Dimension Switchable Auto-fluorescent Peptide based 1D and 2D Nano-assemblies and Their Self-influence on Intracellular Fate and Drug Delivery

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Methodology:

S1. Peptide Synthesis:

Fmoc-HCKF-OH was synthesized using Microwave assisted Automated Solid Phase Peptide Synthesizer (Liberty Blue CEM, Matthews, NC, USA). To start the synthesis, Fmoc-Phe-Wang resin was kept for swelling in sufficient amounts of dimethylformamide (DMF) for 1h. During peptide synthesis, Fmoc group was deprotected using 20 % piperazine in 10 % ethanol containing DMF. Fmoc-amino acid coupling was accomplished in DMF with microwave assisted heating using diisopropylcarbodiimide (DIC) as an activator and oxyma as the base. After synthesis, the resin was filtered followed by washing with dichloromethane and air drying. Cleavage of the peptide from the resin was done by stirring the peptide-loaded resin in trifluoroacetic acid (TFA)/thioanisole/1, 2-ethanedithiol/anisole (90: 5: 3: 2, v/v/v/v) for 3 h at room temperature. Thereafter, the cleaved peptide was precipitated from the TFA solution by dropwise addition to ice cold (-20°C) diethyl ether. The precipitated product was centrifuged at 7500 rpm for 15 min and further the pellet was washed with cold diethyl ether for at least three times. Then the peptide was kept for drying and the dried peptide was dissolved in 20 % glacial acetic acid and then lyophilized to be obtained in powdered form.

The peptide was analysed for purity using high performance liquid chromatography (HPLC). Analysis was performed using Waters HPLC system with Photodiode-array detector (PDA). For HPLC analysis, the dried peptide was injected into an analytical reverse phase C18 column at ACN-H_2O linear gradient of 5-95% with .1% TFA at a flow rate of 1 mL/min over 55 min. Further molecular weight of the peptide was analyzed using Mass Spectrometry (MS).

S2. Confirmation of disulphide bond formation in NSs using LCMS:

Initial confirmation of the disulphide bond formation in NS was done using HPLC/MS. HPLC was performed using a linear gradient of ACN/H_2O on a C18-reverse phase column as described above. A shift in HPLC peak or change in the retention time of the non-oxidized nanofibers (NFs) vs oxidized nanosheets (NSs) with their corresponding mass, confirmed the formation of disulphide bond.

S3. Ellman's test and fourier transform infrared spectroscopy (FTIR) analysis of NSs to confirm disulphide bond formation:

Elman's test was carried out to check the presence of -SH in the samples. Ellman's reagent, 5, 5'-dithio-bis-(2-nitrobenzoic acid), known as DTNB. It is a water-soluble compound utilized for quantifying available sulfhydryl groups. DTNB reacts with free sulfhydryl groups to produce a yellow-colored product, 2-nitro-5-thiobenzoic acid (NTB), that can be quantified using a UV-Vis spectrometer with a peak at 412 nm. First, DTNB stock (1M) solution was prepared in 50 mM sodium acetate and was diluted with Tris buffer (pH 8) and Milli Q water to prepare a working solution (60 µM). Test sample was added to the working solution. The solution was mixed properly, and UV-Vis spectra were recorded at 412 nm to check the presence or absence of free -SH groups (Shimadzu UV-2600).

In addition, the formation of disulphide bonds was also confirmed using FTIR. FTIR was performed using a Cary Agilent 660 IR spectrophotometer in the wavenumber ranging from

400-4000 cm^{-1}. Self-assembled nanostructures (NFs and NSs) were prepared and lyophilized in dry powder form. Then dried powder was used to record the spectra.

S4. Determination of multi-stimuli responsive behaviour of NFs and NSs

S4.1. *Confirmation of pH responsive behaviour of NFs and NSs:*

As Fmoc-HCKF-OH self-assembled to form NFs in water (pH 6), we further investigated the influence of change in media pH on the overall NFs morphology. 2 mM stock solution of NFs was first prepared in water and then the pH of the pre-assembled NFs solution was changed to pH 3 using HCl and pH 8 using NaOH. Following pH change, the solution was kept for 24 h incubation at room temperature and subsequently characterised using atomic force microscopy (AFM).

Similarly, as NSs were formed at pH 8. Then to determine their pH responsive behaviour, the pH of pre-assembled NSs was changed to pH 6 and pH 3 and finally the solution was kept for 24 h incubation at room temperature before being analysed using AFM.

S4.2. Determining *redox responsive behaviour of the nanostructures :*

Redox responsive behaviour of NFs and NSs was determined in presence of glutathione (GSH). 2 mM stock solutions of NFs and NSs were prepared and co-incubated with 10 mM of GSH (pH 4-5) for 24 h. However, after adding GSH to the nanosheets, the pH of the final solution was found to be around pH ~8. After incubation, AFM studies were performed to carry out morphological analysis of the nanostructures.

S4.3. Determination of *thermal responsive behaviour of the nanostructures:*

Thermal responsive behaviour of NFs and NSs were studied at varied temperatures (25°C, 40°C, 55°C and 80°C). Self-assembled solutions of NFs and NSs were prepared and kept in a

water bath at different temperatures. Samples were then drop casted on silicon wafer for being characterised using an AFM.

S5. Structural analysis of the nanostructures

S5.1. *UV-Vis absorption spectroscopy of the peptide nanostructures:*

The spectral properties of NFs, NSs and SNPs in water (2 mM) was monitored using a UV-Vis spectrophotometer (Shimadzu UV-2600).

S5.2. *Circular dichroism (CD) study of the peptide nanostructures:*

Secondary structural analysis of our self-assembled nanostructures (NFs, NSs and SNPs) was done using CD analysis (JASCO J-1500 Circular Dichroism Spectrometer, Easton, MD, USA). A quartz cell of 1mm path length was used to record the spectra and data pitch of 0.1 nm with a scanning speed of 50 nm/min was used. Each spectrum was the average of two consecutive scans. Secondary structural analysis was carried out using Spectra Manager II Spectroscopy Software.

S6. Fluorometric analysis of the peptide nanostructures

For fluorometric analysis, pre-assembled solutions of NFs, NSs and SNPs were added into a 1.0 cm path-length quartz cuvette and spectra were collected using a fluorimeter (FS5 Spectrofluorometer). The emission spectra from 300−600 nm were recorded at an excitation wavelength of 260 nm with a slit width of 2 nm.

S6.1. Determination of the effect of *addition of Zn (II) metal ions to the peptide nanostructures:*

Pre-assembled solutions of NFs, NSs and SNPs were incubated with zinc acetate solution (50 mM) at a peptide/metal ion concentration ratio 1:2 for 24 h. Further samples were analyzed using AFM and fluorimetry.

S6.2. *Determination of optical properties of the auto-fluorescent nanostructures:*

The photostability of the auto-fluorescent peptide nanostructures in presence or absence of Zn (II) was evaluated by subjecting them to UV light at 260 nm for 5 minutes at room temperature and their fluorescence emission spectra was recorded from 300-600 nm post every 5 minutes using a fluorimeter (FS5 Spectrofluorometer). Photostability of tryptophan was also determined for comparing the fluorescence properties of the nanostructures and tryptophan.

Quantum yield (QY) of the nanostructures was determined as per an earlier reported method.[1] Tryptophan was taken as standard (with the reported QY of 0.01). OD of both standard sample and self-assembled nanostructures (OD value between 0.01 and 0.1 nm) were determined at an excitation wavelength of 260 nm by using UV-Vis spectrophotometer and their fluorescence emission spectra (300-600 nm) was also recorded at an excitation of 260 nm by using a fluorimeter. Absolute value of QY of the auto-fluorescent nanostructures was calculated as per the following equation:

$$QYs = QY_R \frac{I_S A_R n_s^2}{I_R A_s n_R^2}$$

In the above-mentioned equation, subscripts S represent sample and R reference, QY represents the quantum yield, I represent the integrated fluorescence intensity, A is the OD of the fluorophore and n represents the refractive index of solvent.

The time-resolved photoluminescence studies of NFs, NSs and SNPs were performed using a picosecond time correlated single photon counting (TCSPC) system from Edinburgh instruments (Model: FL920). A diode laser was used with an excitation wavelength of 260 nm to excite the sample.

S7. Confocal microscopic studies of Zn (II) dopped auto-fluorescent nanostructures:

Zn (II) containing peptide nanostructures were further analysed using confocal microscopy. Studies were carried out using the confocal microscope Zeiss LSM 880 Model. Briefly, 10 x

diluted peptide samples (2 mM) were drop casted on glass slides and were observed under the microscope in the blue and green channels.

C6 glioma cells were seeded on cover slip embedded in 6-well plate at a density of 50000 cells/well and cultured for 24 h at 37°C. For observing nanostructures uptake, cells were treated with Zn (II) dopped auto-fluorescent nanostructures (NFs, NSs and SNPs) in serum free media and were incubated for 8 h at 37 °C. After incubation, cells were rinsed with phosphate buffer saline (PBS) and afterwards cells were fixed with 4% paraformaldehyde for 20 min at 37 °C. Cells were washed with PBS and coverslips were fixed on the glass slides to be imaged under a confocal microscope.

S8. Determination of uptake of the nanostructures in C6 cells using fluorescence-activated cell sorting (FACS) or flow cytometry analysis:

Further to quantify the uptake of auto-fluorescent nanostructures in C6 cells, FACS based studies were carried out. In brief, C6 cells were seeded in 6 well plates at a density of 50000 cells/well and the plate was incubated at 37 °C for 24 h. Further, particles (NFs, NSs and SNPs) dispersed in cell culture media were added to the cells and the cells were incubated at 37°C for 8 h. After incubation, cells were washed with PBS and were trypsinized. Cells were centrifuged at 1500 rpm for 5 min to pellet down and washed twice with 1X PBS. Finally, pellets were resuspended in PBS and analyzed by flow cytometry (BD, Aria Fusion) at corresponding wavelengths.

S9. Uptake studies of nanostructures performed in C6 spheroids using confocal microscopy:

C6 cells were seeded at a density of (5000 cells/well) in a 48-well plate coated with 2% (w/v) low-melting-temperature agarose to prevent cell adherence and adhesion. After that, the plate was incubated at 37 °C for 7 days. Media was subsequently replaced with fresh Dulbecco's

Modified Eagle Medium (DMEM) media after 2-3 days. 200-300 mm size and visible spheroids were formed after 5 days. After spheroid formation, a single spheroid was separated using a 1 mL tip and added to another fresh 96 well plate containing serum free DMEM media. Further spheroids were treated with the NFs, NSs and SNPs and were incubated at 37 °C for 24 h. After treatment, spheroids were carefully washed with PBS and were observed under the confocal microscope (Zeiss LSM 880 Confocal Microscope).

S10. Confocal microscopy-based uptake studies of the nanostructures in C6 cells performed in the presence of endocytosis and phagocytosis inhibitors:

In order to predict the cellular efficacy of the nanostructures, it is essential to understand their mechanisms of cellular uptake, which would likely determine their final sub-cellular fate and cellular localisation. To study the pathway-dependent uptake behaviour of the nanostructures, cellular uptake studies were carried out in presence of the cell uptake/transport inhibitors, cytochalasin D (cyt D) and chlorpromazine HCl (CPZ). Briefly, C6 cells were seeded on cover slip embedded in 6 well plates at a density of 50000 cells/well and kept in the incubator at 37 °C for 24 h. Cells were pre-treated with the inhibitors (10 μM) and incubated for 1 h at 37°C followed by PBS wash (three times). Cells were then co-incubated with NFs, NSs and SNPs either in the presence or absence of the inhibitors for 4 h. After 4 h of incubation cells were washed with PBS and fixed with 4% paraformaldehyde. For confocal imaging, cell laden cover slips were fixed on glass slides and observed under a microscope.

S11. FACS based cellular uptake studies of the nanostructures performed in the presence of endocytosis and phagocytosis inhibitors:

Further to gain deeper insights into the cellular uptake mechanism of the peptide nanostructures, FACS studies were performed in presence of the inhibitors. In brief, C6 cells were seeded in 6 well plates at a density of 50000 cells/well and incubated at 37 °C for 24 h.

Before being treated with the peptide nanostructures (NFs, NSs and SNPs), cells were pre-treated with inhibitors (10 μM) and incubated for 1 h at 37°C. After 1 h of incubation, cells were washed thrice with PBS. Cells were then co-incubated with NFs, NSs and SNPs either in the presence or absence of the inhibitors for 4 h. After incubation, cells were washed with PBS and further trypsinized. Then cells were centrifuged at 1500 rpm for 5 min and washed twice with PBS. Finally, pellets were resuspended in PBS and were analyzed in flow cytometry (BD, Aria Fusion) at corresponding wavelengths.

S12. Assessment of energy dependence of the uptake pathway of the peptide nanostructures by estimating the degree of their uptake at different temperatures

For investigating whether the uptake of the peptide nanostructures was an active or passive process, nanostructures' uptake studies in C6 cells were carried out parallelly at 4 °C and 37 °C. It is well known that some proteins or enzymes are sensitive to temperature, which further affect their active cellular uptake processes at lower temperatures.[2] Therefore, to further understand the detailed mechanism of cellular uptake of our peptide-based nanostructures', C6 cells were first seeded in two coverslips loaded 6-well plates, at a density of 50000 cells/well and kept for incubation at 37 °C for 24 h. Afterwards, cells were treated with NFs, NSs and SNPs and one plate was kept at 4 °C and other was kept at 37 °C for 8 h. After completion of the incubation period, cells were washed with PBS and fixed with 4% paraformaldehyde. After fixation, cells were imaged using confocal microscopy to analyze the effect of temperature on the uptake behaviour of the nanostructures.

S13. Lysosomal colocalization studies of the nanostructures performed in C6 cells using lysotracker red:

The ability of nanostructures for getting colocalized with cellular lysosomes in C6 cells was investigated with the dye, lysotracker red DND 99, which is a membrane-diffusible red

fluorescent dye that accumulates in acidic organelles such as lysosomes.[3] For the study, C6 cells were cultured on coverslips embedded in 6 well plates at a density of 50000 cells/well. Cells were incubated with NFs, NSs and SNPs in serum free media for 4 h. Afterwards culture media was removed and fresh phenol free media containing lysotracker red (100 nM) was added for 30 min. After 30 min of incubation, cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min at 37 °C. Finally, cover slips were fixed on glass slides to image cells under a confocal microscope. Colocalization analysis was performed using Zeiss Zen Black Software. Pearson's correlation coefficient (PCC) was used as a key parameter to measure the colocalization of nanostructures with lysosomes.

S14. Drug loading and release studies in the peptide nanostructures:

NFs, SNPs and NSs were first prepared at a concentration of 2 mM and were incubated with 1 mg of Doxorubicin hydrochloride (Dox) at room temperature with gentle shaking for 24 h to produce Dox loaded self-assembled nanostructures. After incubation, samples were centrifuged at 10000 rpm for 20 min to remove unbound Dox molecules. Amount of Dox was deduced from sample absorbance taken at 485 nm against a linear fitted Dox standard curve. Dox encapsulation efficiencies in the nanostructures were calculated as per the following equation:

$$PE = \ (IR - FR)/IR \ \times 100$$

Here, PE represents percent encapsulation, IR represents initial Dox absorbance and FR represents final Dox absorbance in the formulations.

Further for the Dox release studies, 1 mL solution of Dox-loaded nanostructures was dialyzed against 50 mL of phosphate buffer saline (PBS with pH 7.4) under constant stirring. At different time intervals, 1000 µL of the dialysates from each sample was collected. The release media were replaced with the same volume of fresh PBS after each sampling. The absorbance of the

collected samples was taken at 485 nm. The concentration of Dox in the collected samples was also determined using the same above-mentioned standard curve.

S15. Cytotoxicity of the peptide nanostructures determined in C6 cells:

Viability of C6 glioma cells were assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test. First, C6 cells were seeded in 96 well plates at a density of 10000 cells/well in complete DMEM medium and kept in an incubator at 37 °C for 24 h to properly adhere. After 24 h of incubation, media were removed and fresh media of 200 µL were added to the wells containing different formulations (only Dox, NFs-Dox, NSs-Dox and SNPs-Dox) at varied concentrations (2.5, 5, 10, 15, 20 µg/mL). After incubation of 24 h at 37°C, 20 µL of MTT reagent (5 mg/mL) was added to each well and the plates were further kept for incubation of 4 h at 37 °C. After this, the MTT containing media was aspirated thoroughly and 100 µL of DMSO was added to each well for dissolving the formazan crystals. Next the absorbance of the solution was measured at 570 nm with a microplate reader (Tecan Multimode).

S16. Cell migration study:

C6 glioma cells were seeded onto a 6 well plate at a density of 1×10^5 cells/mL and incubated for 24 h. Wounds were made by scratching the centre of each well using 200-µl sterile tip to form a cross. This was followed by nano formulations treatment (NFs-Dox, NSs-Dox and SNPs-Dox) at a concentration of 10 µg/ml for an additional 24 h. Further the images of the migrated cells into the cell-free scratch area were captured using an inverted microscope at different time points i.e., 0 h and 24 h. Quantitative analysis was done using ImageJ software.

Results:

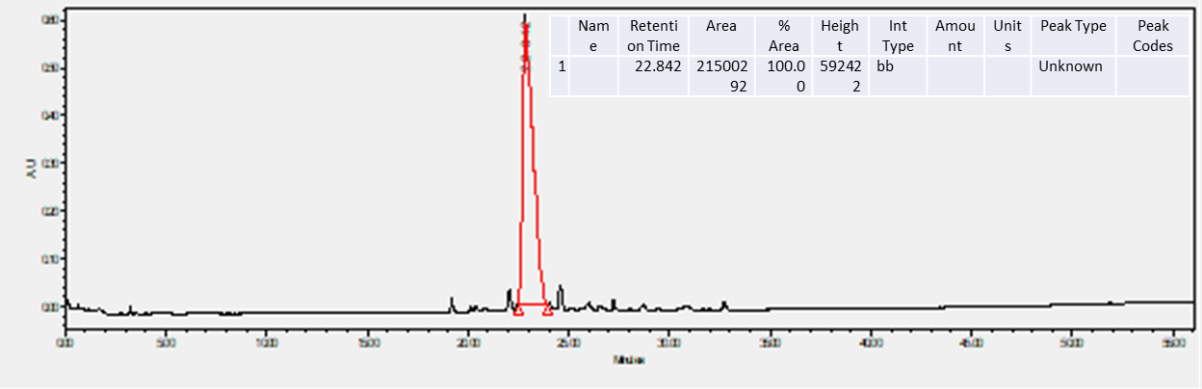

Fig. S1: HPLC chromatogram of Fmoc-HCKF-OH.

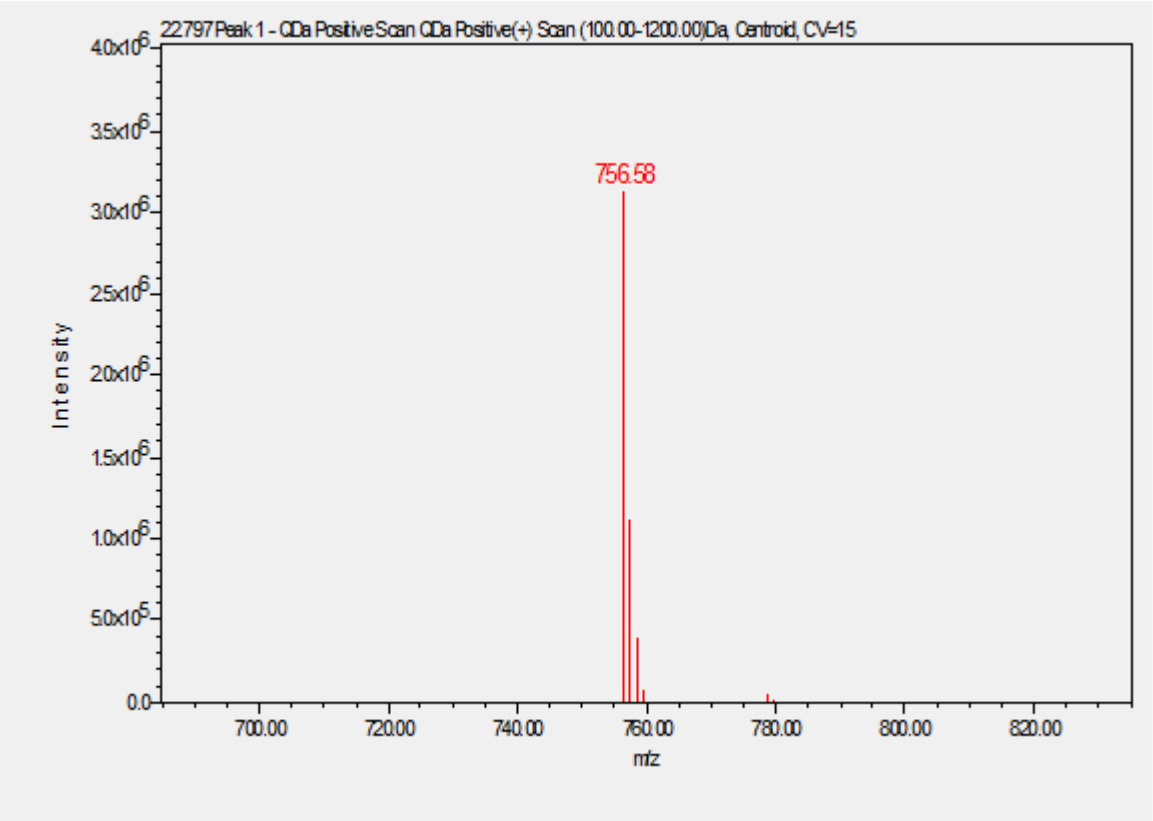

Fig. S2: Molecular weight (m/z ratio) of Fmoc-HCKF-OH determined by mass spectrometric analysis.

Furthermore, the purity of the peptide was calculated using Waters Empower software as shown in **Fig S3**. The peptide used for this study was greater than equal to 96% pure.

	Name	Retention Time (min)	Purity1 Angle	Purity1 Threshold	PDA/FLR Match1 Spect. Name	PDA/FLR Match1 Angle	PDA/FLR Match1 Threshold	PDA/FLR Match1 Lib. Name	Area (µV*sec)	% Area	Height (µV)
1		19.187							119570	0.45	21272
2		22.063							340198	1.29	47545
3		22.846							25527486	96.45	707637
4		24.586							429891	1.62	50404
5		27.213							48562	0.18	12164

Fig. S3: Purity of the peptide calculated by area under the curve method through HPLC Waters Empower software. Results showed the peptide to be more than 96% pure.

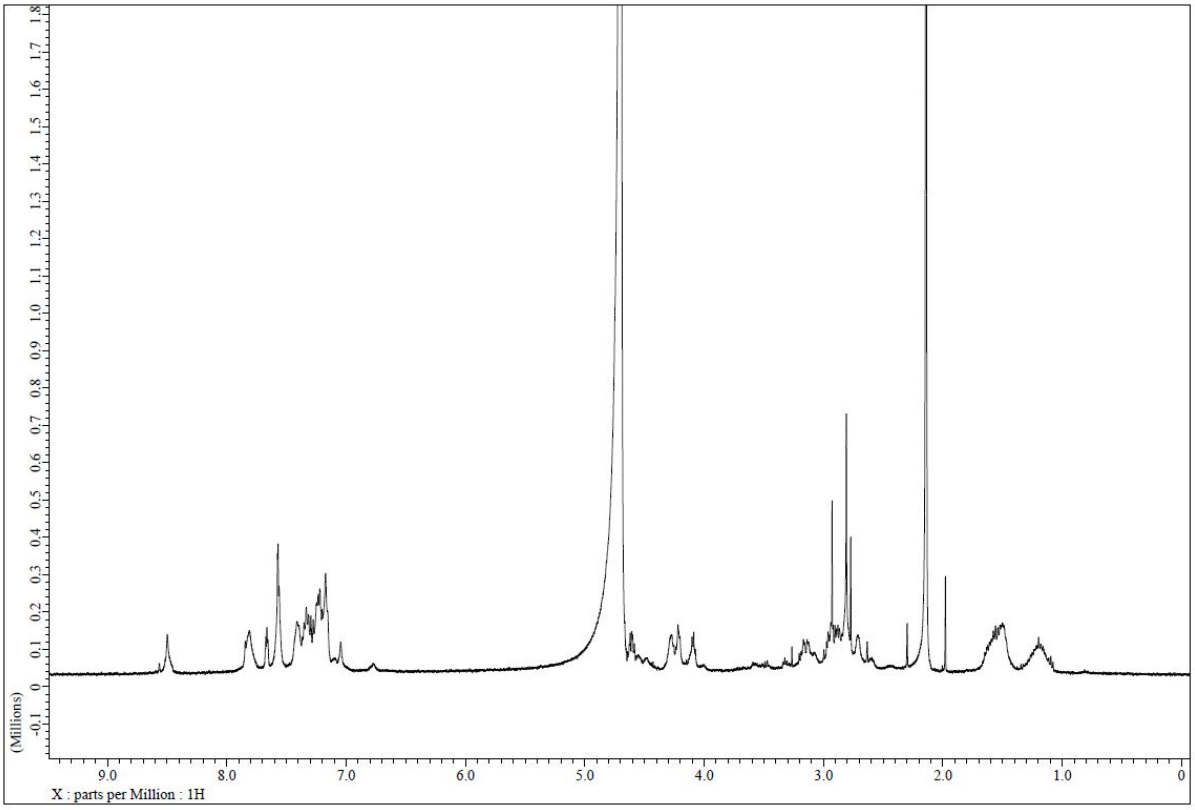

Fig. S4: ^{1}H NMR (450 MHz, DEUTERIUM OXIDE) of the peptide. δ = 8.57 - 8.51 (m, 1 H), 7.93 - 7.79 (m, 2 H), 7.75 - 7.68 (m, 1 H), 7.67 - 7.56 (m, 3 H), 7.50 - 7.44 (m, 2 H), 7.42 - 7.14 (m, 10 H) 4.67 - 4.64 (m, 1 H), 4.38 - 4.23 (m, 4 H), 4.19 - 4.05 (m, 2 H), 3.28 - 3.05 (m, 3 H), 3.01 - 2.96 (m, 2 H), 2.93 - 2.82 (m, 4 H), 2.79 - 2.75 (m, 1 H), 1.71 - 1.55 (m, 4 H), 1.34 - 1.15 (m, 3 H).

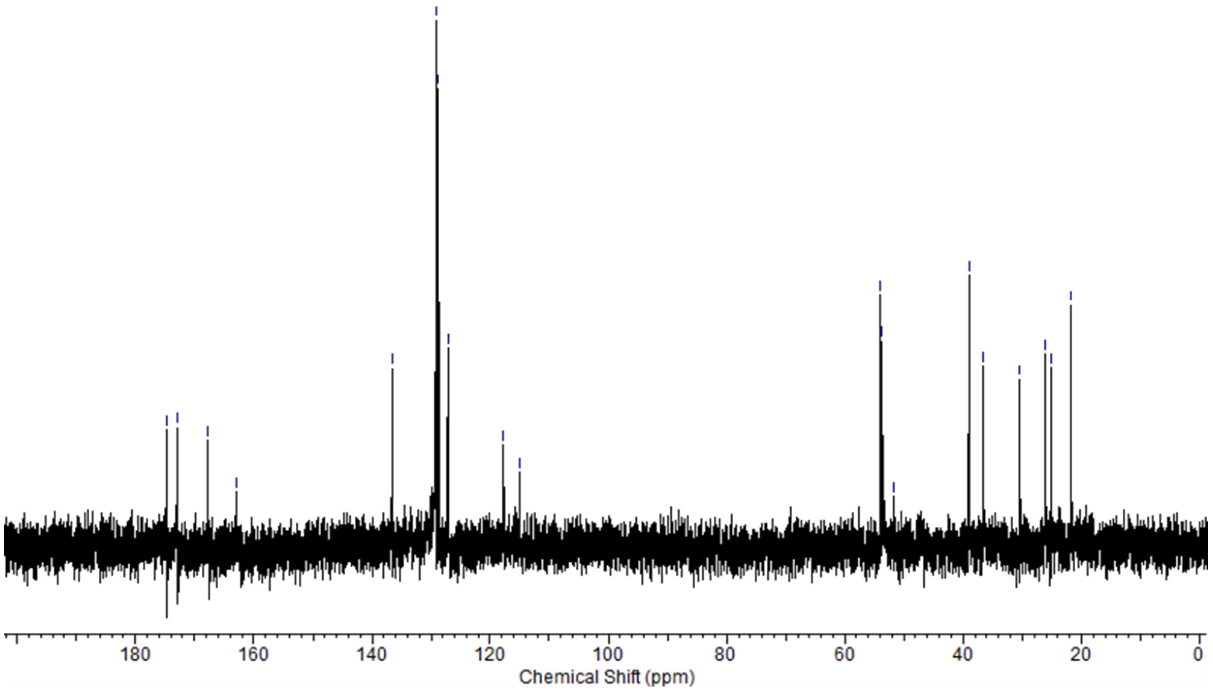

Fig. S5: ^{13}C NMR (101MHz, DEUTERIUM OXIDE) of the peptide. δ = 174.7, 172.8, 167.6, 163.0, 136.5, 129.2, 128.7, 127.1, 117.8, 114.9, 54.1, 54.0, 53.7, 51.8, 39.1, 36.6, 30.5, 26.2, 25.0, 21.8.

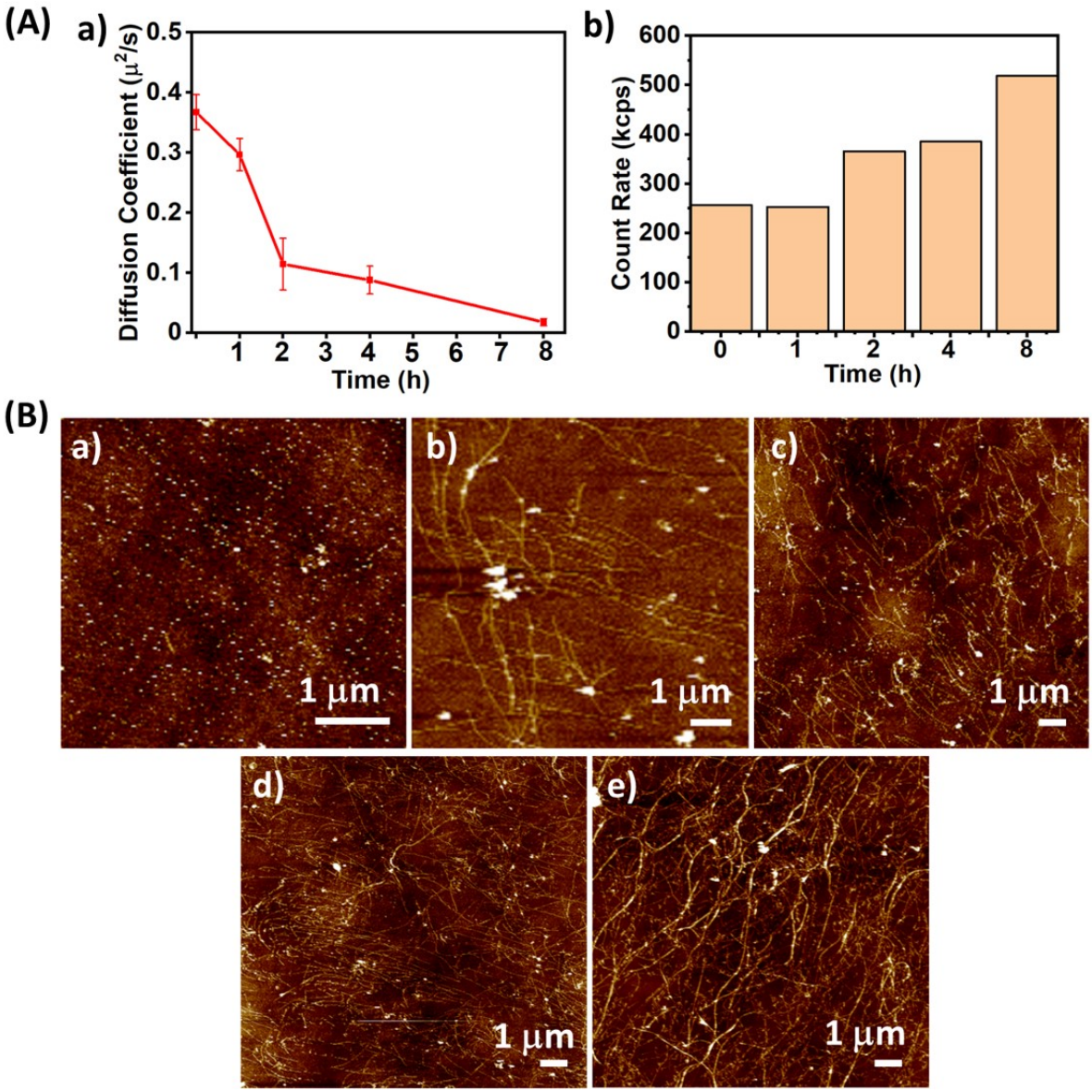

Fig. S6: Kinetics of formation of self-assembled NFs. (A) (a) Diffusion coefficient of self-assembled nanostructures obtained at different time points determined using DLS (b) Counts per seconds of nanostructures, determined at different time points using DLS (B) Morphological analysis of self-assembled nanostructures determined at different time points, 0 h, 1 h, 2 h, 4 h and 8 h (a-e) respectively using AFM.

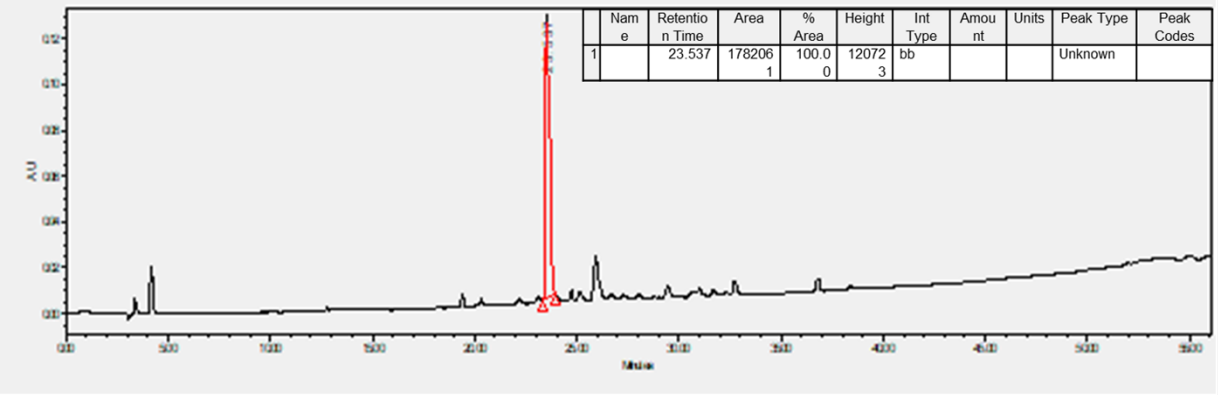

Fig. S7: HPLC chromatogram of oxidized peptide-NSs.

The confirmation of disulphide bond formation among the peptide monomers was done using HPLC. HPLC chromatogram of the oxidised peptide demonstrated a retention time of 23.53 min as compared to 22.84 min retention time obtained for the unoxidized NFs (**Fig. S7**). This increased retention time confirmed the formation of S-S bond in NSs which was due to increase in the hydrophobicity of the structure.

Confirmation of S-S formation in the NSs using Ellman's test and FTIR analysis:

One of the most important functions of thiols is the formation of disulphide bonds through an oxidation reaction at basic pH. We assumed that the morphological transition from NFs to NSs was due to the formation of disulphide bonds in the cysteine residue after the peptide was purged in the presence of pure O_2 at basic condition. Formation of disulfide bonds in the peptide NSs, were validated by carrying out Ellman's test. Ellman's reagent 5, 5'-dithiobis [2-nitrobenzoic acid] (DTNB) was used to determine the presence of sulfhydryl groups in a molecule as it reacts with the sulfhydryl groups to produce a colored product that can be quantified by taking absorbance at 412 nm as shown in **Fig. S8, A**. Ellman's test was carried out on O_2 purged NSs which was expected to have S-S bond after oxidation. Unpurged Fmoc-HCKF-OH (formed NFs) was taken as control. It was clearly confirmed that oxidation led to the formation of disulphide bond (S-S) among the cysteine residues of the peptide NSs as

evident from the colorless solution **Fig. S8, A.** Whereas yellow color was observed in case of NFs which can be due to the -SH moiety.

Further, the presence of a disulphide bond (S-S) in NSs was also confirmed using FTIR spectroscopy (**Fig. S8, B**). NSs did not show any peak at 2520 $cm^{-1,}$ which is generally attributed to the presence of free -SH group.[4] However, the FTIR spectrum of the control NFs showed a clear characteristic peak for -SH groups at 2520 cm^{-1}. These results indicated the formation of a disulphide bond in the self-assembled NSs at pH 8.

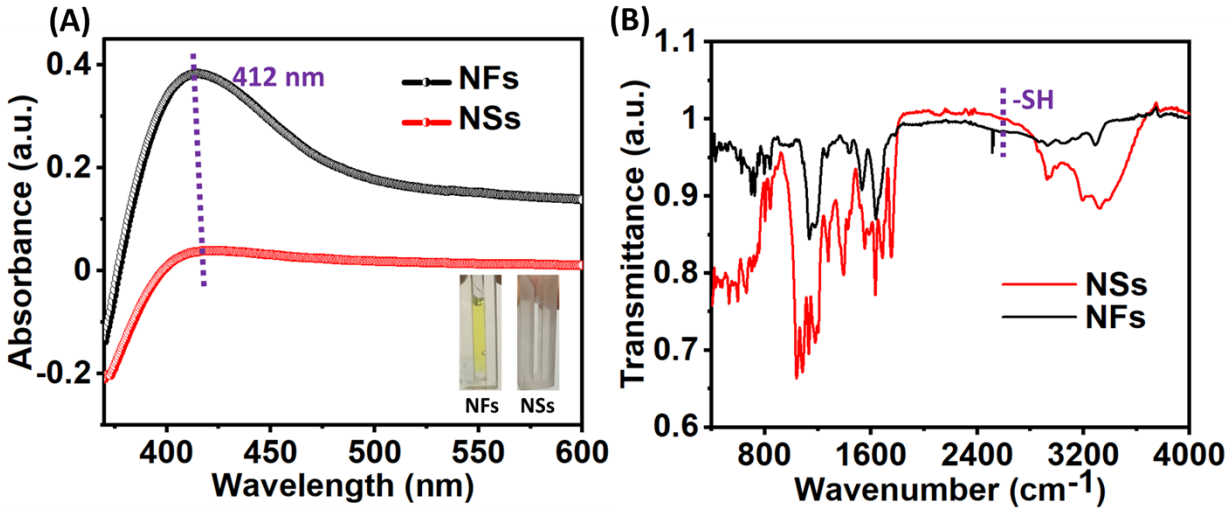

Fig. S8: Confirmation of S-S disulphide bond formation in NSs using (A) Ellman's test and (B) FTIR spectroscopy.

UV-Vis spectroscopy of nanostructures:

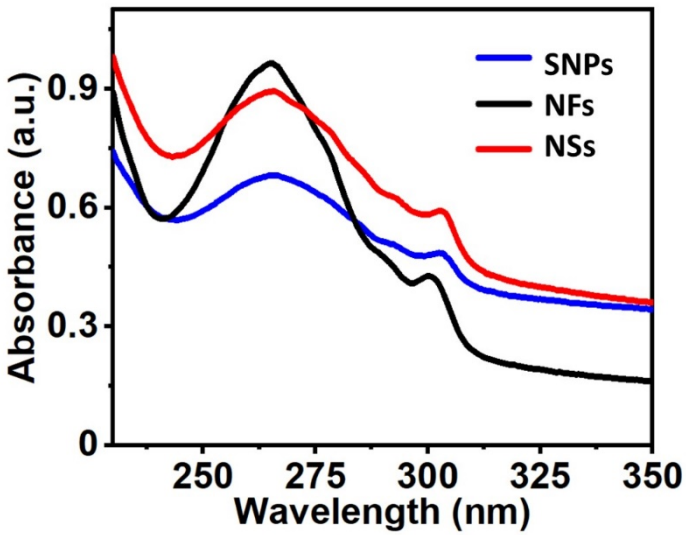

Fig. S9: UV-Vis spectra for NFs, NSs and SNPs showing two peaks at 260 nm and 300 nm.

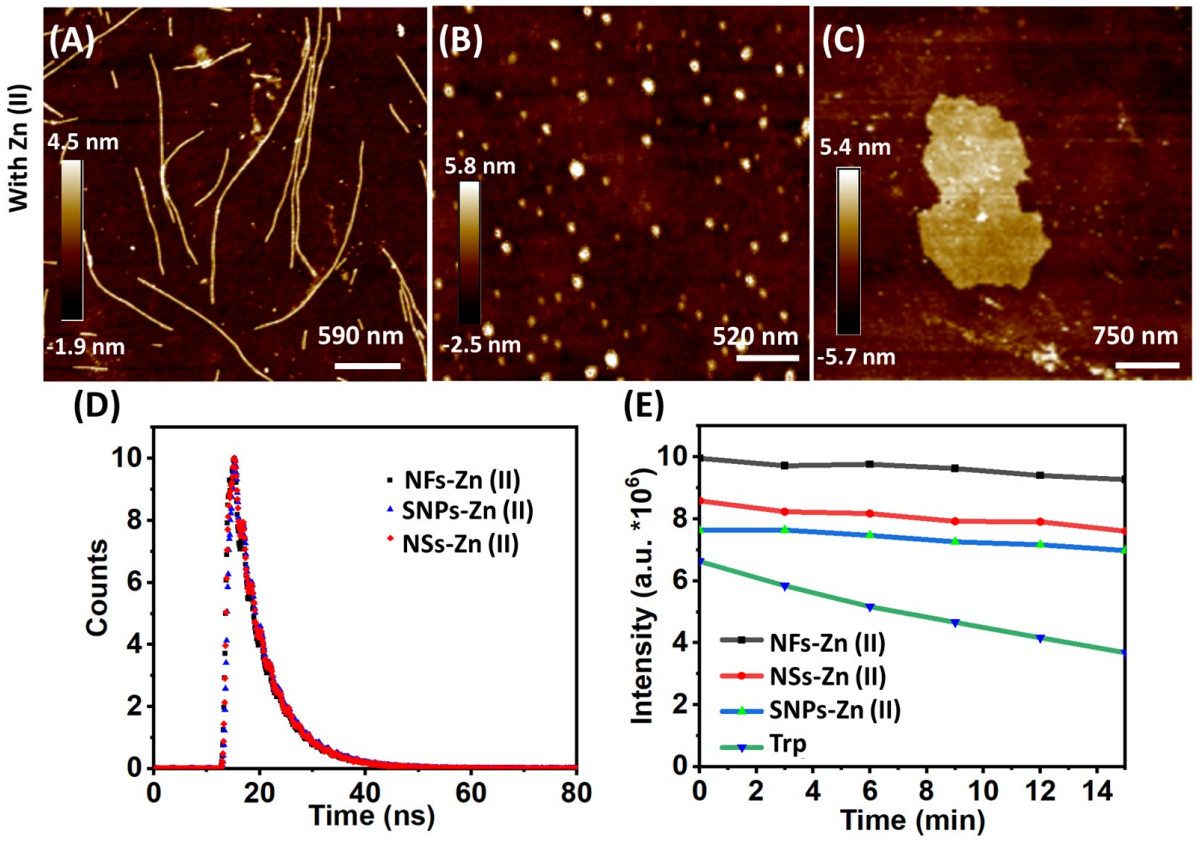

Fig. S10: (A-C) Morphological characterization of Zn (II) doped nanostructures carried out using AFM. (D)Time resolved emission spectra of the Zn (II) dopped nanostructures determined using a picosecond time correlated single photon counting system. (E) Evaluation of the Photostability of Zn (II) dopped nanostructures and its comparison with tryptophan. Samples were irradiated once in three minutes.

Cytocompatibility studies of bare nanostructures:

Briefly, C6 cells were treated with the nanostructures at different concentrations (2.5, 5, 10, 15 and 20 µg/mL) for 24 h. As shown in **Fig. S11,** it was observed that the cell viability of C6 cells was retained upto 90% after being treated with bare nanostructures; which confirmed the cytocompatibility of peptide-based nanostructures.

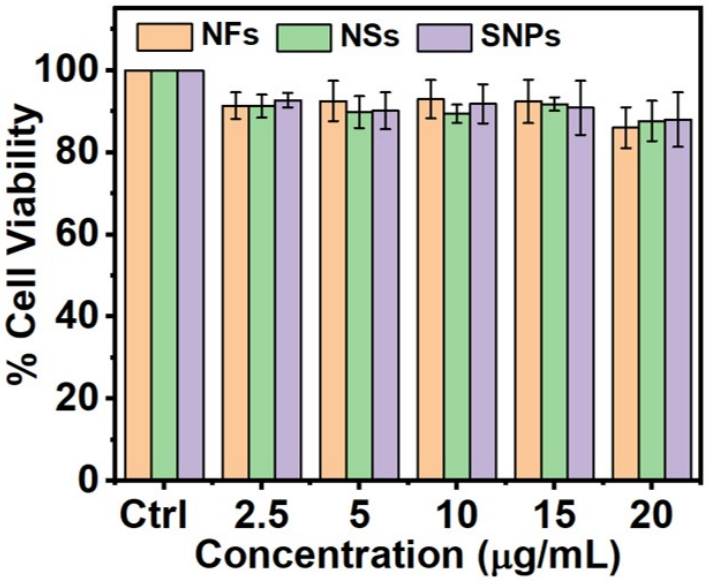

Fig. S11: Cytocompatibility of bare nanostructures; NFs, NSs and SNPs in C6 cells after 24 h.

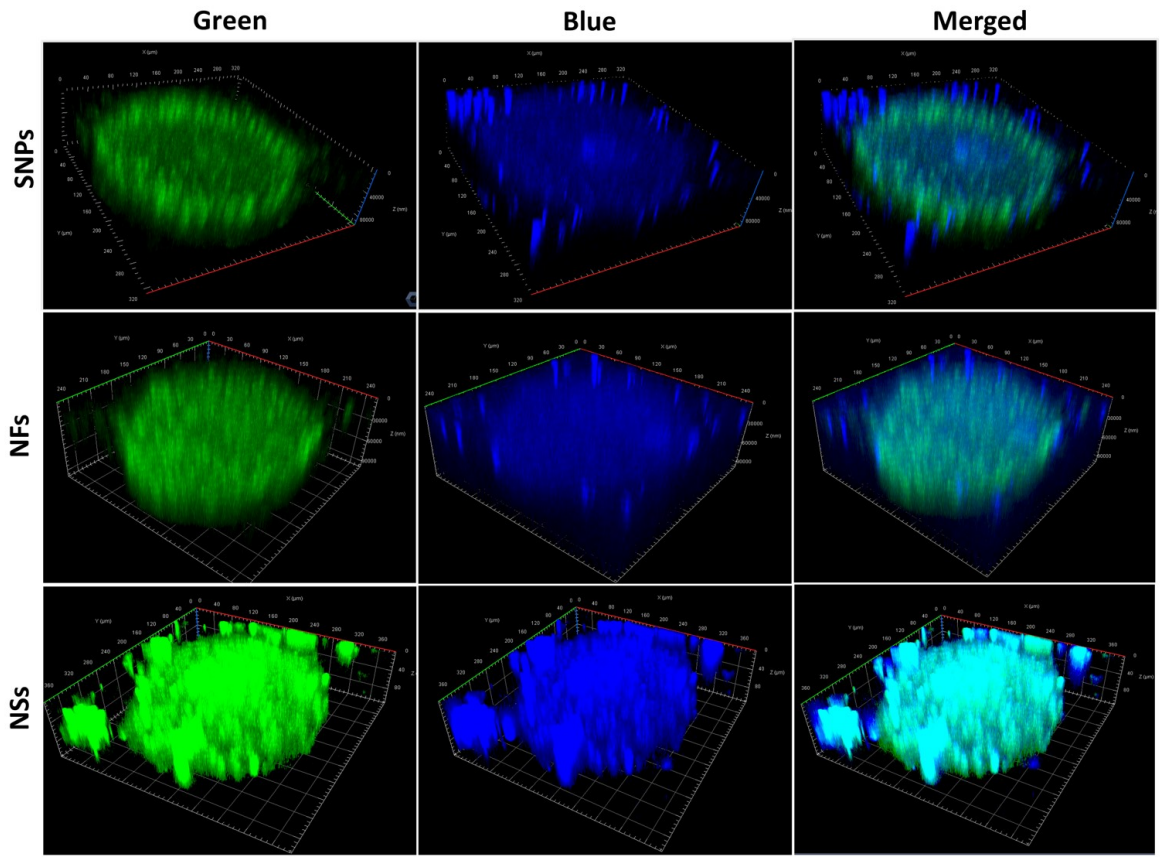

Fig. S12: 3D confocal microscopic images of C6 spheroids determined after they were incubated with the nanostructures.

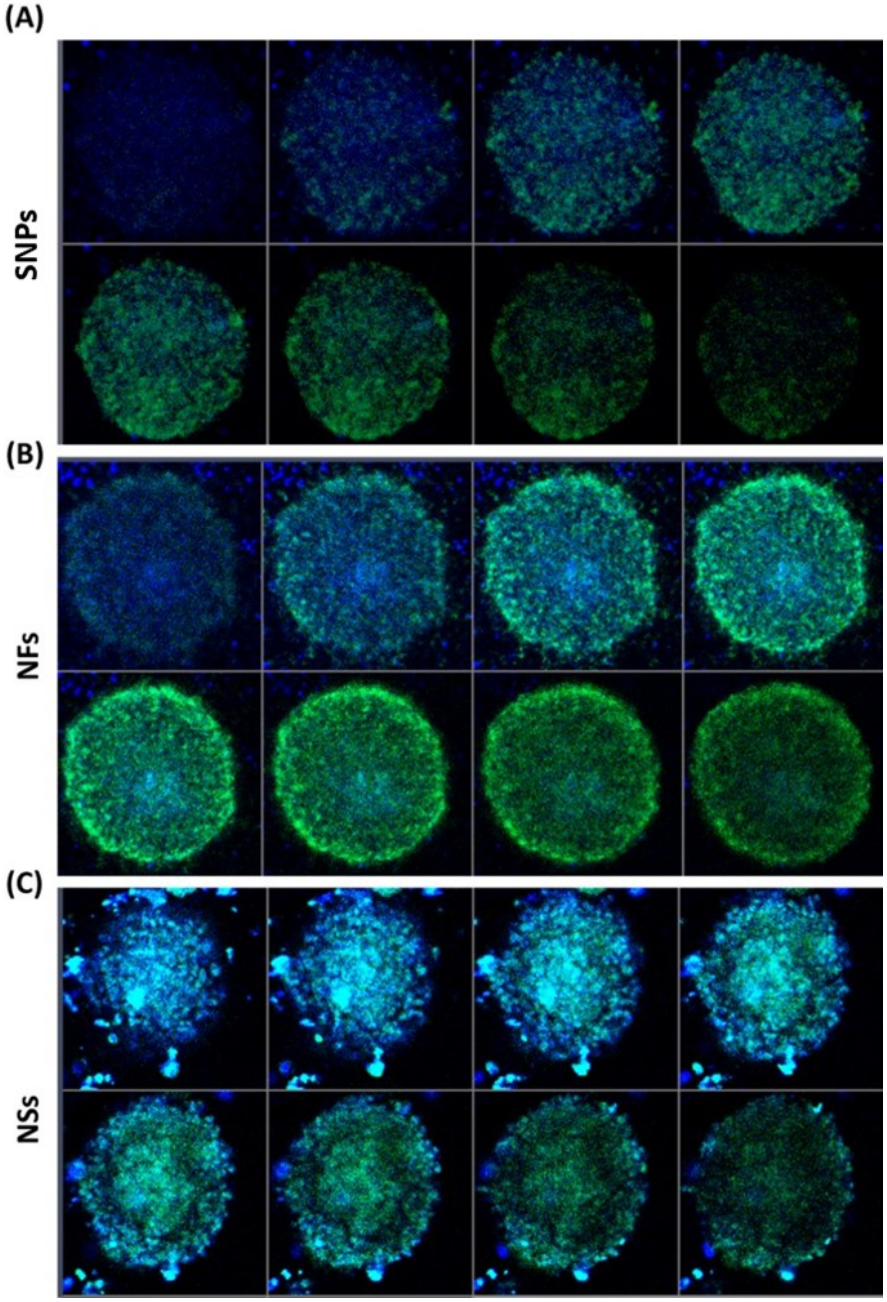

Fig. S13: Z-stack confocal microscopic images of C6 tumor spheroids incubated with the peptide nanostructures.

References:

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