Chapter 2

Materials and Methods

This chapter describes the experimental procedures followed to carry out the studies reported in this thesis.

2.1 Materials

The solid support resin PAL (5-[4-(9-fluorenylmethoxycarbonyl) aminomethyl-3,5-dimethoxy-phenoxy] valeric acid) resin was from Advanced Chemtech (Louisville, KY, USA). 9-fluorenylmethoxycarbonyl (Fmoc) protected amino acids were from Novabiochem AG, Switzerland and Advanced Chemtech. Coupling reagents used in peptide synthesis were N-hydroxybenzotriazole hydrate (HOBr) (Advanced Chemtech), 2-(1-H-benzotriazole-1-yl)-1,1,3,3-tetramethyldiuronium hexa-fluorophosphate (HBTU) (Advanced Chemtech), and N,N-diisopropylethylamine (Sigma). Palmitic acid and myristic acid used for acylation of peptides were from Sigma-Aldrich, MO, USA. 1-Palmitoyl-2-oleoyl-phosphatidylcholine (PC), 1-palmitoyl-2-oleoyl-phosphotidylglycerol (PG), cholesterol (CHL), sphingomyelin (SM), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(5-dimethyl-amino-1-naphtha-lenesulfonyl) (d-PE) were from Avanti Polar lipids, Alabaster, AL, USA. Thioanisole, ethanedithiol, acetic anhydride were either from Fluka AG Chemical Corp, Switzerland or Pierce Chemical Company, USA. Solvents such as dimethylformamide (DMF), N-methyl pyrrolidone (NMP), dichloromethane (DCM), chloroform, methanol, diethylether, isopropanol,
dimethyl sulfoxide (DMSO), 2-mercapto-ethanol were from Spectro Chern Pvt. Ltd., India. HPLC- or UV-grade solvents like methanol, acetonitrile, isopropanol, DMSO were either from Spectro Chem Pvt. Ltd., India or from Qualigens Fine Chemicals, India. Piperidine was from Loba-Chemie Pvt. Ltd., India. Inorganic chemicals such as sodium sulfate, sodium chloride, potassium chloride, mercuric acetate, EDTA, HEPES, potassium iodide, sodium dihydrogen phosphate, disodium hydrogen phosphate, iodine, sodium thiosulfate were from SD Fine Chemicals, India. α-Cyano-4-hydroxycinnamic acid (CHCA) was from Sigma-Aldrich. 2,5-dihydroxybenzoic acid (DHB) and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) were from TCI, Tokyo, Japan. Reversed-phase C18 columns for HPLC were from either Bio-rad or Agilent. Polycarbonate filter membranes were from Avestin, Germany. ZipTips were from Millipore, USA. Solvents used in peptide synthesis such as DMF and NMP were dried over molecular sieves for at least 72 hours before use. DCM was dried over dry sodium sulfate for at least 72 hours before use.

2.2 Synthesis and characterization of synthetic peptides

The peptides were assembled using standard Fmoc-chemistry. Overall three series of peptides were synthesized: Fyn-series, Lck-series, and β2m-series. The synthesis of each series essentially involves generation of a parent peptide which represents a fully protected unmodified peptide. It is this parent peptide which forms the basis for generating differentially modified peptides in each series. Each series of the peptides consists of peptides modified with two fatty acids: myristic acid and palmitic acid. The attachment of these fatty acids was carried out either in the N-terminus or in the side-chain of a Cys or Lys residue. Additionally, in Fyn- and Lck-series, peptides containing fatty acids attached at both N-terminus and side-chain were also synthesized. Peptides were synthesized using standard Fmoc chemistry (Atherton and Sheppard, 1989; Wellings and Atherton, 1997). Fmoc, an amino protecting group is labile to secondary amine and is orthogonal to acid-labile amino acid side-chain protecting groups. The
peptides were assembled on PAL resin (substitution value 0.7 μmol/mg or 0.3 μmol/mg) which results in C-terminally amidated peptides. All three, the Fyn-series, the Lck-series, and the β2m-series peptides were synthesized using routine Fmoc-protected amino acids except the Cys residues which were differentially protected in three series of peptides. Since the Fyn-series peptide were designed to have only one cysteine residue which was later on-resin palmitoylated, Fmoc-Cys(Mmt)-OH, containing very acid-labile Mmt functionality, was used for synthesis of this series of peptides. The Lck-series peptides on the other hand contain two cysteine residues each of which designed to be selectively palmitoylated, hence required orthogonal protection. This was achieved by using Fmoc-Cys(Trt)-OH and Fmoc-Cys(Acm)-OH, which could be selectively deprotected by appropriate iodine treatments. Similarly, two of the Fyn-series members, Pamfyn and Pmmfyn were designed to contain myristoyl chain at Lys side-chains, which was ensured by incorporating Fmoc-Lys(Mmt)-OH at appropriate position in the peptide sequence. Among β2m-series peptides, those acylated at Cys side-chain were synthesized by incorporating Fmoc-Cys(Mmt)-OH while those modified with fatty acids at the side-chain of Lys residue incorporated Fmoc-Lys(Mmt)-OH in the sequence synthesized.

**Attachment of first amino acid**

The desired weight of PAL resin was vacuum dried overnight and then soaked in dry DMF for 1 hour for swelling. Fmoc was removed by treating the resin with 20% piperidine in DMF for 20 minutes. Subsequently, the resin was washed thoroughly with DMF to remove any traces of piperidine which was ascertained by the neutral read-out of the moist pH paper. A three-fold molar excess of HOBt, HBTU, Fmoc-amino acid, and six-fold molar excess of DIEA pre-dissolved in 1 ml solvent and activated for 10 minutes by gentle mixing at room temperature was added to the resin and kept for 1 hour on gentle mixing. Afterwards, the resin was washed two-three times with solvent. The second coupling was carried out by adding two-fold molar excess of HOBt, HBTU, Fmoc-amino acid, and four-fold molar excess of
DIPEA and keeping the reaction mix on gentle mixing for next 1 hour. The resin was then washed two-three times with solvent.

**Determination of amino acid substitution**

After attachment of the first amino acid, Fmoc was removed by treating a known weight of resin with 20% piperidine and reading the amount of Fmoc released spectrophotometrically. It has been reported that 1 mol of Fmoc in 3 ml of 20% piperidine gives an absorbance of 1.650 at 290 nm (Kawakami et al., 1999), thus giving molar absorption coefficient of 4950 M\(^{-1}\) cm\(^{-1}\) at 290 nm. Another convenient way to confirm the substitution of an amino acid at any point during synthesis uses the absorbance of Fmoc at 301 nm (\(\epsilon_{301} = 7800\) M\(^{-1}\) cm\(^{-1}\)) (Meienhofer et al., 1979). The substitution values were calculated using following formulae:

\[
x = \frac{A_{290} \times V \times \text{dilution factor} \times 1000}{4950 \times w}
\]

\[
= \frac{A_{301} \times V \times \text{dilution factor} \times 1000}{7800 \times w}
\]

where, \(x\) is the substitution value in mmol/gm, \(A_{290}\) and \(A_{301}\) are absorbances at 290 nm and 301 nm, respectively, \(V\) is the volume of 20% piperidine in ml used for Fmoc removal, and \(w\) is the weight of resin in mg.

**Subsequent attachments of amino acids**

After attachment of the first amino acid, the resin was washed with solvent and then subject to Fmoc removal by treating the resin with 20% piperidine for 20 minutes at room temperature. After extensive subsequent washes with the solvent and ensuring neutral pH of resin, the pre-activated amino acid mix containing three-fold molar excess of HOBT, HBTU, Fmoc-amino acid, and six-fold molar excess of DIPEA in minimal solvent was added to the resin and kept on gentle-mixing for 45 minutes at room temperature. The second coupling was carried out for 30 minutes with two-fold molar
excess of HOBt, HBTU, Fmoc-amino acid, and four-fold molar excess of DIPEA. Subsequent amino acid attachments were carried out by repeating the Fmoc-removal and amino acid coupling steps till the entire sequence is assembled. After the final attachment, the resin was washed with DMF and dried with diethyl ether and finally kept for air-drying for few hours.

**N-terminal acetylation/acylation**

The covalent modification to generate the Fyn series peptides was carried out on-resin using the strategy developed by Harish Chandran (Harishchandran, 2005), however, with slight modification. Instead of Acm protected cysteine, Mmt protected cysteine was used to generate the peptide. Additionally, low loading capacity PAL resin (0.3 mmol/gm) was used for assembling the peptides as high loading PAL resin (0.7 mmol/gm) yielded unsatisfactory results. Finally, all coupling reactions were performed at elevated temperature (35 °C) to avoid environmental fluctuations of temperature biased towards lower temperature of <30 °C.

The dried resin containing protected peptide chain attached on to it was soaked in DMF for 1 hour for swelling. The DMF was then replaced with 20% piperidine and left on gentle-shaking to remove the N-terminal Fmoc functionality. Afterwards, the resin was washed thoroughly with DMF to remove traces of piperidine (as judged by neutral pH of resin). It was then subjected to N-terminal acetylation or acylation.

For N-acetylation, 5 equivalents each of acetic anhydride and triethylamine or diisopropylethylamine prepared in minimum volume of DMF were added to the resin and kept for 1 hour on gentle mixing. The resin was then washed with DMF and the acetylation reaction was repeated one more time. The resin was washed thoroughly first with DMF and then with diethyl ether and subsequently either kept for air-drying at room temperature or subjected to side-chain acylation.

For N-acylation, a 10-fold molar excess each of HOBt, HBTU, and a 20 fold molar excess of DIPEA prepared in minimum volume of DMF was added to a 10 fold molar excess of myristic/palmitic acid dissolved in dry
DCM and kept at mild-mixing at room-temperature. Within 10 minutes
the activated fatty acid formed a gel which was reconstituted in DMF and
added to the resin containing free amino terminus. The reaction mix was
kept on gentle-mixing for 5 hours at 35 °C. The process was repeated two
more times. Finally, the resin was washed thoroughly first with DMF and
then with diethyl ether and kept for air-drying at room temperature, or
alternatively subjected to side-chain acylation.

Side-chain acylation

The side-chain acylation of Fyn-series peptides was performed either at
Lys or at Cys residue each protected with acid-labile Mmt functionality. After
performing N-terminal modification, the resin was subject to Mmt-cleavage
protocol as follows: A 2% TFA was prepared in dichloromethane:ethanedi­
thiol (95:5 v/v) and 1 ml of this was added to the resin pre-washed with
dichloromethane and kept on mild-shaking for two minutes. The resin was
then quickly washed with plenty of dichloromethane. This was repeated two
more times subsequent to which the resin was washed with dichloromethane
and then with NMP. The activated fatty acid (myristic acid or palmitic acid)
prepared as described in section 2.2 was then added to the resin and the
reaction mix was left on gentle-mixing for 10 hours at 35 °C. Two more
couplings with activated fatty acid each for 5 hours were given. At the end
of the third coupling, the resin was thoroughly washed first with DMF and
then with diethyl ether and left for air-drying at room temperature

Side-chain palmitoylation of Lck-series peptide

The Lck-series peptides selectively palmitoylated at one of the two cysteine residues or dipalmitoylated at both of the cysteines were generated
as following. For generation of dually acylated peptides myristoylated at
N-terminus and palmitoylated at either Cys-3 or Cys-5 residue, the resin-
bound peptide myristoylated at N-terminus and containing trityl-protected
Cys (Cys-Trt) residue at either Cys-3 or Cys-5 respectively was soaked in
NMP for one hour and then washed with HFIP:chloroform (3:1 v/v) mix
two-three times. Now the resin was treated with 10 equivalents of iodine prepared in HFIP:chloroform (3:1 v/v) mix for five minutes at room temperature. The resin was then quickly washed with HFIP:chloroform (3:1 v/v) mix till iodine stains go off. This procedure selectively deprotects the trityl-protected cysteine while leaving the Acm-protected Cys intact. Subsequently, the resin was washed with NMP and to it was added activated palmitic acid prepared as described in section 2.2. The first coupling of palmitoylation was given for 10 hours followed by two additional couplings each lasting for 5 hours. All couplings were carried out at 35 °C. The resin was then sequentially washed with NMP and diethyl ether and left for air-drying at room-temperature.

For generating the triply acylated peptide myristoylated at N-terminus and palmitoylated at both of the Cys residues, the resin-bound peptide already myristoylated at N-terminus was soaked in NMP for one hour. The resin was then treated with 10 equivalents of iodine per cysteine prepared in minimum volume of DMF for 10 minutes. This procedure removes both Trt- and Acm- functionalities at the side-chains of Cys within 25-35 seconds of treatment (Annis et al., 1997). The resin was subsequently washed extensively first with DMF and then with NMP and finally subjected to disulfide reduction by treating with 20 equivalents of dithiothreitol (DTT) per Cys for 1 hour. Disulfide reduction reaction was repeated once more to ensure the reduction of any disulfide bond which might have formed during on-resin deprotection of Cys residues. The resin was then washed thoroughly with NMP and mixed with activated palmitic acid prepared as described in section 2.2. Three couplings of palmitoylation lasting for 10 hours, 5 hour, and 5 hours respectively were given. All couplings were carried out at 35 °C. At the end of palmitoylation the resin was washed first with NMP and then with diethyl ether and finally left at room temperature for air-drying.
**Covalent modification of β2m-series peptides**

The N-terminal modification of β2m-series peptides was carried out in the same way as of Fyn-series peptides described in section 2.2. Similarly, side-chain myristoylated and palmitoylated β2m-series peptides were generated in the same way as side-chain modified Fyn-series peptides, the procedure for which has been described in section 2.2.

**Cleavage and precipitation of resin-bound peptides**

Side-chain deprotection and cleavage of peptides from the resin was achieved by soaking the resin in a cleavage-cocktail consisting of TFA (72.73%), m-cresol (10.91%), thioanisole (10.91%), and ethanedithiol (5.45%) for 10-12 hours. About 500 μl volume of this cleavage mix was added to about 20-30 mg of resin. The cleavage mix was then separated from the resin and precipitated in excess of ice-chilled diethyl ether. The peptide precipitate was centrifuged at 800x g for 5 minutes and supernatant discarded. The peptide pellet was washed 5-6 times with ice-chilled diethyl ether to completely remove the scavengers. The peptide pellet was finally air-dried to obtain crude peptide. The crude peptides were evaluated qualitatively for their solubility in different solvents by sequentially exposing them to different solvents starting from deionized water followed by water-methanol (1:1 v/v) mix, methanol, trifluoroethanol, and finally DMSO.

**Purification and characterization of synthetic peptides**

The crude peptides were dissolved in appropriate solvents and subjected to purification with reversed-phase high-performance liquid chromatography (RP-HPLC). The peptides were purified on a Hewlett-Packard 1100 series high-performance liquid chromatography system using a Zorbax 300SB C18 reversed-phase column under the gradient of acetonitrile containing 0.1% TFA. The HPLC elution of peptides was monitored at 214 nm and at 280 nm since each of the peptides contains a Trp residue. The purified
peptide fractions were characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) on a Voyager DE STR mass spectrometer (PerSeptive Biosystems, MA) at the in-house Proteomics Facility. All purified peptides yielded correct $m/z$ values in mass characterization. While Fyn- and Lck-series peptides yielded correct $m/z$ values by spotting with CHCA matrix alone, the $\beta_2$m-series peptides required addition of the nonionic detergent octyl glucoside in a final concentration of 5 mM along with CHCA matrix (as described by Jagannadham and Nagaraj (2005)) for detecting the MALDI-TOF mass spectra. The purified and MALDI-TOF-MS characterized peptide fractions were dried in a speed vacuum drier and stored till further use. The peptide stocks were prepared by dissolving the required amount of the dry peptide in appropriate solvent immediately before use.

2.3 Preparation of liposomes

For preparing lipid vesicles of various compositions including those doped with fluorescent tagged lipid, appropriated volumes of lipid stocks were drawn from respective chloroform/methanol stocks and transferred to glass tubes and dried either under nitrogen stream or in a rotavapor assembly to yield uniform lipid films. The lipid films were desiccated under vacuum for 6 hours and then hydrated for 10 hours with appropriate buffer. The hydrated films were vortexed vigorously to give the suspension of lipid in buffer which was subsequently used for preparation of either large unilamellar vesicles (LUV) or small unilamellar vesicles (SUV). Large unilamellar vesicles were obtained by extruding the suspension through 200 nm polycarbonate filter membranes. Alternatively, small unilamellar vesicles were obtained by sonication of vortexed lipid suspension till it became clear and subsequent centrifugation at 8000 rpm for 5 min to get rid of metal debris. Homogeneity of lipid vesicles was verified by dynamic light scattering (DLS).
2.4 90° Scatter recording

To assess the aggregating propensity of peptides over time, 90° scatter of all peptides in buffer and methanol was read for 30 minutes on a F4500 fluorescence spectrophotometer (Hitachi) by setting both excitation and emission wavelengths at 450 nm and slit widths at 5 nm for both excitation and emission.

2.5 Tryptophan fluorescence spectroscopic studies

Lipid binding ability of Fyn-series and Lck-series peptides was assessed by monitoring the fluorescence of intrinsic Trp residue in absence and in presence of lipid vesicles with the compositions PC, PC:PG (1:1), PC:CHL (7:3), PC:CHL (1:1), and PC:SMCHL (1:1:1). Tryptophan was excited at 280 nm and emission spectra recorded between 300-400 nm in either a Hitachi F4500 Fluorescence Spectrometer (Hitachi, Tokyo, Japan) or a Fluorolog 3-22 Fluorescence Spectrophotometer (Horiba Jobin Yvon, Park Avenue Edison, NJ). The excitation and emission bandwidths were 2 and 5 nm, respectively. All spectra for Fyn-series peptides were recorded in 5 mM HEPES buffer, pH 7.4 while in 5 mM HEPES buffer, pH 7.4 containing 150 mM NaCl in case of Lck-series peptides. Peptide concentration was 1-2 μM. Accessibility of tryptophan in different peptides to solvent was assessed by monitoring quenching of Trp fluorescence by iodide in absence and presence of lipid vesicles. Increasing amounts of 4 M KI (containing 1 mM Na2S2O3) was added from 0 to 50 mM to a fixed peptide concentration of 1-2 μM. In some cases, another aqueous quencher, acrylamide was used for assessing the solvent exposure of Trp residue. The data for fluorescence quenching were analyzed by Stern-Volmer plots using following equation:

\[
\frac{F_0}{F} = 1 + K_{sv}[Q]
\]  \hspace{1cm} (2.2)

Where, \(F_0\) and \(F\) are the fluorescence intensities of Trp in absence and presence of quencher respectively at quencher concentration \([Q]\), and \(K_{sv}\) is
the Stern-Volmer quenching constant. The normalized accessibility factor (NAF) was calculated from the ratios of $K_{sv}$ obtained from quenching of Trp fluorescence in the presence and absence of liposomes.

The steady-state tryptophan emission of $\beta_2$m-series peptides was recorded at a fixed peptide concentration of 10 $\mu$M in a Fluorolog 3-22 Fluorescence Spectrophotometer (Horiba Jobin Yvon, Park Avenue Edison, NJ). Peptides were diluted from DMSO stocks to 50 mM phosphate buffer (pH 7.0) containing 150 mM NaCl. The Trp residue was excited 280 nm and emission recorded between 300-400 nm with slit widths for excitation and emission set at 2 nm and 5 nm, respectively. To discriminate for any contribution from the intrinsic tyrosine residues, emission was also recorded for excitation at 295 nm. For comparison with the emission of free tryptophan in solution, emission spectra of NATA were also recorded for both excitation wavelengths at 10 $\mu$M concentration. To rule out the possibility of tyrosine quench of tryptophan fluorescence in these peptides, emission were also recorded of a mixture of NATA and N-glycyl-L-tyrosine in 1:3 (mol/mol) ratio which represents the ratio of Trp to Tyr residues in each of the $\beta_2$m-series peptides.

### 2.6 Fluorescence resonance energy transfer (FRET) study

The energy transfer from Trp residue in Fyn- and Lck-series peptides to the DNS chromophore was determined by setting the excitation monochromator at 280 nm (slit width 2 nm) and recording the emission between 300-550 nm (slit width 5 nm) in either a Hitachi F4500 Fluorescence Spectrometer (Hitachi, Tokyo, Japan) or a Fluorolog 3-22 Fluorescence Spectrophotometer (Jobin Yvon, USA). Lipid vesicles were doped with 2 mol% of DNS-PE. To a fixed concentration of the labeled vesicles (50 $\mu$M) taken in 5 mM HEPES buffer, pH 7.4 (for Fyn-series peptides) or 5 mM HEPES buffer, pH 7.4, containing 150 mM NaCl (for Lck-series peptides), an increasing amount of peptide (0.5 $2\mu$M of Fyn-series peptides or 0.5 $5\mu$M of Lck-series peptides) was added and FRET recorded after a 5 minute incubation after each addition. Finally, either 1 mM of unlabeled vesicles (for Fyn-series peptides) or 0.5 mM of unlabeled vesicles (for Lck-series
peptides) were added to the cuvette and FRET recorded after 5 minutes of incubation at room temperature. Appropriate blanks were subtracted from the sample data before plotting the FRET spectra.

2.7 Surface activity measurements

Surface activity of the unacylated and acylated Fyn-series peptides was evaluated by spreading them on air-water interface in a Langmuir-Blodgett Trough (NIMA Technology, Coventry, UK). Peptide monolayers were prepared by spreading increasing amounts of peptides over the surface of 5 mM HEPES buffer, pH 7.4 at room temperature. These monolayers were then symmetrically compressed at a constant speed of 30 cm$^2$/min and pressure-area isotherms recorded in terms of change in surface pressure versus surface area with the help of NIMA software provided with the system. The pressure-area isotherms of a fixed amount of PC spread either over buffer or over the pre-formed monolayers of acylated peptides were also recorded for assessing the interaction of PC with acylated peptides at air-water interface.

2.8 Circular Dichroism spectroscopy

To investigate the secondary structure of peptides, far-UV CD spectra were recorded on a Jasco J-815 spectropolarimeter. The measurements were done in a 0.1 cm path length cell using a step size of 0.2 nm, band width of 1 nm, and scan rate of 100 nm/sec. The spectra were accumulated by averaging 8 scans and corrected by subtracting the solvent or buffer blank. The final spectra were reported as mean residue ellipticity versus wavelength. The mean residue ellipticity ($\theta_{mre}$) was calculated using following formula:

$$\theta_{mre} = \frac{Mr \times \theta_{mdeg}}{100 \times l \times c}$$

Where, $Mr$ is the mean residue weight, $\theta_{mdeg}$ is ellipticity in millidegrees,
\( l \) is pathlength in decimeter, and \( c \) is the peptide concentration in mg/ml.

The secondary structural elements were estimated using CDSSTR, CONTINLL, and Selcon3 deconvolution programs provided in CDPro package (Sreerama and Woody, 2000, 2004) using SMP56, SP43, or SDP48 reference data-sets.

### 2.9 Thioflavin T fluorescence spectroscopy

Thioflavin T (ThT) is a benzothiazole dye which exhibits enhanced fluorescence emission centered at around 482 nm in presence of amyloid fibrils when excited at 450 nm (Naiki et al., 1989). ThT fluorescence assays were carried out with \( \beta_2m \)-series peptides using 10 \( \mu \)M of ThT in aqueous solution buffered at neutral pH with 50 mM phosphate buffer with or without 150 mM NaCl. The spectra were recorded on a Fluorolog-3 Model FL3-22 spectrofluorometer (Horiba Jobin Yvon, Park Avenue Edison, NJ) with excitation at 450 nm and emission between 460-550 nm. The slit widths for excitation and emission were set at 2 nm and 5 nm, respectively.

### 2.10 ANS binding assay

In order to assess the presence of hydrophobic patches or pockets on the surface of aggregates formed by \( \beta_2m \)-series peptides, their ANS (8-anilino-1-naphthalenesulfonic acid) binding ability was monitored in both low and high ionic strength buffers. ANS exhibits enhancement in fluorescence intensity when bound to apolar surfaces and this increase in the intensity is associated with a shift in emission maximum from 545 nm for unbound dye to 470 nm for bound form (Stryer, 1965; Turner and Brand, 1968). Using a F4500 fluorescence spectrophotometer (Hitachi) the fluorescence emission of ANS alone and ANS in presence of peptide was recorded between 420-600 nm for excitation at 350 nm. Slit widths for both excitation and emission were 5 nm. ANS concentration was 25 \( \mu \)M while peptide concentration was 10 \( \mu \)M. Peptides were diluted from respective DMSO stocks to 50 mM phosphate buffer, pH 7.0 either with or without 150 mM NaCl. The spectra
were recorded within 5 minutes of dilution from stock to buffer.

2.11 **Thioflavin T fluorescence microscopy**

To visualize the superstructures formed by ThT-positive aggregates by $\beta_{2m}$-series peptides, ThT fluorescence microscopy was carried out from dilutions prepared in both low and high ionic strength buffers from DMSO stocks of $\beta_{2m}$-series peptides. In case of low ionic strength buffer, a 50 $\mu$M solution of each peptide was incubated at room temperature for 24 hours without shaking and used for preparing the microscopic slides. However, when high ionic strength buffer was used, a detailed concentration-dependent ThT imaging study was carried out for a range of peptide concentration from 1 $\mu$M to 20 $\mu$M and a time window covering from zero to 72 hours. The peptide solutions containing ThT-positive aggregates (as determined by ThT fluorescence spectroscopy) were taken on glass slides and mixed with ThT solution buffered at neutral pH in such a way that final ThT concentration was 10 $\mu$M. The peptide-ThT mix were covered by glass cover-slips which were sealed to prevent evaporation loss and mounted on a Zeiss Axiovert 200 fluorescence microscopy system. The filter set with 450/50 nm excitation and 510/50 nm emission was used for visualization and recording the images. The images were acquired using the Axiovision software and presented after pseudocoloring.

2.12 **Scanning Electron Microscopy (SEM)**

To examine the aggregate morphologies of $\beta_{2m}$-series peptides in dried conditions, SEM imaging was performed. A 20 $\mu$M dilution of each peptide was prepared in 50 mM phosphate buffer, pH 7.0, containing 150 mM NaCl, from respective DMSO stocks. Within 5 minutes of dilution, the peptide solutions were layered over the glass cover slips and allowed to dry at room temperature. Once dried, these cover slips were carefully flushed with deionized water to remove the salt deposits, and again left for drying after soaking the excess water along the edges of the cover slip with tissue paper.
The dried cover-slips were then spur-coated with gold and mounted on the SEM sample holder. The SEM imaging was done with Hitachi SEM at varying magnifications and varying accelerating voltages.

2.13 Atomic force microscopy

The atomic force microscopy of peptide aggregates was carried out to obtain high resolution ultrastructural details of peptide aggregates. The samples were deposited on the freshly cleaved mica surface. Since the peptide aggregates were prepared in high ionic strength buffer, the mica slides were gently flushed with deionized water once the samples were dry to remove the salt crystals. The excess water was soaked along the edge of the slide with a tissue paper. The slides were air-dried and flushed with compressed air before mounting on the AFM system. The images were acquired using tapping mode AFM (Multimode, Digital Instruments, Santa Barbara, CA) with a silicon probe oscillating at 275-310 KHz. The scan speed was optimized for individual samples. Images were processed using Nanoscope (R) III 5.30 rl. The images presented are second order flattened and are in height mode.

2.14 Membrane potential perturbation assay

To assess the ion channel forming ability of the peptides, membrane potential perturbation studies were carried out employing the cyanine dye (Hladky and Rink, 1976). The cyanine dye (di-S-c3-5) is a highly fluorescent dye which partitions between buffer and liposomes in a potential-dependent manner (Sims et al., 1974). In a control experiment, the cyanine dye is added to a dispersion of large unilamellar vesicles containing 150 mM KCl in 5 mM HEPES buffer in 5 mM HEPES buffer containing 150 mM NaCl. The fluorescence of cyanine is followed for few minutes while the intensity stabilizes. Addition of valinomycin, a K⁺-selective neutral ionophore to this system facilitates outward movement of K⁺ down the electrochemical potential gradient thus hyperpolarizing the membrane leading to inward
movement of positively charged cyanine dye into liposomes consequently self-quenching of cyanine fluorescence. Addition of a membrane disrupting agent such as gramicidin allows free movement of ions across the membranes and thus depolarizes the liposome membranes resulting in release of the cyanine dye into buffer and consequent enhanced fluorescence. In an actual experiment, gramicidin is replaced with the peptide under investigation. Membrane potential perturbation assay were performed with each peptide in presence of liposomes of varying compositions by recording the time-dependent fluorescence of cyanine dye on a Fluorolog-3 model FL3-22 spectrofluorometer (Horiba Jobin Yvon, Park Avenue Edison, NJ) with excitation at 622 nm and emission at 670 nm. The slit widths for excitation and emission were set at 2 nm and 5 nm, respectively. The experiment was performed in the same way as control experiment described above except that peptides were used instead of gramicidin in actual experiment.