Electronic Supplementary Information

Matrix metalloproteinase targeted peptide vesicles for delivering anticancer drugs

Debmalya Bhunia[a], Krishnangsu Pradhan[a], Gaurav Das[a,b], Subhajit Ghosh[a], Prasenjit Mondal[a,b], Surajit Ghosh*[a,b]

1. Organic and Medicinal Chemistry Division, CSIR-Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Road, Kolkata-700032, India
2. Academy of Scientific & Innovative Research (AcSIR), CSIR-IICB Campus, 4, Raja S. C. Mullick Road, Kolkata-700032, India
   Correspondence author: sghosh@iicb.res.in

Experimental Section

Materials: All the fmoc protected amino acids and wang resin were purchased from Novabiochem (MERCK). O-(Benzotriazol-1-yl)-*N, N, N′, N′*-tetramethyluronium hexafluorophosphate (HBTU), piperidine, diisopropylethylamine (DIPEA), diethylether, dimethylsulfoxide (DMSO), methanol and trifluoroacetic acid (TFA) were procured from Spectrochem. Phenol, dichloromethane (DCM), ethanedithiol (EDT), hydrogen peroxide (30% solution), acetone and *N, N′*-dimethyl formamide (DMF) were purchased from Merck. TritonX-100 was brought from SRL. *N, N′*- Diisopropylcarbodiimide (DIC), 5(6)-carboxyfluorescein, 5-diphenyltetrazolium bromide (MTT), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), doxorubicin hydrochloride, dulbecco's modified eagle's medium (DMEM), trypsin-EDTA solution, DMSO for cell culture and formaldehyde solution (molecular biology grade) were procured from Sigma Aldrich. Neutravidin and fetal bovine serum (FBS) were purchased from Invitrogen. MMP9 (enz-438-b) was procured from PROSPEC. Annexin V and propidium iodide (PI) apoptosis detection kit were bought from Santa Cruz Biotechnology. Bisbenzimide H 33258 (Hoechst) was purchased from Calbiochem. Cover glass bottom dishes were purchased from SPL. For purification, we used shimadzu HPLC system with C-18 semi preparative reverse phase column. Pure product was lyophilized in Vertis 4K freeze drier after column purification. HPLC grade water and acetonitrile were purchased from J. T. Baker. All the chemicals were used without further purification.

Cell culture: Human breast carcinoma (MCF7), human epithelioid cervix carcinoma (HeLa), human lung carcinoma (A549) and non-tumorigenic epithelial cell line from breast (MCF10A) cells were purchased from National Centre for Cell Science (NCCS) Pune, India. Cells were cultivated in 5 % CO_2 incubator at 37 ^{o}C using DMEM containing fetal bovine serum (FBS) (10 %), kanamycin sulphate (110 mg/L), penicillin (50 units/mL) and

streptomycin (50 µg/mL) in our lab. Trypsin-EDTA (1X) solution was used for cell detachment during cell splitting.

Docking: Autodock-Vina software version 1.1.2 was used for blind docking [1]. 50×82×68 affinity grid box was centered on the receptor matrix metalloproteinase 9 (MMP9) (PDB ID:1L6J) [2] for docking with HWGF peptide.

Methods:

Synthesis of HWGF peptide: 300 mg of wang resin was placed in a peptide vessel and swelled for overnight in DMF-DCM (1:1) solvent. Five equivalent of excess fmoc protected phenylalanine, glycine, tryptophan and histidine were coupled successively followed by fmoc deprotection using 20% piperidine solution in CEM microwave peptide synthesizer (Liberty 1). Coupling and deprotection steps were maintained for eight and five minutes respectively. *N, N′*-Diisopropylethylamine (DIEA) and HBTU were used as an activator base and activator respectively. DMF was used as solvent. After that, peptide attached resin was washed by DMF and DCM solvent. Prepared peptides were cleaved by standard resin cleavage cocktail solution containing 92.5% trifluoroacetic acid (TFA), 2.5% milli Q water, 2.5% EDT and 2.5% phenol. Resin attached peptide was kept for 3 h containing peptide cleavage solution in a peptide vessel on shaker (Labnet international). Then, TFA was removed from the filtrate by nitrogen gas flow. Remaining filtrate was added gradually to the cold diethyl ether solvent to ensure complete precipitation and then it was separated by centrifugation. Peptide was purified by using C-18 reverse phase HPLC column and was confirmed by MALDI-TOF mass spectrometry, ^{1}H and ^{13}C NMR respectively.

Characterization of HWGF peptide: ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 2.89-3.17 (6 H, m) 4.05 (3 H, d, *J*=5.12 Hz) 4.40-4.52 (1 H, m) 4.59-4.68 (1 H, m) 6.94-7.02 (1 H, m) 7.07 (1 H, t, J=7.50 Hz) 7.13-7.39 (7 H, m) 7.65 (1 H, d, J=7.68 Hz) 8.27 (1 H, d, J=8.05 Hz) 8.68-8.78 (2 H, m).

^{13}C NMR (150 MHz, DMSO-d_6) δ ppm 26.91 ,28.12, 37.13, 42.06, 51.51, 53.93, 54.18, 109.71, 111.75, 115.51, 118.41, 118.73, 118.81, 121.43, 124.27, 126.93, 127.41, 128.63, 129.48, 134.85, 136.45, 137.65, 158.72, 158.93, 167.75, 168.94, 172.37, 173.25.

Synthesis of fluorescein-HWGF peptide: 150 mg wang resin was placed in a peptide vessel and swelled for overnight in DMF:DCM solvent (1:1). After that, fmoc-Phe-OH, fmoc-Gly-OH, fmoc-Trp(boc)-OH and fmoc-His(trt)-OH were coupled successively followed by fmoc deprotection by 20% piperidine solution in CEM microwave peptide synthesizer. Coupling and deprotection steps were continued for 8 min and 5 minutes respectively. After complete synthesis of HWGF tetrapeptide attached with wang resin, 5(6)-carboxy fluorescein (3 equivalent excess) was covalently coupled on the N-terminal of HWGF tetrapeptide for 30 min by nitrogen gas bubbling. *N, N′*-Diisopropylethylamine (DIEA) and HBTU were used as activator base and activator respectively for all the coupling steps. After complete synthesis of 5(6)-carboxyfluorescein attached HWGF tetrapeptide, resins were washed thoroughly with DMF and DCM solvents. Then it was cleaved by standard resin cleavage cocktail solution

(mentioned in previous procedure) for 3 h. After TFA removal by N_2 gas flow, filtrate was collected and precipitated out in the cold diethyl ether. Finally, synthesized peptide was separated out by centrifugation (5000 rpm, 5 min) and lyophilized to obtain our desired fluorescein attached HWGF tetrapeptide. The peptide was purified and characterized by C18 reverse phase HPLC and MALDI-TOF mass spectrometry.

Preparation of HWGF peptide vesicle: HPLC purified and lyophilized HWGF peptide was used for the formation of peptide vesicle. HWGF peptide was dissolved in milli Q water [0.2% DMSO v/v maintained], sonicated for 10 min (Takashi electronic, japan) and incubated for 4 days at 37 ºC incubator (AccuBlock, Digital Dry Bath, Labnet International) in a 2 mL centrifuge tube. After 4 days incubation, the peptide solution forms peptide vesicle. The formation of peptide vesicles were confirmed by transmission electron microscope (TEM), Scanning electron microscope (SEM) respectively.

Fourier Transform Infrared Spectroscopy (FTIR): Freshly prepared HPLC purified and lyophilized HWGF tetrapeptide in solid form was used for the FT-IR study considering it as zero day sample. Subsequently, 400 μM HWGF peptide solutions was incubated with milli Q water (0.2% DMSO) for 4 days at 37 ºC incubator (AccuBlock, Digital Dry Bath, Labnet International) in a 2 mL centrifuge tube. After 4 days, it was lyophilized and FT-IR spectrums were recorded after mixing with KBr. FT-IR spectroscopic analysis was performed in Perkin-Elmer Spectrum 100. FT-IR spectrum was recorded with the speed of 0.2 cm/s at a resolution of 1.6 cm^{-1}. Then data plotting was performed in the $LiTaO_3$ detector. Freshly prepared HWGF peptide (0 day, 1669 cm^{-1}) indicates the β-turn structure. Interestingly, after 4 days incubation of peptide (400 μM) exhibited characteristics β-sheet structure (1639 cm^{-1}).

Circular Dichroism (CD): Circular dichroism (CD) spectra were recorded on a J-815 model of JASCO. The freshly prepared and 4 days incubated HWGF peptide solution (400 μM) was placed in a rectangular quartz cuvette of 0.5 cm path-length. Recording parameters for data acquisition were maintained as following, scan speed of 50 nm/min., bandwidth of 1 nm, and sensitivity of 100 milli-degrees. The ellipticity (molar) values are expressed HWGF peptide solution (400 μM) between 190-250 nm wave lengths.

DOX/PI loading and purification by gel filtration: 10 μL solution of DOX (1 mM) was dissolved with freshly prepared HWGF tetra peptide (400 μM, 1000 μL). Then the resulting solution was mixed uniformly followed by sonication for five minutes. Then it was kept at 37 ºC incubator for 4 days for proper encapsulation of DOX. Purification of DOX loaded HWGF tetrapeptide from the free DOX was performed by gel filtration following previously reported procedure [3]. Initially, a uniform sepharose 4B bed was prepared. Then it was washed with PBS (Phosphate Buffer Saline) for four times. After that the incubated sample was added drop wise on sepharose 4B bed. The sample was adsorbed on the sepharose 4B bed. Then, PBS was added and the fractions were collected. The fractions were analysed with UV using their characterization absorbance wave length (Peptide: 220 nm; DOX: 480 nm). Our desired fraction (drug loaded HWGF) was collected and concentration of loaded drug was measured.
DOX loading inside the HWGF peptide vesicle ([HWGF] = 100 μM) has been performed and purified through sepharose 4B column upon addition of PBS buffer to examine the loading efficacy at different peptide concentrations. Likewise, PI loaded HWGF peptide vesicle has been prepared.

Transmission electron microscope (TEM) study: About 2 μL of HWGF and HWGF-DOX incubated (5 μM or 100 μM, 4 days at 37 ^{o}C) peptide solution were placed on cupper coated TEM grid separately. 2% aqueous solution of the uranyl acetate (adjust the pH to 4.2 to 4.5, filtered through 0.22 μm filter paper) was prepared previously and stored at 4 ºC refrigerator. After that, 2 μL of filtered uranyl acetate solution was placed on the cupper grid and pipetting gently followed by absorbing the staining solution from the opposite side using a wedge of filter paper. After absorbing the staining solution completely, the cupper grids were allowed to dry for overnight under vacuum condition. Finally, imaging of the cupper grids was performed using TECNAI G2 SPIRIT BIOTWIN CZECH REPUBLIC 120 KV electron microscope operating at 80 kV. Basically, uranyl acetate stains the positively charged DOX with electrostatic absorption. However, it negatively stains the peptide vesicles [4]. Therefore, the TEM images show a DOX core and peptide shells.

Fluorescence microscopy: 5 μL incubated HWGF tetrapeptide (5 μM, 4 days at 37 ^{o}C) and fluorescein-HWGF encapsulated with DOX ([DOX] = 1 μM) was placed on a cleaned glass slide separately and dried in a desiccator. After that, glass slide was observed under inverted fluorescence microscope (Olympus IX83) in 60× magnification using ANDOR iXON3 camera. Fluorescence microscopic images reveal nice hollow peptide vesicles.

Atomic Force Microscopy (AFM) sample preparation and imaging: 5 μL incubated (5 μM**,** 4 days at 37 ºC) solution of the HWGF and HWGF-DOX (Peptide concentration maintained 5 μM) were deposited onto freshly cleaved muscovite by Ru mica sheet (ASTM V1 Grade Ruby Mica from MICAFAB, Chennai). As Mica sheets are basically negatively charged, our peptide binds strongly on the mica surface placed on a clear glass slide and dried in desiccator using vacuum dryer. AAC mode AFM was performed using a Pico plus 5500 AFM (Agilent Technologies USA) with a piezoscanner maximum range of 9 μm. Micro fabricated silicon cantilevers of 225 μm in length with a nominal spring force constant of 21-98 N/m were used from Nano sensors. Cantilever oscillation frequency was tuned into resonance frequency. The cantilever resonance frequency was 150-300 kHz. The images (512 by 512 pixels) were captured with a scan size of between 0.5 and 5 um at the scan speed rate of 0.5 lines/S. Images were processed by flatten using Pico view1.4 version software (Agilent Technologies, USA).

Dynamic Light Scattering (DLS) study: To characterize the average size of HWGF and DOX encapsulated HWGF peptide vesicle, dynamic light scattering experiment has been performed. First, sepharose 4B column purified HWGF peptide vesicle (100 μM) and DOX encapsulated HWGF peptide vesicle (100 μM) were chosen and performed the dynamic light scattering experiment (Model no. ZEN 3690 ZETASIZER NANO ZS 90). DLS study indicates that the average size of HWGF peptide vesicle and DOX encapsulated enhanced HWGF peptide vesicle are 657 and 863 nm.

Zeta potential study for the characterization of vesicle size: The stability of tetrapeptide vesicle and DOX encapsulated peptide vesicle have been determined by Malvern particle size analyzer (Model no. ZEN 3690 ZETASIZER NANO ZS 90) maintaining 100 μM concentration.

Analysis of DOX loading efficacy: Fluorescence of DOX was measured by fluorimeter (PTI, Quanta Master Spectrofluorimeter, QM-40) and concentration measured by UV spectrophotometer. After that, HWGF tetrapeptide and DOX were co-incubated at 37 ºC for 4 days. After proper encapsulation of DOX inside the peptide vesicle, free DOX was removed by passing the incubated solution through uniform sepharose 4B column. To get the loading capacity of peptide vesicle, free DOX and HWGF-DOX emission spectra (Excitation wave length 480 nm, emission range 510-700 nm) have been observed at 10 and 20 h time interval respectively.
Drug loaded efficacy (DLE) = $[FL_{HWGF\text{-}DOX}]/[FL_{free\text{-}DOX}]\times100$. Here, $FL_{HWGF\text{-}DOX}$ indicates the fluorescence intensity of DOX inside the tetrapeptide vesicle and $FL_{free\text{-}DOX}$ indicates fluorescence intensity of DOX.

Time dependent drug release study: After sepharose column purification, a small fraction (15 μL, analyzed through UV, Peptide: 220 nm; DOX: 480 nm) containing DOX encapsulated HWGF peptide vesicle in phosphate buffer was collected in cuvette and 2% TritonX-100 was added to it and subsequently reading was recorded in Quanta Master Spectrofluorimeter (QM-40) [5]. Due to addition of TritonX-100, higher fluorescence intensity (HWGF-DOX or HWGF-PI) was observed than control (without TritonX-100 addition). Finally different time interval readings were recorded with enhanced intensity till the saturation was reached. Drug loaded efficacy (DLE) = $[FI_{lip}]/[FL_{free}]\times100$. Here, FI_{lip} indicates fluorescence intensity of peptide encapsulated DOX and FL_{free} indicates fluorescence intensity of free DOX.

Intrinsic tryptophan quenching study for binding constant (K_b) determination: The binding constant of our designed HWGF peptide with Matrix metalloproteinase (MMP9) was determined by PTI Quanta Master Spectrofluorimeter (QM-40) with 1 mm path length quartz cuvette and 5 nm slit width for excitation and emission. To determine MMP9 and HWGF binding interaction, we have monitored MMP9 enzymes tryptophan fluorescence with increasing concentration of HWGF (excitation wavelength 280 nm, emission range 290-450 nm). The K_b value was determined by calculating this decrease in MMP9 enzyme's fluorescence emission intensity at using Stern-Volmer equation and plotted against HWGF concentration in μM range.

Isothermal Titration Calorimetric analysis study: Isothermal titration calorimetric analysis has been performed between matrix metalloproteinase, MMP9 (10 μM) and peptide, HWGF (1000 μM) at 281 K in presence of 0.1 mM PBS following standard procedure [6]. Each peak of the binding isotherm in the upper panel represents that peptide injects into the MMP9 receptor protein solution. The amount of heat absorbed by successive addition of peptide is plotted against the molar ratio of peptide to MMP9 protein in the below panels (Figure 4, main text). Standard nonlinear least squares regression binding model for one site binding has been applied to fit the data.

ITC profiles for the binding of HWGF and MMP9 protein have been observed in positive peaks in the plot of power versus time revealing the binding to be endothermic in this case.

For the control dilution experiment the HWGF peptide solution of the same concentration was injected to the experimental buffer following protocol as employed for the samples. The heat absorbed in each injection for the dilution was subtracted from the corresponding heat absorbed for MMP9-HWGF association to yield the heat change for the metalloproteinase-peptide reaction. The thermodynamic parameters were evaluated by fitting all the data to a single set of identical sites model. Heat of dilution has been subtracted from the evolved ITC data using MicroCal LLC ITC data processing software. The ITC data for the binding of HWGF with MMP9 generates an association constant (K_a) of (1.82±0.79) × 10^5 M^{-1}, an enthalpy change (ΔH) of (9910±1601) cal mol^{-1}, an entropy change (ΔS) of 59.3 kcal mol^{-1} and binding stoichiometry (N) of 2.25±0.283. The Gibbs energy change (ΔG°) and the entropic contribution for the binding (TΔS°) were calculated by the following equations,

$\Delta G° = -RT \ln K_a$ (equation 1)

and $\Delta G° = \Delta H° - T\Delta S°$ (equation 2)

Here T is the temperature in Kelvin and R is the universal gas constant (1.9872041 cal mol^{-1} K^{-1}). The negative Gibbs energy change (ΔG°=-6.753 kcal/mol) indicates energetically favourable binding process.

Cell viability assay of tetrapeptides: MCF7 (human breast adenocarcinoma cell line) cells were cultured in DMEM media containing 10% fetal bovine serum. Cell viability assay was performed following standard procedure taking 400 μM, 200 μM, 100 μM, 50 μM, 25 μM and 12.5 μM concentrations of tetrapeptide in DMEM medium [7]. These were treated on the cultured MCF7 cells in ninety six well plates. MTT is a popularly known colorimetric technique where yellow tetrazole compound gets reduced to purple coloured formazen by reductase enzymes present in living cells while the dead cells are unable to interfere. Similarly, comparative cell viability assay of HWGF-DOX and free DOX has been performed for 24 h treatment up to 5 μM concentration of DOX. Percentage viability has been calculated as [(A_{550} Treated Cells - A_{550} Backgrounds)/ (A_{550}Untreated Cells - A_{550} Backgrounds) × 100. Further, cell viability assay was performed following above described procedure for HeLa, A549 and MCF10A using 0.625, 1.25, 2.5 and 5 μM concentrations of DOX.

Cellular uptake study for microscopic imaging: Approximately, 2000 MCF7 cells were seeded in DMEM medium containing 10% fetal bovine serum on cover glass bottom disc for overnight before the treatment. Fluorescein attached HWGF peptide (F-HWGF) undergoes cellular uptake (MCF7 cells) for 1 h treatment. Nucleus was stained with Hoechst 33258 (1 μg/mL) for 45 min. Images were captured in DIC, 405 nm and 488 nm wave lengths respectively.

Free DOX and DOX encapsulated HWGF peptide vesicle was treated in DMEM containing media and incubated for 1 h (DMSO concentration maintained 0.2%) in MCF7 cells. Next, 4% formaldehyde in PBS buffer was added for 30 min to fix the cells in each cover glass. Next, formaldehyde solution was removed and washed with PBS buffer. Finally, Hoechst 33258 solution was removed and washed by PBS buffer for three times. Thus, each cover slip

was ready for the microscopic imaging. Cell imaging was performed by an Andor spinning disc confocal microscope with 40X objective (Olympus) equipped with Andor iXon 3897 EMCCD camera in bright field, 561 and 405 nm wavelength light. Similarly, propidium iodide (PI) encapsulated HWGF peptide vesicle study has been performed.

Flow cytometry for cellular uptake: MCF7 cells were cultured in a 6-well plate at density of ~5×10^5 cells each well prior to 24 h of treatment. Cells were treated with DOX (1 µM) in serum free media for 1 h. Next, cells were washed with phosphate buffer and trypsinized. Comparative cellular uptake of DOX and HWGF-DOX has been performed in MCF7 cells. It was analyzed by FACS (E_x - 488 and E_m -500 to 600) with respect to control MCF7 cells.

Cellular internalization study using FACS: Mechanism of cellular internalization of DOX encapsulated HWGF peptide vesicle (HWGF-DOX) has been analysed using previously described method [7, 8]. Briefly, seeded MCF7 cells (1×10^6) were detached and collected in a suspension containing serum free DMEM (colourless) culture medium. After that cells were incubated at 37 ^oC and 4 ^oC for 1 h separately. Next, these cell suspensions were treated with F-HWGF of final concentration at about 5 µM and incubated at 37 ^oC or 4 ^oC for 1 h separately. After that, the cell suspension was centrifuged to remove excess HWGF-DOX from the solution and resuspended in DMEM culture medium containing trypsin (1 mg/mL) and incubated for 15 min. Cells were washed with serum free DMEM (colorless) culture medium and fluorescent signal was analyzed using 568 nm channels of BD LSRFortessa™ flow cytometer.

Interaction of HWGF with cell surface receptors determined by molecular docking study:

(a) Endothelin receptor: Blind docking between HWGF peptide with endothelin receptor (PDB ID-5GLH) [9] was performed by Autodock version 1.1.2 software. The grid box 68×100×74 was centered on the receptor. It shows significant binding of this peptide with this receptor (binding energy -7.1 kcal/mol). The >C=O gr. (-COOH) of THR 1026 is in H-bonding interaction with –OH gr. (-COOH) of C-terminal acid. The -OH gr. of GLN 1105 and the –OH gr. (-COOH) of PHE 1104 is in H-bonding interaction (receptor) with >C=O gr. of GLY 3. Also the >C=O gr. (-COOH) of ASP 1070 with -NH gr. (secondary ring amine) of TRP 3 are in H-bonding interaction.

(b) Opioid receptor: We performed docking of HWGF peptide with mu opioid receptor (PDB ID-4DKL) [10]. We have performed blind docking with this receptor. The grid box 52×98×66 was centred on the receptor. The binding energy (-8.0 kcal/mol) shows that this peptide has very good binding with this receptor. The -SH group of CYS 217 with -NH gr. (imidazole ring) of HIS 1, >C=O gr. (-COOH) of ASP 147 with N-terminal free –NH2 gr. of tetrapeptide, the >C=O gr. (-CONH2) of GLN 124 has H-bonding interaction with –NH gr. of (-CONH) TRP 2. Also, the >C=O gr. (-CONH2) of GLN 124 and the –OH gr. of TYR 128 are in H-bonding interaction with >C=O gr (-CONH-) TRP 2.

Apoptosis study: Cells (~5 × 10^5 cells/mL) were harvested overnight in a 6-well plate and treated with of HWGF-DOX ([DOX] = 2 μM) separately for 24 h. MCF7 cells were suspended having 100 μL solution of binding buffer contained with propidium iodide (PI) and annexin V and incubated at 37 ºC for 15 min. Emission of annexin V and PI has been detected using FITC and PI channels of BD LSRFortessa™ flow cytometer using emission filters at 530 and 610 nm respectively. Cells in Q1, Q2 and Q4 are considered as necrotic, early and late apoptosis. Q3 quadrant cells are considered as healthy cell population.

Cell cycle study: Cell cycle study has been performed by treatment with either HWFG-DOX or free DOX having [DOX] of 2 μM. Next, cells were incubated with PI (100 μg/mL) and RNase (10 μg/mL) for 45 min at 37 ºC temperature. After treatment, MCF7 cells were fixed with 70% ethanol at 20 ºC for overnight. Finally, cell cycle analysis was performed using PI channels of BD LSRFortessa™ flow cytometer having emission filters at 610 nm.

Migration assay: MCF7 cell spheroids have been prepared. Next, cellular spheroids have been placed in the confocal disk both treated and untreated condition. Next, time dependent spheroid dispersion has been measured up to 24 h. After that, significant migration has been observed in untreated spheroids whereas HWGF-DOX treated spheroids show significant inhibition of migration.

Serum stability: HWGF peptide stability was checked in horse serum by following standard procedure [11, 12]. Concentration of HWGF peptide was 50 μM and stability was checked up to 24 h by C18 reverse phase HPLC system. It was observed that HWGF peptide remained intact almost 40% after 24 h incubation with horse serum.

HWGF-DOX effect on 3D spheroid: After successful *in vitro* study, we were interested to check the effect of HWGF-DOX on tumor mimicking 3D spheroid. Hence, we have used our recently developed tumor mimicking multicellular spheroid study in MCF7 [5]. After complete formation of spheroid, these were treated with LLCPD along with control. Untreated (control) and HWGF-DOX treated spheroid volume were checked using 10X (Olympus IX83) microscope equipped with EMCCD camera upto 7 days from the day of treatment. We observed significant increase in volume of spheroid of untreated group, while significant inhibition of growth has been observed for HWGF-DOX treated spheroids. All these *in vitro* experiments clearly showed that HWGF acts as an excellent MMP9 targeted delivery vehicle.

Data Analysis: Microscopic images were analysed by Image J and cellSens software. FACS data was analysed using FACS Diva software. Entire ITC titration experiment was monitored by Origin 7.0 software.

References:

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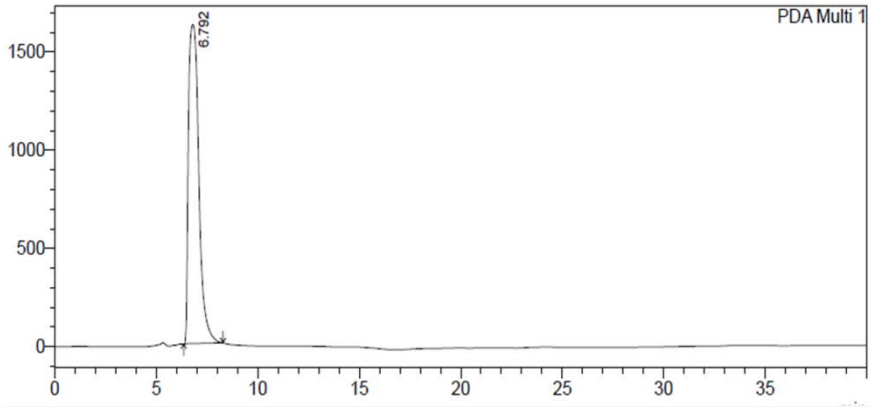
Electronic Supplementary Figures

(a)



Peak#	Ret. Time	Area	Height	Area %	Height %
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Total		57696062	1624016	100.000	100.000

(b)

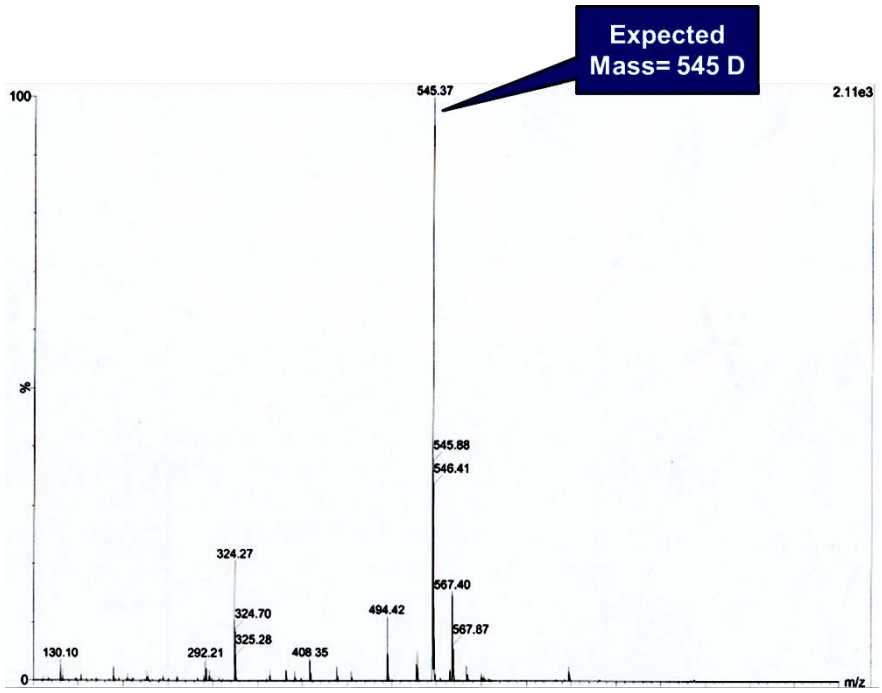


Figure S1. (a) HPLC chromatogram and (b) Mass spectrum of HWGF.

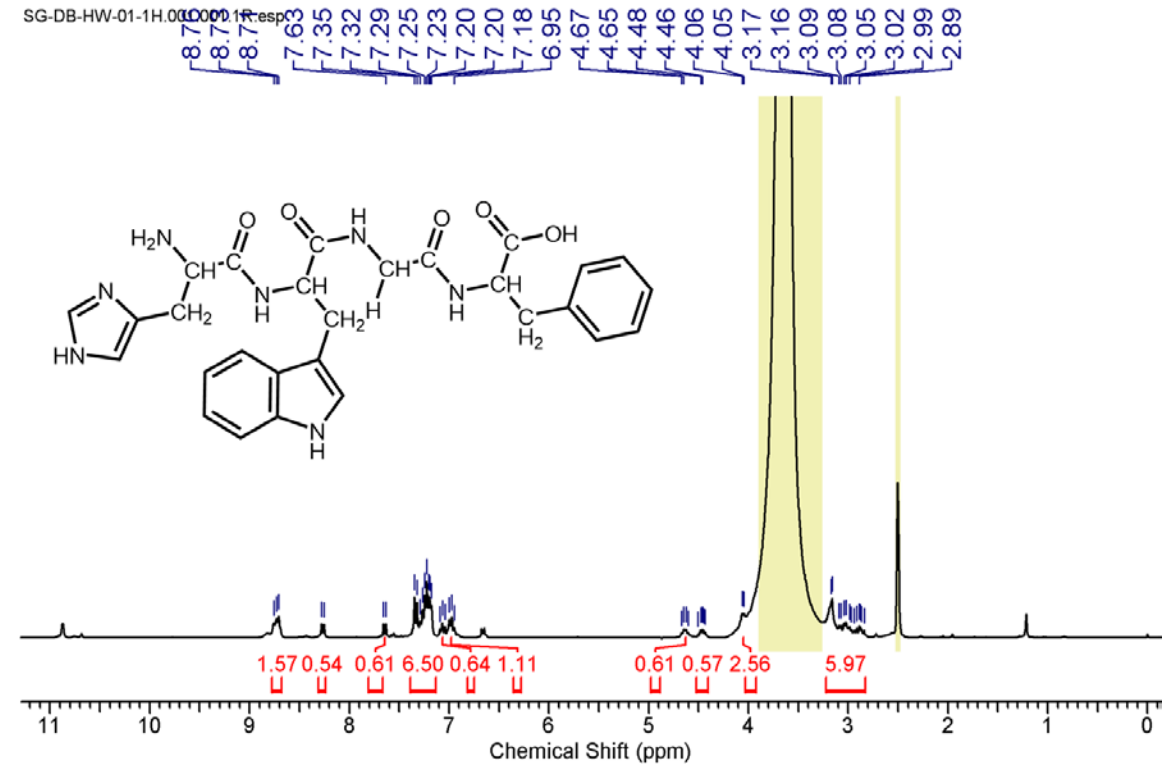


Figure S2. ^{1}H NMR spectra of HWGF tetrapeptide performed in DMSO-d^{6}.

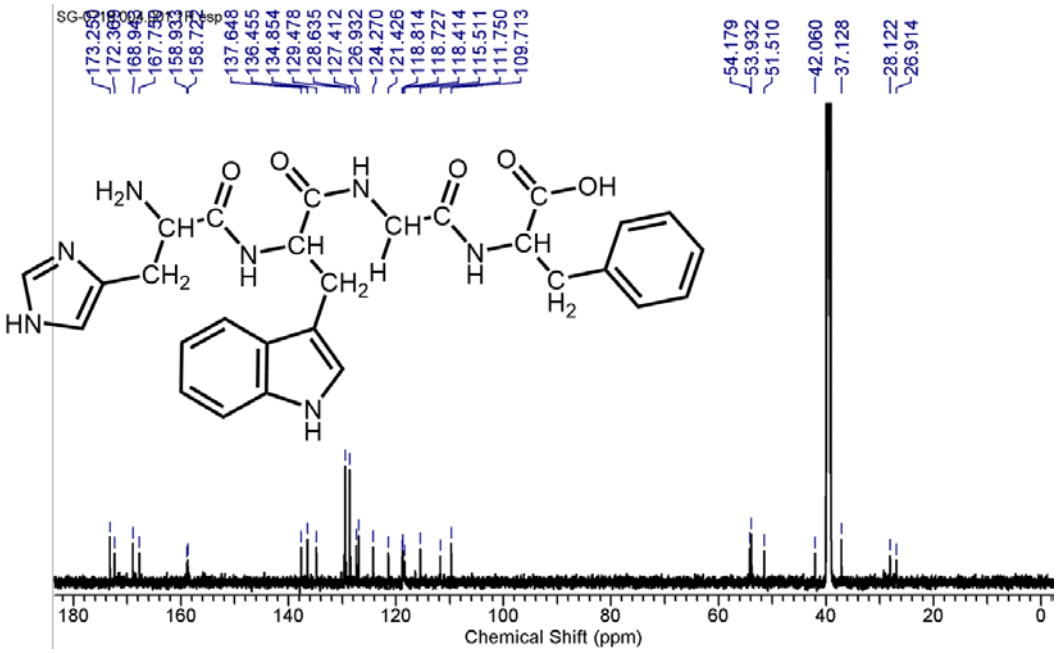
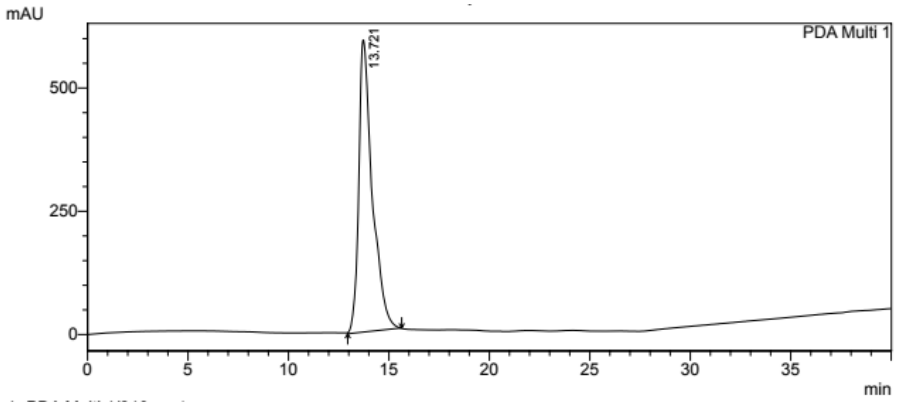


Figure S3. ^{13}C NMR spectra of HWGF tetrapeptide performed in DMSO-d^{6}.

(a)



PDA Ch1 210nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
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Total		27664491	592509	100.000	100.000

(b)

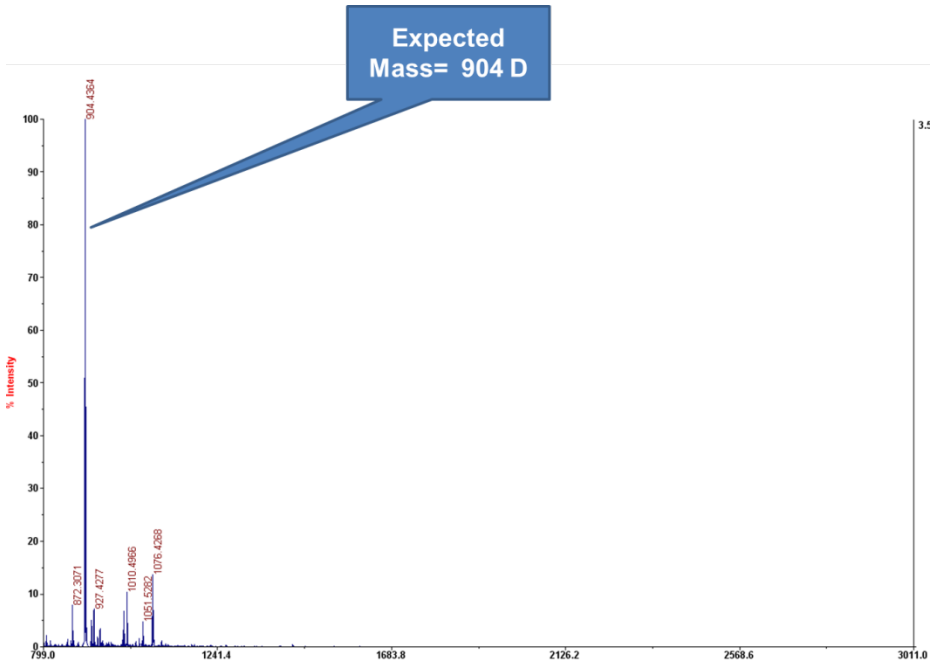

Figure S4. (a) HPLC chromatogram and (b) Mass spectrum of Fluorescein-HWGF.

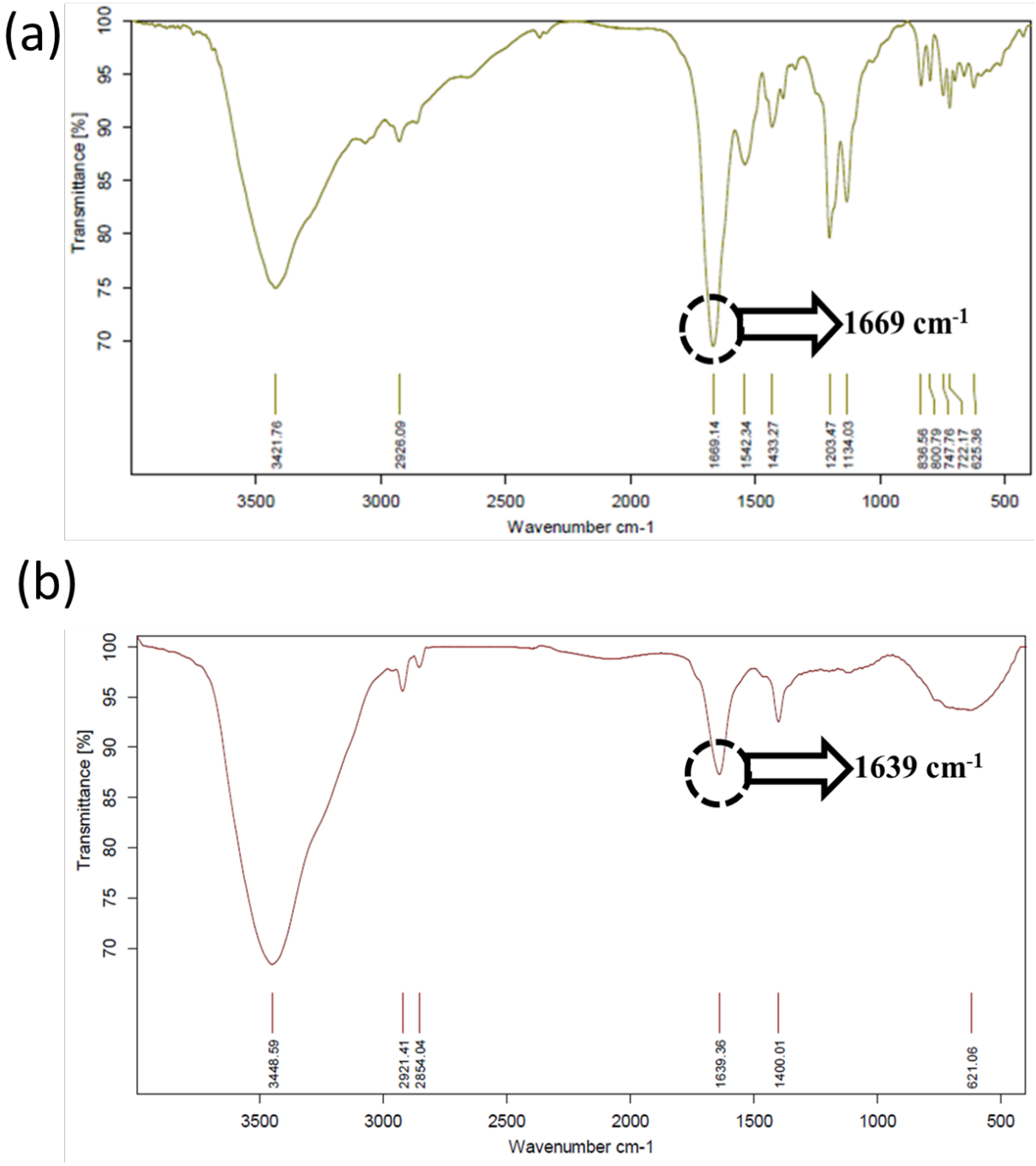

Figure S5. FT-IR spectra of HWGF tetrapeptide upon (a) 0 day incubation and (b) 4 days incubation. Significant peak shift from 1669 cm^{-1} to 1639 cm^{-1} indicates the formation of characteristics β-sheet structure of tetrapeptide upon incubation.

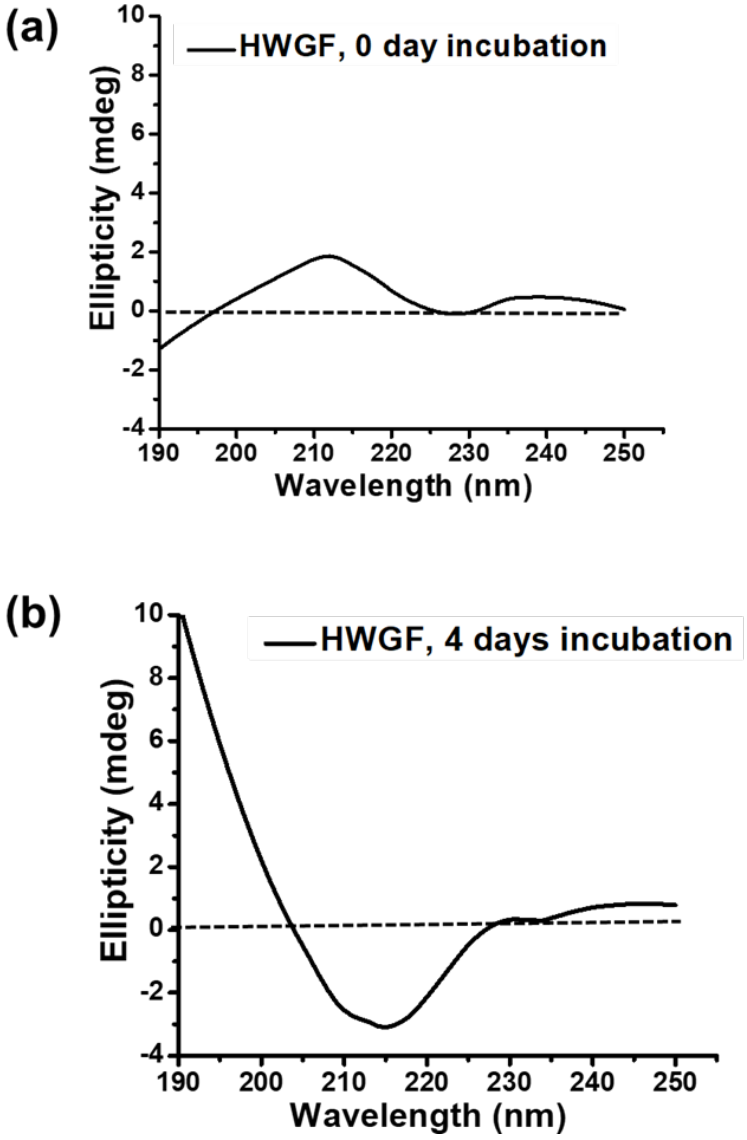

Figure S6. Circular dichroism (CD) study indicates the positive ellipticity at 217 nm and negative ellipticity at 190 nm which determines the characteristic β turn structure (a) upon 0 day incubation. The negative ellipticity at 216 nm and positive ellipticity at 190 nm determines the β sheet structure of HWGF upon 4 days incubation with milli Q water (0.2% DMSO).

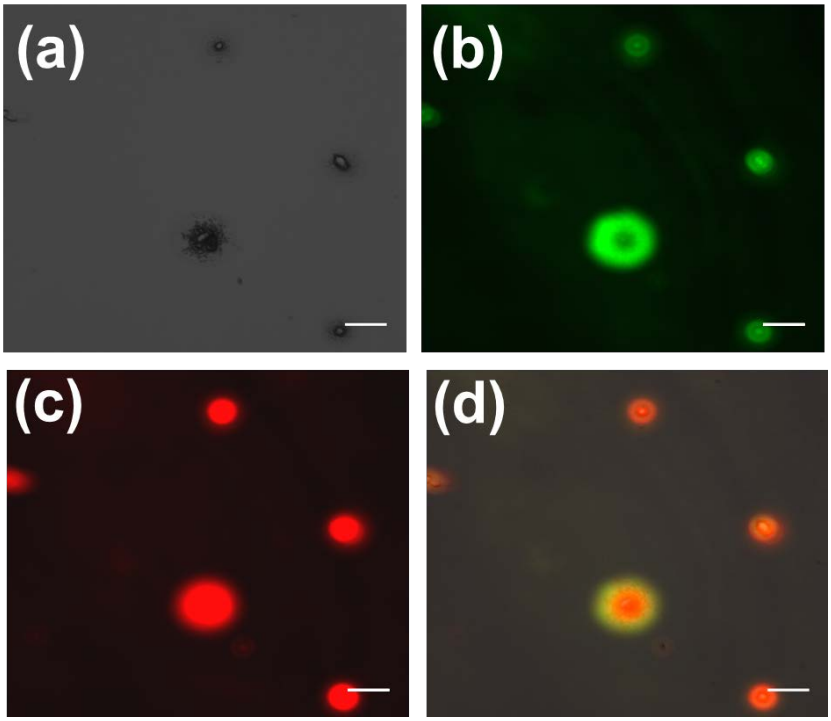

Figure S7. Fluorescein attached HWGF peptide vesicle observed at DIC (A), 488 nm (B), 561 nm (C) and merged (D) image. Scale bars correspond to 1000 nm.

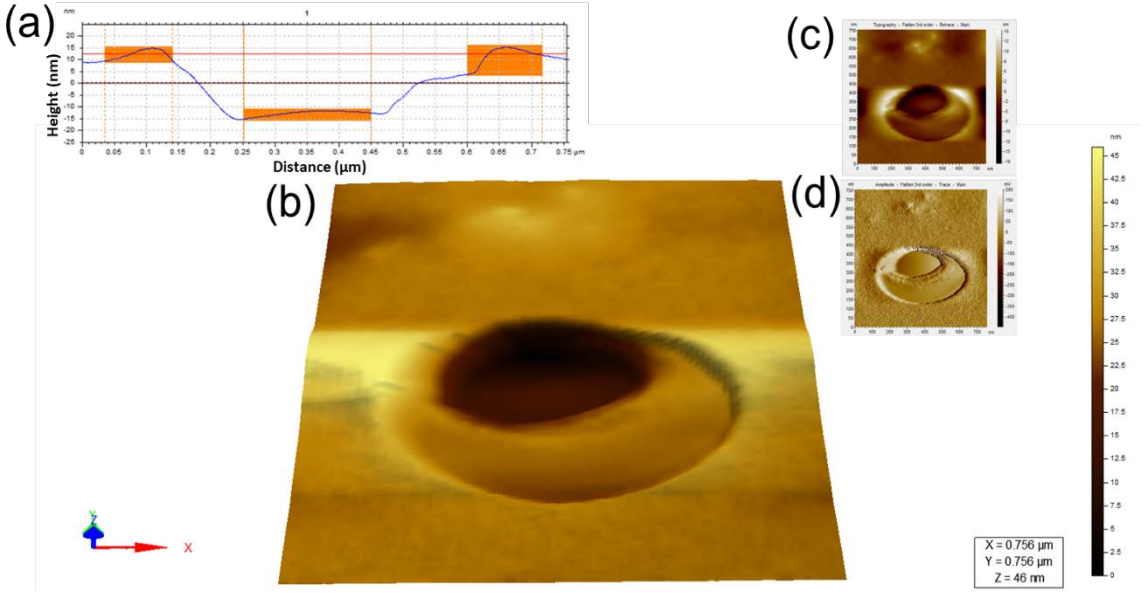

Figure S8. Formation of HWGF peptide vesicle. (a) Graphical presentation of HWGF peptide vesicles, Height (nm) vs Distance (µm). (b) 3D representation of HWGF peptide vesicle. (c) 2D and (d) amplitude images of HWGF peptide vesicle.

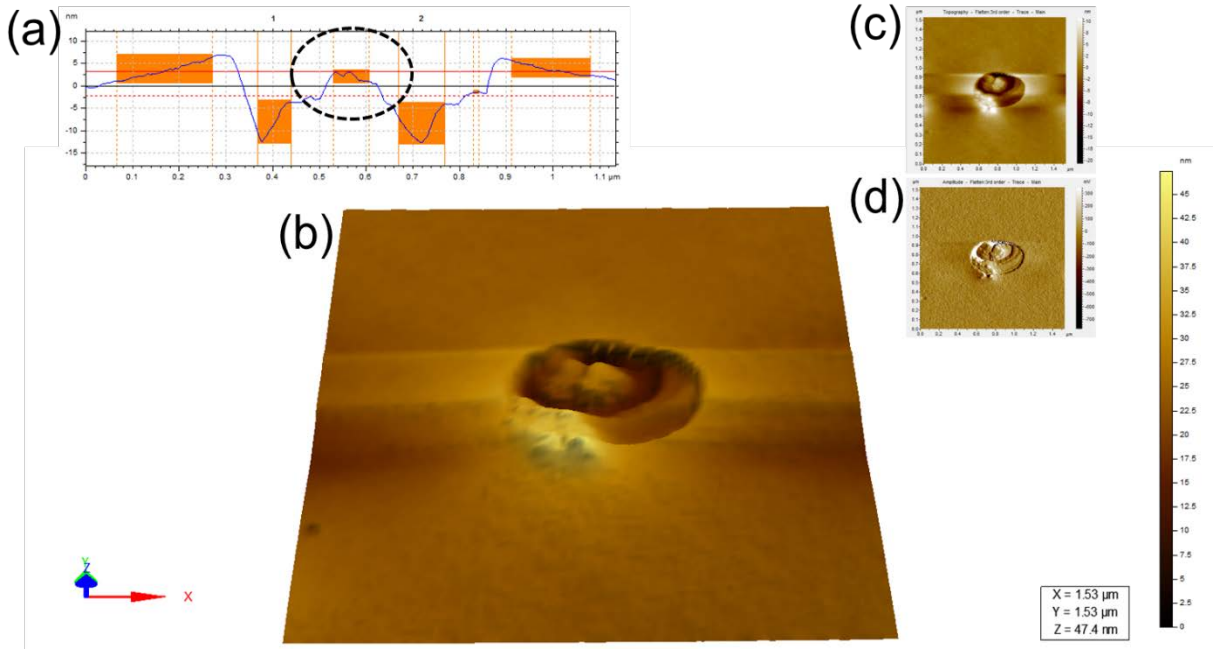

Figure S9. Formation of DOX encapsulated HWGF peptide vesicle. (a) Graphical presentation of DOX encapsulated HWGF peptide vesicles, Height (nm) vs Distance (µm). (b) 3D representation of HWGF-DOX peptide vesicle. (c) 2D and (d) amplitude images of HWGF-DOX peptide vesicle.

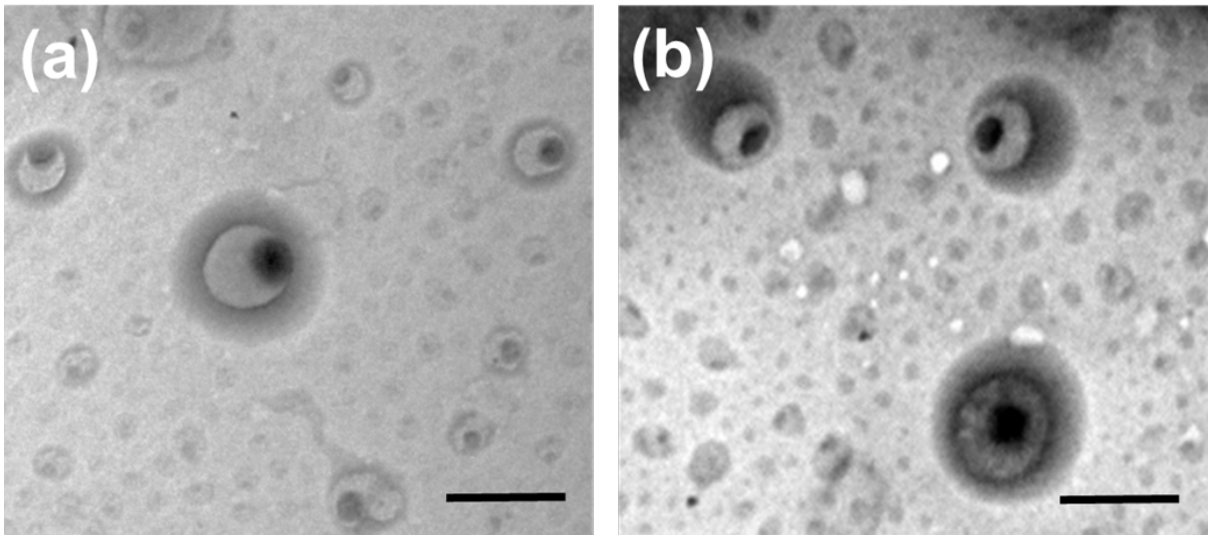

Figure S10. Transmission electron microscope (TEM) study indicates the size of (a) HWGF peptide and (b) DOX encapsulated HWGF peptide vesicle at 100 µM peptide concentration. Scale bars correspond to 1 µm.

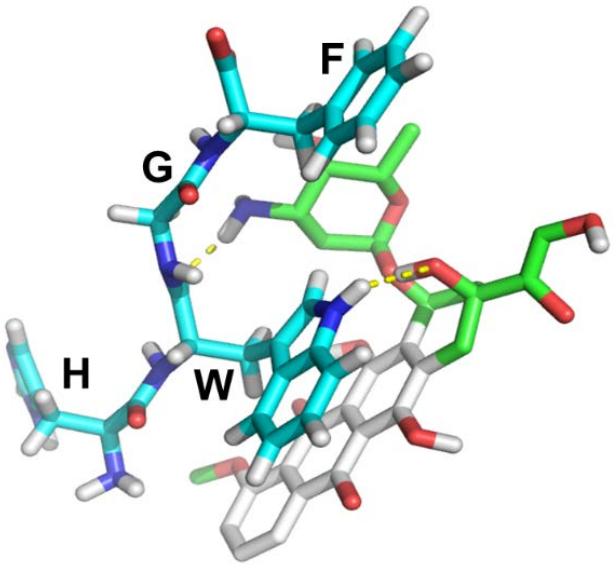

Figure S11. Docking study performed between HWGF tetrapeptide and doxorubicin (BE=-4.1 kcal/mol). In tetrapeptide, the tryptophan indole -NH interacts with the -OH group of cyclohexane ring in doxorubicin, glycine -NH interacts with the pyranose -NH_2 group of doxorubicin (Grid box size is 20×12×12).

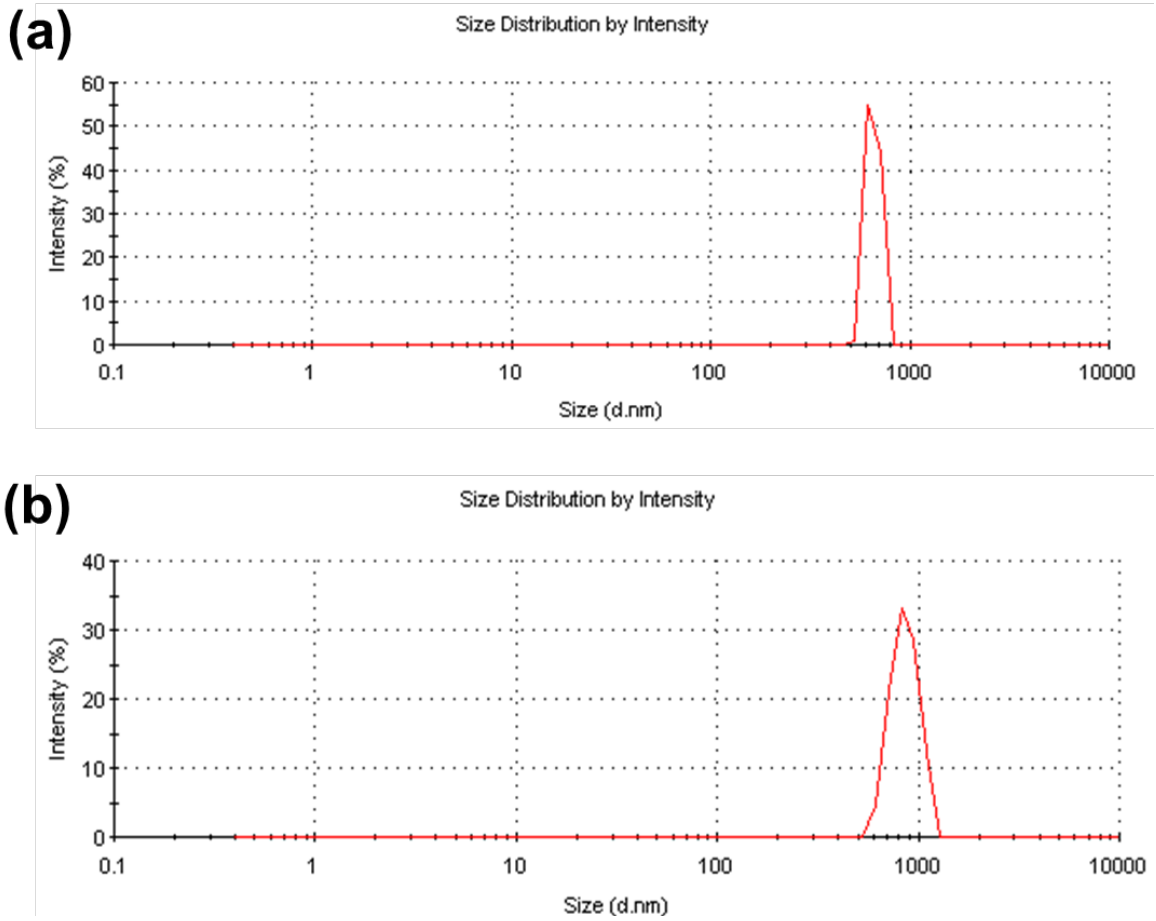

Figure S12. The dynamic light scattering (DLS) result indicates the size of HWGF peptide vesicle is 657 nm (a) and DOX encapsulated HWGF peptide vesicle is 863 nm respectively.

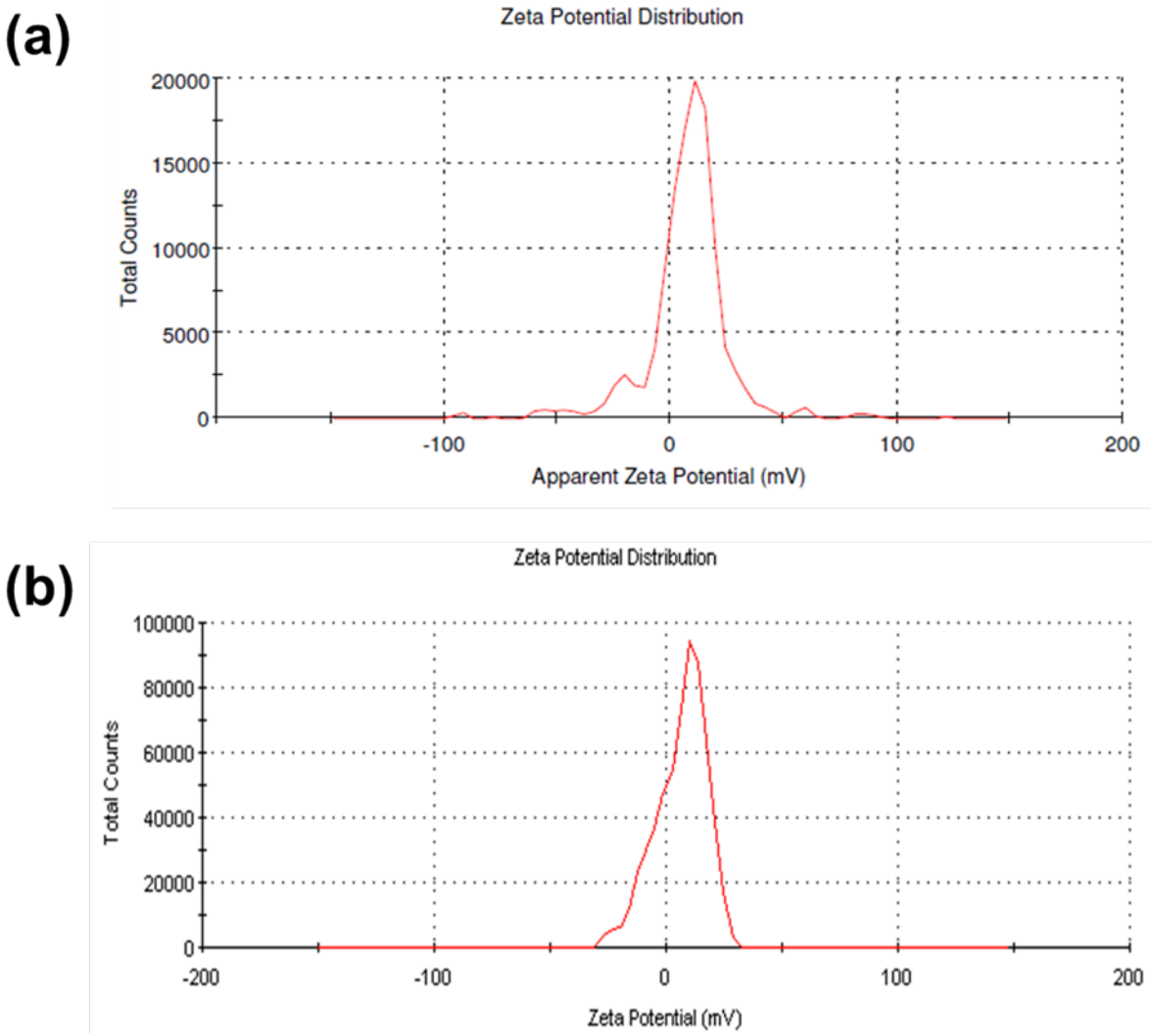

Figure S13. Higher zeta potential of (a) HWGF vesicle (7.97 mV) and (b) HWGF-DOX (6.74 mV) indicates the stability of peptide vesicle and DOX encapsulated peptide vesicle.

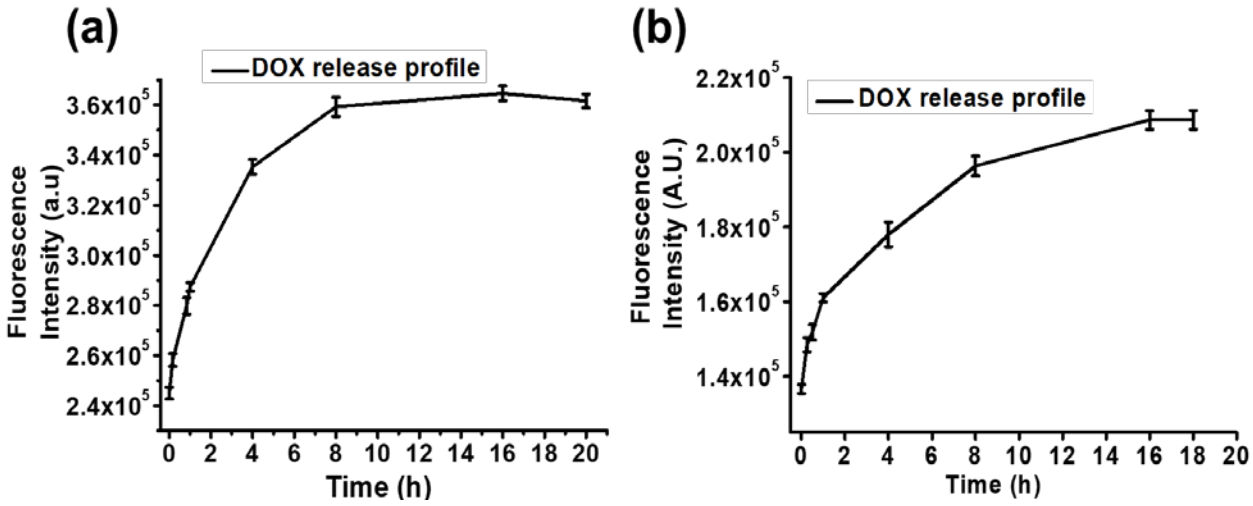

Figure S14. Time dependent DOX release profile graph obtained up to 20 h from (a) 400 µM and (b) 100 µM HWGF peptide vesicle.

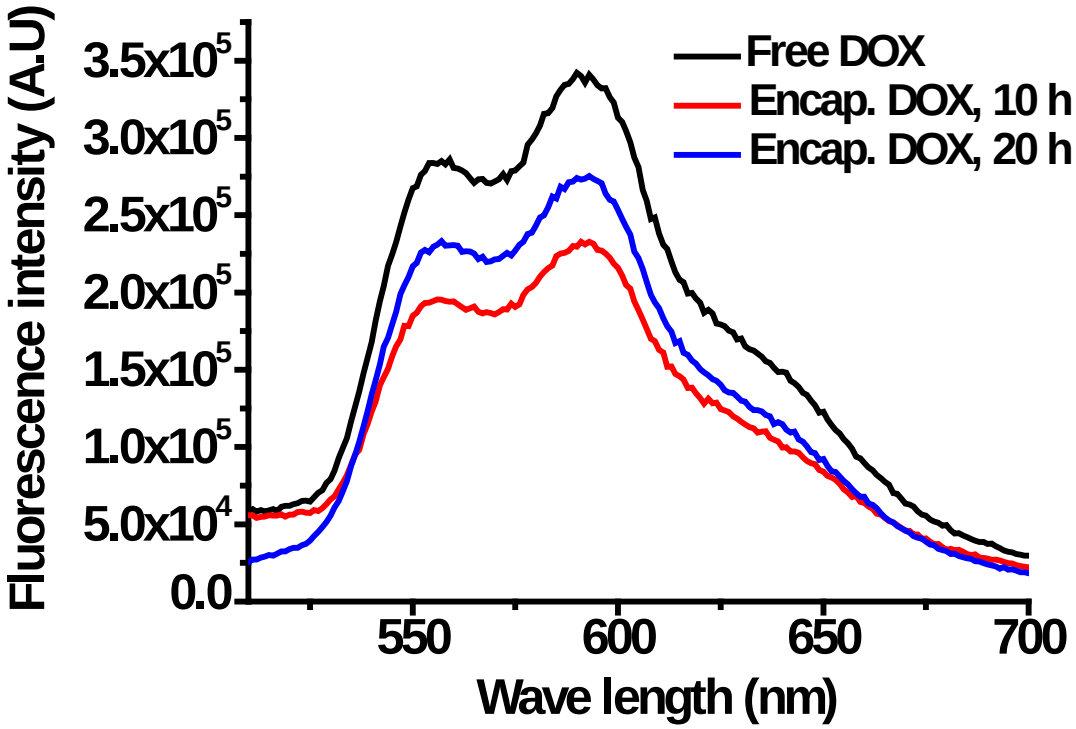

Figure S15. Drug loading capacity has been determined from enhanced DOX fluorescence intensity at 20 h.

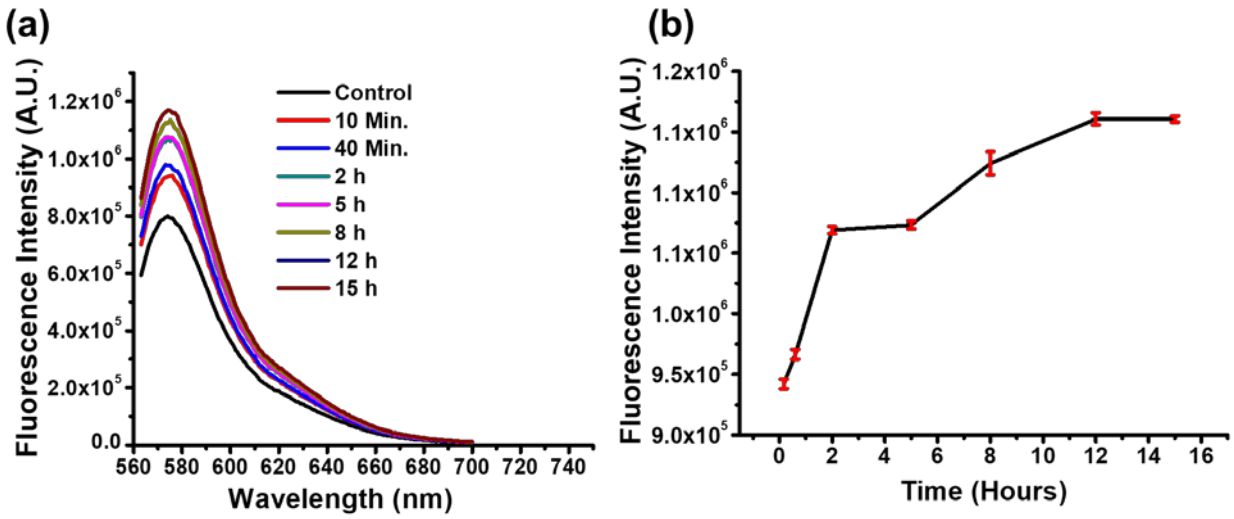

Figure S16. Time dependent release of Propidium Iodide (PI) from PI loaded HWGF tetrapeptide vesicle observed up to 15 h (a) and it's release kinetics (b).

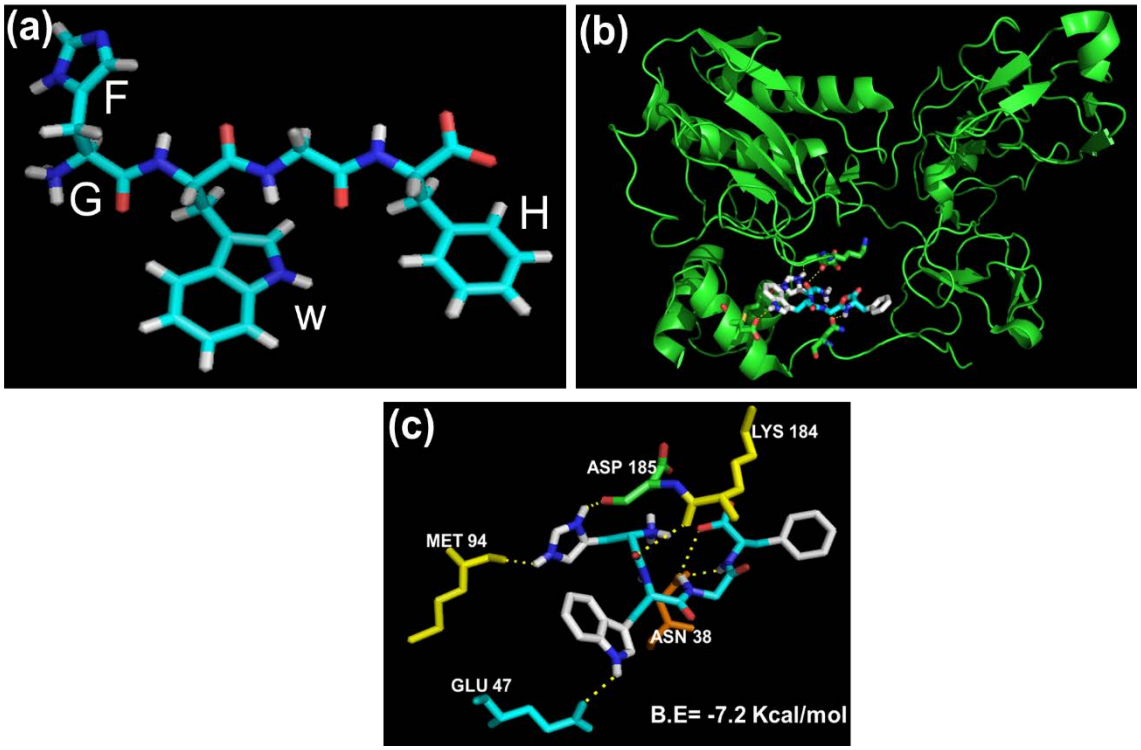

Figure S17. (a) Structure of HWGF peptide, (b-c) Docking study performed between MMP9 (matrix metalloproteinase enzyme) and HWGF peptide indicates significant binding energy (BE=-7.2 kcal/mol).

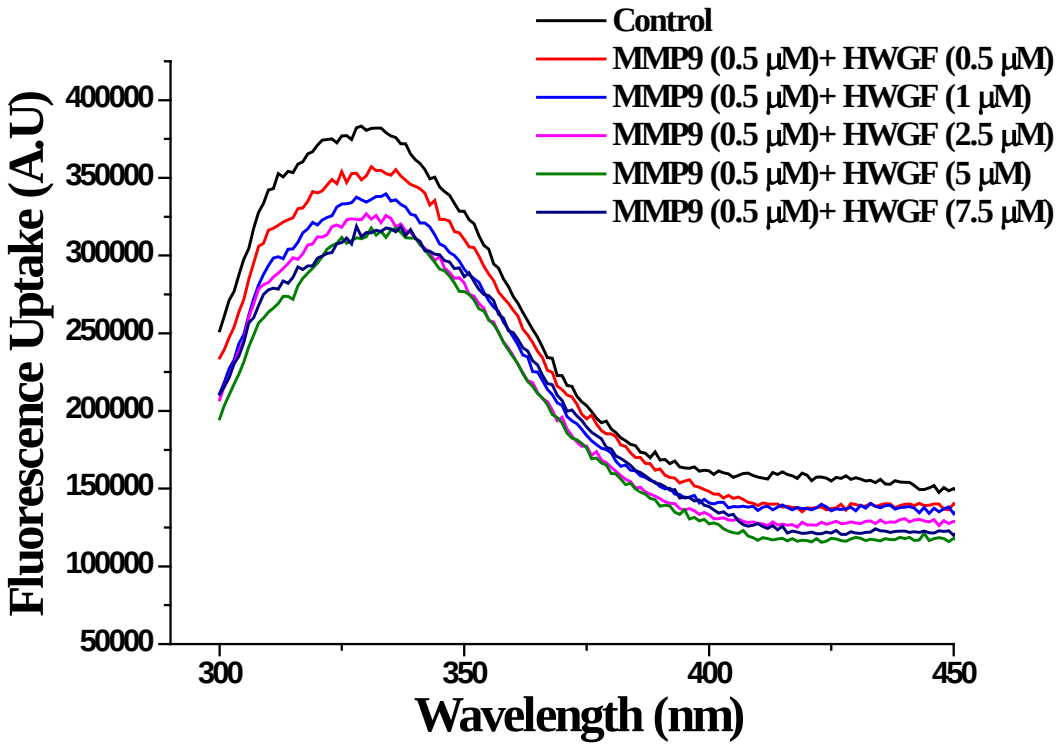

Figure S18. Tryptophan fluorescence quenching experiments were performed at different concentrations of HWGF.

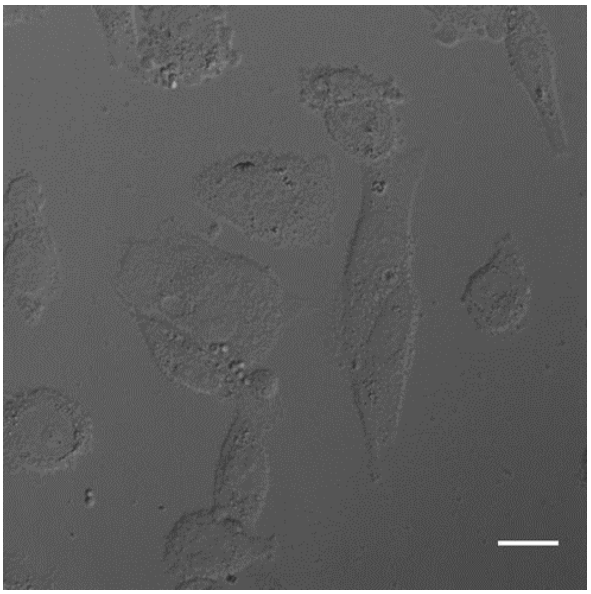

Figure S19. DIC image of MCF7 cells. Scale bar correspond to 20 μm.

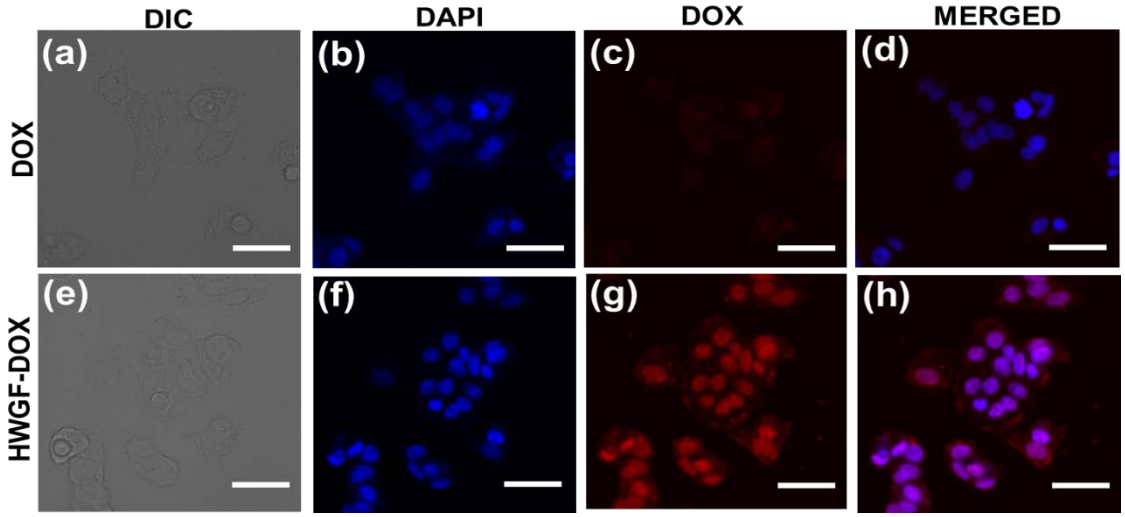

Figure S20. Microscopic images reveals differential uptake of DOX in (a) DIC, (b) 405 nm, (c) 561 nm, (d) MERGED and HWGF-DOX in (e) DIC, (f) 405 nm, (g) 561 nm and (h) MERGED. Scale bars correspond to 20 µm.

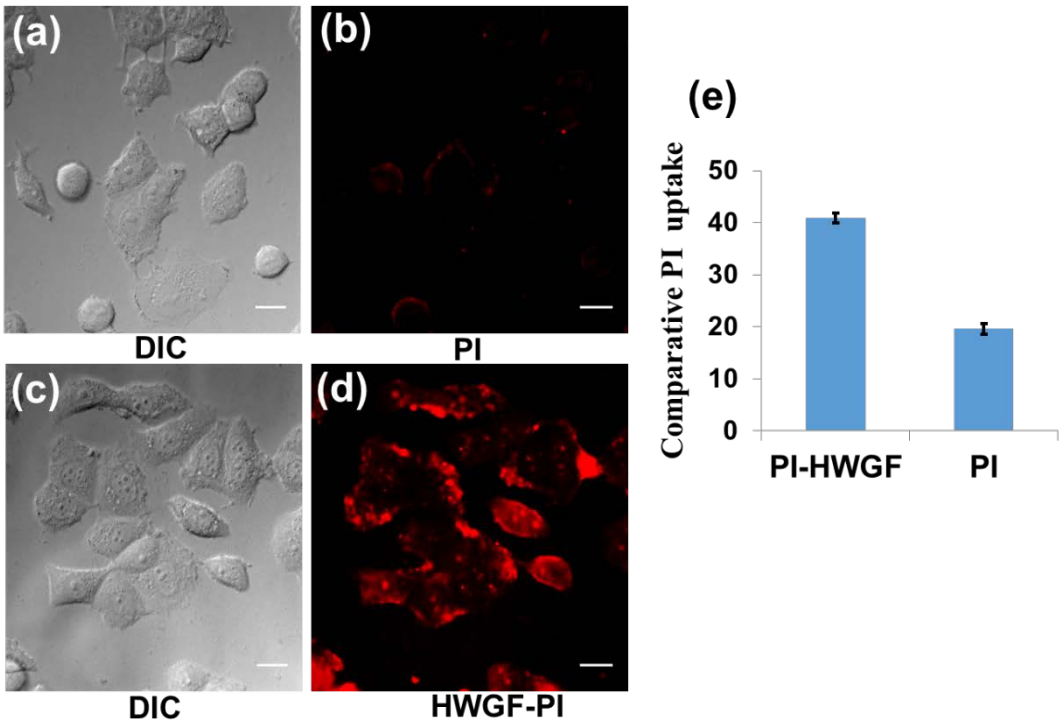

Figure S21. Microscopic images reveal cellular uptake of PI observed in DIC (a), 561 nm (b) and PI-HWGF in DIC (c) and 561 nm (d). Bar diagram represents quantitative uptake of PI in case of encapsulated vs PI alone (e).

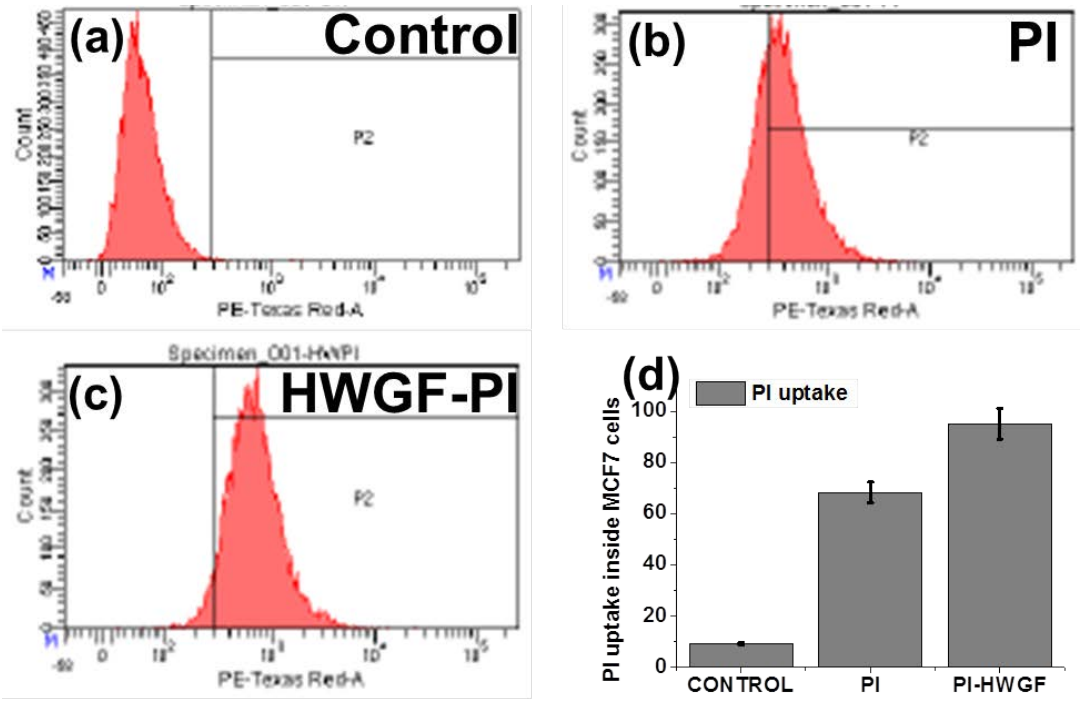

Figure S22. Flow cytometric data indicates the uptake of PI. Control (a), propidium iodide (PI) alone (b), encapsulated PI inside the HWGF vesicles (c) and bar diagram represent quantitative uptake of PI (d).

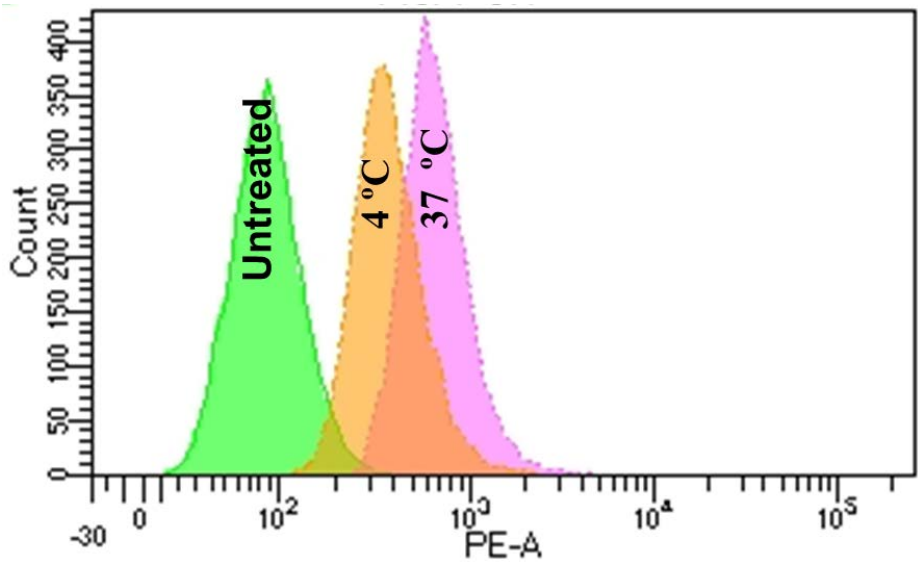

Figure S23. Flow cytometric assay indicates that cellular uptake of DOX, encapsulated in HWGF vesicles ([DOX]=1 μM) follows endocytosis pathway.

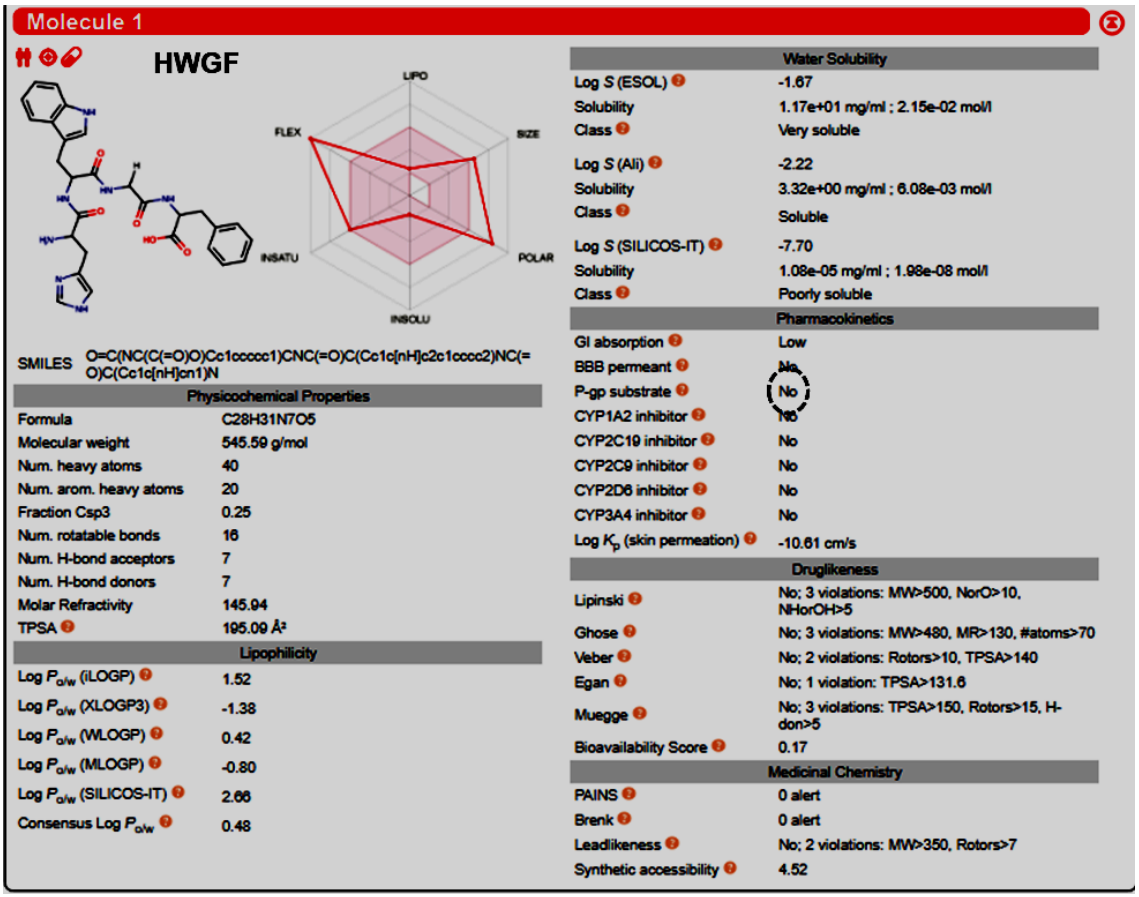

Figure S24. "SwissSimilarity" tool (www.swissadme.ch) determines that HWGF peptide is a non-Pgp substrate.

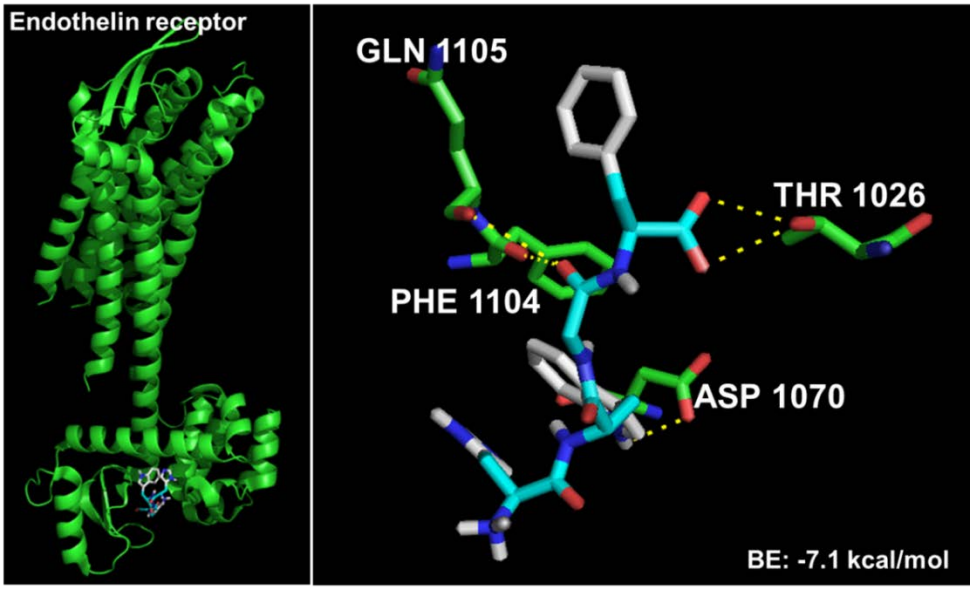

Figure S25. HWGF interacts with Endothelin receptor protein. Docking images indicate that HWGF tetrapeptide involved in the hydrogen bonding interaction with the various amino acid residues of Endothelin receptor.

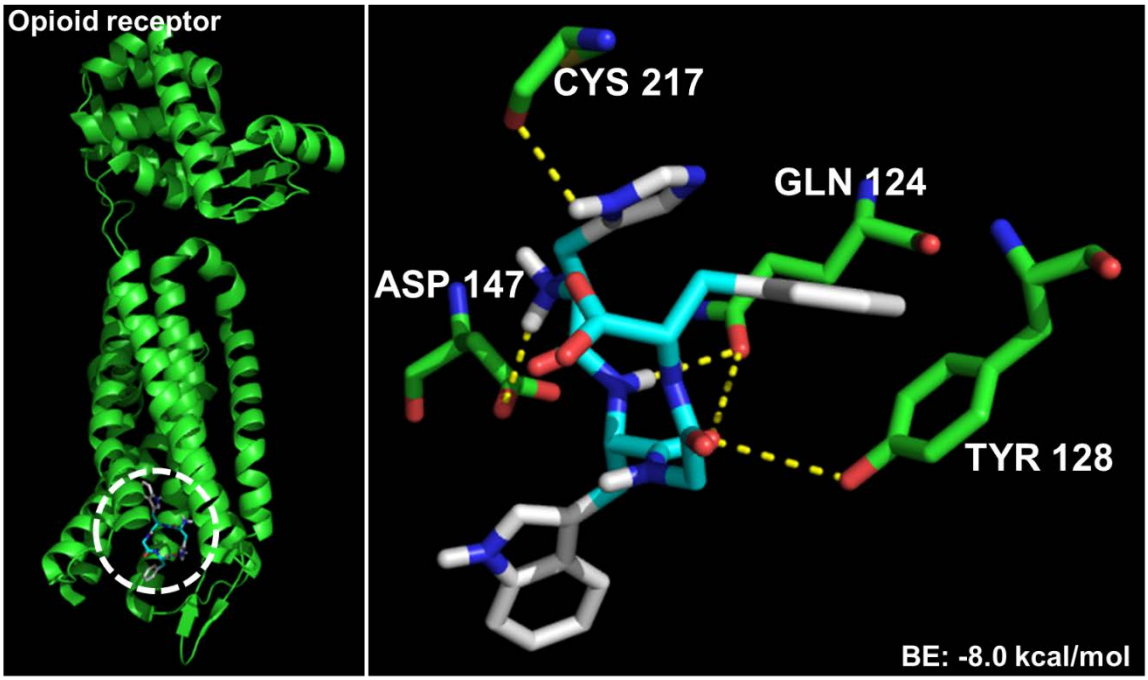

Figure S26. HWGF interacts with Opioid receptor protein. Docking images indicate that HWGF tetrapeptide involved in the hydrogen bonding interaction with the amino acid residues of Opioid receptor.

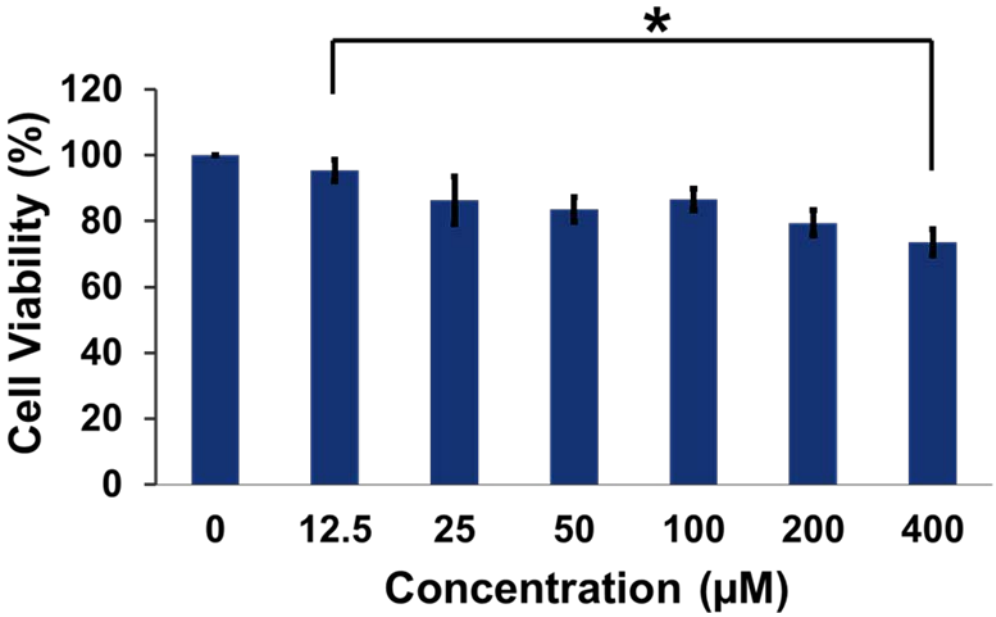

Figure S27. Cell viability assay of HWGF peptide on MCF7 cells up to 400 µM concentrations (*$p < 0.001$, performing student's t-test).

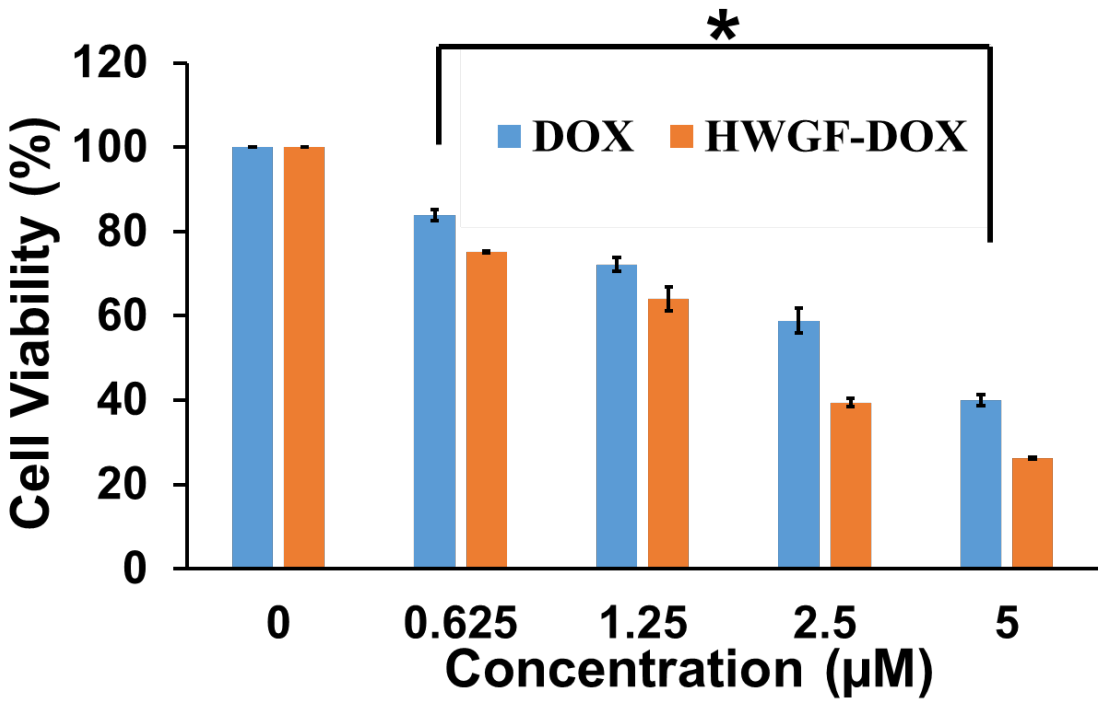

Figure S28. Bar diagram represents cell viability assay of DOX alone and DOX encapsulated inside the HWGF peptide vesicles in HeLa cells up to 24 h (*p < 0.001, performing student's t-test).

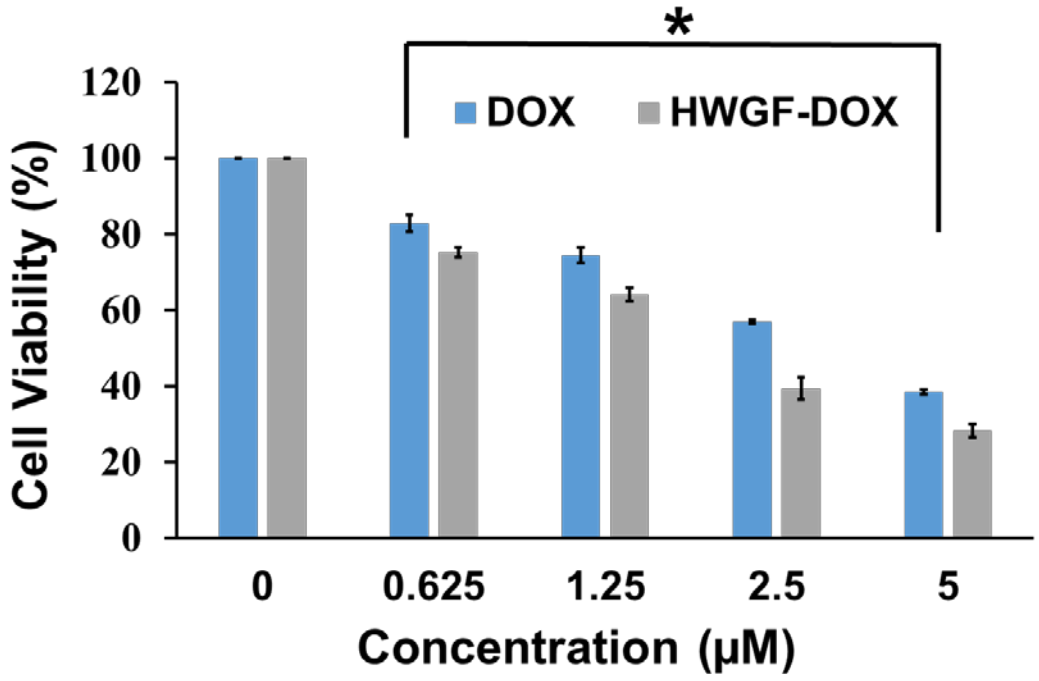

Figure S29. Bar diagram represents cell viability assay of DOX alone and DOX encapsulated inside the HWGF peptide vesicles in A549 cells up to 24 h (*p < 0.001, performing student's t-test).

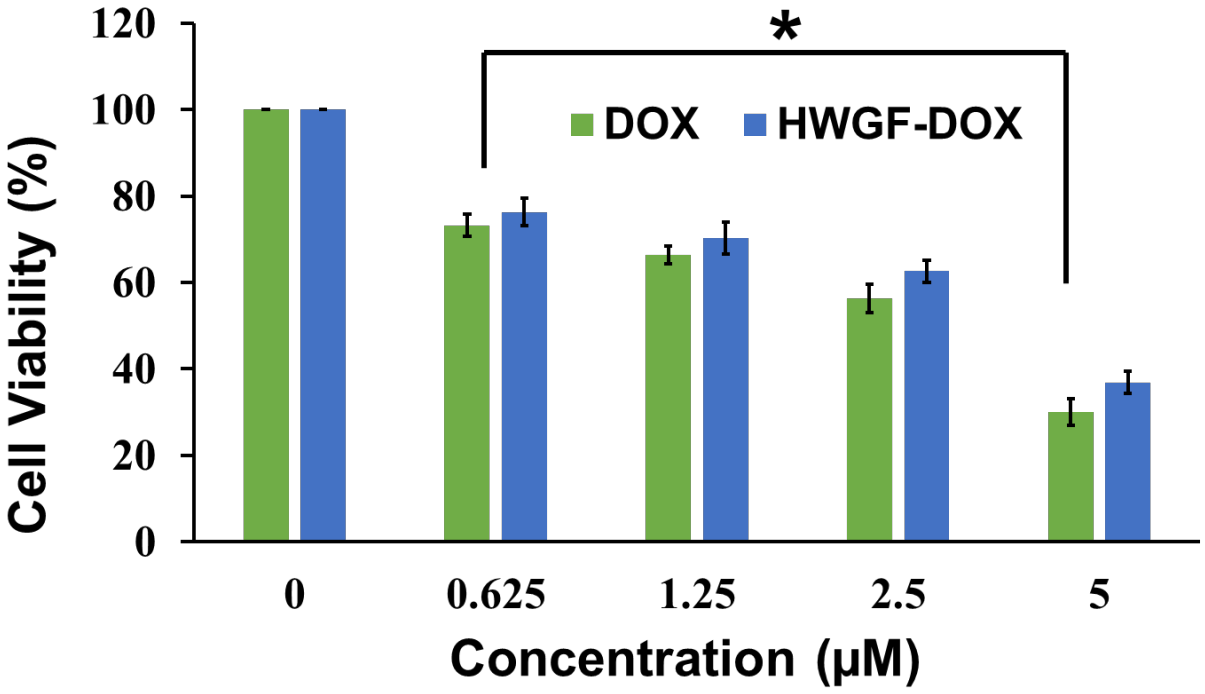

Figure S30. Bar diagram represents cell viability assay of DOX alone and DOX encapsulated inside the HWGF peptide vesicles in MCF10A cells up to 24 h (*p < 0.001, performing student's t-test).

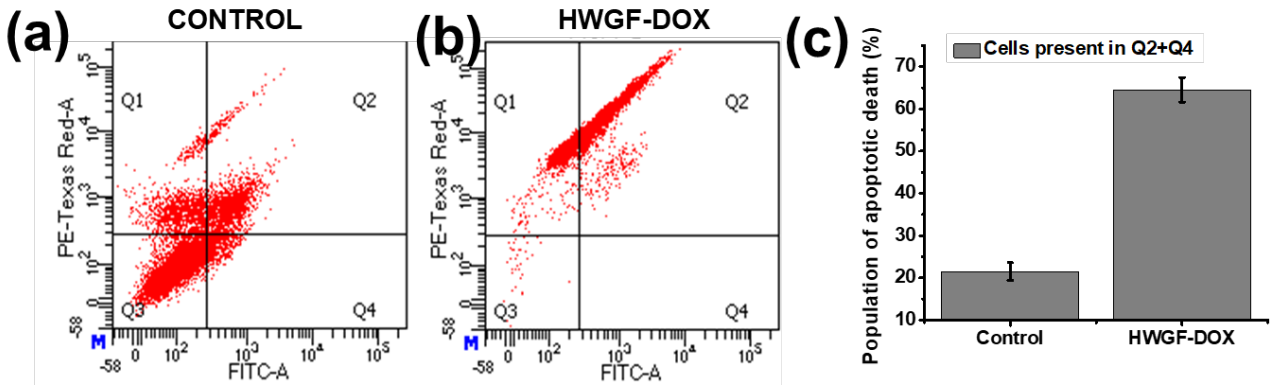

Figure S31. Apoptosis assay using FACS. MCF7 cells treated with (a) control, (b) HWGF-DOX, (c) Bar diagram shows quantitatively that HWGF-DOX induces higher apoptotic death (early and late) of MCF7 cells.

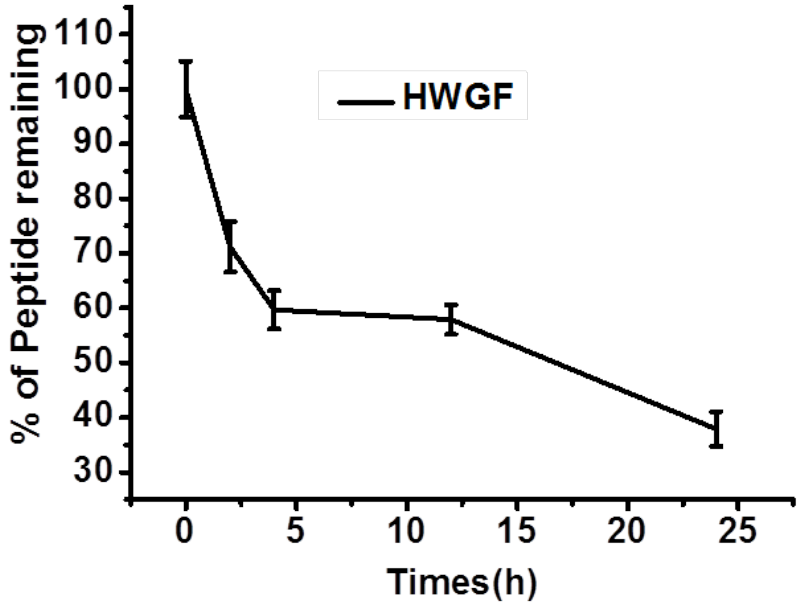

Figure S32. Graph shows the serum stability of HWGF peptide up to 24 h.

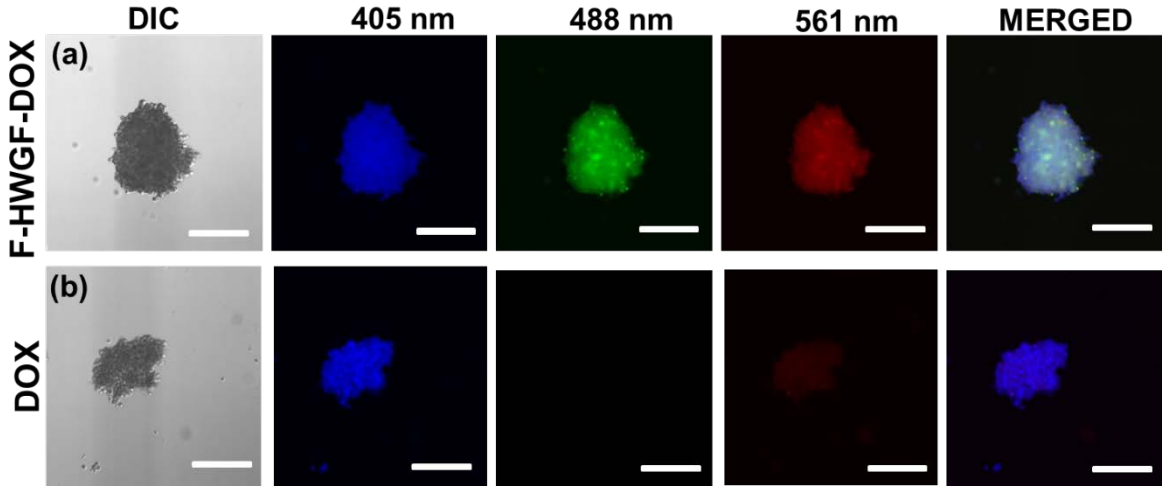

Figure S33. Spheroid image reveals the penetration of DOX encapsulated in F-HWGF vesicles (a) compared to free DOX (b). Merged images of F-HWGF-DOX indicates higher uptake of DOX compared to free DOX. Scale bars correspond to 100 µm.

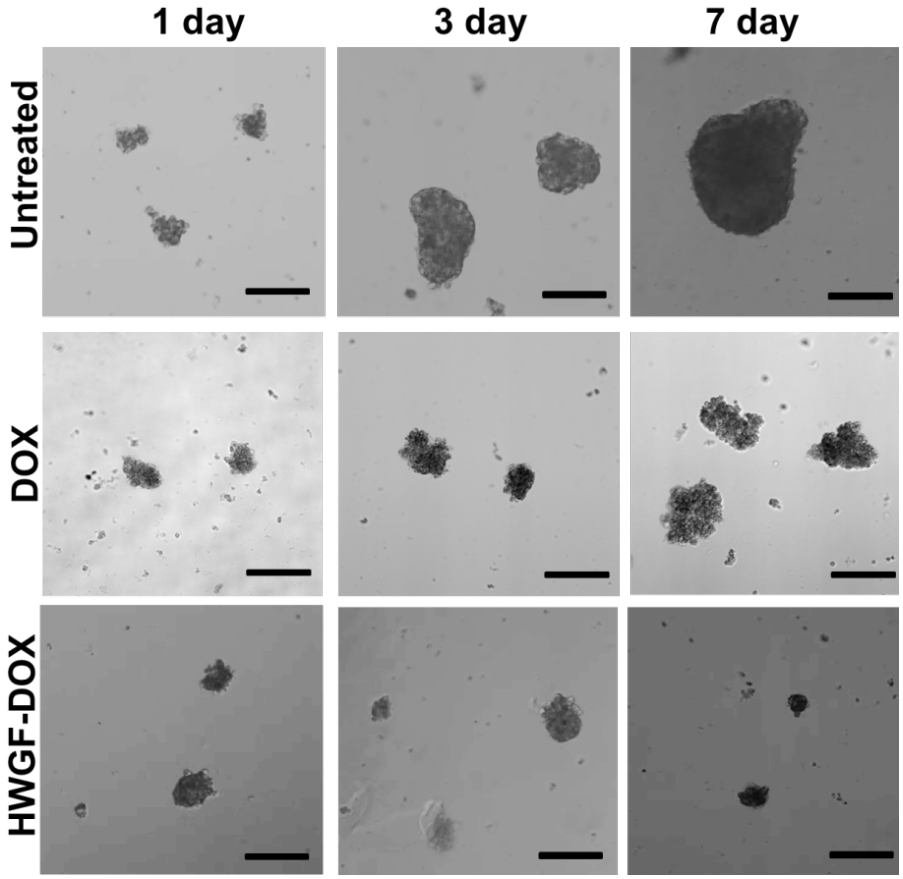

Figure S34. Spheroid growth inhibition assay upon treatment with DOX encapsulated in HWGF peptide vesicles and DOX alone was monitored for 0, 3 and 7 days. Result indicates the significant inhibition of spheroid growth in case of HWGF encapsulated DOX. Scale bars correspond to 100 μm.