Supplementary Information

Concentration Dependent Fabrication of Short Peptide Based Different Self-assembled Nanostructures with Various Morphologies and Intracellular Delivery Property

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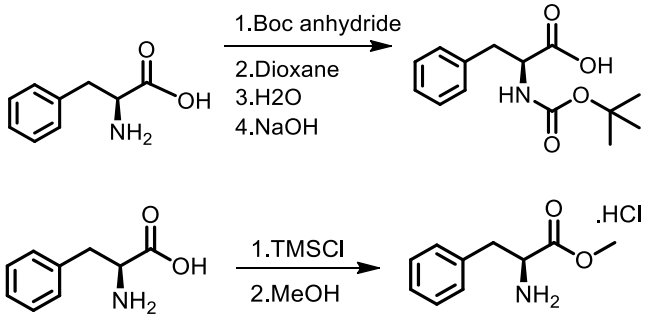
Experimental Section

Peptide synthesis: Peptides were synthesized by conventional solution-phase methods. Peptide coupling was mediated by dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBt). The products were purified by column chromatography using silica gel (100–200 mesh) as the stationary phase and an n-hexane–ethyl acetate mixture as an eluent. The final compounds were fully characterized by Bruker 500 MHz ^{1}H-NMR spectroscopy, and mass spectroscopy (Shimadzu, Japan, LCMS-2020 Spectrometer).

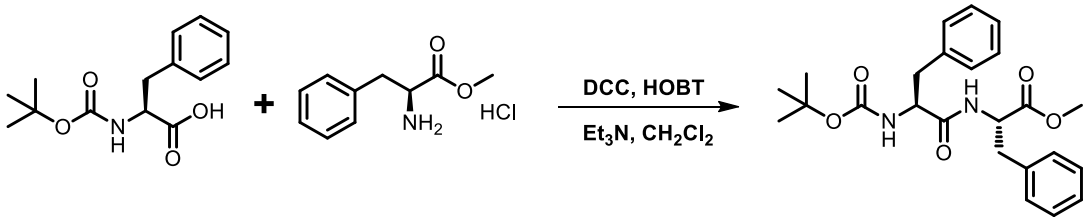


Scheme S1: Synthetic methodologies adopted for the synthesis of BOC-protected phenylalanine and NH_2-Phe-OMe Hydrochloride

Synthesis of BOC-NH-Phe-OH: A solution of L-phenylalanine (3 g, 18 mmol) in a mixture of dioxane (45 mL), water (25 mL), and 1 M NaOH (18 mL) was stirred and cooled in an ice-water bath. 4.35 g of di-tert-butyl dicarbonate was added and stirred continuously at room temperature (RT) for 6 hours. Then, the solution was concentrated using rotary evaporator to

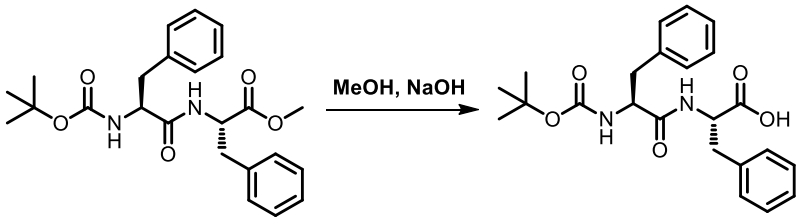
about 10–15 mL, cooled in an ice-water bath, covered with a layer of ethyl acetate (about 50 mL), and acidified with a dilute solution of $KHSO_4$ to pH 2–3 (determined by congo red). The aqueous phase was extracted with ethyl acetate and this operation was performed repeatedly. The ethyl acetate extracts were pooled, washed with water, dried over anhydrous Na_2SO_4, and evaporated under vacuum. The pure material was obtained as a waxy solid. Yield: 4.63 g (17 mmol, 96%) (Scheme S1)

Synthesis of NH_2-Phe-OMe Hydrochloride: L-Phenylalanine 4g (24.21 mmol) was taken in a round bottom flask and dissolved in 70 mL MeOH. Then, 6.16 mL (67.76 mmol) of Trimethyl chlorosilane (TMSCl) was added to the resulting solution slowly in a drop wise manner and stirred for 8 hours at room temperature. After the completion of reaction (as monitored by TLC), the excess solvent was evaporated on a rotary evaporator to get the solid desired product L-Phenylalanine methyl ester hydrochloride. Yield: 4.15 g (23 mmol, 81.6%) (Scheme S1).



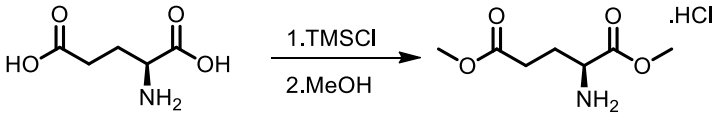
Scheme S2: Synthetic methodologies adopted for the synthesis of BOC-Phe-Phe-OMe.

Synthesis of BOC-Phe-Phe-OMe: 4.63 g (17.46 mmol) of Boc-Phe-OH were dissolved in 40 mL dry DCM in an ice-water bath. NH_2-Phe-OMe.HCl 3.71 g (20.95mmol) and Et_3N 2 ml, 15 mmol) were then added to the reaction mixture, followed immediately by the addition of 4.32 g (20.93 mmol) dicyclohexylcarbodiimide (DCC) and 3.2 g (20.89 mmol) of HOBt. The reaction mixture was allowed to warm-up to RT and stirred for 48 hours. DCM was evaporated and the residue was dissolved in ethyl acetate (45 mL). The dicyclohexylurea (DCU) was filtered off. The organic layer was washed with 2M HCl (3 X 50 mL), brine (2 X 50 mL) followed by 1 M sodium carbonate (3 X 50 mL) and brine (2 X 50 mL), and finally dried over anhydrous sodium sulfate. It was then evaporated under vacuum to yield Boc-Phe-Phe-OMe as a white solid. The product was purified by silica gel (100–200 mesh) using n-hexane–ethyl acetate (3:1) as eluent. Yield: 6.06 g (14.2083mmol, 81%) (Scheme S2).



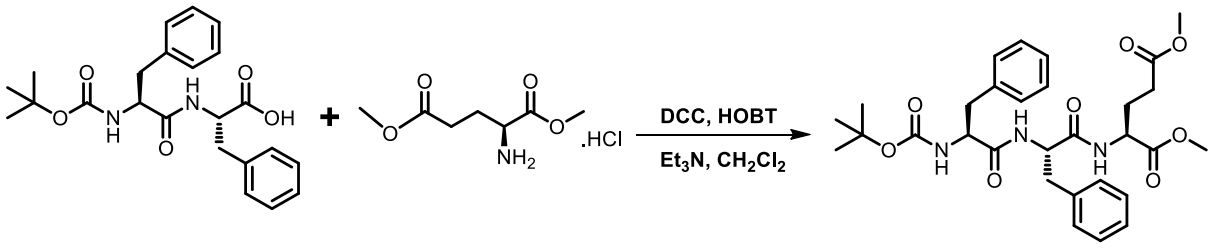
Scheme S3: Synthetic methodologies adopted for the synthesis of BOC-Phe-Phe-OH.

Synthesis of BOC-NH-Phe-Phe-OH: To 6.06 g (14.22 mmol) of Boc-Phe-Phe-OMe, 40 mL MeOH and 2M 23 mL NaOH were added and the progress of saponification was monitored by thin layer chromatography (TLC). The reaction mixture was stirred. After 10 hours, the methanol was removed under vacuum; the remaining residue was dissolved in 50 mL of water, and washed with diethyl ether (2 X 50 mL). Then, the pH of the aqueous layer was adjusted to 2 using 1M HCl and extracted with ethyl acetate (3 X 50 mL). The extracts were pooled, dried over anhydrous sodium sulfate, and evaporated under vacuum to obtain the compound as a waxy solid. Yield: 5.80 g (14 mmol, 98%). (Scheme S3).



Scheme S4: Synthetic methodologies adopted for the synthesis of NH_2-Glu-$(OMe)_2$ Hydrochloride.

Synthesis of NH_2-Glu-$(OMe)_2$ Hydrochloride: L-glutamic acid 4.8 g (27.18 mmol) was taken in a round bottom flask and dissolved in 70 mL MeOH. Then, 13 mL (67.76 mmol) of Trimethyl chlorosilane (TMSCl) was added to the resulting solution slowly in a drop wise manner and stirred for 8 hours at room temperature. After the completion of reaction (as monitored by TLC), the excess solvent was evaporated on a rotary evaporator to get the solid desired product L-glutamic acid methyl ester hydrochloride. Yield: 6.37 g (25 mmol, 93%) (Scheme S4).



Scheme S5: Synthetic methodologies adopted for the synthesis of **BOC-Phe-Phe-Glu-$(OMe)_2$**.

Synthesis of BOC-Phe-Phe-Glu-(OMe)$_2$: 3.8 g (9.21 mmol) of Boc-Phe-Phe-OH were dissolved in 40 mL dry DCM in an ice-water bath. NH_2-Glu-(OMe)$_2$.HCl 2.7 g (11.05 mmol) and Et_3N (24 ml) were then added to the reaction mixture, followed immediately by the addition of 2.28 g (11.05 mmol) dicyclohexylcarbodiimide (DCC) and 1.69 g (11.03 mmol) of HOBt. The reaction mixture was allowed to warm-up to RT and was stirred for 48 hours. DCM was evaporated and the residue was dissolved in ethyl acetate (45 mL). The dicyclohexylurea (DCU) was filtered off. The organic layer was washed with 2M HCl (3 X 50 mL), brine (2 X 50 mL), 1 M sodium carbonate (3 X 50 mL), and brine (2 X 50 mL), and finally dried over anhydrous sodium sulfate. It was then evaporated using vacuum to yield Boc-Phe-Phe-Glu-(OMe)$_2$ as a white solid. Then it was purified by column chromatography. Yield 3.19 g (5.6008 mmol, 60%) (Scheme S5). Elemental analysis calcd (%): ($C_{30}H_{39}N_3O_8$): C 63.25, H 6.90, N 7.38, O 22.47; found: C 62.89, H 6.73, N 7.44. (^{1}H NMR, $CDCl_3$, 500 MHz, δ_{ppm}): 7.25-7.10 (m, 8H, ArH of Phe), 6.99 (d, J= 5.5 Hz, 2H, ArH of Phe) 6.61 (broad peak, 1H, NH), 6.41 (broad peak, 1H, NH), 4.83 (broad peak, 1H, NH), 4.57 (d, J= 6.5 Hz, 1H, Phe CαH), 4.44-4.40 (m, 1H, CαH, Phe) 4.24 (d, J= 4.5 Hz, 1H, Glu CαH), 3.64 (s, 3H, OMe), 3.58 (s, 3H, OMe), 3.06- 2.83(m, 4H, CβH, Phe), 2.28-2.14 (m, 2H, Glu), 1.86-1.79 (m, 2H, Glu) 1.26 (s, 9H, Boc). ^{13}C NMR (125 MHz, $CDCl_3$, δ_{ppm}): 173.11, 171.46, 171.04, 170.36, 136.09, 129.34, 129.29, 128.80, 128.70, 127.16, 127.10, 80.51, 55.89, 54.06, 52.50, 51.82, 51.77, 37.71, 37.69, 29.87, 28.19, 27.03. FTIR (KBr, Nujol $\nu_{max,}$ cm^{-1}): = 697, 745, 851, 1030, 1051, 1162, 1250, 1367, 1457, 1516, 1649, 1723, and 2941. ESI-MS (m/z): = ESI-MS (m/z): $[M+Na]^+$=592.64 (calculated); 592.33 (observed), $[M+K]^+$=608.74 (calculated); 608.25 (observed).

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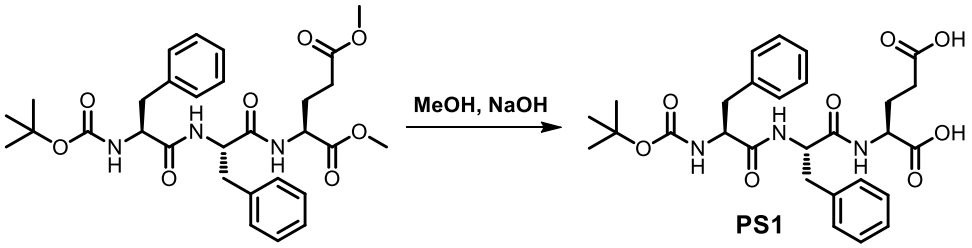


Scheme S6: Synthetic methodologies adopted for the synthesis of BOC-Phe-Phe-Glu-(OH)2.

Synthesis of BOC-NH-Phe- Phe-Glu-(OH)$_2$ (PS1): To 1 g (1.75 mmol) of Boc-Phe-Phe-Glu-(OMe)$_2$, 40 mL MeOH and 2M 6 mL NaOH were added and the progress of saponification was monitored by thin layer chromatography (TLC). The reaction mixture was stirred. After10 hours, the methanol was removed under vacuum; the residue was dissolved in

30 mL of water, and washed with diethyl ether (2 X 50 mL). Then, the pH of the aqueous layer was adjusted to 2 using 1M HCl and extracted with ethyl acetate (3 X 50 mL). The extracts were pooled, dried over anhydrous sodium sulfate, and evaporated under vacuum to obtain the compound as a white solid. Yield 950 mg (1.73mmol, 98%) (Scheme 6). Elemental analysis calcd (%): ($C_{28}H_{35}N_3O_8$): C 62.09, H 6.51, N 7.76, O 23.63; found: C 61.73, H 6.38, N 7.87.^{1}H NMR (DMSO-d6, 500 MHz, δppm): 7.97 (d, J= 8.0 Hz, 1H, NH), 7.24-7.15 (m, 10H, ArH of Phe), 6.82 (d, J= 8.0 Hz, 1H, NH), 4.55 (m, 1H, CαH, Phe), 4.21 (m CαH, Phe), 4.08-4.06 (m, CαH, Glu), 3.05-3.02 (m, 1H, CβH, Phe), 2.84- 2.81(m, 2H, CβH, Phe), 2.60-2.50 (m, 1H, CβH, Phe), 2.27 (m, 2H, Glu), 1.96-1.78 (m, 2H, Glu) 1.25 (s, 9H, Boc). ^{13}C NMR (125 MHz, DMSO-d_6, δ_{ppm}): 174.01, 173.14, 171.26, 155.31, 137.99, 137.41, 129.49, 129.27, 128.28, 126.62, 126.47, 78.70, 55.87, 53.59, 51.32, 38.86, 36.69, 30.05, 28.21, 26.39. FTIR (KBr, Nujol $\nu_{max,}$ cm^{-1}): = 699, 744, 853, 917, 1026, 1048, 1167, 1252, 1290, 1521, 1650, 1712, 2936 and 3232. ESI-MS (m/z): $[M]^+$=541.59 (calculated); 542.08 (observed).

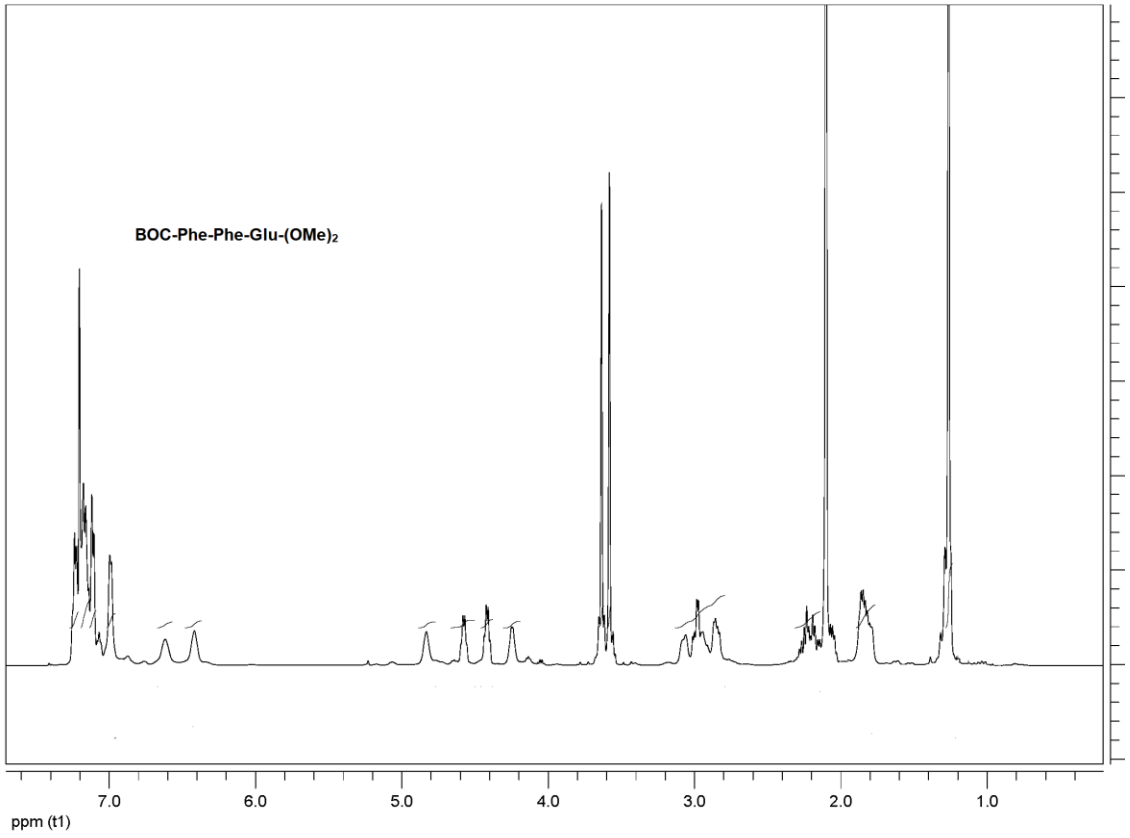


Figure S1. ^{1}H NMR ($CDCl_3$, 500 MHz, δppm) of Boc- Phe-Phe-Glu-$(OMe)_2$.

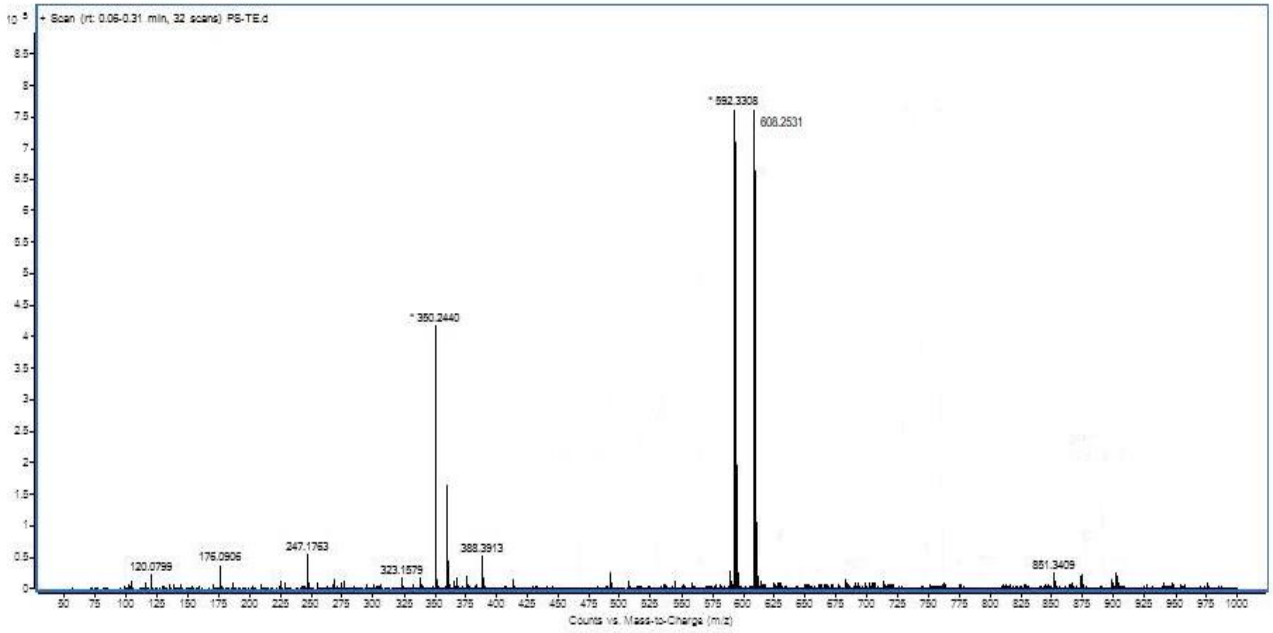


Figure S2. ESI Mass spectra of Boc-NH-Phe-Phe-Glu-$(OMe)_2$.

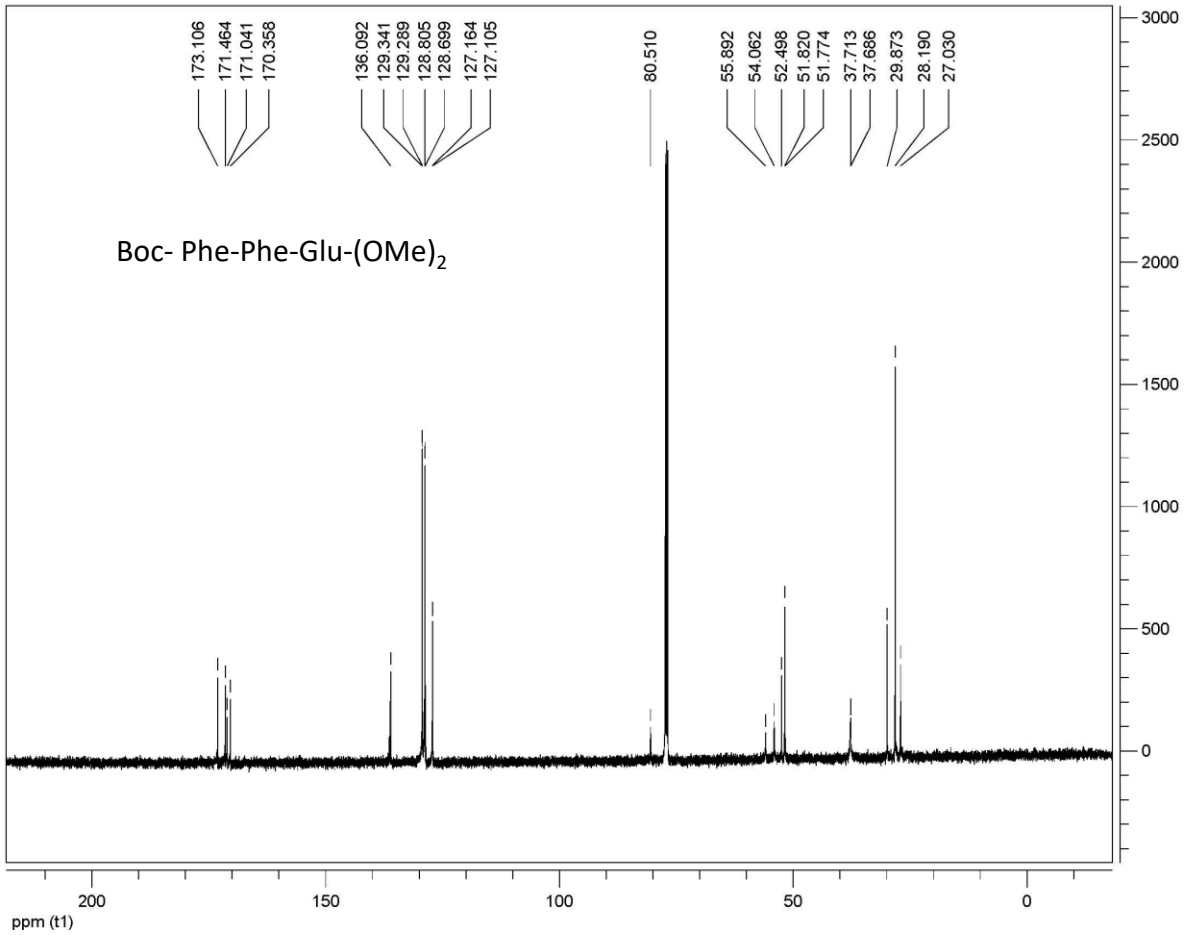


Figure S3. ^{13}C NMR ($CDCl_3$, 125 MHz, δppm) of Boc- Phe-Phe-Glu-$(OMe)_2$.

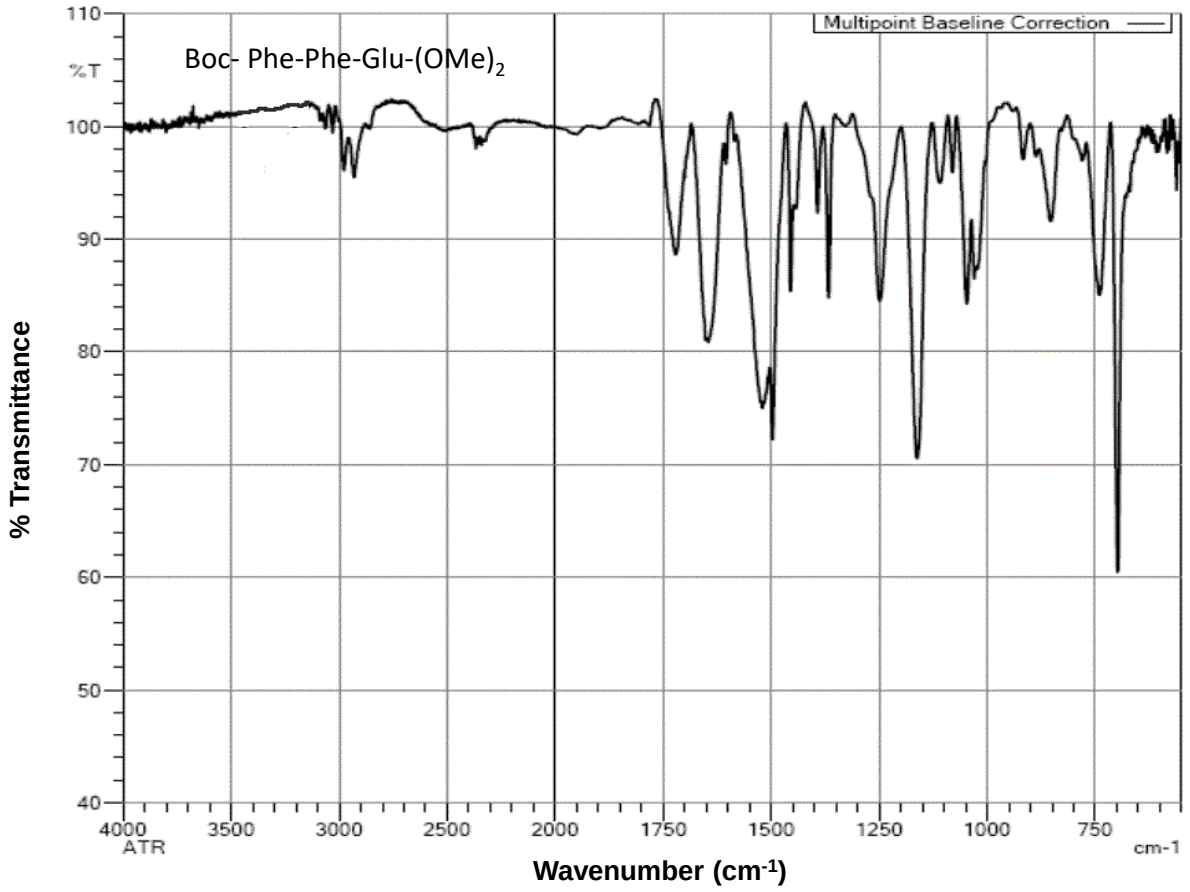


Figure S4. FTIR spectra of Boc- Phe-Phe-Glu-$(OMe)_2$ in monomeric form recorded as KBr pellet.

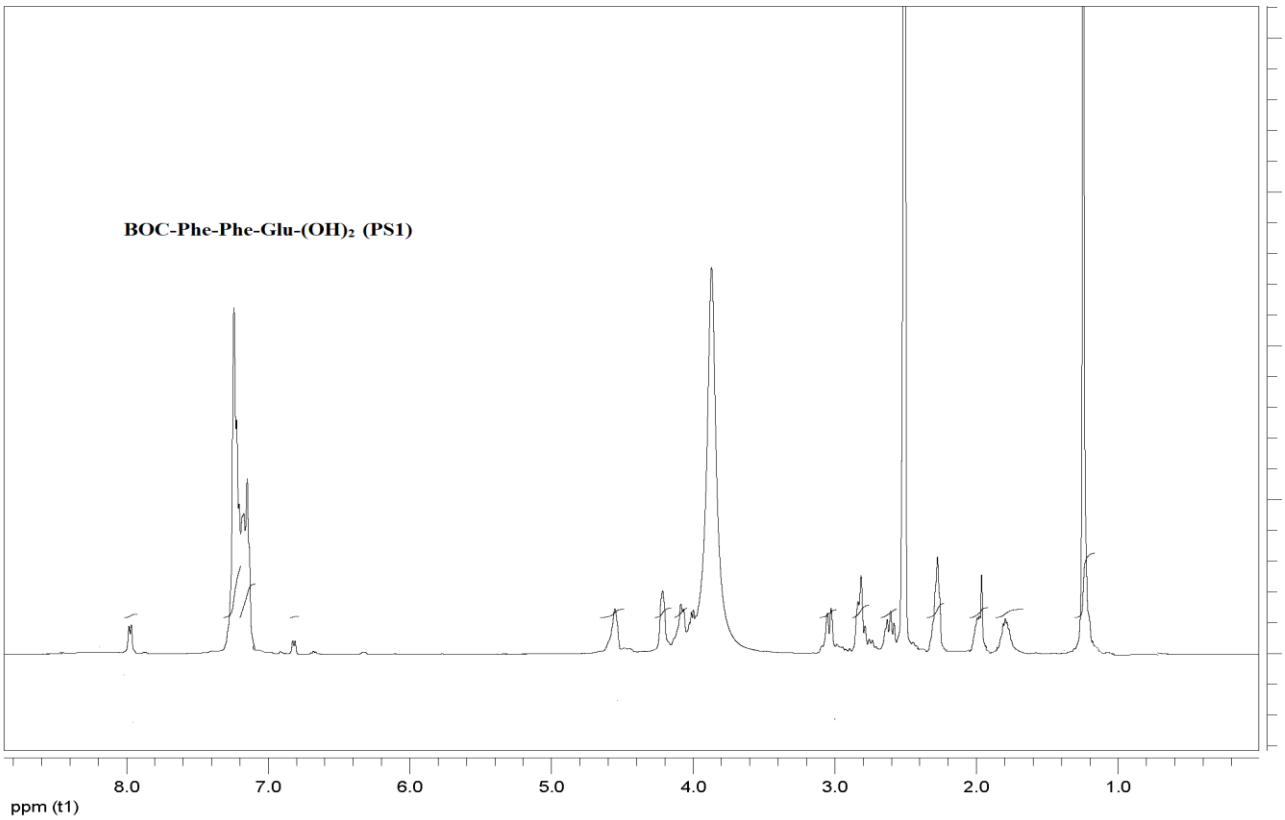


Figure S5. ^{1}H NMR (DMSO d_6, 500 MHz, δppm) of Boc- Phe-Phe-Glu-$(OH)_2$ (**PS1**).

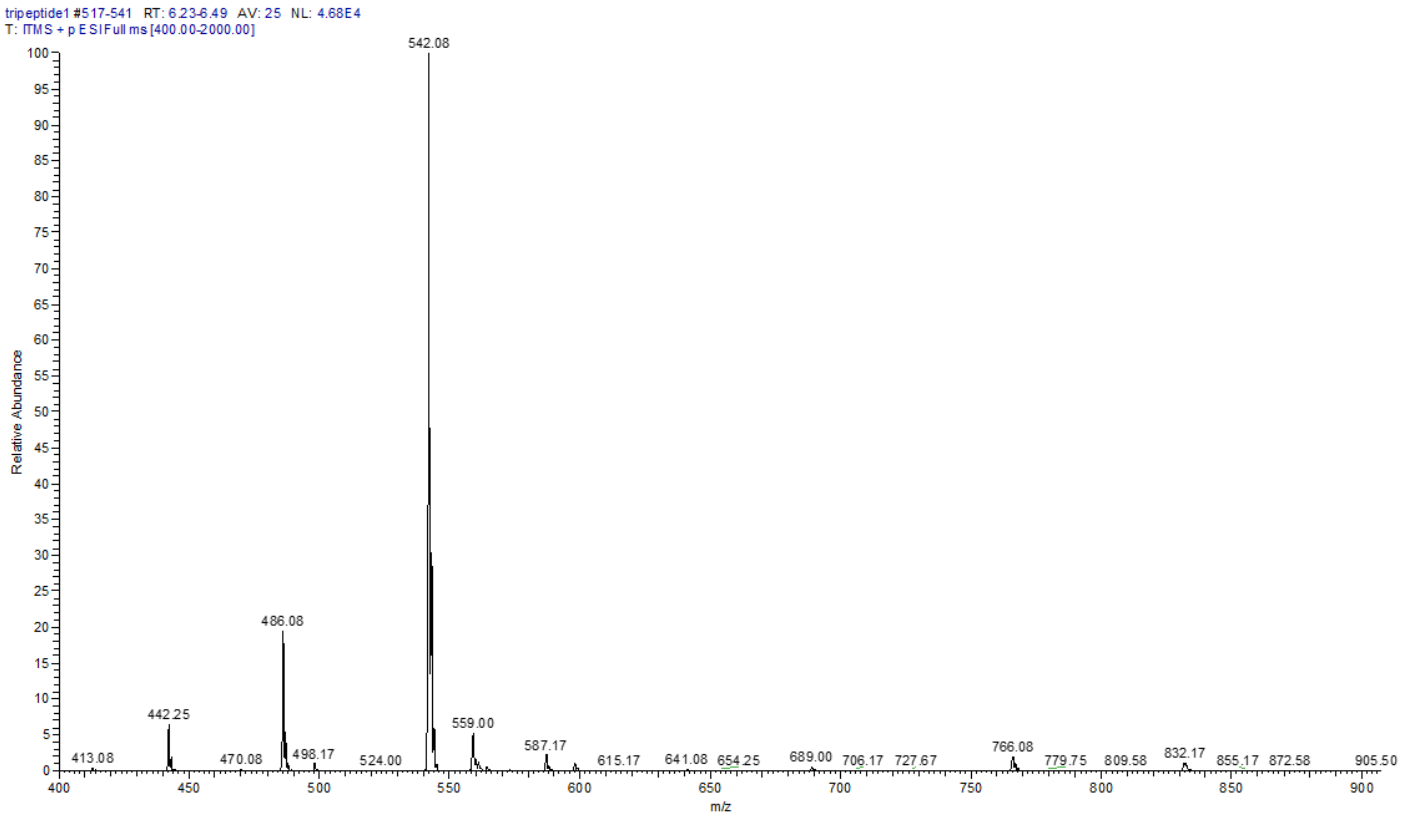


Figure S6. ESI Mass spectra of Boc-NH-Phe-Phe-Glu-$(OH)_2$ (**PS1**).

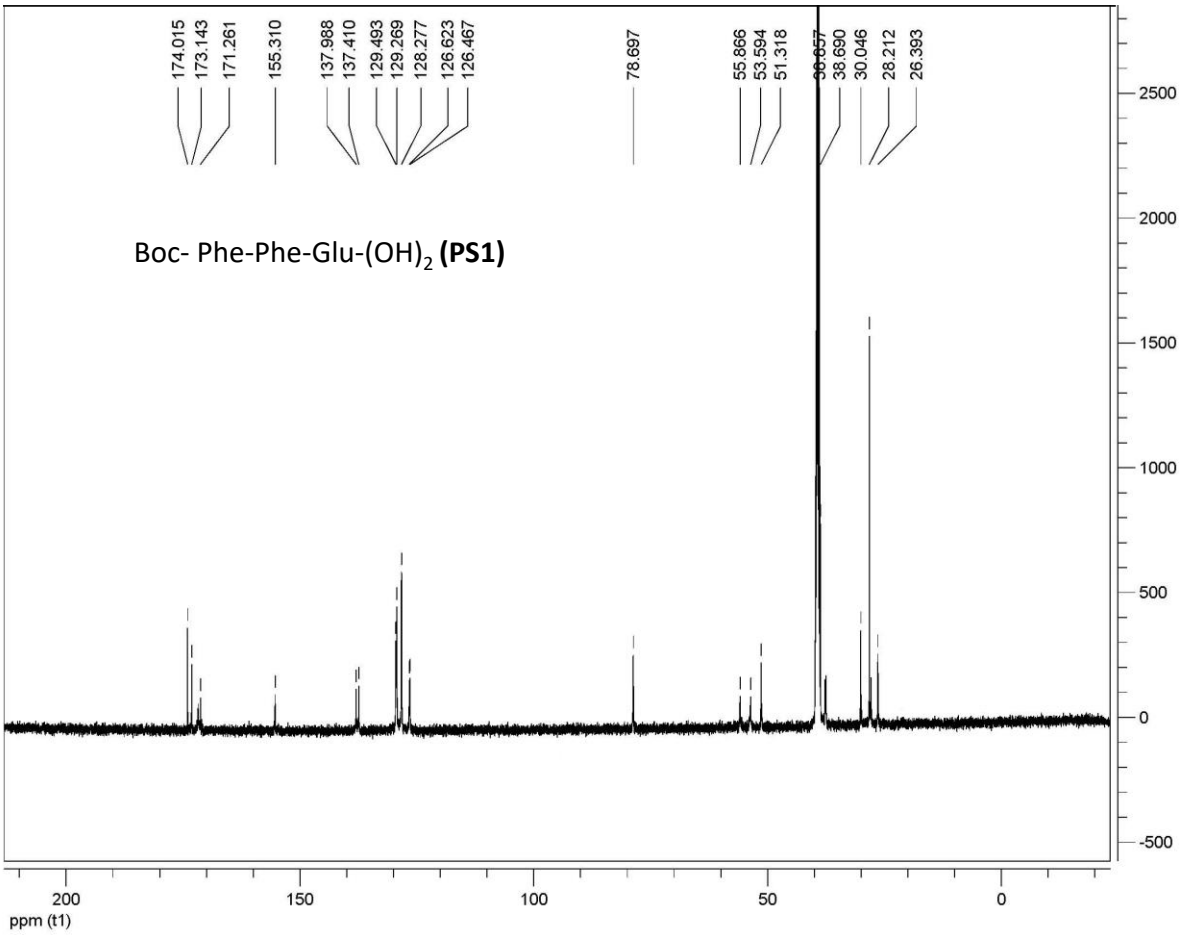


Figure S7. ^{13}C NMR (DMSO d_6, 125 MHz, δppm) of Boc- Phe-Phe-Glu-$(OH)_2$ (**PS1**).

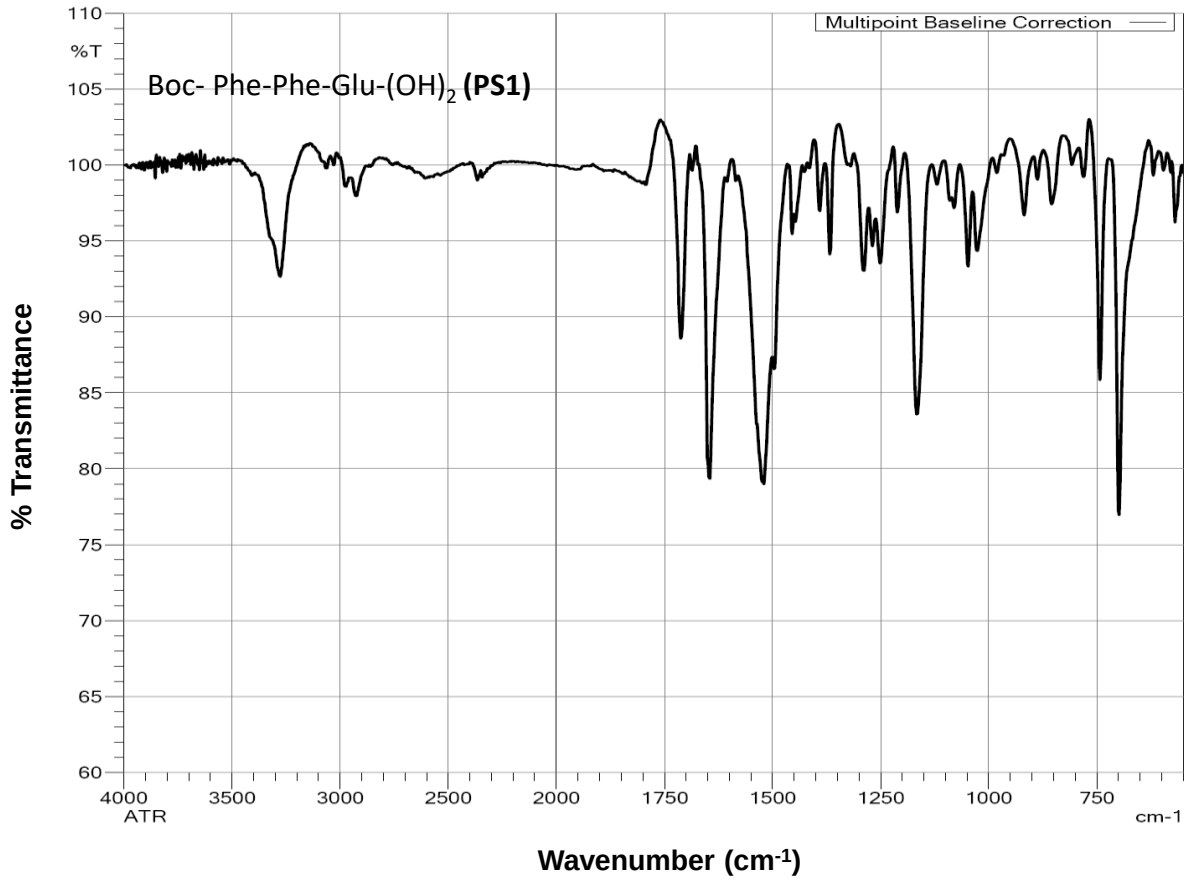


Figure S8. FTIR spectra of Boc- Phe-Phe-Glu-$(OH)_2$ (**PS1**) in monomeric form recorded as KBr pellet

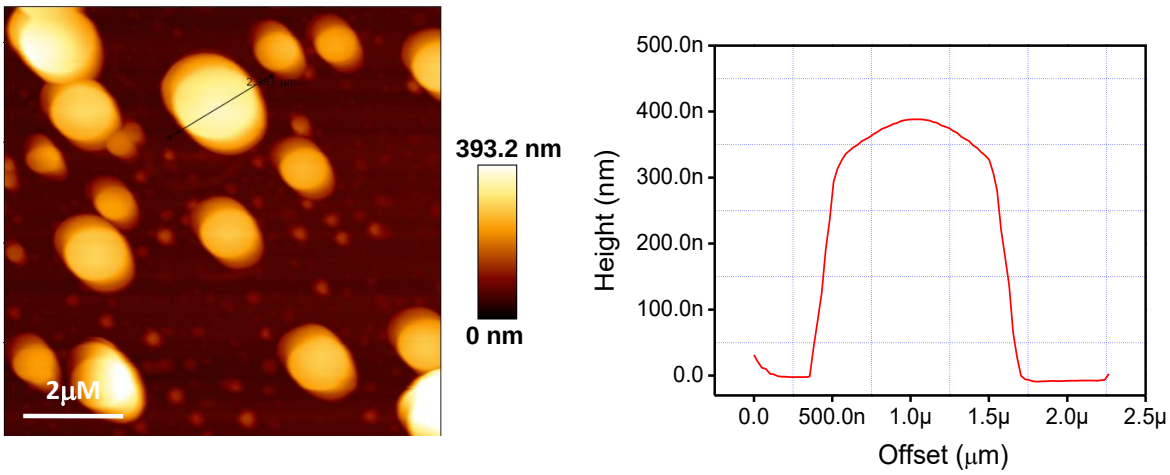


Figure S9. AFM topographic analysis: Two-dimensional representation and height analysis of the spherical self-assembled structures formed by **PS1** in aqueous ethanol medium.

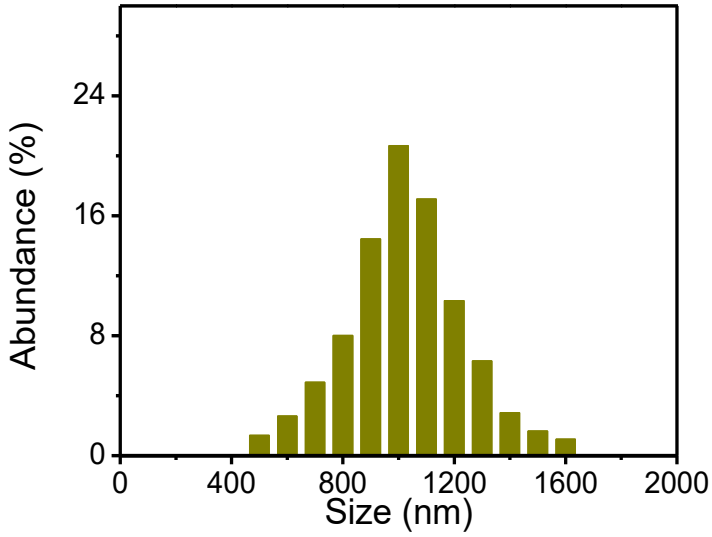


Figure S10. Size distribution obtained from DLS measurements for the spherical particles formed by **PS1** in aqueous ethanol medium.

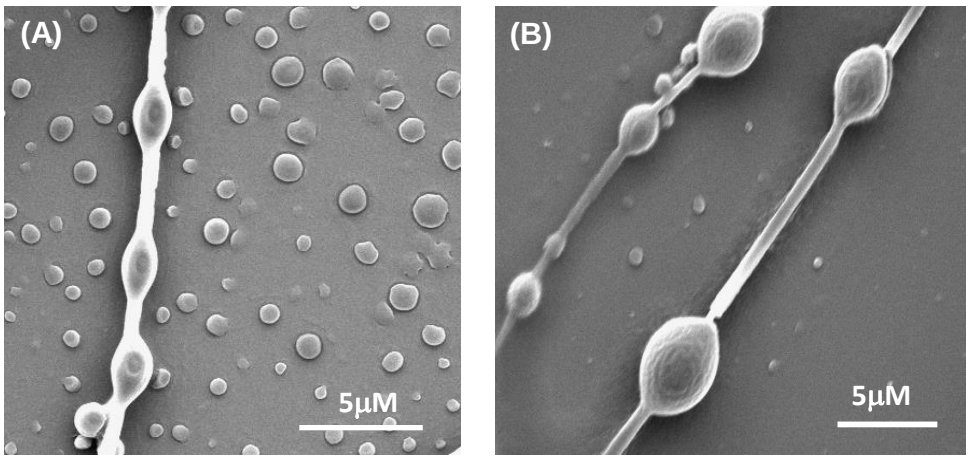


Figure S11. HR-SEM micrographs of the "necklace-like" supramolecular assembly formed at higher peptide (**PS1)** concentration the presence of NaCl after (A) 18h (B) 24h of incubation (pH =4) .

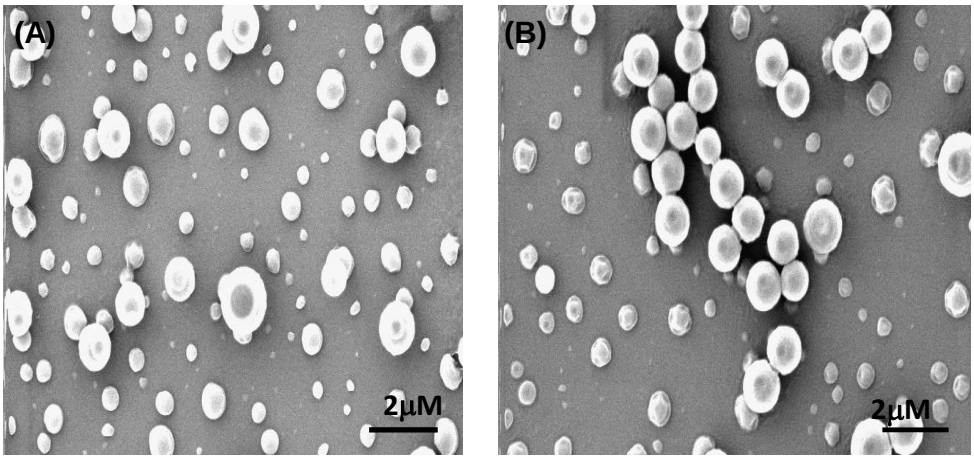


Figure S12. Thermal stability of the spherical assembly: HR-SEM micrographs of the "spherical assembly formed by peptide (**PS1)** after kept at 100°C for 4h.

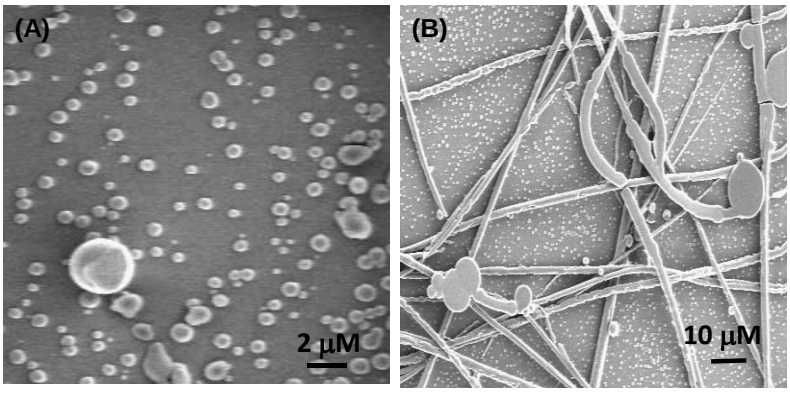


Figure S13. Thermal stability of the tubular structure: (A) HR-SEM analysis of self-assembled solution having nanotubes at room temperature heated to 80°-90°C for 30 mins; (B) and further cooling to room temperature.

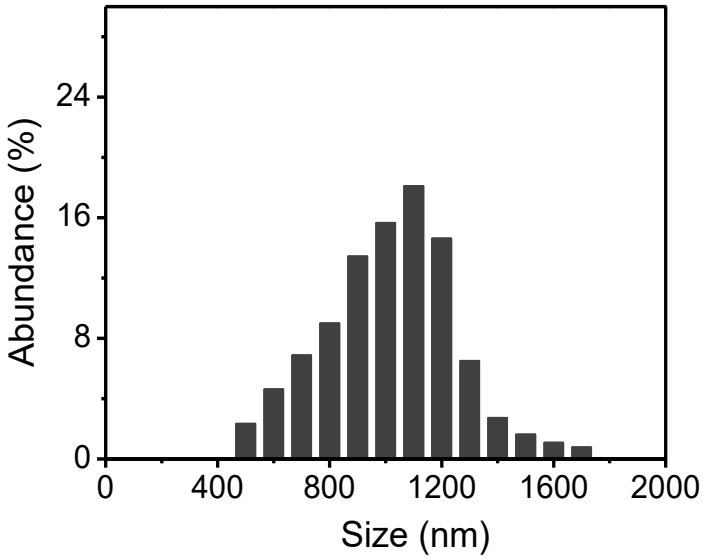


Figure S14. Size distribution obtained from DLS measurements for the RhB incorporated spherical particles formed by **PS1** in aqueous ethanol medium.

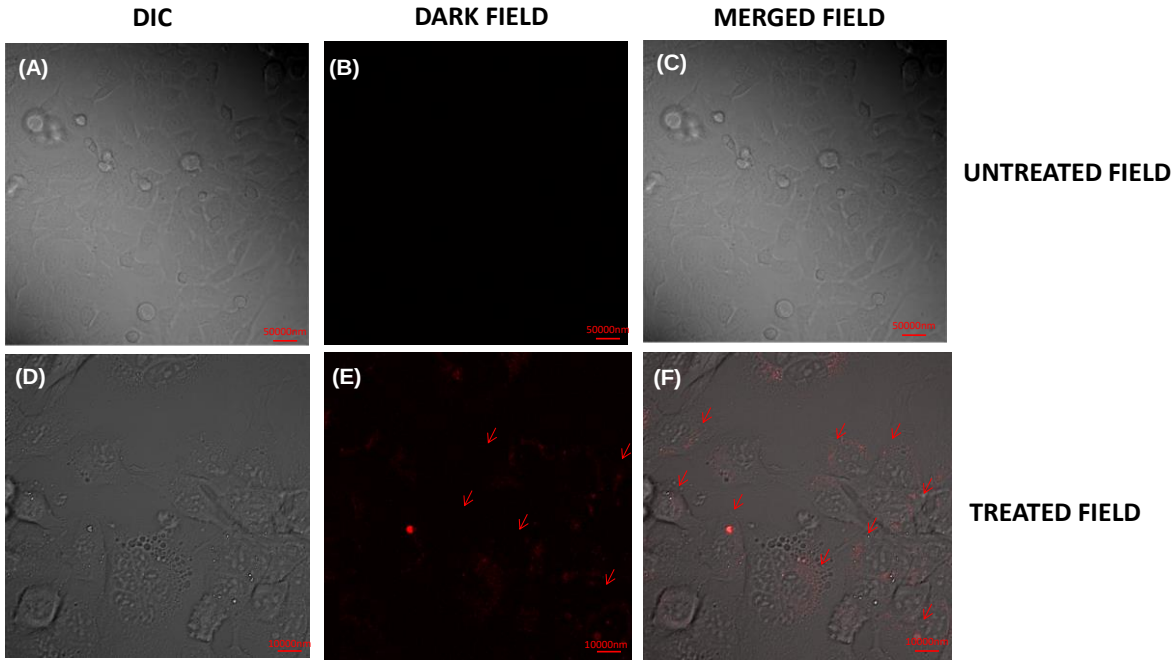
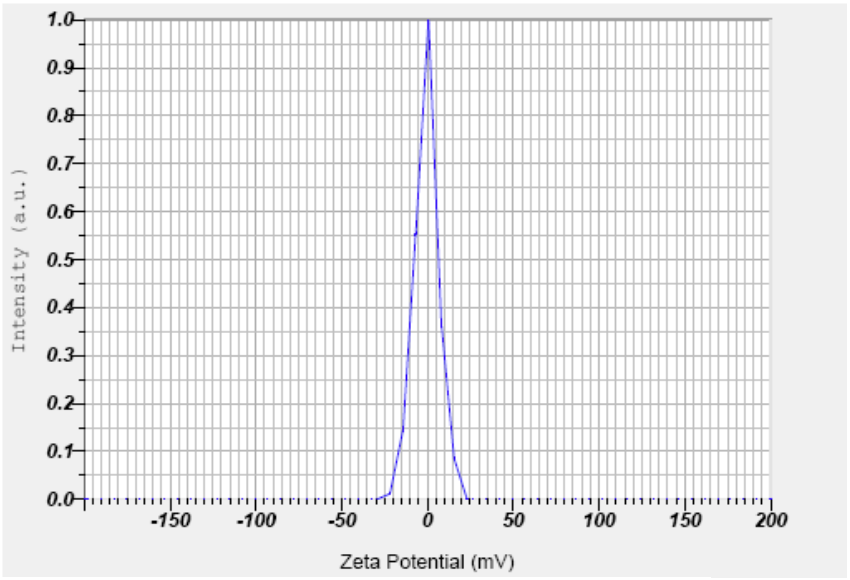
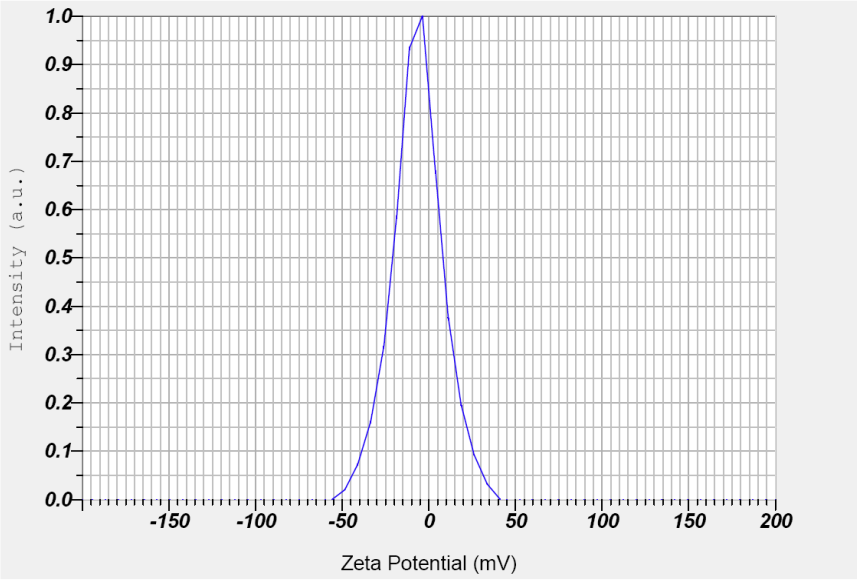


Figure S15. (A) Bright-field transmission; (B) Darkfield fluorecence; C) merging of bright and dark-field transmission images of untreated living HCT 116 cells **(D)** Bright-field transmission; (E) Darkfield fluorecence; (F) merging of bright and dark-field transmission images of living HCT 116 cells treated for 2h with RhB-PS1 conjugate based spherical assembly.



Zeta Potential (Mean) : -0.6 mV
Electrophoretic Mobility Mean : -0.000004 cm^2/Vs

Figure S16. Zeta potential analysis of spherical self-assemblies formed by PS1 at pH=7.2



Zeta Potential (Mean) : -6.4 mV
Electrophoretic Mobility Mean : -0.000050 cm^2/Vs

Figure S17. Zeta potential analysis of spherical self-assemblies formed by PS1 at pH=3.5

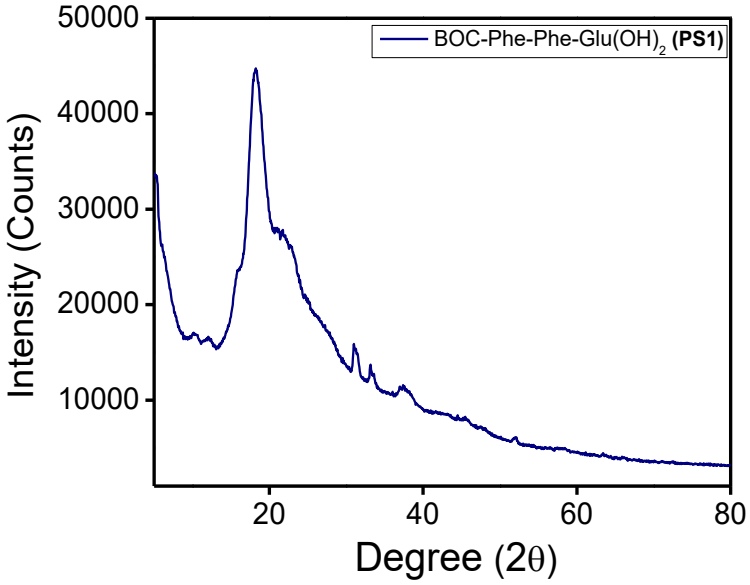


Figure S18. X-ray diffraction pattern of the tripeptide BOC-Phe-Phe-Glu $(OH)_2$ (**PS1).**

Peak List

Pos.[°2Th.]	Height [cts]	FWHMLeft[°2Th.]	d-spacing [Å]	Rel. Int. [%]
5.3731	4117.87	0.3385	16.44782	17.00
6.4368	807.43	0.4231	13.73186	3.33
10.1591	825.54	0.3385	8.70738	3.41
12.1153	563.97	0.8462	7.30545	2.33
15.7409	4922.46	0.4231	5.63001	20.32
17.7939	22709.28	0.9309	4.98480	93.73
18.4304	24229.33	0.4231	4.81406	100.00
20.9045	8479.51	0.3385	4.24955	35.00
22.8436	6861.89	0.4231	3.89302	28.32
24.9017	2408.16	0.3385	3.57575	9.94
28.4206	902.62	0.5077	3.14050	3.73
31.0049	2672.27	0.5077	2.88438	11.03
33.4463	1296.59	0.5077	2.67921	5.35
37.7330	1054.87	0.5077	2.38411	4.35
43.1218	211.21	0.3385	2.09785	0.87
45.5420	506.70	0.3385	1.99183	2.09
47.6549	265.08	0.5077	1.90834	1.09
51.7708	326.78	0.4231	1.76589	1.35
56.2893	57.98	0.6770	1.63438	0.24
57.3376	227.81	0.3385	1.60697	0.94
58.6949	169.22	0.6770	1.57301	0.70
61.2743	110.35	0.3385	1.51283	0.46
63.3382	206.95	0.5077	1.46842	0.85
66.4460	157.28	0.8462	1.40708	0.65
67.7937	81.30	0.5077	1.38235	0.34
70.0648	117.73	0.3385	1.34300	0.49
72.5300	52.29	0.6770	1.30331	0.22
81.5790	97.63	0.5077	1.18010	0.40
83.7305	93.65	0.3385	1.15517	0.39
92.2801	236.26	0.5077	1.06921	0.98

MTT assay for assessing the cytotoxicity of the Spherical assembly formed by peptide PS1 towards HCT116 cells: The in-vitro cytotoxicity of the PS1 peptide based spherical assembly was tested by HCT116 cells using conventional MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide, a yellow tetrazole) assay. HCT116 cells in their exponential growth phase were trypsinized and seeded in 96-well flat-bottom culture plates at a density of 3 x 10^3 cells per well in 100 μL DMEM complete medium (Biological Industries, Beit Haemek, Israel). The cells were allowed to adhere and grow for 24 hours at 37ºC in a 5% CO_2 incubator and then the medium was replaced with 100 μL fresh incomplete medium containing various concentrations of spherical assembly of PS1 (0 to 200 μM). Next, the assay was performed in quadruplet for each concentration. Cells were then incubated for 48 hours, after which the culture medium was removed and 100 μL of 1 mg/mL MTT reagent in PBS was added to each well. Thereafter, it was incubated for 4 hours; during this period active mitochondria of viable cells reduce MTT to purple formazan. Unreduced MTT was then discarded and DMSO (100 μL) was added into each well to dissolve the formazan precipitate, which was then measured spectrophotometrically using a microplate reader (Biorad, USA) at 570 nm. The cytotoxic effect of each treatment was expressed as the percentage of cell viability relative to the untreated control cells. The following formula was used to calculate the viability of cell growth. Cell viability (%) = (means of absorbance value of treated group/ means of Absorbance value of untreated control) X 100.