

γ -Amino Acid Mutated α -Coiled Coils as Mild Thermal Triggers For Liposome Delivery

Sandip V. Jadhav, Sumeet K. Singh, Rahi M. Reja and Hosahudya N. Gopi*

Department of Chemistry, Indian Institute of Science Education and Research, Dr. Homi Bhabha Road, Pune-411021, India. Fax: (+) 91 (20) 2589 9790;

E-mail: hn.gopi@iiserpune.ac.in

Experimental: General Information

All *N*-Fmoc protected amino acids were purchased at their synthesis purity grade from Sven Genetech. MBHA knorr amide resin (substitution value 0.2 mmol/g) was purchased from Novabiochem. Following compounds bought from Sigma-Aldrich: O-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU), dimethyl formamide (DMF), *N*-methyl-2-pyrrolidone (NMP), diisopropylethylamine (DIPEA), piperidine, trifluoroacetic acid (TFA), triisopropyl silane (TIPS), α -phosphatidylcholine (PC), monobasic potassium and dibasic sodium phosphate, sodium chloride, sephadex G-50 and carboxyfluorescein. HPLC grade acetonitrile was purchased from Merck. Water was obtained from Milli-Q water purification system (Millipore). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)-2000] (ammonium salt) i.e. DSPE-PEG(2000)Amine was purchased from Avanti Polar Lipids Inc. A stock solution of 0.1 M phosphate buffer was adjusted to pH 7.4. 10 mM phosphate buffer was prepared as needed by diluting the 0.1 M stock and adjusting to pH 7.4. All biophysical characterizations were performed in phosphate-buffered saline (PBS: 10 mM phosphate, 150 mM NaCl, pH 7.4). Final concentrations of all peptides were prepared from their respective stock solution as needed.

Instrumentation

High Performance Liquid Chromatography (HPLC) was performed on a Waters 600 system. C18 reverse phase column (5 μ m, 10 X 250 mm) from Waters was used for purification of peptides. The gradient applied was from 95% A to 95% B in 60 min, where A was water (0.1% TFA) and B was acetonitrile (0.1% TFA). Pure fractions of peptide were collected monitoring UV-Vis trace at 220 nm. Mass of pure peptides was confirmed by Matrix Assisted LASER Desorption Ionisation (MALDI) TOF/TOF (4800 *Plus* from Applied Biosystems). Fluorescent measurement experiments were carried out in triplicate using

FluoroMax-4 HORIBA fluorimeter, with 490 nm excitation and 500-650 nm emission range using 1/3 slit and 1 nm data interval. JASCO J-815 spectropolarimeter was used for circular dichroism (CD) analysis. Isothermal titration calorimetry (ITC) experiments were performed on iTC₂₀₀ calorimeter from MicroCal at 25 °C. Liposomes were extruded using mini-extruder from Avanti Polar Lipids (USA). Dynamic light scattering (DLS) analysis of liposomes was carried out using *NanoZS90* Zetasizer (Malvern from UK). Scanning electron microscopic (SEM) imaging was performed using ZEISS ULTRA PLUS electron microscope operating at 30 kV.

Chemical synthesis of Fmoc- γ^4 -Ile-OH¹: The suspension of activated Pd/C (20% by weight) and benzyl esters of *N*-Cbz-protected vinylogous isoleucine (1.52 g, 4 mmol), which was synthesized using the reported method,² in 10% acetic acid in THF (20 mL) was stirred overnight at room temperature in the presence of hydrogen. After completion of the reaction, Pd/C was filtered through the bed of celite and the filtrate was evaporated to dryness under vacuum to get free γ^4 -isoleucine (γ^4 -Ile). The pure γ^4 -Ile was isolated as a white powder after trituration with cold diethyl ether in excellent yield (0.508 g, 80%). Further, to the solution of free γ^4 -Ile (0.477 g, 3 mmol) in 20% Na₂CO₃ (10 mL) was added Fmoc-OSu (1.11 g, 3.3 mmol, dissolved in 8 mL of THF) and the reaction mixture was stirred overnight. After completion of the reaction, the reaction mixture was acidified with 10% HCl and the precipitated Fmoc protected γ^4 -Ile was extracted with EtOAc (3 × 25 mL). The combined organic layer was washed with 10% HCl (3 × 15 mL), brine solution (2 × 10 mL), dried over Na₂SO₄ and concentrated under reduced pressure to give gummy Fmoc- γ^4 Ile-OH. The gummy Fmoc- γ^4 Ile-OH was precipitated using ethyl acetate/pet.ether (fraction: 60-80 °C) to give 0.946 g (83%) as a white powder and used directly in the solid phase peptide synthesis without further purification.

Peptide Synthesis: The *N*-acetylated peptides were synthesized on a MBHA Knorr amide resin on a 0.1 mmol scale on a CEM microwave-assisted synthesizer. The synthesis was carried out in NMP by a standard Fmoc protocol using HBTU as coupling reagent. Fmoc deprotection was accomplished by a solution of 20% piperidine in DMF. *N*-Acetylation of the peptides was carried out using acetic acid anhydride/pyridine (1:9). Acidic cleavage from the resin was achieved by treatment of the resin with a mixture of trifluoroacetic acid (TFA)/triisopropylsilane/water (90:5:5, 2 h). The resin was extracted with additional TFA (5 mL),

and the combined extracts were concentrated under vacuum. The crude peptide was then precipitated in cold diethyl ether (30 mL) and isolated by centrifugation and decantation of the ether. The precipitate was redissolved in 5 mL of a 1:1 mixture of acetonitrile/water and then lyophilized to give a fine white solid.

Peptide purification: Peptides were purified by reversed-phase HPLC using a Waters C18 column (5 μm , 10 X 250 mm). The gradient applied was from 95% A to 95% B in 60 min; where A was water (0.1% TFA) and B was acetonitrile (0.1% TFA) at a flow rate of 2 mL/min. Pure fractions of peptide were collected monitoring UV-Vis trace at 220 nm.

Peptide characterization: The peptides were characterized by mass spectrometry on an Applied Biosystems 4800 *Plus* MALDI-TOF/TOF instrument (matrix: α -cyano-4 hydroxycinnamic acid (CHCA), external calibration). Purity of peptides was also tested by HPLC trace.

Circular Dichroism (CD) Spectroscopy: CD spectra and thermal-denaturation curves were recorded using a JASCO J-815 spectropolarimeter fitted with a Peltier temperature controller. CD spectra were measured at 30 μM total peptide concentration (15 μM each) in PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) at 20 $^{\circ}\text{C}$ in 2 mm quartz cuvettes at 50 nm/min scanning speed. Thermal-denaturation experiments were performed by heating from 20 to 90 $^{\circ}\text{C}$ at a rate of 1 $^{\circ}\text{C}/\text{min}$. The CD signal at 222 nm was recorded at 5 $^{\circ}\text{C}$ intervals. Each experiment was performed in triplicate.

Isothermal titration calorimetry (ITC): ITC experiments were performed on iTC200 calorimeter from MicroCal. Following conditions are applied for experiment: auto baseline equilibration at 25 $^{\circ}\text{C}$ temperature, 1000 rpm stirring speed, 60 s initial delay, 18 injections of 2 μL spaced by 120 s, 5 s filter period. Both the cell and syringe were washed and dried thoroughly at the beginning of experiment. All peptides solutions were prepared in PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) buffer. Concentration of peptide in cell (P3) and syringe (P2 or P4) were fixed at 30 μM and 600 μM , respectively. Final concentration of peptides was prepared by dilution of their respective stock solutions. The centrifuged peptide solutions stored at 20 $^{\circ}\text{C}$ and degassed for 5 min prior to the experiments. Results were reproduced thrice for each experiment. MicroCal Origin software was used to analyse the data. Sigmoidal curve fitting was performed with “one binding site” model.

Thermodynamic parameter measurements from CD-thermal denaturation analysis:

Thermodynamic parameters were determined by nonlinear least square fitting of the normalized CD melting curves to five parameters (a , $[\theta]_M(0)$, $[\theta]_D(0)$, ΔH_m , and T_m). Ellipticity was normalized to fraction monomer using the equation (1).

$$\theta = (\theta_M - \theta_D)f_M + \theta_D \quad (1)$$

where θ_M and θ_D represent the ellipticity values for the fully unfolded monomer and fully folded dimer species respectively at each temperature. θ_M was found to be constant at the temperatures higher than the melting region for all the peptides studied. θ_D was approximated by a linear function of temperature $\theta_D = \theta_D[0] + aT$. The fraction monomer (f_M) was expressed in terms of the equilibrium constant after solving the equation for a bimolecular reaction $2M \rightarrow D$:

$$P_M = [(8KC + 1)^{1/2} - 1]/4KC \quad (2)$$

where K is the equilibrium constant and C is the total peptide concentration. K was assumed to be temperature dependent according to the equation (3):

$$K = e^{-\Delta G/RT} \quad (3)$$

The Gibbs–Helmholtz equation can be used to express the temperature dependence of ΔG in terms of ΔH_m and T_m as given by Equation (4):

$$\Delta G = \Delta H_m \cdot (1 - T/T_m) + \Delta C_p \cdot (T - T_m - T \cdot \ln(T/T_m)) \quad (4)$$

where ΔH_m is the enthalpy change at the melting temperature T_m , that is defined as the temperature at which $P_m = 0.5$. ΔC_p is the change in heat capacity that was initially assumed to be zero for the purpose of fitting because due to the high interdependence of ΔH_m and ΔC_p these parameters cannot be fitted simultaneously. Equations (1) through (4) were combined and the data fitted directly. ΔC_p was calculated afterwards from the dependence of ΔH_m from T_m and the standard free energy of unfolding ΔG_o (1 M standard state) was then calculated at $T_o = 37$ °C according to Equation (5):

$$\Delta G_o = \Delta H_m \cdot (1 - T_o/T_m) + \Delta C_p \cdot \{T_o - T_m - T_o \cdot \ln(T_o/T_m)\} - RT_o \ln(C) \quad (5)$$

Peptide-liposome hybrid vesicle preparation: Egg yolk L- α -phosphatidylcholine (EYPC, 22.6 mg, 29.4 μ mol) and 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Amino(Polyethylene Glycol)2000](DSPE-PEG2000, 0.17 mg, 0.6 μ mol) were dissolved in 1 mL DCM. The 200 μ L solution of P3/P4 (1:1, 3 μ mol) was prepared by diluting their stock solution in MeOH. Both the organic solutions (DCM and MeOH) were mixed together in small round bottom flask. Thin and uniform lipid-peptide bilayer was formed by evaporation of organic solvent on rota-evaporator at 30 °C with 50 rpm rotations. Last traces of organic solvent were completely removed by applying high vacuum to the bilayer for 2 hrs. Bilayer

film was hydrated with 1 mL solution of 0.1 mM carboxyfluorescein in PBS buffer (10 mM phosphate, 150 mM NaCl, and pH 7.4). Hydration process was carried out for 1 hour with continuous agitation at room temperature and occasional sonication (total time 120 s). Size exclusion chromatographic separation was carried out using Sephadex G-50 column to remove extra-vesicular carboxyfluorescein (Eluent: 10 mM PBS, 150 mM NaCl at pH 7.4). Liposome containing aliquots were collected and small unilamellar liposomes were obtained after 20 times extrusion through 100 nm polycarbonate membrane using mini-extruder (Avanti Polar Lipids, Alabaster, AL). Size and morphology of liposomes were confirmed by using Zetasizer *NanoZS90* and FE-SEM respectively. Liposomes were stored at 8 °C for further use.

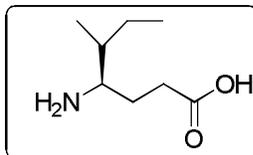
Size distribution analysis of hybrid nanoparticles by DLS: Mean diameter of liposomes was measured by dynamic light scattering (DLS) experiment using 90° scattering angle. Samples were prepared by diluting 50 µL liposome solution to 1mL with PBS buffer at pH 7.4.

Field Emission -Scanning Electron Microscopy (FE-SEM): Morphology analysis of liposomes: 10 µL of liposome solution was dropcasted on silicon vapor. Samples were allowed to dry at room temperature and then coated with gold. Scanning electron microscopic imagings were performed using ZEISS ULTRA PLUS electron microscope operating at 30 kV.

Procedure for fluorescent leakage study: Lipid-peptide hybrid liposome (500 µL) loaded with carboxyfluorescein was sealed in dialysis membrane having molecular weight cut off 500 Daltons. This dialysis bag was suspended in agitating PBS buffer (3 mL, 10 mM phosphate, 150 mM NaCl, and pH 7.4) at the 25 °C and 40 °C, separately. 100 µL aliquot of suspension medium was timely collected and quantification of released carboxyfluorescein was carried out by fluorimeter. All experiments were repeated in triplicates.

Fluorescence measurement: Fluorescent measurement experiments were carried out using FluoroMax-4 HORIBA fluorimeter, with 490 nm excitation and 500-650 nm emission range using 1/3 slit and 1 nm data interval. 100 µL aliquots obtained from leakage assay were diluted to 500 µL with PBS buffer (pH 7.4). 10 mm quartz cuvette (from Hellma) was used for fluorescence measurement. Emission at 514 nm was monitored.

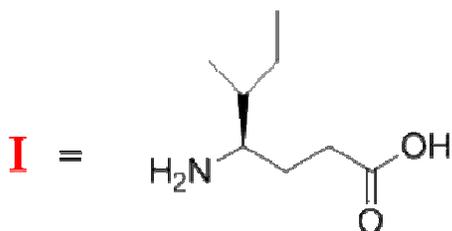
Characterization of H- γ^4 Ile-OH :



(*4R*, *5R*)-4-amino-5-methylheptanoic acid : white solid, mp 154 °C; $[\alpha]_D^{25} = + 1.0$ (c = 1, H₂O); ¹H NMR (400 MHz, D₂O) δ 3.15 (m, 1H), 2.26 (m, 2H), 1.85 (m, 1H), 1.69 (m, 2H), 1.39-1.15 (m, 2H), 0.90 (m, 6H) ; ¹³C NMR (100 MHz, D₂O) δ 181.71, 55.95, 36.53, 33.90, 24.94, 24.46, 13.35, 10.79; MALDI TOF/TOF- *m/z* calcd. for C₈H₁₇NO₂ [M+Na]⁺ 182.1157, obsrvd. 182.0959.

Peptide Sequences:

- P1 Ac-LKEIEDK LEEIESK LYEIE NE LAEIEKL-NH₂
P2 Ac-LKKIKDK LEKIKSK LYKIKNE LAKIKKL-NH₂
P3 Ac-LKEIKDK LEEIESK LYEIE NE LAEIEKL-NH₂
P4 Ac-LKKIKDK LEKIKSK LYKIKNE LAKIKKL-NH₂
P5 Ac-LKEIKDK LEEIESK LYEIE NE LAEIEKL-NH₂
P6 Ac-LKEIKDK LEEIESK LYEIE NE LAEIEKL-NH₂
P7 Ac-LKEIKDK LEEIESK LYEIE NE LAEIEKL-NH₂



References

- 1 S. V. Jadhav, A. Bandyopadhyay and H. N. Gopi, *Org. Biomol. Chem.*, 2013, **11**, 509.
- 2 S. M. Mali, A. Bandyopadhyay, S. V. Jadhav, M. G. Kumar and H. N. Gopi, *Org. Biomol. Chem.*, 2011, **9**, 6566.

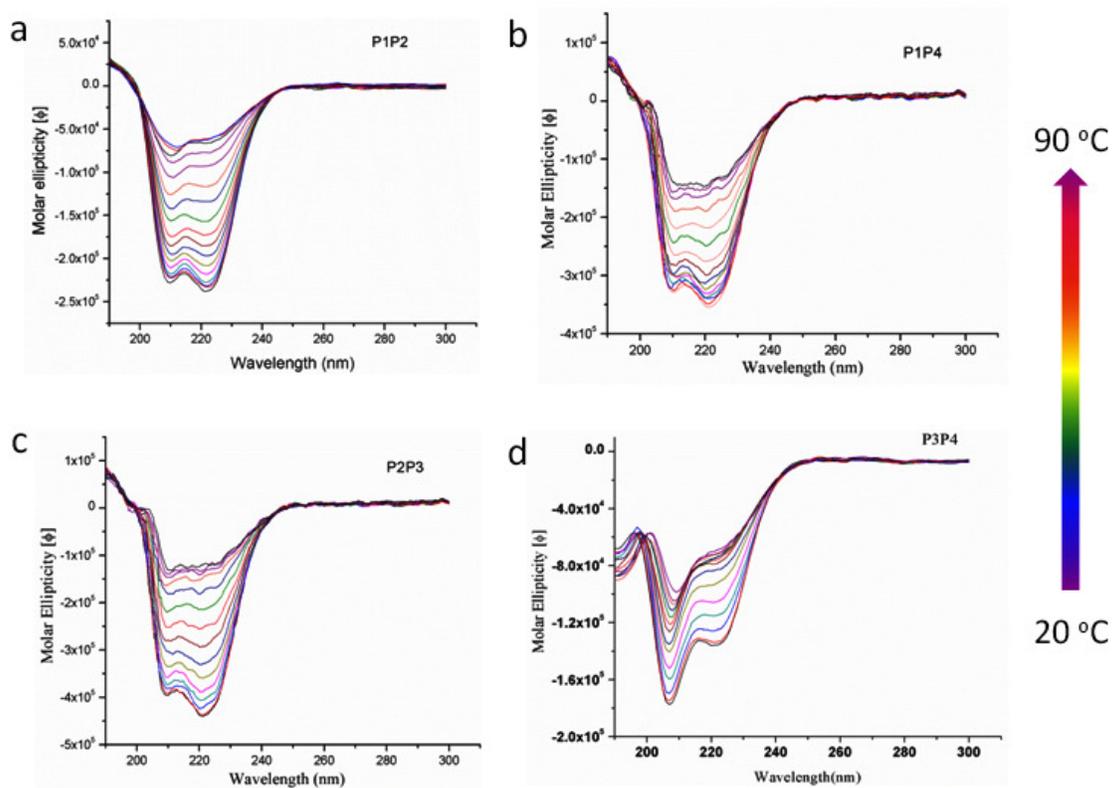


Fig. S1 CD analysis depicting temperature-dependent conformational changes of coiled coil peptide hybrids. Concentration of each peptide is 15 μ M (10 mM PBS, 150 mM NaCl) at 7.4 pH

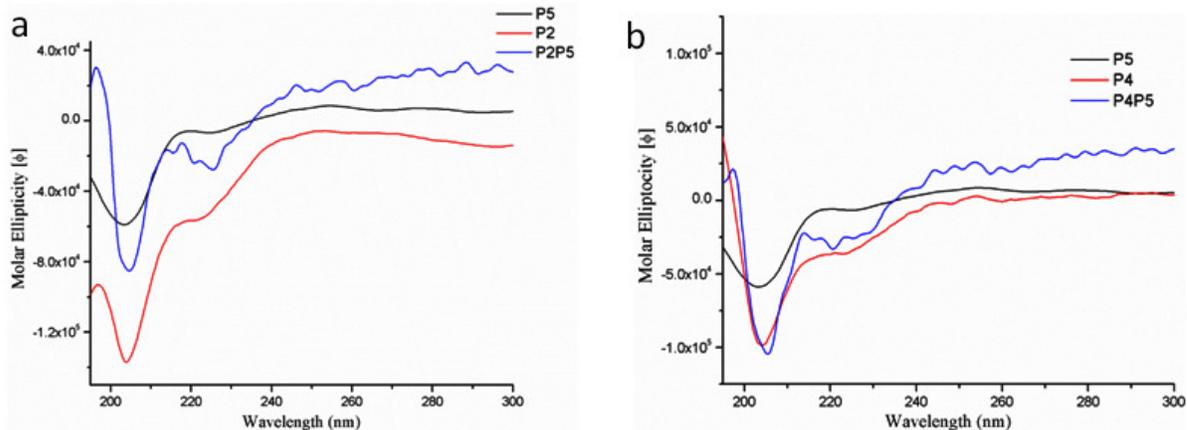


Fig. S2 CD analysis suggest that **P5** with two mutation sites at hydrophobic stripe is reluctant to form coiled coil structures with a) **P2** and b) **P4**. Concentration of each peptide is 15 μ M (10 mM PBS, 150 mM NaCl) at 7.4 pH and 20 °C.

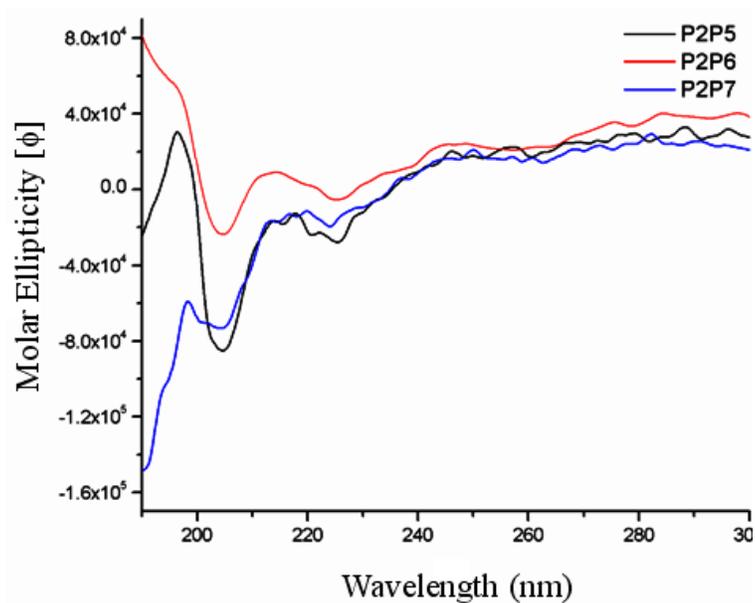


Fig. S3 CD analysis depicting that peptides **P5**, **P6** and **P7** are unable to form coiled coil structures with complementary peptide **P2**. Concentration of each peptide is 15 μ M (10 mM PBS, 150 mM NaCl) at 7.4 pH and 20 °C.

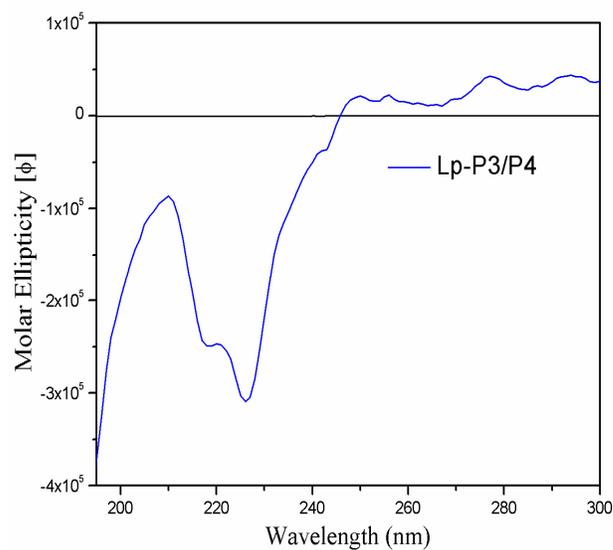


Fig S4. CD Spectrum of carboxyfluorescein loaded P3/P4 liposome composite at 20 °C.

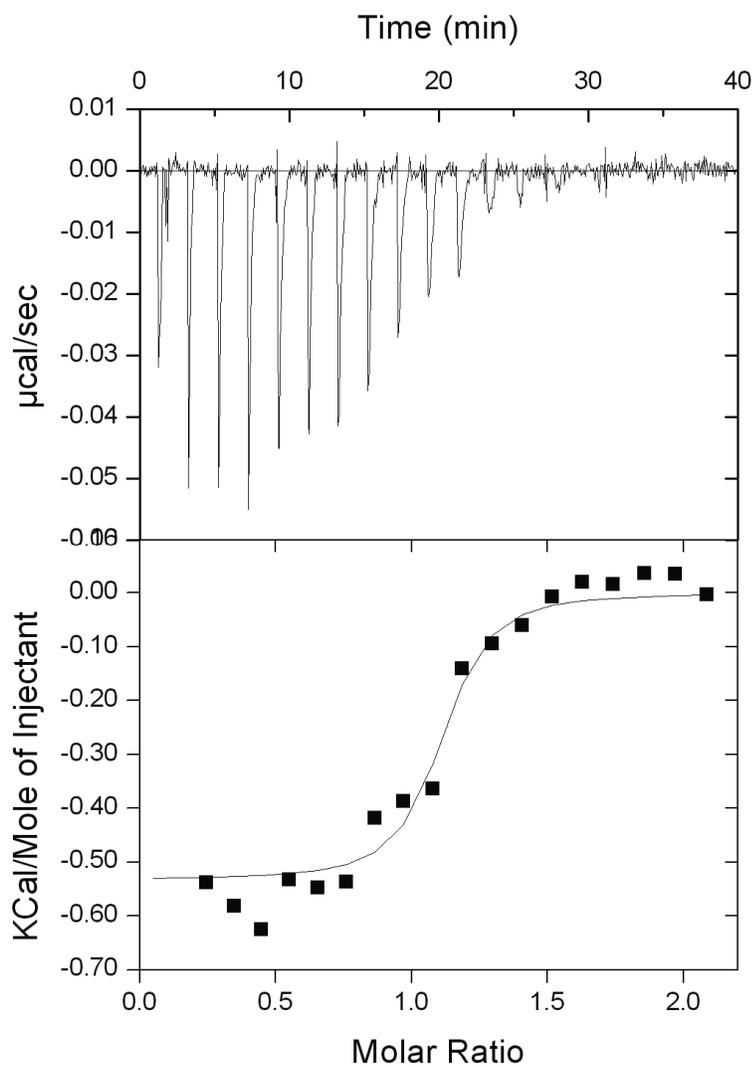


Fig. S5 Binding isotherm obtained from isothermal titration calorimetry (ITC) study for coiled coil formation between **P2** and **P3** at 25 °C suggests that binding event is exothermic and stoichiometry of complex formation is one (heterodimer). Results are reproduced thrice by repeating the experiment.

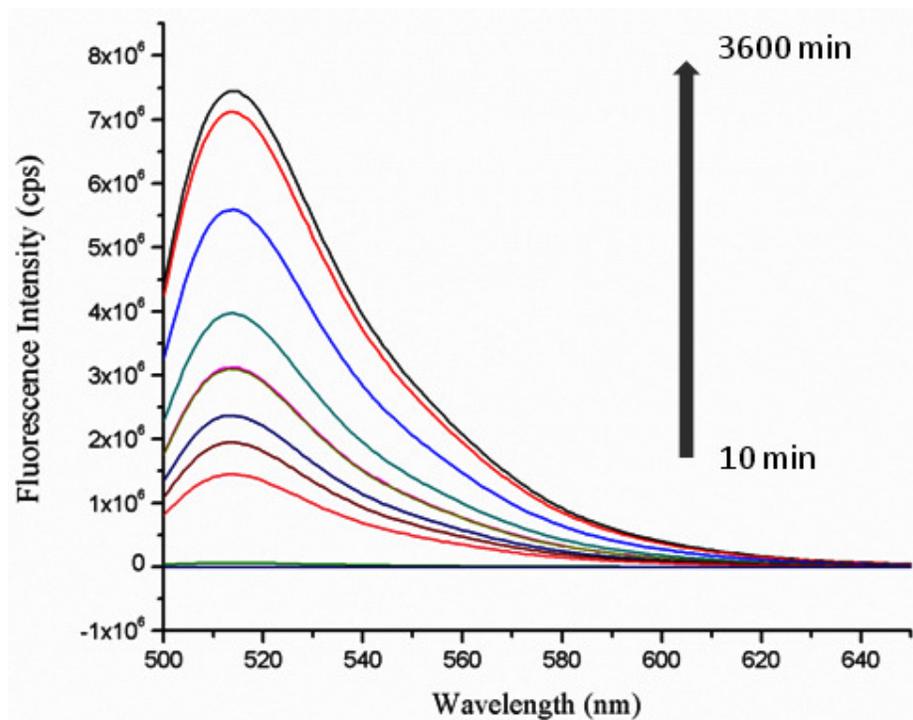


Fig. S6 Fluorescence emission spectra at 514 nm indicates controlled release of carboxyfluorescein from **P3/P4** liposome composite at 40 °C.

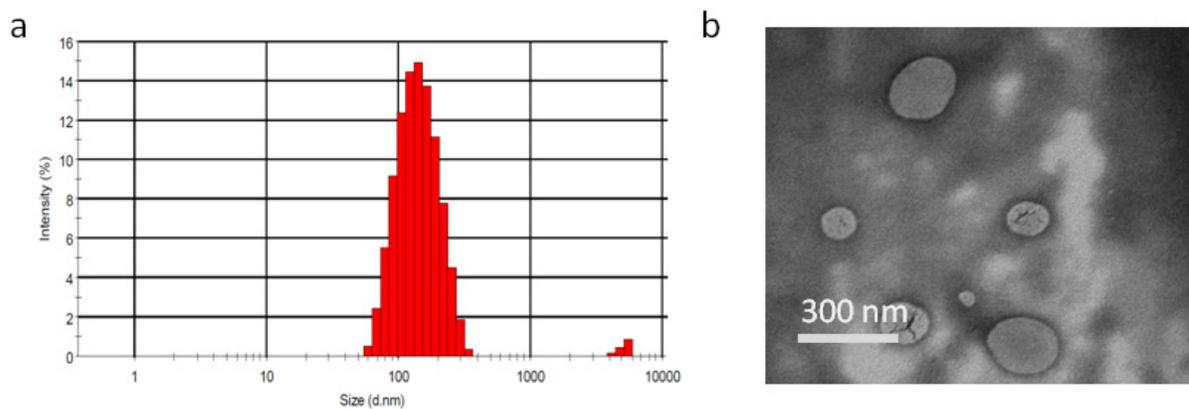
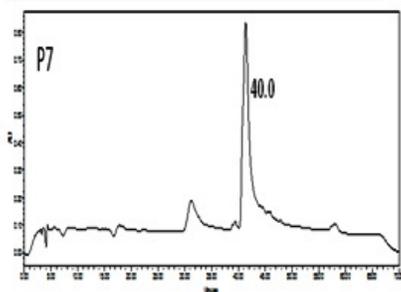
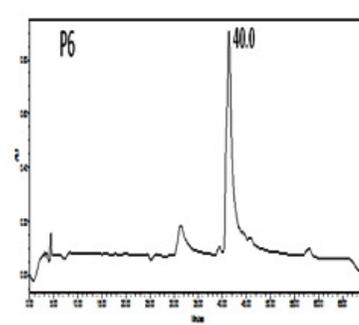
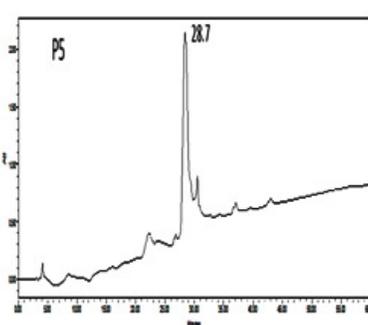
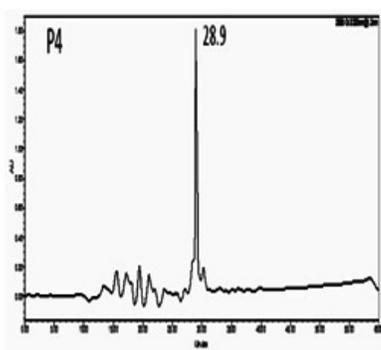
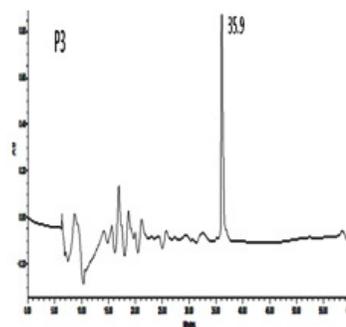
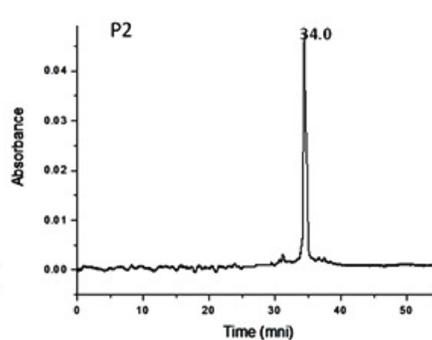
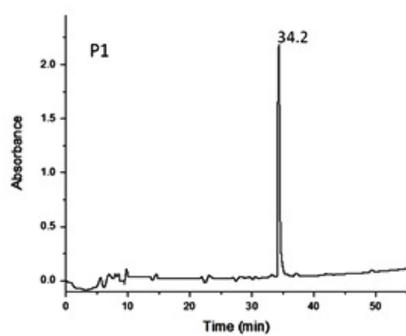
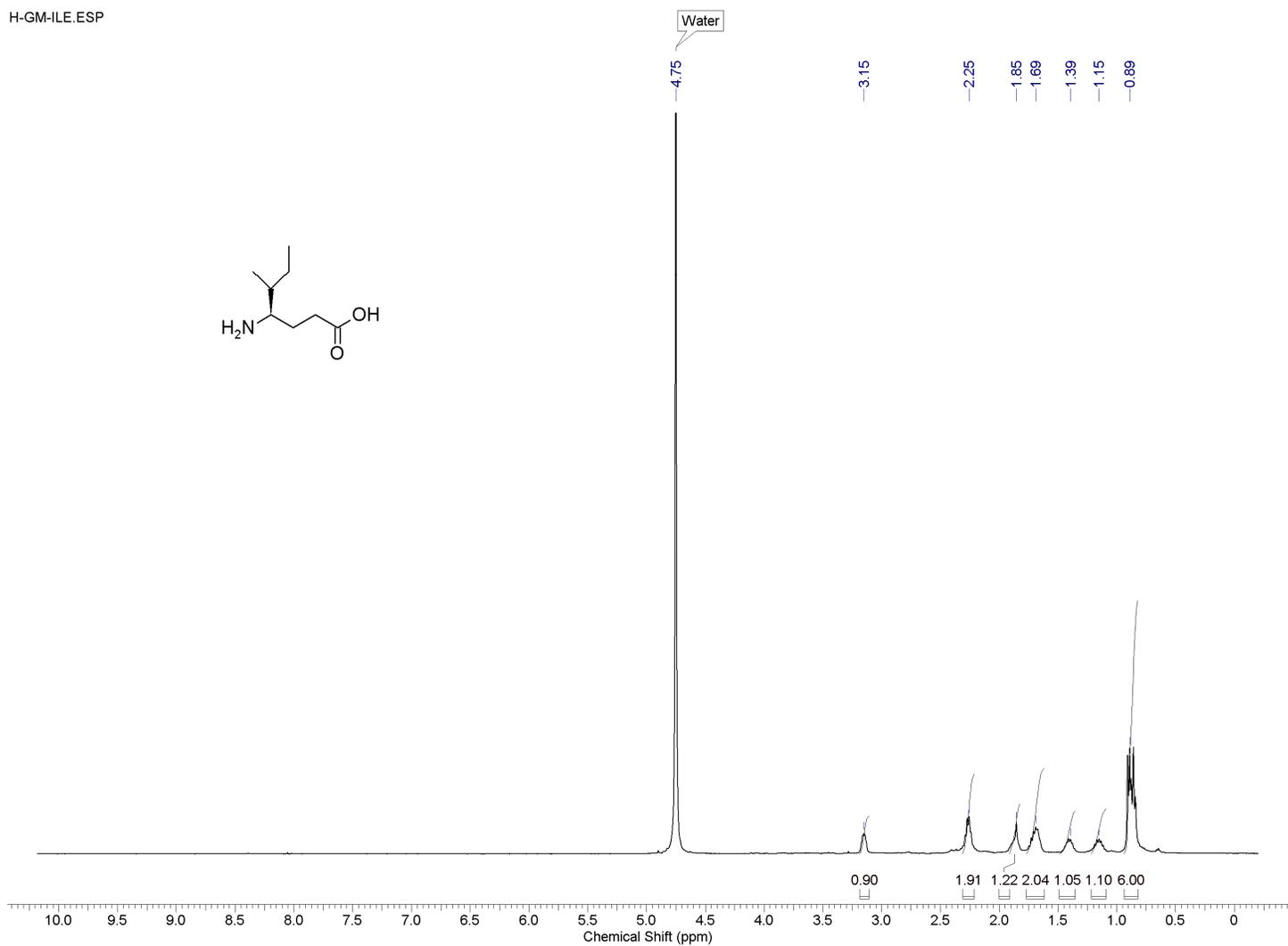
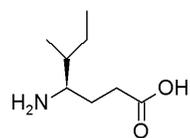


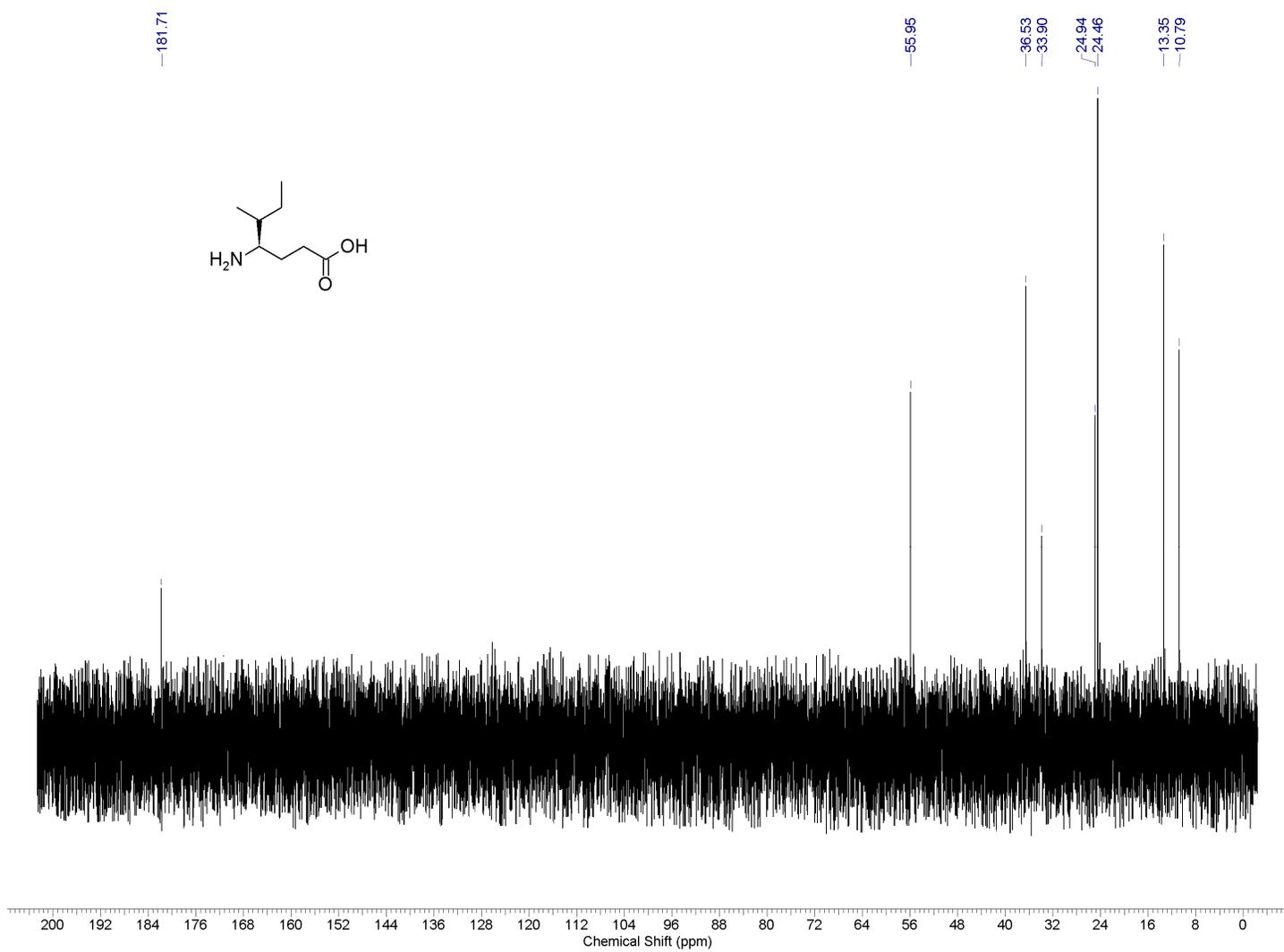
Fig. S7 a) DLS and b) FE-SEM analysis for **P1/P2** liposome composite.

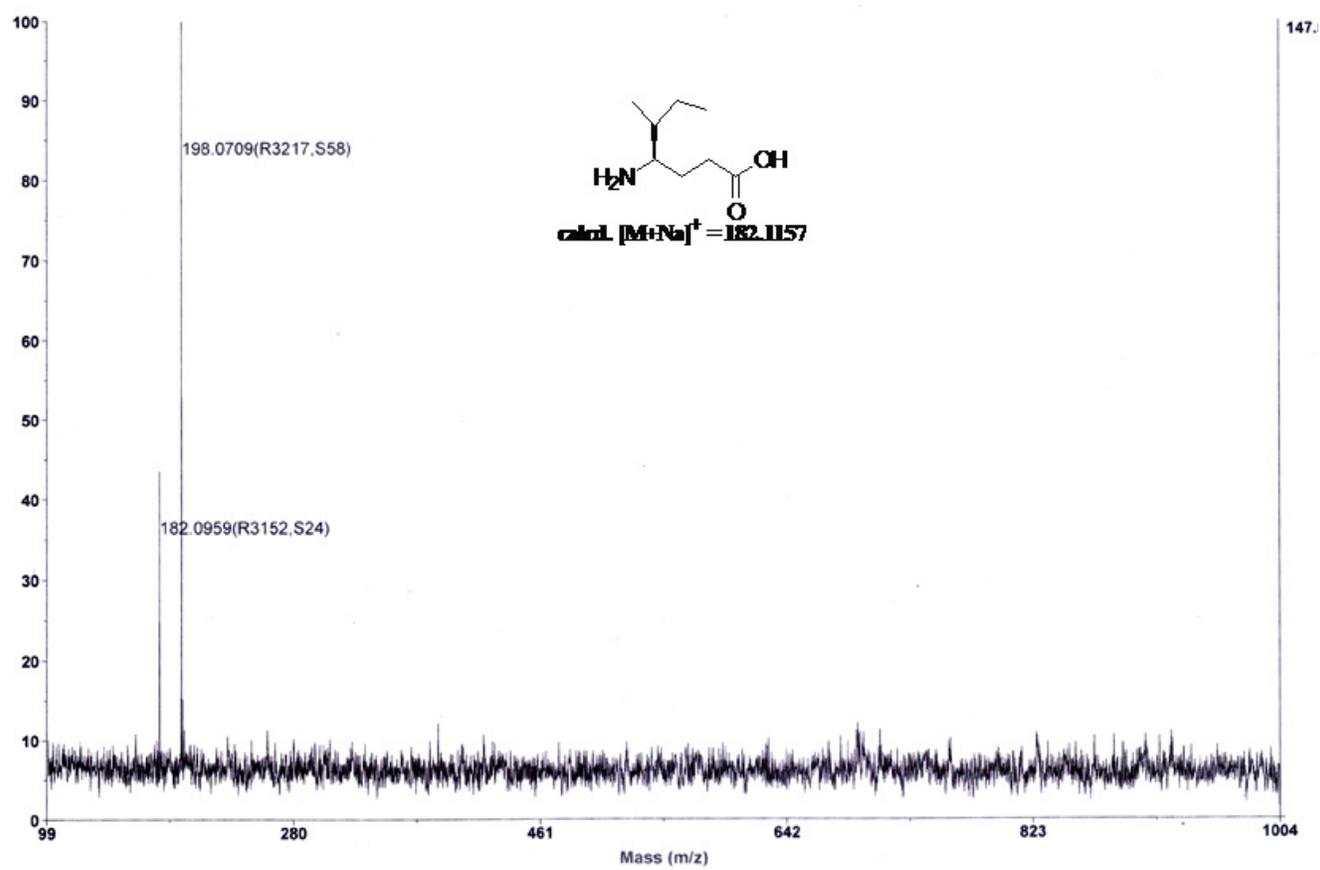
HPLC traces for peptides

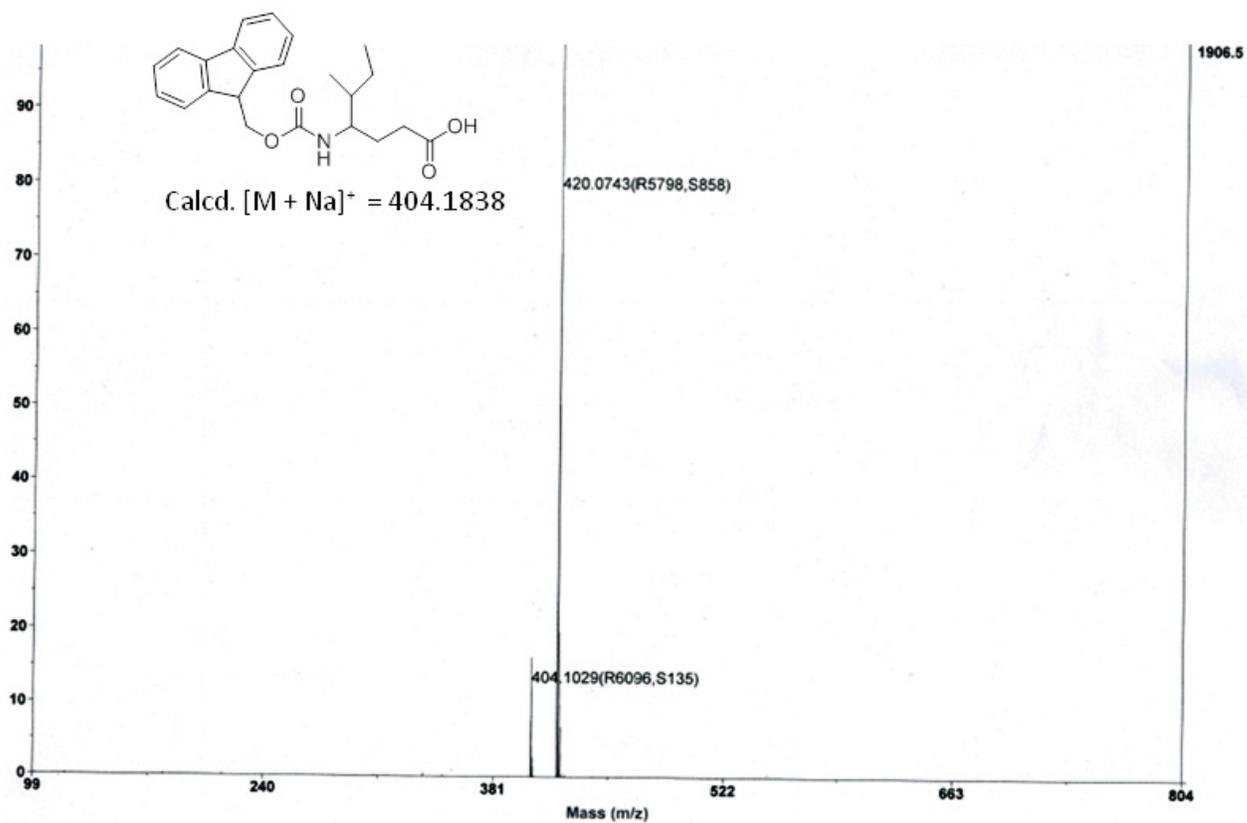


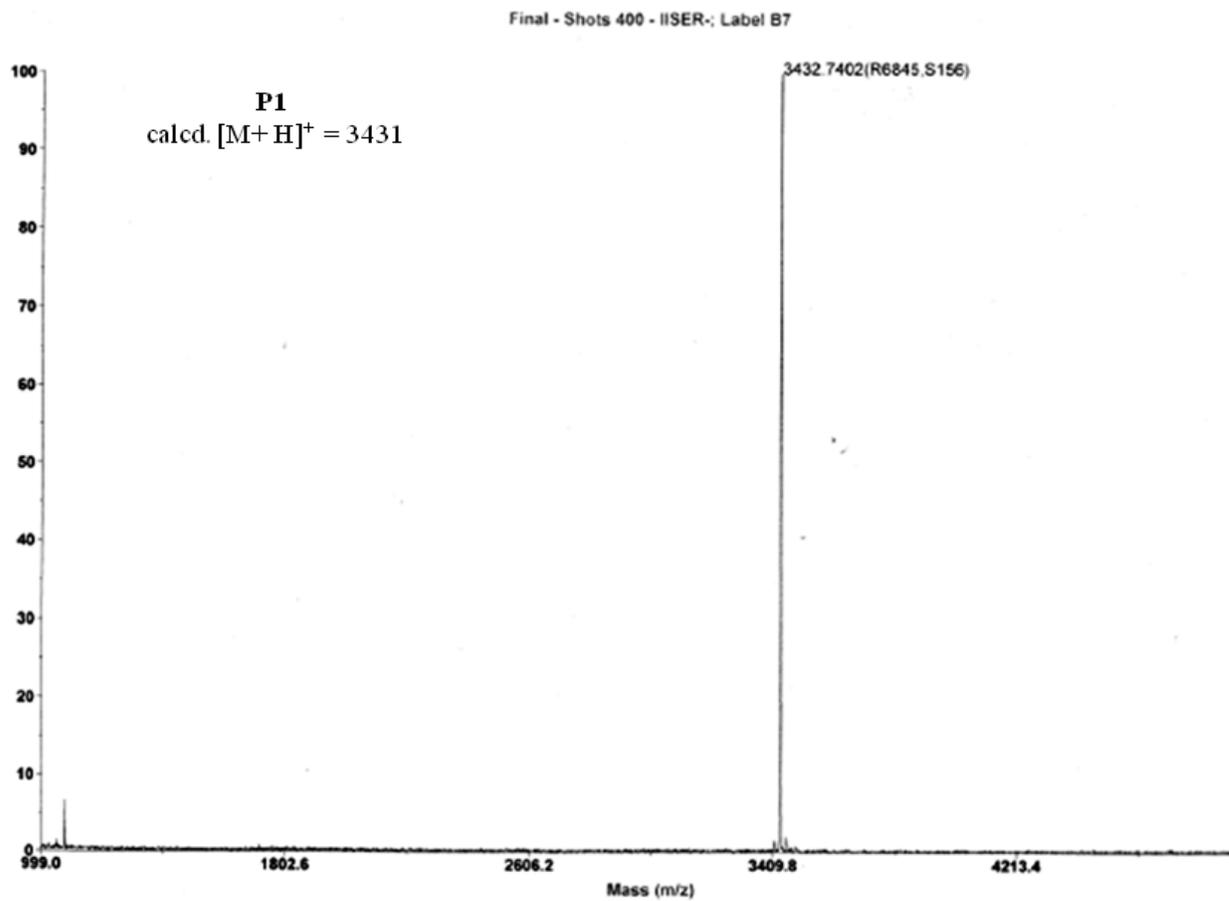
H-GM-ILE.ESP



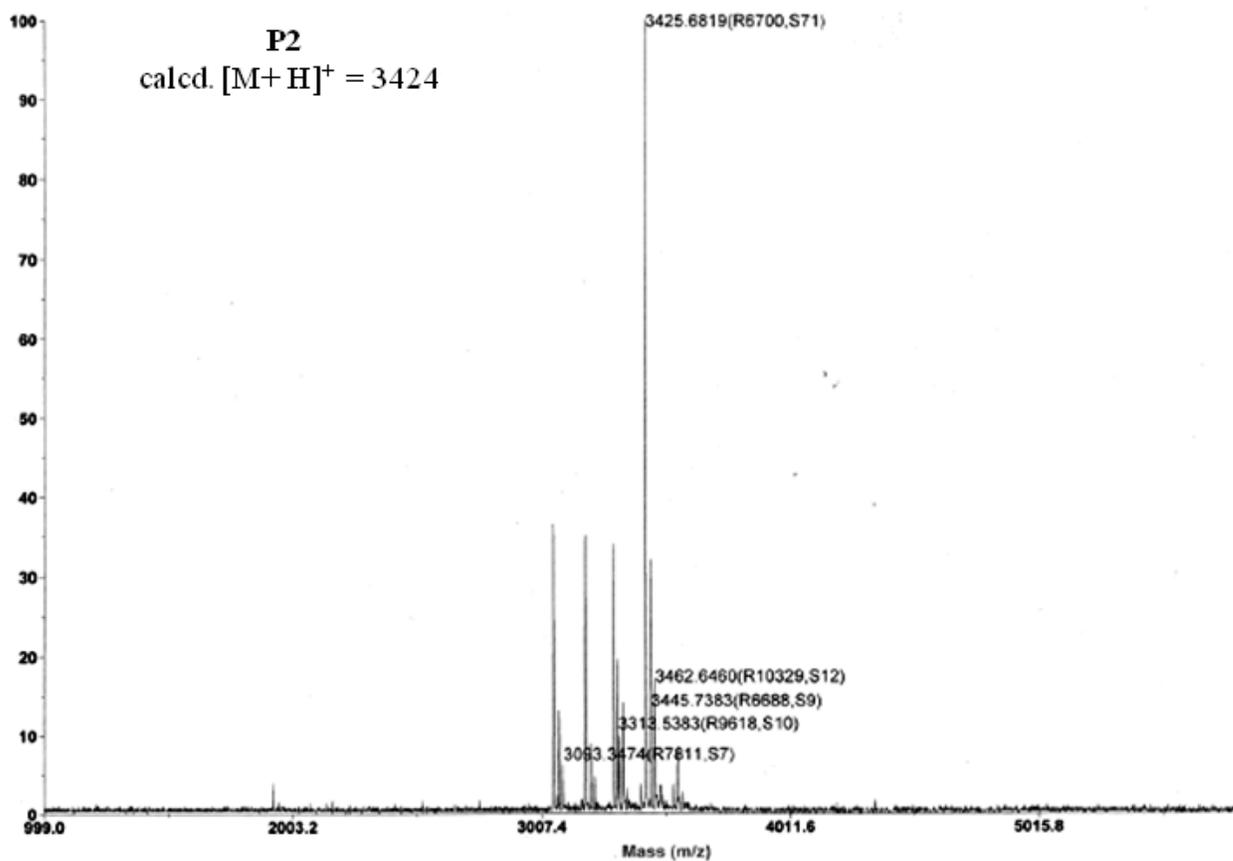




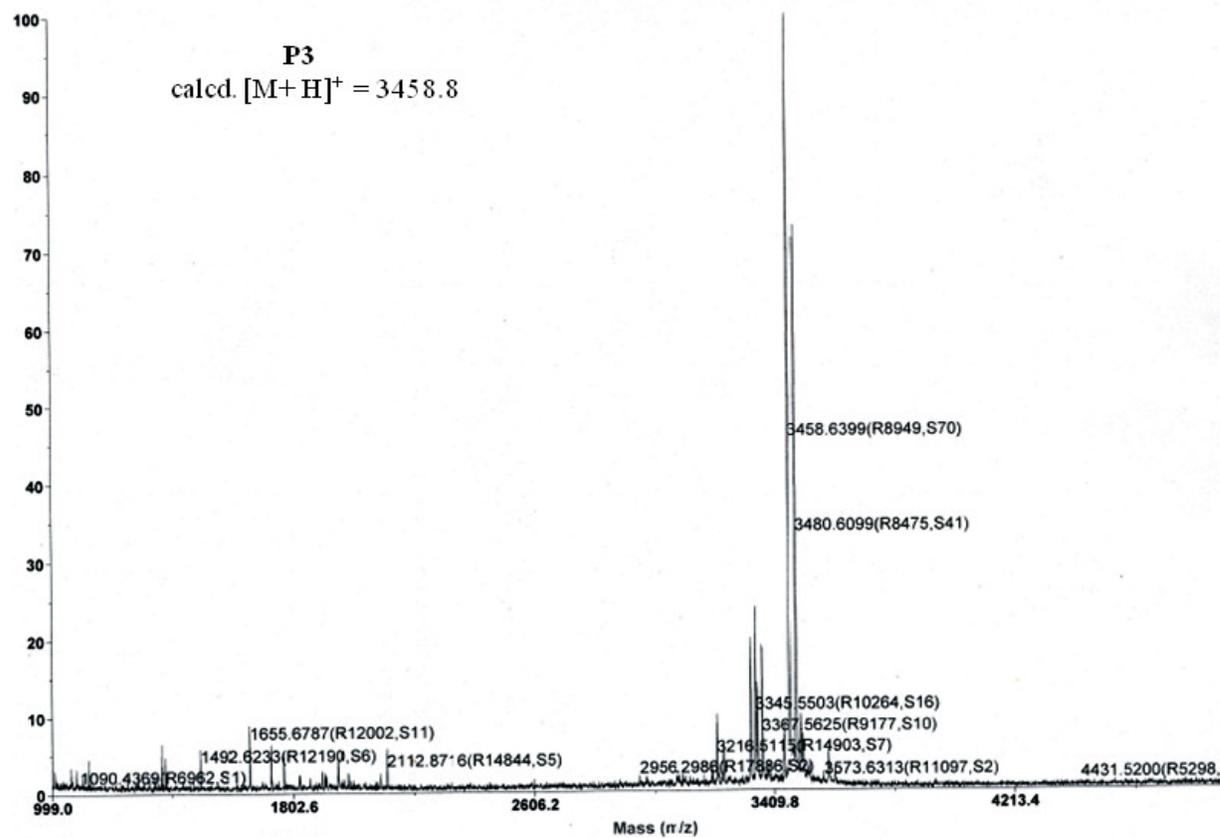




Final - Shots 400 - IISER; Run #1013; Label O19



Final - Shots 1000 - HNG GROUP; Label D10



Final - Shots 400 - HNG GROUP; Run #456; Label O19

