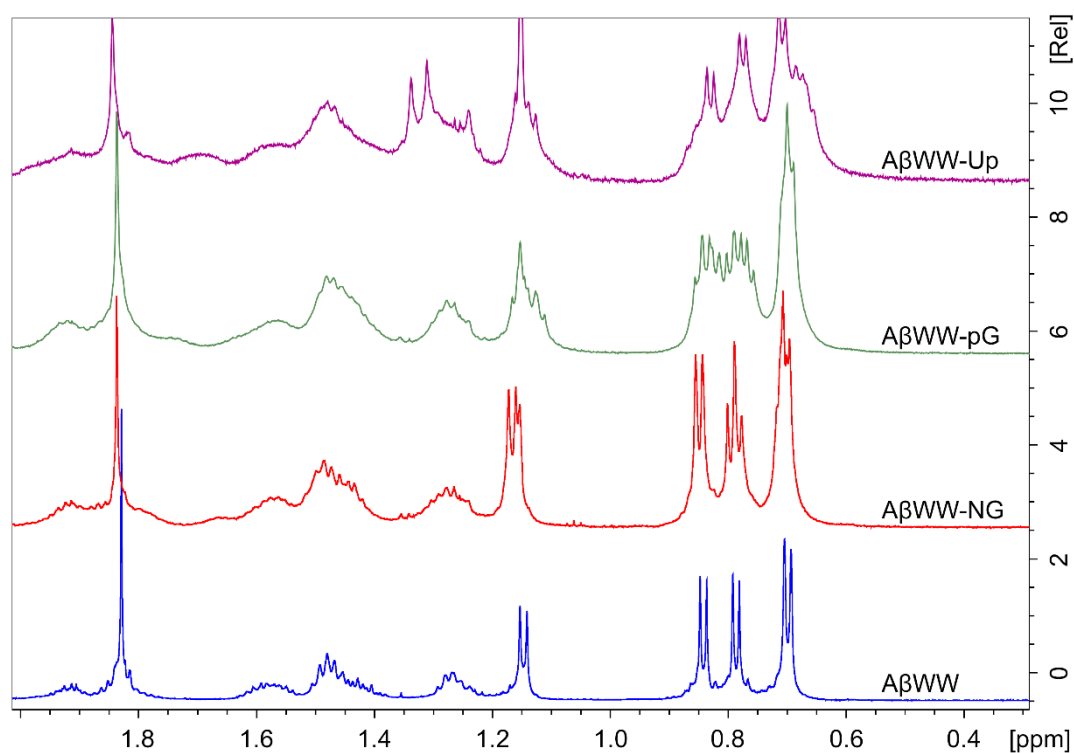


Figure 5.5A The aliphatic region of the proton NMR spectra of AβWW and its turn-connected repeats in DMSO-d₆.

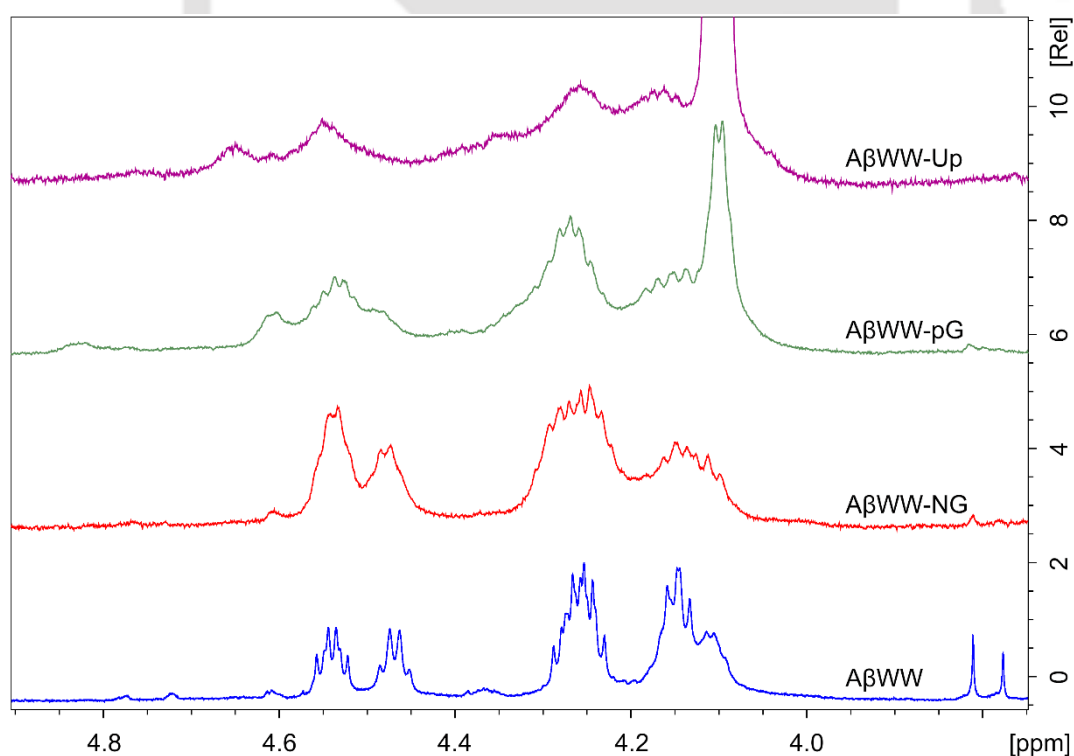


Figure 5.5B The C_α region of the proton NMR spectra of AβWW and its turn-connected repeats in DMSO-d₆.

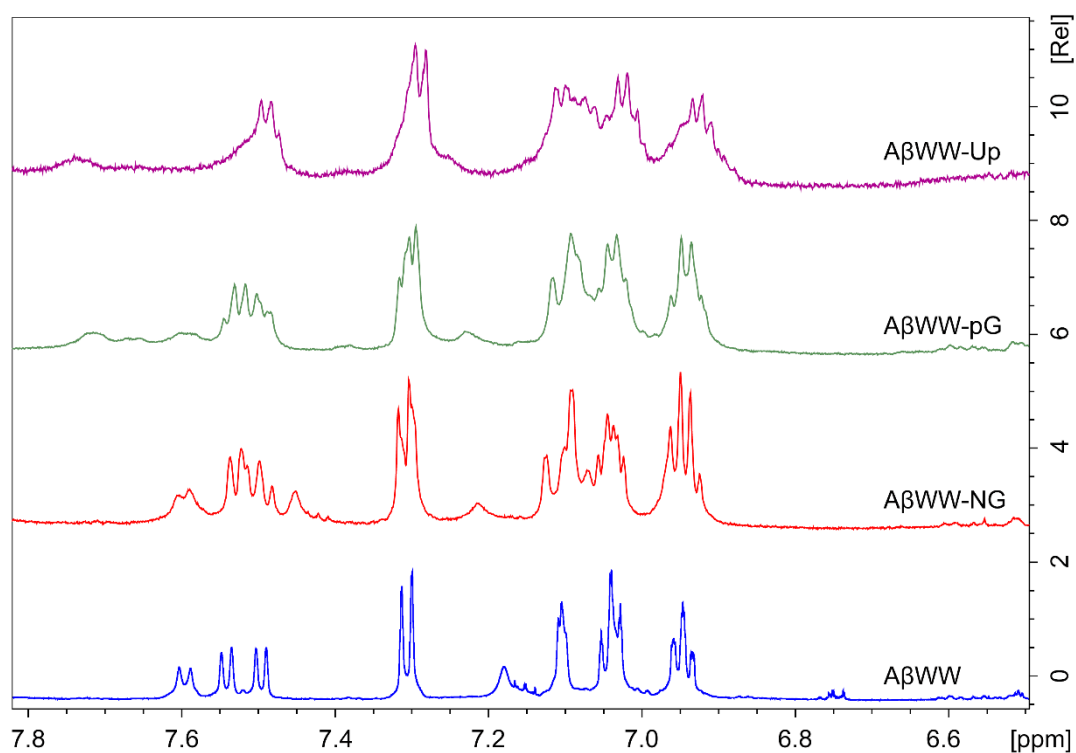


Figure 5.5C The aromatic region of the proton NMR spectra of A β WW and its turn-connected repeats in DMSO-d₆.

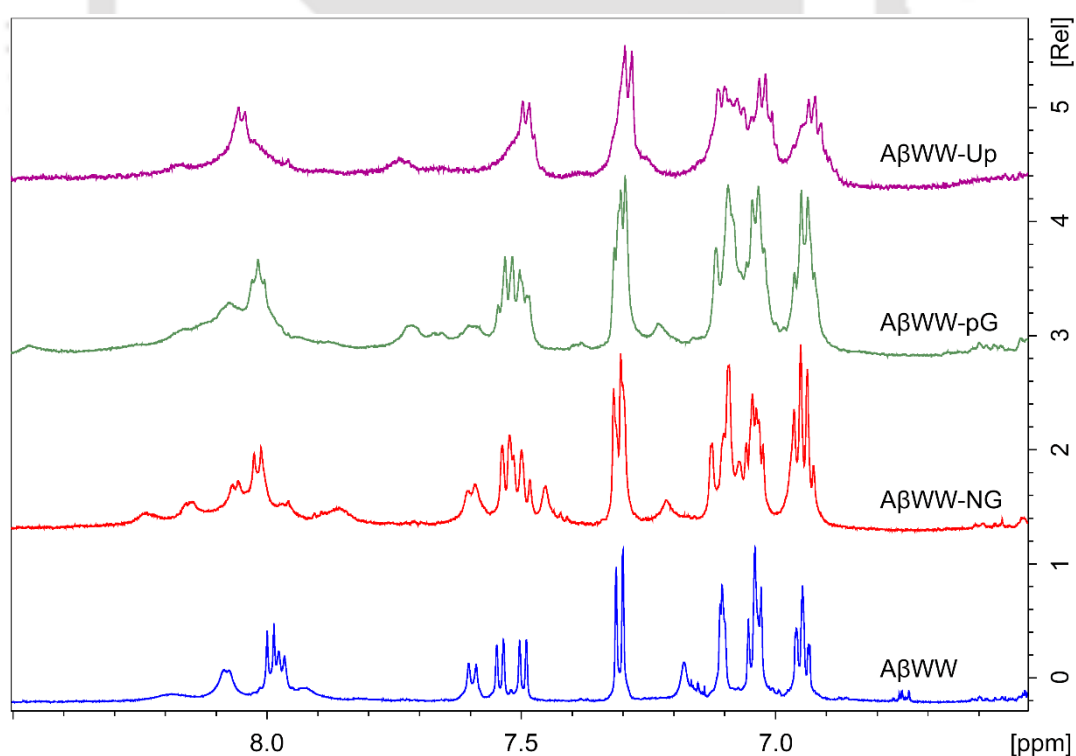


Figure 5.5D The amide region of the proton NMR spectra of A β WW and its turn-connected repeats in DMSO-d₆.

Chapter 5

As the control peptides, *i.e.* the 7-residue native peptides could be dissolved in water to achieve 1.66 mg/ml concentration, a concentration good enough for NMR spectroscopy, the 1D NMR spectra were recorded in 2% D₂O. The spectra recorded in water are plotted with those recorded in DMSO-d₆ (at identical peptide concentration) in figure 5.6. It is interesting to note that the A β FF, A β YY, and A β WW spectra in DMSO are broader compared to the corresponding spectra in water suggesting that the peptides form larger oligomers in DMSO compared to those in water (Panels A, B, E, F, G, and H). A β FY, on the other hand, shows broader peaks for water sample (Panels C and D). This substantiates the rather high aggregation propensity for A β FY in the water among the aromatic analogs.

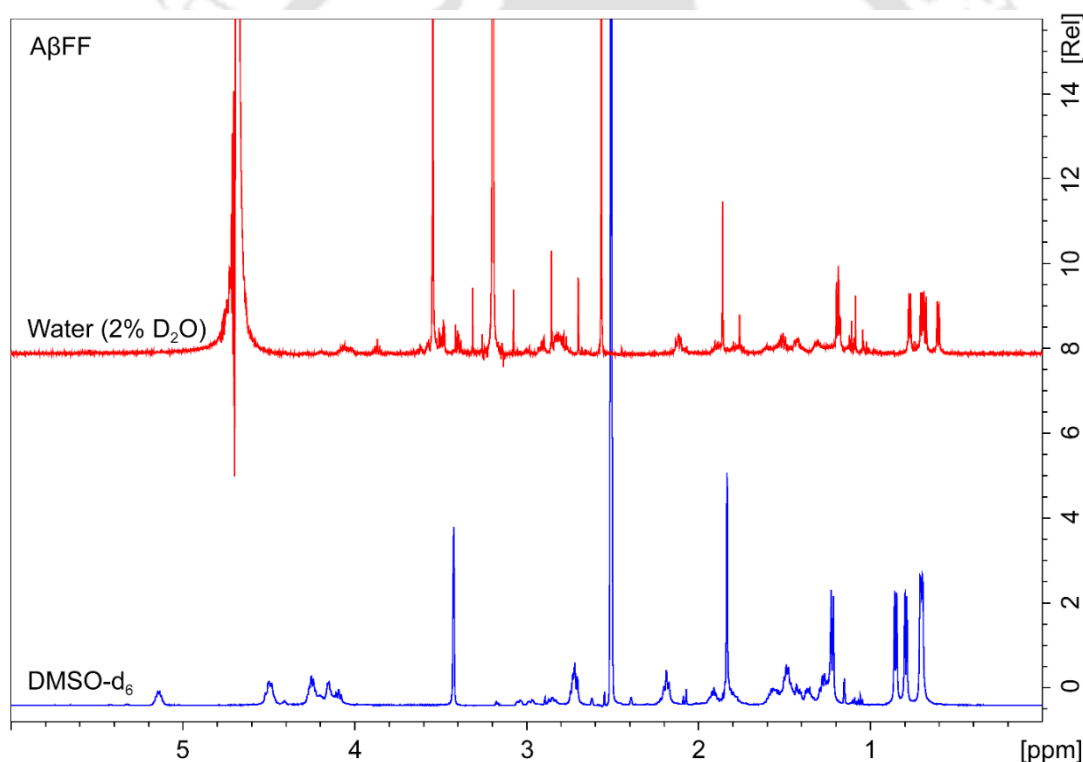


Figure 5.6A The aliphatic region of the 1D proton NMR spectra of A β FF in water and DMSO.

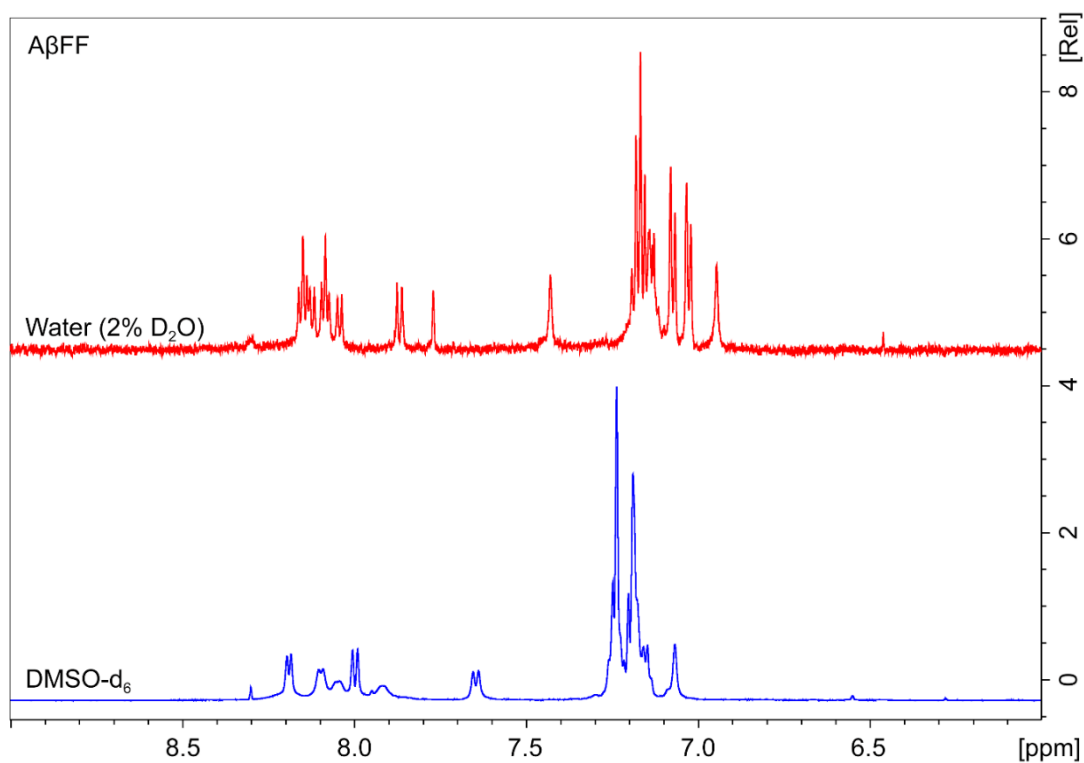


Figure 5.6B The aromatic and amide region of the 1D proton NMR spectra of AβFF in water and DMSO.

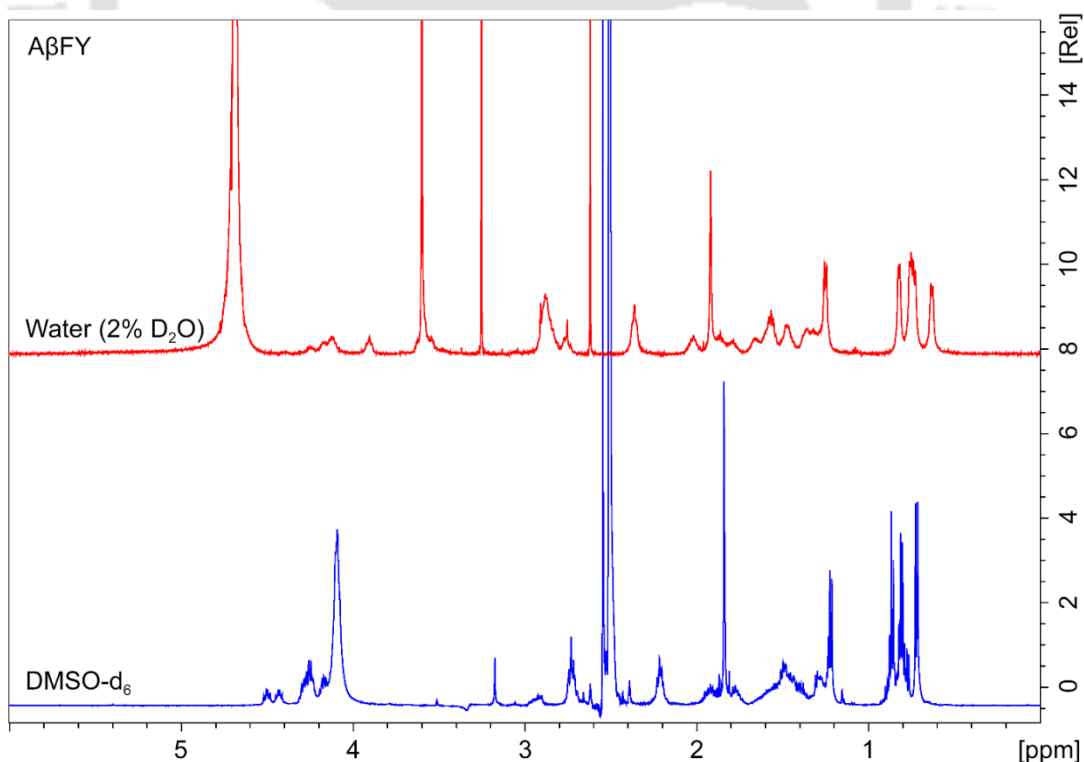


Figure 5.6C The aliphatic region of the 1D proton NMR spectra of AβFY in water and DMSO.

Chapter 5

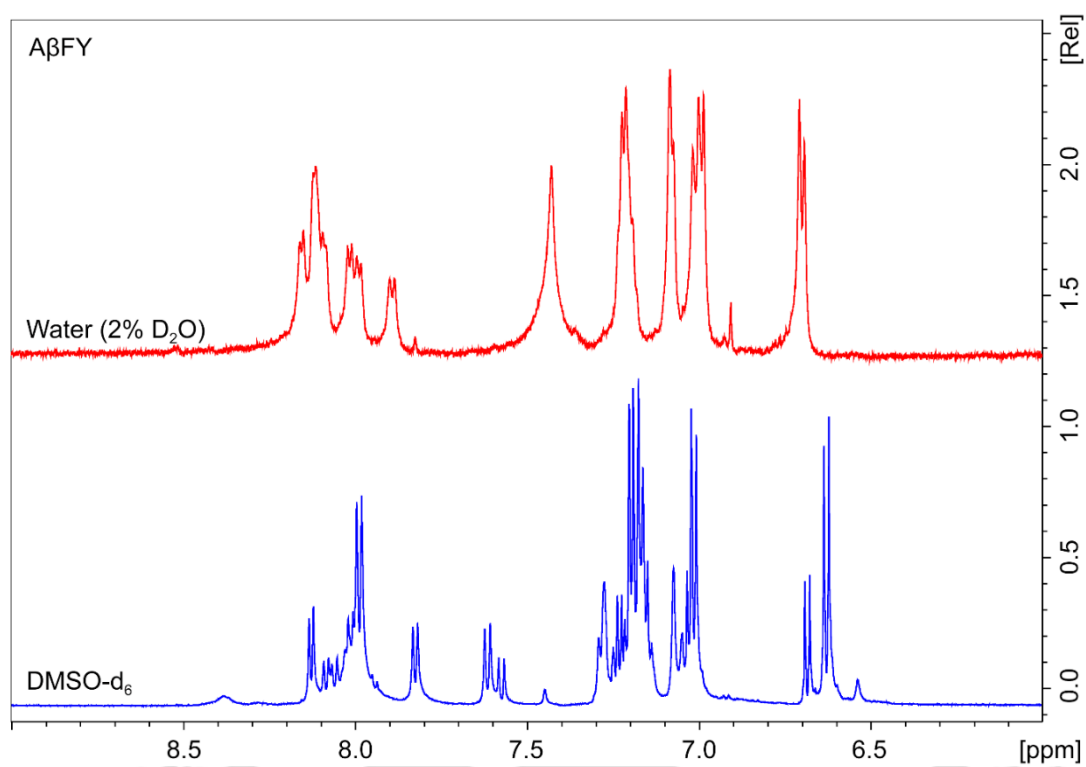


Figure 5.6D The aromatic and amide region of the 1D proton NMR spectra of AβFY in water and DMSO.

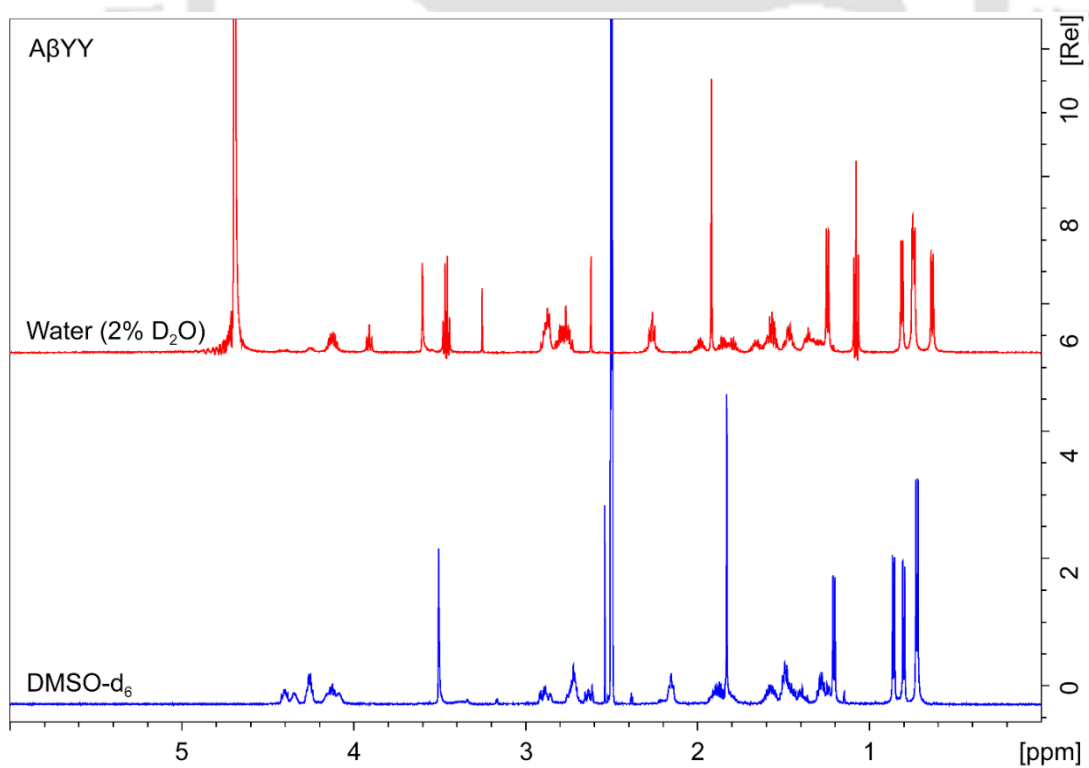


Figure 5.6E The aliphatic region of the 1D proton NMR spectra of AβYY in water and DMSO.

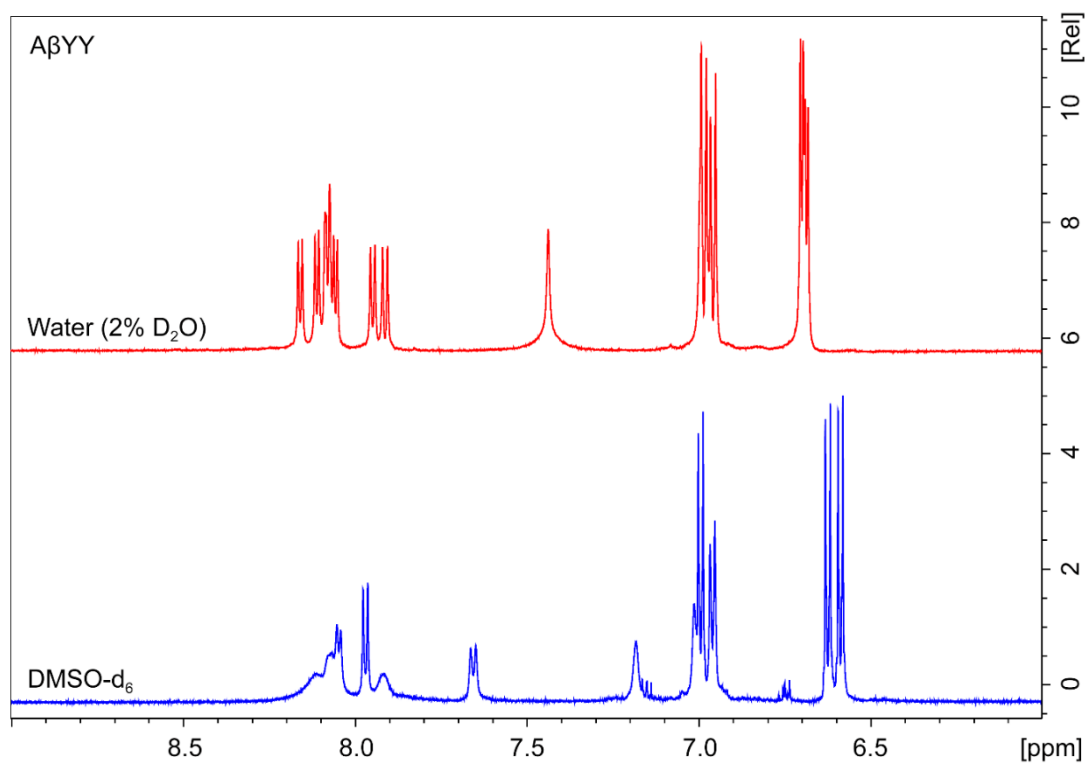


Figure 5.6F The aromatic and amide region of the 1D proton NMR spectra of A β YY in water and DMSO.

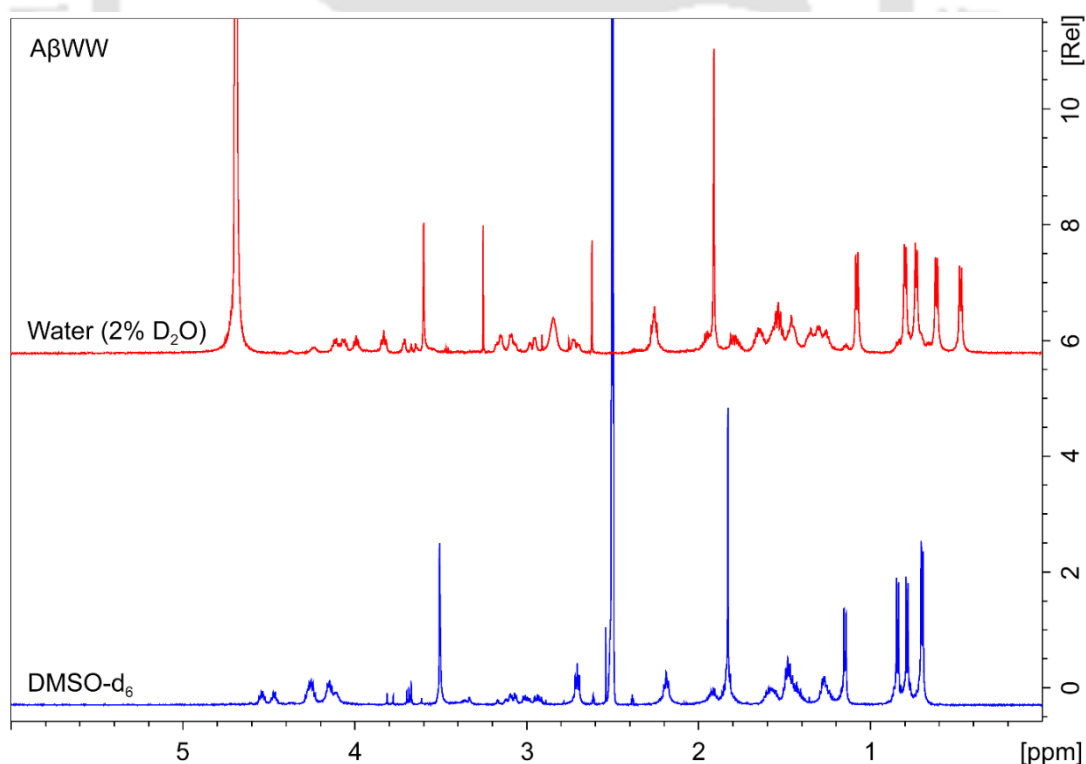


Figure 5.6G The aliphatic region of the 1D proton NMR spectra of A β WW in water and DMSO.

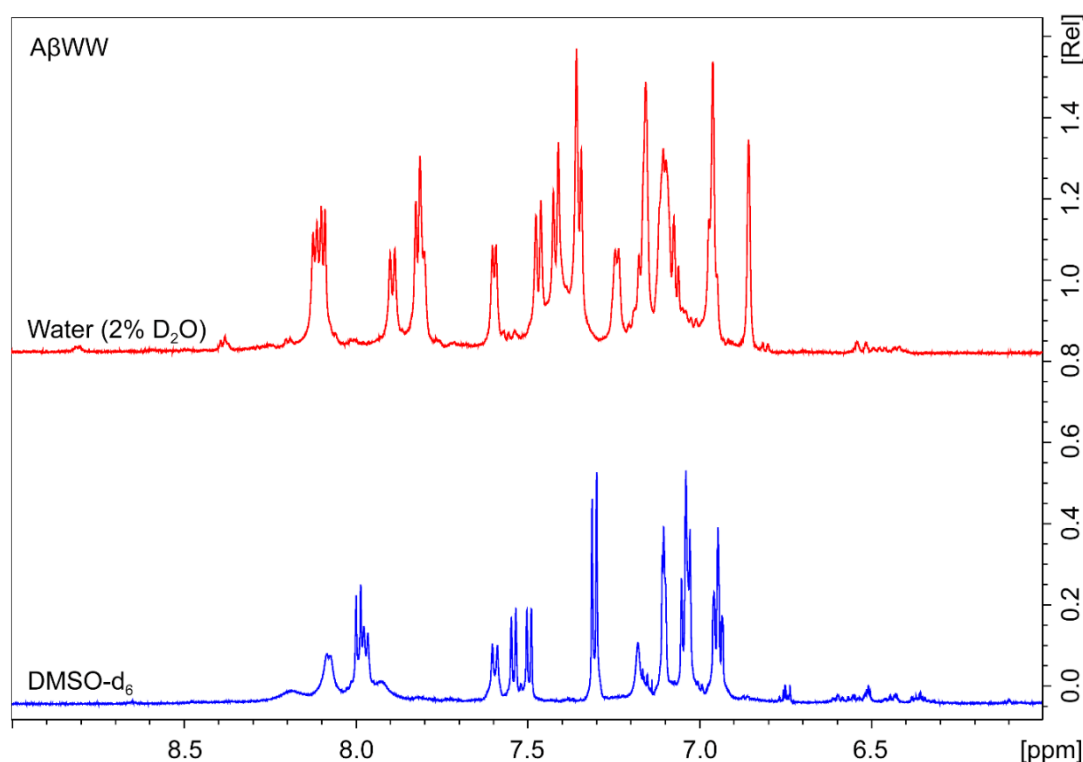


Figure 5.6H The aromatic and amide region of the 1D proton NMR spectra of A β WW in water and DMSO.

As the turn motif-harbouring peptides were poorly soluble in water, the 2D TOCSY and NOESY experiments for all the peptides were carried out in DMSO- d_6 only. The residual water signal obtained for the DMSO- d_6 samples was suppressed, as mentioned in section 5.2.3. Assignments were made from the TOCSY spectra (Figures 5.7-5.10). Large spectral overlap, however, made it difficult to carry out sequential assignment from the NOESY spectra. Individual amino acids or pairs of amino acids such as AA, KK, LL, VV, Aromatic-Aromatic, EE, on the other hand, could be unambiguously identified. The chemical shifts were compared with those of model peptides. The chemical shifts index (CSI) is extensively used to delineate helical and β -conformation in peptides and proteins [15, 16]. Furthermore, shift characteristics of helical and β -conformation are not solvent dependent [5]. Excellent correlations have been obtained between NOE data and CSI on the structures of model peptides adopting a β -hairpin conformation in DMSO [5]. Hence, the NMR spectra are largely interpreted based on the assignment of KK, LL, VV, AA, EE pairs; assignment of Asn, Gly, and D Pro are also unambiguous.

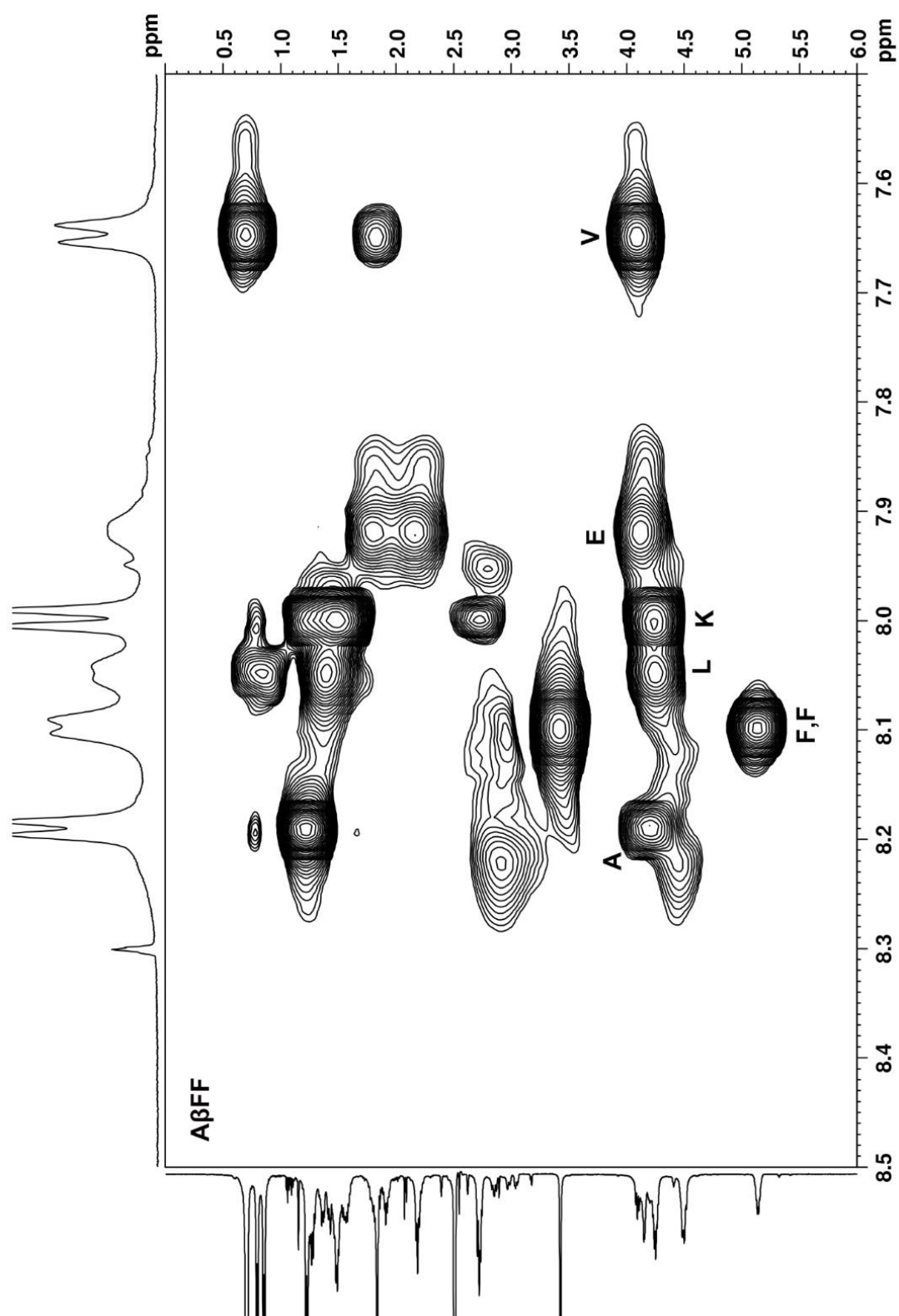


Figure 5.7A ^1H - ^1H TOCSY spectra of A β FF recorded in DMSO- d_6 .

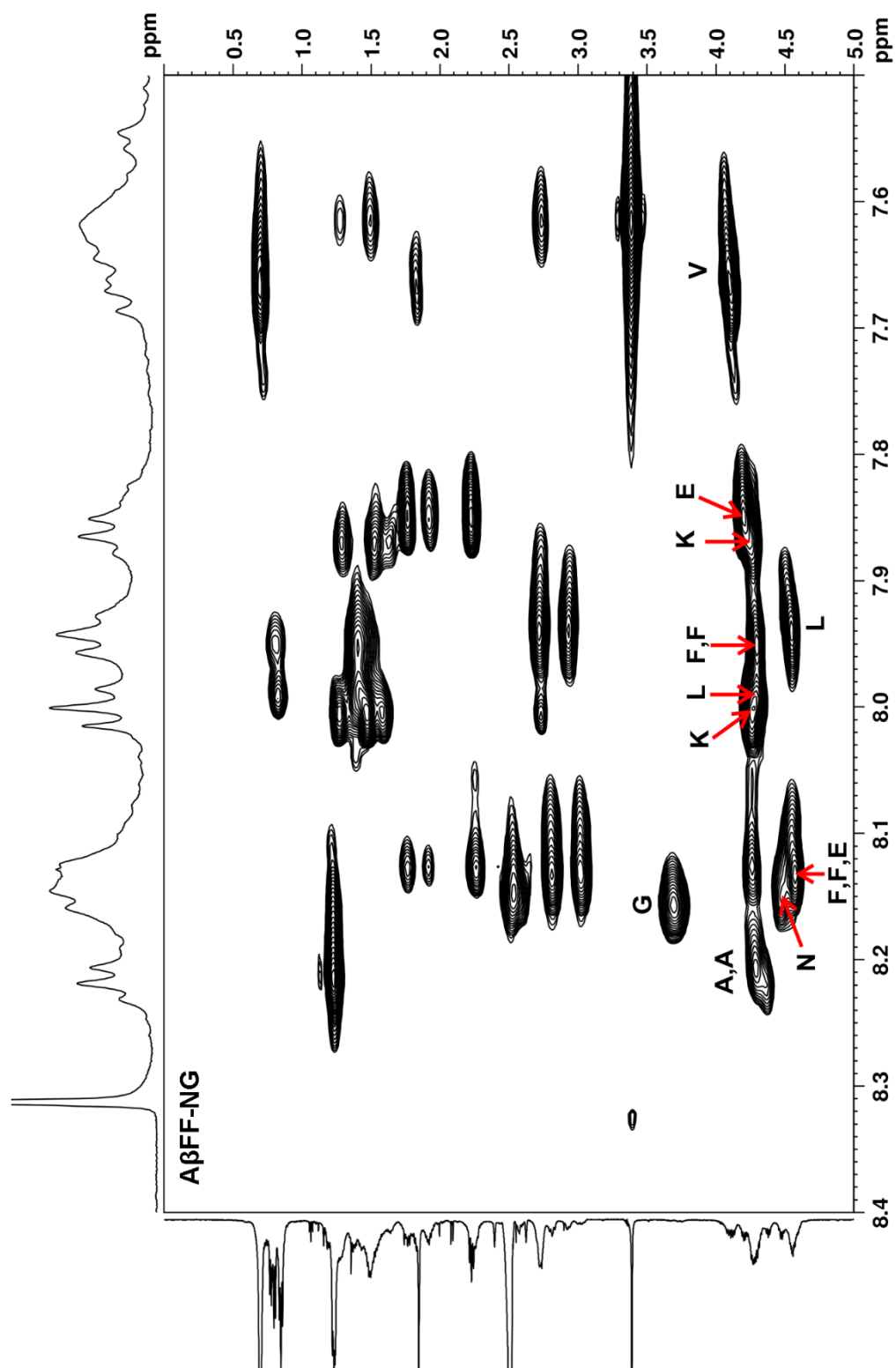


Figure 5.7B ^1H - ^1H TOCSY spectra of A β FF-NG recorded in DMSO- d_6 .

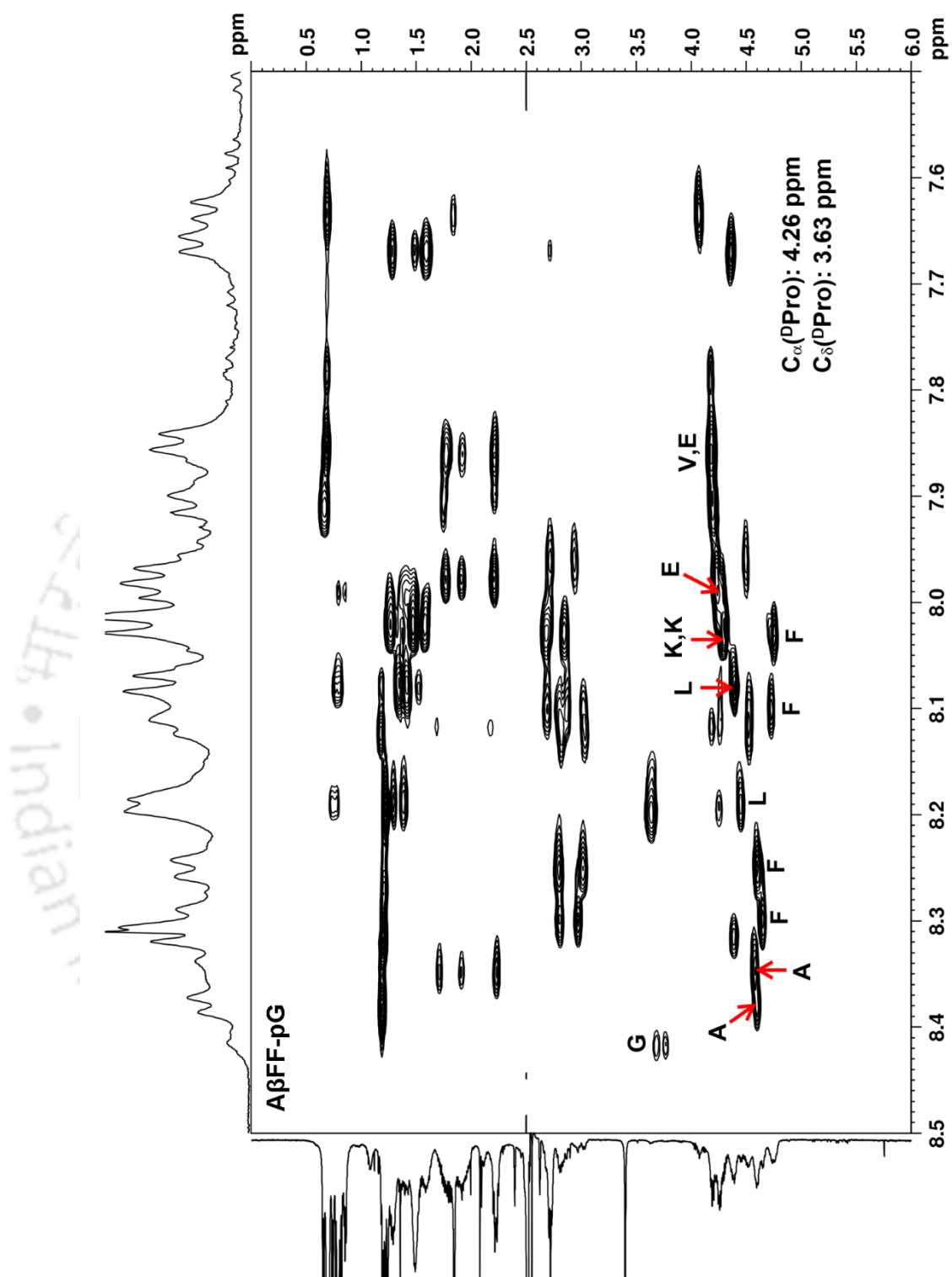


Figure 5.7 ^1H - ^1H TOCSY spectra of AβFF-pG recorded in DMSO- d_6 .

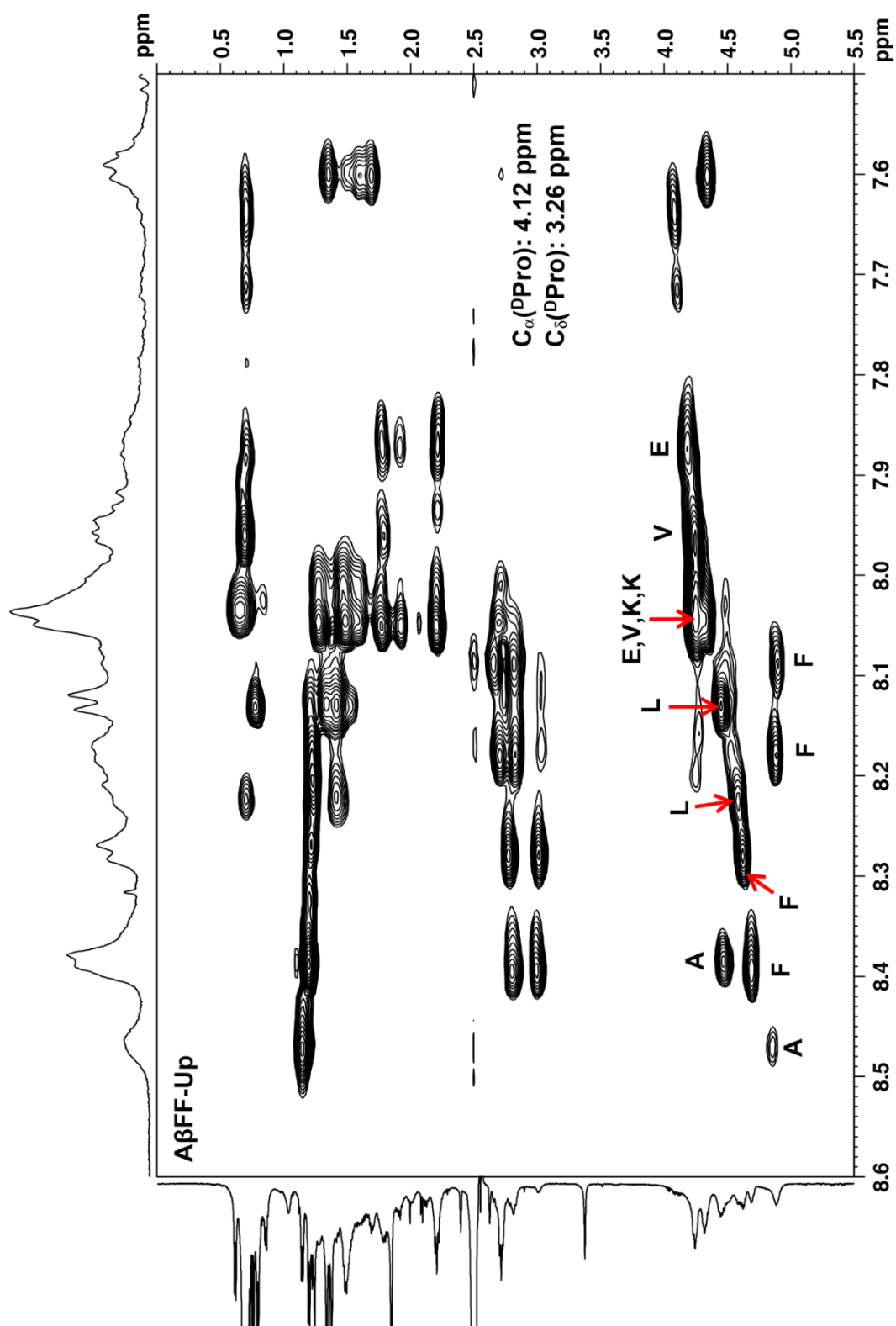


Figure 5.7D ^1H - ^1H TOCSY spectra of A β FF-Up recorded in DMSO- d_6 .

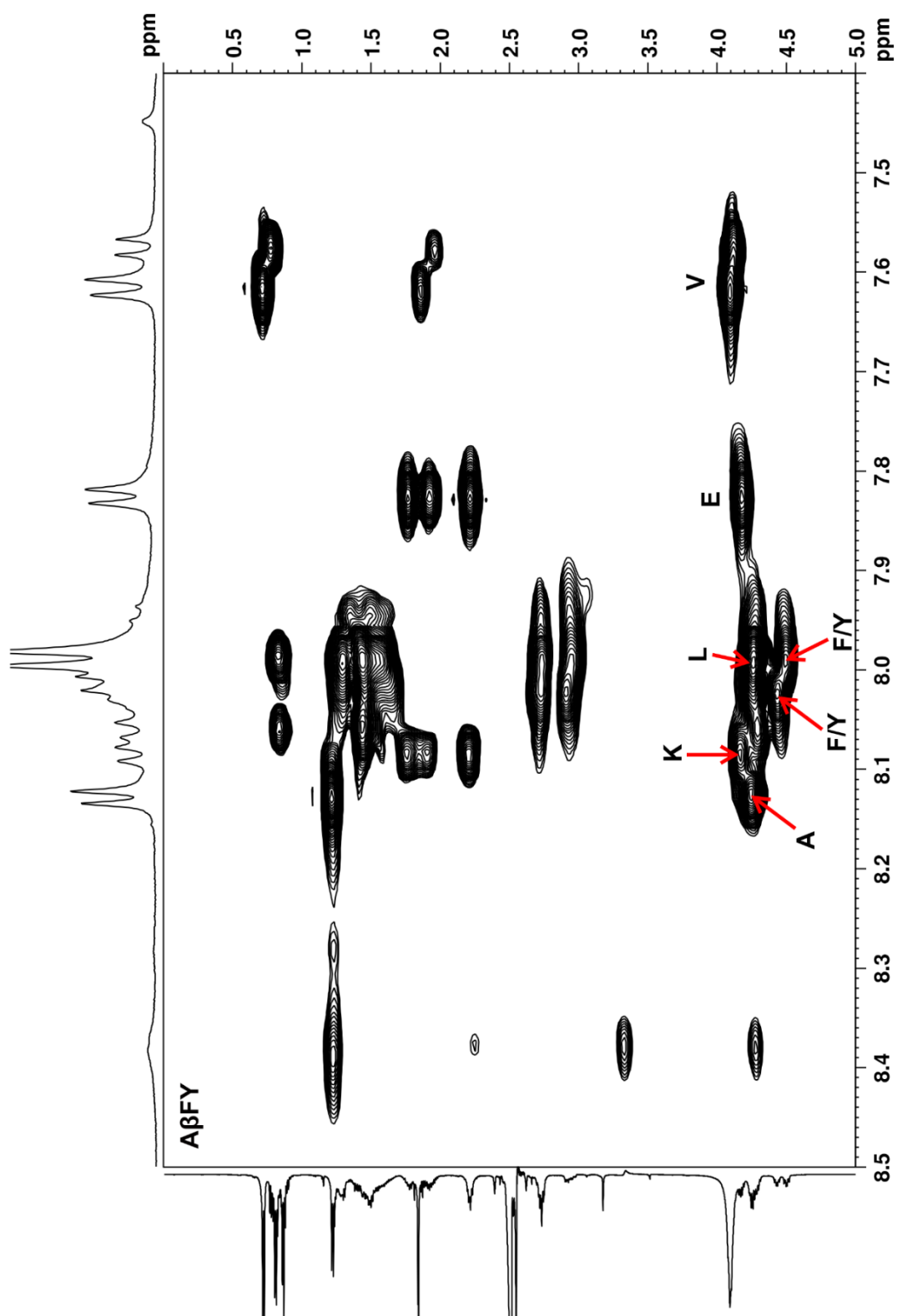


Figure 5.8A ^1H - ^1H TOCSY spectra of A β FY recorded in DMSO- d_6 .

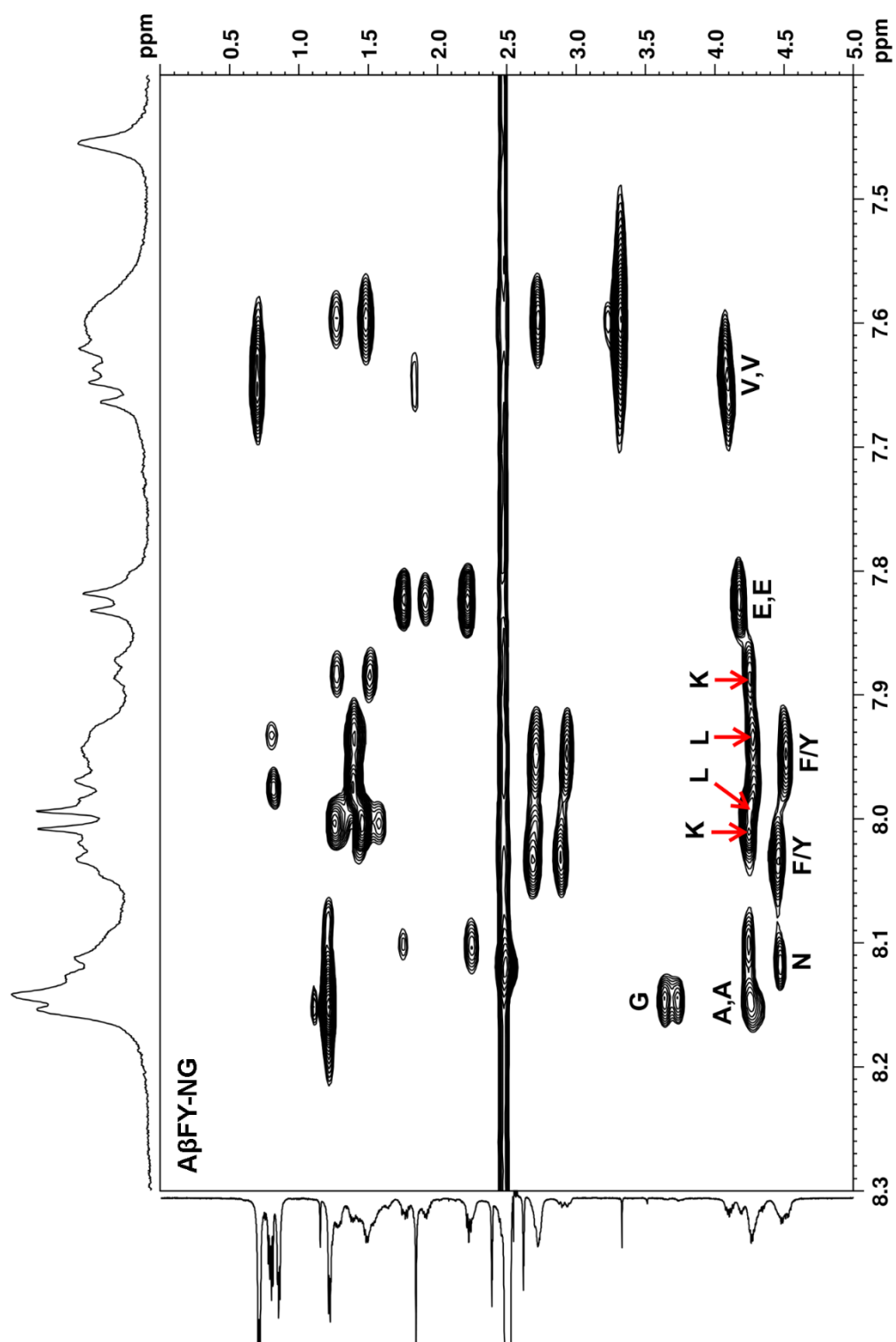


Figure 5.8B ^1H - ^1H TOCSY spectra of A β FY-NG recorded in DMSO- d_6 .

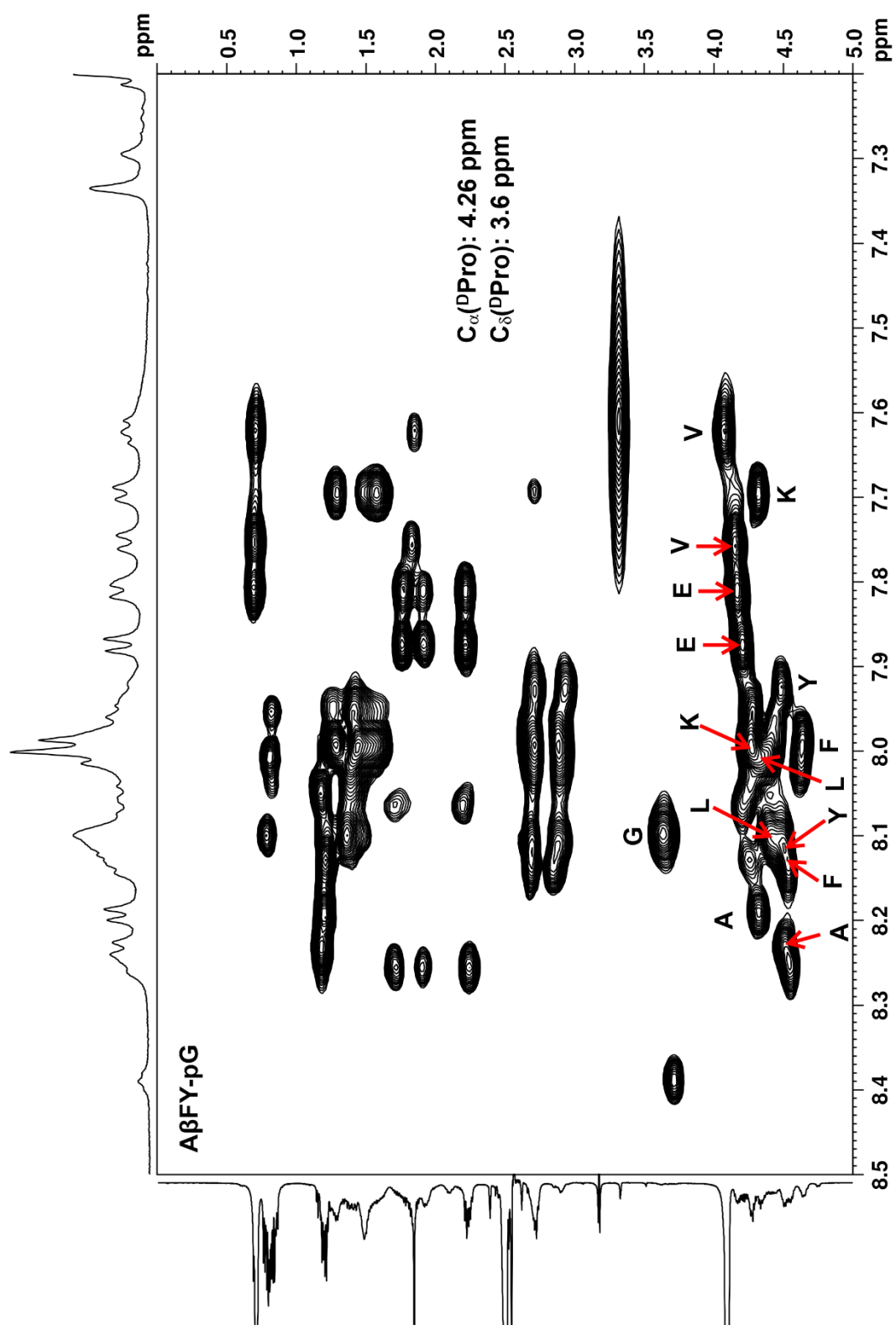


Figure 5.8C ^1H - ^1H TOCSY spectra of AβFY-pG recorded in DMSO- d_6 .

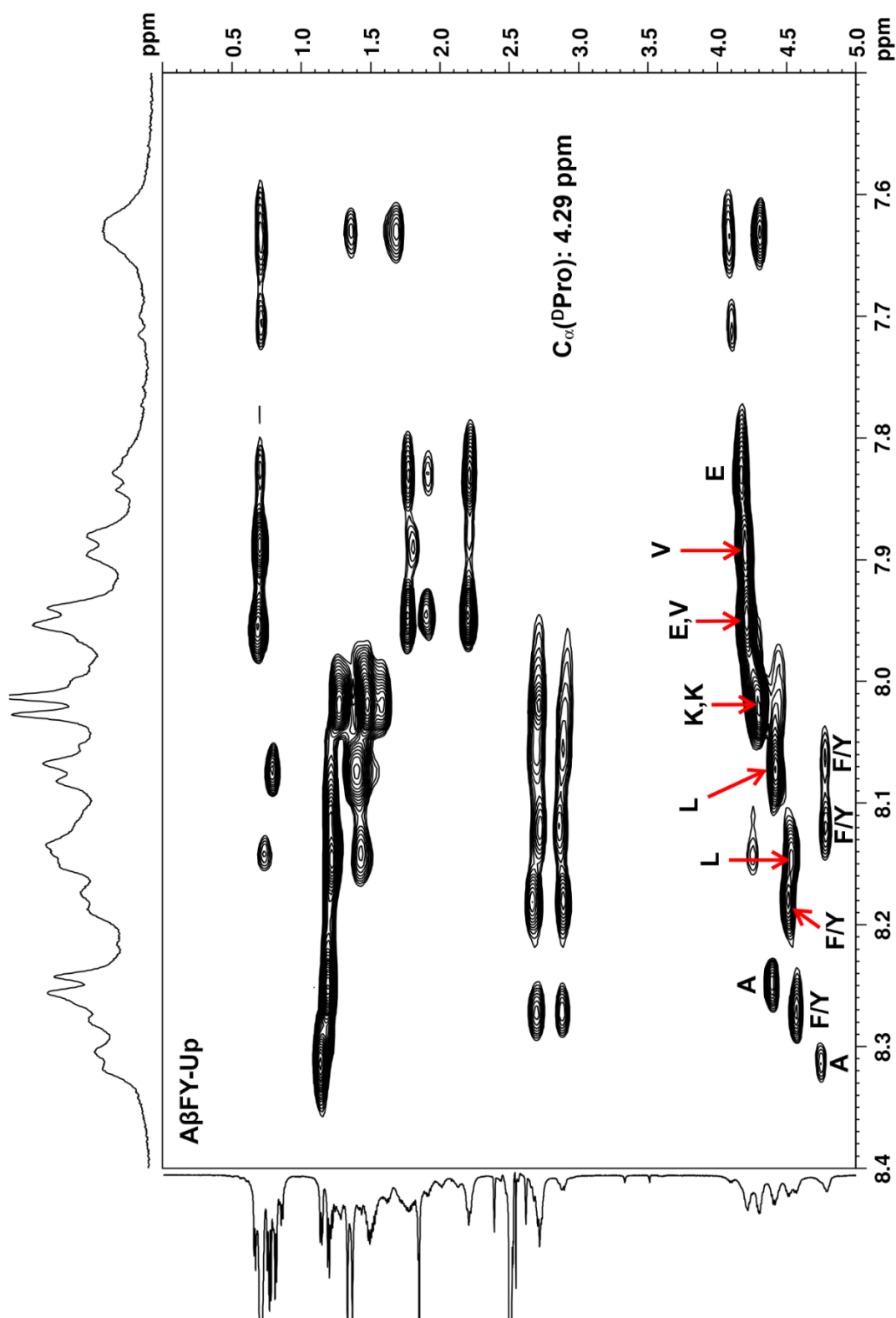


Figure 5.8D ^1H - ^1H TOCSY spectra of A β FY-Up recorded in DMSO- d_6 .

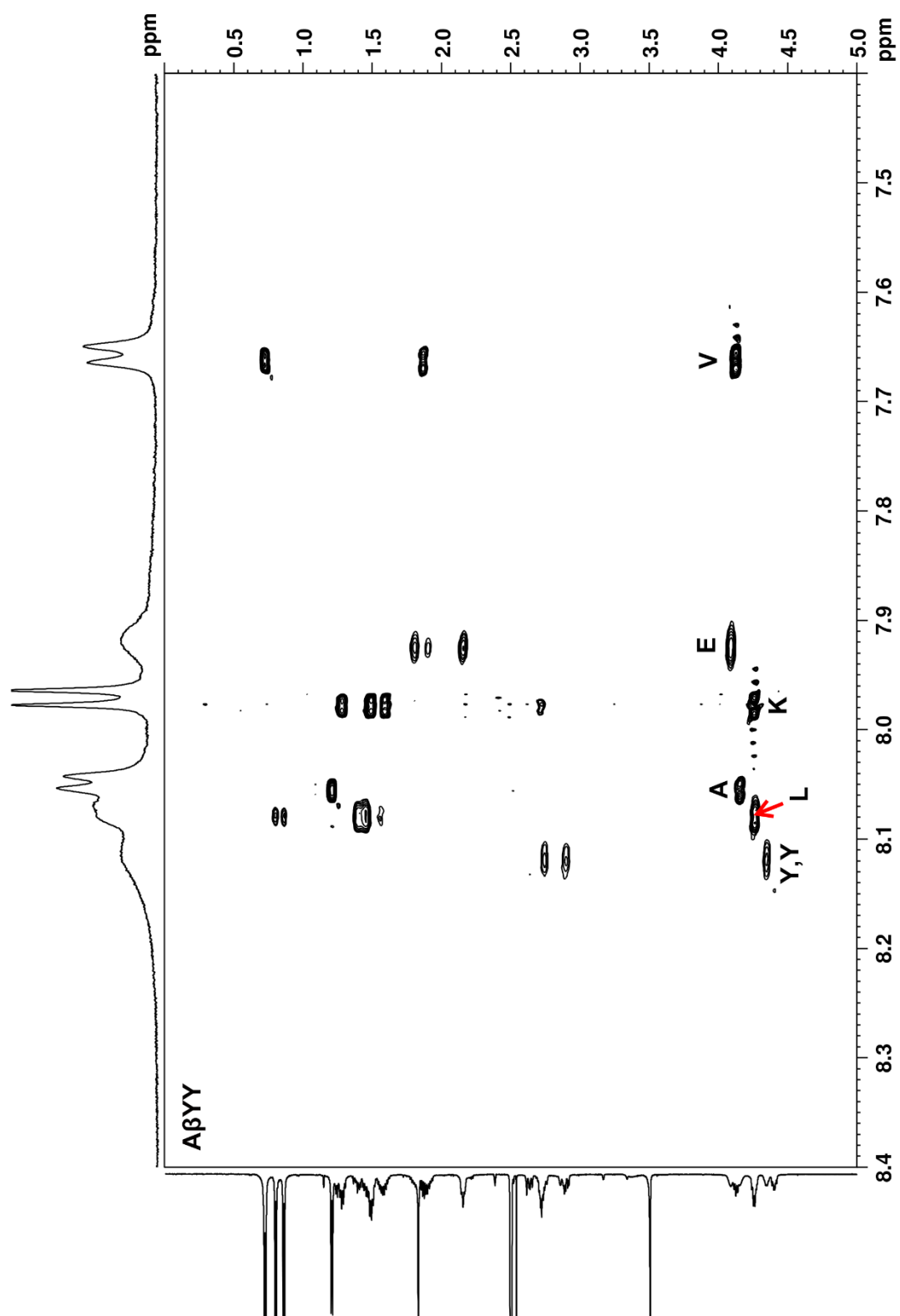


Figure 5.9A ^1H - ^1H TOCSY spectra of A β YY recorded in DMSO- d_6 .

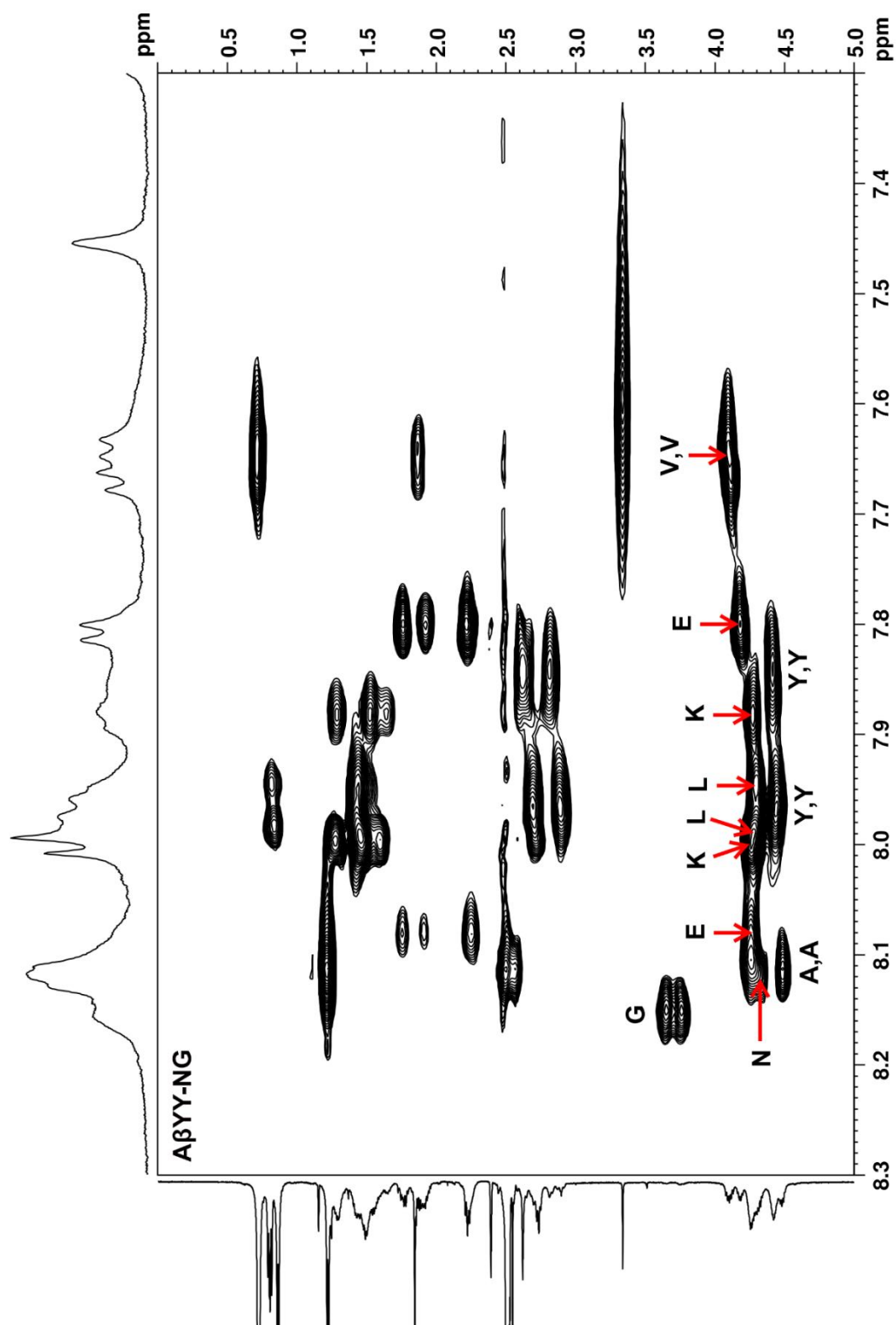


Figure 5.9B ^1H - ^1H TOCSY spectra of A β YY-NG recorded in DMSO- d_6 .

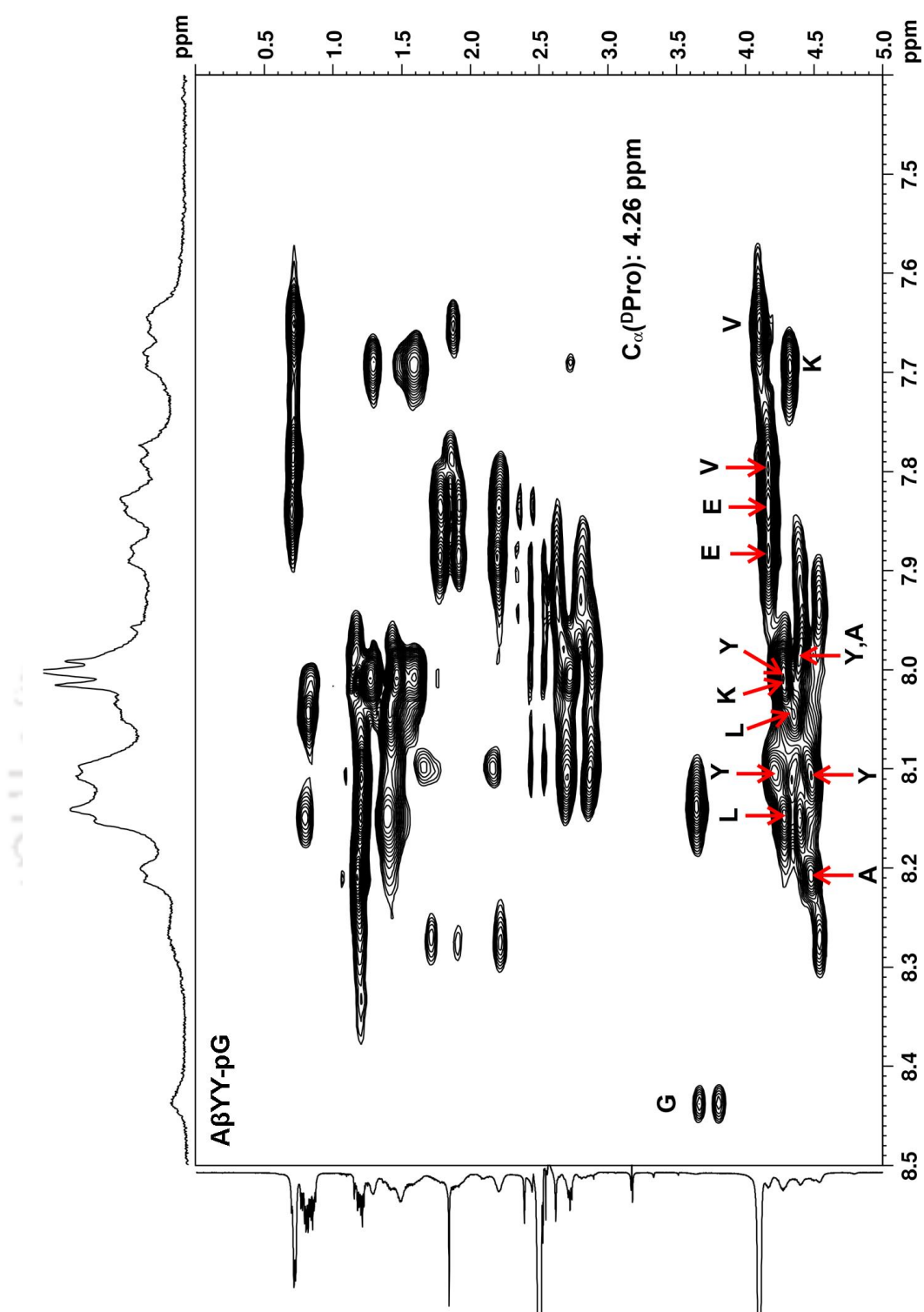


Figure 5.9 ^{13}C - ^1H - ^1H TOCSY spectra of A β YY-pG recorded in DMSO- d_6 .

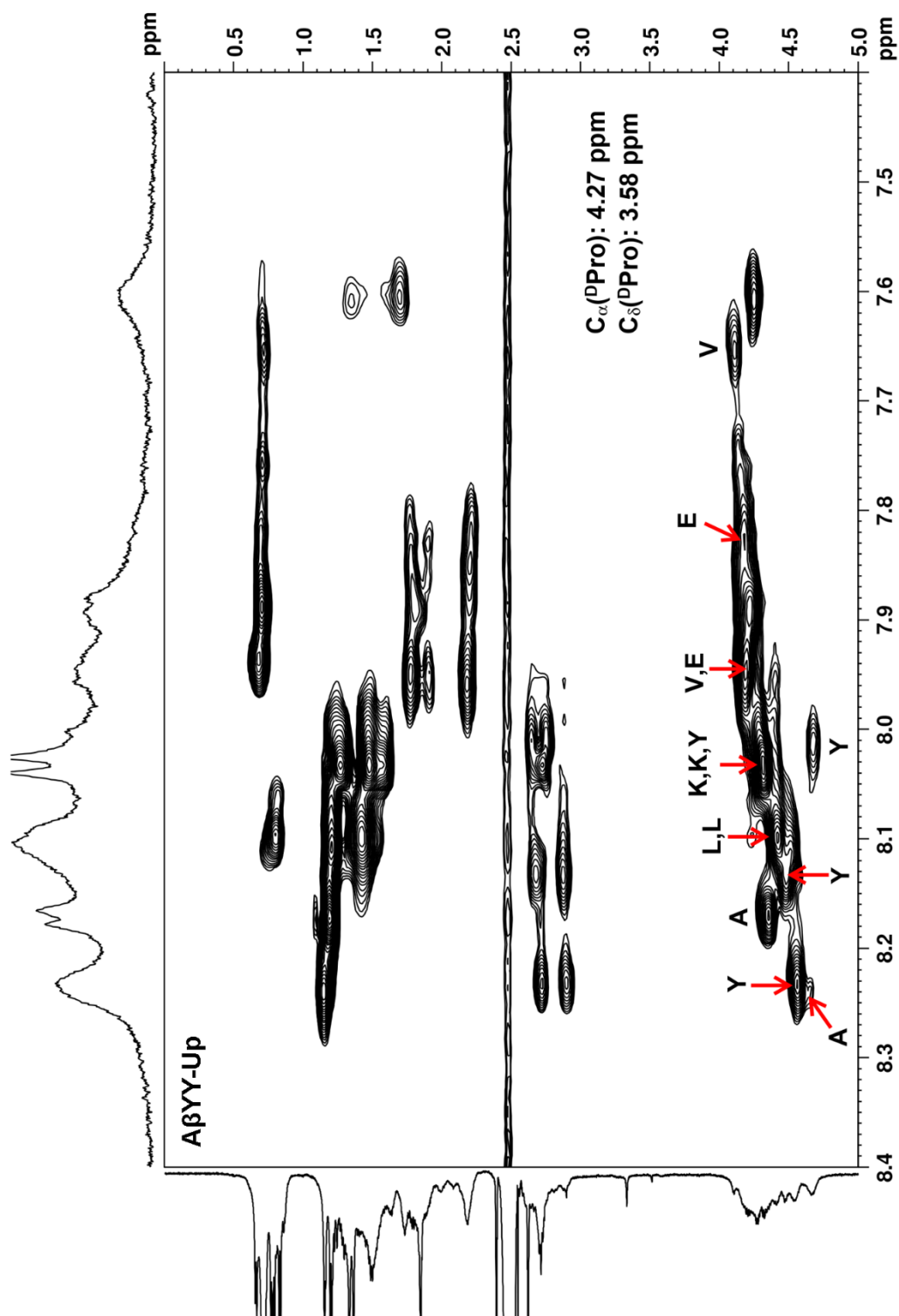


Figure 5.9D ^1H - ^1H TOCSY spectra of A β YY-Up recorded in DMSO- d_6 .

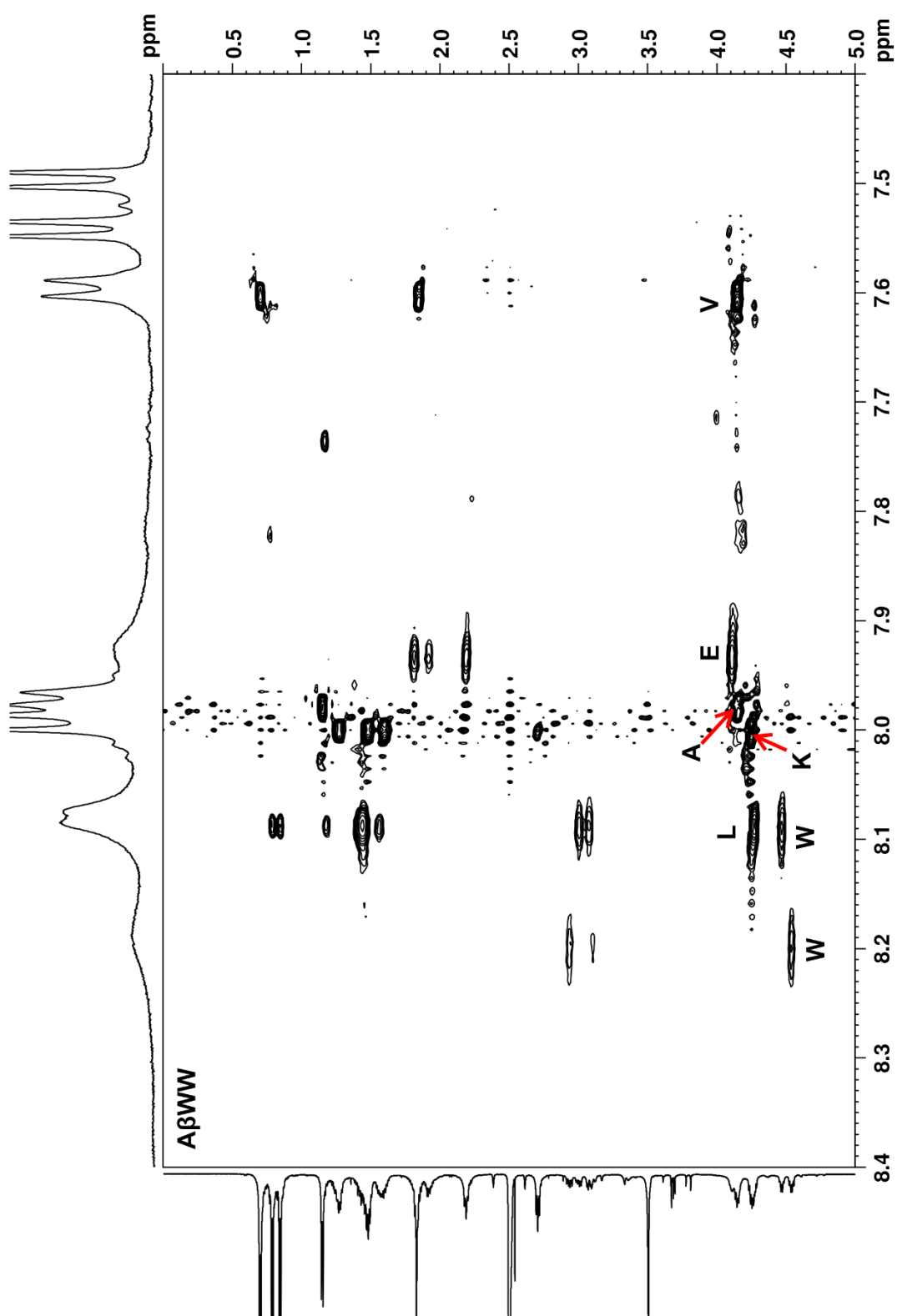


Figure 5.10A ^1H - ^1H TOCSY spectra of A β WW recorded in DMSO- d_6 .

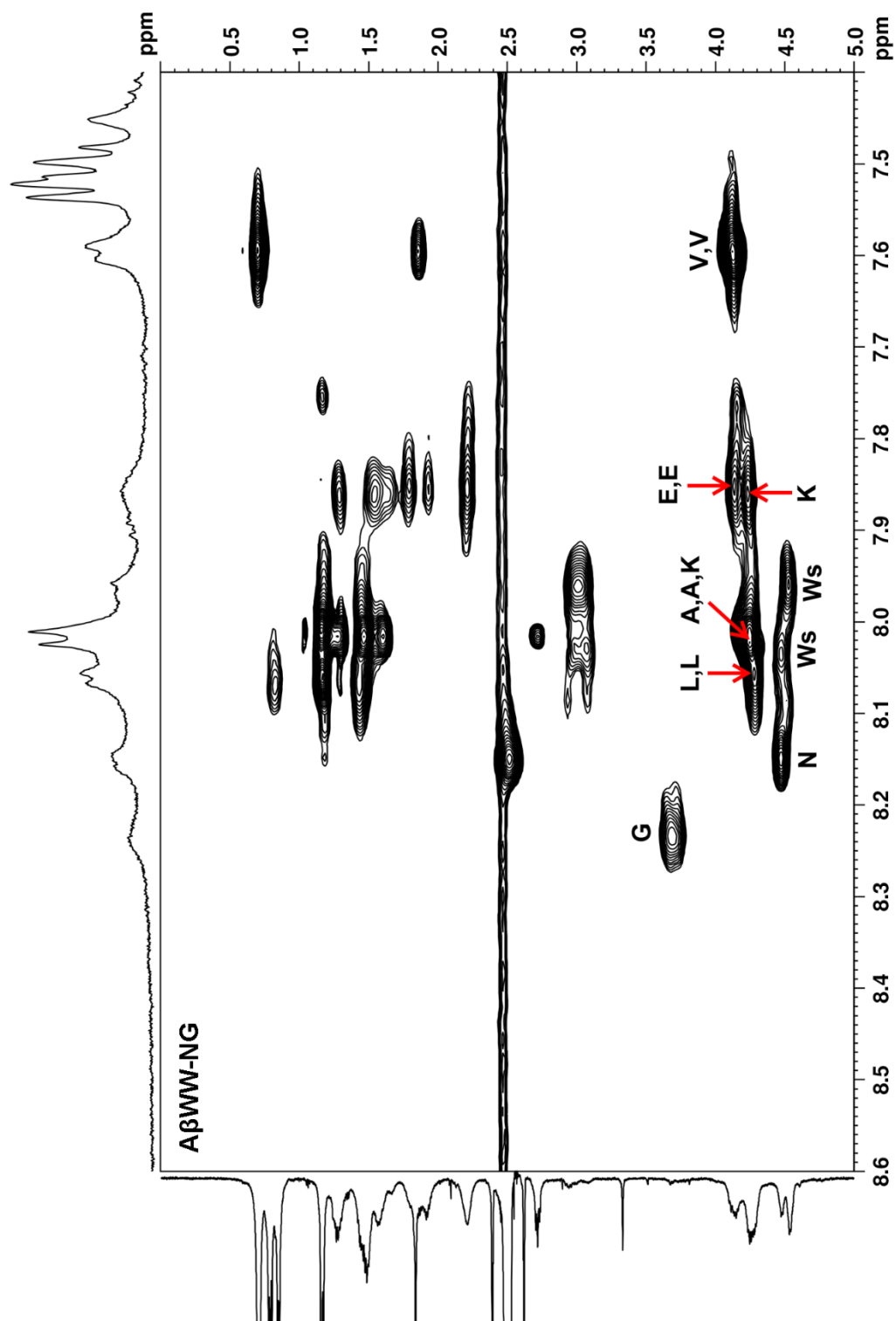


Figure 5.10B ^1H - ^1H TOCSY spectra of A β WW-NG recorded in DMSO- d_6 .

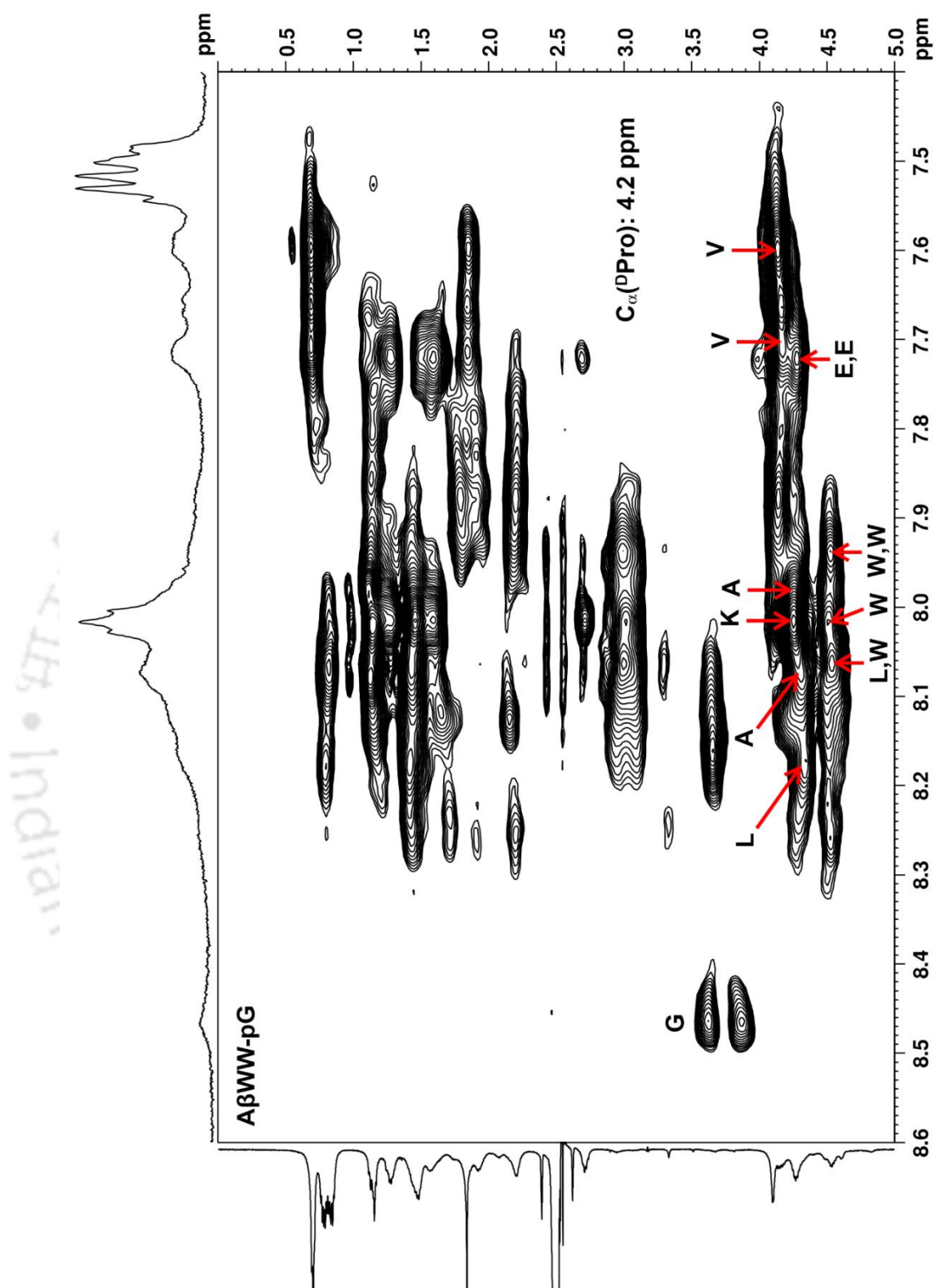


Figure 5.10C ^1H - ^1H TOCSY spectra of A β WW-pG recorded in DMSO- d_6 .

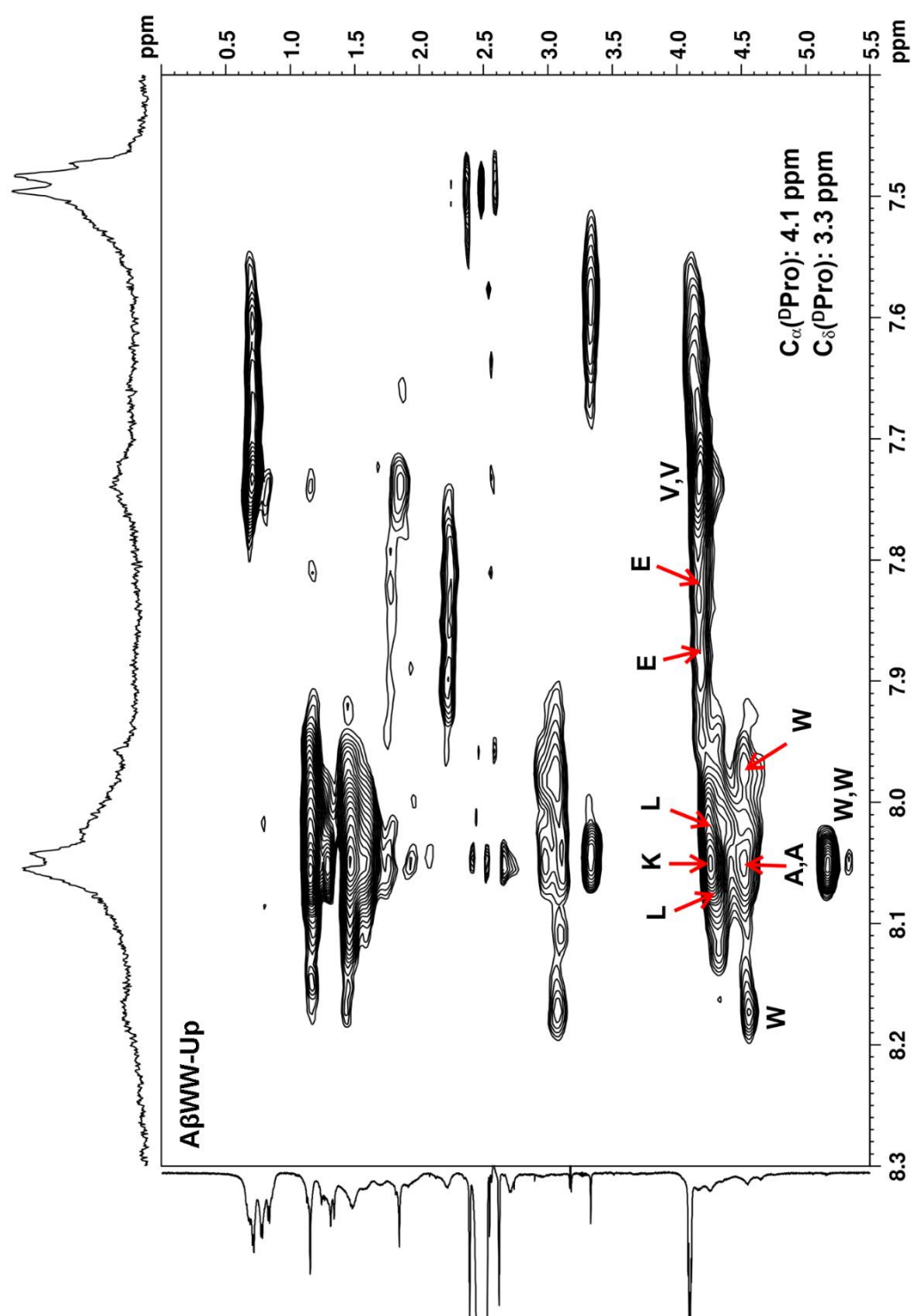


Figure 5.10D ^1H - ^1H TOCSY spectra of A β WW-Up recorded in DMSO- d_6 .

The chemical shifts of C_{α} protons ($H_{C_{\alpha}}$) and the amide protons (H_N) in random-coil conformation are shown in table 5.1. The random-coil chemical shifts have been adapted from the study carried out by Peter Wright and coworkers on model GGXGG peptides in water (10% D_2O) where 'X' is one of the 20 amino acids [17].

Table 5.1 The random coil chemical shifts of $H_{C_{\alpha}}$ and H_N for the amino acids used in this study. The values have been adapted from [17].		
Amino acid	$\delta (H_{C_{\alpha}})$ ppm	$\delta (H_N)$ ppm
Lys	4.32	8.67
Leu	4.35	8.64
Val	4.13	8.51
Phe	4.62	8.61
Tyr	4.56	8.59
Trp	4.67	8.46
Ala	4.34	8.67
Glu	4.29	8.83
Asn	4.76	8.76
Gly	4.01	8.66
DPro	4.44*	—
*The value indicated was essentially reported for LPro and has been adapted here for DPro .		

As the H_N protons can engage in H-bonding interactions; the H_N chemical shifts are solvent-dependent. The analysis of the spectra using CSI, therefore, is best carried out solely based on the $H_{C_{\alpha}}$ chemical shifts alone, unless the solvent used for all the samples is the same. The $H_{C_{\alpha}}$ and H_N chemical shifts obtained for the peptides are shown in table 5.2.

Chapter 5

Table 5.2 The chemical shifts of the H_{Cα} and H_N obtained from the TOCSY assignment. The protons that are upfield compared to the random coil chemical shifts are marked with yellow colour whereas the downfield ones are marked with green colour.

Amino acid	AβFF		AβFF-NG				AβFF-pG				AβFF-Up			
	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N
K	4.24	8.00	4.28	8.00	4.24	7.87	4.24	7.99	4.30	8.02	4.33	8.04	4.25	8.04
L	4.25	8.05	4.27	8.00	4.55	7.95	4.39	8.08	4.45	8.18	4.59	8.22	4.45	8.13
V	4.09	7.65	4.10	7.67	4.10	7.67	4.19	7.90	4.18	7.85	4.25	8.03	4.25	7.96
F	5.14	8.10	4.58	8.13	4.55	7.93	4.65	8.29	4.60	8.25	4.69	8.39	4.62	8.28
F	5.14	8.10	4.58	8.13	4.55	7.93	4.72	8.09	4.75	8.03	4.89	8.18	4.90	8.08
A	4.20	8.19	4.30	8.21	4.30	8.21	4.59	8.38	4.58	8.35	4.47	8.38	4.86	8.47
E	4.12	7.92	4.57	8.13	4.25	7.87	4.18	7.86	4.23	7.96	4.25	8.04	4.18	7.87
N	-	-	4.48	8.15	-	-	-	-	-	-	-	-	-	-
G	-	-	3.70	8.16	-	-	3.68	8.41	-	-	-	-	-	-
p	-	-	-	-	-	-	3.77	-	-	-	4.12	-	-	-
							4.26	-	-	-				
	AβFY		AβFY-NG				AβFY-pG				AβFY-Up			
	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N
K	4.16	8.08	4.25	8.00	4.26	7.88	4.28	7.99	4.33	7.69	4.31	8.02	4.27	8.00
L	4.25	7.99	4.28	7.93	4.29	7.97	4.44	8.10	4.38	8.00	4.52	8.14	4.42	8.07
V	4.09	7.61	4.10	7.65	4.09	7.64	4.16	7.75	4.07	7.62	4.22	7.95	4.21	7.89
F	4.43	8.02	4.46	8.03	4.46	8.03	4.54	8.13	4.64	7.99	4.57	8.27	4.51	8.18
Y	4.49	7.99	4.52	7.94	4.52	7.94	4.50	8.11	4.50	7.92	4.79	8.12	4.78	8.05
A	4.24	8.13	4.26	8.15	4.26	8.15	4.33	8.18	4.55	8.24	4.75	8.31	4.40	8.24
E	4.17	7.82	4.18	7.82	4.18	7.82	4.18	7.81	4.21	7.87	4.18	7.83	4.22	7.94
N	-	-	4.48	8.11	-	-	-	-	-	-	-	-	-	-
G	-	-	3.74	8.14	-	-	3.64	8.09	-	-	-	-	-	-
p	-	-	-	-	-	-	4.26	-	-	-	4.29	-	-	-
	AβYY		AβYY-NG				AβYY-pG				AβYY-Up			
	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N
K	4.26	7.98	4.26	8.00	4.26	7.89	4.32	7.69	4.27	8.00	4.30	8.03	4.30	8.03
L	4.27	8.08	4.28	7.99	4.30	7.95	4.28	8.15	4.35	8.04	4.42	8.09	4.42	8.09
V	4.13	7.66	4.11	7.66	4.09	7.64	4.16	7.78	4.10	7.65	4.10	7.65	4.19	7.94
Y	4.35	8.12	4.44	7.97	4.44	7.97	4.47	8.10	4.21	8.10	4.56	8.23	4.48	8.13
Y	4.35	8.12	4.42	7.85	4.42	7.85	4.40	7.98	4.27	8.00	4.31	8.03	4.67	8.00
A	4.15	8.05	4.49	8.12	4.49	8.12	4.48	8.20	4.40	7.98	4.65	8.23	4.35	8.17
E	4.09	7.92	4.18	7.80	4.26	8.08	4.16	7.88	4.12	7.83	4.19	7.95	4.17	7.85
N	-	-	4.33	8.12	-	-	-	-	-	-	-	-	-	-
G	-	-	3.62	8.15	-	-	3.64	8.13	-	-	-	-	-	-
p	-	-	3.76	-	-	-	4.26	-	-	-	4.27	-	-	-
	AβWW		AβWW-NG				AβWW-pG				AβWW-Up			
	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N	H _{Cα}	H _N
K	4.26	8.00	4.24	7.86	4.25	8.01	4.25	8.01	4.25	8.01	4.27	8.05	4.27	8.05
L	4.28	8.09	4.28	8.07	4.28	8.07	4.33	8.18	4.53	8.06	4.25	8.08	4.24	8.01
V	4.15	7.60	4.12	7.59	4.12	7.59	4.16	7.70	4.13	7.59	4.18	7.73	4.18	7.73
W	4.55	8.20	4.53	7.96	4.53	7.96	4.54	8.06	4.51	8.01	4.58	8.17	4.52	7.97
W	4.48	8.09	4.48	8.03	4.48	8.03	4.52	7.93	4.52	7.93	5.17	8.05	5.17	8.05
A	4.16	7.98	4.25	8.02	4.25	8.02	4.23	7.98	4.31	8.08	4.52	8.05	4.52	8.05
E	4.12	7.93	4.14	7.85	4.14	7.85	4.27	7.72	4.27	7.72	4.20	7.89	4.15	7.81
N	-	-	4.48	8.15	-	-	-	-	-	-	-	-	-	-
G	-	-	3.69	8.23	-	-	3.62	8.46	-	-	-	-	-	-
p	-	-	-	-	-	-	4.20	-	-	-	4.14	-	-	-

Comparison of Cα chemical shifts of various residues in the 7-residue peptides with random coil chemical shifts indicates that sequential shifts for three or more

residues are observed only for A β YY, which indicates a helical fold. Although the 7-residue peptides appear to aggregate in DMSO, there is no evidence for oligomers in β -strand conformation. The H_N protons may be involved in intermolecular hydrogen bonding. Hydrogen bonding only with the solvent would have resulted in considerably more downfield shifts.

As sequential assignment could not be made due to the large spectral overlap, the chemical shifts of turn-supporting dipeptidic regions *viz.* Asn-Gly, ^DPro-Gly, and Aib-^DPro were analyzed. The chemical shift values already reported in table 5.2 for these residues have been reproduced in table 5.3 to make comparison with the chemical shift values reported in the literature for these residues in turns. The H_N chemical shifts for Aib have not been included as Aib H_N could not be unambiguously assigned.

Model peptides harbouring Asn-Gly, ^DPro-Gly, and Aib-^DPro dipeptide motifs have been investigated using NMR spectroscopy [5, 18-22]. The H_{C α} chemical shift analyses of the ^DPro and Gly residues in the designed peptides wherein the turn is mediated by the ^DPro-Gly motif suggest a negative $\Delta\delta_{\alpha H}$ (observed $\delta_{\alpha H}$ - random-coil $\delta_{\alpha H}$) for the ^DPro residue whereas a small positive $\Delta\delta_{\alpha H}$ for Gly [20]. Balaram and coworkers have reported the ^DPro H_{C α} chemical shift of 4.27 ppm in type II' β -turn conformation mediated by ^DPro-Gly motif in a designed octapeptide in DMSO-d₆ [19]. The $\Delta\delta$ of -0.17 ppm is in agreement with the values reported in the literature for spectra recorded in water. Interestingly, however, the H_{C α} and H_N chemical shifts for the Gly residue in the same peptide were '3.70, 3.47' ppm ($\Delta\delta$ = -0.31, -0.54) and 8.18 ppm, respectively. The H_{C α} chemical shift values obtained for ^DPro and Gly in the A β ₁₆₋₂₂-derived peptides are in very good correlation with the chemical shifts reported for the octapeptide. The ^DPro-Gly motif, therefore, seems to be mediate a turn-like conformation in the ^DPro-Gly containing A β ₁₆₋₂₂-derived peptides.

Chapter 5

Table 5.3 The $H_{C\alpha}$ and H_N chemical shifts of the amino acids in the turn-supporting dipeptide motifs.						
Peptide	Asn8			Gly9		
	$H_{C\alpha}$	H_N	$\Delta\delta_{\alpha H}$ (observed $\delta_{\alpha H}$ – random-coil $\delta_{\alpha H}$)	$H_{C\alpha}$	H_N	$\Delta\delta_{\alpha H}$ (observed $\delta_{\alpha H}$ – random- coil $\delta_{\alpha H}$)
A β FF-NG	4.48	8.15	-0.28	3.70	8.16	-0.31
A β FY-NG	4.48	8.11	-0.28	3.74,3.64	8.14	-0.27,-37
A β YY-NG	4.33	8.12	-0.43	3.62,3.76	8.15	-0.39,-0.25
A β WW-NG	4.48	8.15	-0.28	3.69	8.23	-0.32
	D Pro8			Gly9		
	$H_{C\alpha}$	H_N		$H_{C\alpha}$	H_N	
A β FF-pG	4.26	–	-0.18	3.68,3.77	8.41	-0.33,-0.24
A β FY-pG	4.26	–	-0.18	3.64	8.09	-0.37
A β YY-pG	4.26	–	-0.18	3.64	8.13	-0.37
A β WW-pG	4.2	–	-0.24	3.62	8.46	-0.39
	Aib8			D Pro9		
	$H_{C\alpha}$	H_N		$H_{C\alpha}$	H_N	
A β FF-Up	–	–	–	4.12	–	-0.32
A β FY-Up	–	–	–	4.29	–	-0.15
A β YY-Up	–	–	–	4.27	–	-0.17
A β WW-Up	–	–	–	4.14	–	-0.30

Searle and coworkers have reported the chemical shift indices for Asn and Gly $H_{C\alpha}$ in the model peptides wherein the Asn-Gly motif supports a type I' turn [21, 23]. The $H_{C\alpha}$ of both the residues have negative $\Delta\delta$ values. The negative $\Delta\delta_{\alpha H}$ values observed for the A β_{16-22} -derived peptides suggest that the Asn-Gly motif supports a turn-like structure in these peptides. Rai *et al.* have reported the $H_{C\alpha}$ of 4.33 ppm for D Pro ($\Delta\delta = -0.11$) in DMSO- d_6 for a designed octapeptide that folds into a β -hairpin wherein Aib- D Pro mediate a tight type I' turn [22]. The upfield shifts obtained for the Aib- D Pro containing peptides suggest turn-like conformation.

The NOESY spectra recorded for the peptides did not allow complete assignment due to large structural overlaps and aggregation. NOESY spectra, however, supported the turn conformation for some of the peptides. In the D Pro-Gly mediated turns, the Gly H_N shows a strong NOE with the H_N of the next residue

in the sequence. Such NOE peaks were indeed observed for A β FF-pG and A β YY-pG peptides (Figure 5.11).

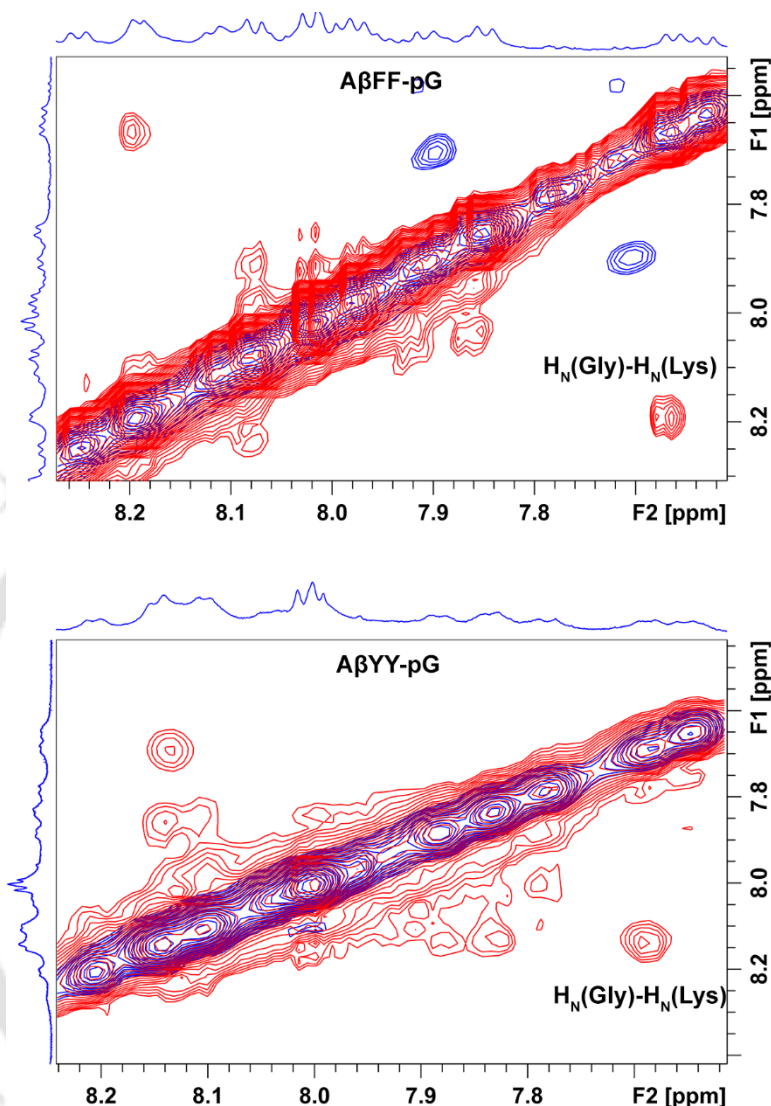


Figure 5.11 The NOE cross-peaks between the H_N of Gly with that of Lys observed for A β FF-pG (top) and A β YY-pG (bottom). The NOESY spectrum was overlaid on the TOCSY spectrum. The red contours are from the NOESY spectra. The blue contours are from the TOCSY spectra.

The H_N peaks in A β FY-pG, however, were overlapped, thereby not allowing this analysis. In A β WW-pG, such an NOE was absent, indicating that the D Pro-Gly motif might not mediate a defined β -turn. In fact, the $H_{C\alpha}$ of D Pro in A β WW-pG is somewhat more upfield compared to that in the other three peptides.

Chapter 5

The A β_{16-22} -derived β -turn supporting motif-harboring peptides have been shown to be highly amyloidogenic and hydrogelating in chapters 2 and 4. MD simulations carried out in water (Chapter 2) clearly indicate their propensity to fold into β -hairpin structures. The peptides, unfortunately, turned out to be poorly soluble in water. The NMR studies, therefore, were carried out in DMSO- d_6 . DMSO is a solvent that is often employed to dissolve the preformed peptide aggregates. It was of interest to examine whether the turn motif-harboring peptides adopted β -strand conformation in DMSO as observed for several model hydrophobic peptides with these turn-initiating motifs. Extensive spectral overlap made it difficult to do sequential assignment. The C α chemical shifts of non-turn promoting amino acids in the turn peptides do not suggest folded β -hairpin conformation as observed for the model peptides in DMSO. However, the H C_{α} CSI of the assigned amino acids suggests that the dipeptide motifs chosen to employ the β -turn in the peptides lie in the turn region, even in DMSO. The lack of β -hairpin structure for the A β_{16-22} -derived turn containing peptides reflects the intrinsic conformation propensity rather than solvent effect, as hydrophobic model peptides with these turn containing motifs do fold into well-defined β -hairpin structures in DMSO [1, 6]. The conformational propensities as deduced from NMR is an average overall structure in the conformational ensemble due to the relatively slow time scales in NMR experiments [24]. Hence, the transient β -hairpin conformations that are fast on the NMR time scale would not be detected. These transient structures, however, are likely to be important for initiating self-assembly and form cross- β structures when peptides from DMSO are transferred to aqueous solutions, as observed for these turn-containing peptides. Hence, when amyloid-forming peptides such as the ones investigated in this study, are dissolved in DMSO, they do not form β -hairpin structures but have a turn conformation at the turn potentiating residues that then assist the formation of cross- β structures in the aqueous environment. A β_{16-22} (A β FF), though gives sharp spectrum in the aqueous medium, indicating the absence of aggregates, it aggregates over a period of time as reported in the literature [25]. The other three 7-residue peptides, though largely unstructured in DMSO, form cross- β structures when transferred to aqueous medium [26].

This NMR study provides insights into the conformational propensities of amyloidogenic A β peptides in solution, prior to forming well-defined cross- β structures when assembled into amyloid fibrils.

5.4 CONCLUSIONS

The conformation of the turn-supporting dipeptide motifs in the repeats of A β_{16-22} and its aromatic analogs were investigated using NMR spectroscopy. The peptides, sadly, did not dissolve in water and methanol thereby hampering these studies. All the turn containing peptides could readily be dissolved in DMSO up to concentrations ≥ 0.8 mM which was good enough for NMR spectroscopic investigation. NMR studies were, therefore, carried out in DMSO- d_6 . Sequential assignment could not be carried out, largely due to the extensive spectral overlap alongside the peptide aggregation. The amino acid pairs and the β -turn supporting dipeptide motifs, however, could be assigned. The chemical shift index analysis indicated the turn conformations for these turn-supporting motifs.

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Chapter 5

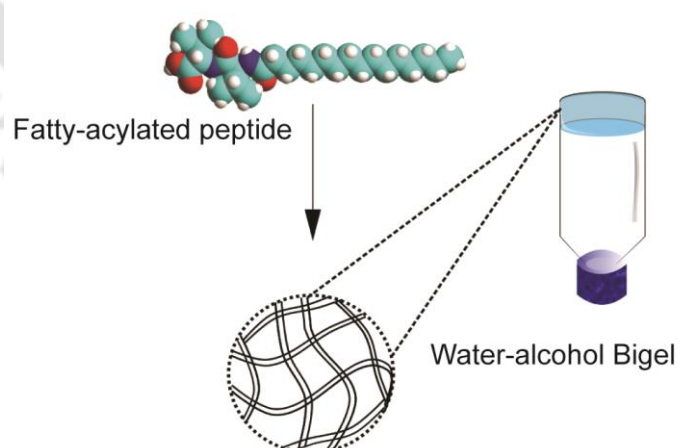
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WATER-ALCOHOL BIGELS FROM FATTY-ACYLATED DIPEPTIDES





6.1 INTRODUCTION

Chapters 3 and 4 discuss the entrapment and release of doxorubicin in the peptide hydrogels. A large number of drug molecules, however, are hydrophobic and consequently sparingly soluble in water. The poor water solubility of such drugs causes them to precipitate in hydrogels. Such drugs can readily dissolve in the organogels (gels of organic solvents), but the organogels may not be biocompatible for drug-delivery applications. Bigels, *i.e.* the gels of both water and an organic solvent, is an interesting class of materials. Bigels made up of bio-acceptable organic solvent, in particular, could be interesting drug-delivery vehicles. Bigels can hold hydrophobic drugs to high concentrations. The subsequent release of the drug would depend on the bigel network and solubility of the drug in water.

Self-assembly of very short peptides (2-3 amino acids) has resulted in a variety of superstructures that include fibers, tubes, rods, tapes, spheres, and vesicles [1-9]. Many of these peptides can self-assemble to form hydrogels and bigels, particularly those with free COOH. Peptide amphiphiles constitute yet another class of peptide-based gelators [10, 11]. The amphiphilicity is conferred either by suitably incorporating the hydrophilic and hydrophobic residues in the peptide sequence [12, 13] or by attaching long alkyl chains with the peptide molecules [11, 14]. Bhattacharya and coworkers reported the two-component gels; wherein fatty acids mixed with di- and multi-amines resulted in distinct hydrogels [15]. On a similar line, Xu and coworkers reported hydrogelation caused by sodium salts of fatty acids mixed with oligolysines [16]. Fatty-acylated amino acids and peptides are reported in the literature to cause hydrogelation and organogelation [17-19]. The idea is to obtain an amphiphilic molecule wherein the peptide contributes to the polar region, and the fatty-acyl chain contributes to the non-polar region. The gelator amount required for causing gelation of a solvent determines how good a gelator is. Good gelators would typically cause gelation at concentrations $\leq 2\%$ (w/w) concentrations, *i.e.* can fix at least 50 times the solvent [20]. In this chapter, an attempt has been, made to investigate the investigations into the self-assembly as well as bigel (alcohol/water gel) formation by the fatty-acylated

Chapter 6

dipeptides. Unlike previous studies wherein a polar peptide is linked to an acyl chain, in this case, hydrophobic aliphatic residues (Ile, Leu, and Val) has been chosen to make the peptidic region. Nagaraj and co-workers have previously reported highly ordered superstructures such as nanotubes and nanospheres from Boc-protected dipeptide methyl esters *viz.* Boc-Val-Ile-OMe, Boc-Val-Leu-OMe, Boc-Leu-Val-OMe, and Boc-Val-Val-OMe [21]. The permutation of the three amino acids in a dipeptide gives six different peptides. Three fatty acids *viz.* lauric acid, myristic acid, and palmitic acid were chosen for N-terminal acylation. This resulted in a total of 27 fatty-acylated peptides, wherein the C-terminal carboxyl group is the only polar group other than the peptide backbone (Figure 6.1). Amino acids with hydrophobic aliphatic side-chains are cheap resource material as side-chains need not be protected during synthesis. In addition to that, the high hydrophobicity of the peptides facilitates ready purification through water/dichloromethane extraction. Out of the 27 fatty-acylated peptides, 8 resulted in distinct alcohol/water gels. The gels were identified by tube-inversion and subsequently characterized by DLS-microrheology, infrared spectroscopy, and electron microscopy.

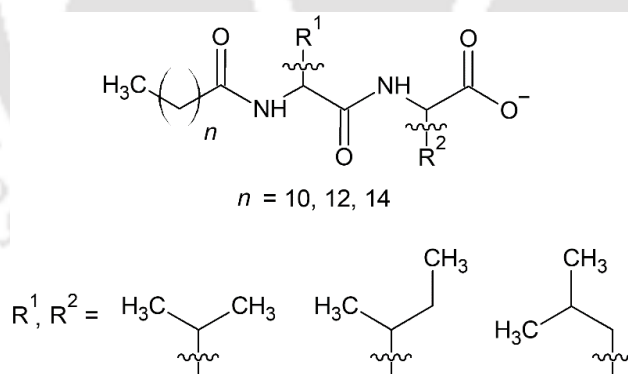


Figure 6.1 The fatty-acylated peptides employed in this study.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Fmoc-protected amino acids and HBTU were purchased from Novabiochem (Darmstadt, Germany). HMPB resin, myristic acid, palmitic acid, DIPEA, 4-dimethylaminopyridine (DMAP), *N,N'*-dicyclohexylcarbodiimide (DCC),

piperidine, TFA, and docetaxel were procured from Sigma-Aldrich Chemicals Pvt. Ltd. HOBt was obtained from Sisco Research Laboratory, India. Lauric acid was purchased from Himedia. *N,N*-dimethylformamide, glacial acetic acid, isoamyl alcohol, and rhodamine B were obtained from Merck, India. All other reagents were of the highest grade available.

6.2.2 Peptide synthesis and characterization

Fatty-acylated peptides were synthesized manually on HMPB resin by employing Fmoc-chemistry. The C-terminal amino acid was attached to the resin using DCC/DMAP-mediated coupling [22]. Coupling of the N-terminal amino acid and subsequent fatty-acylation was carried out using HBTU/HOBt/DIPEA activation. The synthesized peptides were cleaved from the resin using 95% TFA/5% water. The cleaved peptides were dried by nitrogen purging. The peptides were dissolved in DMSO, and the purity was assessed on a reversed-phase C₁₈ column on a Shimadzu Prominence Modular HPLC instrument using a linear gradient of acetonitrile containing 0.1% TFA. The peptides were subsequently characterized by MALDI-TOF mass spectrometry.

6.2.3 Self-assembly

The fatty-acylated peptides were weighed as required, and the 40 mg/ml stock solutions were prepared in methanol as well as ethanol. An equal volume of water was added subsequently, and the vials were left undisturbed for 24 h. Formation of self-supporting gels was ascertained by inverting the tubes and keeping them inverted for at least 12 h. The effective peptide concentration in the gels was 20 mg/ml in 50% alcohol/water co-solvent mixture.

6.2.4 TEM imaging

All the peptide samples, including non-gelating ones, were investigated using TEM. Briefly, 5 μ l of the 24 h-old samples were drop-casted on 200 mesh Formvar/Carbon coated copper grids. The excess sample was blotted out from the periphery of the grid by Whatman I filter paper. The grids were then stained with saturated uranyl acetate solution, which was blotted out after 30 seconds.

Chapter 6

The samples were dried overnight, and images recorded on a JEOL-2100F field emission TEM at 200 kV.

6.2.5 Scanning Electron Microscopy (SEM) imaging

The morphology of the eight 50% ethanol-water bigels was investigated using field emission SEM. Briefly, 10 μ l of the 24 h-old samples were drop-casted on clean glass slides and air dried. The samples were coated with gold prior to analysis. Images were recorded using a Zeiss field emission SEM (Model: Sigma 300) at 2–3 kV.

6.2.6 FTIR spectroscopy

FTIR spectra were recorded for the 24 h-old peptide samples as described in section 2.2.5, chapter 2.

6.2.7 Microrheology

DLS-Microrheology measurements were carried out for the bigels on a Malvern Zetasizer Nano ZS instrument with 633 nm laser and 173° non-invasive back-scattering detector configuration. Carboxylated melamine beads (GmbH, 0.6 μ m) were used as the tracer particle. The concentration of the tracer particles was optimized to prevent tracer aggregation and interaction with the sample. The temperature for all the experiments was fixed at 25 °C. The tracer particles were added to the fatty-acylated peptide samples such that the final peptide concentration was 20 mg/ml, and the final tracer volume fraction was 0.01%. The mean squared displacement (MSD) data were recorded after 24 h of incubation. Subsequently, the elastic and viscous moduli were derived from the MSD data using the inbuilt Malvern Zetasizer instrument software based on the generalized Stokes-Einstein equation as introduced by Mason [23].

6.2.8 Rhodamine B release assay

Rhodamine B release assay was carried out for the 50% methanol/water bigels. Rhodamine B was added during the formation of the gels in a quartz cuvette. Briefly, 40 mg/ml peptide solutions were prepared in methanol while a 400 μ M

rhodamine B solution was prepared in water. The peptide solutions were transferred to the quartz fluorescence cuvette. An equal volume of rhodamine B was subsequently added so as to obtain 20 mg/ml peptide bigels with 200 μ M rhodamine B. The samples were left undisturbed for 24 h for allowing gelation and dye uptake. Water (9-times volume) was gently added to the cuvette without disturbing the gel. The fluorescence emission intensity at 573 nm was recorded for the water phase continuously for 60 min after exciting the samples at 545 nm. The excitation and emission slit widths were 2.5 and 5 nm, respectively.

6.2.9 Docetaxel release assay

Docetaxel is a chemotherapeutic drug that is practically insoluble in water. The drug entrapment and subsequent release were studied using Ma-IV ethanol/water bigel as the representative bigel. Briefly, docetaxel dissolved in ethanol (20 μ M) was added to the peptide Ma-IV. After the peptide's dissolution, an equal volume of deionized water was added, and the sample was left undisturbed for 24 h to achieve gelation and drug uptake. The effective concentration of the peptide in the gel was 20 mg/ml, and the docetaxel concentration was 10 μ M in the final volume of 50 μ l. Release of docetaxel in 30% ethanol and PBS (9-times volume) was monitored by measuring absorbance at 230 nm.

6.3 RESULTS AND DISCUSSION

Fatty-acylated dipeptides were synthesized using valine, leucine, and isoleucine (amino acids) and lauric acid, myristic acid, and palmitic acid (fatty acids). Three choices for the amino acids in dipeptides and three choices for the N-terminal acylation resulted in 27 different peptides (Figure 6.1 and Table 6.1). The peptides were synthesized and characterized, as described in section 6.2.2. The sequences of the peptides, their IDs, the calculated monoisotopic masses, and the observed m/z in the MALDI-TOF MS are given in table 6.1.

Table 6.1 List of the fatty-acylated peptides employed in this study; their sequence, ID, calculated monoisotopic mass, and the m/z values obtained in MALDI mass spectrometry.

S. No.	Sequence	Peptide ID	Calculated monoisotopic mass (Da)	Observed m/z
1	Palmitoyl-Val-Val-COOH	Pa-VV	454.55	476.59
2	Myristoyl-Val-Val-COOH	Ma-VV	426.50	448.29
3	Lauroyl-Val-Val-COOH	La-VV	398.44	413.12
4	Palmitoyl-Ile-Val-COOH	Pa-IV	468.57	490.60
5	Myristoyl-Ile-Val-COOH	Ma-IV	440.52	462.44
6	Lauroyl-Ile-Val-COOH	La-IV	412.46	434.22
7	Palmitoyl-Leu-Val-COOH	Pa-LV	468.57	490.78
8	Myristoyl-Leu-Val-COOH	Ma-LV	440.52	462.40
9	Lauroyl-Leu-Val-COOH	La-LV	412.46	415.0
10	Palmitoyl-Leu-Leu-COOH	Pa-LL	482.58	504.77
11	Myristoyl-Leu-Leu-COOH	Ma-LL	454.53	476.57
12	Lauroyl-Leu-Leu-COOH	La-LL	426.47	448.34
13	Palmitoyl-Ile-Leu-COOH	Pa-IL	482.58	504.78
14	Myristoyl-Ile-Leu-COOH	Ma-IL	454.53	476.54
15	Lauroyl-Ile-Leu-COOH	La-IL	426.47	448.34
16	Palmitoyl-Val-Leu-COOH	Pa-VL	468.57	490.68
17	Myristoyl-Val-Leu-COOH	Ma-VL	440.52	462.57
18	Lauroyl-Val-Leu-COOH	La-VL	412.46	434.21
19	Palmitoyl-Ile-Ile-COOH	Pa-II	482.58	504.86
20	Myristoyl-Ile-Ile-COOH	Ma-II	454.53	476.59
21	Lauroyl-Ile-Ile-COOH	La-II	426.47	448.38
22	Palmitoyl-Val-Ile-COOH	Pa-VI	468.57	490.69
23	Myristoyl-Val-Ile-COOH	Ma-VI	440.52	462.49
24	Lauroyl-Val-Ile-COOH	La-VI	412.46	434.26
25	Palmitoyl-Leu-Ile-COOH	Pa-LI	482.58	504.86
26	Myristoyl-Leu-Ile-COOH	Ma-LI	454.53	476.57
27	Lauroyl-Leu-Ile-COOH	La-LI	426.47	448.40

The peptides, expectedly, were insoluble in water but readily dissolved in methanol and ethanol. The peptides were weighed and dissolved in methanol to achieve 40 mg/ml stock solutions. Self-assembly/gelation was initiated by adding an equal volume of deionized water to the peptide stock solutions; the 20 mg/ml (2 wt%) peptide solutions thus obtained in 50% methanol/water were kept undisturbed for 24 h at room temperature. The vials were subsequently examined for gelation by tilting and inverting the tubes.

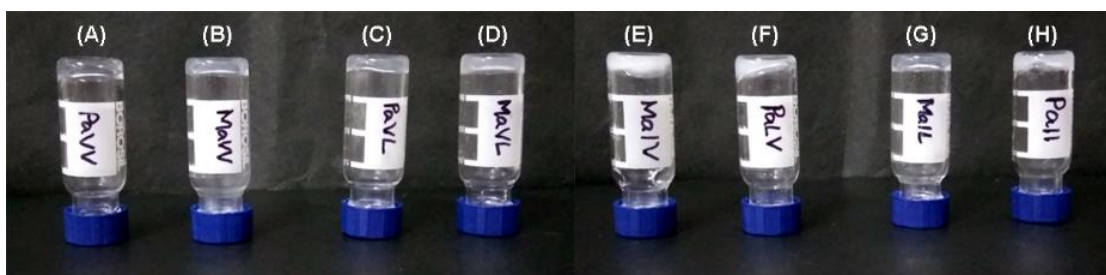


Figure 6.2 Images of the inverted tubes containing 24 h-old 20 mg/ml peptides in methanol/water; Pa-VV (A), Ma-VV (B), Pa-VL (C), Ma-VL (D), Ma-IV (E), Pa-LV (F), Ma-IL (G), and Pa-II (H).

None of the lauroyl peptides caused gelation of 50% methanol/water up to 20 mg/ml peptide concentration. Among the rest of the 18 peptides, eight viz. Pa-VV, Ma-VV, Pa-VL, Ma-VL, Ma-IV, Pa-LV, Ma-IL, and Pa-II resulted in self-supporting gels (Figure 6.2). Length of the acyl chain, therefore, plays a deciding role in the gelation of these peptides. Other than Pa-VV, which resulted in a transparent gel, the gels by other 7 peptides were either translucent or opaque. Out of the six homodipeptides with myristoyl/palmitoyl chains, only Pa-VV, Ma-VV, and Pa-II turned out to be gelating at 20 mg/ml concentration.

The gelation of 50% ethanol/water was also investigated at identical peptide concentration. The same set of 8 peptides that cause gelation of 50% methanol/water, when tested under the same condition, caused gelation of 50% ethanol/water as well (Figure 6.3).

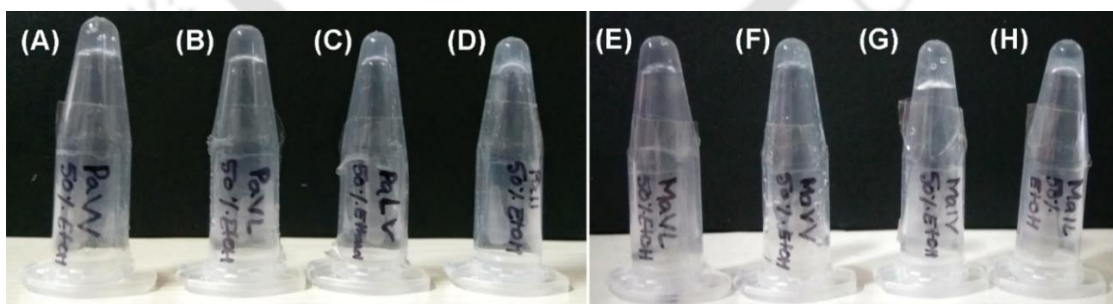


Figure 6.3 The tube inversion test for gelation of 50% ethanol/water; Pa-VV (A), Pa-VL (B), Pa-LV (C), Pa-II (D), Ma-VL (E), Ma-VV (F), Ma-IV (G), and Ma-IL (H).

6.3.1 Electron microscopy

To gain further insights into the self-assembled structures underlying gelation,

Chapter 6

the samples were analysed using TEM (Figure 6.4). All the gelating peptides except Pa-II form long fibrils that can entangle to form a mesh-like structure thereby trapping the solvent molecules. Pa-II forms a network of rod and ribbon-like structures. The non-gelating peptides, on the other hand, form short fibrous or non-fibrous structures. It is interesting to note that Pa-IV, Pa-IL, and Pa-VI form flake/ribbon-like structures. These data suggest both palmitoyl and myristoyl acyl chains, but not lauroyl, favour bigel formation. In addition, Val, an amino acid with branched β -carbon, is preferred over Ile and Leu to form fibrous aggregates and bigel.

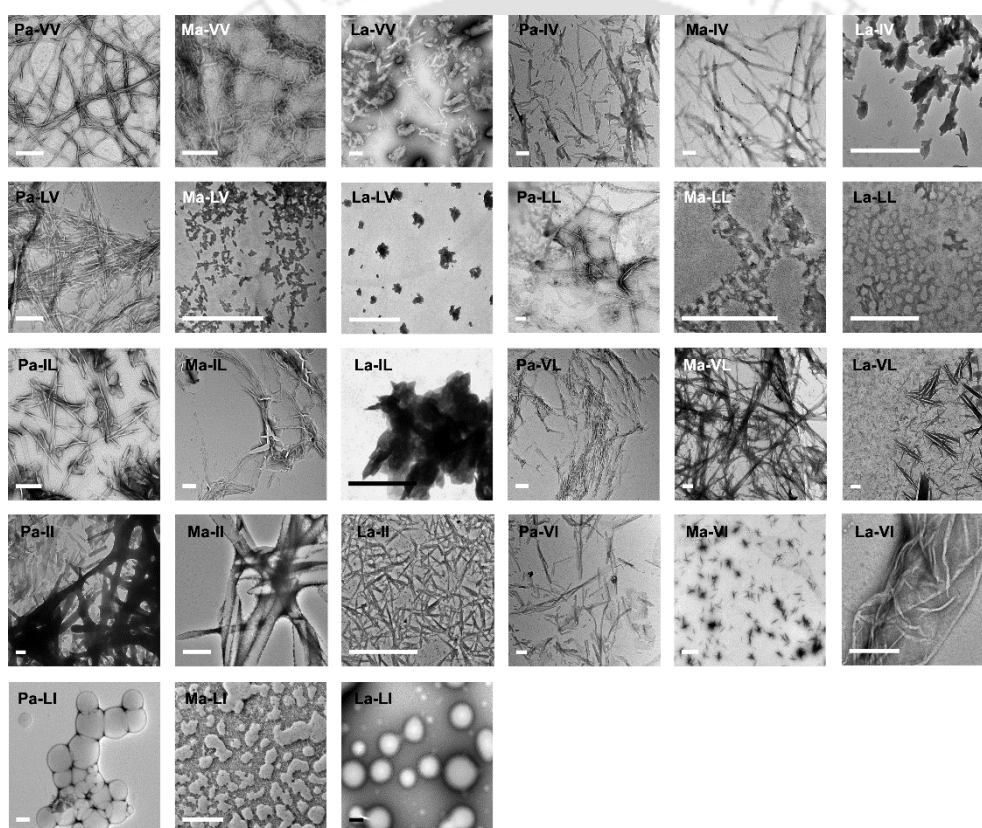


Figure 6.4 TEM images of the 24 h-old fatty-acylated dipeptide samples (20 mg/ml) in 50% methanol/water. The scale bar corresponds to 0.5 μm .

The 50% ethanol/water gels formed by the peptides were also investigated using SEM; all the 8 peptides show a dense network of fibrous aggregates (Figure 6.5).

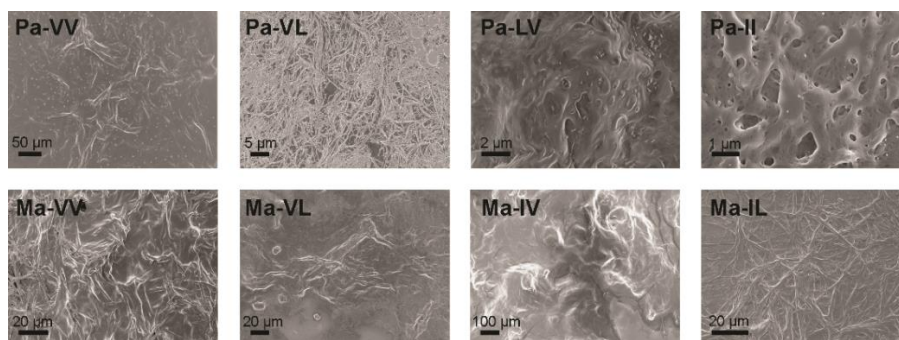


Figure 6.5 SEM images of 50% ethanol/water bigels.

6.3.2 FTIR spectroscopy

The secondary structure of the peptidic region in the self-assembled fatty-acylated peptides was investigated using FTIR spectroscopy of the dried peptide samples. The 24 h-old 20 mg/ml peptide samples in 50% methanol/water were deposited on the ZnSe ATR crystal and dried. The spectra obtained are shown in figure 6.6. The peptides Pa-VV, Ma-VV, Ma-IV, and Pa-II, showed distinct IR peaks around $1633\text{--}1634\text{ cm}^{-1}$, suggesting β -sheet formation [24]. Ma-VV, in addition to the $\sim 1633\text{ cm}^{-1}$ peak, shows a broad peak around 1681 cm^{-1} . Infrared bands around $1680\text{--}1695\text{ cm}^{-1}$ have been assigned to antiparallel β -sheets [25, 26]. Such bands have been reported in short self-assembling peptides [27–29]. Besides, the infrared bands around 1680 cm^{-1} have been assigned to turns and bands as well [30]. Infrared bands around $1680\text{--}1685\text{ cm}^{-1}$ have also been observed for the self-assembled structures of Boc-protected aliphatic dipeptide methyl esters [21]. The bands in such peptides have been assigned to the urethane (carbamate) group. As the fatty-acylated peptides investigated in this study lack urethane moiety and the turns/bands are unlikely for such small peptides, it is tempting to assign the $\sim 1681\text{ cm}^{-1}$ band to the antiparallel β -sheet conformation. It is, however, important to mention that the $1680\text{--}1695\text{ cm}^{-1}$ peak arises from the vibrational excitons running along the β -strands ($\nu_{||}$) [31]. The intensity of the band, consequently, does not depend on the number of strands. An extended antiparallel β -sheet made up through the self-assembly of small peptides is not expected to give the high frequency peak ($1680\text{--}1695\text{ cm}^{-1}$) with an intensity comparable to the low frequency ($1610\text{--}1635\text{ cm}^{-1}$) peak. However, such peaks

Chapter 6

have been assigned to the antiparallel β -sheets for the self-assembled structures of Fmoc-protected very short peptides [32-34]. Tuttle and coworkers investigated the hydrogels made up of Fmoc-AA and Fmoc-AA (9-fluorene-methylcarbonyl dialanine) and showed a high frequency peak to be arising from the carbamate group [35]. The IR spectra of the fatty-acylated peptides are considerably different from those typical of the amyloid fibril forming proteins/peptides [24]. It is, therefore, inappropriate to assign the 1681 cm^{-1} band to the antiparallel β -sheet conformation.

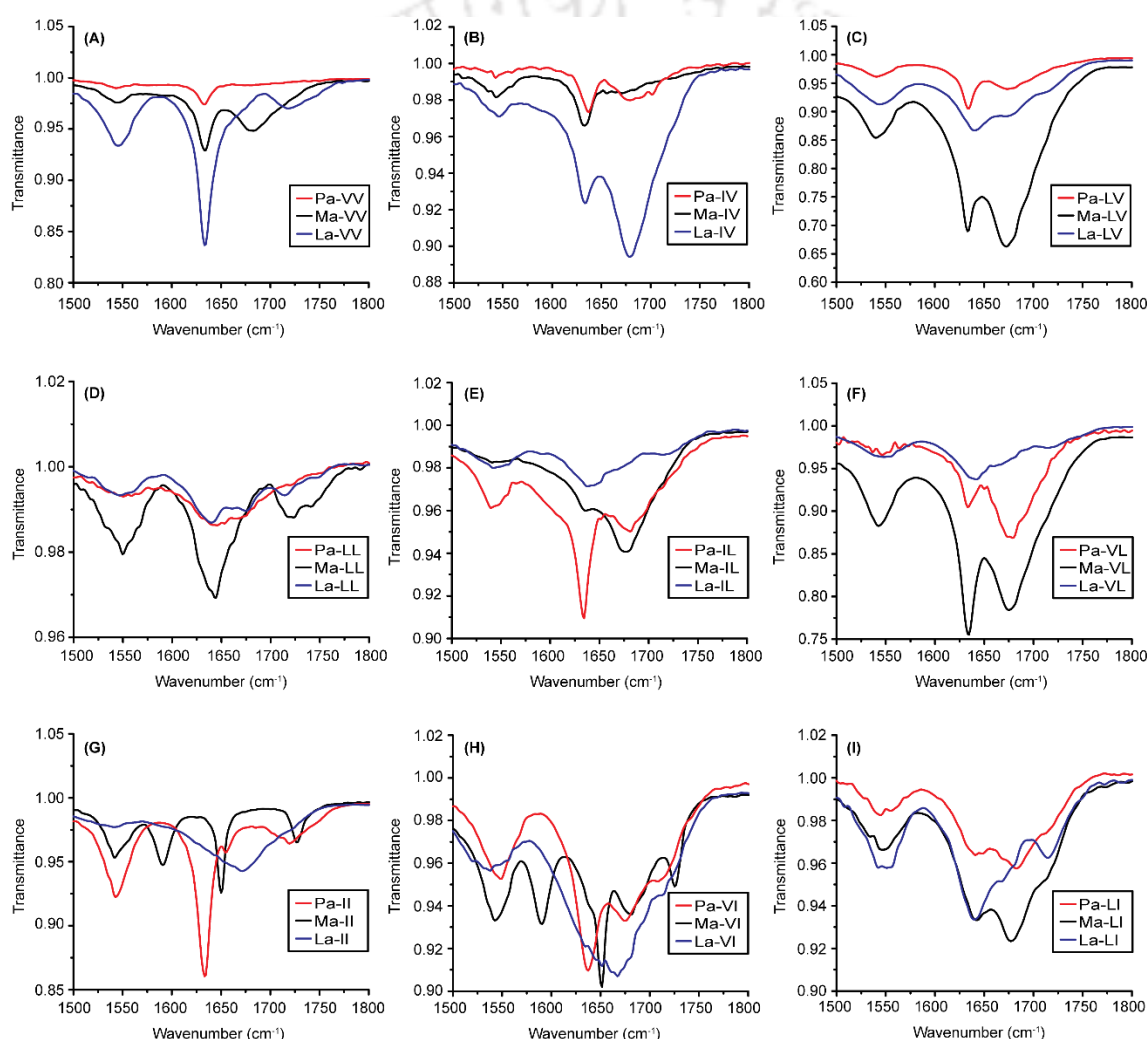


Figure 6.6 ATR-FTIR spectra recorded for the fatty-acylated peptide samples, dried from the 20 mg/ml methanol/water bigels.

All the leucine containing gelators viz. Pa-VL, Ma-VL, Ma-IL, and Pa-LV display bands around 1634 cm^{-1} and 1675 cm^{-1} in the amide I region. The 1634 cm^{-1} peak is

assigned to the β -sheet conformation. The high frequency peak (1675 cm^{-1}) is likely to be arising from the residual TFA present in the peptides. TFA strongly absorbs in the amide I region, giving a peak around 1673 cm^{-1} . The peptides, as mentioned in section 6.2.2, were cleaved from the resin using 95% TFA. TFA was subsequently evaporated under nitrogen stream to obtain the dried peptides. Therefore, the peaks obtained around $1672\text{--}1681\text{ cm}^{-1}$ are assigned to TFA. Among the non-gelating peptides, none of the VI, LI and LL fatty-acylated peptides displayed a peak $\sim 1634\text{ cm}^{-1}$. However, it is to be noted that the peptide Pa-VI that shows an infrared band at 1638 cm^{-1} with a shoulder around 1634 cm^{-1} forms short fibrous aggregates. La-VI displays an unusually broad amide I band and forms fibrous structures. La-VV, interestingly, shows a spectrum very similar to Pa-VV but forms shorter aggregates that possibly accounts for its non-gelation property. In the isoleucylvaline peptide series, Ma-IV causes gelation and displays a distinct peak at 1634 cm^{-1} . La-IV does show the 1634 cm^{-1} peak alongside an intense 1678 cm^{-1} peak but fails to form fibrillar structures. Pa-IV displays the IR band at 1637 cm^{-1} and fails to cause gelation. Pa-LV, the sole gelating member of the leucylvaline series, shows IR bands at 1633 cm^{-1} and 1672 cm^{-1} . The non-gelating member Ma-LV displays the bands at same wavenumber, albeit with higher intensity for the 1672 cm^{-1} band. The other non-gelating member La-LV shows the lower energy IR band at 1640 cm^{-1} instead of $1633\text{--}1634\text{ cm}^{-1}$. The only non-gelating member of the valylleucine series, La-VL also displays the IR band around $1640\text{--}1641\text{ cm}^{-1}$. Lauroylated and myristoylated di-isoleucine, unlike palmitoyl di-isoleucine that displays an IR band at 1634 cm^{-1} and causes gelation, display amide I band at wavenumber 1650 cm^{-1} or higher. The myristoylated isoleucylleucine displays IR bands around 1636 cm^{-1} and 1675 cm^{-1} and forms gel. Pa-IL with IR bands around 1634 cm^{-1} and 1675 cm^{-1} , however, fails to cause gelation. The other non-gelating member, La-IL displays infrared band $\sim 1638\text{ cm}^{-1}$. These data provide useful insights into the gelation propensity of the peptides. All the gelators display an amide I infrared band around $1634\text{--}1636\text{ cm}^{-1}$. These data indicate that β -conformation of the peptidic region is necessary but not sufficient for the peptide to be gelating. The length of the acyl chain and the amino-acid side chain chemistry do play a critical role in the assembly and

Chapter 6

gelation. The infrared spectra were also recorded for the 50% ethanol/water gel samples. Each of the gel samples showed an infrared band around 1632–1634 cm⁻¹ (Table 6.2).

Table 6.2 FTIR data of gel-forming fatty-acylated peptides in ethanol-water.		
S. No.	Fatty-acylated peptide	Major FTIR peaks in amide I region (cm ⁻¹)
1.	Pa-VV-COOH	1633.42
2.	Ma-VV-COOH	1632.89, 1682.07
3.	Pa-VL-COOH	1633.78, 1681.01
4.	Ma-VL-COOH	1634.36, 1682.05
5.	Ma-IV-COOH	1633.61
6.	Pa-LV-COOH	1634.49
7.	Ma-IL-COOH	1633.35
8.	Pa-II-COOH	1634.48

6.3.3 Microrheology

The viscoelastic nature of the gels was investigated using DLS-microrheology (DLS- μ R) that allows determination of the viscoelastic properties of a medium by measuring the thermally-driven displacement of the probe particles in the medium. The mean squared displacement (MSD) of the particles is dictated by the rheological properties of the medium. A plot of log (MSD) against log (time) gives a straight line with slope 1 in an ideally viscous medium. The slope is zero in a purely elastic medium and will lie between 0 and 1 for a viscoelastic medium [36, 37]. The viscoelastic properties of the gels are examined using DLS- μ R measurements with 600 nm carboxy-melamine tracer particles. The MSD of the probe particles in the 50% methanol/water gels are shown in figure 6.7. The MSD of the palmitoylated peptide gels show roughly linear plot at smaller time scale but eventually flatten out at longer time points indicating elastic properties. The myristoylated gelators, like palmitoylated ones, show linear MSDs at lower time scale but the slope decreases gradually with time.

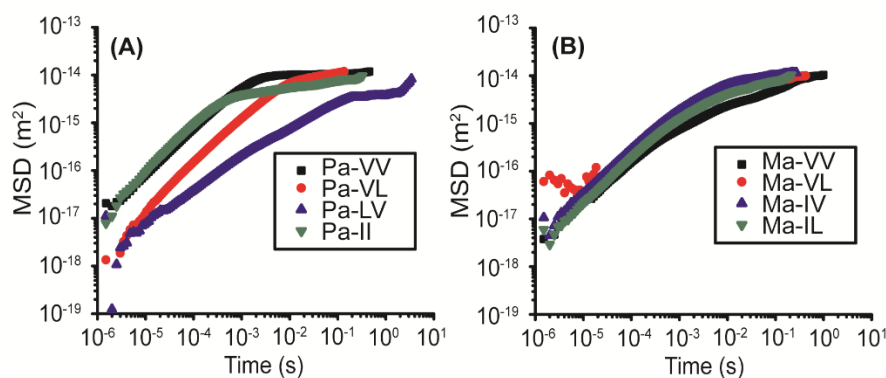


Figure 6.7 MSD data for Pa-VV, Pa-VL, Pa-LV, Pa-II (panel A) and Ma-VV, Ma-VL, Ma-IV, Ma-IL (panel B) gels in 50% methanol/water.

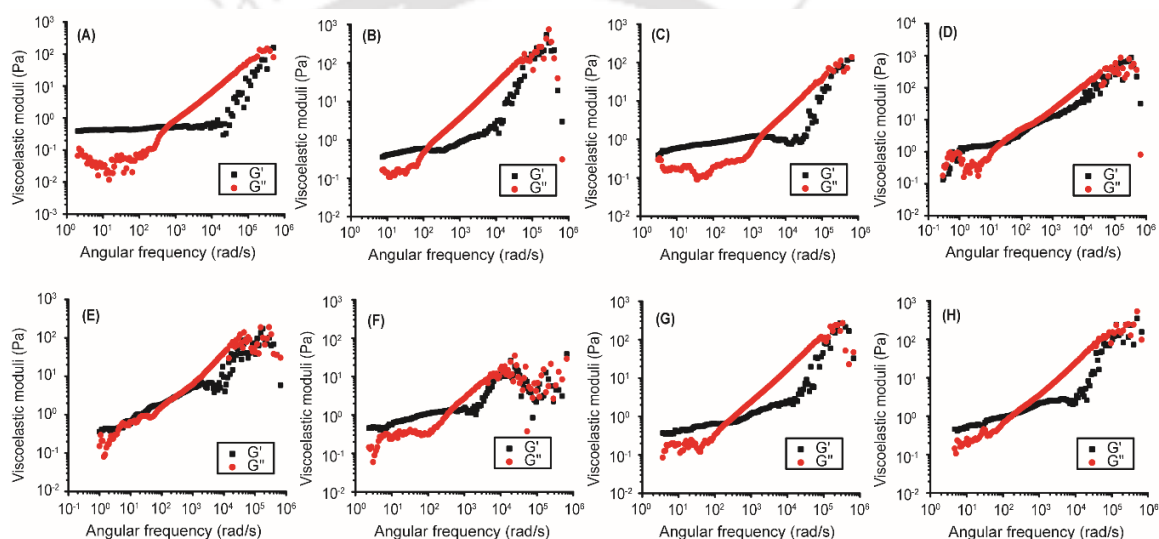


Figure 6.8 The G' (black squares) and G'' (red circles) data, as determined from the MSD data using generalized Stokes-Einstein relation; Pa-VV (A), Pa-VL (B), Pa-II (C), Pa-LV (D), Ma-VV (E), Ma-VL (F), Ma-IV (G), and Ma-IL (H) in 50% methanol/water.

The storage (G') and loss (G'') moduli for the gels were derived from the MSD data using the generalized Stokes-Einstein relation and are shown in figure 6.8 [26]. The top panels correspond to the palmitoylated peptides and show G' values higher than G'' ones at lower frequencies. Pa-VV shows G' values higher than G'' up to ~ 200 rad/s (panel A) while Pa-II shows up to at least 1000 rad/s (panel C). The high G' - G'' crossover frequencies indicate shorter relaxation times. The other gelators except Ma-VV show G' slightly higher than the G'' supporting their gel-like nature. MaVV, interestingly shows comparable G' and G'' values in the ~ 1 -1000 rad/s frequency range, indicating comparable elastic and viscous properties.

Chapter 6

The DLS- μ R data for the 50% ethanol/water gels are given in figures 6.9 and 6.10. The viscoelastic properties of the peptides Pa-VV, Pa-II, Pa-LV, and Ma-VV, have rather notable differences when compared to the gels in 50% methanol/water. The 50% methanol/water Pa-VV gel shows a storage modulus of ~ 0.4 - 0.5 Pa in the 2-10,000 rad/s range. The 50% ethanol/water gel, however, shows a G' value of 4 Pa at 2 rad/s that gradually increases with frequency and goes beyond 10 Pa at 10^4 rad/s. In 50% ethanol, PA-II displays comparable G' and G'' values from ~ 50 rad/s onward whereas the distinct difference between G' and G'' values was obtained from ~ 5 – 1000 rad/s in 50% methanol/water. The 50% methanol/water gel of Pa-LV displays gel-like properties, *i.e.* G' higher than G'' within ~ 1 -10 rad/s (panel D, Figure 6.8). The 50% ethanol/water gel, on the other hand, displays gel-like properties from <1 -500 rad/s. Ma-VV that shows comparable G' and G'' values in the ~ 1 -1000 rad/s frequency range in 50% methanol (panel E, Figure 6.8), displays G' values higher than G'' values in the 1-1000 rad/s range.

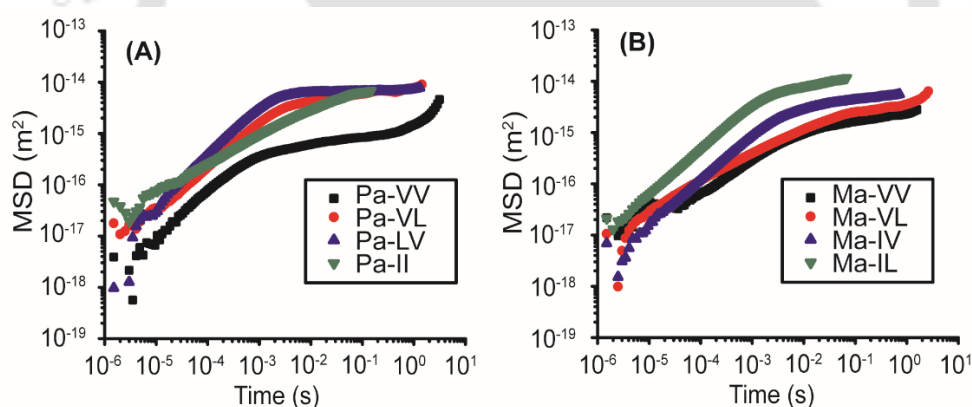


Figure 6.9 MSD data of Pa-VV, Pa-VL, Pa-LV, Pa-II (A), and Ma-VV, Ma-VL, Ma-IV, Ma-II (B) 50% ethanol/water bigels.

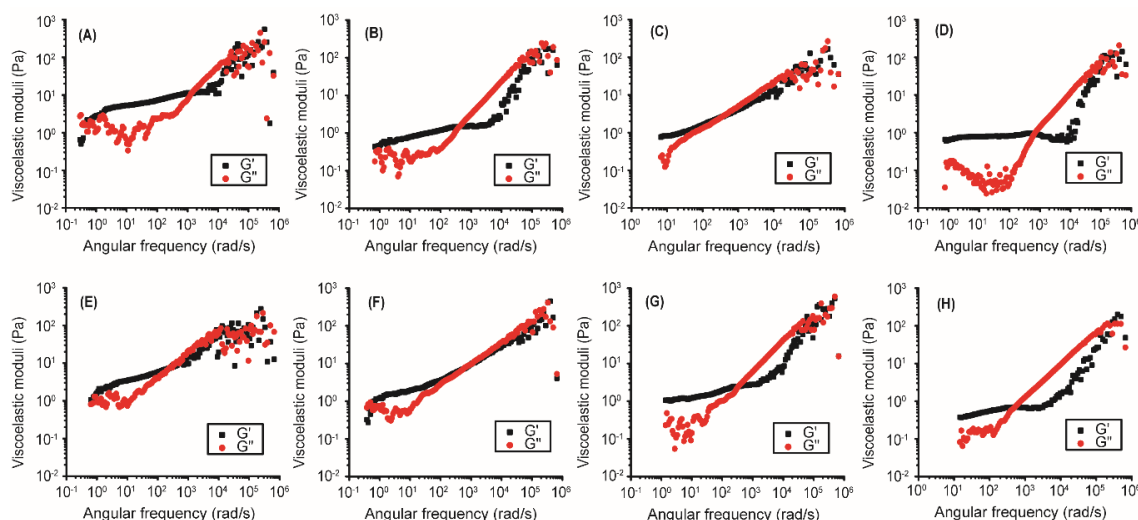


Figure 6.10 The G' (black squares) and G'' (red circles) data, as determined from the MSD data using generalized Stokes-Einstein relation; Pa-VV (A), Pa-VL (B), Pa-II (C), Pa-LV (D), Ma-VV (E), Ma-VL (F), Ma-IV (G), and Ma-IL (H) 50% ethanol/water bigels.

6.3.4 Rhodamine B release assay

The entrapment of rhodamine B was investigated with the methanol/water gels. The peptides were allowed to gel in the presence of 200 μM dye; uptake of dye by the gels rendered it red in colour. The subsequent release of the dye was observed by gently adding water on top of the gel and recording rhodamine B fluorescence at 573 nm over time. The rhodamine B release data is shown in figure 6.11. All the gels resulted in a rather quick release of dye; 50% or more rhodamine B is released in water within 20 minutes.

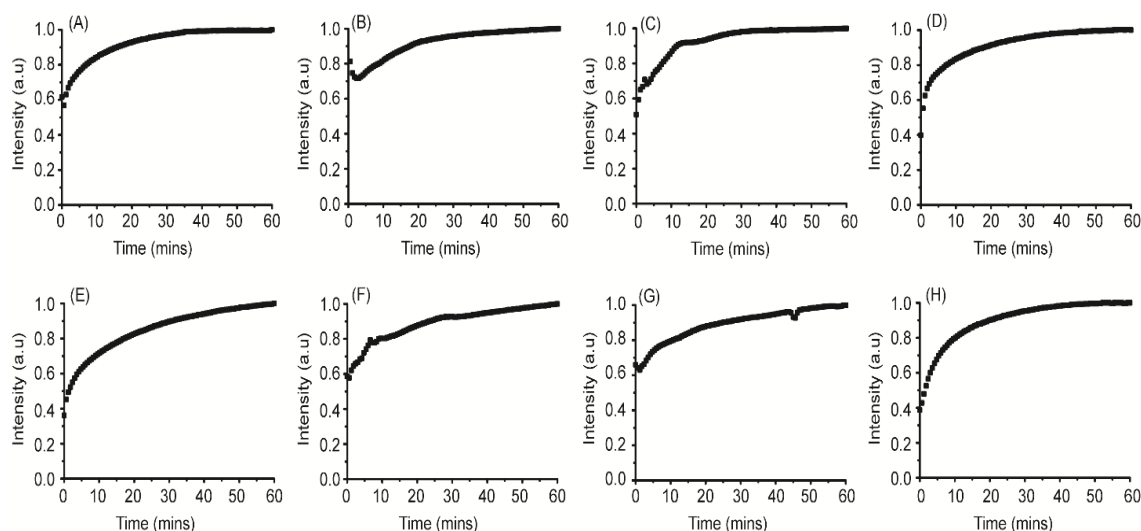


Figure 6.11 Rhodamine B release from Pa-VV (A), Pa-VL (B), Pa-LV (C), Pa-II (D), Ma-VV (E), Pa-VL (F), Ma-IV (G), and Ma-IL (H) 50% methanol/water bigels, as monitored by recording fluorescence emission at 573 nm.

6.3.5 Docetaxel release

As it is not appropriate to use methanol containing gel for drug delivery application, the release of the anticancer compound, docetaxel was investigated from 50% ethanol/water Ma-IV bigel as a representative example. The drug was loaded during the gelation process, and subsequent release was monitored in 30% ethanol and PBS by recording absorbance at 230 nm. The drug showed a rapid release in 30% ethanol but a sustained release for 3 days was observed in PBS (Figure 6.12).

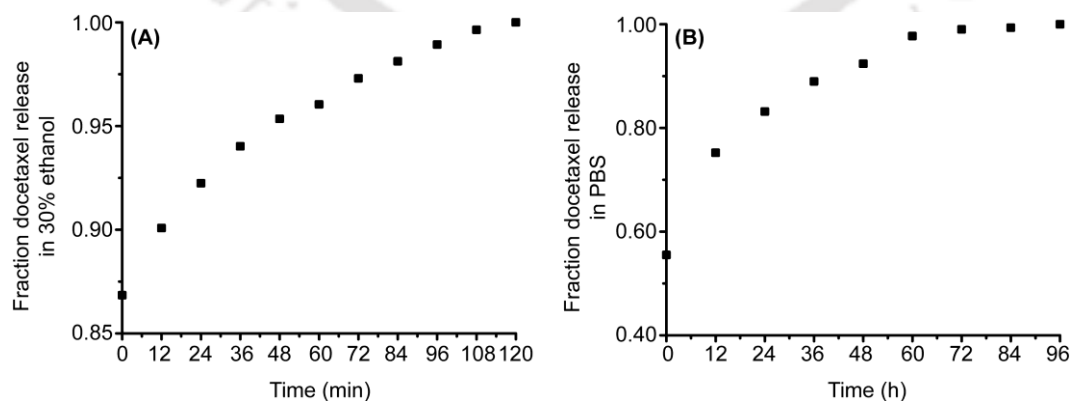


Figure 6.12 Fraction docetaxel release from Ma-IV 50% ethanol/water bigel.

6.4 CONCLUSIONS

Peptide-based gels have shown promising applications in drug delivery and regenerative medicine. Their utility, however, is hampered by the high cost associated with the synthesis of peptides composed of several amino acids as in the case of the hydrogel-forming peptides [38]. It is, therefore, imperative to design synthetically easily amenable peptide-based gelators. Nagaraj and coworkers have reported the self-assembly of Boc-protected aliphatic dipeptide methyl esters Boc-Val-Ile-OMe, Boc-Val-Leu-OMe, Boc-Leu-Val-OMe, Boc-Val-Val-OMe in 10%, 50%, and 100% methanol [21]. All the four peptides formed tubular or needle-like nanostructures. No fibrillar morphology was discernible. In this chapter, the self-assembly and gelation potential of the fatty-acylated dipeptide acids wherein the peptidic region is made up of Val, Leu, and Ile has been explored. Due to their high hydrophobicity, the peptides can be easily purified by extracting them in dichloromethane. The peptides turned out to be insoluble in water but readily dissolved in methanol and ethanol. Addition of water to these solutions resulted in distinct gels after 24 h incubation for eight of the peptides viz. Pa-VV, Ma-VV, Pa-VL, Ma-VL, Ma-IV, Pa-LV, Ma-IL, and Pa-II. It is interesting to note that three of the dipeptides, viz. Val-Val, Val-Leu, and Leu-Val caused gelation when palmitoyl is the fatty acid chain. Even though Boc-Val-Ile-OMe, like other three peptides, formed tubes, none of the fatty-acylated Val-Ile peptides caused gelation of 50% methanol/water and 50% ethanol/water solutions up to 20 mg/ml concentration. The self-assembled structures underlying the fatty-acylated peptide bigels show infrared amide I band around 1634 cm^{-1} , an IR band characteristic of β -sheets. The Boc-dipeptide esters, on the other hand, primarily display the major amide I band between $1643\text{--}1653\text{ cm}^{-1}$ suggesting a very different self-association mode.

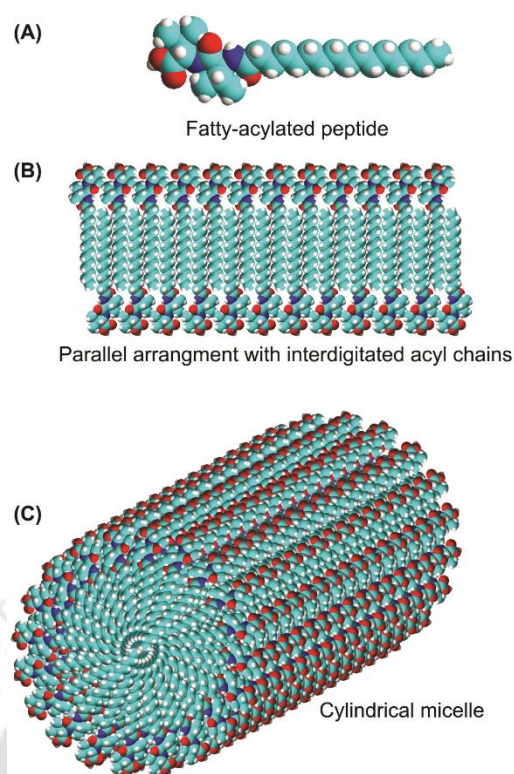


Figure 6.13 Schematic models of probable self-assembling modes of the fatty-acylated dipeptides; A fatty-acylated dipeptide (A), a parallel arrangement of the peptide with interdigitated acyl chains (B), and a cylindrical micellar arrangement of the peptide chains with acyl chains buried in the core (C).

Amphiphiles wherein the polar head group are bulkier than the hydrophobic tail, *i.e.* the ones that have a somewhat conical geometry, tend to self-assemble into micelles. Such conical peptide amphiphiles have been reported to self-assemble into spherical and cylindrical micelles [18, 39]. The fatty-acylated peptides investigated here harbour the C-terminal carboxylate group and the backbone amides as the only polar moiety. As the peptidic region is bulkier than the acyl chains, the peptide could self-assemble with the modes shown in figure 6.13. Association shown in panel B is the parallel-arrangement of the peptides wherein the acyl chains are interdigitated. Such an arrangement can lead to superstructures as a result of strong hydrophobic interactions between the fatty acyl chains and side chains of hydrophobic amino acids. This mode of assembly can lead to the formation of thin sheet-like structures. The sheet/flake-like structures observed for La-VV, La-IV, Pa-IV, Pa-II and the ribbon-like structures found for Pa-IL are likely to originate through this mode of assembly. The

association mode shown in panel C is that of a cylindrical micelle, similar to that reported for peptide amphiphiles by Stupp and coworkers [18]. Such an assembly mode would result in long fibrous structures as observed for Pa-VV, Ma-VV, Ma-IV, Pa-VL, and Pa-LV.

To conclude, this chapter presents the study showing the formation of the water/methanol and water/ethanol bigels from simple fatty-acylated dipeptide acids. The low solubility of many hydrophobic drugs in water limits the applications of the hydrogels. The ethanol/water bigels reported here could serve as drug delivery vehicles for hydrophobic drugs as demonstrated here by trapping the anticancer drug, docetaxel in the Ma-IV gel. The drug displayed a gradual release for over three days. This study shows that rather simple amphipathic peptides are attractive gelator candidates with potential application in drug delivery.

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Chapter 6

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CONCLUSIONS AND FUTURE DIRECTIONS





7.1 CONCLUSIONS AND FUTURE DIRECTIONS

Soft matter, that includes colloids, foams, gels, surfactants, liquid crystals, and biological materials, constitute an important class of materials that exhibit large response to relatively small forces. Such materials have very diverse applications such as food additives, paints, adhesives, packaging materials, lubricants, drug-delivery agents, and many more. Gels have gained considerable attention of the material scientists. They are characterized by their distinct viscoelastic properties. Hydrogels have been shown to find applications in sustained drug delivery and regenerative medicine as far as biomedical applications are concerned. This thesis explored and unveiled some of the potent gelator molecules based on short self-assembling peptides and fatty-acylated peptides.

The highlights of the research findings of the chapters and their potential future possibilities are discussed below.

Chapter 2

Taking the well-studied amyloidogenic fragment from β -amyloid, $A\beta_{16-22}$ as an example, this study shows that connecting two repeats of the self-assembling sequences through β -turn-inducing motifs can have dramatic effects on the self-assembling propensities of the peptides. $A\beta_{16-22}$ aromatic analog $A\beta YY$ turned out to be particularly interesting. The peptide did not self-assemble under the conditions tested in this study. The β -turn motif inducing connected, repeats, however self-assembled to form fibrous structures. It can therefore be concluded that the turn-inducing motifs can have determining effects on the self-assembly of the peptides that have little or no-self-assembling propensity. While only three types of turn-inducing motifs were viz. Asn-Gly, D Pro-Gly, and Aib- D Pro were employed in this study, the studies can further be extended to the other turn-inducing motifs as well. Motifs like Aib- D Ala and Aib-Gly units are known to adopt a type I' β -turn conformation whereas D Pro- L Pro takes well-defined type II' β -turn. In fact, if the residues constituting the strands have some intrinsic self-assembling propensity, a rather flexible turn-supporting dipeptide motif like Gly-Gly would be interesting to explore. Such flexibility would allow to minimize the

Chapter 7

inter-strand steric clashes and optimize the cross-strand interactions. Even though A β_{16-22} -derived peptides have been investigated here, the idea should be applicable to other peptides as well that self-assemble with an antiparallel strand conformation. In addition, it would be interesting to see if the peptides that self-assemble into parallel β -sheet structure can assemble with antiparallel arrangement, when connected by a tight-turn inducing motif. Such an assembly could result in superstructures with very different emergent properties. Additionally, several turns containing peptides are known to have antimicrobial activity. The possibility of the β -turn-harboring peptides to show such activity could also be tested against microbial pathogens.

Chapter 3

This chapter discusses the hydrogelating potential of the A β_{16-22} aromatic analog, A β FY and its potential as a drug delivery vehicle and as cell culture scaffold. It is interesting to note that a subtle substitution wherein Phe₂₀ in A β_{16-22} is replaced by Tyr becomes a very potent hydrogelator with gelating concentration as low as 0.18 wt%. This study shows that the amyloidogenic peptides or other non-gelating self-assembling short peptides could be engineered to give hydrogels. A number of very short amyloidogenic stretches have been identified from proteins of human origin. Tailoring such peptides, with minimum perturbation, to form gels could have interesting biomedical applications as such self or self-like sequences are likely to exhibit no or little immunogenicity.

Chapter 4

The findings of chapter 4 showed A β FF(β -turn)A β FF and A β FY(β -turn)A β FY peptides to be hydrogelators. Few notable things include: gelation by A β FF-turn containing repeats (A β FF was non-gelating in itself), gelation by A β YY-turn containing repeats (A β YY was non-amyloidogenic), and mechanoresponsiveness of the gels (A β FY was non-responsive; the sheared gels did not heal.). The turn-harboring peptides, though resulted in fibrillary structures only, the emergent fibrillary properties are distinct: they cause gelation. Studies with other short self-assembling peptides, rich in polar residues in particular, would be worth

investigating. Such peptides might result in hydrogels at further lower concentrations. Low gelating concentration adds a high commercial value to hydrogelating peptides.

Chapter 5

Largely because of the peptides' poor solubility in water, the NMR spectroscopic analysis of the peptides having turn-supporting motifs could not be investigated in water. The chemical shift index analysis of the peptides dissolved in DMSO indicted turn for the dipeptide motifs. In fact, if the peptides were soluble to high concentrations in water, they are expected to self-assemble to give large aggregates that would lead to further spectral broadening. As the peptides readily dissolve in HFIP, the conformation of the turn-supporting motifs can be investigated using solid-state NMR techniques for the dried self-assembled structures obtained in 10% HFIP. The exact conformation of the turn-inducing residues could help in optimizing these motifs.

Chapter 6

Chapter 6 presents the self-assembly and bigel formation by fatty-acylated dipeptides. Literature is abounding with the self-assembly and gelation of alkylated and lipidated peptides. A great majority of these studies employ polar residues to impart amphipathicity. The study reported in this chapter shows gelation from the fatty acylated peptides wherein simple hydrophobic aliphatic amino acids constitute the peptidic region. Such amino acids are rather cheap compared to the polar residues as side-chain protection is not required. Even though, only bigel formation has been investigated in this study with alcohol/water as the cosolvent mixture, the potential of causing organogelation can also be explored. Only peptide acids were shown to cause gelation of alcohol/water mixtures in this study. It would be interesting to explore the C-terminal amide and methyl ester chemistry as well. In fact, dipeptide methyl esters of such dipeptides has been shown to form ordered structures. In addition, it would also be interesting to study the coassembly of peptides having alkyl chains at amino and C-termini.

Chapter 7

The research work presented in this thesis explores the rather unharnessed potential of the short amyloidogenic peptides as potential gelators. The data suggest that the self-assembling and gelating propensities of such peptides can be modulated through subtle modifications or through incorporation of fold-inducing motifs. In addition, the rather simple peptide conjugates are reported to cause gelation. The short self-assembling peptides reported in literature, including the amyloidogenic peptides, therefore can be tailored to modulate the emergent properties of the resulting superstructures.



FULL PAPER

Self-assembly of β -turn motif-connected tandem repeats of A β_{16-22} and its aromatic analogs

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Abstract

Self-assembly of peptides and proteins into aggregates with a signature of cross- β conformation is a hallmark of amyloids. Short peptides have provided important insights in understanding the various interactions that drive self-assembly as well as the molecular architecture of the self-assembled structures. The short amyloidogenic-stretch of β -amyloid, A β_{16-22} (Ac-KLVFFAE-am), is a good model peptide to study the aspects of β -amyloid fibril formation. In order to investigate how a turn-supporting sequence could modulate the interaction of the Ac-KLVXZAE-am chains, where X and Z are the aromatic amino acids, Phe, Tyr, or Trp, we investigated the self-assembly of Ac-KLVFFAE-am, Ac-KLVFYAE-am, Ac-KLVYYAE-am, and Ac-KLVWWAE-am separated by turn-inducing dipeptide motifs, Asn-Gly, ^DPro-Gly, and Aib-^DPro. The peptides harboring β -turn-inducing motifs aggregate rapidly causing large enhancements in thioflavin T (ThT) fluorescence compared to control, β -turn motif lacking peptides. The morphology of fibrils strongly depends on the type of β -turn. Ac-KLVFYAE-am repeats separated by Aib-^DPro and ^DPro-Gly have the highest aggregation propensity among all the peptides studied; they caused very large enhancement in ThT fluorescence. Ac-KLVYYAE-am is largely non-amyloidogenic; the ^DPro-Gly and Aib-^DPro connected repeats, however, resulted in distinct fibrils that bind ThT. The study indicates that β -turn motifs can be exploited to modulate and control the aggregation propensity of peptides and the morphology of aggregates.

KEYWORDS

amyloids, beta-turn, fibrils, self-assembly

1 | INTRODUCTION

Amyloid fibrils are β -sheet rich fibrous aggregates of proteins and peptides. Although initially identified as toxic protein/peptide deposits associated with various diseases, disease-unrelated amyloids have been identified in bacterial biofilm formation, aerial hyphae formation in filamentous bacteria, memory consolidation in *Drosophila*, and melanin synthesis in humans.^[1-4] Amyloid fibrils are formed by peptides and proteins that differ from each other in their sequence, amino acid composition, and length. On the molecular level, however, they possess striking similarity with interdigitated β -sheets. Many amyloidogenic proteins need to denature, at least partially, to yield amyloid aggregates and aggregation kinetics could be characterized by a lag phase. Short amyloidogenic peptides, on the other hand, could aggregate rather rapidly as they would be largely unordered in the

monomeric state and structural perturbation is not required as in the case of folded proteins. The rate of assembly would depend on the intrinsic amyloidogenic propensity and stability of the oligomeric nucleus. Several short amyloidogenic peptides self-assemble through antiparallel β -sheet formation. The β -turn, originally identified by Venkatachalam in 1968, happens to be the most prevalent nonrepetitive structure in proteins.^[5] A β -turn motif involves 4 amino acid residues and various types have been defined based on the backbone torsion angles of the second and third residues.^[6] Type I ($\varphi_2 = -60^\circ$, $\psi_2 = -30^\circ$, $\varphi_3 = -90^\circ$, $\psi_3 = 0^\circ$) and type II ($\varphi_2 = -60^\circ$, $\psi_2 = 120^\circ$, $\varphi_3 = 90^\circ$, $\psi_3 = 0^\circ$) are the most common β -turns found in proteins.^[7] β -turns are often found in proteins connecting two antiparallel strands of β -sheets.^[8] Mirror image turns, that is, the turns wherein the conformations are mirror images of regular turns are also observed to a small extent for type I and type II turns. Sibanda and Thornton noticed

that the mirror image turns, type I' and type II', are the preferred turns for β -hairpin structures.^[8] β -hairpins can conceivably aggregate wherein multiple hairpins can assemble into a larger β -sheet. Designed β -hairpin sequences have been utilized to obtain responsive hydrogels through self-assembly.^[9,10] The basic propagating unit of β -amyloid fibers is a dimer wherein the peptide molecules take hairpin-like structures albeit with strands rotated by 90°, that is, the two strands of the monomeric unit belong to two different sheets that are stacked through side-chain interdigitation.^[11]

A β_{16-22} , an amyloidogenic fragment from β -amyloid peptide, self-assembles into amyloid-like aggregates with an antiparallel organization of β -strands.^[12] The peptide has a "Phe-Phe" cassette which is believed to be important in its self-assembly.^[13,14] Self-assembly of the tandem A β_{16-22} repeats connected through a type I' turn nucleating sequence Asn-Gly has recently been investigated.^[15] Pachahara and Nagaraj investigated the role of specific aromatic residues by substituting the Phe residues with Trp and Tyr and identified highly amyloidogenic A β_{16-22} variants.^[16] Tight turns in proteins cause reversal of the polypeptide backbone thereby playing critical role in protein folding.^[17] Aggregation of short amyloidogenic peptides could conceivably be facilitated by connecting the two strands through a tight turn. We investigated the aggregation of A β_{16-22} , Ac-KLVFFAE-am (A β FF), and its aromatic analogs, Ac-KLVFYAE-am (A β FY), Ac-KLVYYAE-am (A β YY), and Ac-KLVWWAE-am (A β WW), connected in tandem through type I' and type II' β -turn inducing motifs. Asn-Gly and Aib-^DPro are chosen as the type I' β -turn-inducing motifs^[18-20] while ^DPro-Gly is chosen as the type II' turn-inducing motif.^[21,22] Introduction of the turn-inducing motifs rendered the peptides more amyloidogenic as indicated by thioflavin T (ThT) fluorescence spectroscopy and transmission electron microscopy (TEM). Morphology of the self-assembled structures and their ThT binding properties strongly depend on the nature of β -turn motif.

2 | MATERIALS AND METHODS

2.1 | Materials

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

2.2 | Peptide synthesis and characterization

Peptides were synthesized on solid support using Fmoc chemistry by employing HBTU/HOBt/DIPEA activation strategy. For 16-residue

peptides, HATU activation was used for coupling the turn-inducing amino acids (eighth and ninth amino acids). On-resin acetylation of the *N*-terminus was carried out using 5 equivalents of acetic anhydride and 10 equivalents of DIPEA. The peptides were cleaved from the resin using a cocktail mixture containing TFA:*m*-cresol:thioanisole:ethanedithiol (20:2:2:1) and precipitated in ice-cold diethyl ether. The crude peptides were dried, dissolved in dimethyl sulfoxide, and purified on a reversed-phase C-18 column on a Shimadzu Prominence Modular HPLC instrument (Shimadzu, Kyoto, Japan) using a linear gradient of acetonitrile containing 0.1% TFA (2.25% acetonitrile/min). Chromatograms of the purified peptides are shown in Supporting Information (Figure S1). The purified peptides were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Autoflex Speed MALDI-TOF-TOF, USA) at Central Instruments Facility, IIT Guwahati.

2.3 | Peptide aggregation

Peptides were dissolved in HFIP and their concentrations estimated by measuring absorption at 280 nm for peptides containing tyrosine or tryptophan and at 254 nm for peptides lacking these amino acids. Molar absorption coefficients of peptides were calculated by adding the molar absorption coefficients of the aromatic amino acids at corresponding wavelengths, that is, Tyr ($\epsilon_{280} = 1280 \text{ M}^{-1} \text{ cm}^{-1}$), Trp ($\epsilon_{280} = 5690 \text{ M}^{-1} \text{ cm}^{-1}$), and Phe ($\epsilon_{254} = 143 \text{ M}^{-1} \text{ cm}^{-1}$). The concentrations of the peptides were adjusted to 1.25 mM in HFIP. Subsequently, the peptides were diluted in water to obtain 250 μM concentration having 20% HFIP and their aggregation studied.

2.4 | Circular dichroism (CD) spectroscopy

Far-UV CD spectra were recorded on a Chirascan CD spectropolarimeter (Applied Photophysics, Leatherhead, UK) for the 24-hour-old samples with suitable dilution in 20% HFIP. The spectra were recorded in a 1 mm path length cell with a step size of 0.5 nm, corrected by subtracting the blank spectrum, that is, spectrum for 20% HFIP, and smoothed. The data are presented as mean residue ellipticity, $[\theta]_{\text{MRE}}$, calculated using formula: $[\theta]_{\text{MRE}} = (M_r \times \theta_{\text{obs}}) / (100 \times l \times c)$, where M_r is the mean residue weight (peptide molecular weight/number of amino acid residues), θ_{obs} is the observed ellipticity in millidegrees, l is the length in decimeters, and c is the peptide concentration in mg/mL.^[23]

2.5 | Fourier transform infrared (FTIR) spectroscopy

FTIR spectra were recorded on a Bruker Alpha-E spectrometer (Bruker Optik GmbH, Germany) with Eco attenuated total reflection (ATR) single reflection ATR sampling module equipped with ZnSe ATR crystal. Peptides (10 μL) were spread out and dried as films on ZnSe crystal and ATR-FTIR spectra were recorded at a resolution of 4 cm^{-1} .

2.6 | Molecular dynamics (MD) simulations

Peptides in fully extended conformation ($\varphi = \psi = 180^\circ$) were constructed using Ribosome.^[24] The dihedral angles φ and ψ for ^DPro, wherever applicable, were fixed to 70° and -45° , respectively. The amino-terminus was acetylated while C-terminus was amidated for each of the peptides. The lysine and glutamate side-chains were electrically charged rendering the peptide electrically neutral; no counter-ion was added separately. Energy minimization and MD simulation were performed using the Gromacs software package (v 5.0.4)^[25] with the GROMOS96 54a7 forcefield^[26] at the CDAC-IIT Guwahati Supercomputing facility. The ribosome-constructed structures were energy-minimized in vacuum using Steepest Descent algorithm. The vacuum energy-minimized peptide structures were solvated with water using simple point-charge^[27] water model in a cubic box with a distance of 1.5 nm between the peptide and the edge of the box. The systems comprising of 1 peptide molecule and 25,148 water molecules were energy-minimized. A small position restrained dynamics was performed before the final production of MD run. MD simulation was carried out for 100 ns at 300 K and 1 bar by using leap-frog integration method.^[28] Temperature and pressure couplings were done with v-rescale^[29] and Berendsen^[30] methods, respectively. Long-range electrostatics was calculated with fast smooth Particle mesh Ewald (PME) method with cubic interpolation and Fourier grid spacing of 0.1 nm.^[31,32] The Verlet cutoff-scheme was followed for short-range electrostatics and van der Waals interactions, with the cutoff value set to 1.0 nm for both rcoulomb and rvdw. Bond lengths were constrained using LINCS algorithm.^[33] All the simulations were carried out using periodic boundary conditions in all directions. The results were analyzed using the built-in Gromacs utilities. Discovery Studio Visualizer was used for visualization.^[34]

2.7 | ThT fluorescence assay

ThT fluorescence spectra for the 24-hour-old peptide samples in 20% HFIP were recorded on a Jasco FP8500 spectrofluorometer (Jasco, Japan). The peptides were diluted to 20 μ M concentration in 5 mM phosphate buffer, pH 7.0, containing 10 μ M ThT. The samples were excited at 440 nm and fluorescence recorded from 460 to 550 nm. Excitation and emission slit widths were 2.5 and 5 nm, respectively. Spectra of the peptides without ThT were recorded as blank and subtracted. Fluorescence intensity at 482 nm is reported in the results.

2.8 | Transmission electron microscopy

The peptide samples (72 hours old) were diluted 2.5-fold in deionized water and drop-casted on Formvar/Carbon coated 200-mesh copper grids. After 2 minutes, the solvent was blotted out from the periphery of the grid by Whatman I filter paper. The grids were stained with saturated uranyl acetate negative stain solution which was blotted out after 30 seconds. The grids were then dried and images recorded on JEM-2100 LaB₆ (JEOL, Japan) transmission electron microscope at 120 kV.

3 | RESULTS

We investigated the self-assembly of A β ₁₆₋₂₂, its aromatic analogs, and their tandem analogs wherein β -turn-inducing motifs have been incorporated between two peptide repeats. In order to understand how β -turn can modulate peptide assembly, we investigated 12 peptides containing β -turn-inducing motifs between the two peptide repeats of A β FF, A β FY, A β YY, and A β WW (Table 1). Self-assembly of

TABLE 1 List of the peptides studied: their ID, sequence, calculated monoisotopic mass, and the m/z values obtained in MALDI mass spectrometry

Peptide ID ^a	Sequence ^a	Calculated monoisotopic mass	Observed m/z
A β FF	Ac-KLVFFAE-am	893.49	895.35
A β FY	Ac-KLVFYAE-am	909.48	911.29
A β YY	Ac-KLVYYAE-am	925.48	927.36
A β WW	Ac-KLVWWAE-am	971.51	973.42
A β FF-Up	Ac-KLVFFAE-U ^D P-KLVFFAE-am	1910.06	1911.02
A β FF-pG	Ac-KLVFFAE- ^D PG-KLVFFAE-am	1882.03	1884.29
A β FF-NG	Ac-KLVFFAE-NG-KLVFFAE-am	1899.02	1900.09
A β FY-Up	Ac-KLVFYAE-U ^D P-KLVFYAE-am	1942.05	1944.05
A β FY-pG	Ac-KLVFYAE- ^D PG-KLVFYAE-am	1914.02	1916.27
A β FY-NG	Ac-KLVFYAE-NG-KLVFYAE-am	1931.01	1933.79
A β YY-Up	Ac-KLVYYAE-U ^D P-KLVYYAE-am	1974.04	1975.91
A β YYpG	Ac-KLVYYAE- ^D PG-KLVYYAE-am	1946.01	1947.78
A β YY-NG	Ac-KLVYYAE-NG-KLVYYAE-am	1963.00	1964.68
A β WW-Up	Ac-KLVWWAE-U ^D P-KLVWWAE-am	2066.10	2068.29
A β WW-pG	Ac-KLVWWAE- ^D PG-KLVWWAE-am	2038.07	2040.21
A β WW-NG	Ac-KLVWWAE-NG-KLVWWAE-am	2055.06	2057.02

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^a D-Proline is indicated by lower case "p" in the ID and by ^DP in the sequence.

^a Letter U in the peptide ID and sequence represents Aib.

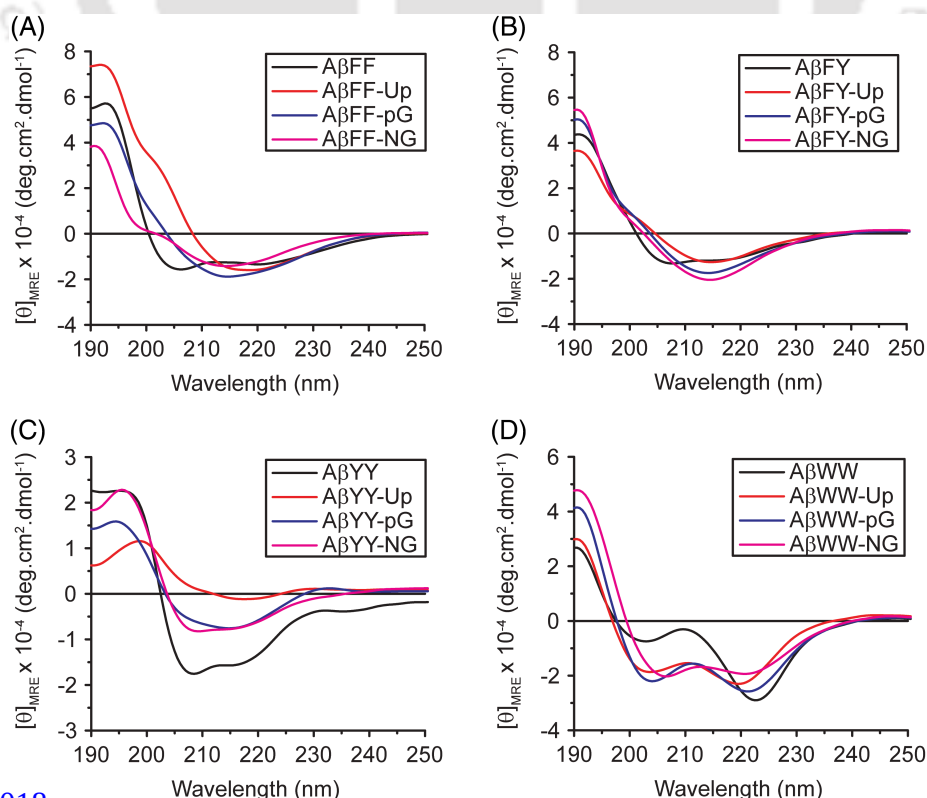
A β_{16-22} aromatic analogs has been studied previously.^[13,14,16] Pachahara and Nagaraj studied aggregation of the aromatic analogs in 20% HFIP; aromatic cassette was found to have deciding role in the self-assembly and dictates the morphology of the self-assembled structures.^[16] We chose to study the β -turn analogs under same conditions: the peptides were dissolved in 20% HFIP and analyzed using CD spectroscopy and ATR-FTIR spectroscopy. ThT fluorescence spectroscopy and TEM imaging were carried out by diluting the peptides into water immediately before the assays.

3.1 | CD spectroscopy

We examined the secondary structures of tandem repeats of A β FF, A β FY, A β YY, and A β WW connected via β -turn-inducing motifs. Peptides dissolved in HFIP (1.25 mM) were diluted in deionized water to obtain 250 μ M peptide solutions in 20% HFIP. Far-UV CD spectra were recorded for the 24-hour-old peptide samples (Figure 1).

A β FF displays negative bands at 206 and 220 nm with an intense positive band $\sim$ 193 nm (panel A). Such helix-like spectra have been obtained in trifluoroethanol for tripeptide to hexapeptide methylamides that harbor Asn-Pro-Gly sequence and have been assigned to β -turns.^[27] A β FY (panel B) and A β YY (panel C) show spectra similar to that of A β FF, albeit with the long wavelength band blue-shifted to $\sim$ 216 nm. The spectra suggest β -sheet conformation alongside turns. A β WW on the other hand displays an intense negative band $\sim$ 223 nm, a positive band of comparable intensity $\sim$ 191 nm, and a weak negative band $\sim$ 203 nm (panel D). The spectrum is similar to that reported by Pachahara and Nagaraj for A β WW in 20% HFIP.^[16] Similar spectra have been reported for indolicidin in SDS micelles and

tryptophan-rich self-assembling peptides and are believed to arise due to stacking of Trp residues.^[35,36] Tryptophan stacking could take place for A β WW through intermolecular interaction. The Asn-Gly β -turn motif-harboring peptides, A β FF-NG (panel A) and A β FY-NG (panel B), display broad negative band centered $\sim$ 214 nm and a shoulder around 202 nm suggesting largely β -sheet conformation. CD spectrum with a weak negative band between 220 and 230 nm and a positive band $\sim$ 200 nm arises from β -turns.^[37] A positive shoulder $\sim$ 203 nm could therefore be a contribution from β -turn motif. A β YY-NG displays a spectrum with negative bands around 209 and 216 nm, suggesting β -sheet conformation with an appreciable contribution from turn (panel C). A β WW-NG displays negative bands around 207 and 221 nm, a spectrum characteristic of distinct α -helix (panel D). The data indicate that A β WW-NG folds into largely α -helical conformation in 20% HFIP, whereas other Asn-Gly motif harboring peptides largely populate β -sheet structure. Fluorinated alcohols are well known to promote α -helical conformation in peptides.^[38,39] Furthermore, Asn-Gly dipeptide motif supports type I' β -turn formation; but is not an obligatory turn-nucleating motif. Aib-DPro, on the other hand, is an obligatory type I' β -turn inducing motif. A β FF-Up displays a broad negative band around 218 nm and a positive band around 192 nm along with a 203 nm shoulder (panel A). The spectrum is suggestive of β -sheet and β -turn conformations. A β FY-Up spectrum also displays β -sheet and β -turn conformations (panel B). A β YY-Up shows a very weak band around 218 nm and a positive band $\sim$ 199 nm (panel C). Such spectra have been assigned to turns in designed β -turn peptides.^[37] A β WW-Up displays a spectrum with negative bands around 219 and 204 nm. The positive band $\sim$ 190 nm is of comparable intensity. Spectra similar to these have been assigned to β -turns in



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FIGURE 1 CD spectra of 24-hour-old peptide solutions in 20% HFIP

literature.^[40] Assignment of the secondary structure to the A β WW and its analogs' CD spectra is complicated by aromatic exciton coupling. The A β WW-Up spectrum appears to be influenced by aromatic exciton coupling of tryptophan. The type II' β -turn nucleating motif ^DPro-Gly appears to nucleate β -turn in A β FF-pG (panel A) and A β FY-pG (panel B). Both the peptides display a negative band ~214-215 nm, a positive band around 192 nm with a shoulder around 203 nm. A β YY-pG displays a broad negative band ~215 nm with a hump ~207 nm (panel C). The spectrum is suggestive of β -sheet conformation with turn signature as well. A β WW-pG spectrum (panel D) is qualitatively similar to that of A β WW-Up. All the analogs of A β FF and A β FY that harbor β -turn-inducing motifs display distinct β -conformation with a negative band around 214-218 nm.

3.2 | FTIR spectroscopy

ATR-FTIR spectra were recorded for the 24-hour-old peptide samples to investigate the secondary structure in the dried films. The spectra of A β FF, A β FY, A β YY, and their turn containing analogs show intense bands around 1624-1626 cm⁻¹, a characteristic signature of β -sheets (Figure 2). Tryptophan-containing peptides A β WW, A β WW-NG, A β WW-Up, and A β WW-pG though displayed a major band ~1625 cm⁻¹, a shoulder ~1650 cm⁻¹ is observed. The results suggest that the β -sheet conformation of the peptides is retained in the dried films.

3.3 | MD simulations

Molecular dynamics simulations (100 ns) were carried out in water to gain insights about the type of turn formed by the Asn-Gly and ^DPro-Gly harboring peptides. The trajectory files obtained following

MD simulations were analyzed to examine folding of the peptides. Distance between the α -carbons of terminal amino acid residues and that between the α -carbons of the residues preceding and succeeding the β -turn dipeptide motifs were calculated. Furthermore, the dihedral angles of the β -turn dipeptide motifs and the possibility of H-bonding between C=O of preceding residue and NH of the succeeding residue investigated. The data for A β FY-NG is shown in Figure 3.

A β FY-NG simulation was started with an extended peptide structure ($\phi_{all} = \psi_{all} = 180^\circ$). The peptide folds to take a β -hairpin conformation after ~30 ns; the distance between K1C α and E16C α is ~0.6 nm and shows only small variations (black trace, panel A). The distance between the E7C α and K10C α is also ~0.6 nm and little variation is observed through the simulation (red trace, panel A), wherein a hydrogen-bond is formed between Glu7 C=O and Lys10 NH, suggesting a β -turn.^[5,7] At 34 ns, the dihedral angles ϕ and ψ for Asn are -56° and 91° , respectively. The angles show variations around ϕ and ψ values of $\sim -57^\circ$ and $\sim 105^\circ$ for the largest cluster (panel B). Gly, on the other hand, adopts ϕ and ψ values of 56° and 51° after 34 ns. After 34 ns, Gly9 ϕ samples values largely between 40° and 120° , spread around $+66^\circ$ (panel C). The values of ψ range from -60° to $+60^\circ$ with $\psi = 30^\circ$ as the largest cluster. The dihedral angles of the largest cluster indicate a type II β -turn. At 34 ns, the distance between E7CO and K10NH is ~0.19 nm (panel D) with angle HNO of 19° (panel E), indicating a H-bond. Panels F-K show the snapshots at 20 ns interval supporting hairpin structures. A β FY-pG simulation was started with extended conformation; ϕ and ψ values for ^DPro, however, were taken 70° and -45° , respectively. The peptide folds into a β -hairpin-like structure around 19 ns (Figure 4). The distance between K1C α and E16C α as well as that between E7C α and K10C α is

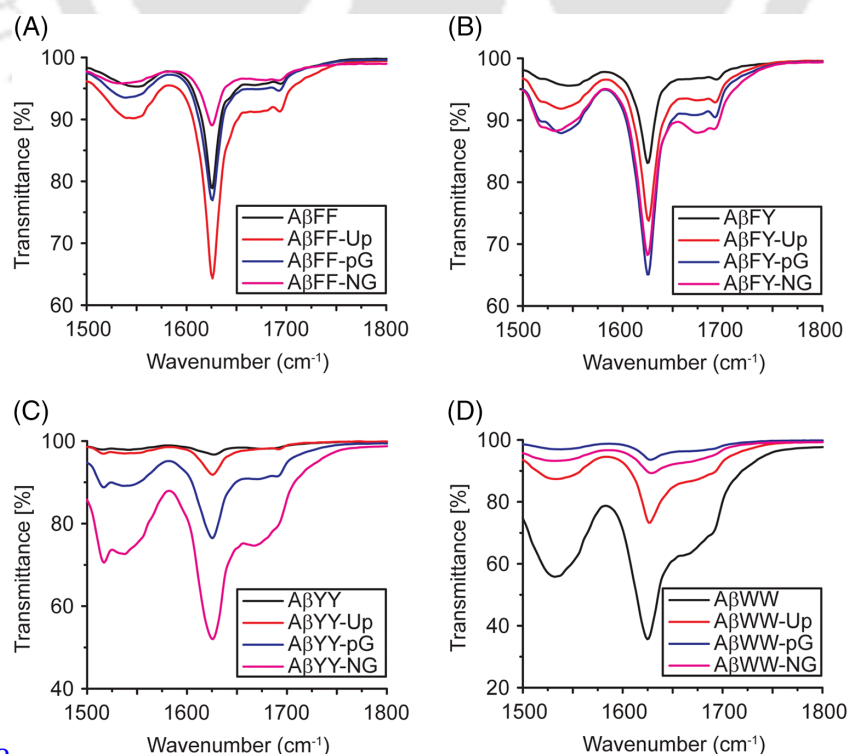


FIGURE 2 ATR-FTIR spectra of the dried films from 24-hour-old peptide solutions in 20% HFIP

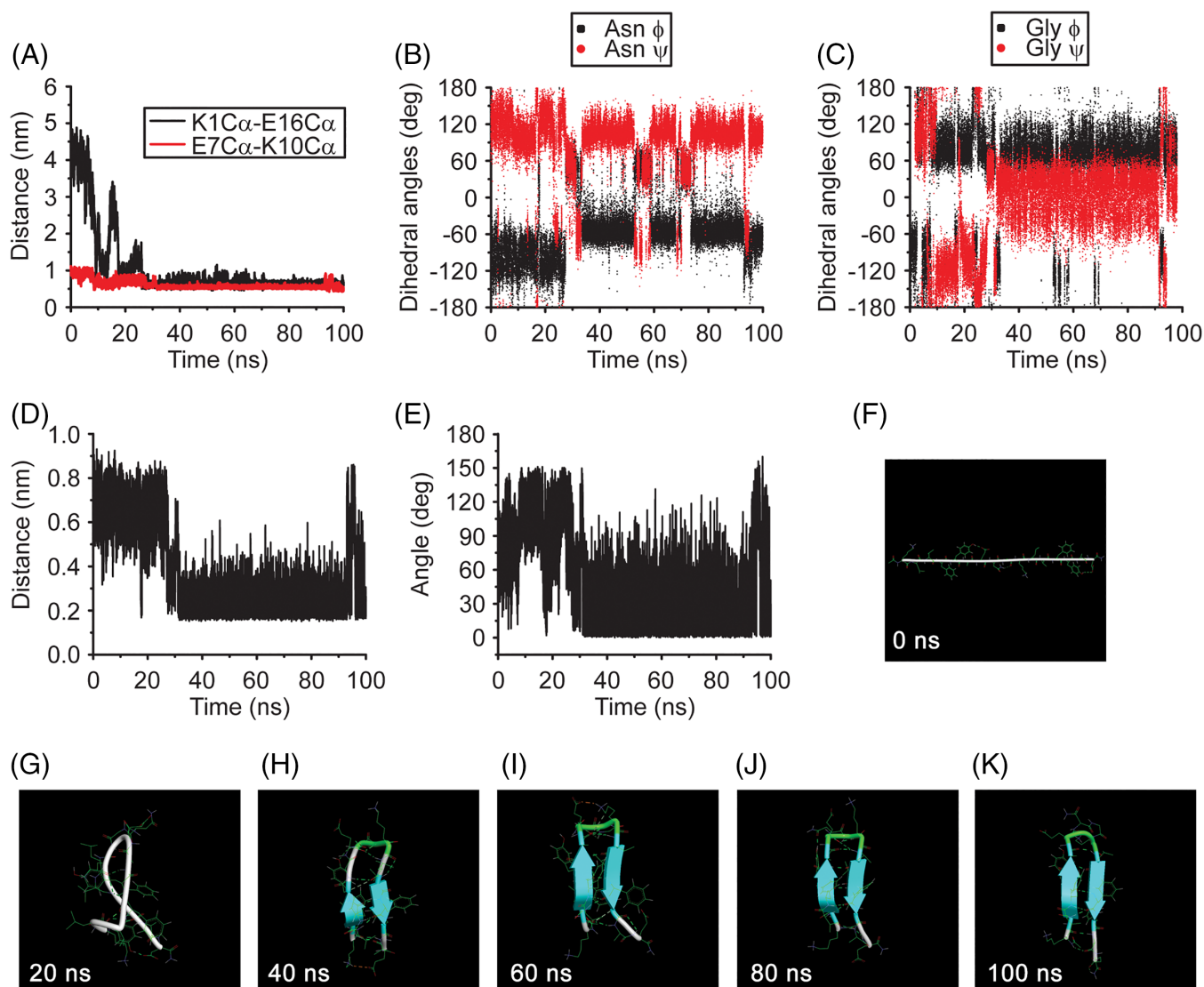


FIGURE 3 MD simulation of AβFY-NG. A, Distance between K1Cα and E16Cα (black) and that between E7Cα and K10Cα (red); B, dihedral angles, ϕ (black) and ψ (red) of Asn residue; C, dihedral angles, ϕ (black) and ψ (red) of Gly residue; D, distance between amide oxygen of Glu 7 and amide proton of Lys10; E, angle HNO, wherein HN belongs to Lys 10 amide whereas O belongs to the Glu7 amide; after ~32 ns, the distance and the angle are, mostly, within 0.35 nm and 60°, respectively; F-K, snapshots from the MD trajectory at 20 ns interval

~0.5-0.6 nm and is invariant over time. After 19 ns, the largest cluster takes the ϕ and ψ values for ^DPro spread around +60° and ~-110°, respectively (panel B). Gly9 takes ϕ and ψ values around -120° and 30°, respectively (panel C). The ^DPro and Gly dihedral angles conform to a type II' turn. The hairpin appears to be stabilized by an *i* to *i* + 3 hydrogen-bond in the β -turn region as suggested by the distance between E7CO and K10NH (panel D) and angle HNO (panel E).

MD simulation data for AβYY-NG are shown in Figure 5. The data show a K1Cα and E16Cα distance of ~0.6-0.7 nm within 10 ns of simulation (panel A). However, β -turn is not mediated by residues 7-10, but by residues 8-11. Instead of Asn8 and Gly9, Gly9 and Lys10 happen to be the *i* + 1 and *i* + 2 turn residues, respectively. This results in a shift in β -strand registry (panels F-K). Large fluctuations observed in the distance between K1Cα and E16Cα could be attributed to the flexible N-terminal residue.

Around 40 ns, Gly9 takes up ϕ and ψ values spread around +65° and -105°, respectively (panel B). Lys10 takes ϕ and ψ values around -90° and -30°, respectively (panel C). The distance between N8Cα and L11Cα are also within 0.6 nm. The distance between N8CO and L11NH is largely within 0.3 nm (panel D) with angle HNO within 60° (panel E) indicating a H-bond. The presence of H-bond between *i*th and (*i* + 3)th residues and the dihedral angles of the *i* + 1 and *i* + 2 residues indicate a type II' β -turn.

AβYY-pG rapidly folds into a hairpin structure (Figure 6). Around 7 ns, the distance between K1Cα and E16Cα and that between E7Cα and K10Cα are within 0.6 ns and are largely invariant throughout the simulation (panel A). ^DPro8 takes up ϕ and ψ values spread around +60° and -110°, respectively (panel B). Gly9 takes up ϕ and ψ values spread around -90° and 0°, respectively (panels C); ψ values largely

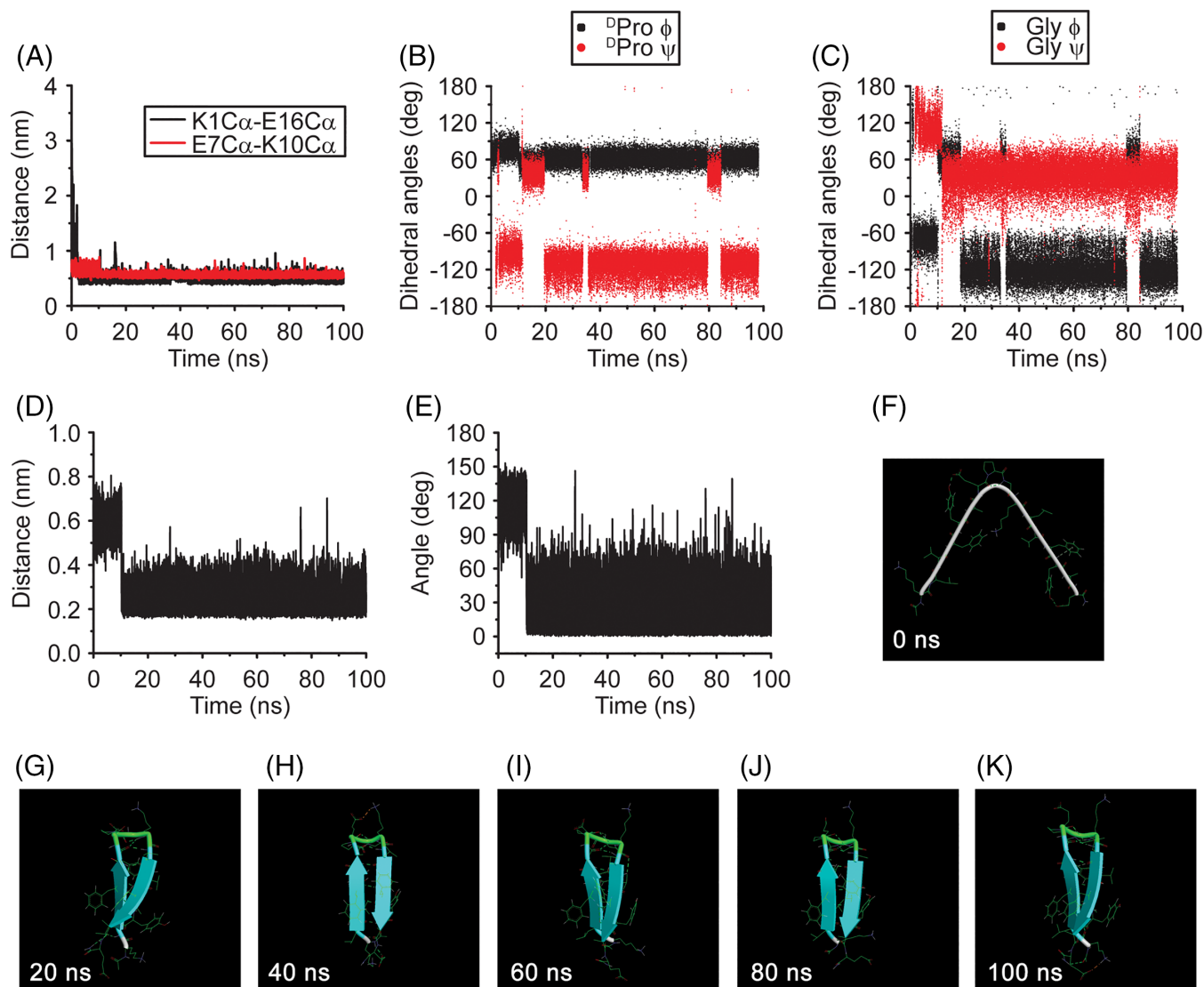


FIGURE 4 MD simulation of AβFY-pG. A, Distance between K1Cα and E16Cα (black) and that between E7Cα and K10Cα (red); B, dihedral angles, ϕ (black) and ψ (red) of ^DPro residue; C, dihedral angles, ϕ (black) and ψ (red) of Gly residue; D, distance between amide oxygen of Glu 7 and amide proton of Lys10; E, angle HNO, wherein HN belongs to Lys 10 amide whereas O belongs to the Glu7 amide; after ~11 ns, the distance and the angle are, mostly, within 0.35 nm and 60°, respectively; F-K, snapshots from the MD trajectory at 20 ns interval

reside around 30° and −30° suggesting two major clusters around 0°. The data suggest a classic type II' turn mediated by ^DPro8 and Gly9. The hairpin appears to be stabilized by an *i* to *i* + 3 hydrogen-bond in the β-turn region as suggested by the distance between E7CO and K10NH (panel D) and angle HNO (panel E). Panels F-K show the snapshots at 20 ns interval and confirm the β-hairpin structure.

Snapshots of the MD simulations of AβFF-NG, AβFF-pG, AβWW-NG, and AβWW-pG are shown as Supporting Information (Figures S2-S5). AβFF-NG folds into a hairpin structure. The turn, however, is a nonclassical one (Supporting Information Figure S2). AβFF-pG takes up a β-hairpin structure mediated by a β-turn within 20 ns (Supporting Information Figure S3E). ^DPro mostly takes up the dihedral angles characteristic of a type II' turn but Gly shows high conformational flexibility (Supporting Information Figure S3). AβWW-NG took up a hairpin-like conformation only around 96 ns

(Supporting Information Figure S4I). AβWW-pG takes up a hairpin structure but the turn is neither of the classical β-turns (Supporting Information Figure S5).

3.4 | ThT fluorescence spectroscopy

Peptide solutions at 250 μM in 20% HFIP were incubated at room temperature and analyzed using ThT fluorescence spectroscopy after 24 hours incubation. AβFF causes small enhancement (~3-fold) in ThT fluorescence in agreement with the previous reports (Figure 7). AβFY caused ~5-fold enhancement in ThT fluorescence. The other two peptides, AβYY and AβWW caused only little enhancement (<2-fold) in ThT fluorescence. All the turn-containing peptides, on the other hand, cause larger enhancement in ThT fluorescence compared to control peptides. The effect of the turn-inducing motifs, however, is sequence

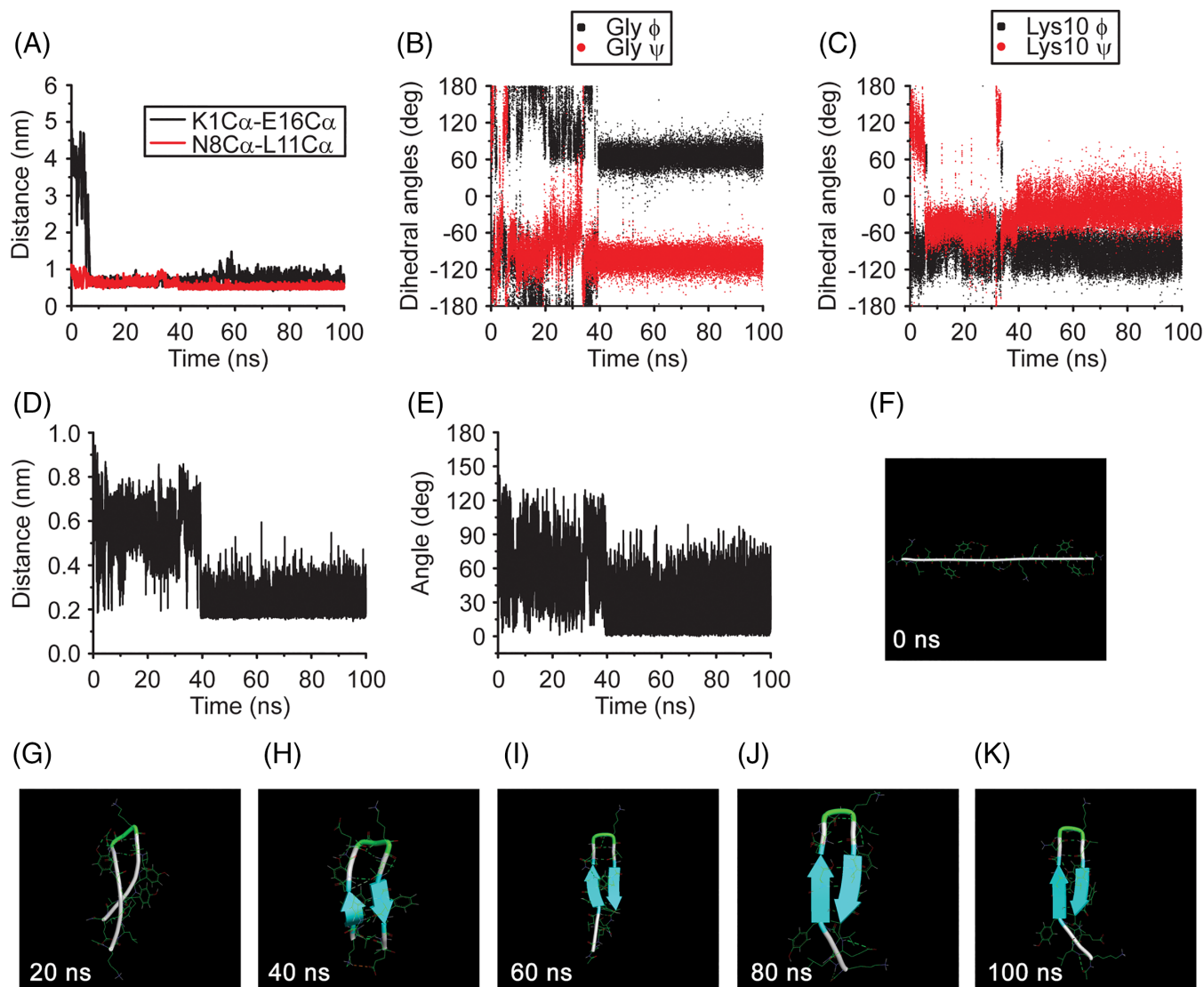


FIGURE 5 MD simulation of AβYY-NG. A, Distance between K1Cα and E16Cα (black) and that between N8Cα and L11Cα (red); B, dihedral angles, φ (black) and ψ (red) of Gly residue; C, dihedral angles, φ (black) and ψ (red) of Lys10 residue; D, distance between backbone amide oxygen of Asn8 and amide proton of Leu11; E, angle HNO, wherein HN belongs to Leu11 amide whereas O belongs to the Asn8 backbone amide; after 40 ns, the distance and the angle are, mostly, within 0.35 nm and 60°, respectively; F-K, snapshots from the MD trajectory at 20 ns interval

dependent. These results are unsurprising as control peptides have different aggregation properties by themselves.^[16] More than 10-fold enhancement in ThT fluorescence was observed for the AβFF-derived turn-harboring peptides. The AβFY-derived peptides turned out to be highly amyloidogenic; around 15 and 25-fold enhancement was observed for AβFY-Up and AβFY-NG, respectively. AβFY-pG turned out to be the most amyloidogenic one, causing ~50-fold enhancement in ThT fluorescence intensity. AβYY and AβWW are largely non-amyloidogenic as is suggested by the ThT fluorescence. Turn-harboring AβYY repeats, on the other hand, caused ~4 to 5-fold enhancement in ThT fluorescence. AβWW-derived peptides caused ~7-10-fold enhancement in ThT fluorescence suggesting their higher self-assembling propensity.

3.5 | Transmission electron microscopy

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TEM images of the 72-hour-old peptide samples are shown in Figure 8. AβFF, AβFY, and their β-turn analogs show distinct fibrillar

structures. The fibril morphology, however, depends on the type of β-turn motif present in the peptide. The control AβFF shows thick short fibers. AβFF-Up forms long ribbon-like structures whereas long needle-like aggregates were obtained from AβFF-pG. AβFF-NG shows thin long fibers; the fibers show lateral assembly and entanglement. AβFY and its analogs resulted in long thin fibrils; AβFY forms thin sub-micrometer fibers as well (inset, panel AβFY). Very extensive networks of fibrils were obtained for all the β-turn motif harboring peptides that corroborates with the large enhancement in ThT fluorescence (Figure 7). AβYY does not show any fibrillar aggregates while AβYY-NG showed few fibrous structures suggesting that peptides are sluggish to aggregate. MD simulation of AβYY-NG shows a β-turn mediated by Gly9-Lys10 (Figure 5). This renders the two ends of the β-hairpin cationic and could diminish the self-assembly through electrostatic repulsions. AβYY-Up and AβYY-pG, on the other hand, resulted in distinct fibrillar structures. The data suggest that introduction of rigid β-turn could impart amyloidogenicity to the otherwise non-amyloidogenic peptides. A tight β-hairpin essentially brings two

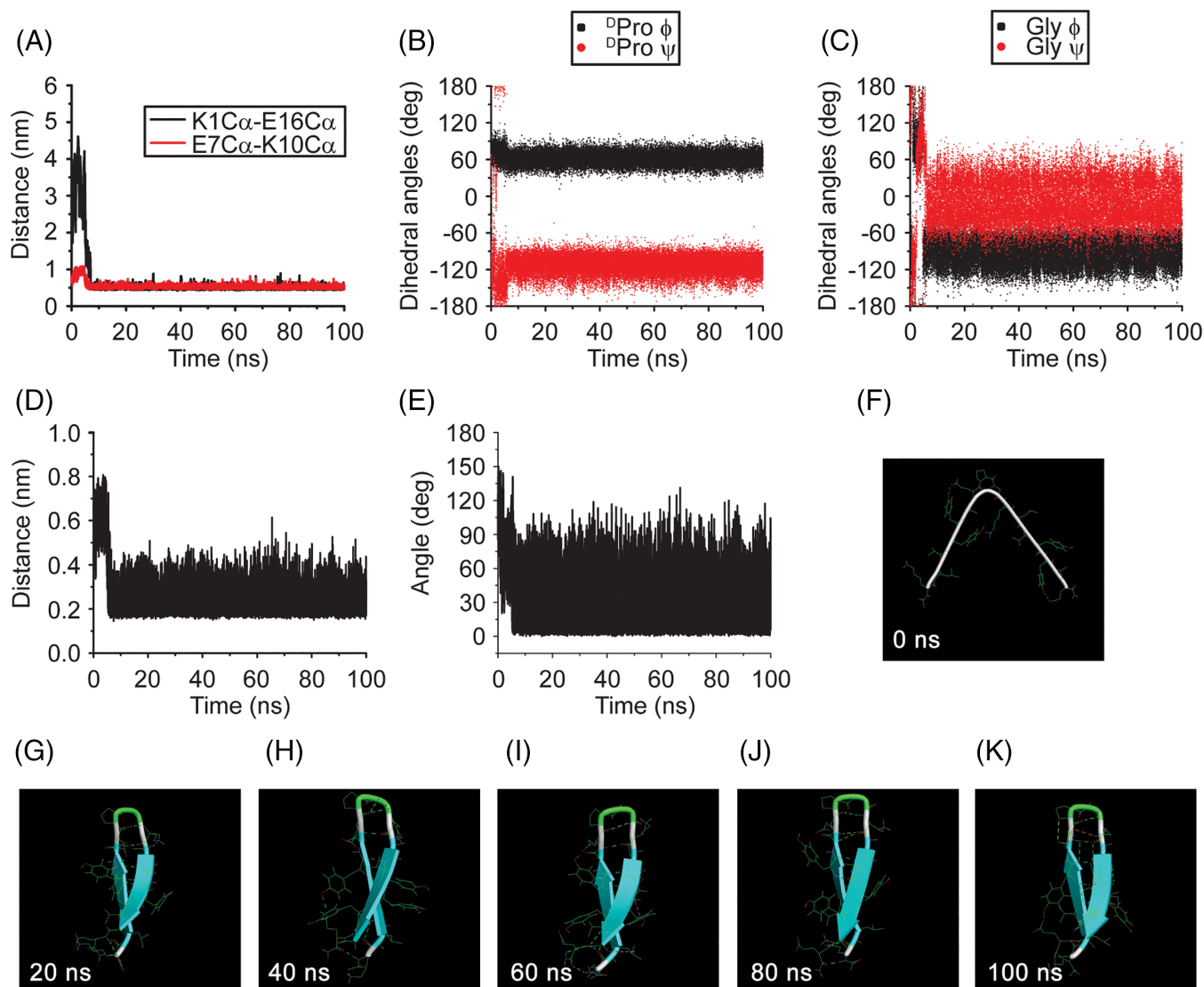


FIGURE 6 MD simulation of AβYY-pG. A, Distance between K1Cα and E16Cα (black) and that between E7Cα and K10Cα (red); B, dihedral angles, ϕ (black) and ψ (red) of D Pro residue; C, dihedral angles, ϕ (black) and ψ (red) of Gly residue; D, distance between amide oxygen of Glu 7 and amide proton of Lys10; E, angle HNO, wherein HN belongs to Lys 10 amide whereas O belongs to the Glu7 amide; after ~6 ns, the distance and the angle are, mostly, within 0.35 nm and 60°, respectively; F-K, snapshots from the MD trajectory at 20 ns interval

self-assembling motifs (β -strands) in close proximity. Such a structure is a small β -sheet that could nucleate growth of fibrils by incorporating monomers. AβWW and its analogs resulted in largely spherical clumps. Thin long fibrils were also obtained from AβWW-Up. MD simulations of AβWW-NG and AβWW-pG suggest formation of hairpin-like structures, albeit not mediated by classical β -turns. Such a structure could result in self-assembly through tryptophan-zipper interactions; the structures formed by AβWW turn analogs, in fact, caused large enhancement in ThT fluorescence suggesting formation of β -sheet rich aggregates.

4 | DISCUSSION

Amyloid aggregates have been long known to be the causative agents of many human diseases.^[41] Despite several decades of

research on amyloid aggregates, we are far from completely understanding the process of protein assembly into amyloid fibrils and consequently development of therapeutic agents. Growing evidence in past decade about the functional roles of amyloids indicates that it is not an alien-fold.^[42–44] There is evidence that cytotoxicity in amyloid diseases is imparted by prefibrillar oligomers. Many of the functional amyloidogenic proteins aggregate rapidly such that the prefibrillar structures are only short-lived.^[1,2] Turn-inducing motifs play important role in protein folding.^[17] We show in this study that β -turns can have deciding roles on the aggregation propensity and morphology of the self-assembled structures. Compared to AβFF, that formed short fibers after 3 days, the β -turn analogs form distinct long fibers that cause large enhancement in ThT fluorescence. AβFY is more amyloidogenic than AβFF as shown previously^[16] and is evident from ThT fluorescence and TEM imaging data in this study. β -turn connected

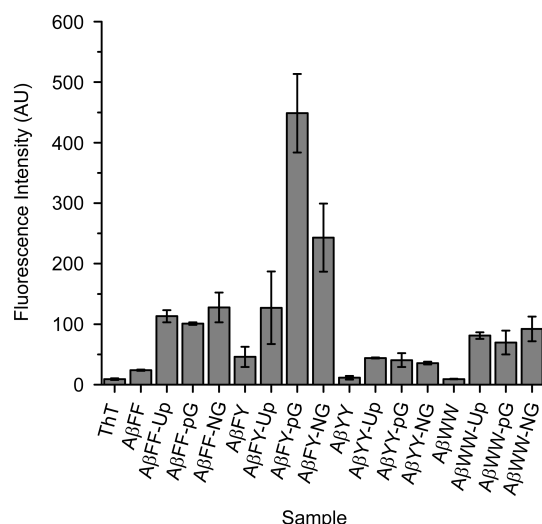


FIGURE 7 ThT fluorescence intensity at 482 nm for 24-hour-old samples in 20% HFIP. The 24-hour-old samples were diluted in 5 mM phosphate buffer, pH 7.0 and spectra recorded. The error bars represent SD

AβFY repeats resulted in very large enhancement in ThT fluorescence and resulted in distinct fibrils as revealed by TEM imaging. Among all the β-turn analogs, AβFY-derived peptides turned out to be highly amyloidogenic, forming distinct fibrillar mesh and causing very large enhancement in ThT fluorescence. Phe19 has been shown to be critical for the assembly of Aβ₁₆₋₂₂ in previous studies; substitution of Phe19 with Tyr or Trp severely diminishes its aggregation propensity suggesting its role in cross-strand interactions.^[13,14] Consequently, neither AβYY nor AβWW causes any significant ThT fluorescence. β-turn analogs of AβYY, however, caused enhancement in ThT fluorescence and resulted in fibrillar structures indicating that a non-amyloidogenic peptide could be rendered amyloidogenic by inducing β-hairpin formation. The role of β-hairpin in amyloid formation has been studied using model polyQ peptides.^[45,46] A model polyQ peptide harboring a ^DPro-Gly motif in the middle of polyQ region displays faster aggregation kinetics suggesting that β-turn formation could be the rate-determining step in the aggregation.^[46] The same study reveals that the polyglutamine core formed by Huntingtin exon-1 is based on interdigitated hairpin-like structures.^[46] These data suggest that hairpin-like structures could be crucial in the assembly of other polyQ diseases as well. A molecular dynamics simulation study with the amyloidogenic fragments of murine Prion protein, PrP₁₀₉₋₁₂₂, PrP₁₀₆₋₁₂₆, and their Ala117Val mutants suggests that the assembly is mediated by β-hairpin structures.^[47] Even though AβWW is not amyloidogenic, AβWW β-turn analogs cause ~7-10-fold enhancement in ThT fluorescence intensity. MD simulation data indicate β-turn mediated hairpin formation for AβYY-NG and AβYY-pG. AβWW-pG, on the other hand, takes up hairpin-like structures via unconventional turns. For AβWW-NG, a hairpin-like structure could be obtained around 96 ns in MD simulation. However, AβWW β-turn analogs cause higher ThT fluorescence enhancement compared to AβYY analogs suggesting that the AβWW

analogs have higher self-assembling propensity possibly through tryptophan zipper formation.

The structure of Aβ₁₄₋₂₃ fibrils has been investigated using solid-state NMR.^[48] The fibrils are made up of antiparallel β-sheets with a registry that aligns residue 17+k with residue 22-k, where k is an integer. This implies that the adjacent residues are out-of-register by two residues. Connecting two Aβ₁₄₋₂₃ peptides with a tight turn would change the registry of β-strands by two residues thereby diminishing their aggregation propensity as observed by Lyubchenko and coworkers.^[49] Polymorphism is another interesting aspect of amyloid fibrils. It is now believed that amyloid fibrils are structurally polymorphic at the molecular level and such polymorphism could lead to variations in the disease development.^[50,51] The heterogeneous nature of the fibrils has turned out to be a major obstruction in the structural characterization of the fibrils. In addition to that, this hampers the potential applications of amyloid fibrils as nanomaterials. A recent study on Aβ42 employed organic solvents and optimized pH to obtain highly homogeneous amyloid fibrils.^[52,53] The combined cryoelectron microscopy and solid state NMR analysis identifies a novel topology for the Aβ42 fibrils wherein the C-terminal residues from two filaments are buried right in the fibrillar core.^[52] In this context, intelligent incorporation of the β-turn inducing motifs at the structurally relevant sites could result in highly homogeneous fibrils enabling structure determination. Other than aggregation, β-hairpin conformation has a therapeutic aspect as well. Härd and coworkers showed that Aβ40 binds to the affibody protein Z_{Aβ3}.^[54] Aβ40 binds to the Z_{Aβ3} dimer by adopting a β-hairpin conformation. Z_{Aβ3} completely abolishes Aβ40 aggregation by trapping the Aβ40 monomers with stoichiometric concentration of dimeric Z_{Aβ3}. Hoyer and coworkers engineered a β-wrapin that could bind α-synuclein. The designed affibody β-wrapin AS69 binds α-synuclein wherein α-Syn₃₇₋₅₄ takes a β-hairpin conformation thereby abolishing its aggregation.^[55] Similar inhibition of IAPP aggregation could be achieved by another engineered affinity protein, β-wrapin, HI18.^[56] IAPP monomer binds to two subunits of HI18 by taking a β-hairpin conformation.

Our results indicate that β-hairpin conformation modulates the aggregation propensity of short amyloidogenic sequences, making them more aggregation-prone. Largely non-amyloidogenic sequences were rendered amyloidogenic suggesting β-hairpin to be an important conformation in the context of amyloid formation. The aggregation of amyloidogenic peptides could be modulated by suitably incorporating the β-turn motifs. The β-turn motifs in short peptides define the registry of the strands in β-hairpins thereby defining the structures of the fibrils. Highly aggregating peptides such as AβFY-Up, AβFY-pG, and AβFY-NG could be useful models in studying amyloidogenesis.

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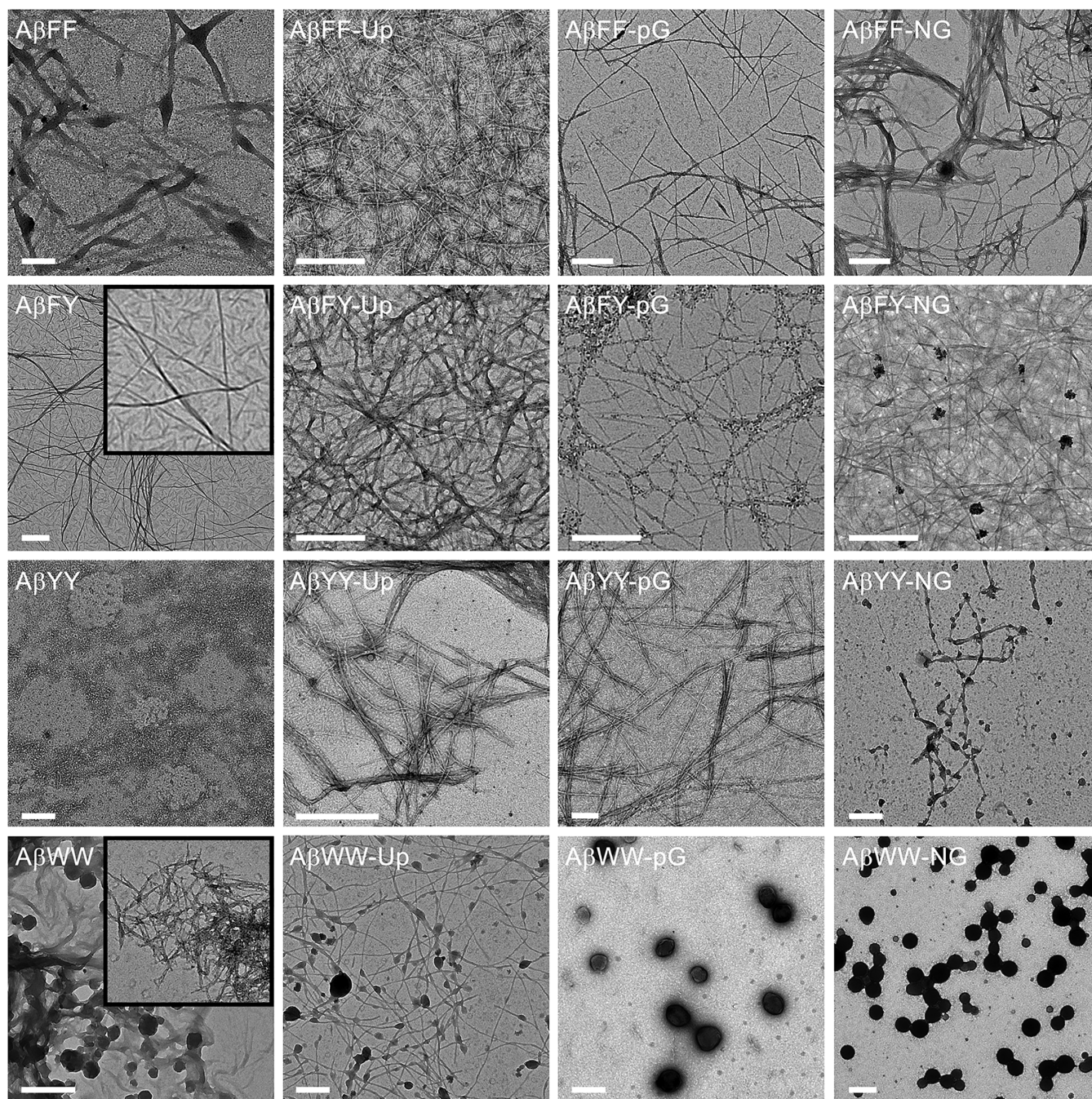


FIGURE 8 TEM analyses of 72-hour-old peptide samples in 20% HFIP. The scale bars represent 0.5 μm

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Hydrogel Formation by an Aromatic Analogue of a β -Amyloid Fragment, $A\beta_{16-22}$: A Scaffold for 3D Cell Culture

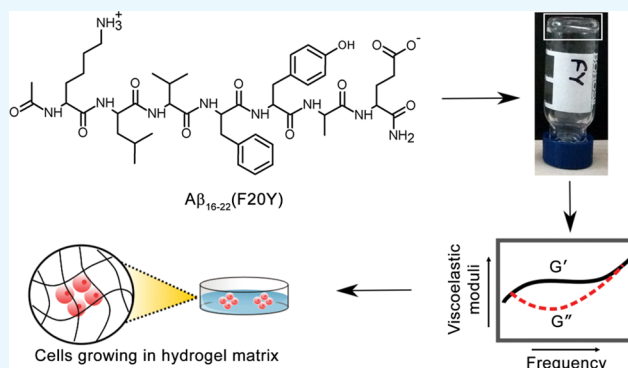
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Supporting Information

ABSTRACT: A short 7-residue stretch from β -amyloid, $A\beta_{16-22}$ (Ac-KLVFFAE-am), yields typical amyloid-like fibrils at neutral pH. The Phe–Phe cassette present in the peptide is believed to be critical for its self-assembly. We report that the aromatic analogue $A\beta_{16-22}$ (F20Y) forms self-supporting soft gels at concentrations ≥ 2 mM even though the end-capped parent peptide does not form hydrogel up to 20 mM (1.8% w/w) concentration. The hydrogel is made up of distinct amyloid-like fibers. The storage modulus of 20 mM gel is ~ 3 – 5 -fold higher than the loss modulus in the 2–3000 rad/s angular frequency range, indicating distinct elastic properties. The hydrogel supports the growth of rat pancreatic cells (RIN-5F), human embryonic kidney cells (HEK-293), baby hamster kidney cells (BHK-21), and human neuroblastoma cells (IMR-32). The cells grow in clusters as is anticipated in a three-dimensional matrix. The rat pancreatic cells produced insulin, suggesting that they are functional inside the gel.



INTRODUCTION

Gels play an important role in our day-to-day life; toothpaste gels, shaving gels, and shampoos are some of the examples. They constitute an important class of soft materials. They are characterized by a large amount of solvent, typically $\geq 98\%$ w/w, fixed by a small amount of solid. Supramolecular gels or physical gels are characterized by the self-assembly of gelator molecules through noncovalent interactions. Water-based gels (hydrogels) have gained considerable attention over the past 2 decades and turn out to be promising materials for biomedical applications.^{1–4} Among the various classes of molecules that have been explored for biocompatible hydrogels, peptides stand out as the material of choice.^{1,5,6} Peptides are biocompatible, easily synthesized using standard methods, and readily hydrolyzed by proteases.⁷ In addition to that, the amino acids obtained through hydrolysis could serve as a nutrient pool. Peptide-based hydrogels are promising candidates as drug-delivery vehicles and scaffolds for three-dimensional (3D) cell culture.^{2,8} Their potential has quickly been realized, and several peptide-based hydrogels have already been commercialized as media for cell culture.⁹

A large majority of peptide-based hydrogels are based on β -sheet architecture.¹⁰ Non- β -sheet peptide hydrogels, however, are also known.^{11,12} The peptide molecules self-assemble into β -sheet-rich fibrillar structures, much like amyloid fibrils. The fibrils entangle to make a three-dimensional network trapping water molecules. In fact, hydrogels have been obtained from amyloidogenic proteins^{13,14} and Fmoc-capped amyloidogenic

peptides.¹⁵ We investigated if a short amyloidogenic peptide could be engineered to obtain hydrogels through minimal modifications. $A\beta_{16-22}$ (Ac-KLVFFAE-am), an amyloidogenic fragment from β -amyloid, has been studied in great detail. Depending on the aggregation conditions, the peptide self-assembles to form amyloid-like fibers,¹⁶ nanotubes,¹⁷ and annular rings.¹⁸ The aromatic cassette is believed to play an important role in its self-assembly.^{19–22} Phe19, in particular, is very critical as it resides in the intersheet space and is involved in hydrophobic interactions. The Phe19–Phe19 and Leu17–Phe19 interactions are believed to be important in driving the self-assembly.¹⁹ Phe20, on the other hand, is less important for intersheet interactions but could be involved in the interprotofilament interactions. Phe and Tyr feature in various peptide-based hydrogelators.^{5,9,23,24} In fact, Fmoc-Tyr and Fmoc-Phe are reported to cause hydrogelation at slightly acidic pH.⁸ Interestingly, Fmoc-Tyr forms hydrogels with a storage modulus at least 2 orders of magnitude larger than that of Fmoc-Phe hydrogels. The reasons underlying different hydrogelation potentials are not clear. Packing of the bulkier Tyr side chain and the possibility of forming hydrogen bonds with water molecules could be the reason. These observations led us to investigate the hydrogelating potential of the Phe20Tyr analogue of $A\beta_{16-22}$.

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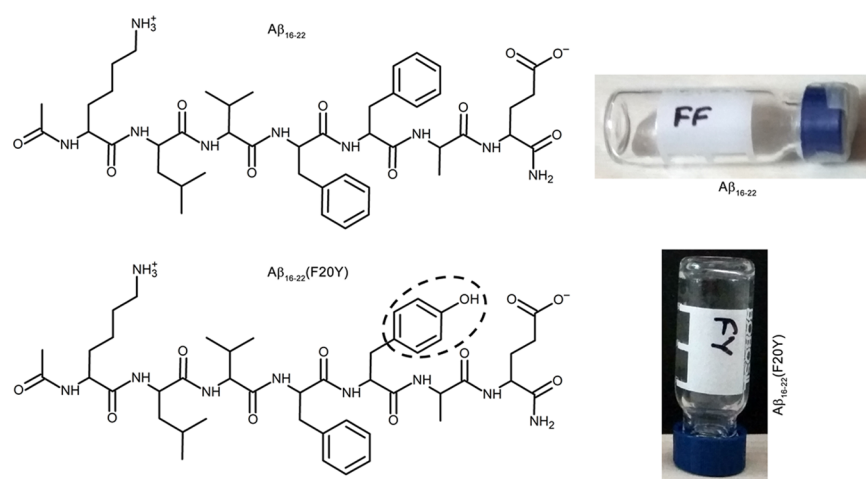


Figure 1. Gelation by peptides as assessed by tube inversion. The 12 h old $A\beta_{16-22}$ (20 mM) flows when the tube is tilted (top), whereas $A\beta_{16-22}$ (F20Y) (2 mM) supports its weight in the inverted tube (bottom).

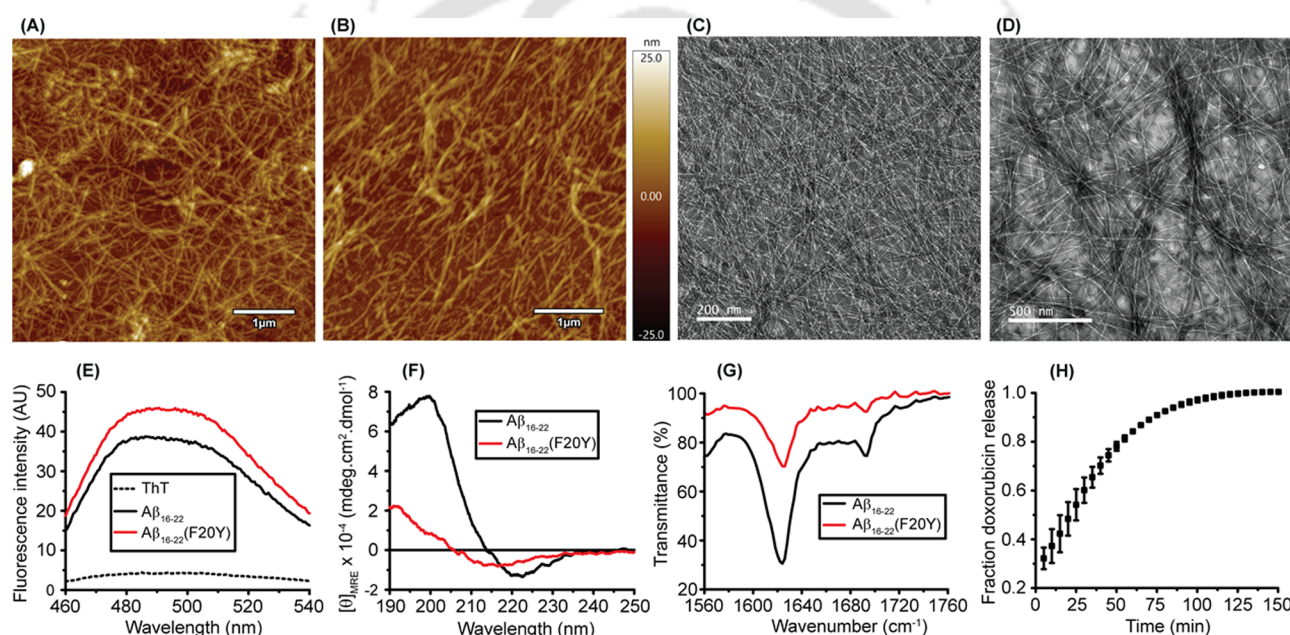


Figure 2. Characterization of self-assembled $A\beta_{16-22}$ structures and the $A\beta_{16-22}$ (F20Y) gel. Self-assembled fibrillar structures formed by $A\beta_{16-22}$ (panels A and C) and the $A\beta_{16-22}$ (F20Y) gel (panels B and D) as observed using AFM (panels A and B) and TEM (panels C and D). ThT fluorescence spectra (panel E), CD spectra (panel F), and attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) spectra (panel G) of the self-assembled peptides. Release of doxorubicin from the gel, as monitored by absorbance at 490 nm (panel H). Error bars indicate standard deviation for three independent experiments.

RESULTS AND DISCUSSION

The peptides were synthesized, purified, and characterized as described in the [Experimental Section](#). The peptide stock solutions were prepared in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). For gelation, peptides were diluted in water to the desired concentrations such that the final HFIP concentration is 10%. Dilution in water resulted in clear transparent solutions. The peptide solutions thus obtained were left undisturbed at room temperature for 12 h. Formation of the gel was examined by inverting the tubes. $A\beta_{16-22}$ (F20Y) resulted in self-supporting transparent gels in the concentration range from 2 to 20 mM (~ 0.18 – 1.8% w/w). [Figure 1](#) shows the picture of the inverted tube for 2 mM $A\beta_{16-22}$ (F20Y) gel. $A\beta_{16-22}$, on the other hand, failed to form gels up to 20 mM

concentration; tilting the tube caused the sample to flow, indicating largely viscous behavior.

As the peptide $A\beta_{16-22}$ (F20Y) is derived through subtle modification of the amyloidogenic peptide $A\beta_{16-22}$, the structural makeup of the self-assembled structures was investigated using non-contact mode atomic force microscopy (AFM) and transmission electron microscopy (TEM) imaging, thioflavin T (ThT) fluorescence spectroscopy, circular dichroism (CD) spectroscopy, and infrared spectroscopy. As anticipated, both the peptides resulted in entangled fibrous structures ([Figure 2A–D](#)).

Fibrils formed by the two peptides are very similar. The data show that the substitution of Phe20 by Tyr does not compromise the self-assembling propensity of the β -amyloid-derived peptide. Aggregation of both the peptides resulted in

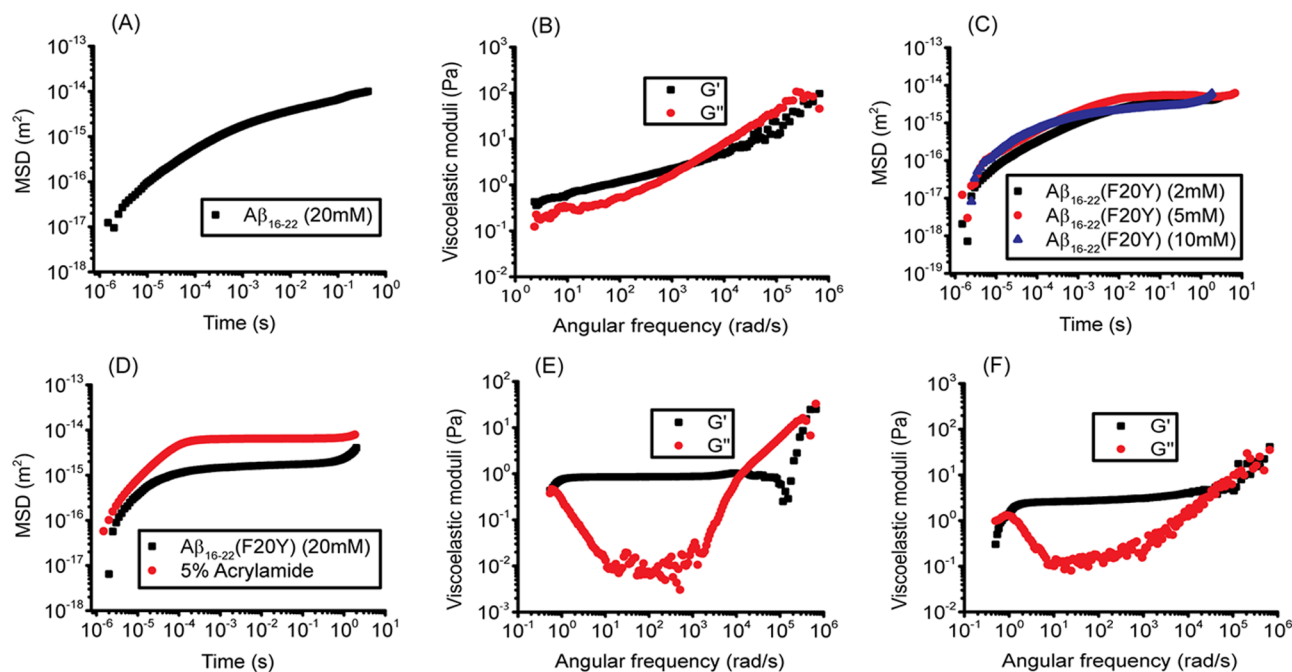


Figure 3. DLS microrheology characterization. Plot of MSD against time for 20 mM $A\beta_{16-22}$ (panel A). Plots of G' and G'' against angular frequency for 20 mM $A\beta_{16-22}$ (panel B). Plots of MSD against time for 2, 5, and 10 mM $A\beta_{16-22}$ (F20Y) gels (panel C). Plots of MSD against time for 20 mM $A\beta_{16-22}$ (F20Y) and 5% acrylamide (panel D) gels. Plots of G' and G'' against angular frequency for 5% acrylamide (panel E) and 20 mM $A\beta_{16-22}$ (F20Y) (panel F) gels.

micrometer-long fibers. However, analysis of TEM images identified a considerable difference in their width. $A\beta_{16-22}$ fibrils turned out to be 7.6 ± 1.6 nm wide, whereas the average width of $A\beta_{16-22}$ (F20Y) fibrils was found to be 14.3 ± 4.2 nm. The ThT fluorescence assay carried out in 5 mM phosphate buffer, pH 7.4, shows ~ 10 -fold enhancement in fluorescence intensity in the presence of 20 μ M peptide. Both the peptides caused similar enhancement in fluorescence intensity (Figure 2E). The data confirms the cross- β nature of the fibrillar structures, if any, at the secondary structure level. The $A\beta_{16-22}$ (F20Y) spectrum displays a negative band centered at ~ 216 nm and a positive band at ~ 191 nm, indicating a distinct β -sheet conformation (Figure 2F). The CD spectrum of $A\beta_{16-22}$, on the other hand, displays a negative band at ~ 221 nm and an intense positive band at ~ 199 nm. The spectrum is suggestive of a twisted β -sheet.²⁵ Distinct CD spectra indicate differences at the secondary structure level for the self-assembled peptides. Both $A\beta_{16-22}$ and $A\beta_{16-22}$ (F20Y), however, harbor dipeptide aromatic motifs, and clusters of aromatic residues can have a significant contribution to far-UV CD spectra of short peptides.^{26,27} Aromatic residues constitute the core of $A\beta_{16-22}$ fibrils.²⁸ In fact, intermolecular aromatic interactions are believed to play a critical role in $A\beta_{16-22}$ self-assembly. Such a cluster of aromatic residues can cause marked perturbation in far-UV CD. The secondary structure of the self-assembled peptides was, therefore, further characterized using ATR-FTIR spectroscopy. Amide I band at ~ 1626 cm^{-1} indicates a β -sheet conformation, even for peptides rich in aromatic residues.²⁹ Both $A\beta_{16-22}$ and $A\beta_{16-22}$ (F20Y) showed the amide I band at 1624 cm^{-1} , a signature characteristic of β -sheet-rich fibrils.³⁰ The high-frequency peak often observed for amyloid-like fibrils³⁰ was observed at ~ 1692 cm^{-1} for both the peptides. The Tyr side chain is bulkier than that of Phe. In addition to that, Tyr is less hydrophobic than Phe and could be

more solvent-exposed. These characteristics alongside the electronic effects of the electron-withdrawing hydroxyl group in the Tyr side chain and the possibility of H-bonding could result in distinct hydrogelation propensity. H-bonding has recently been shown to play a critical role in the gelation of a small peptide hydrogelator, GHG.³¹ Involvement of the Tyr side chain hydroxyl group in H-bonding is often characterized using Raman spectroscopy. Tyrosine generates a pair of fairly intense Raman bands at ~ 850 and 830 cm^{-1} .³² The ratio of the intensities of these bands (I_{850}/I_{830}) depends on the H-bonding status of the phenolic OH group.^{32,33} An I_{850}/I_{830} ratio of 0.3 suggests the hydroxyl group to be a strong H-bond donor, whereas a ratio of 2.5 suggests it to be a strong H-bond acceptor. An intermediate value suggests the hydroxyl group to be both a H-bond donor and an acceptor. The Raman spectrum recorded from the $A\beta_{16-22}$ (F20Y) hydrogel resulted in distinct bands centered at ~ 853 and 830 cm^{-1} (Supporting Information, Figure S1). The ratio I_{853}/I_{830} turns out to be ~ 0.98 , indicating that the Tyr hydroxyl group acts as both a H-bond donor and an acceptor in the $A\beta_{16-22}$ (F20Y) hydrogel. We got curious to see if the peptide could cause gelation at acidic pH values. We found that the peptide resulted in self-supporting gels at pH values 2 and 4 (Figure S2). These gels were not further investigated.

Peptide hydrogels based on a fibrillar network can trap small molecules and hold promise to be used as drug-delivery vehicles. The entrapment of an anticancer drug, doxorubicin, in the $A\beta_{16-22}$ (F20Y) gel and subsequent release in water were studied. Doxorubicin was trapped inside the gel during the gelation process itself. The release of doxorubicin was monitored by gently adding water (19-fold volume) on the gel and measuring the absorbance at 490 nm as a function of time. Doxorubicin displayed a slow release (Figure 2H); saturation in absorbance, an indication of equilibrium, was achieved after around 150 min, indicating a potential

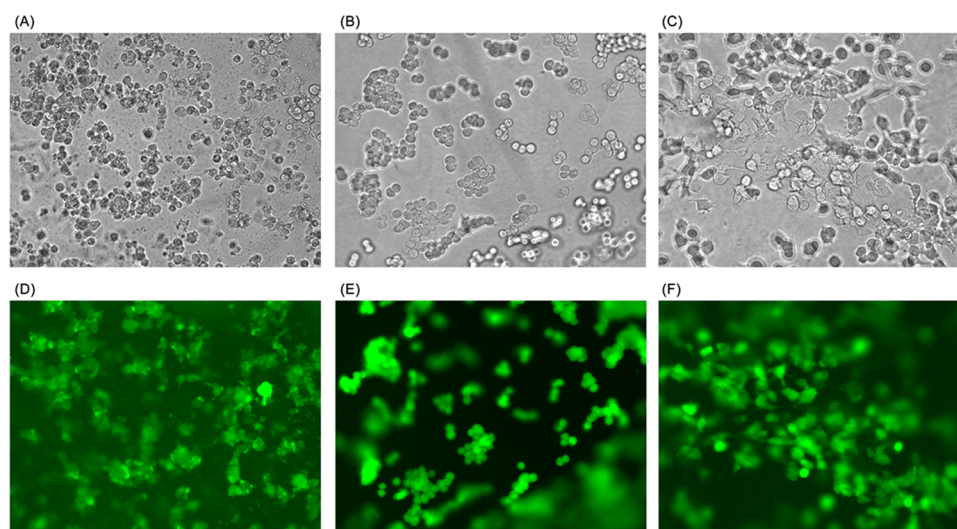


Figure 4. Brightfield (top panels) and calcein fluorescence (bottom panel) images of RIN-5F (panels A and D), HEK-293 (panels B and E), and BHK-21 (panels C and F) cells grown for 24 h in the $A\beta_{16-22}$ (F20Y) gel.

application of the gel as a drug-delivery vehicle. Release of a fluorescent dye, rhodamine B, was also examined by monitoring its fluorescence as a function of time. The dye displayed a rather slow release for 3 days (Supporting information, Figure S3). As $A\beta_{16-22}$ (F20Y) formed transparent gels, the rheological properties could readily be investigated using dynamic light scattering (DLS) microrheology.^{34,35} The polyacrylamide gel (5%) was used as a reference gel material. Diffusion of spherical colloid particles is the principle underlying DLS microrheology. For a Newtonian fluid, the mean-squared displacement (MSD) of the colloid particles increases linearly over time. A nonlinear increase in MSD is indicative of a viscoelastic material. MSD can be related to the viscoelastic modulus of the fluid using the generalized Stokes–Einstein relation.³⁶ Figure 3 shows the microrheology data recorded using 600 nm diameter carboxylated melamine beads.

$A\beta_{16-22}$ (20 mM) displays a nonlinear MSD plot, suggesting a viscoelastic behavior (Figure 3A). Plots of storage (G') and loss (G'') moduli against frequency indicate comparable storage and loss moduli across the frequency range (Figure 3B). Figure 3C shows the MSD plots of 2, 5, and 10 mM $A\beta_{16-22}$ (F20Y) samples. The traces indicate viscoelastic behavior at all three concentrations. The peptide at 10 mM concentration exhibits distinct elastic behavior. The MSD trace of 20 mM $A\beta_{16-22}$ (F20Y) is similar to that of the 5% acrylamide gel (Figure 3D). Such MSD traces have been reported in the literature for Fmoc-Tyr hydrogels.³⁷ Plots of G' and G'' against angular frequency for 5% acrylamide (Figure 3E) and 20 mM $A\beta_{16-22}$ (F20Y) gels (Figure 3F) indicate a higher storage modulus than the loss modulus in the 1–10 000 rad/s frequency range. The storage modulus for the 5% acrylamide gel is ≥ 10 -fold higher than the loss modulus in the 2–3000 rad/s frequency range, whereas that for 20 mM $A\beta_{16-22}$ (F20Y) is at least 3–5 times higher in the same frequency range. The storage modulus is at least 1 order of magnitude higher than the loss modulus in the 5–500 rad/s frequency range. The sensitivity of the gel to changes in temperature was assessed for the 5 mM $A\beta_{16-22}$ (F20Y) gel by monitoring the changes in microrheological properties at different temperatures. MSD for the $A\beta_{16-22}$ (F20Y) gel prepared at 25 °C was recorded. Subsequently, the temper-

ature was increased up to 55 °C with a step size of 10 °C and brought back to 25 °C with the same step size. MSD data recorded at these temperatures were used to determine G' and G'' against angular frequency (Figure S4, Supporting Information). An increase in temperature caused subtle changes in MSD, which was also reflected in the viscoelastic moduli wherein G' and G'' decrease a little. No distinct melting temperature was observed up to 55 °C. Increasing the temperature beyond 55 °C caused considerable solvent evaporation; data beyond 55 °C, therefore, could not be recorded. A subsequent decrease in temperature did not cause any appreciable change in MSD and viscoelastic moduli. As viscoelastic moduli for most peptide hydrogels reported in the literature were determined using bulk rheology, we did not attempt comparing them with those of the $A\beta_{16-22}$ (F20Y) gel. In fact, Frith and co-workers have shown that DLS microrheological properties of the Fmoc-Tyr hydrogel do not reproduce the bulk rheological properties.³⁷

Peptide hydrogels find much interest in regenerative medicine as they can act as scaffolds to support cell growth in three dimensions. $A\beta_{16-22}$ (F20Y) gelation at 5 mM concentration was tested at 37 °C. The peptide formed a distinct transparent gel. The potential of the gel as a 3D cell culture scaffold was investigated using the rat pancreatic cell line RIN-5F, human embryonic kidney cells (HEK-293), and baby hamster kidney cells (BHK-21). Gels (5 mM) were prepared in the wells of a 96-well plate. The cells were seeded as described in the Experimental Section and examined after 24 h using microscopy (Figure 4). All of the three cells grow in three-dimensional clumps as has been reported in the literature for three-dimensional cell cultures.³⁸ Viability of cells was assessed through calcein acetoxymethyl ester (calcein AM) uptake. Calcein AM is a cell-permeable nonfluorescent derivative of calcein. Following cellular uptake, ester bonds are hydrolyzed by the intracellular esterases, resulting in green fluorescence.³⁹ All of the three cells display green fluorescence upon calcein AM treatment, indicating that the cells are alive inside the gel (Figure 4D–F).

RIN-5F cells were assayed for insulin expression using polymerase chain reaction (PCR). The cDNA synthesized from total mRNA was amplified using rat insulin-1 primers and

analyzed using agarose gel electrophoresis. The cells were found to express insulin-1, suggesting that they were functional while growing in the $A\beta_{16-22}$ (F20Y) gel (Figure 5). Amyloid-

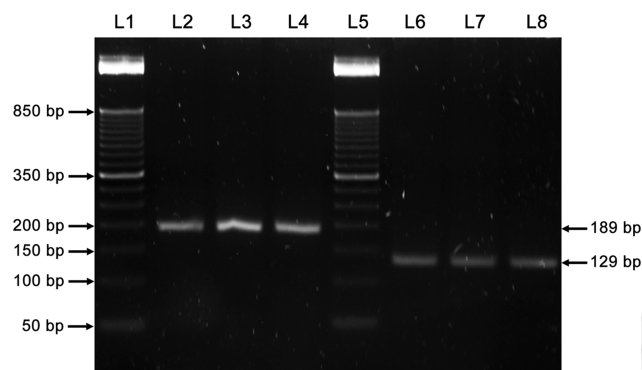


Figure 5. Expression of glyceraldehyde 3-phosphate dehydrogenase (L2–L4) and insulin-1 (L6–L8) from three independent samples. L1 and L5 show the bands from the molecular weight marker. L2, L6; L3, L7; and L4, L8 correspond to the three independent samples. The ~ 189 bp bands in L2–L4 correspond to glyceraldehyde 3-phosphate dehydrogenase, whereas ~ 129 bp bands in L6–L8 correspond to insulin-1.

based hydrogels have the ability to act as matrices for three-dimensional cell culture and tissue engineering.^{13,15} Clearly, the $A\beta_{16-22}$ (F20Y) hydrogel supports the growth and function of RIN-SF cells. In addition to RIN-SF, HEK-293, and BHK-21 cells, the gel also supported the growth of human neuroblastoma cell line, IMR-32 (Supporting Information, Figure S5).

Physical gels constitute an important class of soft materials. Large-molecular-weight biopolymers, polysaccharides in particular, are known for long to form hydrogels. Proteins and peptides have emerged as another important class of hydrogelating biopolymers over the past 2 decades. Peptides make an interesting class of molecules. Physical and chemical properties that determine the structure, self-assembling propensities, and the characteristics of the self-assembled structures can be modulated by suitably modifying the sequence. Most peptide-based hydrogels are characterized by β -sheet-rich fibrillar structures that entangle to form a three-dimensional network similar to that of amyloid fibrils. Amyloidogenic proteins and peptides can form hydrogels.^{14,40,41} This study presents a hydrogelating analogue of the well-studied amyloidogenic fragment from β -amyloid, $A\beta_{16-22}$. The analogue, $A\beta_{16-22}$ (F20Y), self-assembles to yield a soft gel at a concentration as low as 2 mM ($\sim 0.18\%$ w/w), whereas $A\beta_{16-22}$ fails to form hydrogels even at a 10-fold higher concentration. Aromatic amino acids are present in various amyloidogenic peptides and are believed to play a critical role in their self-assembly through aromatic stacking interactions; Phe has received special attention.⁴² End-capped amino acids and small peptides that harbor one or more aromatic moieties are reported to self-assemble through stacking interactions and cause hydrogelation. Fmoc is a well-studied N-terminal aromatic moiety; a number of Fmoc-protected peptide hydrogelators are reported. In our study, $A\beta_{16-22}$ and $A\beta_{16-22}$ (F20Y), both lacking Fmoc, showed fibrillar aggregates that caused comparable enhancement in ThT fluorescence. The $A\beta_{16-22}$ (F20Y) fibrillar network favored gelation even at a very low concentration of 2 mM.

Even though the fibrils formed by the two peptides are very similar, the CD spectra are quite distinct. The difference in the secondary structure alongside the H-bonding potential of Tyr could possibly account for $A\beta_{16-22}$ (F20Y) gelation propensity. These data indicate that Tyr could be as promising as Phe in designing small peptide-based hydrogelators. Furthermore, this study shows that the native amyloidogenic peptides hold the promise to be developed into nontoxic hydrogelators through subtle modifications.

EXPERIMENTAL SECTION

Materials. Fmoc-protected amino acids and N,N,N',N' -tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) were procured from Novabiochem (Darmstadt, Germany). Rink amide resin, piperidine, N,N -diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), thioanisole, ethanedithiol, thioanisole, acetic anhydride, 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), thioflavin T (ThT), and calcein AM were procured from Sigma-Aldrich Chemicals Pvt. Ltd. 1-Hydroxybenzotriazole hydrate (HOBt) and trypan blue were obtained from Sisco Research Laboratory, India. N,N -dimethylformamide, diethyl ether, *m*-cresol, and rhodamine B were obtained from Merck, India. Doxorubicin hydrochloride was procured from TCI Chemical Pvt. Ltd. All of the cell lines were obtained from the National Centre for Cell Science (Pune, India). All other reagents were of the highest grade available.

Peptide Synthesis. Peptides were synthesized manually on Rink amide resin by employing Fmoc chemistry and HBTU/HOBt/DIPEA activation. N-terminal acetylation of peptides was carried out on resin using 5 equiv of acetic anhydride and 10 equiv of DIPEA. The synthesized peptides were cleaved from the resin by overnight incubation of the peptide-bound resin in a cleavage cocktail containing TFA/*m*-cresol/thioanisole/ethanedithiol (20:2:2:1). The peptides were precipitated in ice-cold diethyl ether, washed with diethyl ether to remove the scavengers, and dried. The peptides were dissolved in dimethyl sulfoxide and purified on a reversed-phase C18 column, as described previously.⁴³ The purified peptides were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF).

Self-Assembly/Hydrogelation. The peptide stock solutions were prepared in HFIP. $A\beta_{16-22}$ concentration was estimated using a molar absorption coefficient of $286 \text{ M}^{-1} \text{ cm}^{-1}$ at 254 nm, whereas $A\beta_{16-22}$ (F20Y) concentration was estimated using the molar absorption coefficient of tyrosine ($\epsilon_{280} = 1280 \text{ M}^{-1} \text{ cm}^{-1}$). The peptides were subsequently diluted in water to obtain the desired peptide concentrations (final HFIP concentration = 10%). The peptides were left undisturbed at room temperature; self-supporting gels were obtained for $A\beta_{16-22}$ (F20Y) after 12 h.

Atomic Force Microscopy. Peptide samples (24 h old; 2 mM concentration in 10% HFIP) were 100-fold diluted and quickly deposited on clean glass slides. The excess solution was blotted out after 2 min using Whatman I filter paper, and slides were allowed to air-dry. AFM images were acquired in non-contact mode using a silicon probe on an Asylum MFP-3D Origin atomic force microscope (Oxford Instruments).

Transmission Electron Microscopy. Peptide samples (2 mM; incubated for 24 h) were diluted 100-fold, and 5 μL volume was deposited on carbon-coated 200-mesh copper grids. The excess solution was blotted out from the periphery

of the grid by Whatman I filter paper after 2 min. The grids were subsequently stained with saturated uranyl acetate solution; the uranyl acetate solution was blotted out after 30 s. The grids were then air-dried, and images were recorded at 200 kV on a JEM-2100F (JEOL, Japan) transmission electron microscope at Central Instruments Facility, IIT Guwahati. Widths of the fibrils were determined using ImageJ software. At least 20 fibrils were measured for each of the samples.

ThT Fluorescence Spectroscopy. The ThT fluorescence assay was carried out at 10 μ M ThT concentration in 5 mM phosphate buffer, pH 7.4, on a Jasco FP8500 spectrofluorometer. Peptide samples (24 h old, 2 mM) were diluted to 20 μ M concentration and excited at 440 nm, slit width 2.5 nm. Fluorescence emission spectra were recorded from 460 to 540 nm with a 5 nm emission slit width.

Circular Dichroism Spectroscopy. Far-UV circular dichroism (CD) spectra were recorded on a Jasco J-1500 spectropolarimeter. The 24 h old peptide samples (2 mM) were diluted to obtain 35–50 μ M concentration, and spectra were recorded in a 0.1 cm path length cell with a scan rate of 100 nm/min. Each spectrum was an average of four scans and was corrected by subtracting the solvent spectrum. The data were converted to mean residue ellipticity, as previously described.⁴³

Fourier Transform Infrared (FTIR) Spectroscopy. FTIR spectra were recorded for the 24 h old 2 mM peptide samples on a Shimadzu IRAffinity-1S Fourier transform infrared spectrophotometer on the attenuated total reflection (ATR) module equipped with a diamond ATR crystal. Peptide samples (10 μ L) were spread out and dried as films on the crystal, and ATR-FTIR spectra were recorded at a resolution of 4 cm^{-1} .

Doxorubicin Release Assay. Doxorubicin at 0.5 mg/mL concentration was trapped inside the $A\beta_{16-22}$ (F20Y) gel (5 mM peptide concentration) during the gelation process. Briefly, 5 μ L of 50 mM $A\beta_{16-22}$ (F20Y) stock solution in HFIP was added to a quartz cuvette; 45 μ L of 0.55 mg/mL doxorubicin stock solution prepared in water was subsequently added and left undisturbed for 12 h, resulting in the gel. The release of drug was assayed by gently adding 950 μ L of water on the doxorubicin-loaded gel and monitoring absorbance at 490 nm as a function of time.

Microrheology. Microrheology measurements were carried out on a Malvern Zetasizer Nano ZS instrument using the 173° non-invasive backscattering configuration. Carboxylated melamine beads (0.6 μ m) were used as the tracer particle. The concentration of the tracer particles was optimized so as to prevent tracer aggregation as well as the interaction between the sample and the tracer. All of the microrheology experiments were carried out at 25 °C in a quartz cuvette having a final tracer volume fraction of 0.025% in the sample. Briefly, the tracer particles were added along with the peptide during the gel formation. Each gel was set in the DLS cuvette and left undisturbed for 12 h. The mean-squared displacement (MSD) data were recorded for the 12 h old peptide samples. The elastic (G') and viscous (G'') moduli were determined using Malvern Zetasizer instrument software from the mean-squared displacement data using the generalized Stokes–Einstein equation, as introduced by Mason.³⁵

Cell Seeding and Cultivation. The human embryonic kidney (HEK-293), baby hamster kidney (BHK-21), rat pancreas (RIN-5F), and human neuroblastoma (IMR-32) cells were procured from the National Centre for Cell Science,

Pune, India. The cells were cultured in T25 culture flasks containing Roswell Park Memorial Institute medium (RPMI 1640) (RIN-5F) or Dulbecco's modified Eagle's medium (DMEM) (HEK-293, BHK-21, and IMR-32) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic solution (Gibco, Life Technologies). The cells were incubated at 37 °C containing 5% CO_2 and were subsequently re-cultured and maintained for further assays. Cells were harvested by trypsinization, stained with trypan blue, and counted using Countess II FL automated cell counter (Thermo Fisher Scientific). $A\beta_{16-22}$ (F20Y) gels (5 mM concentration) were prepared in the wells of a 96-well plate at 37 °C in 5% CO_2 . After 12 h of gelation, RPMI or DMEM (2-fold volume) was gently added to the gel and decanted carefully after 60 min; this step was repeated once more. Cells (2×10^5) were then seeded in the 96-well plate and incubated at 37 °C in 5% CO_2 .

Cell Viability Assay. RIN-5F, HEK-293, and BHK-21 cells grown in the gels for 24 h were washed twice with phosphate-buffered saline (PBS, pH 7.4) and incubated with 5 μ M calcein AM in plain RPMI/DMEM medium (without FBS) for 30 min at 37 °C. After staining, cells were washed three times with PBS and visualized under white light and images were recorded on an EVOS FLoid cell imaging station (Thermo Fisher Scientific). Subsequently, fluorescence images were recorded using a green channel. Green fluorescence indicates hydrolysis of the nonfluorescent calcein AM by cellular esterases to fluorescent calcein. RIN-5F cells were assayed for the insulin-1 expression as well.

Insulin Gene Expression Assay. Functional characterization of RIN-5F cells grown in the gel was carried out by assaying for insulin-1 gene expression by PCR. Total RNA was extracted from the RIN-5F cells grown for 24 h using an RNeasy mini kit (QIAGEN) according to the manufacturer's protocol. Genomic DNA contamination, if any, was removed by treating with DNase I (NEB) for 30 min at 37 °C. cDNA was synthesized from 500 ng of RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and rat insulin-1 was confirmed by PCR amplification. The sequences of primers used for PCR amplification are shown in the Supporting Information (Table S1). The expected PCR product sizes for insulin-1 and GAPDH are 129 and 189 bp, respectively.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02771.

Raman spectroscopic analysis of the $A\beta_{16-22}$ (F20Y) gel, thermal response of the gel, gelation at acidic pH values, rhodamine B release kinetics, growth of IMR-32 cells, and PCR primer sequences (PDF)

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Author Contributions

N.C. and R.N. conceived the idea. D.D., R.N., and N.C. designed the experiments. D.D. performed the hydrogelation, AFM and TEM imaging, spectroscopic characterization, and microrheology experiments. V.K. and S.K. designed, conducted, and analyzed cell culture experiments. All of the authors contributed in manuscript writing. All of the authors approve the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

HBTU, *N,N,N',N'*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)-uronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; DIPEA, *N,N*-diisopropylethylamine; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; ThT, thioflavin T; CD, circular dichroism; AFM, atomic force microscopy; TEM, transmission electron microscopy; ATR-FTIR, attenuated total reflectance-Fourier transform infrared spectroscopy; DLS, dynamic light scattering

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Lipid hydrogels from β -turn motif-connected tandem repeats of $A\beta_{16-22}$ [†]

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Self-assembling peptides constitute an important class of functional biomaterials. A number of short amyloidogenic stretches have been identified from amyloid proteins. Such peptides, as such or through subtle modifications, can turn out to be promising candidates for functional biomaterials. End-capped $A\beta_{16-22}$, the well-studied amyloidogenic stretch from β -amyloid, is reported to be non-hydrogelating up to 20 mM concentration. Here we investigated the hydrogelation propensity of $A\beta_{16-22}$ repeats connected through β -turn-supporting motifs. The peptide repeats connected through Asn-Gly, Aib-^DPro, and ^DPro-Gly formed transparent hydrogels at concentrations ≥ 2 mM. The repeats of the aromatic analog $A\beta_{16-22}$ (F20Y) also resulted in similar hydrogels. Like other peptide-based gels reported earlier, these gels could trap the anticancer drug doxorubicin and displayed steady release in water. In addition, the gels supported the growth of mammalian cell lines, HEK-293 and RIN-5F. These data show that turn-inducing motifs can have marked effects on the hydrogelating propensity of self-assembling peptides.

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Introduction

Amyloid fibers are insoluble protein/peptide deposits found in various neurodegenerative diseases *viz.* Alzheimer's disease, Parkinson's disease, type II diabetes, Huntington's disease, *etc.* Initially suspected to be the causative agents for the disease, fibrillar aggregates subsequently turned out to be rather benign. Research carried out in the past two decades identified soluble oligomeric species as the real culprits behind cytotoxicity and disease progression.^{1–3} In fact, shifting the equilibrium of the protein aggregation pathway towards fibrils should diminish the cytotoxicity, albeit at the cost of higher protein deposition, which could have its own implications. The non-cytotoxic nature of the amyloid-like fibrils is further substantiated by the discovery of native, functional amyloids. Initially identified in lower organisms such as bacteria and fungi, such functional amyloids have now been discovered in higher organisms including humans.⁴ Growing evidence suggesting amyloids as non-toxic functional materials eased their way for technological applications.⁵ Amyloid fibrils possess impressive material properties and have drawn the attention of the material science community. They have

been used as scaffolds for catalysis, casting nanowires, and tissue engineering.^{6–9} Amyloid-like fibrils formed by designed peptides have been reported to possess intrinsic conductivity.¹⁰ In addition, amyloidogenic proteins/peptides can be functionalized with molecules to obtain fibrous aggregates that are decorated with the tagged molecules.¹¹ Such functionalization has resulted in fibers with biological, catalytic, optical, electrical, and electronic properties.^{12–20}

Hydrogels happen to be an important class of soft materials that find applications in enzyme immobilization, drug delivery, tissue engineering, catalysis, and waste treatment.^{7,21–36} Largely due to their biocompatibility and ease of synthesis, short peptides have gained huge attention as hydrogelating materials.³⁷ It is noteworthy that most, but not all, of these peptides self-assemble into β -sheet rich amyloid-like fibrous structures. The fibrils make a three-dimensional network that can fix water molecules. It is, therefore, imperative to investigate if naturally-occurring amyloidogenic proteins and peptides could be tuned to form such gels. Amyloid-like fibers formed by *de novo* designed peptides, amyloidogenic peptides and proteins, and Fmoc-protected amyloidogenic peptides have indeed been reported to form hydrogels that may find applications in drug delivery and regenerative medicine.^{38,39} The 7-residue amyloidogenic stretch of β -amyloid peptide, $A\beta_{16-22}$ (Ac-KLVFFAE-am), is reported to form amyloid fibrils at neutral pH.⁴⁰ Self-assembly of $A\beta_{16-22}$ has been investigated in great detail. The peptide molecules self-assemble to form in-register antiparallel β -sheets that give rise to amyloid-like fibrils. At pH 2, however, the peptide molecules

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self-assemble with a one-residue shift in the β -strand registry, resulting in nanotubes.⁴¹ We have previously reported that two $A\beta_{16-22}$ repeats connected *via* β -turn-supporting dipeptide motifs *viz.* Asn-Gly, Aib-^DPro, and ^DPro-Gly turn out to be more amyloidogenic than the parent peptide.⁴² In addition, the Phe20Tyr mutant and the β -turn motif-harboring repeats of $A\beta_{16-22}$ (F20Y) are, by and large, more amyloidogenic than their $A\beta_{16-22}$ counterparts. Schneider and coworkers designed short peptides that self-assemble, through β -turn mediated hairpin formation, to form responsive hydrogels.^{43,44} Here we investigated the hydrogelating propensity of β -turn motif-harboring $A\beta_{16-22}$ repeats. We have previously reported $A\beta_{16-22}$ to be non-hydrogelating up to 20 mM concentrations under our working conditions.⁴⁵ $A\beta_{16-22}$ repeats connected *via* Asn-Gly, Aib-^DPro, and ^DPro-Gly dipeptide motifs, however, resulted in limpid hydrogels that, like many peptide-based gels, hold promise as drug delivery vehicles and cell culture scaffolds. These data highlight the dramatic influence the turn-inducing motifs can have on the hydrogelating propensity of self-assembling peptides including amyloidogenic peptides.

Materials and methods

Materials

Fmoc-protected amino acids and *N,N,N',N'*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) were purchased from Novabiochem (Darmstadt, Germany). Rink amide resin, *N,N*-diisopropylethylamine (DIPEA), piperidine, trifluoroacetic acid, ethanedithiol, thioanisole, acetic anhydride, 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), and thioflavin T (ThT) were procured from Sigma-Aldrich Chemicals Pvt. Ltd. 1-Hydroxybenzotriazole hydrate (HOBt) was obtained from Sisco Research Laboratory, India. *N,N*-dimethylformamide, diethyl ether, and *m*-cresol were obtained from Merck, India. Doxorubicin hydrochloride was purchased from TCI Chemicals Pvt. Ltd. All the cell lines were obtained from the National Centre for Cell Science (Pune, India).

Peptide synthesis

Peptides were synthesized manually on Rink amide resin by employing Fmoc chemistry and HBTU/HOBt/DIPEA activation. N-terminal acetylation was carried out on-resin using 5 equivalents of acetic anhydride and 10 equivalents of DIPEA. The synthesized peptides were cleaved from the resin, precipitated in ice-cold diethyl ether, and purified on a reversed-phase C18 column as described previously.⁴² The purified peptides were characterized by MALDI-TOF mass spectrometry on a Bruker Autoflex Speed MALDI-TOF mass spectrometer at Central Instruments Facility, IIT Guwahati.

Self-assembly/hydrogelation

The peptide stock solutions were prepared in HFIP. For the peptides lacking Tyr, *i.e.* $A\beta$ FF-NG, $A\beta$ FF-pG, and $A\beta$ FF-Up, the concentrations were estimated using the Phe molar absorption coefficient at 254 nm as previously reported.⁴² The concentrations of $A\beta$ FY-NG, $A\beta$ FY-pG, and $A\beta$ FY-Up were estimated using

the molar absorption coefficient of tyrosine ($\epsilon_{280} = 1280 \text{ M}^{-1} \text{ cm}^{-1}$). For self-assembly and gelation, the peptides were diluted using water to obtain the desired peptide concentrations; the final HFIP concentration in all the reactions was 10%. Gelation was examined by inverting the tubes after 12 h incubation at room temperature.

Circular dichroism (CD) spectroscopy

Far-UV CD spectra were recorded on a Jasco J-1500 spectropolarimeter. 24 h old peptide samples were diluted in 10% HFIP and spectra recorded in a 0.1 cm path length cell with a scan rate of 100 nm min^{-1} . Each spectrum is an average of 4 scans and corrected by subtracting the solvent spectrum. The data were converted to mean residue ellipticity as previously described.⁴⁶

Fourier transform infrared (FTIR) spectroscopy

FTIR spectra were recorded for 24 h old samples on a Shimadzu IRAffinity-1S Fourier transform infrared spectrophotometer in attenuated total reflection (ATR) mode, equipped with a diamond ATR crystal. Peptide gels were sheared by pipetting, deposited (10 μl) on the crystal, and allowed to air-dry. ATR-FTIR spectra were recorded at a resolution of 4 cm^{-1} .

Atomic force microscopy (AFM)

Peptide samples (incubated for 24 h) were sheared by pipetting, diluted 100-fold in deionized water, and quickly deposited on clean glass slides. Excess solution was blotted after 2 minutes and the samples were allowed to air dry. AFM images were acquired in non-contact mode using a silicon probe on an Asylum MFP-3D Origin atomic force microscope (Oxford Instruments).

Thioflavin T (ThT) fluorescence

ThT fluorescence assay was carried at a 10 μM ThT concentration in 5 mM phosphate buffer, pH 7.4 on a Jasco FP8500 spectrofluorometer for 24 h old samples. Peptide samples were diluted to 20 μM concentration and excited at 440 nm, slit width 2.5 nm. Fluorescence emission spectra were recorded from 460–540 nm with a 5 nm slit width.

DLS microrheology (DLS μR)

Microrheology measurements were carried out on a Malvern Zetasizer Nano ZS instrument equipped with a 633 nm laser and 173° non-invasive back-scattering detector configuration. Carboxylated melamine beads (GmbH, 0.6 μm) were used as the tracer particle. The concentration of the tracer particles was optimized so as to prevent tracer aggregation as well as its interaction with the samples. The temperature for all the experiments was fixed at 25°C and a small volume quartz cuvette was used. The tracer particles in water were added to the peptide stock solutions in HFIP to obtain a final peptide concentration of 5 mM and tracer volume fraction of 0.025%. The samples were allowed to gel in the cuvette and the mean squared displacement (MSD) data were recorded after 12 h of incubation. The elastic (G') and viscous (G'') moduli were subsequently derived from the MSD data using the inbuilt Malvern Zetasizer instrument software based on the generalized Stokes–Einstein equation as introduced by Mason.⁴⁷

Doxorubicin release assay

Doxorubicin was trapped inside the 5 mM peptide gels at a 0.5 mg ml⁻¹ concentration during the gelation process. Briefly, 5 µl of 50 mM peptide stock solution in HFIP was added to a quartz cuvette. Subsequently, 45 µl of 0.55 mg ml⁻¹ doxorubicin hydrochloride solution prepared in water was added and the cuvette left undisturbed for 12 h resulting in gels. Water (950 µl) was gently loaded on top of the drug-loaded gel (50 µl) and absorbance monitored at 490 nm with respect to time.

Cell seeding and cultivation

Rat pancreatic cell line (RIN-5F) and human embryonic kidney cells (HEK-293) were cultured in T25 culture flasks containing RPMI 1640 and DMEM media, respectively, supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution. The culture flasks were incubated at 37 °C in a humidified atmosphere having 5% CO₂. Cells were subsequently harvested using trypsin and stained with trypan blue for counting. Counting was carried out on a Countess II FL automated cell counter (Thermo Fisher Scientific). AβFF-NG and AβFY-NG gels (2 mM peptide concentration) were prepared in a 96-well plate at 37 °C in 5% CO₂. RPMI or DMEM media (2-fold volume) was gently added to the gels obtained after 12 h incubation and carefully decanted after 60 minutes; this step was repeated once more. Approximately 2 × 10⁵ cells were then seeded on the gel and incubated at 37 °C in 5% CO₂.

Cell viability assay

RIN-5F and HEK-293 cells grown in the gels for 24 h were washed with phosphate-buffered saline (PBS, pH 7.4) twice. Subsequently, 5 µM calcein AM dye dissolved in plain RPMI/DMEM medium (without FBS) was added to the cells and incubated for 30 min at 37 °C. After staining, the cells were washed thrice with PBS and visualized under white light on an EVOS FLoid cell imaging station (Thermo Fisher Scientific). Fluorescence images were recorded using the green channel.

Insulin gene expression assay

Expression of the insulin-1 gene by the RIN-5F cells grown in hydrogels was studied as previously described.⁴⁵ Briefly, RNA was extracted from the gel-grown cells using an RNeasy mini kit (QIAGEN) as per the manufacturer's protocol. Complementary DNA was subsequently synthesized using a high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). The expression of insulin-1 was examined through PCR amplification wherein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control. The primers used for PCR amplifications are listed in the ESI† (Table S1).

Results

The IDs and the sequences of the β-turn motif-harboring peptides employed in this study are shown in Table 1. The peptides were synthesized, purified, and characterized as described in the Materials and methods section.

Table 1 The peptide IDs and their sequences. Letters 'U' and 'p' in peptide IDs represent Aib and ^DPro, respectively

Peptide ID	Sequence ^a
AβFF-NG	Ac-KLVFFAE-NG-KLVFFAE-am
AβFF-pG	Ac-KLVFFAE- ^D PG-KLVFFAE-am
AβFF-Up	Ac-KLVFFAE-Aib ^D P-KLVFFAE-am
AβFY-NG	Ac-KLVFYAE-NG-KLVFYAE-am
AβFY-pG	Ac-KLVFYAE- ^D PG-KLVFYAE-am
AβFY-Up	Ac-KLVFYAE-Aib ^D P-KLVFYAE-am

^a 'Ac-' represents N-terminal acetylation whereas '-am' represents C-terminal amide.

The peptides self-assemble to form transparent hydrogels

The β-turn motif-harboring peptides are poorly soluble in water and ethanol. Self-assembly/hydrogelation was, therefore, investigated in 10% HFIP. The peptides were dissolved in HFIP; water was subsequently added to achieve the desired peptide concentration such that the final HFIP concentration is 10%. The tubes were left undisturbed at room temperature and gelation examined by inverting the tubes after 12 h of incubation (Fig. 1). All three Aβ₁₆₋₂₂ β-turn analogs *viz.* AβFF-NG, AβFF-pG, and AβFF-Up resulted in self-supporting hydrogels at 2 mM (~0.4 wt%) peptide concentration (panels A–C). AβFY-NG also resulted in hydrogels at 2 mM peptide concentration (panel D). AβFY-pG and AβFY-Up, on the other hand, failed to cause gelation at 2 mM concentration; at a higher concentration (5 mM, ~1 wt%), however, both the peptides resulted in clear gels (panels E and F). All six peptides resulted in limpid hydrogels at 5 mM peptide concentration.

The viscoelastic properties of the 5 mM gels were examined by DLS-microrheology measurements using 600 nm carboxy-melamine tracer particles. The MSD of the tracer particles in a purely viscous material displays a linear increase with time; any deviation from linearity indicates non-viscous behavior. The MSD traces obtained for the peptide hydrogels are shown in Fig. 2 (panels A and E). The tracer particles display non-linear displacement indicating distinct viscoelastic properties.

The complex shear modulus $G^*(\omega) = G'(\omega) + iG''(\omega)$ is obtained from the MSD data using the generalized Stokes–Einstein relation. It is important to note that DLS-microrheology makes it possible to investigate the rheological properties at very high frequencies; frequencies higher than a few hundred Hz are otherwise inaccessible using bulk rheology.⁴⁸ The storage and loss moduli obtained from the MSD data are shown in Fig. 2 (panels B–D and F–H). All six peptides display distinct elastic properties; the storage moduli are higher than the loss moduli in at least the 2–2000 rad s⁻¹ angular frequency range. AβFF-pG, AβFF-Up, AβFY-NG, and AβFY-pG display storage moduli higher than the loss moduli in the 1–10⁴ rad s⁻¹ range.

The gels are composed of β-sheet rich fibrous aggregates

Structural characterization of the hydrogels was carried out using AFM, TEM, CD spectroscopy, FTIR spectroscopy, and ThT fluorescence spectroscopy. The secondary structure of the peptides in the hydrogels was examined using far-UV CD spectroscopy (Fig. 3). The peptides display a negative band centered

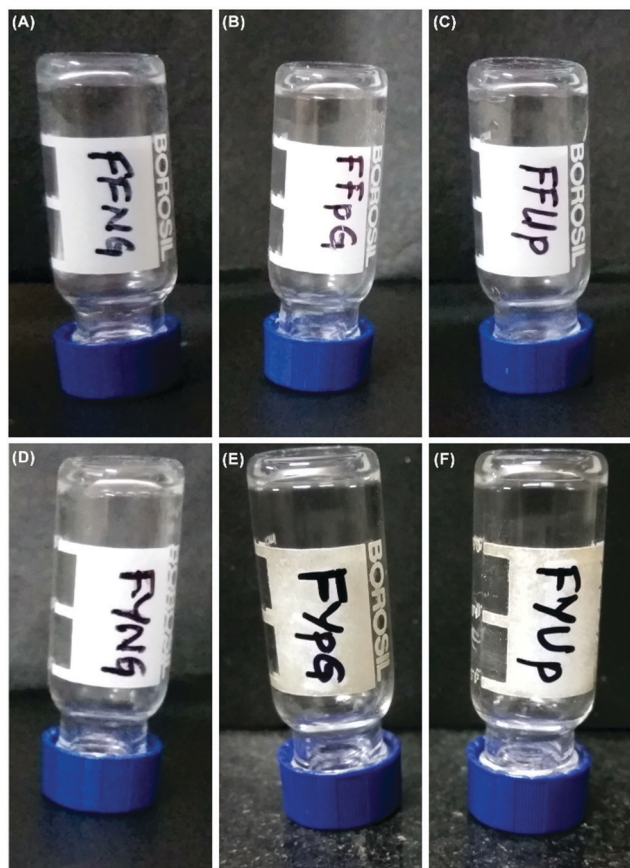


Fig. 1 Images of the inverted tubes showing self-supporting gels. Panels (A–D) represent the gels formed by AβFF-NG, AβFF-pG, AβFF-Up, and AβFY-NG, respectively, at a 2 mM peptide concentration. Panels (E) and (F) represent the gels formed by AβFY-pG and AβFY-Up, respectively, at a 5 mM peptide concentration.

around 215–218 nm with a positive band near 190 nm suggesting a distinct β -sheet conformation. Far-UV CD spectra can have a significant contribution from the aromatic side-chains if the peptide's aromatic content is high or the peptide folds to form aromatic clusters.^{49,50}

As the peptides are designed to fold into β -hairpins that place aromatic residues in the β -sheet core, the secondary structure was further characterized using ATR-FTIR spectroscopy of the dried peptide films (Fig. 4). The amide I band shown by proteins/peptides largely arises from carbonyl stretching vibrations. The transition is sensitive to the backbone conformation thereby allowing secondary structure determination. The amide I band for all 6 peptides appeared around 1624 cm^{-1} , supporting a β -sheet conformation.

Fig. 5 shows the morphology of the self-assembled peptide structures as determined using AFM. The peptides, predictably, formed amyloid-like fibrous aggregates. Networks of such fibrillar structures are known to cause hydrogelation by fixing water molecules. The fibrous structures were characterized by TEM as well (Fig. S1, ESI[†]). It is interesting to note that the end-capped A β_{16-22} , though amyloidogenic by itself, fails to cause hydrogelation.⁴⁵ Repeats connected *via* β -turn-supporting motifs

turn out to be promising hydrogelators. Amyloid-like fibrils are routinely characterized by monitoring the fluorescence of ThT, a benzothiazole dye that exhibits a higher quantum yield when bound to amyloid fibrils. The hydrogel fibrils caused a very large enhancement in ThT fluorescence (Fig. 6).

The A β_{16-22} -turn-based peptides, by and large, caused higher enhancement compared to A β_{16-22} (F20Y)-turn-based peptides. In addition, the peptides harboring Asn-Gly as the turn-supporting motif caused at least 2-fold higher enhancement compared to the peptides harboring ^DPro-Gly and Aib-^DPro as the turn-supporting motifs. Asn-Gly mediates a flexible turn compared to the rather tight turns mediated by ^DPro-Gly and Aib-^DPro. A flexible turn would provide conformational flexibility to the two strands; such flexibility might be important for optimal packing of the intra- and inter-sheet side chains. The mechano-responsive behaviour was studied using AβFF-NG and AβFY-NG as representative gels.⁵¹ The gels exhibited shear-thinning when pulled out using a pipette. The sheared gels were transferred to clean empty vials and left undisturbed. Overnight standing of the sheared samples resulted in gels, suggesting reversibility (Fig. S2, ESI[†]). Furthermore, the gelation of AβFF-NG and AβFY-NG was investigated at pH 2 and 4; both the peptides resulted in transparent gels at both the pHs (Fig. S3, ESI[†]).

The hydrogels cause sustained release of doxorubicin, an anticancer drug

Peptide-based hydrogels are promising drug-delivery vehicles.³⁶ The gels can trap drug molecules, which can be released at the target site in a sustained manner. In order to investigate if, like many peptide-based hydrogels reported in the literature, the gels obtained from the β -turn motif-harboring amyloidogenic peptides can trap and show sustained release of a drug, we examined the release of anticancer drug doxorubicin from the hydrogels. Fig. 7 shows the release of doxorubicin (trapped at a 0.5 mg ml^{-1} concentration during gelation) from 5 mM AβFF-NG (panel A) and AβFY-NG (panel B) gels. Both the gels show sustained release of doxorubicin for around 2.5 hours. Similar release was observed from the AβFF-pG and AβFF-Up gels as well (Fig. S4, ESI[†]).

Using AβFF-NG as a representative peptide, the gelation was studied in the presence of a higher doxorubicin concentration (3.2 mg ml^{-1}). The peptide gelation was unaffected and doxorubicin up to at least a 3.2 mg ml^{-1} concentration could be trapped by the gel (data not shown).

The hydrogels support 3-D cell culture

Supporting cell-growth in 3-dimensions is yet another biomedical application of peptide-based hydrogels. In fact, many peptide-based hydrogels have been commercialized for 3-D cell cultures. We investigated if the hydrogels described in this study can support the growth and function of mammalian cells. Peptides harboring the Asn-Gly β -turn motif *i.e.* AβFF-NG and AβFY-NG were chosen as representative peptides for cell culture studies. Human embryonic kidney cells, HEK-293, and rat pancreatic cells, RIN-5F, were seeded on the 2 mM peptide gels

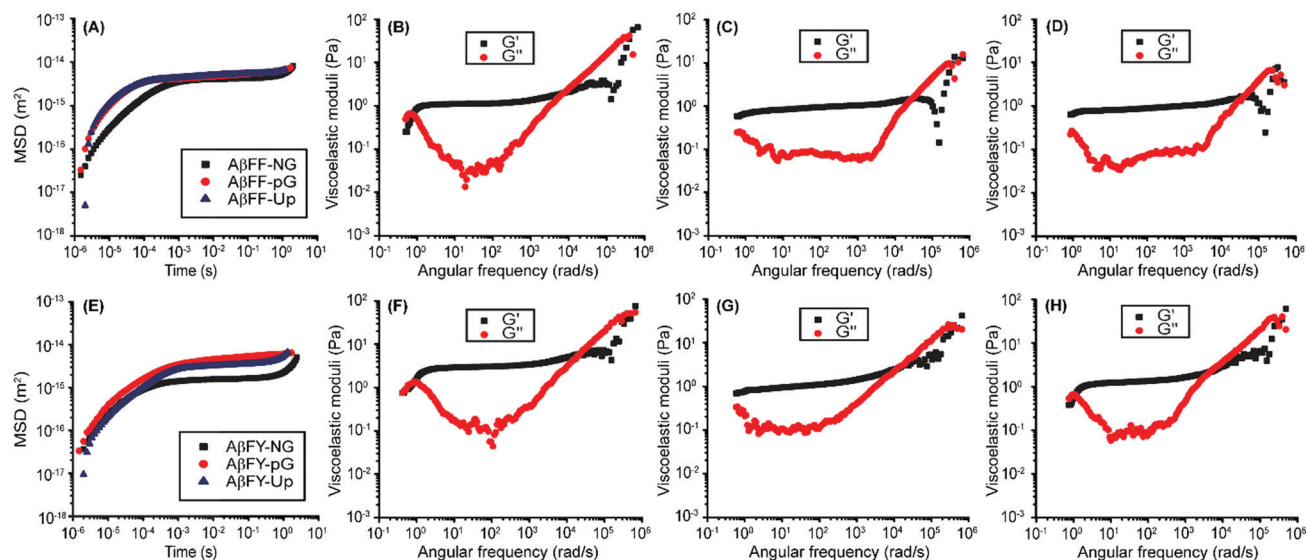


Fig. 2 DLS-microrheology data recorded for 5 mM hydrogels. Panels (A) and (E) show the MSD data for the A β_{16-22} and A β_{16-22} (F20Y) turn-based peptides, respectively. The G' (storage modulus, black squares) and G'' (loss modulus, red circles) data, as determined from the MSD data using the generalized Stokes–Einstein relation: A β FF-NG (B), A β FF-pG (C), A β FF-Up (D), A β FY-NG (F), A β FY-pG (G), and A β FY-Up (H).

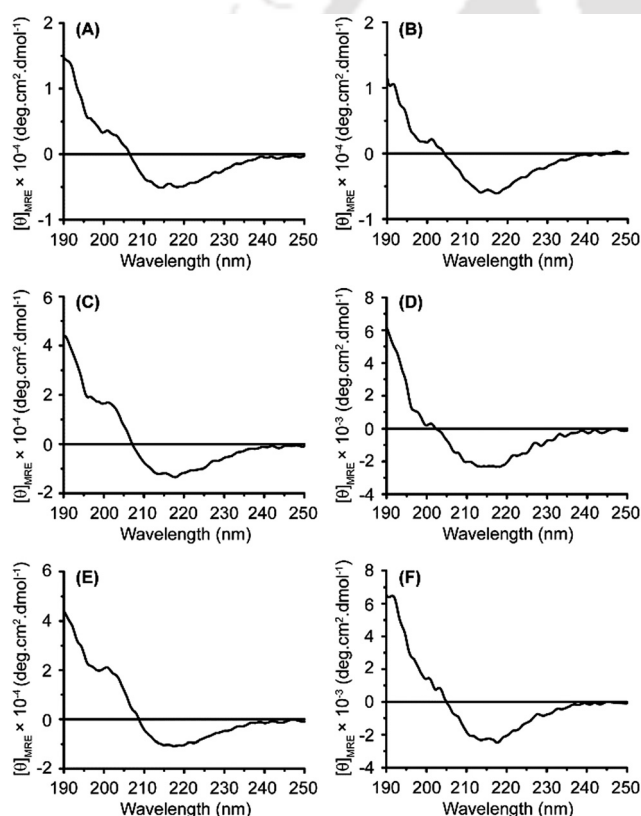


Fig. 3 Far-UV CD spectra recorded for A β FF-NG (A), A β FY-NG (B), A β FF-pG (C), A β FY-pG (D), A β FF-Up (E), and A β FY-Up (F).

prepared in 96-well plates as described in the Materials and methods section. The cells grow in the hydrogel matrix as ascertained by the uptake of calcein AM. Calcein AM is a cell-permeable, non-fluorescent acetoxymethyl ester of calcein. Ester hydrolysis caused by the cellular esterases results in green

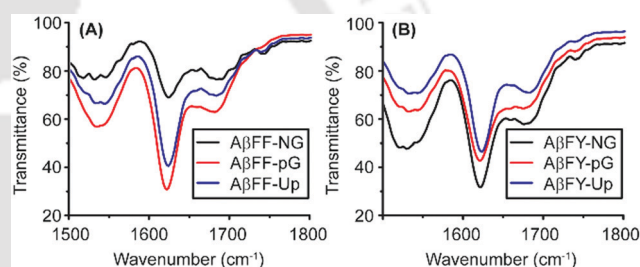


Fig. 4 ATR-FTIR spectra of A β FF-NG, A β FF-pG, and A β FF-Up (A) and A β FY-NG, A β FY-pG, and A β FY-Up (B).

fluorescence, indicating cell viability. Both HEK-293 and RIN-5F cells grow in clumps and yield green fluorescence upon calcein AM treatment (Fig. 8).

As RIN-5F is a pancreatic cell line, expression of the insulin-1 gene by the cells grown in the gels was examined. The total mRNA isolated from the RIN-5F cells grown in the hydrogels for 24 h was reverse-transcribed to cDNA and amplified by PCR using insulin-1 and GAPDH primers. The cells growing in the gels were found to be expressing the housekeeping gene GAPDH as well as insulin-1 (Fig. 9). These data indicate that the peptide hydrogels reported here can serve as scaffolds for 3-D cell culture.

Discussion

Assembly of proteins into highly ordered superstructures is essential for life and has been known for a long time. Such assembly is strictly dictated by the native three-dimensional structure of the protein and is highly sensitive to subtle changes in the environment like pH, temperature, and ionic strength. Even though such molecules constitute an interesting class of materials, they are difficult and uneconomic to synthesize chemically and need specialized storage conditions to keep

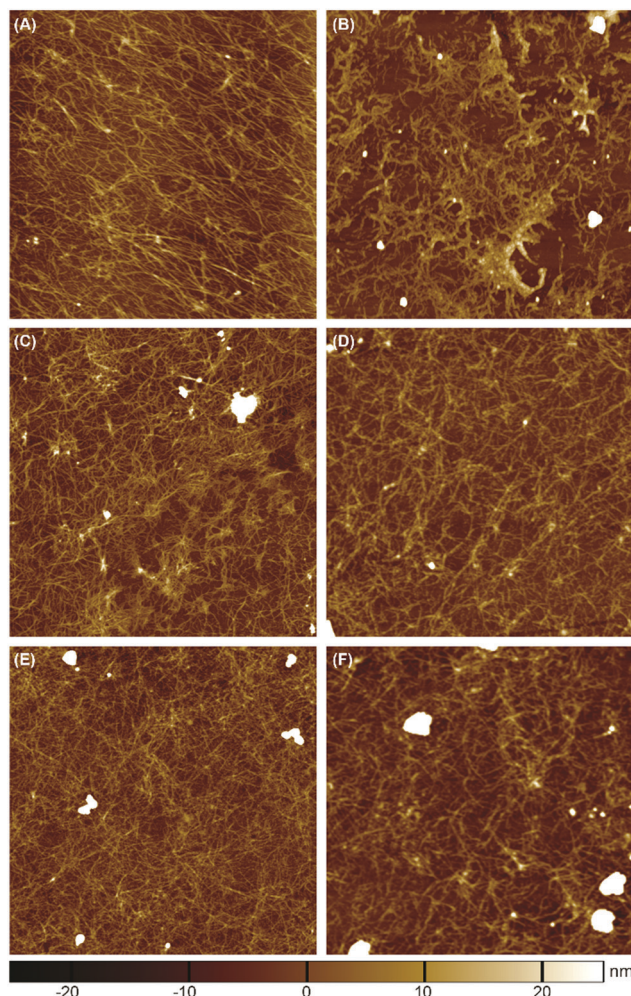


Fig. 5 AFM images of the self-assembled structures that underlie hydrogel formation. AβFF-NG (A), AβFF-pG (B), AβFF-Up (C), AβFY-NG (D), AβFY-pG (E), and AβFY-Up (F). Each panel represents a $10\ \mu\text{m} \times 10\ \mu\text{m}$ scan area.

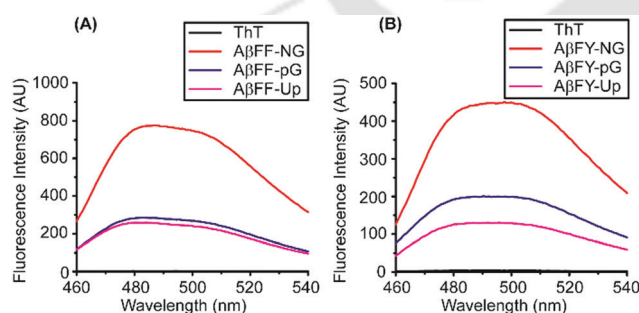


Fig. 6 ThT fluorescence spectra recorded for the sheared hydrogels: (A) Aβ₁₆₋₂₂-turn-based peptides and (B) Aβ₁₆₋₂₂(F20Y)-turn-based peptides. The assay was carried out in 5 mM phosphate buffer, pH 7.4 having $10\ \mu\text{M}$ ThT. The peptide concentration in the assay was $20\ \mu\text{M}$.

their native structures intact. Short peptides, on the other hand, are readily synthesized and can be stored under ambient conditions. Self-assembling peptides happen to be one of the most promising biomaterials. A variety of ordered superstructures that include micelles, solid spheres, hollow spheres, nanotubes, microtubes,

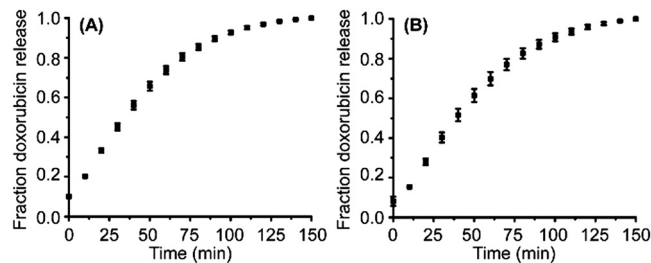


Fig. 7 Doxorubicin release kinetics from AβFF-NG (A) and AβFY-NG (B) gels as determined by monitoring the absorbance at 490 nm.

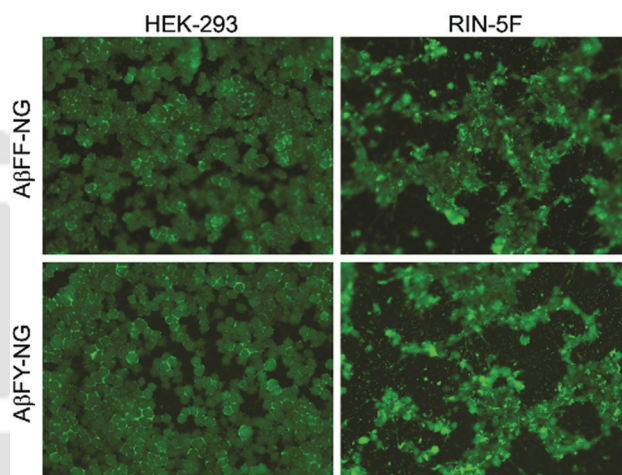


Fig. 8 Fluorescence images of the calcein AM treated HEK-293 (left panels) and RIN-5F (right panels) cells grown for 24 h in peptide hydrogels.

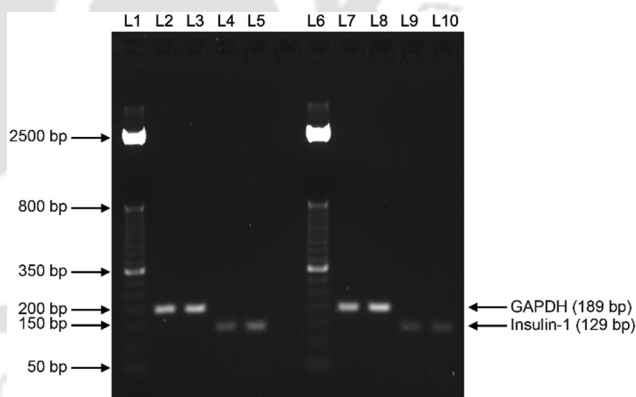


Fig. 9 Agarose gel electrophoretic analysis of GAPDH and insulin-1 mRNA amplification from RIN-5F cells grown for 24 h in AβFF-NG (lanes 2–5) and AβFY-NG (lanes 7–10) hydrogels. Lanes 2, 3, 7, and 8 correspond to the GAPDH PCR amplified product while lanes 4, 5, 9, and 10 correspond to the insulin-1 amplification product. Lanes 1 and 6 represent size markers.

rods, fibers, and ribbons have been obtained through peptide self-assembly.^{25,52–59} Self-assembly into ordered structures can impart interesting material properties to the superstructures that are otherwise absent in the monomeric peptide molecules or their amorphous aggregates. Such properties as well as the ease

to modulate these properties through intelligent modifications in the peptide sequence, chemistry, and self-assembly conditions make peptides an impressive class of materials.⁴³ Amyloid fibrils constitute an important class of ordered protein and peptide aggregates. Ever since they have been identified as the native fold, amyloids have been looked at with great interest as functional materials. A majority of designed hydrogelating peptides self-assemble into amyloid-like β -sheet-rich fibrils. The hydrogelating propensity of short amyloidogenic peptides, however, remains largely unexplored.

A β_{16-22} , the 7-residue fragment from the fibrillar core of the well-studied β -amyloid protein, forms amyloid fibrils by itself. Solid-state NMR studies have revealed that the peptide molecules take up an in-register antiparallel β -sheet conformation in the fibrils.⁴⁰ A β_{16-22} , however, was found to be non-hydrogelating up to a 20 mM peptide concentration.⁴⁵ We explored the hydrogelating propensity of the A β_{16-22} motifs connected by β -turn-supporting motifs. Aib-^DPro and ^DPro-Gly are well-established dipeptide motifs that nucleate tight type I' and type II' β -turns, respectively. Asn-Gly, on the other hand, supports the type I' β -turn but is not an obligatory type I' turn-forming motif. Irrespective of the type of turn-inducing motif, the A β_{16-22} repeats resulted in limp hydrogels at concentrations as low as 2 mM. The structures underlying hydrogelation were found to be amyloid-like fibrils. Even though CD, FTIR, and AFM suggested no marked difference between the fibrils formed by A β FF-NG, A β FF-pG, and A β FF-Up, ThT fluorescence was at least two-fold higher for A β FF-NG fibrils compared to A β FF-pG and A β FF-Up. We have previously reported the hydrogelation by A β_{16-22} (F20Y); the peptide forms transparent hydrogels at concentrations ≥ 2 mM.⁴⁵ When the A β_{16-22} (F20Y) repeats are connected *via* the Asn-Gly motif, the peptide resulted in hydrogels at a 2 mM concentration. Interestingly, however, repeats connected *via* the ^DPro-Gly and Aib-^DPro motifs failed to cause hydrogelation under these conditions. The peptides resulted in hydrogels at a 5 mM concentration. As is the case with the A β_{16-22} β -turn peptides, the Asn-Gly connected A β_{16-22} (F20Y) caused at least a 2-fold higher enhancement in ThT fluorescence compared to the repeats connected *via* the ^DPro-Gly and Aib-^DPro motifs. An Asn-Gly dipeptide motif provides higher conformational flexibility in the turn region compared to ^DPro-Gly and Aib-^DPro. We have previously reported 100 ns MD simulations of A β FF-NG, A β FF-pG, A β FY-NG, and A β FY-pG.⁴² Even though a 100 ns simulation may not be sufficient for sampling the entire conformational space of a peptide, β -hairpins were obtained for all the peptides within 100 ns. Such rapid folding can be attributed, alongside the β -turn inducing motifs, to the self-assembly propensity of the strands. Interestingly, however, the major cluster for A β FY-NG was a type II turn instead of type I'. A β FF-NG and A β FF-pG do fold into hairpin structures but the hairpin is not mediated by a classical β -turn. These data show that the turn-supporting motifs can play a deciding role in the emergent properties of self-assembled structures; A β FY-NG causes gelation at a 2 mM peptide concentration whereas A β FY-pG and A β FY-Up fail. It appears that the bulky Tyr side chain might be difficult to pack, thereby affecting assembly and

consequently gelation at lower concentrations. This is further supported by the fact that A β FY-NG displays a higher G''/G' ratio than that displayed by A β FF-NG. Beta-turn-inducing motifs have been incorporated in designed hydrogelating peptides.²⁶ Schneider and coworkers have designed a series of short peptides, named MAX peptides, that harbor a ^DPro-^LPro dipeptide motif in the middle of the peptide sequence.^{21,43,44} ^DPro-^LPro is a well-established type-II' β -turn-inducing dipeptide motif. The designed peptides fold into β -hairpin structures mediated by the predicted type-II' β -turn triggering self-assembly and consequently hydrogelation. Our results presented in this study show that β -turns can have a marked influence on the hydrogelating propensity of the peptides. End-capped A β_{16-22} , which is previously reported to be non-hydrogelating,⁴⁵ becomes a potent hydrogelator when the two strands are connected *via* a turn-supporting motif. Results with the A β FY-based peptides indicate a preference for Asn-Gly as the β -turn motif over Aib-^DPro and ^DPro-Gly. It would, therefore, be interesting to see the effects of β -turn-inducing motifs other than ^DPro-^LPro for the MAX series of peptides as well.

To conclude, the β -turn motif-connected repeats of A β_{16-22} and its aromatic analog A β_{16-22} (F20Y) form transparent hydrogels at very promising concentrations (≤ 1 wt%). The Asn-Gly β -turn-supporting dipeptide motif was, by and large, found to be more promising for hydrogelation compared to the ^DPro-Gly and Aib-^DPro motifs. Furthermore, these gels are mechano-responsive *i.e.* display shear-thinning upon mechanical shearing and re-gel upon standing. Like various other peptide-based hydrogels reported in the literature, these hydrogels show continual drug release and also act as scaffolds for mammalian cell culture.

Author contributions

NC and RN conceived the idea. DD, RN, and NC designed the experiments. DD performed the hydrogelation experiments, AFM and TEM imaging, spectroscopic characterization, micro-rheology, and drug release experiments. VK and SK designed, conducted, and analyzed the cell culture experiments. All the authors contributed to manuscript writing. All the authors approve the final version of the manuscript.

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Conflicts of interest

The authors declare no competing financial interest.

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Water-Alcohol Bigels from Fatty Acylated Dipeptides

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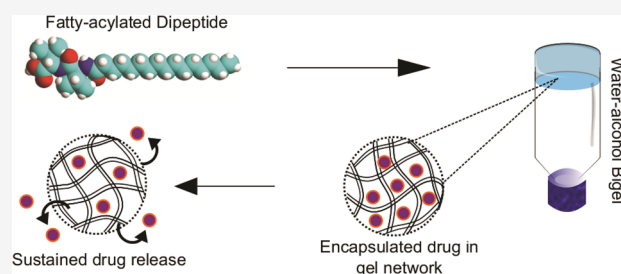


Article Recommendations



Supporting Information

ABSTRACT: Peptide-based gels are emerging as an interesting class of biocompatible soft materials. 9-Fluorenylmethoxycarbonyl-protected amino acids and short peptides have gained considerable attention as promising gelators. Peptide amphiphiles, wherein an alkyl chain is appended to a polar peptidic moiety, are another important class of peptide-based gelators. Here, we report the alcohol/water bigels formed by the rather simple fatty acylated dipeptides wherein the peptidic moiety is made up of hydrophobic amino acids, viz., Val, Ile, and Leu. Lauroyl, myristoyl, and palmitoyl were investigated as the N-terminal fatty acyl groups. None of the lauroylated peptides caused gelation of methanol/water and ethanol/water mixtures up to 2 wt % peptide concentration. Eight out of the 27 peptides resulted in distinct bigels. The gels are composed of fibrous aggregates as characterized by electron microscopy. Infrared spectroscopy suggests the β -sheet conformation of the peptidic region in the gels. Using the Ma-IV ethanol/water bigel as the representative gel, entrapment and steady release of the anticancer drug docetaxel are demonstrated. Such bigels from rather simple amphipathic peptides that are easily synthesized and purified through solvent extraction could be attractive gelator candidates with potential application in drug delivery.



INTRODUCTION

Large numbers of peptides, peptide derivatives, and amino acid derivatives can form hydrogels and bigels (made up of both water and organic solvent).^{1–5} Bigels hold promise as drug delivery vehicles. Self-assembled structures formed by very short peptides (2–3 amino acids) have gained considerable attention in the past two decades. Such peptides have resulted in a variety of superstructures that include fibers, tubes, rods, tapes, spheres, and vesicles.^{6–14} Many of these peptides can self-assemble to form hydrogels and bigels, particularly those with free COOH. Peptides, peptidomimetics, and amino acid derivatives exhibit excellent biocompatibility and biodegradability, making the nanostructures formed by them ideal for biomedical applications. N-terminal-protected amino acids and short peptides constitute an important class of low-molecular-weight gelators. The majority of these amino acids and peptides are rich in aromatic residues, and aromatic stacking interactions are believed to be critical for their assembly. Therefore, it comes as no surprise that an aromatic moiety often constitutes the N-terminal cap for these peptides.^{15–19} Fmoc (9-fluorenylmethoxycarbonyl) happens to be one of the most widely employed N-terminal aromatic moieties in amino acid and peptide gelators.^{14,20–22} Peptide amphiphiles constitute yet another class of peptide-based gelators.^{23,24} The amphiphilicity is conferred either by suitably incorporating the hydrophilic and hydrophobic residues in the peptide sequence^{25,26} or by attaching long alkyl chains with the peptide molecules.^{24,27} Bhattacharya and co-workers reported two-component gels wherein fatty acids mixed with di- and

multiamines resulted in distinct hydrogels.²⁸ On a similar line, Xu and co-workers reported hydrogelation caused by sodium salts of fatty acids mixed with oligolysines.²⁹ Fatty acylated amino acids and peptides are reported in the literature to cause hydrogelation and organogelation.^{30–32} The idea is to obtain an amphiphilic molecule wherein the peptide contributes the polar region and the fatty acyl chain contributes the nonpolar region. The gelator amount required for causing gelation of a solvent determines how good a gelator is. Good gelators would typically cause gelation at concentrations $\leq 2\%$ (w/w) concentrations; that is, they can fix at least 50 times the concentration of the solvent.¹ Here, we report the self-assembly as well as bigel (alcohol/water gel) formation by the fatty acylated dipeptides. Unlike previous studies wherein a polar peptide is linked to an acyl chain, we chose hydrophobic aliphatic residues (Ile, Leu, and Val) to make the peptidic region. Nagaraj and co-workers have previously reported highly ordered superstructures such as nanotubes and nanospheres from Boc-protected dipeptide methyl esters, viz., Boc-Val-Ile-OMe, Boc-Val-Leu-OMe, Boc-Leu-Val-OMe, and Boc-Val-Val-OMe.³³ The permutation of the three amino acids gives six different peptides. Three fatty acids, namely, lauric acid, myristic acid, and palmitic acid, were chosen for N-terminal

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acylation. This resulted in a total of 27 fatty acylated peptides wherein C-terminal carboxyl group is the only polar group other than the peptide backbone (Figure 1). Amino acids with

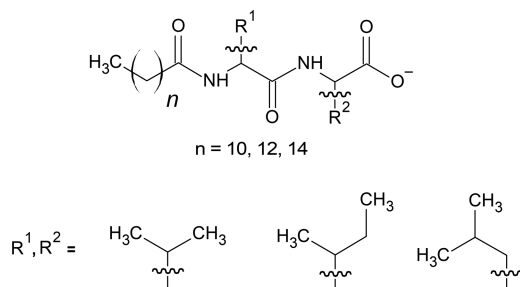


Figure 1. Fatty acylated peptides employed in this study.

hydrophobic aliphatic side chains are a cheap resource material as side chains need not be protected during synthesis. In addition to that, the high hydrophobicity of the peptides facilitates ready purification through water/dichloromethane extraction. Out of the 27 fatty acylated peptides, eight resulted in distinct alcohol/water gels. The gels were identified by tube inversion and subsequently characterized by DLS–microrheology, infrared spectroscopy, and electron microscopy.

METHODS

Materials. Fmoc-protected amino acids and N,N,N',N' -tetramethyl- O -(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) were purchased from Novabiochem (Darmstadt, Germany). HMPB resin, myristic acid, palmitic acid, N,N -diisopropylethylamine (DIPEA), 4-dimethylaminopyridine (DMAP), N,N' -dicyclohexylcarbodiimide (DCC), piperidine, and trifluoroacetic acid (TFA) were procured from Sigma-Aldrich Chemicals Pvt. Ltd. 1-Hydroxybenzotriazole hydrate (HOBt) was obtained from Sisco Research Laboratory, India. Lauric acid was purchased from Himedia. N,N -Dimethylformamide, glacial acetic acid, and isoamyl alcohol were obtained from Merck, India. All other reagents were of the highest grade available.

Peptide Synthesis. Fatty acylated peptides were synthesized manually on HMPB resin by employing Fmoc-chemistry. The C-terminal amino acid was attached to the resin using DCC/DMAP-mediated coupling. Coupling of the N-terminal amino acid and subsequent fatty acylation were carried out using HBTU/HOBt/DIPEA activation. The synthesized peptides were cleaved from the resin using 95% TFA/5% water. The cleaved peptides were dried by nitrogen purging. The peptides were dissolved in DMSO, and the purity was assessed on a reversed-phase C18 column on a Shimadzu Prominence Modular HPLC instrument using a linear gradient of acetonitrile containing 0.1% TFA. The peptides were subsequently characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry on a Bruker Autoflex Speed MALDI-TOF mass spectrometer.

Self-Assembly. The fatty acylated peptides were weighed as required, and the 40 mg/mL stock solutions were prepared in methanol. An equal volume of water was added subsequently, and the vials were left undisturbed for 24 h. Formation of self-supporting gels was ascertained by inverting the tubes and keeping them inverted for at least 12 h. A similar method was followed when the peptides were studied in an ethanol/water cosolvent mixture (Supporting Information).

The effective peptide concentration in the gels was 20 mg/mL in a 50% alcohol/water cosolvent mixture.

Transmission Electron Microscopy (TEM). All the peptide samples, including bigels, were investigated using TEM. Briefly, 5 μL of the 24 h-old samples were drop-cast on 200 mesh Formvar/carbon-coated copper grids. The excess sample was blotted out from the periphery of the grid by Whatman I filter paper. The grids were then stained with saturated uranyl acetate solution, which was blotted out after 30 s. The samples were dried overnight, and images were recorded on a JEOL-2100F field emission transmission electron microscope at 200 kV.

Fourier Transform Infrared (FTIR) Spectroscopy. FTIR spectra were recorded on a Bruker Alpha-E spectrometer with an Eco attenuated total reflection (ATR) single-reflection ATR sampling module equipped with a ZnSe ATR crystal. Around 10 μL of the 24 h-old samples was spread on the crystal and dried. ATR-FTIR spectra were subsequently recorded at a resolution of 4 cm^{-1} .

DLS–Microrheology. DLS–microrheology measurements were carried out for the bigels on a Malvern Zetasizer Nano ZS instrument with a 633 nm laser and a 173° non-invasive back-scattering detector configuration. Carboxylated melamine beads (GmbH, 0.6 μm) were used as the tracer particle. The concentration of the tracer particles was optimized to prevent tracer aggregation and interaction with the sample. The temperature for all the experiments was fixed at 25 °C. The tracer particles were added to the fatty acylated peptide samples such that the final peptide concentration was 20 mg/mL and the final tracer volume fraction was 0.01%. The mean squared displacement (MSD) data were recorded after 24 h of incubation. Subsequently, the elastic and viscous moduli were derived from the MSD data using the inbuilt Malvern Zetasizer instrument software based on the generalized Stokes–Einstein equation as introduced by Mason.³⁴

Critical Aggregation Concentration (CAC). CAC was determined using dynamic light scattering. The CAC was determined in 10% methanol as higher methanol concentrations resulted in highly inconsistent scattering intensity even with neat cosolvent mixtures. Briefly, the peptide stock solutions prepared in methanol were added to water to obtain the desired peptide concentrations in 10% methanol. The photon count rates were obtained from the scattering recorded in a 173° non-invasive back-scattering detector configuration on a Malvern Zetasizer Nano ZS instrument with a 633 nm laser.

Docetaxel Release. The chemotherapeutic drug docetaxel was entrapped in the Ma-IV ethanol/water bigel. Docetaxel was dissolved in ethanol to obtain 20 μM concentration. The 20 μM docetaxel solution was then added to the peptide to achieve a 40 mg/mL peptide solution. Following the peptide's dissolution, a 25 μL volume was transferred to a quartz absorbance cuvette. An equal volume (25 μL) of deionized water was subsequently added, and the sample was left undisturbed for 24 h to achieve gelation and drug uptake, thereby giving a 20 mg/mL bigel having 10 μM docetaxel. Release of docetaxel in 30% ethanol and phosphate-buffered saline (9 times volumes) was monitored by measuring absorbance at 230 nm. There was no notable change in the gel during drug release.

RESULTS

Fatty acylated dipeptides were synthesized using valine, leucine, and isoleucine (amino acids) and lauric acid, myristic acid, and palmitic acid (fatty acids). Three choices for the amino acids in dipeptides and three choices for the N-terminal acylation resulted in 27 different peptides. The peptides were synthesized and characterized as described in the [Methods](#) section. The sequences of the peptides, their IDs, the calculated monoisotopic masses, and the observed m/z in the MALDI-TOF MS are given in [Table 1](#).

Table 1. List of the Fatty Acylated Peptides Employed in This Study

S. no.	sequence	peptide ID	calculated monoisotopic mass (Da)	observed m/z
1	palmitoyl-Val-Val-COOH	Pa-VV	454.55	476.59
2	myristoyl-Val-Val-COOH	Ma-VV	426.50	448.29
3	lauroyl-Val-Val-COOH	La-VV	398.44	413.12
4	palmitoyl-Ile-Val-COOH	Pa-IV	468.57	490.60
5	myristoyl-Ile-Val-COOH	Ma-IV	440.52	462.44
6	lauroyl-Ile-Val-COOH	La-IV	412.46	434.22
7	palmitoyl-Leu-Val-COOH	Pa-LV	468.57	490.78
8	myristoyl-Leu-Val-COOH	Ma-LV	440.52	462.40
9	lauroyl-Leu-Val-COOH	La-LV	412.46	415.0
10	palmitoyl-Leu-Leu-COOH	Pa-LL	482.58	504.77
11	myristoyl-Leu-Leu-COOH	Ma-LL	454.53	476.57
12	lauroyl-Leu-Leu-COOH	La-LL	426.47	448.34
13	palmitoyl-Ile-Leu-COOH	Pa-IL	482.58	504.78
14	myristoyl-Ile-Leu-COOH	Ma-IL	454.53	476.54
15	lauroyl-Ile-Leu-COOH	La-IL	426.47	448.34
16	palmitoyl-Val-Leu-COOH	Pa-VL	468.57	490.68
17	myristoyl-Val-Leu-COOH	Ma-VL	440.52	462.57
18	lauroyl-Val-Leu-COOH	La-VL	412.46	434.21
19	palmitoyl-Ile-Ile-COOH	Pa-II	482.58	504.86
20	myristoyl-Ile-Ile-COOH	Ma-II	454.53	476.59
21	lauroyl-Ile-Ile-COOH	La-II	426.47	448.38
22	palmitoyl-Val-Ile-COOH	Pa-VI	468.57	490.69
23	myristoyl-Val-Ile-COOH	Ma-VI	440.52	462.49
24	lauroyl-Val-Ile-COOH	La-VI	412.46	434.26
25	palmitoyl-Leu-Ile-COOH	Pa-LI	482.58	504.86
26	myristoyl-Leu-Ile-COOH	Ma-LI	454.53	476.57
27	lauroyl-Leu-Ile-COOH	La-LI	426.47	448.40

Self-Assembly. The peptides, expectedly, were insoluble in water but readily dissolved in methanol. The peptides were weighed and dissolved in methanol to achieve 40 mg/mL stock solutions. Self-assembly/gelation was initiated by adding an equal volume of deionized water to the peptide stock solutions; the 20 mg/mL (2 wt %) peptide solutions thus obtained in 50% methanol/water were kept undisturbed for 24 h at room temperature. The vials were subsequently examined for gelation by tilting and inverting the tubes. None of the lauroyl peptides caused gelation of 50% methanol/water up to 20 mg/mL peptide concentration. Among the rest of the 18 peptides, eight, viz., Pa-VV, Ma-VV, Pa-VL, Ma-VL, Ma-IV, Pa-LV, Ma-IL, and Pa-II, resulted in self-supporting gels ([Figure 2](#)). The length of the acyl chain, therefore, plays a deciding role in the gelation of these peptides. Other than Pa-VV, which resulted in a transparent gel, the gels by the other seven peptides were either translucent or opaque. Out of the six homodipeptides with myristoyl/palmitoyl chains, only Pa-VV, Ma-VV, and Pa-

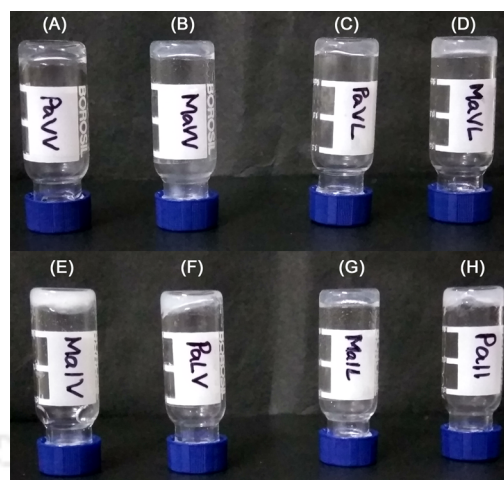


Figure 2. Images of the inverted tubes containing 24 h-old 20 mg/mL peptides in methanol/water; (A) Pa-VV, (B) Ma-VV, (C) Pa-VL, (D) Ma-VL, (E) Ma-IV, (F) Pa-LV, (G) Ma-IL, and (H) Pa-II.

II turned out to be gelating at 20 mg/mL concentration. Gelation was also investigated at 2, 5, 10, and 15 mg/mL concentrations. None of the peptides caused gelation up to 15 mg/mL concentrations. Among the eight peptides that form gels at 20 mg/mL concentration, the solutions of palmitoylated peptides, that is, Pa-VV, Pa-VL, Pa-LV, and Pa-II, became viscous at 15 mg/mL but could not support their weight when the tubes were inverted.

To gain further insights into the self-assembled structures underlying gelation, the samples were analyzed using TEM ([Figure 3](#)).

All the gelating peptides except Pa-II form long fibrils that can entangle to form a mesh-like structure, thereby trapping the solvent molecules. Pa-II forms a network of rod- and ribbon-like structures. The non-gelating peptides, on the other hand, form short fibrous or non-fibrous structures. It is interesting to note that Pa-IV, Pa-IL, and Pa-VI form flake/ribbon-like structures. These data suggest that both palmitoyl and myristoyl acyl chains, but not lauroyl, favor bigel formation. In addition, Val, an amino acid with branched β -carbon, is preferred over Ile and Leu to form fibrous aggregates and bigels. The gelation of 50% ethanol/water was also investigated at identical peptide concentration. The same set of peptides that cause gelation of 50% methanol/water also caused gelation of 50% ethanol/water ([Figure S1](#) in the Supporting Information). The gels formed by the peptides were also investigated using scanning electron microscopy; all the eight peptides show a dense network of fibrous aggregates ([Figure S2](#) in the Supporting Information).

FTIR Spectroscopy. The secondary structure of the peptidic region in the self-assembled fatty acylated peptides was investigated using FTIR spectroscopy of the dried peptide samples. The 24 h-old 20 mg/mL peptide samples in 50% methanol/water were deposited on the ZnSe ATR crystal and dried. The spectra obtained are shown in [Figure 4](#). The peptides Pa-VV, Ma-VV, Ma-IV, and Pa-II showed distinct IR peaks around 1633–1634 cm^{-1} , suggesting β -sheet formation.³⁵ Ma-VV shows, in addition to the $\sim 1633 \text{ cm}^{-1}$ peak, a broad peak around 1681 cm^{-1} . Infrared bands around 1680–1695 cm^{-1} have been assigned to antiparallel β -sheets.^{36,37} Such bands have been reported in the short self-assembling peptides.^{38–40} Besides, the infrared bands around 1680 cm^{-1}

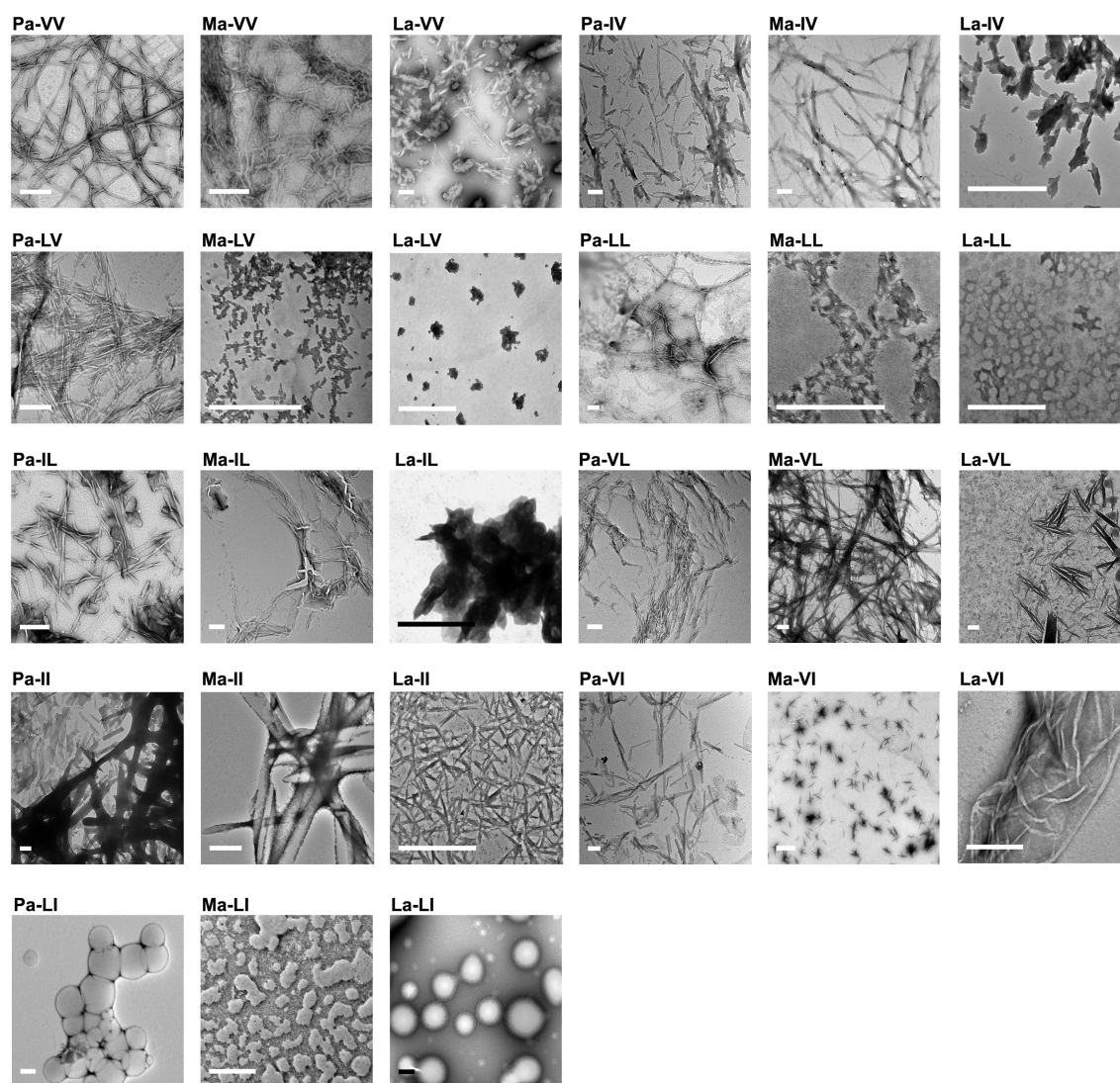


Figure 3. TEM images of the 24 h-old fatty acylated dipeptide samples (20 mg/mL) in 50% methanol/water. The scale bars represent 500 nm.

have also been assigned to turns and bands.⁴¹ Infrared bands around $1680\text{--}1685\text{ cm}^{-1}$ have also been observed for the self-assembled structures of Boc-protected aliphatic dipeptide methyl esters.³³ The bands in such peptides have been assigned to the urethane (carbamate) group. As the fatty acylated peptides investigated in this study lack a urethane moiety and the turns/bands are unlikely for such small peptides, it is tempting to assign the $\sim 1681\text{ cm}^{-1}$ band to the antiparallel β -sheet conformation. It is, however, important to mention that the $1680\text{--}1695\text{ cm}^{-1}$ peak arises from the vibrational excitons running along the β -strands ($\nu_{\parallel}$).⁴² The intensity of the band, consequently, does not depend on the number of strands. An extended antiparallel β -sheet made up through the self-assembly of small peptides is not expected to give the high-frequency peak ($1680\text{--}1695\text{ cm}^{-1}$) with an intensity comparable to the low-frequency ($1610\text{--}1635\text{ cm}^{-1}$) peak. However, such peaks have been assigned to the antiparallel β -sheets for the self-assembled structures of Fmoc-protected very short peptides. Tuttle and co-workers investigated the hydrogels made up of Fmoc-AA and Fmc-AA (9-fluorene-methylcarbonyl dialanine) and showed the high-frequency peak to be arising from the carbamate group.⁴³ The IR spectra of the fatty acylated peptides are considerably

different from the amyloid fibril forming proteins/peptides.³⁷ It is therefore inappropriate to assign the 1681 cm^{-1} band to the antiparallel β -sheet conformation. All the leucine-containing gelators, viz., Pa-VL, Ma-VL, Ma-IL, and Pa-LV, display bands around 1634 and 1675 cm^{-1} in the amide I region. The 1634 cm^{-1} peak is assigned to the β -sheet conformation. The high-frequency peak (1675 cm^{-1}) is likely to be arising from the residual TFA present in the peptides. TFA strongly absorbs in the amide I region, giving a peak around 1673 cm^{-1} . The peptides, as mentioned in the **Methods** section, were cleaved from the resin using 95% TFA. TFA was subsequently evaporated under a nitrogen stream to obtain the dried peptides. Among the non-gelating peptides, none of the VI, LL, and LL fatty acylated peptides displays the $\sim 1634\text{ cm}^{-1}$ peak. However, it is to be noted that the peptide Pa-VI that shows an infrared band at 1638 cm^{-1} with a shoulder around 1634 cm^{-1} forms short fibrous aggregates. La-VI displays an unusually broad amide I band and forms fibrous structures. La-VV, interestingly, shows a spectrum very similar to Pa-VV but forms shorter aggregates that possibly account for its non-gelation property. In the isoleucylvaline peptide series, Ma-IV causes gelation and displays a distinct peak at 1634 cm^{-1} . La-IV does show the 1634 cm^{-1} peak alongside an intense 1678

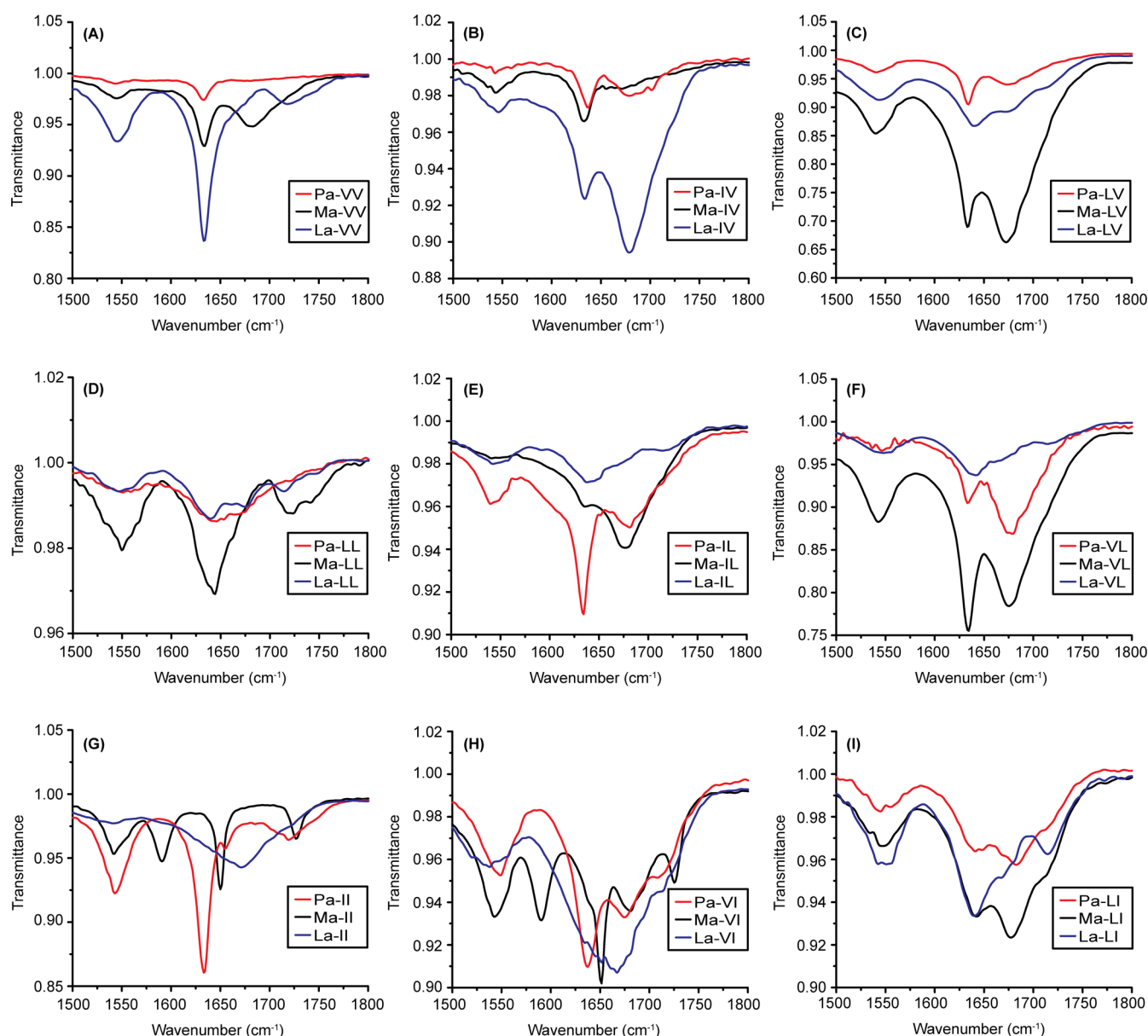


Figure 4. ATR-FTIR spectra recorded for the fatty acylated peptide samples, dried from the 20 mg/mL methanol/water bigels.

cm^{-1} peak but fails to form fibrillar structures. Pa-IV displays the IR band at 1637 cm^{-1} and fails to cause gelation. Pa-LV, the sole gelating member of the leucylvaline series, displays IR bands at 1633 and 1672 cm^{-1} . The non-gelating member Ma-LV displays the bands at the same wavenumbers, albeit with higher intensity for the 1672 cm^{-1} band. The other non-gelating member La-LV displays the lower-energy IR band at 1640 cm^{-1} instead of at $1633\text{--}1634\text{ cm}^{-1}$. The only non-gelating member of the valylleucine series, La-VL, also displays an IR band around $1640\text{--}1641\text{ cm}^{-1}$. Lauroylated and myristoylated diisoleucine, unlike palmitoyl diisoleucine that displays an IR band at 1634 cm^{-1} and causes gelation, display amide I band at wavenumber 1650 cm^{-1} or higher. The myristoylated isoleucylleucine displays IR bands around 1636 and 1675 cm^{-1} and forms a gel. Pa-IL with IR bands around 1634 and 1675 cm^{-1} , however, fails to cause gelation. The other non-gelating member, La-IL, displays infrared band $\sim 1638\text{ cm}^{-1}$. These data provide useful insights into the gelation propensity of the peptides. All the gelators display an

amide I infrared band around $1634\text{--}1636\text{ cm}^{-1}$. These data indicate that the β -conformation of the peptidic region is necessary but not sufficient for the peptide to be gelating. The length of the acyl chain and the amino acid side-chain chemistry do play critical roles in self-assembly and gelation. The infrared spectra were also recorded for the 50% ethanol/water gel samples. Each of the gel samples showed an infrared band around $1632\text{--}1634\text{ cm}^{-1}$ (Table S1 in the Supporting Information).

DLS–Microrheology (DLS- μ R). The viscoelastic nature of the gels was investigated using DLS- μ R that allows determination of the viscoelastic properties of a medium by measuring the thermally driven displacement of probe particles in the medium. The mean squared displacement (MSD) of the particles is dictated by the rheological properties of the medium. A plot of $\log(\text{MSD})$ against $\log(\text{time})$ gives a straight line with slope 1 in an ideally viscous medium. The slope is zero in a purely elastic medium and lies between 0 and 1 for a viscoelastic medium.^{44,45} We examined the viscoelasticity of

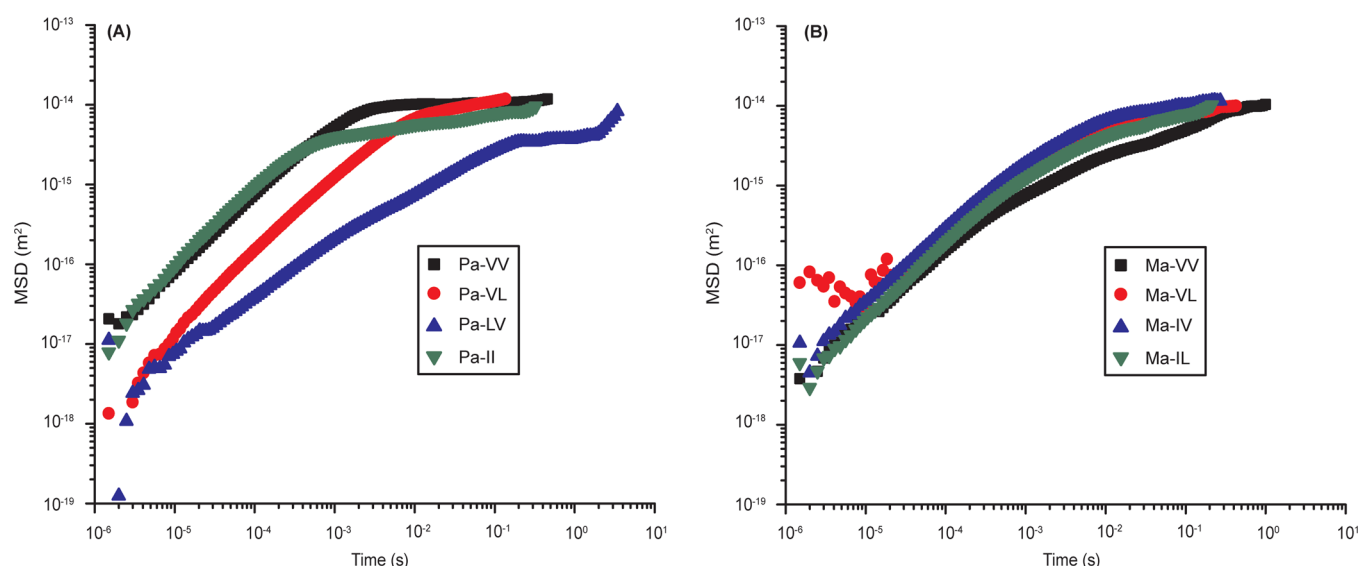


Figure 5. MSD data for (A) Pa-VV, Pa-VL, Pa-LV, and Pa-II and (B) Ma-VV, Ma-VL, Ma-IV, and Ma-IL gels in 50% methanol/water.

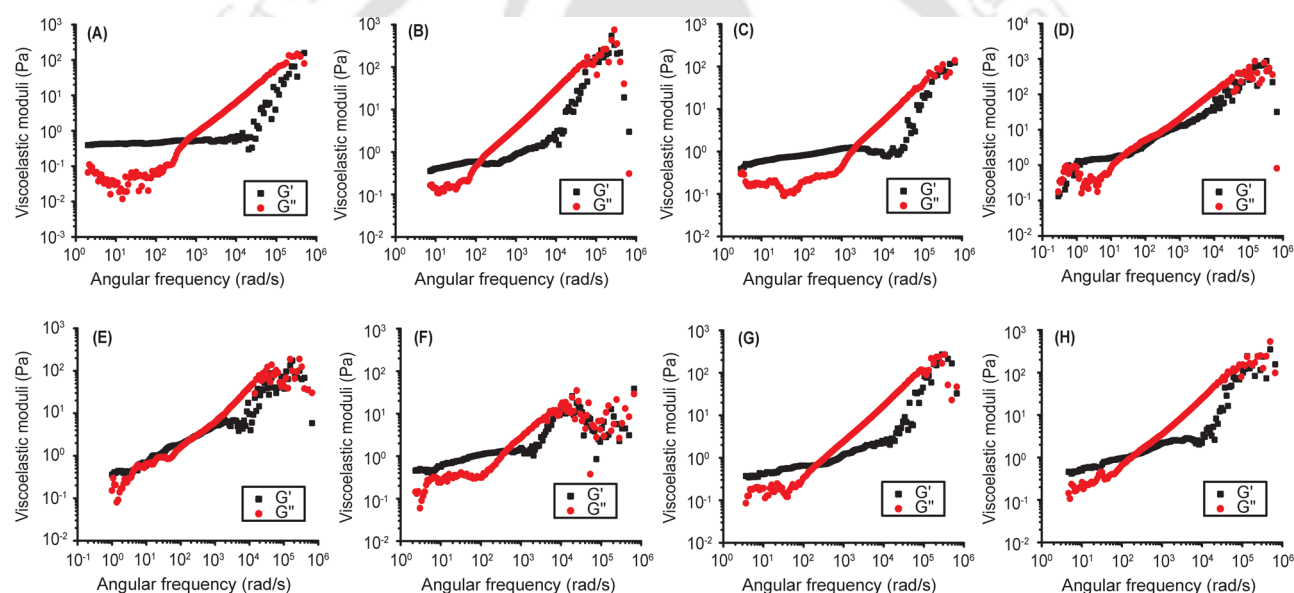


Figure 6. G' (black squares) and G'' (red circles) data, as determined from the MSD data using generalized Stokes–Einstein relation: (A) Pa-VV, (B) Pa-VL, (C) Pa-II, (D) Pa-LV, (E) Ma-VV, (F) Ma-VL, (G) Ma-IV, and (H) Ma-IL in 50% methanol/water.

the gels using DLS- μ R measurements with 600 nm carboxy-melamine tracer particles. The MSDs of the probe particles in the 50% methanol/water gels are shown in Figure 5. The MSDs of the palmitoylated peptide gels show a roughly linear plot at a smaller time scale but eventually flatten out at longer time points, indicating the elastic nature. The myristoylated gelators, like the palmitoylated ones, show linear MSDs at a lower time scale, but the slope decreases gradually with time.

The storage (G') and loss (G'') moduli for the gels were derived from the MSD data using the generalized Stokes–Einstein relation and are shown in Figure 6.³⁴ The top panels correspond to the palmitoylated peptides and show G' values higher than the G'' ones at lower frequencies. Pa-VV shows G' values higher than G'' up to ~ 200 rad/s (Figure 6A), while Pa-II shows higher G' up to at least 1000 rad/s (Figure 6C). The high $G'-G''$ crossover frequencies indicate shorter relaxation times. The other gelators, except Ma-VV, show G' slightly higher than the G'' , supporting their gel-like nature. MaVV

interestingly shows comparable G' and G'' values in the ~ 1 to 1000 rad/s frequency range, indicating comparable elastic and viscous properties (Figure 6E).

The DLS- μ R data for the 50% ethanol/water gels were also recorded (Figures S3 and S4 in the Supporting Information). The viscoelastic properties of the peptides Pa-VV, Pa-II, Pa-LV, and Ma-VV have rather notable differences when compared to the gels in 50% methanol/water. The 50% methanol/water Pa-VV gel shows a storage modulus of ~ 0.4 to 0.5 Pa in the 2–10,000 rad/s range. The 50% ethanol/water gel, however, shows a G' value of 4 Pa at 2 rad/s that gradually increases with the frequency and goes beyond 10 Pa at 10^4 rad/s. In 50% ethanol, Pa-II displays comparable G' and G'' values from ~ 50 rad/s onward, whereas a distinct difference between G' and G'' values was obtained from ~ 5 to 1000 rad/s in 50% methanol/water. The 50% methanol/water gel of Pa-LV displays gel-like properties, that is, G' higher than G'' within ~ 1 to 10 rad/s. The 50% ethanol/water gel, on the

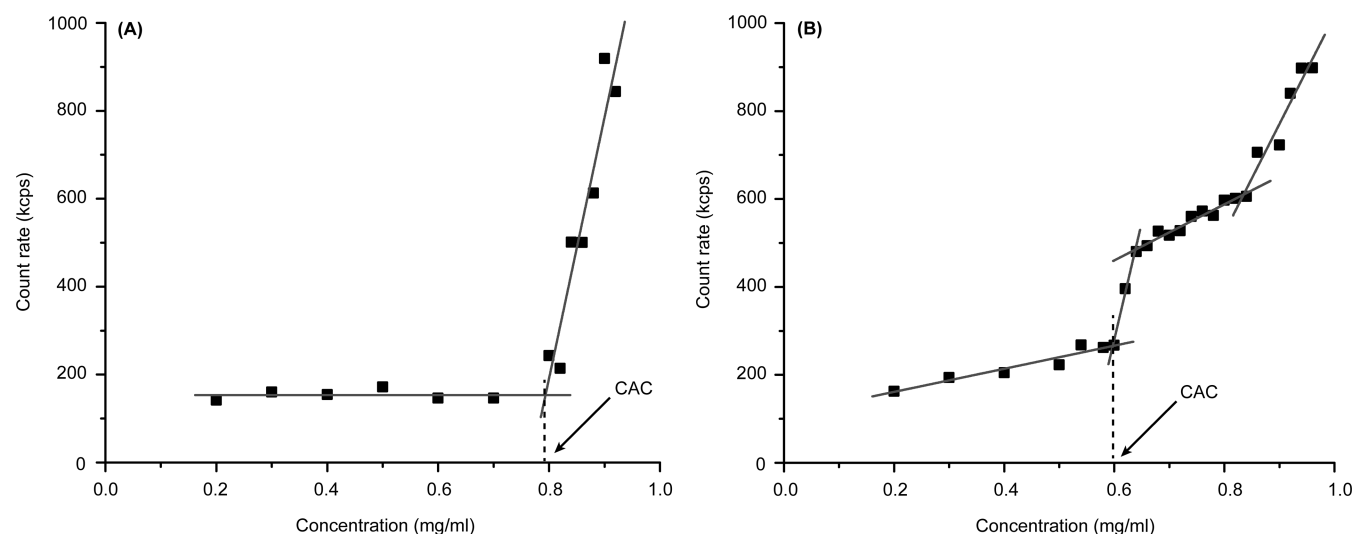


Figure 7. CAC of (A) Ma-VV and (B) Pa-VV in 10% methanol.

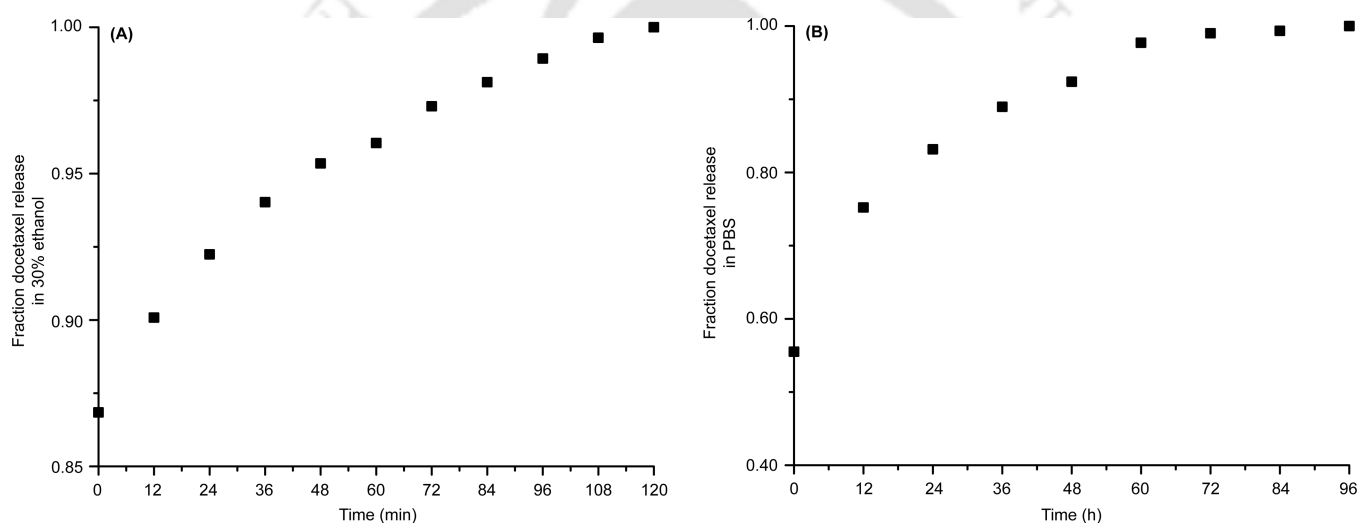


Figure 8. Fraction docetaxel release from the Ma-IV 50% ethanol/water bigel.

other hand, displays gel-like properties from <1 to 500 rad/s. Ma-VV that shows comparable G' and G'' values in the ~ 1 to 1000 rad/s frequency range in 50% methanol displays G' values higher than G'' values in 50% ethanol in the 1–200 rad/s range.

In order to probe the role of the C-terminal carboxyl group's ionization status, gelation reactions in 50% methanol at 20 mg/mL concentration were set up at acidic (0.2% TFA, pH ~ 2) and alkaline pH (10^{-5} N NaOH, pH ~ 9). None of the samples formed a gel even after 24 h of incubation. In addition, considering that the negatively charged carboxylate groups could lead to electrostatic repulsion, thereby causing the gelation at higher concentration, gelation with Ma-VV was investigated at neutral pH (50% methanol/water with 100 mM NaCl) at 10 mg/mL peptide concentration to see if gelation could happen at lower concentration. No gelation was observed under this condition as well.

Critical Aggregation Concentration. CAC was determined for Ma-VV and Pa-VV as the representative peptides. Pa-VV was chosen as it resulted in transparent gels with a distinct fibrillar morphology, whereas Ma-VV was chosen to make a direct comparison of the acyl chain length on the CAC.

Critical micellar concentration (CMC) or CAC of self-assembling amphiphilic molecules is often determined using extrinsic polarity-sensitive fluorescent probes such as 8-anilino-1-naphthalenesulfonic acid (ANS) and Nile red.^{46–50} These methods, however, did not work out for determining the CAC of the fatty acylated dipeptides due to the presence of methanol in our samples. DLS is an excellent method for determining the CMC and has been employed for determining the CAC of lipid-like peptide surfactants.⁵¹ The CAC of Ma-VV and Pa-VV could be estimated in 10% MeOH using DLS. The CAC for Ma-VV is estimated to be around 0.8 mg/mL, while that of Pa-VV was estimated to be around 0.6 mg/mL (Figure 7). None of the samples formed gels in the tested concentration range. The longer chain, understandably, shows a smaller CAC. It is interesting to note that, unlike Ma-VV that shows a distinct single transition point, Pa-VV shows three such points up to around a 0.95 mg/mL concentration range. The further higher concentrations turned out to be unsuitable for DLS analysis and therefore could not be studied. The multiple transition points observed for Pa-VV could possibly be due to higher-order assembly and packing of the smaller aggregates. As a higher methanol percentage resulted in

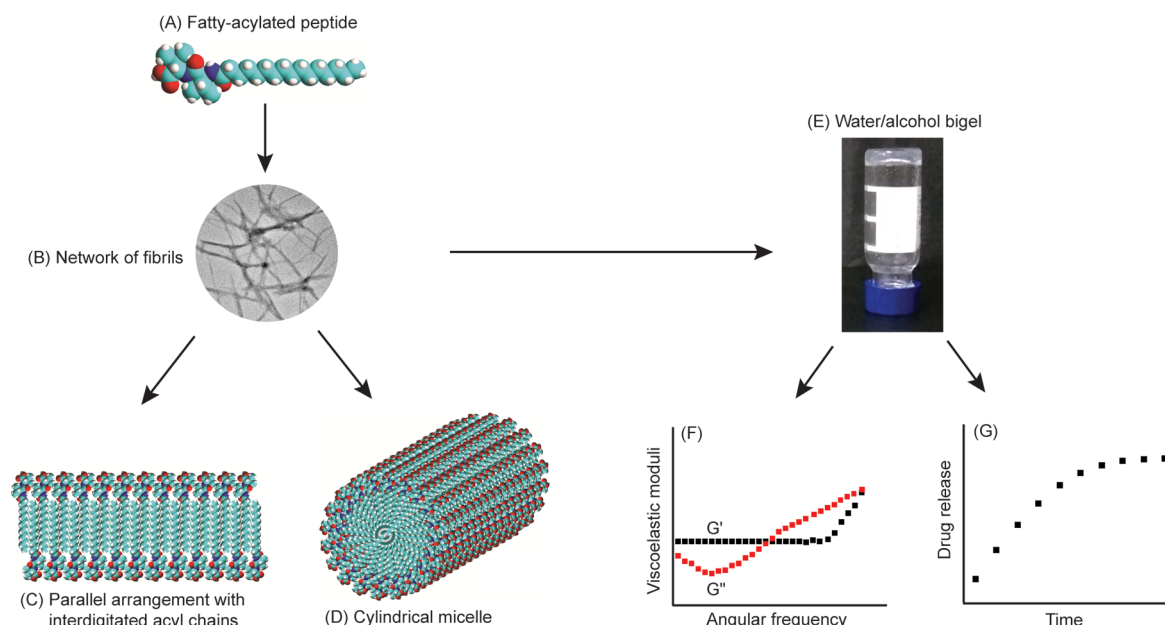


Figure 9. A concise summary of the study. (A) Fatty acylated dipeptides self-assemble to form (B) fibrils, possibly through (C) parallel and/or (D) cylindrical arrangement of monomers, causing gelation of (E, F) the alcohol/water cosolvent mixture that can be used for (G) sustained drug release.

inconsistent scattering, possibly due to solvent clustering, the CAC could not be determined at higher methanol concentrations. It is important to note that the CAC values of Ma-VV and Pa-VV in 10% methanol are less than 1 mg/mL, way below the concentration required for gelation of 50% methanol. This is reasonable because CAC is a measure of the critical concentration for aggregation initiation whereas gelation requires large aggregates that can entangle to trap the solvent molecules, which are formed at concentrations higher than the CACs. In addition, the higher solubility of the fatty acylated peptides in 50% methanol compared to that in 10% methanol could account for the higher gelation concentration.

Dye/Drug Release Assay. Entrapment and subsequent release of small molecules is a useful attribute of gels. Entrapment of rhodamine B, a fluorescent dye, was investigated with the fatty acylated peptide gels. The peptides were allowed to gel in the presence of 200 μ M dye; uptake of dye by the gels rendered them red in color. The subsequent release of the dye was observed by gently adding water on top of the gel and recording rhodamine B fluorescence at 573 nm over time (Figure S5 in the Supporting Information). All the gels resulted in a rather quick release of dye; 50% or more rhodamine B is released in water within 20 min. It would be worth investigating the release of a drug molecule. However, it is not appropriate to use a methanol-containing gel for drug delivery application. Release of the anticancer compound, docetaxel, therefore, was investigated from the 50% ethanol/water Ma-IV bigel as a representative example. The drug was loaded during the gelation process, and subsequent release was monitored in 30% ethanol and phosphate-buffered saline (PBS) by recording absorbance at 230 nm. The drug showed a rapid release in 30% ethanol, but a sustained release for 3 days was observed in PBS (Figure 8). A large number of drug molecules are sparingly soluble in water and can precipitate in hydrogels. Bigels, however, can hold such drugs at higher concentrations. The bigel network and the low solubility in water determine the release kinetics.

DISCUSSION

Gels happen to be an important class of soft materials. Although we encounter them routinely, there is no strict definition of a gel. They can loosely be defined as semisolid materials that entrap a large amount of solvent molecules. The gels can be better described rheologically: if a system displays higher storage modulus than loss modulus under the given conditions, it is a gel. At the molecular level, a gel is composed of a network of molecules that are held together through non-covalent interactions (physical gels) or through covalent bonds (chemical gels). In the past few decades, small molecules that self-assemble through non-covalent interactions have gained considerable interest as gelators. Largely due to their biocompatibility, biodegradability, and chemical functionality, the peptides have been investigated in great detail as gelators. Janmey and co-workers found that certain Fmoc-protected dipeptides can self-assemble, resulting in hydrogels.⁵² The π - π interactions between the fluorenyl moieties and hydrogen-bonding between peptide backbone amides are believed to be critical for the self-assembly of short Fmoc-protected peptides.^{53,54} Subsequently, a large number of Fmoc-protected peptides and unprotected peptides rich in aromatic residues were reported to cause hydrogelation.^{20,21,55–59} Other than peptides with aromatic residues, peptides having amphipathic characteristics is another important class of hydrogelating molecules. The amphipathic character can be imparted to a peptide by appending a series of hydrophobic residues to one or more polar ones^{25,26,60} or through intramolecular folding that segregates polar and non-polar residues.^{61,62} Hydrogelating amphipathic peptides, other than those harboring aromatic residues or protected with bulky aromatic moieties, are generally composed of six or more residues.⁶³ Additionally, attaching a non-peptidic hydrophobic moiety to a polar peptide renders the peptide hybrid amphipathic.^{27,32,64,65}

Peptide-based gels have shown promising applications in drug delivery and regenerative medicine. Their utility, however, is hampered by the high cost associated with the

synthesis of peptides composed of several amino acids as in the case of the hydrogel-forming peptides.⁶³ It is therefore imperative to design synthetically easily amenable peptide-based gelators. Nagaraj and co-workers have reported the self-assembly of Boc-protected aliphatic dipeptide methyl esters Boc-Val-Ile-OMe, Boc-Val-Leu-OMe, Boc-Leu-Val-OMe, and Boc-Val-Val-OMe in 10, 50, and 100% methanol.³³ All the four peptides formed tubular or needle-like nanostructures. No fibrillar morphology was discernible. In this study, we explored the self-assembly and gelation potential of the fatty acylated dipeptide acids wherein the peptidic region is made up of Val, Leu, and Ile. Due to their high hydrophobicity, the peptides can be easily purified by extracting them in dichloromethane. The peptides turned out to be insoluble in water but readily dissolved in methanol and ethanol. Addition of water to these solutions resulted in distinct gels after 24 h of incubation for eight of the peptides, viz., Pa-VV, Ma-VV, Pa-VL, Ma-VL, Ma-IV, Pa-LV, Ma-IL, and Pa-II. It is interesting to note that three of the dipeptides, viz., Val-Val, Val-Leu, and Leu-Val, caused gelation when palmitoyl is the fatty acid chain. Even though Boc-Val-Ile-OMe, like the other three Boc-protected peptide esters, formed tubes,³³ none of the fatty acylated Val-Ile peptides caused gelation of 50% methanol/water and 50% ethanol/water solutions up to 20 mg/mL concentration. The self-assembled structures underlying the fatty acylated peptide bigels show an infrared amide I band around 1634 cm⁻¹, an IR band characteristic of β -sheets. The Boc-dipeptide esters, on the other hand, largely display the major amide I band between 1643–1653 cm⁻¹, suggesting a very different self-association mode.

Amphiphiles wherein the polar head group is bulkier than the hydrophobic tail, that is, the ones that have a somewhat conical geometry, tend to self-assemble into micelles. Such conical peptide amphiphiles have been reported to self-assemble into spherical and cylindrical micelles.^{31,66} The fatty acylated peptides investigated here harbor the C-terminal carboxylate group and the backbone amides as the only polar moieties. As the peptidic region is bulkier than the acyl chains, the peptide could self-assemble with the modes shown in Figure 9C,D. Association shown in Figure 9C is the parallel arrangement of the peptides wherein the acyl chains are interdigitated. Such an arrangement can lead to super structures as a result of strong hydrophobic interactions between the fatty acyl chains and side chains of hydrophobic amino acids. This mode of assembly can lead to the formation of thin sheet-like structures. The sheet/flake-like structures observed for La-VV, La-IV, Pa-IV, and Pa-II, and the ribbon-like structures observed for Pa-IL could originate through this mode of assembly. The association mode shown in Figure 9D is that of a cylindrical micelle, similar to that reported for peptide amphiphiles by Stupp and co-workers.³¹ Such an assembly mode would result in long fibrous structures as observed for Pa-VV, Ma-VV, Ma-IV, Pa-VL, and Pa-LV. The packing in the structures shown in Figure 9C,D would vary depending on the fatty acyl chain and the dipeptide sequence. The differences in the morphology of the self-assembled structures formed by different peptides could arise due to variations in the packing of the fatty acyl chains modulated by the dipeptide sequence and the interactions between the peptidic motifs depending on the nature of the side chains. In order to investigate the possible arrangements of the molecules in the gel-forming self-assembled structures, the Ma-VV bigel was sheared using a pipette, deposited on a glass cover slip as a

thin film, air-dried, and analyzed using thin-film X-ray diffraction (XRD). The XRD data from Ma-VV shows somewhat broad overlapping peaks around 4.17 and 4.5 Å (Figure S6 in the Supporting Information). The XRD of the dipalmitoylphosphatidylcholine gel L β phase is reported to give a sharp peak at 4.2 Å.^{67,68} The peak arises from the spacing between fatty acyl chains. The biological membranes at physiological temperature are found in the liquid crystalline state L α . The XRD of this phase is characterized by a diffuse band around 4.5–4.7 Å. The peak around 4.17 Å for Ma-VV could be due to the gel-like phases of the acyl chains. The 4.5 Å peak is suggestive of the β -sheet conformation. The spacing is smaller than the typical 4.7 Å distance between the β -strands. The smaller distance of 4.5 Å could be due to the tightly packed structures. Hamley and co-workers have reported even smaller interstrand distance of 4.4 Å for the amphiphilic self-assembling oligopeptide A₁₂R₂.⁶⁹ The 4.5 Å reflection could also have contribution from the liquid-crystalline phase of the acyl chains coexisting with the ordered structures.

CONCLUSIONS

The results obtained in this study have been summarized in Figure 9. The simple fatty acylated dipeptide acids self-assemble into fibrillar structures in water/alcohol cosolvent mixtures (Figure 9B). The fibrillar network traps the solvent molecules, resulting in bigels (Figure 9E) that were characterized using DLS microrheology (Figure 9F). The length of the acyl chain plays a critical role in the gelation. None of the lauroylated peptides formed bigels, whereas four each of the myristoylated and palmitoylated peptides formed distinct methanol/water and ethanol/water bigels. The longer acyl chain, as expected, decreases the CAC of the peptide. The role of the peptide moiety in gelation is subtle; whereas Val-Val and Val-Leu turn gelators through myristoylation as well as palmitoylation, Ile-Val and Ile-Leu turn gelators only upon myristoylation, and Leu-Val and Ile-Ile turn gelators only upon palmitoylation. Val and Ile, therefore, happen to be the preferred N-terminal residues for the peptide to be a gelator. The β -sheet structure is critical for the gelation as suggested by the FTIR spectroscopic data, and Val and Ile are among the highest β -sheet-propensity residues of all natural amino acids. Valine has a high self-assembling propensity and has been used as the hydrophobic residue in designed self-assembling amphipathic peptides.⁶¹ The role of the C-terminal residue could be subtler in modulating self-assembly and gelation as suggested by the non-gelation propensity of Ma-VI and Pa-VI up to 2 wt %. The low solubility of many hydrophobic drugs in water limits the applications of the hydrogels for their delivery. The ethanol/water bigels reported here could serve as drug delivery vehicles for hydrophobic drugs as demonstrated by trapping the anticancer drug docetaxel in the Ma-IV gel. The drug displayed a sustained release for over 3 days. This study shows that rather simple amphipathic peptides are attractive gelator candidates with potential application in drug delivery.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jpcb.9b10002>.

Figure S1: Tube inversion test for gelation in 50% ethanol/water; Figure S2: SEM images of 50% ethanol/water bigels; Figure S3: Mean squared displacement data

of 50% ethanol/water bigels; Figure S4: G' and G'' data for 50% ethanol/water bigels; Figure S5: Rhodamine B release from 50% methanol/water bigels, as monitored by recording fluorescence emission at 573 nm; Figure S6: X-ray diffractogram obtained from the dried film of the Ma-VV 50% methanol bigel; and Table S1: FTIR data of gel forming fatty acylated peptides in 50% ethanol/water (PDF)

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Author Contributions

N.C. and R.N. conceived the idea. D.D., R.N., and N.C. designed the experiments. D.D. carried out the experiments. All the authors contributed to manuscript writing. All the authors approve the final version of the manuscript.

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Notes

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