Electronic Supplementary Material (ESI) for Nanoscale Advances.
This journal is © The Royal Society of Chemistry 2022

Supplementary Information

Self-assembled dipeptide based fluorescent nanoparticles as a platform for developing cellular imaging probes and targeted drug delivery chaperones

Subramaniyam Sivagnanam[a] ¶, Kiran Das[b] ¶, Madhuri Basak[b], Tarun Mahata[b], Adele Stewart[c], Biswanath Maity[b]* Priyadip Das[a]*

[a]Department of Chemistry, SRM Institute of Science and Technology, SRM Nagar, Potheri, Kattankulathur, Tamil Nadu-603203

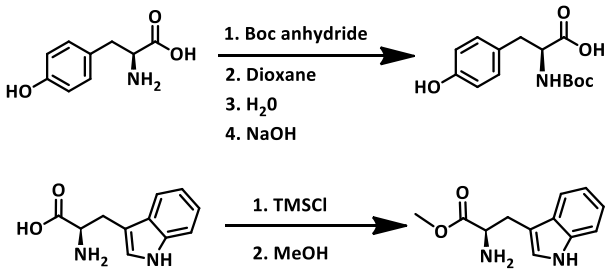
[b]Centre of Biomedical Research, Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGI) campus, Raebareli Road, Lucknow, Uttar Pradesh 226014, India

[c]Department of Biomedical Science, Charles E. Schmidt College of Medicine, Florida Atlantic University, Jupiter, FL 33458, USA

* *Email:* priyadipcsmcri@gmail.com, priyadip@srmist.edu.in
bmaity28@gmail.com, bmaity@cbmr.res.in

Experimental section

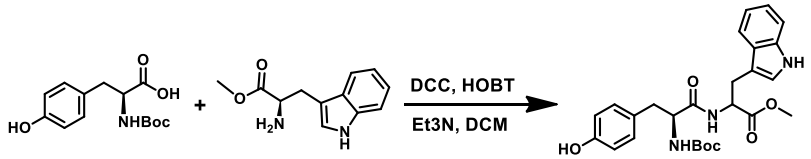
Peptide synthesis: Peptides were synthesized by conventional solution-phase methods. Peptide coupling was mediated by dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBt). The products were purified by column chromatography using silica gel (100–200 mesh) as the stationary phase and a chloroform-methanol mixture as an eluent. The final compounds were fully characterized by Bruker 500 MHz ^{1}H-NMR spectroscopy, and mass spectroscopy (Agilent, High Resolution Mass Spectrometer).



Scheme S1: Synthetic methodologies adopted for the synthesis of BOC-protected Tyrosine and NH_2-Trp-OMe Hydrochloride.

Synthesis of BOC-NH-Tyr-OH: A solution of L-Tyrosine (3g, 16.55 mmol) in a mixture of dioxane (45 mL), water (25 mL), and 1 M NaOH (20 mL) was stirred and cooled in an ice-water bath. 4.73 g of di-tert-butyl dicarbonate was added and stirred continuously at room temperature (RT) for 6 hours. Then, the solution was concentrated using a 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: 4.55 g (16.17 mmol, 97%) (Scheme S1)

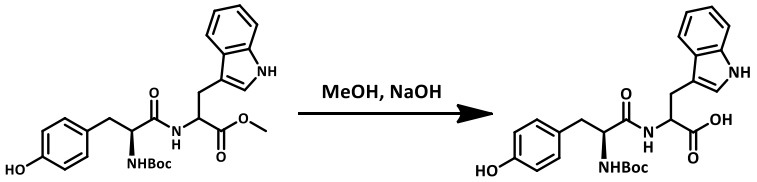
Synthesis of NH_2-Trp-OMe Hydrochloride: L-Tryptophan 4g (19.58 mmol) was taken in a round bottom flask and dissolved in 50 mL MeOH. Then, 10 mL (78.79 mmol) of Trimethyl chlorosilane (TMSCl) was added to the resulting solution slowly in a drop wise manner and stirred for 8 hours at room temperature. 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-Tryptophan methyl ester hydrochloride. Yield: 4.15 g (19.01 mmol, 97%) (Scheme S1).



Scheme S2: Synthetic methodologies adopted for the synthesis of BOC-Tyr-Trp-OMe. **(PS1)**

Synthesis of BOC-Tyr-Trp-OMe (PS1): 4g (14.06 mmol) of Boc-Tyr-OH were dissolved in 50 mL dry DCM in an ice-water bath. NH_2-Trp-OMe.HCl 3.6 g (16.49 mmol) and Et_3N 2 ml, (15 mmol) were then added to the reaction mixture, followed immediately by the addition of 3.5 g (16.96 mmol) dicyclohexylcarbodiimide (DCC) and 2.3 g (17.02 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 1M 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-Tyr-Trp-OMe as a white solid. The product was purified by silica gel (100–200 mesh) using chlorofom–methanol (99%:1%) as eluent. Yield: 5.5 g (11.42 mmol, 82%) (Scheme S2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.49 (s, 1H, Ar-NH), 7.39 (s, 1H, ArH), 7.33-7.32 (d, J=7.75 Hz, 1H, ArH), 7.17 (s, 1H, ArH), 7.08 (s, 1H, ArH), 6.90 (s, 1H, ArH), 6.79 (s, 1H, ArH), 6.63 (s, 2H, ArH), 6.29 (s, 1H, ArH), 5.20 (s, 1H, Ar-OH of Tyr), 4.80 (s, 1H, CαH, Try), 4.26 (s, 1H, CαH, Trp), 3.52 (s, 3H, OMe), 3.22 (s, 2H, CβH, Tyr), 2.9-2.85 (broad peak, 2H, CβH, Trp), 1.42 (s, 9H, Boc). ^{13}C NMR (100 MHz, $CDCl_3$, δ_{ppm}): 171.86, 171.27, 155.56, 155.27, 136.13, 130.39, 127.80, 127.46, 123.14, 122.22, 119.63, 118.36, 115.58, 111.46, 109.27, 80.49, 56.06, 53.10, 52.35, 37.92, 28.26, 27.56. FTIR (CaF_2, ν_{max}, cm^{-1}): 673, 1043, 1087, 1184, 1514, 1647, 2131, 2345, 3352. HRMS MS (m/z): $[M+H]^+$= 482.2291 (calculated); 482.2286 (observed),



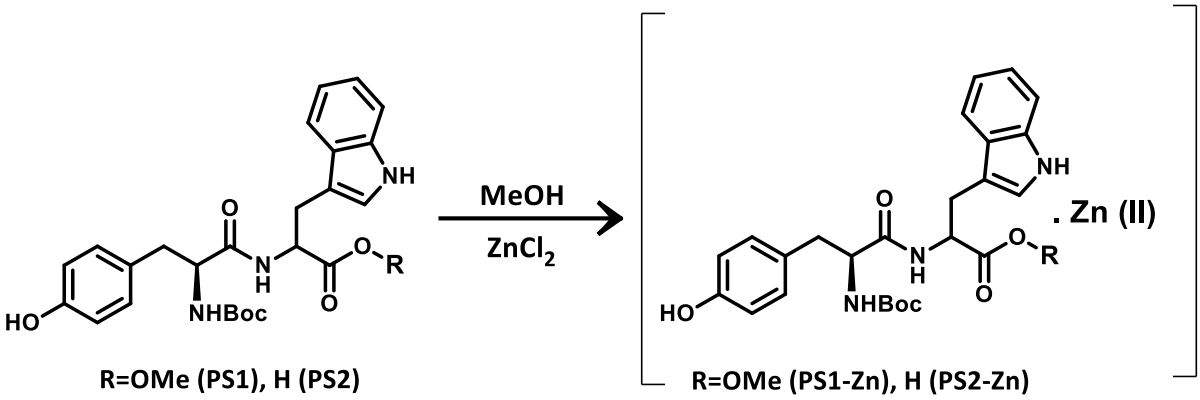
Scheme S3: Synthetic methodologies adopted for the synthesis of BOC-Tyr-Trp-OH **(PS2)**.

Synthesis of BOC-NH-Tyr-Trp-OH (PS2): To 1 g (2.07 mmol) of Boc-Tyr-Trp-OMe, 30 mL MeOH and 2M 3 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

white solid. Yield: 900mg (1.92 mmol, 93%). (Scheme S3). ^{1}H NMR (500 MHz, DMSO-d6) δ 10.60 (s, 1H, -COOH of Trp), 7.54-7.48 (m, 1H, -NH), 7.33-7.28 (m,1H, -NH), 7.11 (s, 1H, ArH of Trp), 7.07-7.04 (m, 1H, ArH), 6.98-6.93 (m, 4H, ArH), 6.61-6.59, (d, J=6.8 Hz, 3H, ArH), 4.50 (s, 1H, Ar-OH of Tyr), 4.44 (s, 2H, CαH), 3.21-3.16 (m, 2H, CβH of Tyr), 3.08-3.04 (m, 1H, CβH of Trp), 2.81-2.79 (d, 1H, CβH of Trp), 1.22 (s, 9H, Boc). ^{13}C NMR (100 MHz, DMSO- d_6, δ_{ppm}): 173.75, 172.13, 156.15, 155.62, 136.50, 130.52, 128.62, 127.83, 124.10, 121.33, 118.79, 115.26, 111.76, 110.19, 78.54, 67.49, 56.62, 53.55, 37.12, 28.60, 28.21, 27.65. FTIR (CaF_2, ν_{max}, cm^{-1}): 677, 1043, 1083, 1186, 1645, 2133, 3346. HRMS MS (m/z): $[M+H]^+$= 468.2135 (calculated); 468.2125 (observed).

Synthesis of Zinc complexes

Synthesis of PS1-Zn and PS2-Zn: The metal-peptide complexes (**PS1-Zn** and **PS2-Zn**) were prepared by dissolving peptides **PS1** (200 mg, 0.41 mmol) or **PS2** (200 mg, 0.42 mmol) in ethanol. The pH of the solution was adjusted to pH 11 with diluted sodium hydroxide solution. In a typical experiment, 0.7 mmol, 100 mg Zinc(II) chloride dissolved in 0.5 mL of triple distilled water were added to the peptide solution at RT with stirring. After the zinc salt addition, the pH was readjusted to 11. The zinc peptide complexes (**PS1-Zn** and **PS2-Zn**) immediately precipitated. To complete the reaction, the reaction mixture was further reacted for 6 hours at RT. Then, the solids were centrifuged, washed with ethanol, water and dried under vacuum overnight. Yields were above 90%. (Scheme S4). FTIR (CaF_2, ν_{max}, cm^{-1}): **PS1-Zn**-680, 827, 1016, 1053, 1101, 1163, 1220, 1365, 1436, 1514, 1595, 1616, 1655, 1730, 2951, 3338. **PS2-Zn-** 742, 827, 1022,1053, 1103, 1161, 1242, 1367, 1452,1514, 1595, 1615, 1658, 2935, 3316. ESI MS (m/z): **PS1-Zn** $[M+K]^+$= 584.1141 (calculated), 583.5300 (observed) and **PS2-Zn** $[M+Na]^+$= 554.1246 (calculated); 553.9500 (observed).



Scheme S4: Synthetic methodologies adopted for the synthesis of metallo-peptides **PS1-Zn** and **PS2-Zn**.

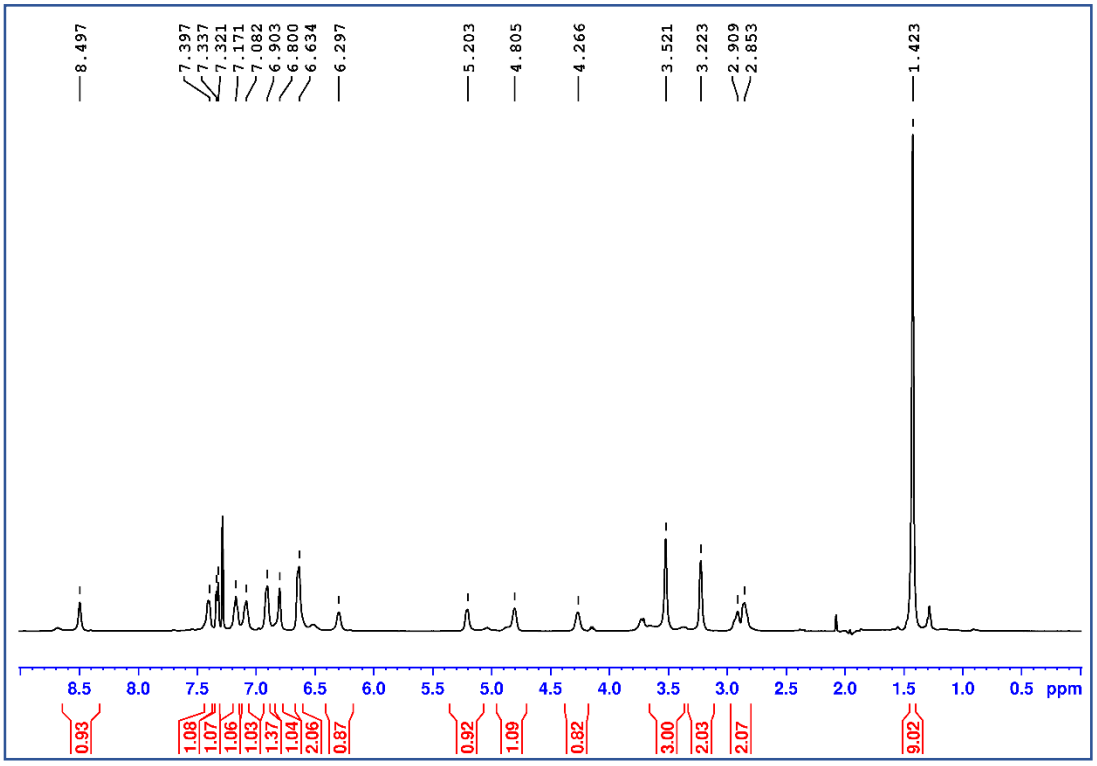


Figure S1. ^{1}H NMR ($CDCl_3$, 500 MHz, δppm) of Boc- Trp-Tyr-OMe (**PS1**).

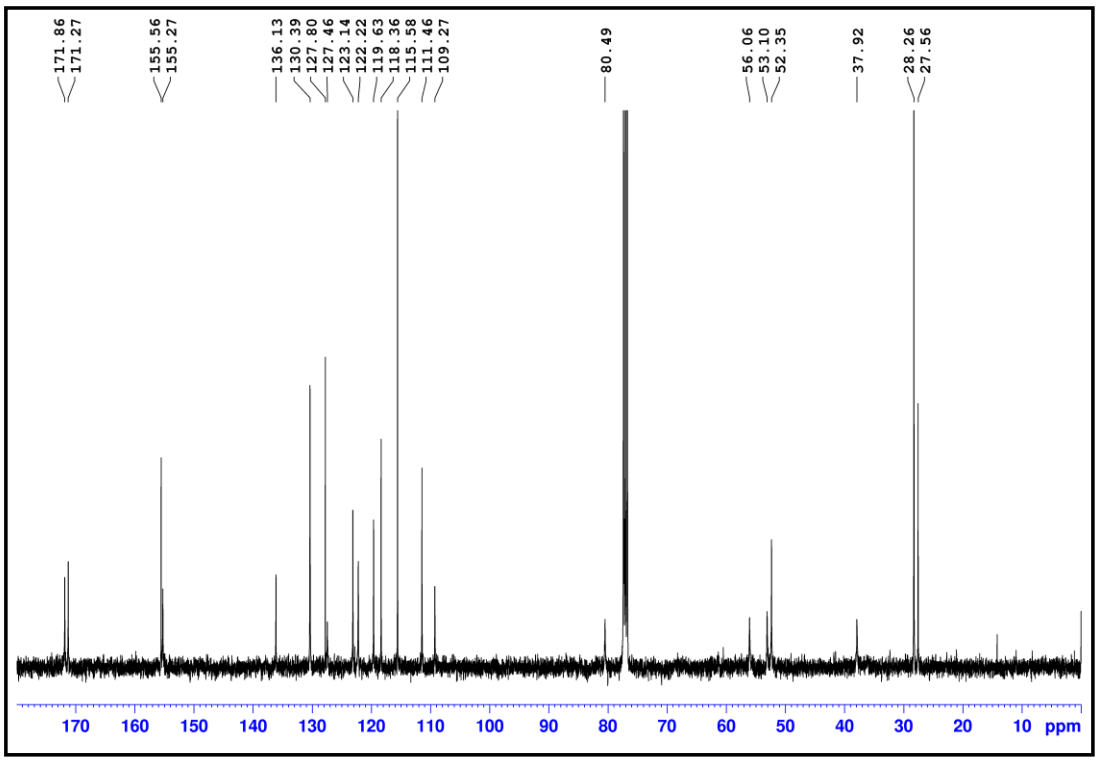


Figure S2. ^{13}C NMR ($CDCl_3$, 100 MHz, δppm) of Boc- Trp-Tyr-OMe (**PS1**).

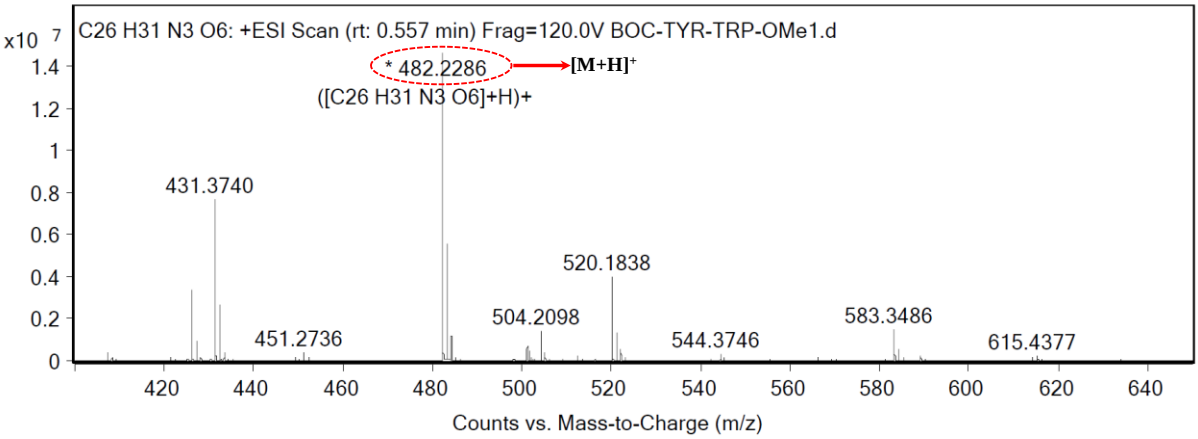


Figure S3. High Resolution Mass spectra of Boc-Trp-Tyr-OMe (**PS1**).

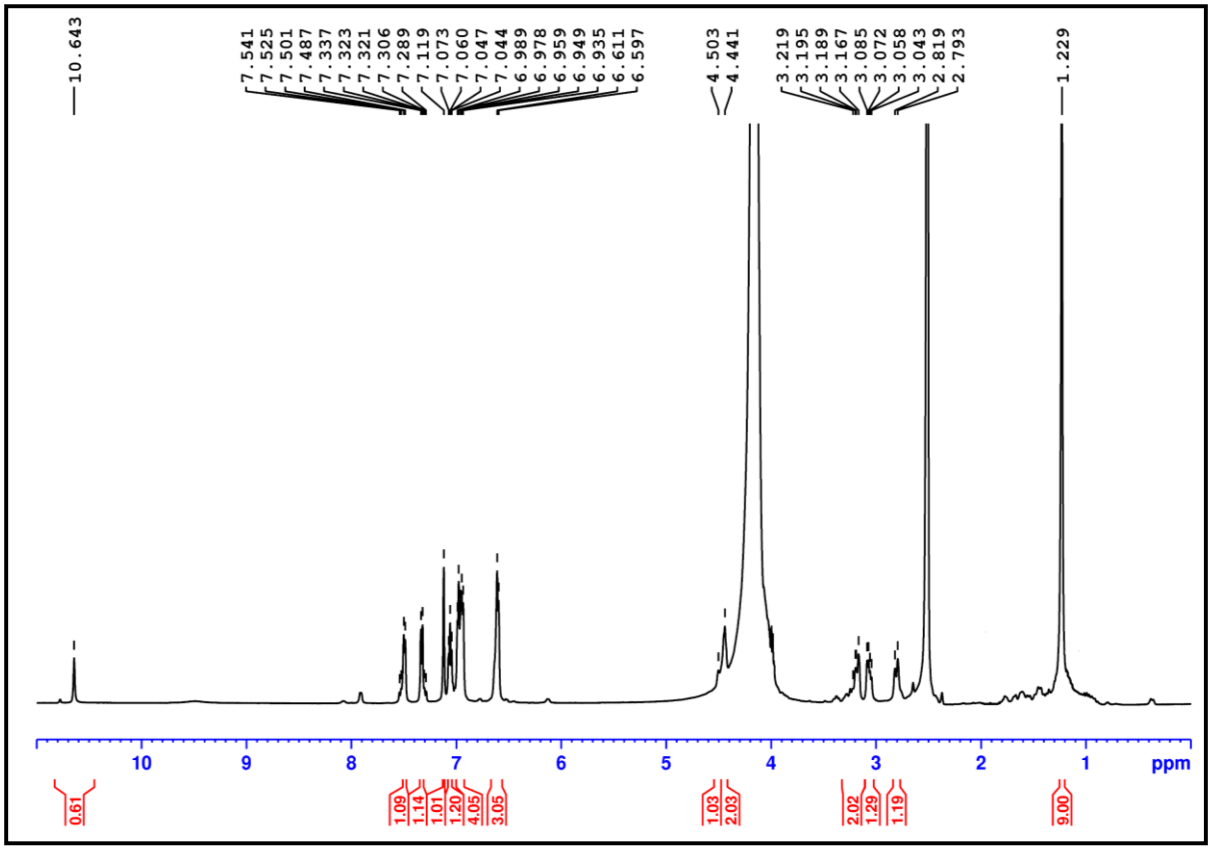


Figure S4. ^{1}H NMR (DMSO-d6, 500 MHz, δppm) of Boc- Trp-Tyr-OH (**PS2**).

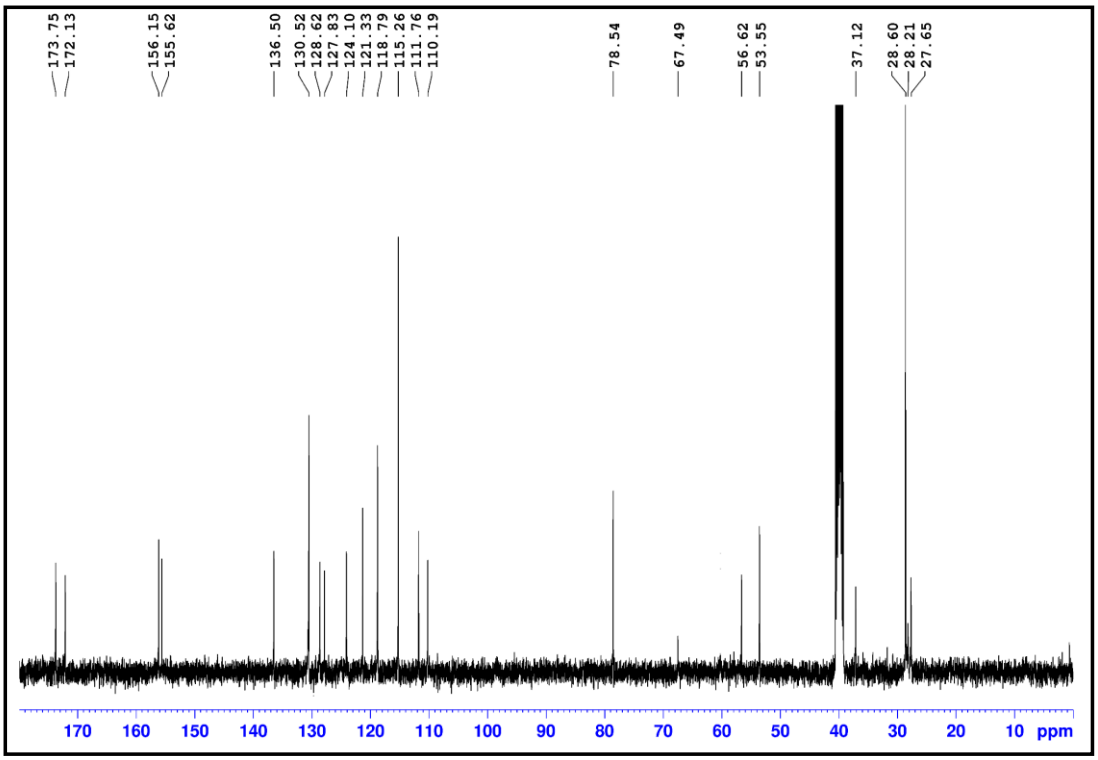


Figure S5. ^{13}C NMR (DMSO-d_6, 100 MHz, δppm) of Boc- Trp-Tyr-OH (P**S2**).

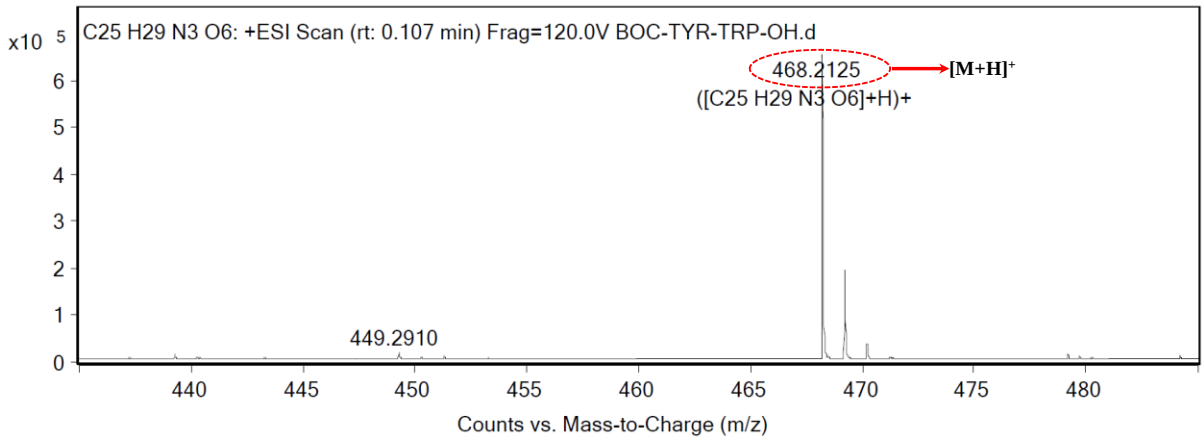


Figure S6. High Resolution Mass spectra of Boc-Trp-Tyr-OH (**PS2**).

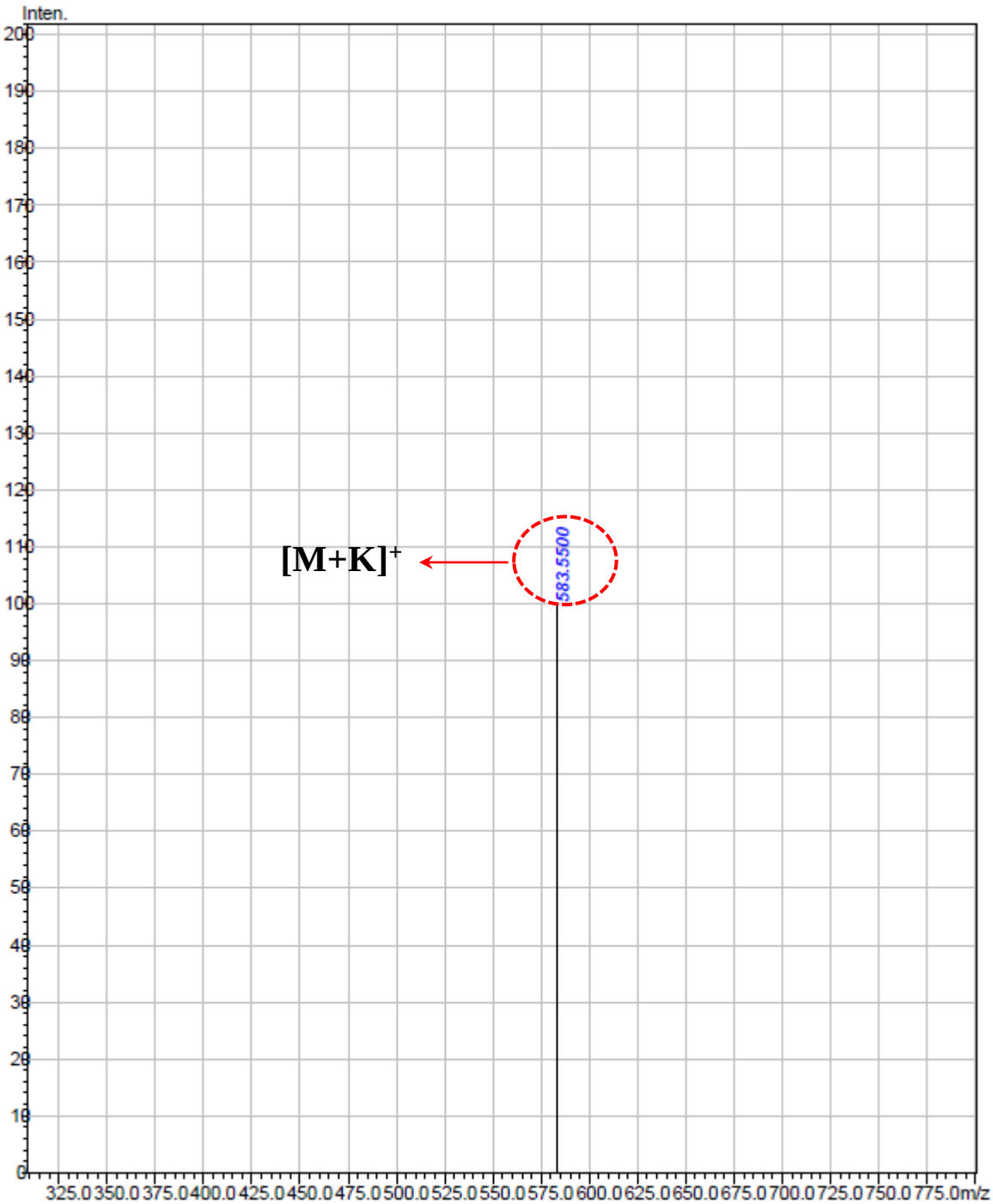


Figure S7. ESI Mass spectra of **PS1-Zn**

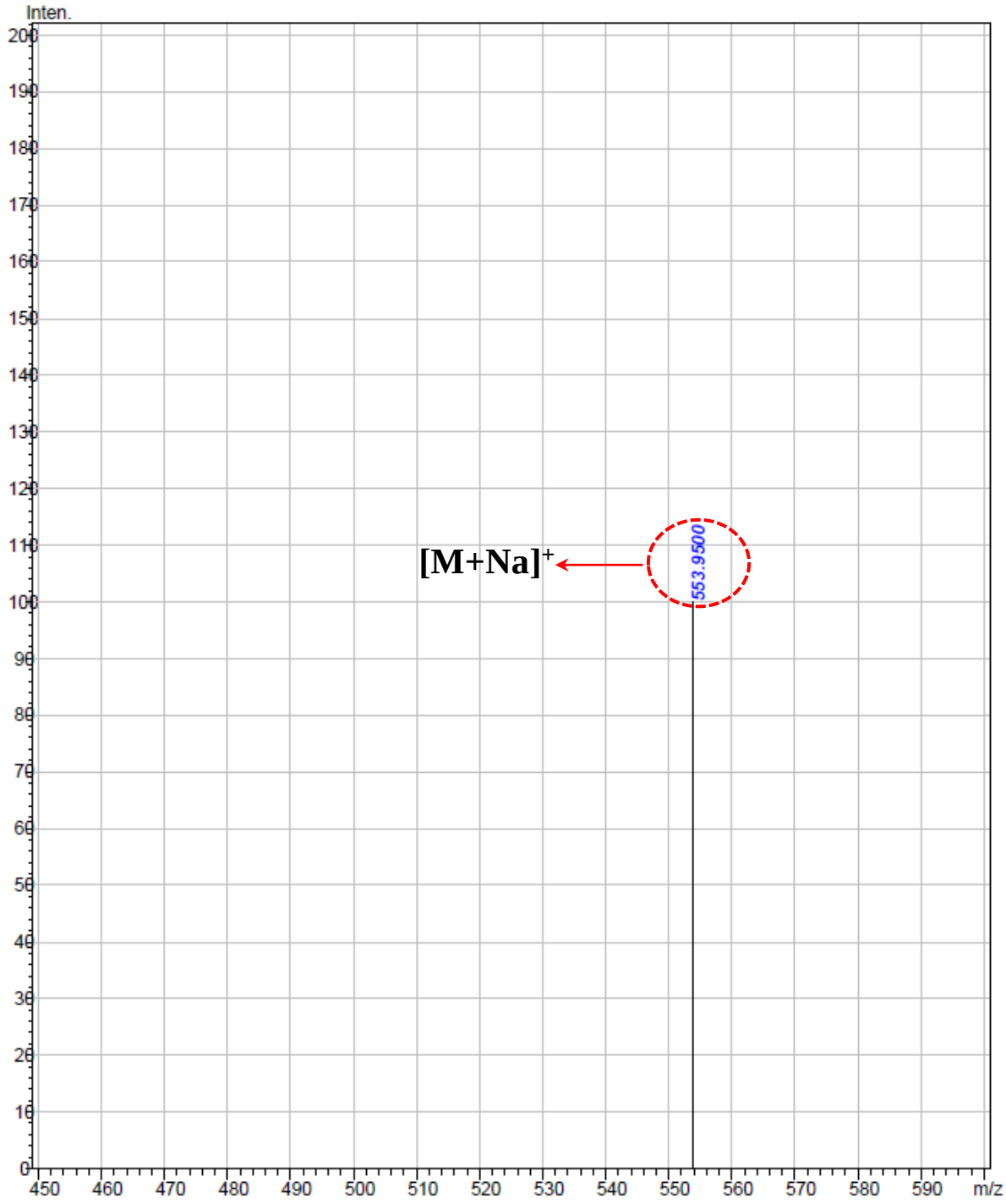


Figure S8. ESI Mass spectra of **PS2-Zn**

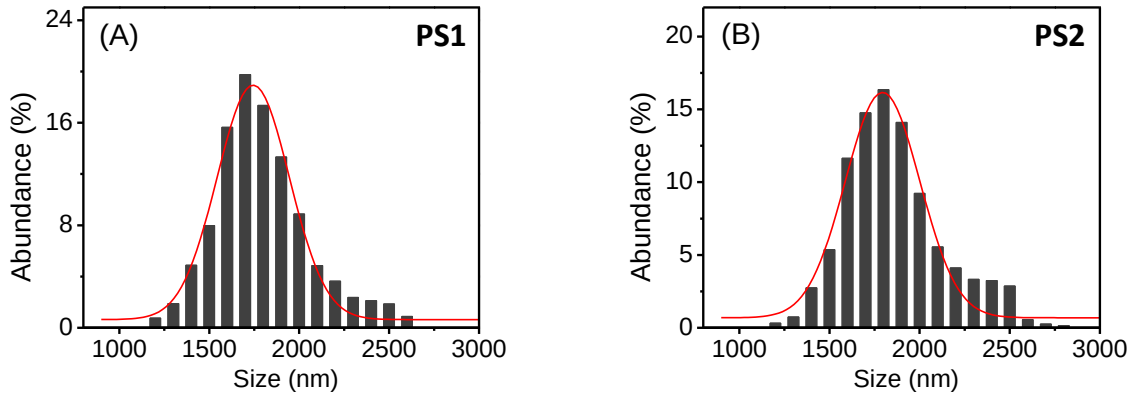


Figure S9. Size distribution obtained from Dynamic lighting scattering measurements for the spherical particles formed by self-assembly of (A) **PS1** without Zn (II). The average hydrodynamic diameter of the spherical structures is 1744±201.04 nm. (B) **PS2** without Zn (II). The average hydrodynamic diameter of the spherical structures is 1794±206.86 nm.

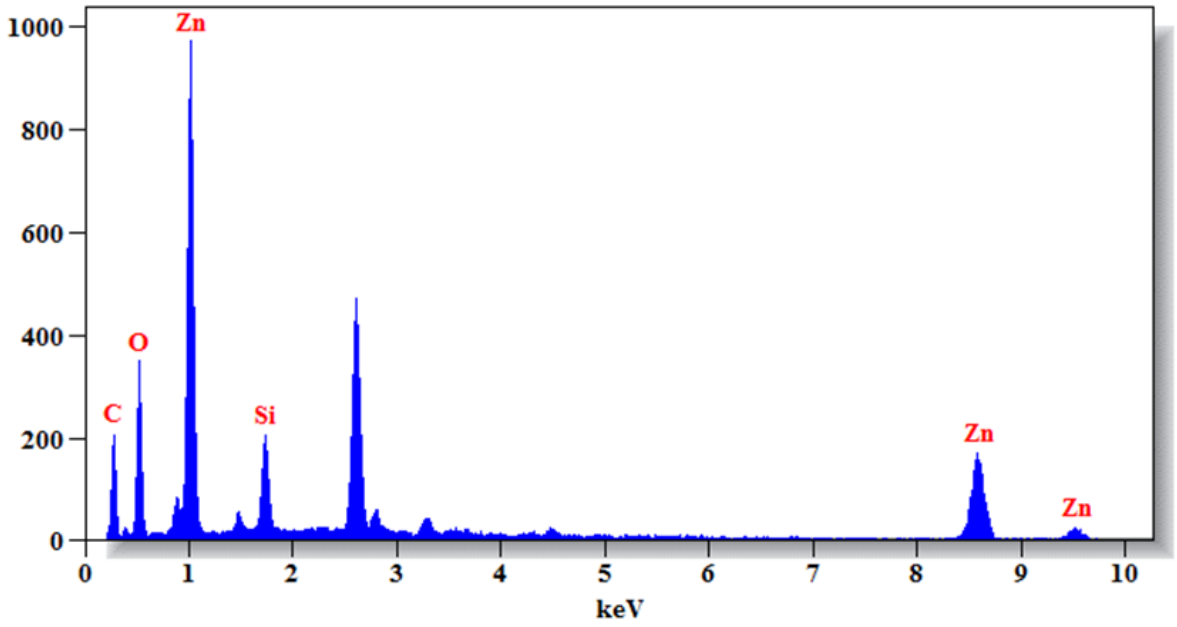


Figure S10. EDS analysis of **PS2-Zn**(II) based DPNPs using SEM.

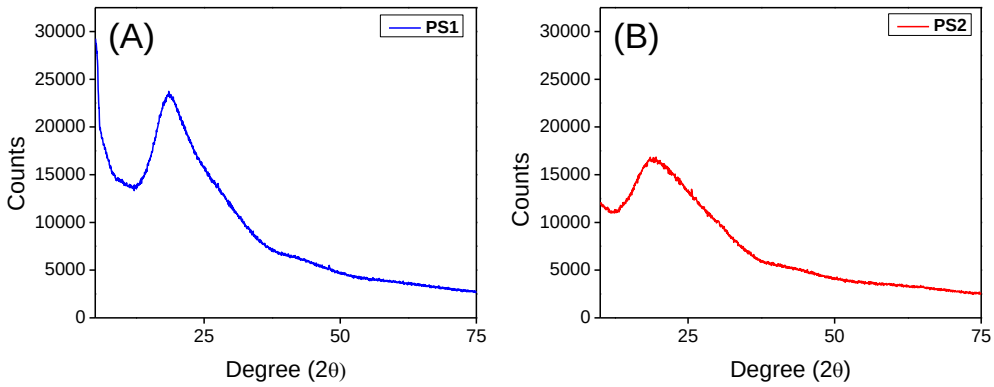


Figure S11. Powder X-ray diffraction spectra of self-assembled nano structures formed by **PS1** (A) and **PS2** self-assembly (B) without Zn (II) coordination. The diffraction pattern indicates the amorphous nature of the self-assembled structures.

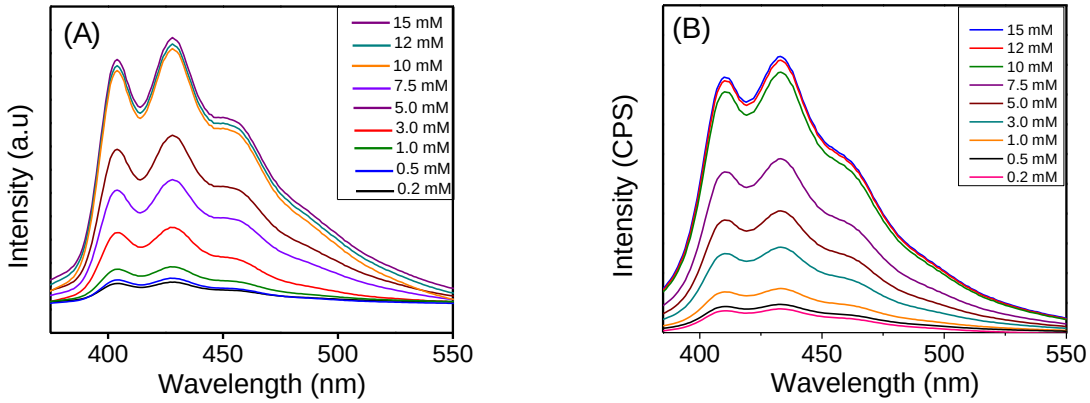


Figure S12. (A) Emission spectra of **PS1** self-assembly in presence of different concentration of Zn (II) with fixed dipeptide (**PS1**) concentration at 2 mM. (B) Emission spectra of **PS2** self-assembly in presence of Zn (II) at different concentrations with fixed dipeptide (**PS2**) concentration at 2 mM.

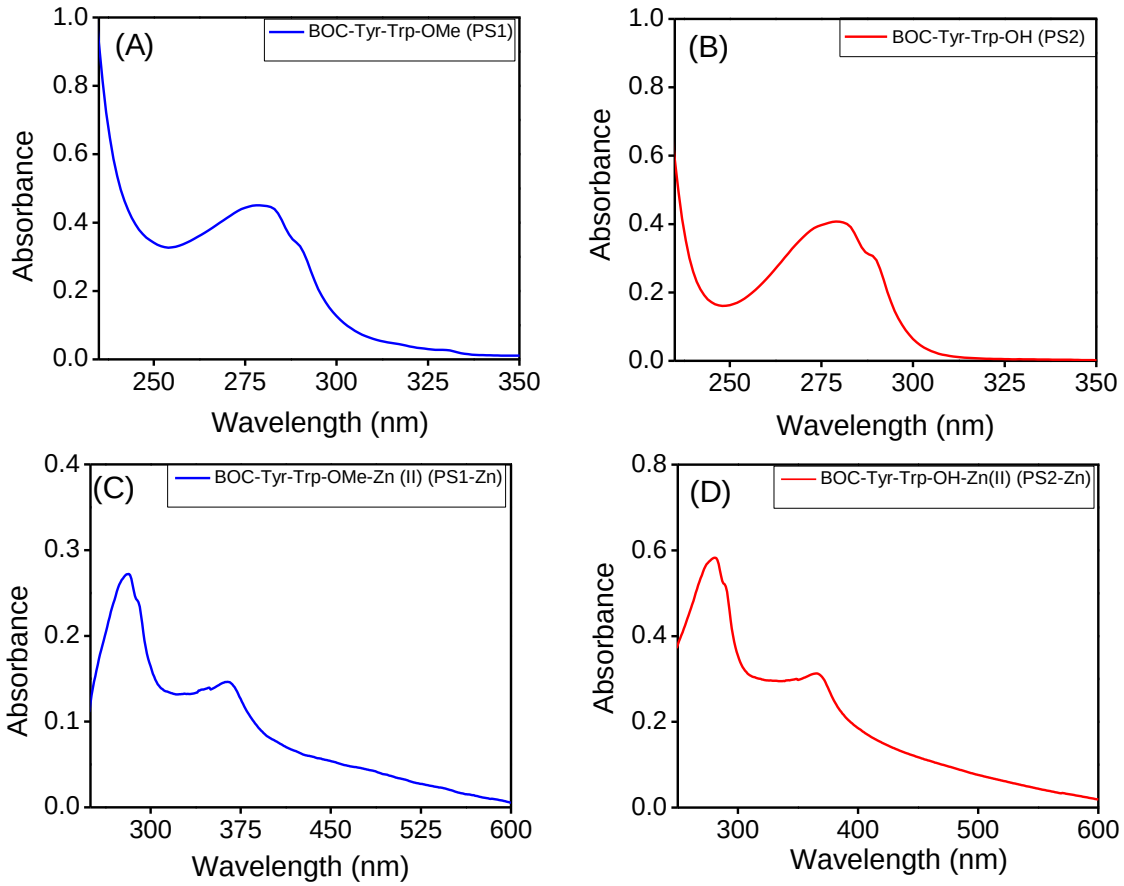


Figure S13. UV-Vis absorption spectra of (A) BOC-Tyr-Trp-OMe (**PS1**), (B) BOC-Tyr-Trp-OH (**PS2**), (C) BOC-Tyr-Trp-OMe (**PS1**)-Self-assembly with Zn (II) coordination and (D) BOC-Tyr-Trp-OH (**PS2**)-Self-assembly with Zn (II) coordination.

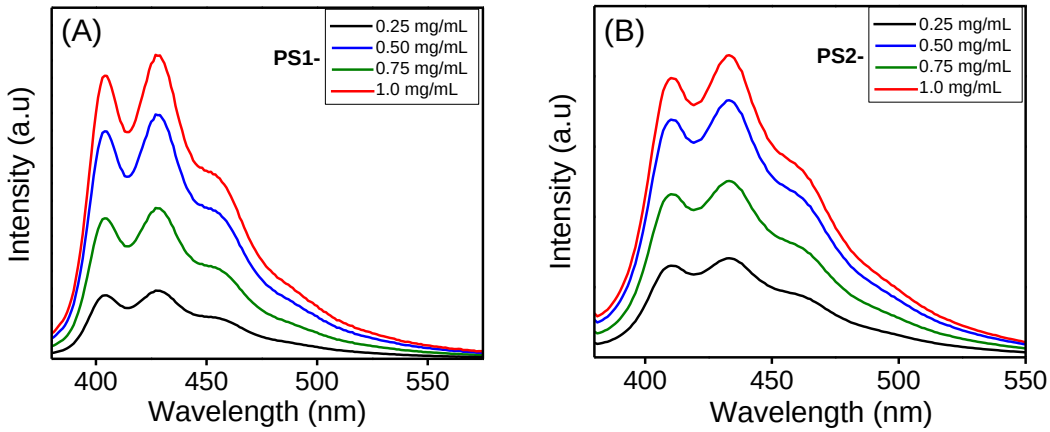


Figure S14. Dynamic fluorescence emission spectra on sequential dipeptide addition: On sequential dipeptide addition (A) **PS1** and (B) **PS2**, the emission intensity showed an increase with the increase in concentration of the dipeptides, when Zn(II) was in excess, the dipeptide concentration was found to affect only the total amount of DPNPs.

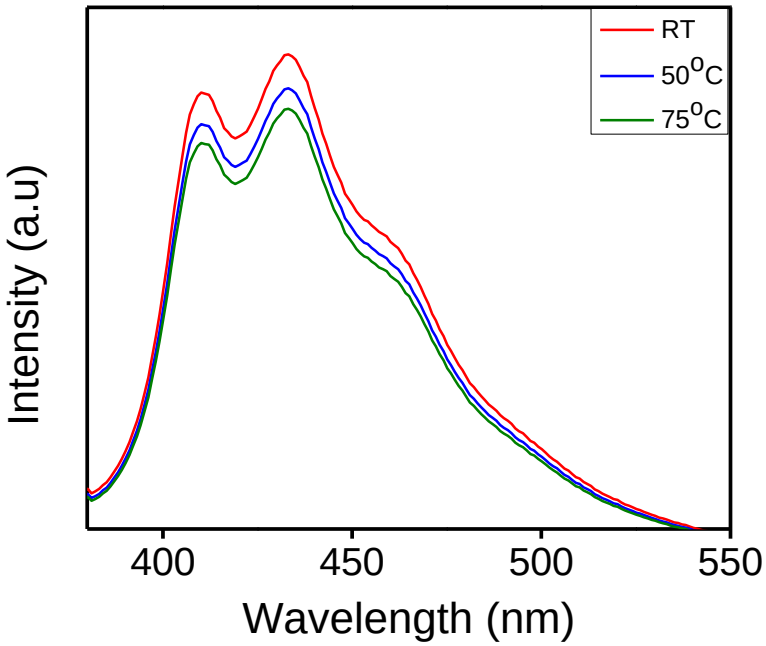


Figure S15. Fluorescence emission spectra of DPNPs formed by the self-assembly of **PS2** with Zn (II) coordination at room temperature, 50°C and 75°C. The emission intensity of the DPNPs remained stable from room temperature to 75°C.

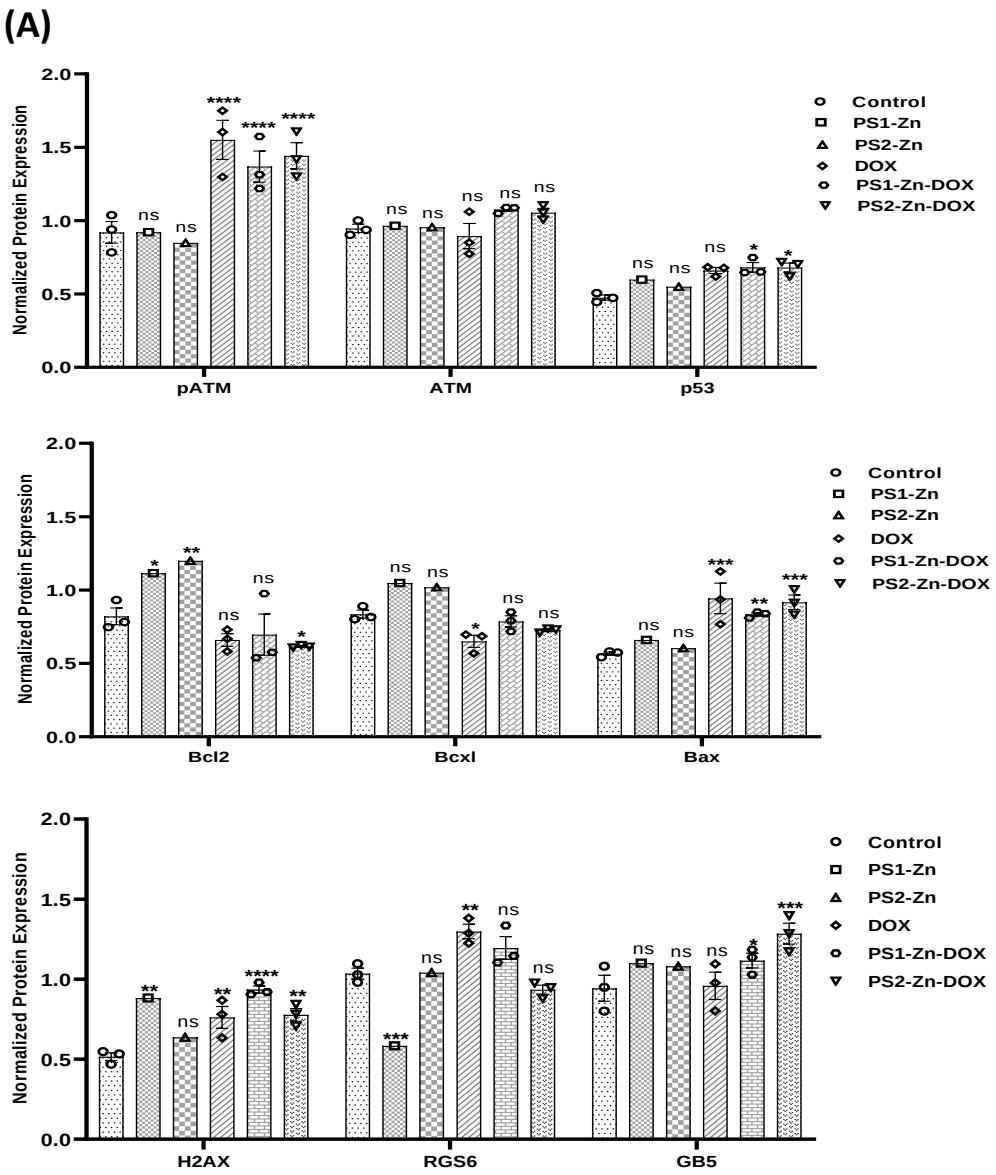


Figure S16: Effects of **PS1-Zn** and **PS2-Zn** and encapsulation of Dox on levels of different apoptosis related proteins in A549 cells. Protein expression in A549 cells was quantified in different treatment groups from blots in Figure 6. Data are presented as mean ± S.E. *$p<0.05$ compared to untreated control group. Data were analyzed by one or two-way ANOVA. Data are presented as mean ± S.E. [Fig 6 quantification]

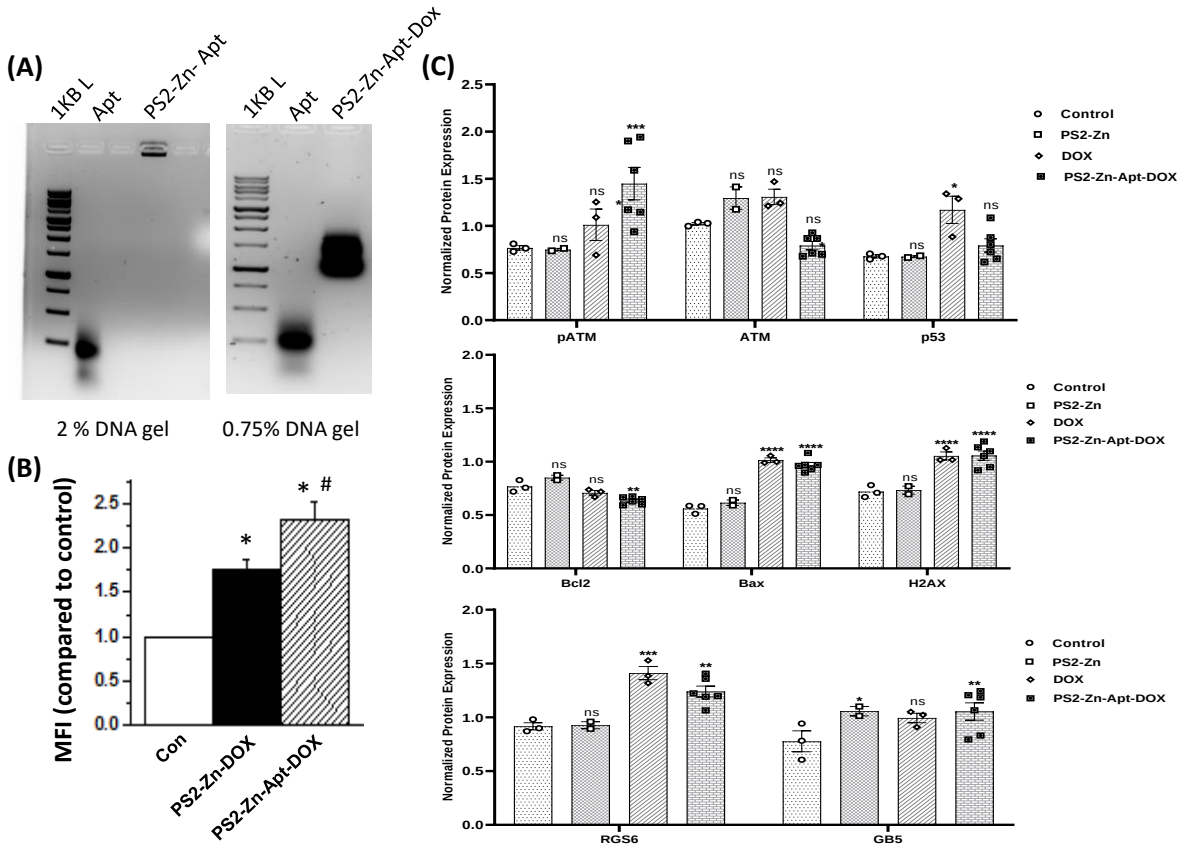


Figure S17: Effect of **PS2-Zn**-EPCAM aptamer on A549 cells. (A) Agarose gel-electrophoresis of **PS2-Zn**-Apt. Gels were visualized in UV light and the wells corresponding to ladder, free aptamer and **PS2-Zn** conjugated aptamers are indicated. (B) Spectrofluorometric analysis of cellular incorporation of **PS2-Zn**-Dox and **PS2-Zn**-Apt-Dox after a 24 h incubation in cells. Results are means + S.E. of multiple experiments (n=5, *p<0.01compared with control, #p<0.05 compared with **PS2-Zn** group). (C) Quantification of protein expression from immunoblots in Figure 7C. Data are presented as mean ± S.E. *p<0.05 compared to untreated control group. Data were analyzed by one or two-way ANOVA. Data are presented as mean ± S.E. [Fig 8 quantification].

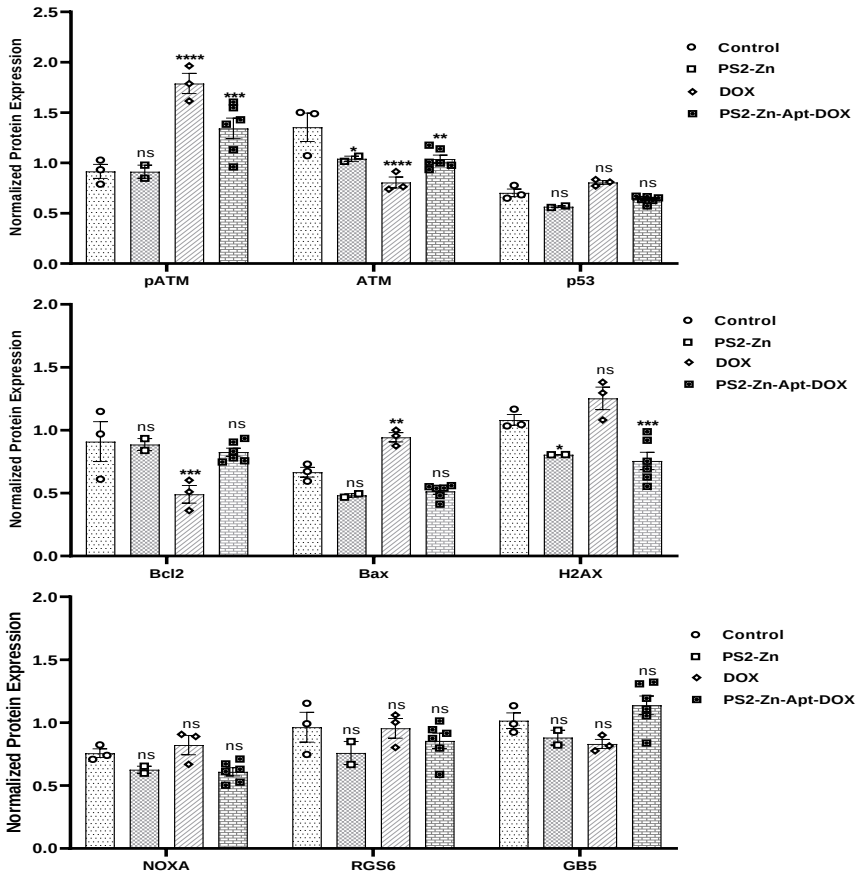


Figure S18: Effect of **PS2-Zn**-EPCAM aptamer on AC16 cells. Quantification of protein expression from immunoblots in Figure 7D. Data are presented as mean ± S.E. *p<0.05 compared to untreated control group. Data were analyzed by one or two-way ANOVA. Data are presented as mean ± S.E. [Fig 8 quantification]

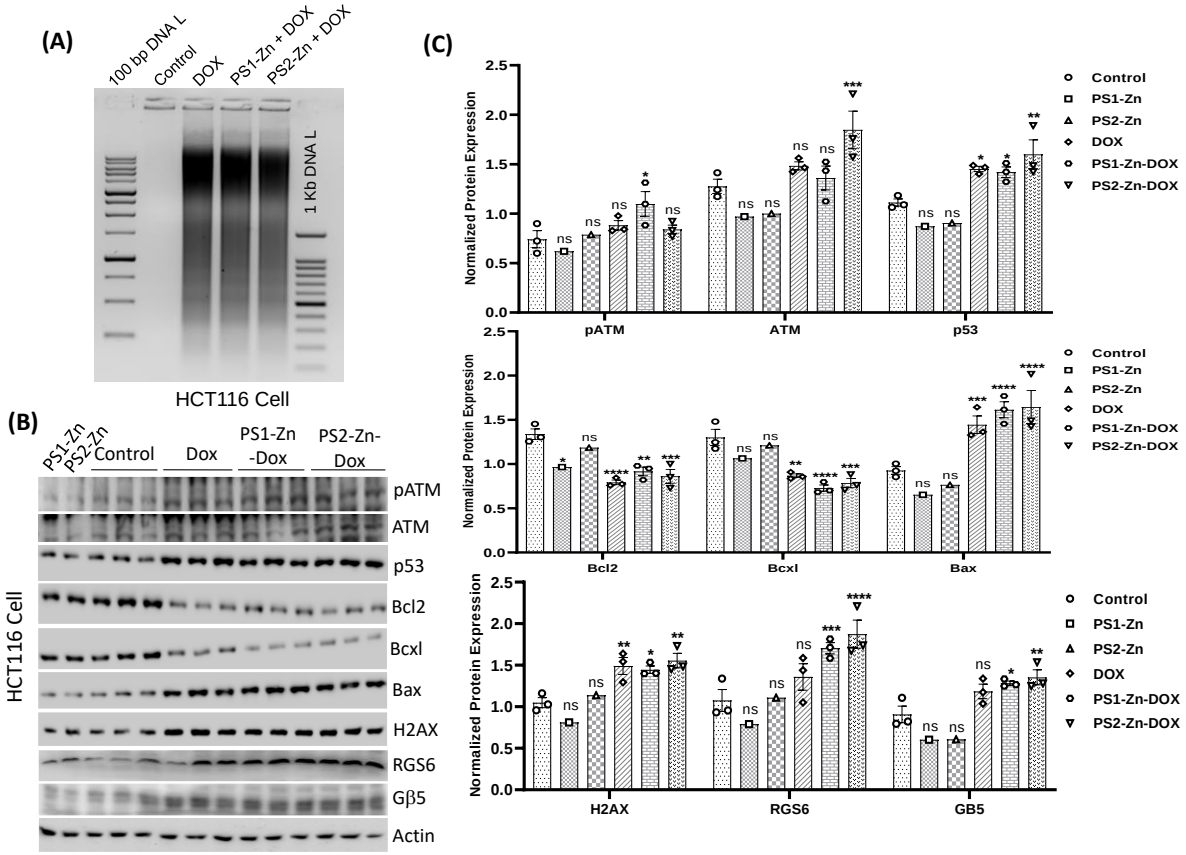


Figure S19: Effects of the test compounds on HCT116 cells. (A) **PS1-Zn**, **PS2-Zn** and DPNP encapsulated Dox induces DNA damage represented by DNA laddering. (B) **PS1-Zn** and **PS2-Zn** DPNPs encapsulated Dox altered expression of various apoptosis related proteins. β-Actin serves as a loading control for all immunoblots. (C) Quantification of protein expression from immunoblots in Figure S19A. Data is presented as mean ± S.E. *$p<0.05$ compared to untreated control group. Data were analysed by one or two-way ANOVA. Data are presented as mean ± S.E.