Supplementary Information for:

Transition from Vesicles to Nanofibres in the Enzymatic Self-Assemblies of an Amphiphilic Peptide as an Antitumour Drug Carrier

Contents:

S1 MATERIALS AND METHODS

S1.1 Materials.

Fmoc-Ala-OH, Fmoc-Lys(Boc)-OH, 1-hydroxybenzotrizole (HOBT), o-benzotriazole-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU) and Rink amide MBHA resin were obtained from GL Biochem Ltd. (Shanghai, China). Deprotection reagents, cracking reagents and solvents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) was provided by HyClone (Logan, USA). Trypsin, foetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and penicillin/streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). DOX was purchased from Apexbio Technology LLC (Houston, USA). Calcein-AM, propidium iodide (PI)

and Hoechst 33258 were purchased from Yeasen Biotech Co., Ltd. (Nanjing, China), while SYTO 9 was purchased from Life Technologies (Carlsbad, USA). Ultrapure water was produced by a Milli-Q Biocel system (Millipore TANKPE030), and it was used in all experiments. Unless specifically noted, all reagents were used without further purification.

The gram-negative (G^-) strain *Escherichia coli* DH5a (*E. coli* DH5a) and the gram-positive (G^+) strain *Staphylococcus aureus* (*S. aureus*) were supplied by the China Center of Industrial Culture Collection. The methods used to culture *E. coli* DH5a and *S. aureus* were reported in a previous work.[1] The human liver cell line L02 and human hepatocellular carcinoma cell line BEL-7402 were purchased from the Shanghai Institute for Biological Science of the Chinese Academy of Sciences. L02 cells and BEL-7402 cells were cultured in DMEM with 10% (v/v) FBS at 37 °C.[1]

S1.2 Synthesis and Characterization of the Peptide.

An amphiphilic peptide with the sequence Ac-AAAAAAKK-NH_2 (A_6K_2) was synthesized from the C-terminus to the N-terminus using a CEM Liberty Blue automatic microwave peptide synthesizer through a standard Fmoc solid-phase peptide synthesis (SPPS) method. The peptide was purified according to a previously described method.[1] The purity of the final product (>95%) was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and reversed-phase high-performance liquid chromatography (RP-HPLC) analysis.

S1.3 Preparation and Enzyme-responsiveness of Peptide Nanovesicles.

A_6K_2 (0.741 mg) was dissolved in 1 mL of 4-(2-hydroxyethyl)- 1-piperazineethanesulfonic acid (Hepes) buffer (25 mM, pH 7.4) to obtain a 1 mM stock solution, which was incubated for 24 h at room temperature prior to use. The enzyme-responsive peptide nanovesicles were prepared in an aqueous solution as follows: A_6K_2 (0.741 mg) was dissolved in 0.91 mL of Hepes buffer (25 mM, pH 7.4), and then 0.05 mL of PAO solution (400 U/mL) and 0.04 mL of $CuCl_2$ solution (250 μM) were added to the stock solution of A_6K_2 after standing for 24 h. After PAO addition, the A_6K_2 solution was incubated at 37 °C for the indicated time. The changes in peptide concentration and pH after these treatments were negligible.

S1.4 Self-assembly of A_6K_2 in Solution.

A 30-μL aliquot of peptide solution was pipetted onto parafilm and then adsorbed onto a pure carbon membrane for 3 min. The excess solution was removed with filter paper, and the surface was dried in air. The sample was stained with a small amount of phosphotungstic acid dye (1.0%) for 3 min, and then the dye was removed with filter paper. The sample was naturally air-dried and

tested. A HITACHI HT7700 instrument (Japan) was used for transmission electron microscopy (TEM) studies.

The peptide solution was freeze-dried to a powder using a vacuum freeze dryer (EYELA FDU-1100, Japan), and a clean, single-crystal silicon wafer was placed on the test copper surface with a conductive paste on the reverse side. A small amount of peptide powder was applied to the surface of the silicon wafer, and then the samples was coated in gold powder for testing. A HITACHI SU8010 (Japan) was used for scanning electron microscopy (SEM) studies.

S1.5 MALDI-TOF MS and RP-HPLC.

The A_6K_2 solution was incubated for 15 days both with and without PAO addition, and then the molecular weight was determined by MALDI-TOF MS and RP-HPLC. The mass spectra were recorded on an Autoflex III mass spectrometer (Bruker Daltonics, Germany) in positive-ion mode over a mass range of m/z 500 to 2000 via a similar method using a 355-nm laser for excitation.[1]

Before and after the addition of PAO, A_6K_2 was analysed by RP-HPLC on a C18 reversed-phase column with an Agilent 1260 Infinity HPLC system (USA). RP-HPLC analyses were performed with the following mobile phases: A: water with 0.1% (v/v) TFA, and B: acetonitrile with 0.1% (v/v) TFA. The gradient was 0-1 min 95% A and 5% B; 1-40 min from 95% A/5% B to 5% A/95% B; 40-45 min from 5% A/95% B to 95% A/5% B. The sample was filtered, and the filtrate was extracted via a similar method.[1] The injection volume was 40 μL.

S1.6 CD Spectra.

A_6K_2 was incubated with PAO for 15 days, and then the secondary structure was analysed using an Applied Photophysics Chirascan spectrometer (APL, England). The CD spectra were scanned from 260 to 190 nm in a 1-mm quartz cell. A_6K_2 solution incubated for 15 days without PAO was used as a control. The obtained ellipticity (θ) data were converted into the mean residue ellipticity ([θ] ($deg \cdot cm^2 \cdot dmol^{-1}$)).

S1.7 Size Distribution.

The mean particle size and size distribution of the peptide self-assemblies were determined using dynamic light scattering (DLS) techniques with a Zetasizer Nano ZS90 instrument (MALVERN Instruments, England). The peptide solutions with and without added PAO were incubated for 15 days and then tested.

S1.8 Cytotoxicity Assay and Antitumour Effect *In Vitro.*

The cytotoxicity of the peptide was tested by an MTT assay. The human liver cell line L02 and human hepatocellular carcinoma cell line BEL-7402 were selected as cell models. L02 and BEL-7402 cells were plated into 96-well plates at a cell density of 1×10^5 per millilitre, and the

samples were cultured in a 37 °C incubator. After 24 h, the medium was changed to solutions containing dissociative DOX, the DOX-encapsulated amphiphilic peptide A_6K_2 or different concentrations of A_6K_2. DOX (0.148 mg) and peptide (0.741 mg) were dissolved in 5 μL of DMSO, and Hepes buffer was added to a volume of 0.5 mL and shaken for 30 min. The same volume of DOX-encapsulated amphiphilic peptide A_6K_2 solution was added to cultured cell medium after standing at room temperature for 24 h in the dark. The final concentration of dissociative DOX was 0.148 mg/mL. All samples were incubated at 37 °C for the indicated time. Ten microlitres of MTT solution (5 mg/mL) was added to each well, and the mixtures were incubated for 4 h. The culture supernatant in each well was carefully removed. Then, 100 μL of DMSO was added to each well, and the plates were shaken for 10 min. The OD value of the sample was measured at 490 nm using a microplate reader (Multiskan go, Thermo Scientific, USA). The cell viability is expressed as a percentage of the control (untreated) cells, and survival in the control group was considered 100%. All experiments were performed in triplicate.

S1.9 Bacterial Viability Assays *In Vitro*.

Suspensions (100 μL) of *E. coli* and *S. aureus* (1×10^6 CFU/mL) were inoculated into 96-well plates, and 20 μL of 6 mM A_6K_2 peptide solution or 20 μL of medium was added to each well and placed in the incubator (HERAcell 150i, Thermo, USA) for 24 h. The OD value of the sample was measured at 600 nm with a microplate reader (Multiskan go, Thermo Scientific, USA). The peptide-free solution was used as a control group, and only 120 μL of the medium was added as a blank group. The survival rates of the bacteria were calculated based on the following equation:

$$\text{Mortality \%} = (1 - (OD_{600\ nm\ peptide} - OD_{600\ nm\ blank}) / (OD_{600\ nm\ TCPS} - OD_{600\ nm\ blank})) * 100$$

where $OD_{600\ nm\ peptide}$ denotes the absorbance value of peptide solution-coated wells in the presence of bacteria measured at 600 nm, and $OD_{600\ nm\ TCPS}$ and $OD_{600\ nm\ blank}$ denote the absorbance values of the wells without peptide solution in the presence and absence of bacteria, respectively, measured at 600 nm.

Further study of the true growth of *E. coli* in the peptide environment was performed as follows. Aliquots (1000 μL) of *E. coli* DH5a suspensions (1×10^6 CFU/mL) and 200 μL of 1 mM A_6K_2 solution were added onto a dish with a borosilicate glass bottom. Moreover, 1200 μL of *E. coli* DH5a incubated in LB medium was added to a dish with a borosilicate glass bottom as a control. After these suspensions were incubated for 24 h at 37 °C, the liquid in the culture dishes was carefully removed and washed 3 times with sterile PBS solution. One hundred microlitres of

solution containing 15 μM SYTO 9 and 30 μM PI was added to the culture dish, and staining occurred for 15 min at ambient temperature. A 3D scan was performed using a confocal laser scanning microscope (CLSM, TCS SP8, Leica, German) at 40× magnification, scanning one layer every 5 μM and scanning 36 layers along the Z axis. The excitation wavelength of 488 nm was selected, and the green fluorescence of SYTO 9 was observed in the emission wavelength range of 500-550 nm. The red fluorescence of PI was observed in the emission wavelength range of 580-630 nm.

S1.10 Drug Release Test *In Vitro*.

To evaluate the nanovesicle encapsulation efficiency, DOX was dissolved in DMSO, and a standard curve (wavelength of 480 nm) of free DOX was prepared with an ultraviolet (UV)-visible spectrophotometer (Thermo Evolution 300, USA). The packaging efficiency was calculated according to the following formula: encapsulation efficiency (%) = (the mass of the drug encapsulated in the nanoparticles / the mass of the added drug) × 100%.

The release kinetics of A_6K_2-encapsulated DOX *in vitro* were measured by dialysis. Briefly, 0.1 mL of DOX (0.148 mg) and peptide (0.741 mg) dissolved in DMSO were sonicated and homogenized, and Hepes buffer was added to bring the volume to 1 mL. The solution was transferred to a dialysis bag with a molecular weight cut-off of 1 kDa. After sealing, the mixture was stirred in 2000 mL of distilled water and continuously dialysed for 24 h, and the distilled water was replaced every 4 h to remove the DMSO and unembedded DOX.

The DOX release kinetics from A_6K_2 with or without PAO incubation were measured by an ultraviolet-visible spectrophotometer. Briefly, dialysis bags with or without PAO (20 U/mL)- and $CuCl_2$ (10 μM)-loaded peptide solutions were immersed in 25 mL of ultrapure water. At predetermined times, aliquots of the samples were taken, and 25 mL of fresh ultrapure water was added after each sampling. The absorbance of the sample at 480 nm was measured using a UV-visible spectrophotometer, and the amount of DOX cumulatively released was calculated. The experiment was repeated three times at room temperature in the dark for each sample.[2,3]

S1.11 Confocal Laser Scanning Microscopy for Cell Endocytosis.

L02 cells and BEL-7402 cells were seeded in glass-bottom culture dishes and incubated in DMEM containing 10% FBS for 24 h. Subsequently, after dialysis to remove the DMSO and unembedded DOX, DOX-encapsulating amphiphilic peptide nanovesicles were added to the cells for a specific time, and then the cells were washed with PBS buffer. Staining was performed by adding Hoechst 33258 and Calcein-AM for 5 min and 15 min, respectively. The cells were washed 3 times with PBS buffer, and then the stained cells were observed under a CLSM (Leica,

TCS SP8 Germany). The excitation wavelength of Hoechst 33258 was 405 nm, and the emission wavelength range was 430-490 nm. The excitation wavelength of DOX and calcein-AM was 488 nm, and their emission wavelength ranges were 580-640 nm and 500-550 nm, respectively.

S2 Supporting Figures

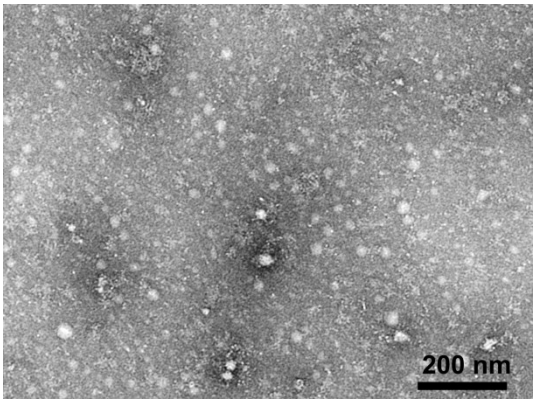

Fig. S1 TEM image of the A_6K_2 self-assembled nanostructures after the addition of PAO and incubation for 7 days at 37 °C.

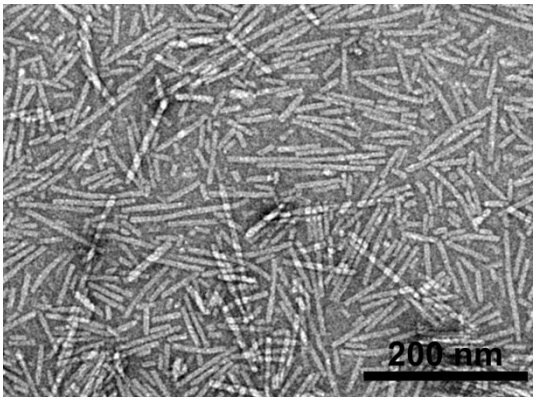

Fig. S2 TEM images of A_9K_2 self-assembled nanostructures. The A_9K_2 solution of 1 mM was left to stand at room temperature for 24 h.

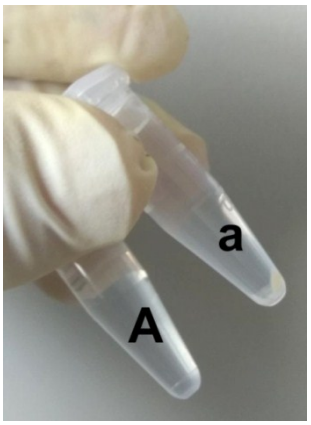

Fig. S3 Photographs of the A_6K_2 solution after incubation (a) with or (A) without PAO for 15 days followed by centrifugation at 3000 rpm for 3 min.

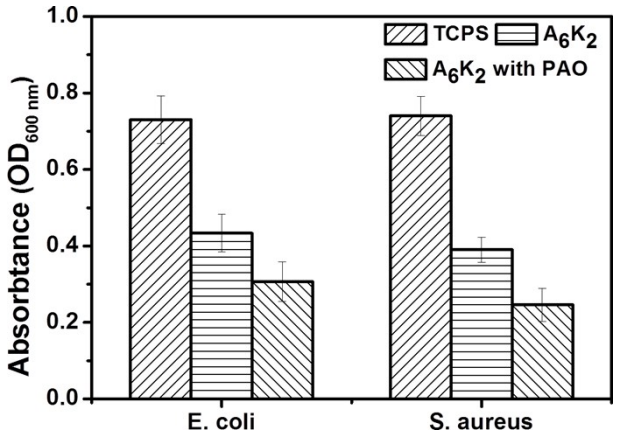

Fig. S4 Bacterial growth in A_6K_2 solution or A_6K_2 with PAO solution after incubation for 24 h. Before the addition of PAO, the peptide solution was aged for 24 h at room temperature, followed by enzymatic incubation at 37 °C for 15 days.

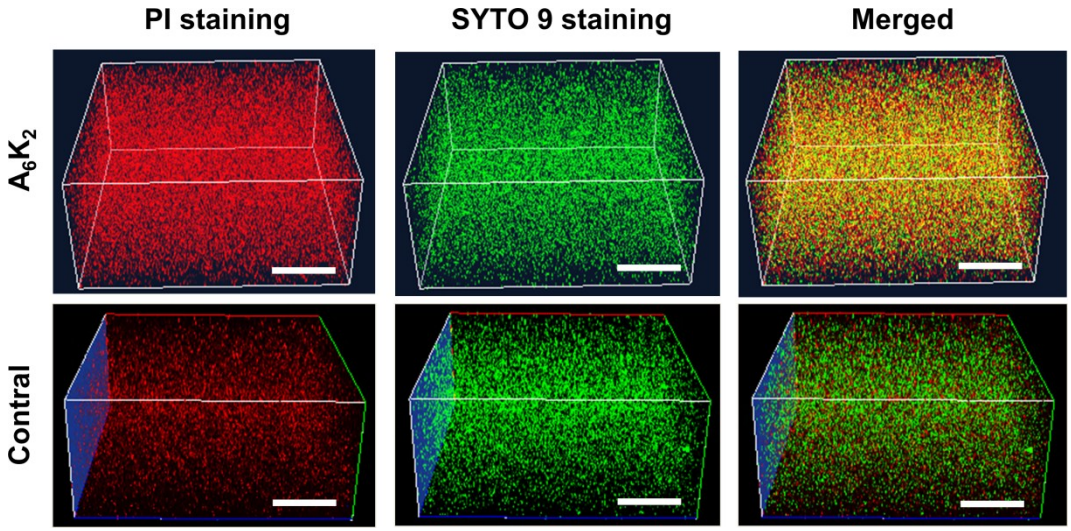

Fig. S5 CLSM images of *E. coli* DH5a in an A_6K_2 solution after incubation for 24 h. Live (green)/dead (red) staining was performed with SYTO 9 and PI. Bacteria incubated on the TCPS plates in the absence of A_6K_2 solution acted as a control. The scale bar is 100 μM.

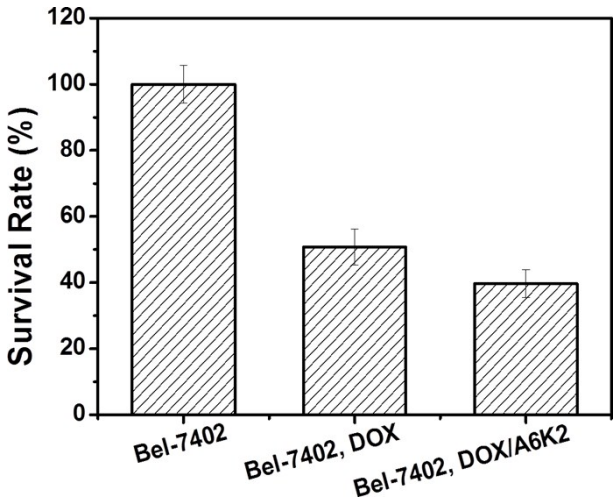

Fig. S6 MTT assay results for BEL-7402 cells cultured for 24 h with DOX or DOX-encapsulated A_6K_2 nanovesicles.

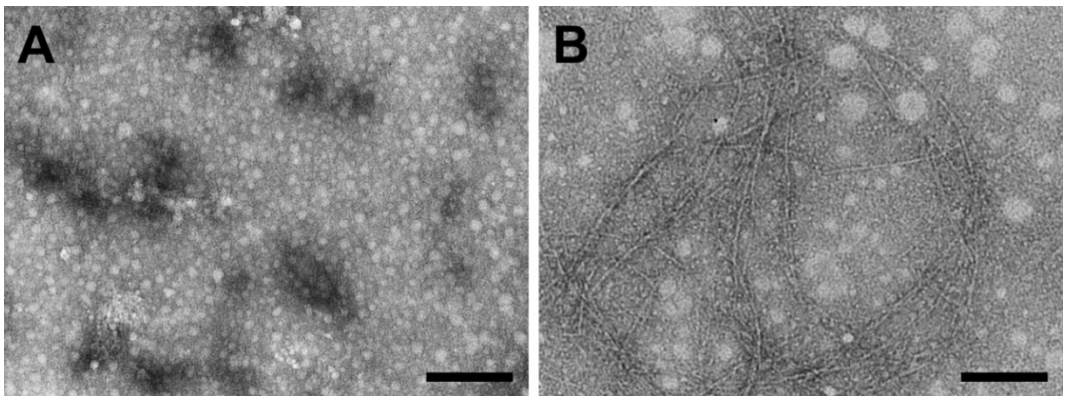

Fig. S7 TEM images of DOX-loaded, self-assembled nanostructures (A) before and (B) after the addition of PAO; the measurements were performed after 120 days of drug release at 37 °C. The scale bar is 200 nm.

S3 References

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