Supporting Information

Cross-linked Colloidal Network of Peptide/Nucleic Base Amphiphiles for Targeted Cancer Cells Encapsulation

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1. Molecular Synthesis and Characterization

1.1 General Methods

All reactions were conducted under an argon atmosphere using anhydrous freshly distilled solvents unless otherwise stated. All organic solvents were dried and distilled before use. Anhydrous dichloromethane (DCM) and dimethyl formamide (DMF) were distilled over $CaCl_2$ and CaH_2 respectively, and kept anhydrous with 4Å molecular sieves. The water was purified by Millipore filtration system. 4-Bromo-3-methylbenzoic acid tert-butyl ester (compound 2) was purchased from Energy Chemical Co., Ltd. (Shanghai); Fmoc-protected amino acids and reagents for peptide synthesis were purchased from GL Biochem (Shanghai) Ltd. All reagents and chemicals were AR grade and used without further purification unless otherwise noted. Analytical thin-layer chromatography (TLC) was performed using silica gel 60 F254 using UV light as visualizing agent.

The ^{1}H and ^{13}C NMR spectra were recorded at room temperature with Bruker AV 400 spectrometer. High-resolution mass spectra were obtained on an LCT Premier XE (electronic spray ionization, ESI) and a Waters Micromass® Q-TOF (ESI) spectrometer. The mass spectra of peptide were tested on an AB Sciex (MALDI-TOF) mass spectrometer. The analytical "High Performance Liquid Chromatography" (HPLC) was performed with the following parameters: reversed phase, RP-C18 HPLC column (10 μm particle size) and UV detector. The mobile phase was a gradient of 10-90% of methanol aqueous solution containing 0.5‰ TFA at a total flow rate of 0.8 mL/min. The UV absorption peaked at 254 nm of the elution was recorded for analysis. FT-IR spectra were taken on NICOLET 380 FT-IR, Thermo Electron Corp. The UV/Vis and fluorescence spectra were recorded with a Varian Cary 100 Conc UV-Visible Spectrometer and a Fluoromax-4 Spectrofluorometer (HORIBA Scientific), respectively. The pH was measured by a Mettler Toledo FE 20K pH meter.

1.2 Synthesis of Fmoc-Lys(Rh)-OH:

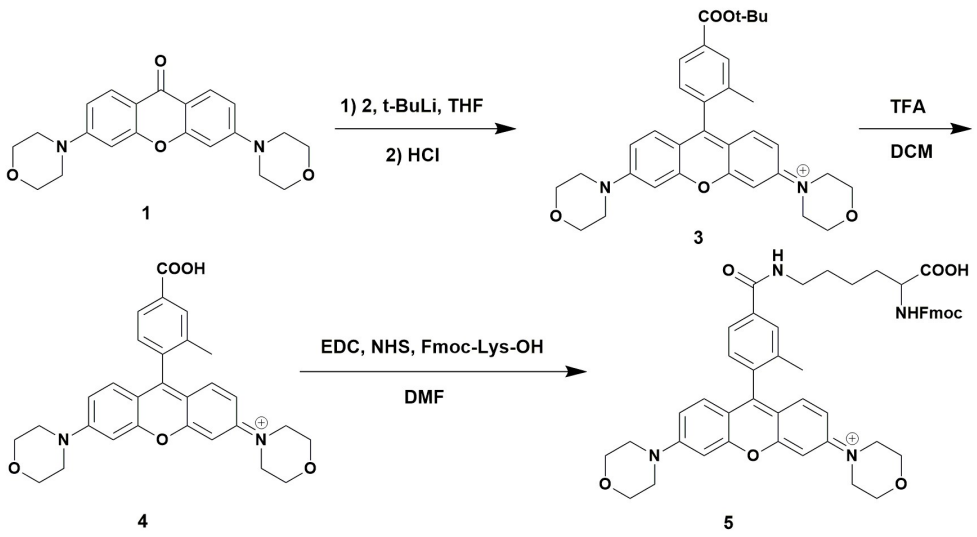


Figure S1 Synthesis route of **Fmoc-Lys(Rh)-OH**.

Synthesis of compound 1: Compound **1** was prepared by the reported procedure in the literature. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.15 (d, *J* = 8.9 Hz, 2H), 6.87 (d, *J* = 8.9 Hz, 2H), 6.67 (s, 2H), 3.87 (s, 8H), 3.34 (s, 8H). HRMS-ESI (m/z): calcd for $C_{21}H_{24}N_2O_4$, 366.1580, found $[M+H]^+$, 367.1559.

Synthesis of compound 3: To a solution of the 4-Bromo-3-methylbenzoic acid *tert*-butyl ester (**2**) (2.8 g, 10.9 mmol) in THF (30 mL) cooled to -78ºC under an argon atmosphere was slowly added *n*-BuLi (2.5 M, 4.2 mL, 10.5 mmol), and the solution was stirred for 30 min. To this solution was added compound **1** (1 g, 2.7 mmol) in THF (15 mL) in a dropwise manner. The reaction mixture was stirred for 1 h. The mixture was further treated with 2 M HCl (10 mL) and stirred for another 10 min. After having been diluted with sat. $NaHCO_3$, the reaction mixture was extracted with DCM (3 × 30 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated by rotary evaporation. Subsequently, the solution was recrystallized from ethyl acetate/petroleum ether to red solid **3** (1.26 g, 89.3%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.03 (s, 1H), 8.00 (d, *J* = 7.9 Hz, 1H), 7.38 (s, 2H), 7.20 (m, 3H), 7.14 (s, 1H), 7.12 (s, 1H), 3.89 (d, *J* = 4.0 Hz, 8H), 3.82 (d, *J* = 3.8 Hz, 8H), 2.06 (s, 3H), 1.64 (s, 9H). HRMS-ESI (m/z): calcd for $C_{33}H_{37}N_2O_5^+$, 541.2697, found $[M]^+$, 541.2675.

Synthesis of compound 4: To a solution of **3** (1.2 g, 2.3 mmol) in TFA/DCM (1/1, 10 mL) at r.t. for 3 h. The solution was concentrated, and the resulting residue exchanged with CH_3OH and dried under high vacuum. The crude product **4** was used for the next step without any purification. ^1H NMR (400 MHz, DMSO-d^6): δ = 13.30 (s, 1H), 8.09 (s, 1H), 8.01 (d, *J* = 7.9 Hz, 1H), 7.45 (d, *J* = 7.9 Hz, 1H), 7.33 (d, *J* = 9.6 Hz, 2H), 7.25 (d, *J* = 2.3 Hz, 2H), 7.09 (d, *J* = 9.5 Hz, 2H), 3.77 (s, 16H), 2.07 (s, 3H). HRMS-ESI (m/z): calcd for $C_{29}H_{29}N_2O_5^+$, 485.2071, found $[M]^+$, 485.2091.

Synthesis of compound 5: Compound **5** (200 mg, 0.41 mmol) was dissolved in the mixture of 100 ml of DMF, followed by addition of the solution of EDC·HCl and NHS in DMF. After incubation at room temperature for 2 h, the solution of Fmoc-Lys-OH (288 mg, 0.62 mmol) with 1 mL of TEA in DMF was added. The reaction was allowed to proceed for 2 h. The mixture was further treated with H_2O (20 ml) and stirred for another 10 min and washed with DCM (3 × 10 mL), the organic layers were combined, dried with $MgSO_4$, filtered and concentrated by rotary evaporation. The product was purified by column chromatography using MeOH/DCM (1:20). The product is a rad solid **5** (648 mg, 64.7% yield). HRMS-ESI (m/z): calcd for $C_{50}H_{51}N_4O_8^+$, 835.3701, found $[M]^+$, 835.3702.

1.3 Synthesis of Fmoc-Lys(Nph)-OH:

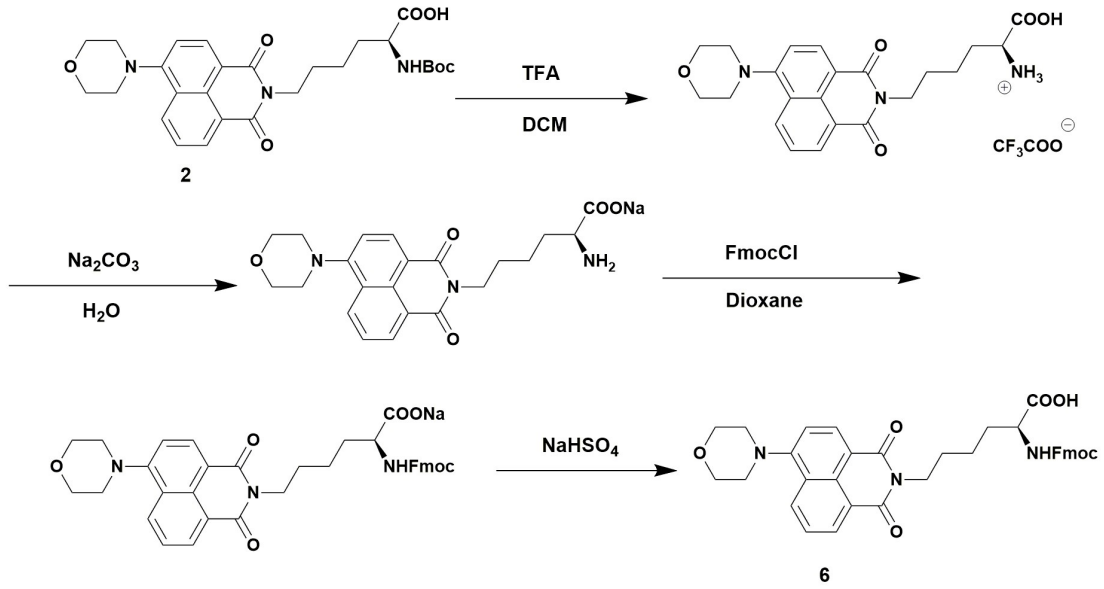


Figure S2 Synthesis route of **Fmoc-Lys(Nph)-OH.**

Synthesis of compound 2: Compound 2 was prepared by the reported procedure in the literature. It was washed with water, dried over anhydrous Na_2SO_4. ^1H NMR (400MHz, CDCl3): δ = 1.44 (s, 9H), 1.49-1.56 (m, 2H), 1.75-1.84 (m, 3H), 1.95-2.00 (m, 1H), 3.36-3.28 (t, J = 4.4 Hz, 4H), 4.01-4.04 (t, J = 4.6 Hz, 4H), 4.12-4.27 (m, 3H), 5.30 (s, 1H), 7.22 - 7.24 (d, J = 8.0 Hz, 1H) , 7.67-7.73 (t, J = 15.6 Hz, 1H), 8.42 - 8.44 (d, J = 8.4 Hz, 1H), 8.53 - 8.55 (d, J = 8.0 Hz, 1H), 8.59-8.61 (d, J = 7.2 Hz, 1H).

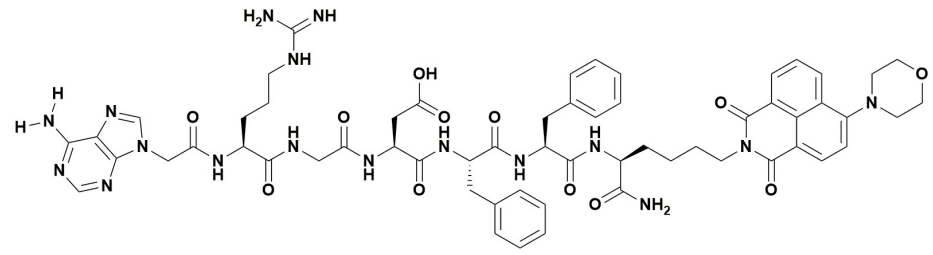
Synthesis of Fmoc-Lys(Nph)-OH 6: The compound 2 (5.42 g, 10.57 mmol) obtained in the above step was dissolved in 60 mL of dichloromethane, and 30 mL of trifluoroacetic acid was slowly added dropwise in an ice water bath, and stirred at room temperature overnight. Spin dry, add 200 mL of water, extract with dichloromethane, collect the aqueous layer, spin off most of the water, add 100 mL of 1,4-dioxane, adjust the pH to 8-9 with Na_2CO_3 aqueous solution. FmocCl (3.28 g, 12.68 mmol) was dissolved in 30 mL of 1,4-dioxane, added dropwise to the mixture and stirred at room temperature overnight. After completion of the reaction, it was acidified with $NaHSO_4$ to pH 5-6, and extracted twice with dichloromethane. It was eluted with a silica gel column gradient (dichloromethane/methanol = 1/0-250/1, 0.5% glacial acetic acid). It was washed with water, dried over anhydrous with Na_2SO_4, dried to give a yellow solid powder 6 (4.8 g, yield 71.8 %). ^1H NMR (400 MHz, $CDCl_3$): δ = 1.42-1.57 (m, 2H), 1.99-2.10 (m, 2H), 3.92 (s, 4H), 3.98-4.00 (t, J = 3.8 Hz, 4H), 4.18- 4.22 (m, 3H), 4.27-4.32 (m, 1H), 4.36-4.41 (m, 2H), 5.30 (s,1H), 5.65-5.67 (d, J = 8.0 Hz, 1H), 7.13-7.15(d , J = 8.0 Hz, 1H), 7.27-7.29 (d, J = 7.6 Hz, 2H), 7.36-7.40 (t, J = 7.4 Hz, 2H), 7.58-7.60 (m, 2H), 7.63-7.65 (t, J = 7.8 Hz, 1H), 7.74-7.76 (d, J = 7.6 Hz, 2H), 8.36-8.39 (d, J = 8.4 Hz, 1H), 8.51-8.53 (d, J = 8.0 Hz, 1H), 8.58-8.60 (d, J = 7.2 Hz, 1H) .

1.4 Synthesis of Peptides:

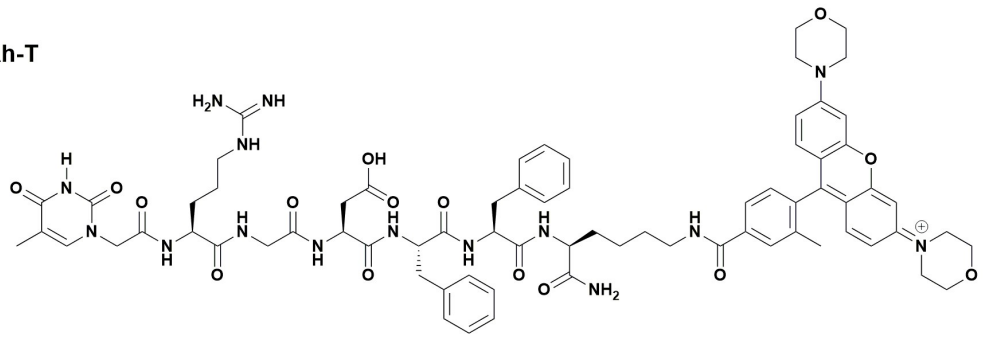
Synthesis of Nph-A, Rh-T and Nph-T: Rink amide resin (390 mg, 0.64 mmol/g, 0.25 mmol, 1 equiv.) was weighed and allow to swelling in DCM/DMF (5.0/5.0 mL) for 2 h in a glass peptide synthesis vessel. Then, the Fmoc protection group was

removed. After an intensive cycle with DMF the following amino acid were attached for SPPS: Fmoc-protected amino acid (0.75 mmol, 3 equiv.), DIC (2.5 mmol, 10.0 equiv.), HOBt (0.75 mmol, 3.0 equiv.) in DMF (15 mL), washed 1×30 mL with DCM, 2×30 mL with DMF. Bases (adenine or thymine acetic acid) were attached at room temperature. The final products were cleaved from the solid support according to the general procedure for the rink amide resin. The final products were cleaved from the solid support by treatment with a cleavage cocktail of trifluoroacetic acid-triisopropylsilane-H_2O (95:2.5:2.5) for 2 h. After removing the solution, the solid for PAs were precipitated with dry diethyl ether and centrifuged at 6500 rpm for 15 min. The crude products were purified and isolated by preparative high performance liquid chromatography (HPLC) on a C-18 column. They were further characterized using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Figure S3-6).

Nph-A



Rh-T



Nph-T

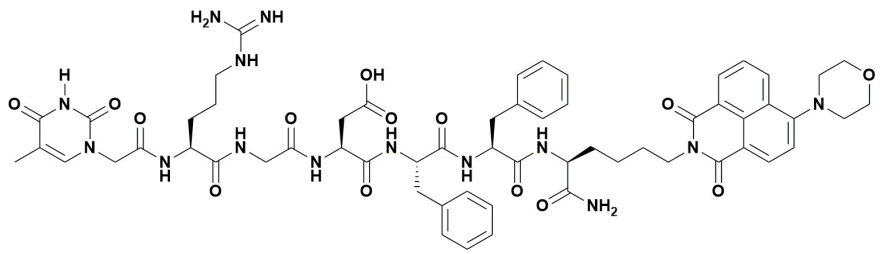


Figure S3. Chemical Structures of **Nph-A**, **Rh-T** and **Nph-T**.

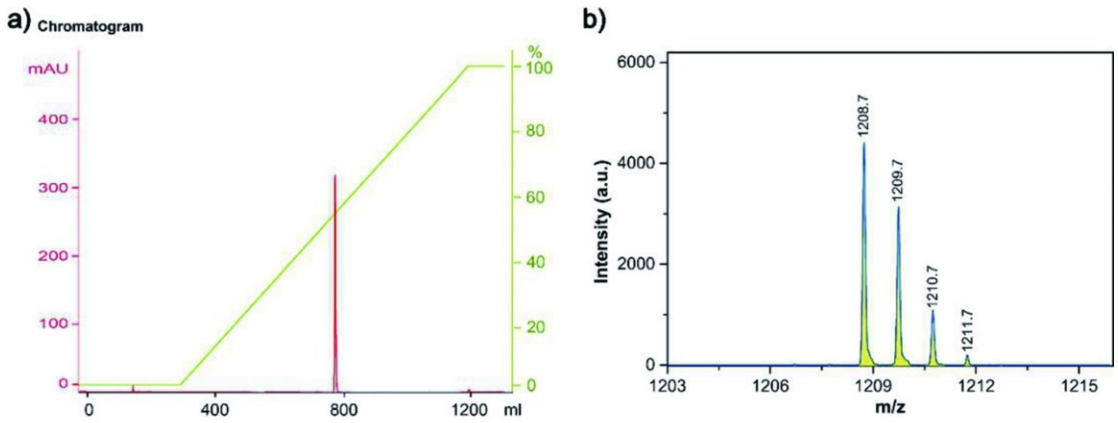


Figure S4. Analytical HPLC and MALDI-TOF spectra of **Nph-A**.

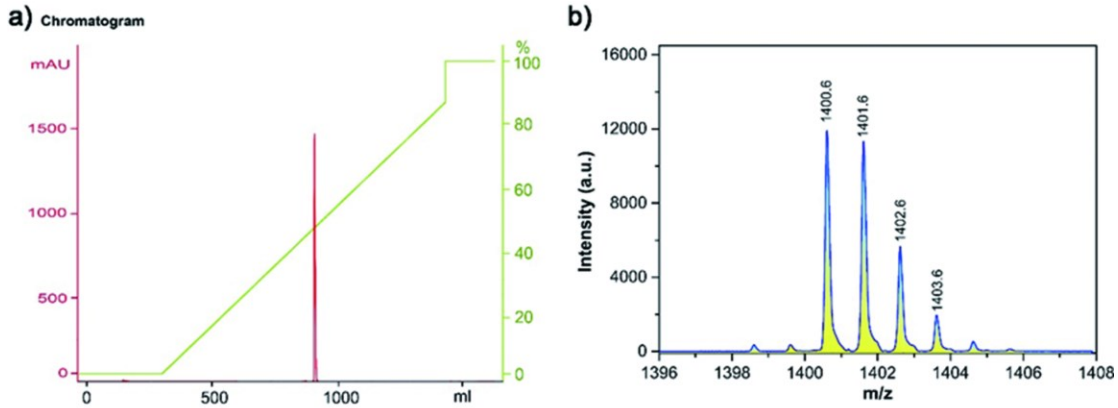


Figure S5. Analytical HPLC and MALDI-TOF spectra of **Rh-T**.

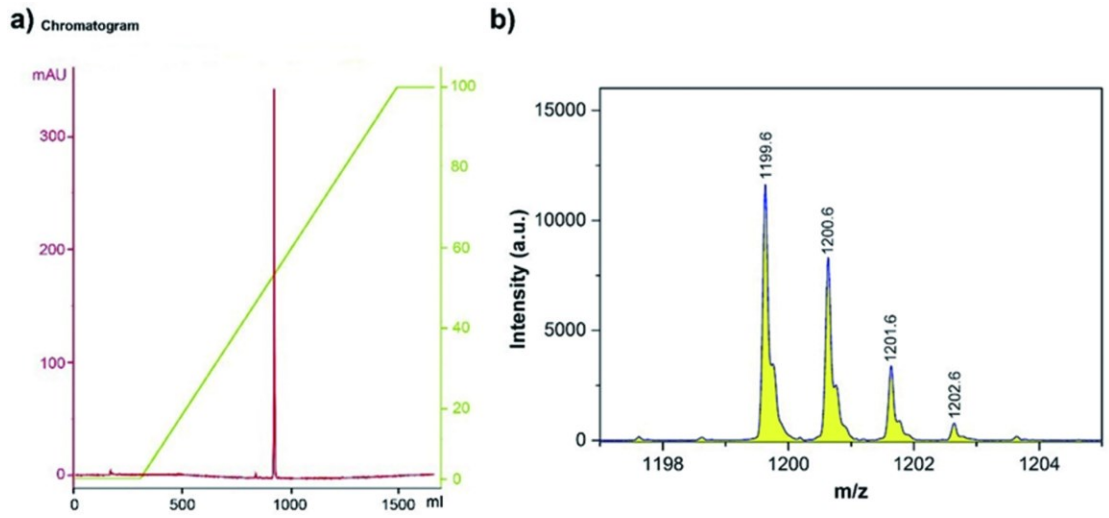


Figure S6. Analytical HPLC and MALDI-TOF spectra of **Nph-T**.

2. Fluorescence Spectroscopy

All fluorescence and absorption spectra were recorded using a VARIAN Cary Eclipse Fluorescence Spectrophotometer at 25 °C. The samples were each excited at the wavelength appropriate for the fluorescent peptide. The slit widths were set to 10 nm for excitation and emission. The data points were collected at 1 nm increments with a 0.1 s integration period. All spectra were corrected for intensity using the manufacturer-supplied correction factors and corrected for background fluorescence and absorption by subtracting a blank scan of the buffer system. The peptides were dissolved in a 50 mM TBS buffer (25 ºC).

3. Circular Dichroism (CD) Spectroscopy

CD spectra were recorded on a spectropolarimeter (*Chirascan*) using a 10 mm path length quartz UV-Vis absorption cell at 25 ºC. The brand widths were set to 5 nm, data points were collected at 0.5 nm increments with a scanning speed of 200 nm/min. Background spectra of the solvents/buffer were acquired and subtracted from the sample spectra.

4. Transmission Electron Microscopy (TEM)

Dissolve the **Nph-A**, **Rh-T**, **Nph-T** directly into hexafluoroisopropanol (HFIP) to gain a stock at a concentration of 10^{-3} mol/L. Peptides were diluted into different ratios with TBS after HFIP was evaporated. Samples were prepared by placing a few droplets onto a carbon-coated grid with holes. Samples were stained with sodium phosphotungstate (2.0 wt% aqueous solutions) for 20 s and dried at room temperature. TEM characterization was performed using a JEM-2100 electron microscope (JEOL, Japan). The diameters distribution of assembled nanostructures of peptides was analysed by ImageJ.

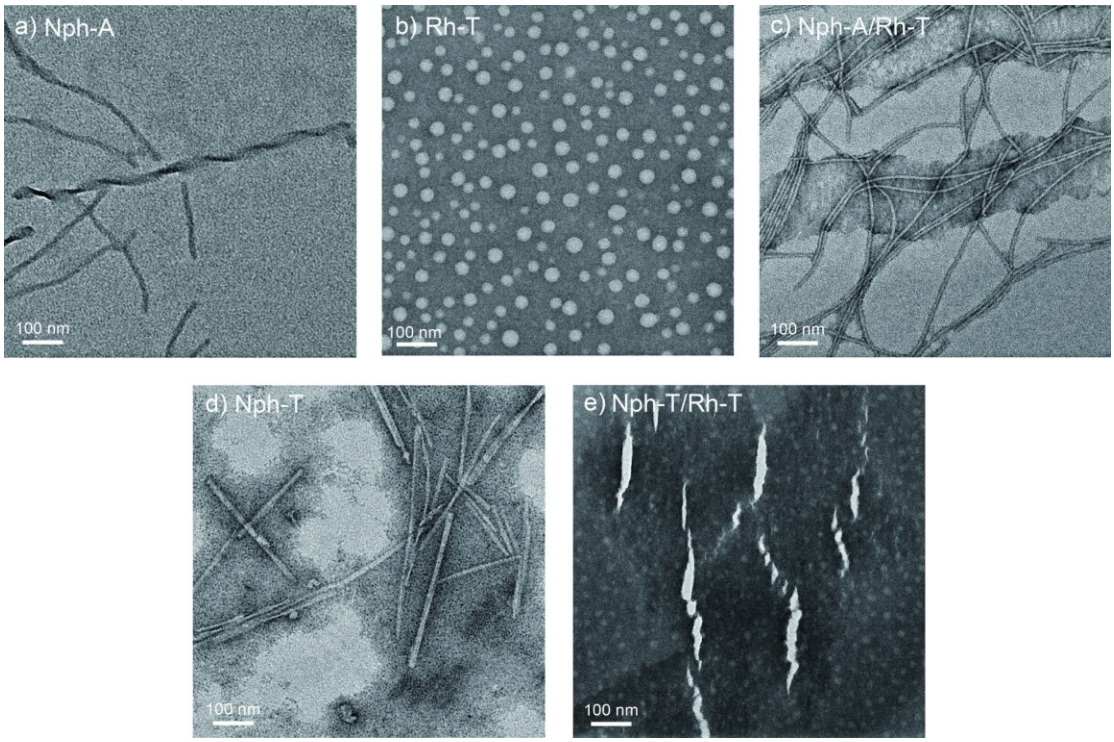


Figure S7. TEM images of a) **Nph-A** (10 μM), b) **Rh-T** (10 μM), c) **Nph-A/Rh-T** (10 μM, 1:1), d) **Nph-T** (10 μM), e) **Nph-T/Rh-T** (10 μM, 1:1) after aging for 2 h in TBS (Tris-HCl = 50 mM, pH = 7.4, 25^{o}C). Scale bar = 100 nm.

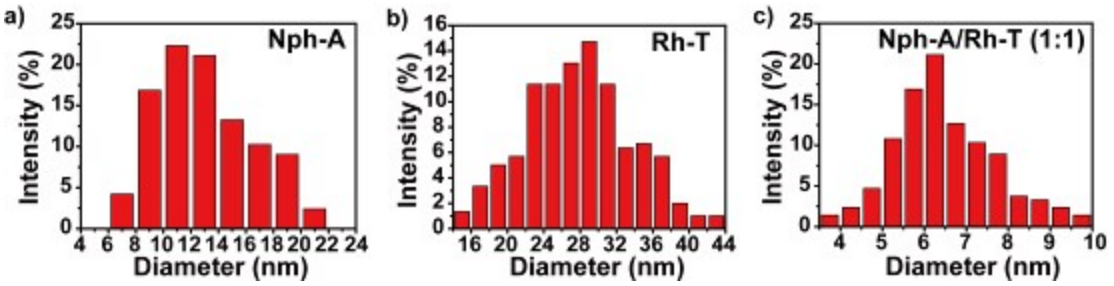


Figure S8. The diameters distribution of assembled nanostructures of peptides a) **Nph-A** (10 μM), b) **Rh-T** (10 μM), c) **Nph-A/Rh-T** (10 μM, 1:1) by statistical analysis of TEM images above.

5. Dynamic Light Scattering (DLS)

The hydrodynamic diameters of the peptides were measured using a dynamic light scattering spectrometer (DLS). The DLS experiments were determined by Nano-ZS (Zatasizer, Malvern) instrument. Samples of **Nph-A** (10.0 μM), **Rh-T** (10.0 μM), **Mixture** of (**Nph-A/Rh-T**=1:1, 10.0 μM) in TBS (pH 7.4, 25 °C) in a total sample volume of 1.0 mL.

6. Fourier Transform Infrared (FT-IR) Spectroscopy

Peptide solids were freeze-dried and prepared at least 24 h before measurements.

Samples for FT-IR spectroscopy were prepared as KBr pellets. Typically, a sample of 1 mg was ground to homogeneity in a mortar together with 200 mg dry KBr (Sigma-Aldrich) and immediately pressed to a pellet (10 mm diameter) with 7.5 tons of pressure. Pellets were stored under dry nitrogen until measured. The FT-IR spectroscopy spectra of the KBr pellets were measured in transmission geometry in a Magna-IR 550 FT-IR spectrometer (Nicolet, USA). A pellet of pure KBr from the same batch was used as a reference for the absorbance measurements. The spectra were collected within the wavelength range of 4000-400 cm^{-1} at 1 cm^{-1} resolution and were used without any further mathematical processing.

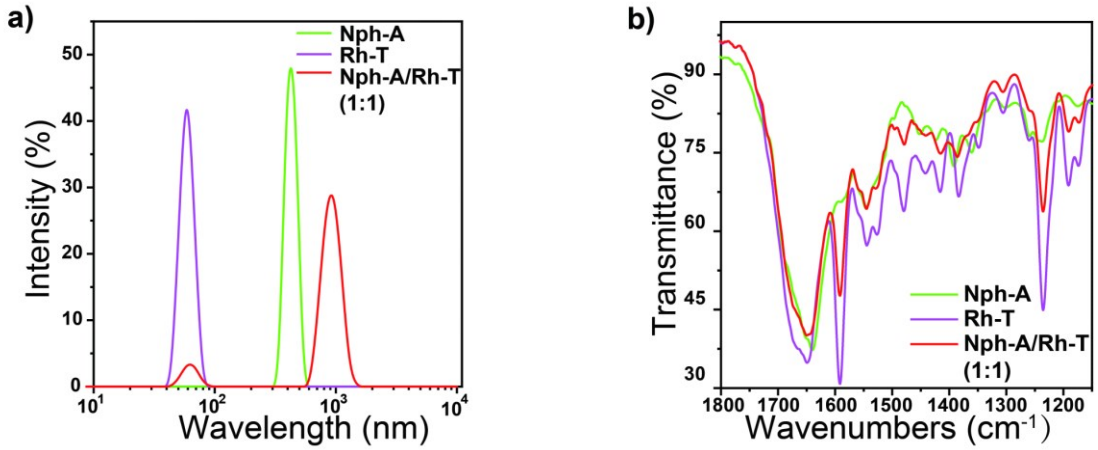


Figure S9. a) DLS profile showing hydrodynamic diameters for **Nph-A** (10.0 μM), **Rh-T** (10.0 μM), **Nph-A/Rh-T** (10 μM, 1:1) in TBS (50 mM Tris, pH 7.4, 25°C). b) Fourier transform infrared (FTIR) spectra of **Rh-T** (10 μM), **Nph-A** (10 μM) and co-assembled **Nph-A/Rh-T** (10 μM, 1:1), respectively.

7. SEM measurement

Nph-A (1.2 mg) and **Rh-T** (1.4 mg) powder were solved in 100 μL deionized water to a concentration of 20 mM. Then the sample was heated under 90 °C until the peptides were totally solved. After cooling down, hydrogel samples were obtained. The hydrogels were kept at -80 °C overnight under vacuum to get xerogels using a FD-1A-50 Freeze Drier. Field emission scanning electron microcopy (FESEM) measurements were carried out using a GeminiSEM 500 with an accelerating voltage of 3 kV. The sample was prepared on a conducting resin. Then the sample was coated with a layer of Pt to increase the contrast before SEM measurement.

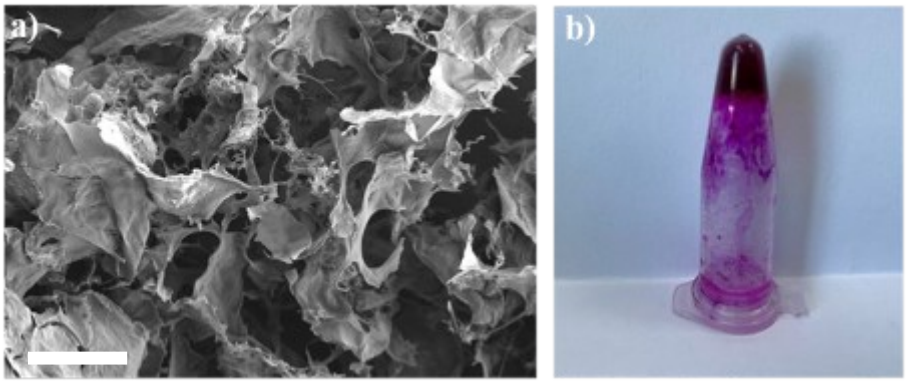


Figure S10. a) SEM image of **Nph-A/Rh-T** (molar ratio = 1:1) at a concentration of 20 mM. Scale bar = 10 μm. b) The photo of the upside-down centrifugal tube with **Nph-A/Rh-T** (molar ratio = 1:1) at a concentration of 20 mM after gelation.

8. Cell Experiments

Cell culture: MDA-MB-231 (human breast cancer cell) and HeLa (human cervical carcinoma cell) were purchased from Shanghai Bogoo Biotech Co., Ltd, China. All cells were cultured in HG-DMEM (Gibco) at 37 °C under humidified conditions of 95% air and 5% CO_2. All media were supplemented with 10% FBS (fetal bovine serum), 100 U penicillin and 0.1 mg of streptomycin (Gibco) per milliliter. The culture media were changed every two days to maintain exponential growth of the cells. Cells were passaged using 0.25% Trypsin/EDTA (Sigma) when they reached 80-90% confluence and seeded for the experiments.

Preparation of culture medium with peptides: The peptides were pretreated with HFIP for shattering assembly system (10^{-3} mol/L). After the HFIP volatilized, the culture solution was added and diluted to certain concentrations using HG-DMEM with 10% FBS and aged for 2 h before further using.

Confocal microscopic imaging: MDA-MB-231/HeLa cells (1×10^5) were seeded into optical dishes and incubated overnight, after which the cells were washed twice with PBS and added with 1 mL HG-DMEM containing 10% FBS and peptide assembly, followed by incubation for 2.5 h at 37 °C under humidified conditions of 95% air and 5% CO_2. Then, the cells were washed 5 times, and cytomembrane staining was performed with DiD ($DiIC_{18}(5)$). Cell fluorescence images were conducted with the confocal laser scanning microscope (Nikon A1, Japan, 60x oil-immersion objective lens). 3D confocal images were performed using Olympus Fluoview FV1000 confocal laser scanning microscope (Olympus Life Science Europa

GmbH, Hamburg, Germany). A UPLSAPO 60x oil objective lens was used to scan cells of 14.4 μm thickness at a step of 0.8 μm. Channel 1 for **Rh-T**: excitation: 561 nm, emission collected: 570-620 nm; Channel 2 for **Nph-A**: excitation: 405 nm, emission collected: 470-550 nm; Channel **DiD** for $DiIC_{18}(5)$: excitation: 640 nm, emission collected: 663-738 nm.

DiD was diluted in HG-DMEM without FBS at a concentration of 20 μM.

Cytotoxicity assay: For adherent cells, cells were seeded at 1×10^4 into a 96-well plate with and incubated overnight. After that, the media were discarded, replaced by fresh media containing a series of concentrations (0 μM, 6.25 μm, 12.5 μM, 25 μM, 50 μM) of various peptides and incubated for the designated time. Cells without treatment were run concurrently as control samples. Then, the supernatants were discarded, and 100 μL of culture media containing 10% CCK-8 was added to every well. The cells were incubated at 37 °C for 1-2 h, and the absorbance at 450 nm was measured by an enzyme plate analyzer (Synergy, Boten Instrument Co., Ltd). The groups added Cilengitide were cultured with Cilengitide (40 μM) for 3 h before adding peptides.

Scratch wound healing assay: Cells were seeded in 6-well plates with 1×10^6 cells per well, and incubated at 37 ℃ until cells reached a density of at least 90%. Wounds were created by scratching cell monolayers with a 200 μL plastic pipette tip. Then the cells were washed with PBS and incubated in fresh medium containing 1% FBS for 24 h. Quantification of the migration rate of cells was calculated by ImageJ:

Migration of cells (%) = $(A_{t=0\ h} - A_{t=24\ h})/A_{t=24\ h}\times100$.

$A_{t=0\ h}$ is the area of the wound measured immediately after scratching (time zero), and $A_{t=24\ h}$ is the area of the wound measured 24 hours after the scratch is performed.

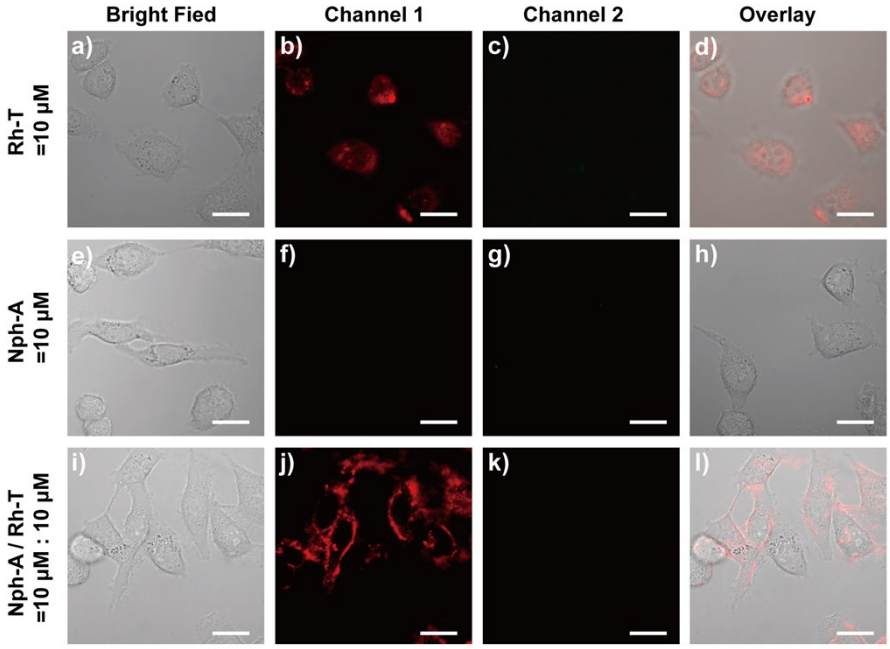


Figure S11. Confocal laser scanning microscopy of MDA-MB-231 cells incubated with assembled a-d) **Rh-T** (10 µM), e-h) **Nph-A** (10 µM), i-l) co-assembled **Nph-A/Rh-T** (1:1; **Rh-T** = 10 µM), respectively. Channel 1 for **Rh-T**: excitation: 561 nm, emission collected: 570-620 nm. Channel 2 for **Nph-A**: excitation: 405 nm, emission collected: 470-550 nm. Scale bar: 20 µm.

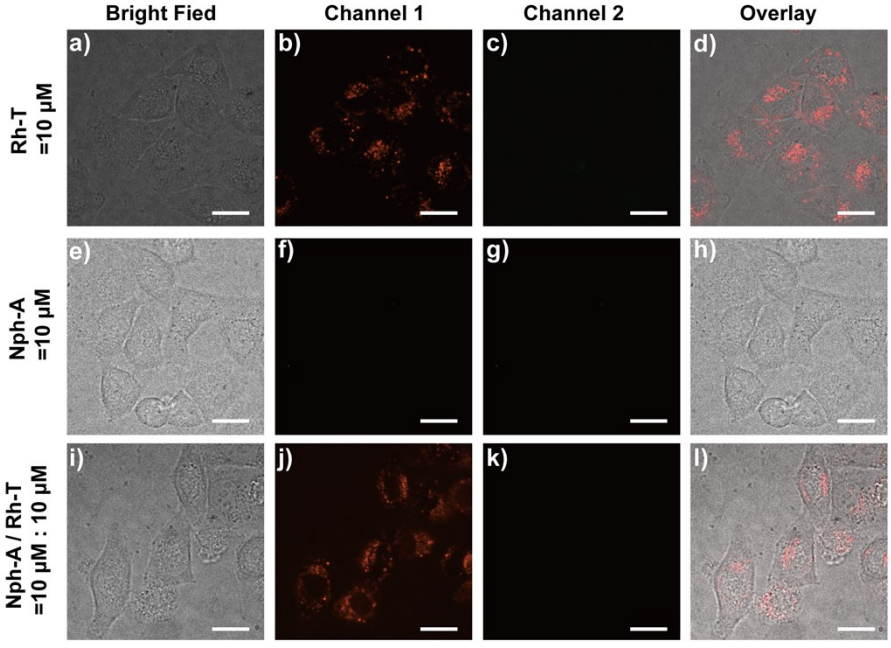


Figure S12. Confocal laser scanning microscopy of HeLa cells incubated with assembled a-d) **Rh-T** (10 µM), e-h) **Nph-A** (10 µM), i-l) co-assembled **Nph-A/Rh-T** (1:1; **Rh-T** = 10 µM), respectively. Channel 1 for **Rh-T**: excitation: 561 nm, emission collected: 570-620 nm. Channel 2 for **Nph-A**: excitation: 405 nm, emission collected: 470-550 nm. Scale bar: 20 µm.

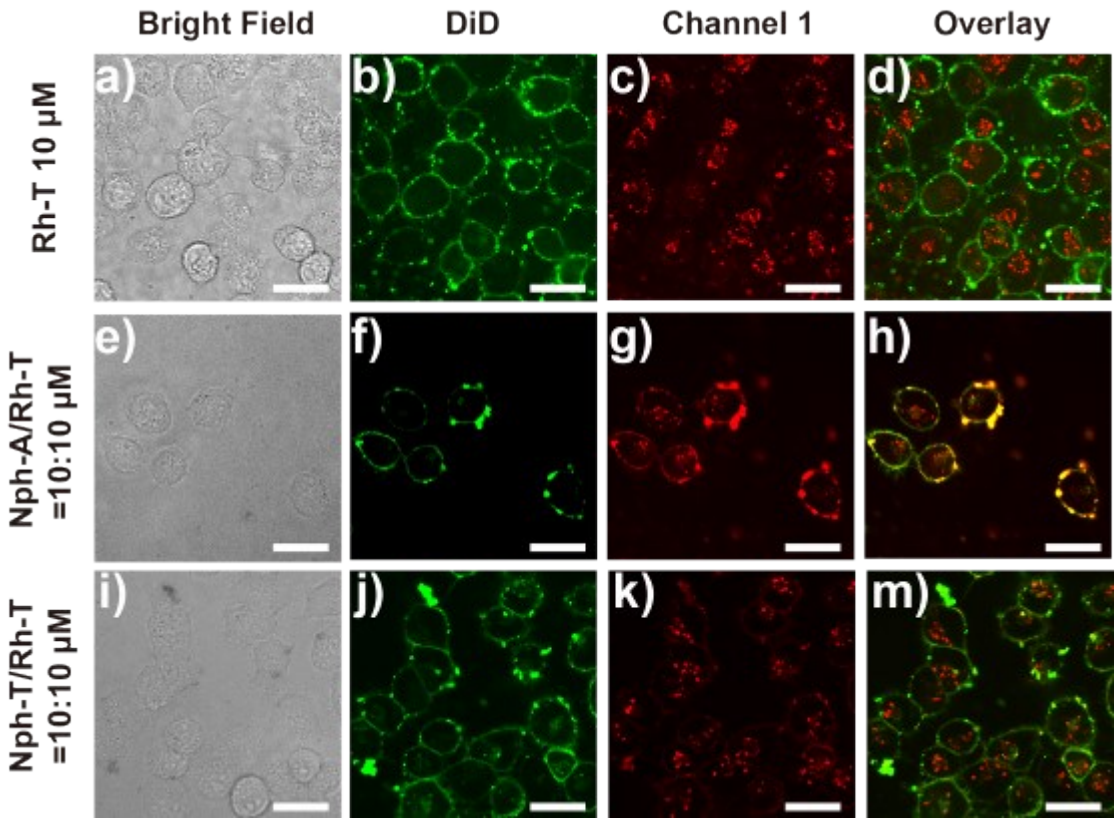


Figure S13. Confocal laser scanning microscopy of MDA-MB-231 cells incubated with assembled a-d) **Rh-T** (10 μM), e-h) **Nph-A** (10 μM), i-l) co-assembled **Nph-A/Rh-T** (1:1; **Nph-A** = 10 μM, **Rh-T** = 10 μM) and m-p) co-assembled **Nph-T/Rh-T** (1:1; **Nph-T** = 10 μM, **Rh-T** = 10 μM), respectively. Channel DiD for $DiIC_{18}(5)$: excitation: 640 nm, emission collected: 663-738 nm. Channel 1 for **Rh-T**: excitation: 561 nm, emission collected: 570-620 nm. Scale bar: 10 μm, DiD: 20 μM.

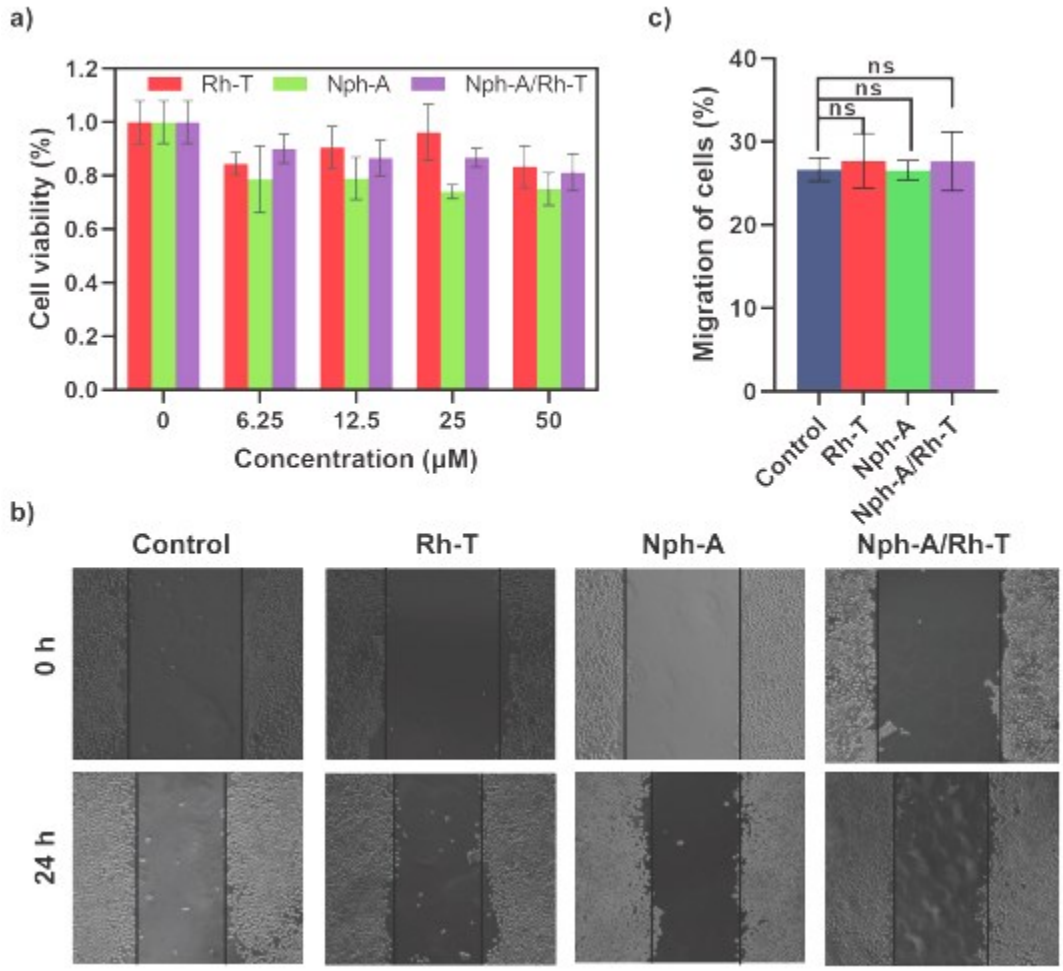


Figure S14. a) CCK-8 assay of HeLa cells incubated with **Rh-T**, **Nph-A**, and co-assembled **Nph-A/Rh-T** (1:1) in various concentrations (0 μM, 6.25 μM, 12.5 μM, 25 μM and 50 μM). b) Scratch wound healing assay of HeLa cells with **Rh-T** (12.5 μM), **Nph-A** (12.5 μM) and the co-assembled **Nph-A/Rh-T** (1:1, 12.5 μM). c) Quantification of the wound closure (%). ns: $p \geqslant 0.05$.