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Supporting Information

of

Complementary hydrogen bonding interaction triggered co-assembly of amphiphilic peptide and anti-tumor drug

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1. Materials

N-Fluorenyl-9-methoxycarbonyl (FMOC) protected amino acids (FMOC-Gly-OH, FMOC-Asp(OtBu), FMOC-Arg(Pbf)-OH and FMOC-Ser(tBu)-OH), 2-chlorotrityl chloride resin (100-200 mesh, loading: 1.32 mmol/g), 9-fluorenylmethoxy carbonyl chloride (Fmoc-Cl), *N*-hydroxybenzotriazole (HOBt), benzotriazole-*N,N,N',N'*-tetramethyluroniumhexafluorophosphate (HBTU) and piperdine were purchased from GL Biochem (Shanghai) Ltd. (China) and used as received. FMOC protected 12-aminododecanoic acid (FMOC-ADDA-OH) was synthesized according our previous reports [1,2]. Triisopropylsilane (TIS) and pyrene were provided by Sigma-Aldrich and used directly. Trifluoroacetic acid (TFA), methotrexate (MTX), 2,2,2-trifluoroethanol, cyanuric acid, chloroacetic acid, 1,8-diazabicyclo[5.4.0]-7-undecene (DBU), dichloromethane (DCM), 1,4-dioxane and ethyl were provided by Shanghai Reagent Chemical Co. (China) and used without purification. Dimethylformamide (DMF) and diisopropylethylamine (DiEA) were obtained from Shanghai Reagent Chemical Co. (China) and distilled prior to use. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT), penicillinestreptomycin and trypsin were purchased from Invitrogen Corp. All other reagents and solvents are of analytical grade and used directly.

2. Synthesis of the amphiphilic peptide (CA-C11-GGGRGDS)

The peptide was synthesized manually in 1.32 mmol scale on the 2-chlorotrityl chloride resin employing a standard FMOC solid phase peptide synthesis (SPPS) technique. Before the reaction, the resin was washed with DCM (three times) and DMF (three times) and then immersed in DMF for 30 min. After draining off DMF solution, a DMF solution of the mixture of FMOC-Ser(tBu)-OH (4 equiv relative to resin loading) and DiEA (6 equiv) was added to the resin and shaken for 2 h at room temperature. After removing the reaction solution, the resin was washed with DMF (three times). Subsequently, 20% piperdine/DMF (V/V) solution was introduced to the resin to remove the FMOC

protected groups. After shaking for 30 min at room temperature, the reaction solution was drained off and the resin was washed with DMF (three times). The presence of free amino groups was indicated by a blue color in the Kaiser test. Thereafter, a DMF solution of the mixture of FMOC-Asp(OtBu)-OH (4 equiv), HBTU (4 equiv), HOBt (4 equiv) and DiEA (6 equiv) was added. After shaking at room temperature for 1.5 h, the reaction solution was drained off and the resin was washed with DMF (three times). The absence of free amino groups was indicated by a yellow color in the Kaiser test. After the repetition of the deprotection and acylation reaction, a DMF solution of the mixture of chloroacetic acid (4 equiv), HBTU (4 equiv), HOBt (4 equiv) and DiEA (6 equiv) was added. After shaking at room temperature for 1.5 h, the resin was washed with DMF (three times) and DCM (three times). Cleavage of the peptide from the resin was performed using a mixture of 2,2,2-trifluoroethanol, acetic acid and DCM in the ratio of 2:1:7. After 2 h shaking at room temperature, the cleavage mixture and DCM washing were collected. The combined solution was concentrated to a viscous solution by rotary evaporation. Cold ether was then added to precipitate the product. After washing with cold ether (three times), the product (fully protected peptide with a sequence of Cl-C11-GGGRGDS) was obtained after drying under vacuum for 24 h.

To synthesize the amphiphilic peptide (CA-C11-GGGRGDS), cyanuric acid (58 mg, 0.45 mmol), DBU (0.24 mL, 1.6 mmol) and the fully protected peptide (0.51 g, 0.41 mmol) prepared above were dissolved in 2.5 mL anhydrous DMF. The mixture was stirred for 7 h at 70 ^{o}C. After removal of DMF under reduced pressure, the residue was dissolved in 10 mL of the mixture of TFA, TIS and H_2O in the ratio of 95:2.5:2.5. After 2 h stirring at room temperature, the solution was concentrated to a viscous solution by rotary evaporation. Cold ether was then added to precipitate the product. After washing with cold ether (three times), the amphiphilic peptide (CA-C11-GGGRGDS) was collected after drying under vacuum for 24 h. ESI-MS (Fig. S1): 969.9 $(M-H)^-$. The purity of the amphiphilic peptide (91.4%) was examined by high-performance liquid chromatography (HPLC, Fig. S2).

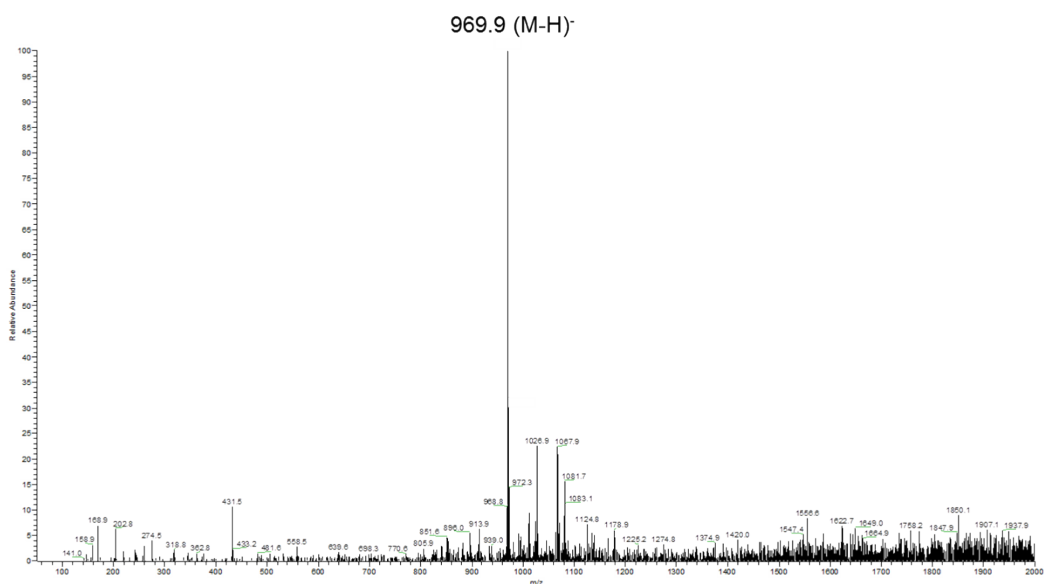

Fig. S1. ESI-MS profile of the amphiphilic peptide.

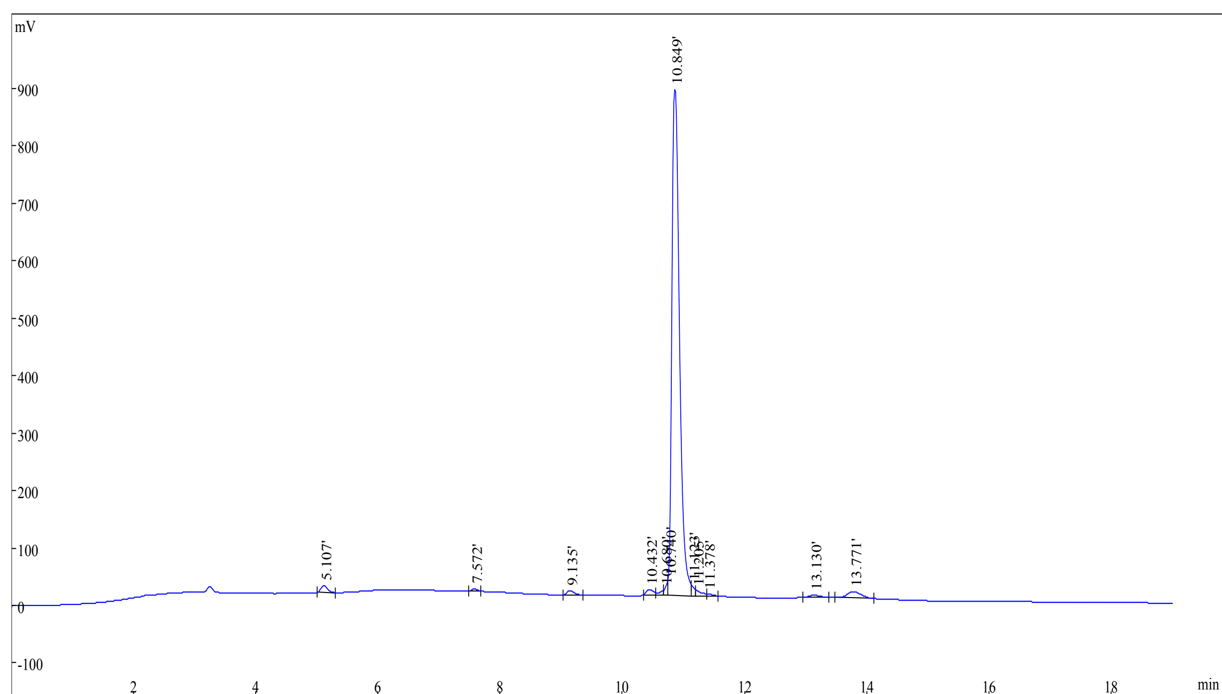

Fig. S2. HPLC profile of the amphiphilic peptide.

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3. Nuclear magnetic resonance (NMR)

The ^{1}HNMR spectra of MTX, cyanuric acid and the mixture of MTX and cyanuric acid were recorded on a Mercury VX-300 spectrometer at 300 MHz (Varian, USA) by using DMSO-*d6* as a solvent. To

prepare the mixture of MTX and cyanuric acid, these two chemicals with equal molar ratio were first dissolved in methanol. After sonication for several seconds, the solvent was removed by rotary evaporation. After dissolution-sonication-evaporation thrice, the sample was dissolved in DMSO-*d6* for NMR analysis. As shown in Fig. S3, the signals at ~6.7 and 7.5 ppm correspond to the protons of
5 the amine groups of MTX [3]. After mixing with cyanuric acid, these two peaks respectively shift to ~7.3 and 7.9 ppm, indicating the formation hydrogen bonds between MTX and cyanuric acid [4-6].

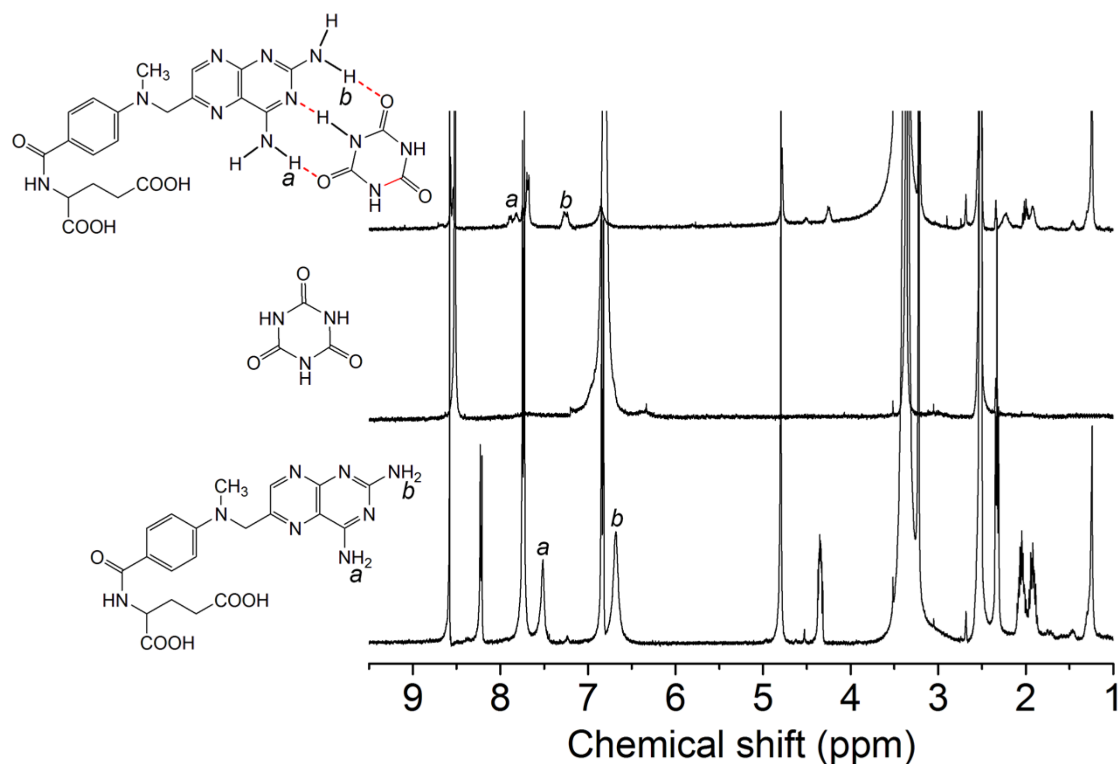
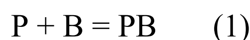


Fig. S3. ^{1}HNMR spectra of MTX, cyanuric acid and the mixture of MTX and cyanuric acid.

10 **4. Determination of the hydrogen bond association constant (K_a)**

The MTX and cyanuric acid with different molar ratio were dissolved in DMSO and the UV absorbance of MTX at 303 nm was collected on UV spectrophotometer (Perkin-Elmer Lambda Bio 40 UV/VIS spectrometer, USA). Assuming that MTX and cyanuric acid form 1:1 complex (equation 1), the K_a between MTX and cyanuric acid was determined by using the Benesi-Hildebrand model
15 (equation 2) [7].



$$P + B = PB \qquad (1)$$

$$C_{P0}/\Delta A = 1/[C_{B0}K(\varepsilon_{PB}-\varepsilon_{P})] + 1/(\varepsilon_{PB}-\varepsilon_{P}) \qquad (2)$$

Herein, C_{P0} and C_{B0} are the initial concentrations of MTX and cyanuric acid, while ε_P and ε_{PB} are the absorbance coefficients of MTX and the cyanuric acid/MTX complex, respectively. With the addition of different amount of cyanuric acid to MTX solution at a concentration of 2.2×10^{-5} M^{-1}, the absorbance (ΔA) at 303 nm will be changed during the complexation. By plotting $C_{P0}/\Delta A$ vs. $1/C_{B0}$, a linear relationship can be obtained. As shown in Fig. S4, the K_a between MTX and cyanuric acid can be calculated as ~664 M^{-1} via the intercept divided by the slope. In this work, the pattern of the hydrogen bonding interaction between MTX and cyanuric acid is the classic DAD-ADA model (D-hydrogen bond donor, A-hydrogen bond acceptor, Fig. S3) and the corresponding K_a value is close to the typical K_as (10^2-10^3 M^{-1}) for hydrogen bonded DAD-ADA pairs [8].

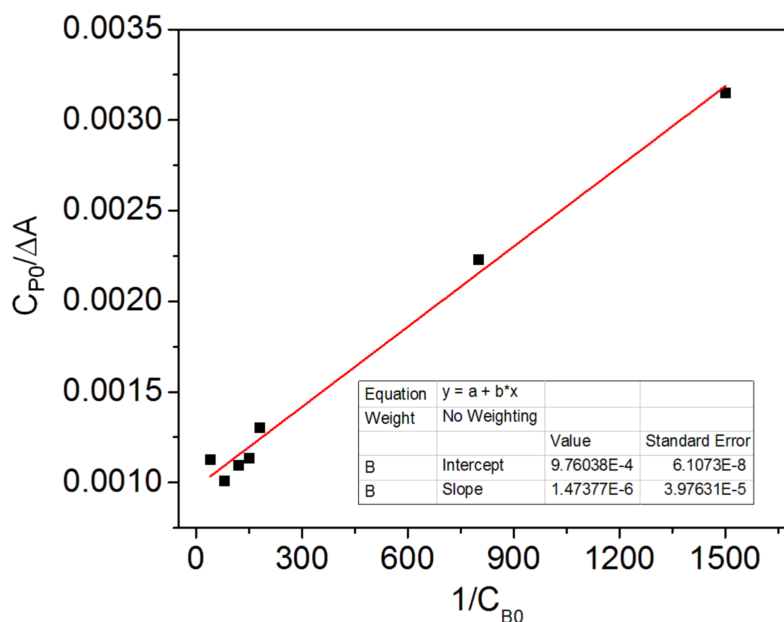


Fig. S4. Dependence of UV absorbance of MTX upon the addition of cyanuric acid.

5. Self-assembly of the amphiphilic peptide or amphiphilic peptide/MTX complex

The aqueous solution of the amphiphilic peptide was prepared by dissolving the peptide in aqueous solution (pH 7.0). And the corresponding self-assembly behavior was monitored using the following

dynamic light scattering (DLS), transmission electron microscopy (TEM), circular dichroism (CD) and fourier transform infrared spectroscopy (FT-IR). For the self-assembly of the amphiphilic peptide/MTX complex, the mixture of the amphiphilic peptide and MTX with equal molar ratio was first dissolved in methanol. After sonication for several seconds, the solvent was removed by rotary evaporation. After dissolution-sonication-evaporation thrice, distilled water was added and solution pH was adjusted to a value of 7.0. After placing at room temperature for 30 min, the corresponding self-assembly behavior was investigated by TEM, CD and FT-IR.

6. Dynamic light scattering (DLS)

The average size and size distribution of the self-assembled amphiphilic peptide were determined using dynamic light scattering (DLS) techniques with a Nano-ZS ZEN3600 instrument (MALVERN Instruments). The average size and size distribution of the self-assembled peptide at a concentration of 4 mg/mL are presented in Fig. S5.

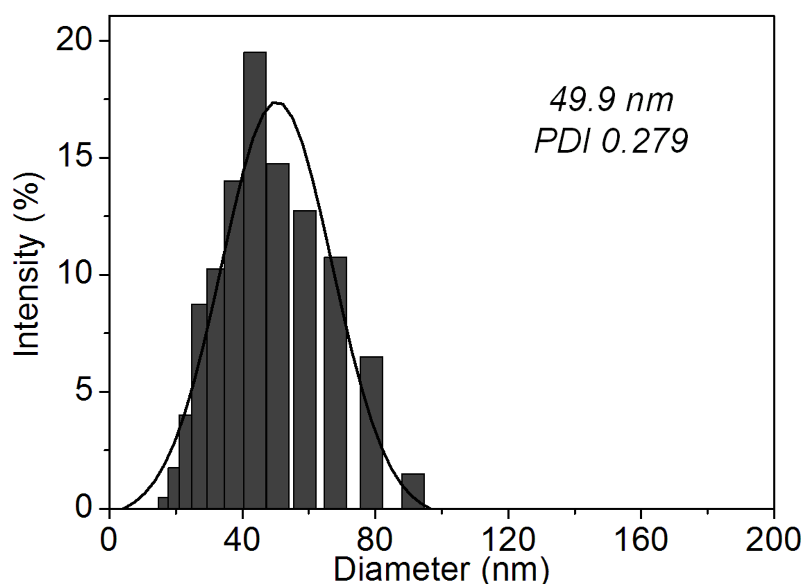
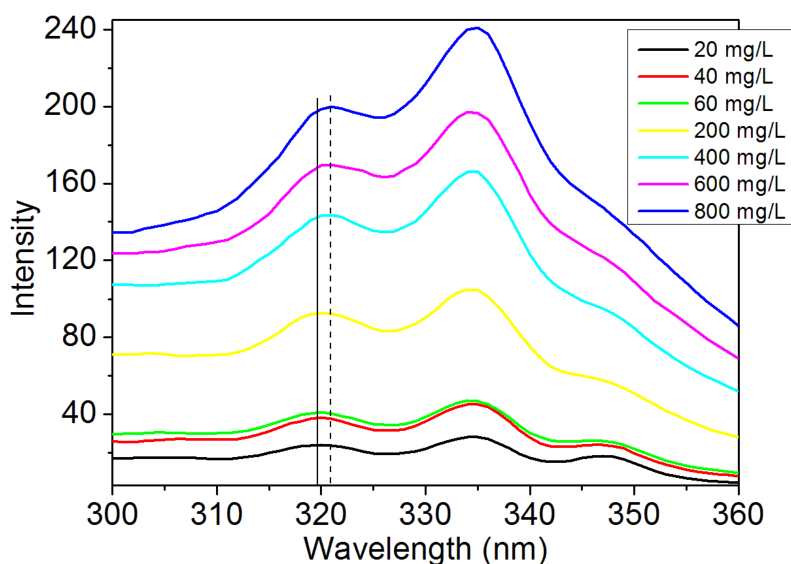


Fig. S5. Size distribution of the self-assembled amphiphilic peptide (4 mg/mL).

7. Determination of critical micelle concentration (CMC) and critical aggregation concentration

(CGC)

By using pyrene as a hydrophobic fluorescent probe, the CMC of the amphiphilic peptide was determined according to the fluorescence spectra recorded on a LS55 luminescence spectrometer (Perkin-Elmer). In brief, aliquots of pyrene solution (6×10^{-6} M in acetone, 0.1 mL) were added to
5 tubes. After the complete evaporation of acetone from the tubes, 1 mL aqueous solution of the amphiphilic peptide with a fixed concentration was added. After gently shaking the tubes at 37 °C for several hours, the tubes were kept in a thermostatic water bath (37 °C) overnight to reach the solubilization equilibrium of pyrene. Subsequently, the solution was fixed in a quartz cell and the excitation spectra were examined with emission at 390 nm and excitation data range between 300 and
10 360 nm. Based on the excitation spectra of pyrene (Fig. S6), the fluorescent intensity ratio of the third and first vibronic bands (I_3/I_1) was plotted against the logarithm of the concentration of the amphiphilic peptide, and the CMC value was calculated from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the point at low concentration.



15 **Fig. S6.** Dependence of the fluorescent excitation spectra of pyrene upon the concentration of amphiphilic peptide.

The CGC of the amphiphilic peptide/MTX complex was measured according to the similar method.

Before the measurement, the amphiphilic peptide/MTX complex was first prepared as described above (dissolution-sonication-evaporation thrice). Fig. S7 shows the intensity of I_1, I_3 in the excitation spectra as a function of the logarithm of the concentration of amphiphilic peptide/MTX complex. The CGC was calculated as ~24.5 mg/L according the aforementioned method.

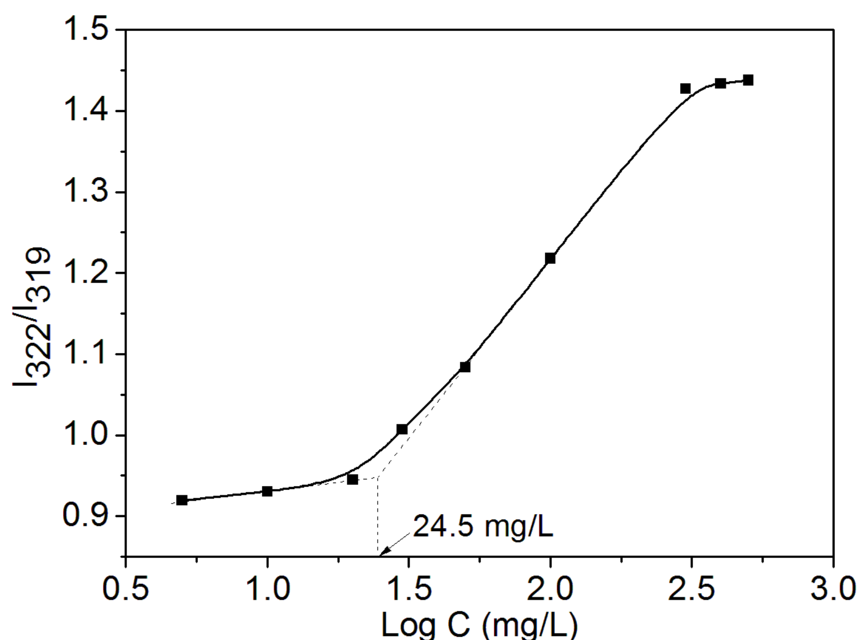


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Fig. S7. Intensity of I_1, I_3 in the excitation spectra as a function of the logarithm of the concentration of amphiphilic peptide

8. Transmission electron microscopy (TEM)

10 The morphology of the self-assembled amphiphilic peptide or the amphiphilic peptide/MTX complex was observed on a Tecnai G20 S-TWIN transmission electron microscope (TEM). Before the observation, the self-assembled amphiphilic peptide or the amphiphilic peptide/MTX complex was first applied to a copper grid with Formvar film and stained by a 0.2% (w/v) phosphotungstic acid solution.

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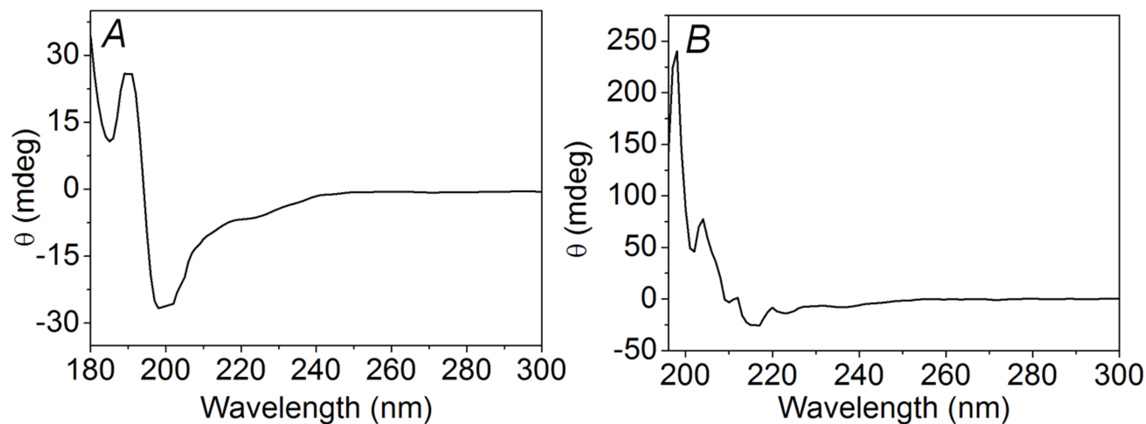
9. Circular dichroism (CD)

The self-assembled amphiphilic peptide or the amphiphilic peptide/MTX complex solution was fixed in a 0.5 mm quartz cell and analyzed on a Jasco J-810 spectropolarimeter with 4 s accumulations every 1 nm and averaged over three acquisitions.



5 **Fig. S8.** CD spectra of the self-assembled amphiphilic peptide (4 mg/mL) (*A*) and the amphiphilic peptide/MTX complex (4 mg/mL) (*B*).

10. Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectrum of the self-assembled amphiphilic peptide or the amphiphilic peptide/MTX complex 10 was collected on a Perkin-Elmer spectrophotometer by pressing the lyophilized sample into KBr pellet.

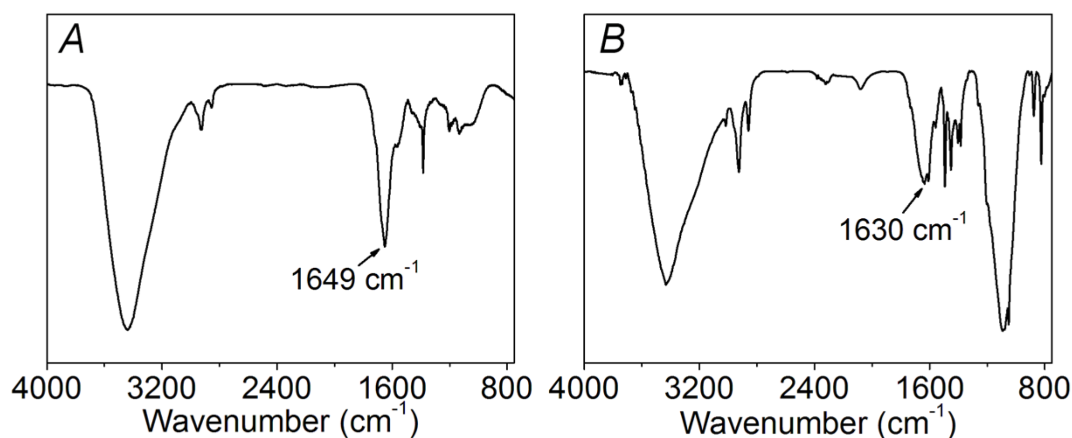


Fig. S9. FT-IR spectra of the self-assembled amphiphilic peptide (4 mg/mL) (*A*) and the amphiphilic peptide/MTX complex (4 mg/mL) (*B*).

11. Determination of encapsulation efficiency (EE) and loading level (LL)

To determine the EE and LL of the MTX in the self-assembled nanostructures, the self-assembled amphiphilic peptide/MTX complex prepared as described above was transferred to a dialysis tube (MWCO 1000 Da) and dialyzed against distilled water for 2 h to remove unloaded MTX and unassembled amphiphilic peptide. Thereafter, the self-assembled nanostructures were freeze-dried and dissolved in 10 mL of DMSO. The amount of loaded MTX was determined according to UV absorbance at ~303 nm. The EE and LL of MTX was calculated as follows (Table S1):

$$EE = (\text{mass of loaded MTX}/\text{mass of feed MTX}) \times 100\%$$

$$LL = (\text{mass of loaded MTX}/\text{mass of MTX loaded nanostructures}) \times 100\%$$

Table S1. EE and LL of MTX in the self-assembled amphiphilic peptide/MTX complex

Complex concentration (mg/mL)	Nanostructure	EE (%)	LL (%)
1	Nanorod	80.4	22.7
2	Nanorod/nanofiber	81.7	25.1
4	Nanofiber	84.9	28.4

12. *In vitro* MTX release

The MTX loaded nanofibers were chosen to examine the drug release behavior. In brief, the MTX loaded nanofibers were prepared and purified as described above. Subsequently, the nanofibers stored in the dialysis tube (MWCO 500-1000 Da) were directly immersed in 10 mL of PBS solution (pH 7.4) at physiological temperature (37 ^{o}C). At a predetermined time interval, the total PBS solution was withdrawn and another 10 mL of fresh PBS solution was added after each sampling. The amount of released MTX was measured by using a UV spectrophotometer (Perkin-Elmer Lambda Bio 40

UV/VIS spectrometer, USA) at 303 nm. The average value of three independent experiments was collected and the cumulative MTX release was calculated as follow:

$$\text{Cumulative MTX release (\%)} = (M_t / M_\infty) \times 100$$

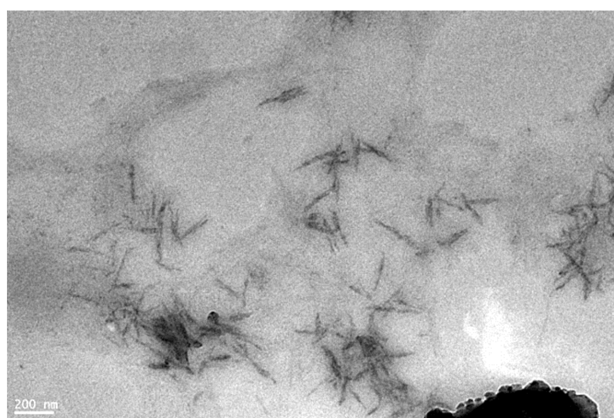
where M_t is the amount of MTX released from the nanofibers at time t and M_∞ is the amount of MTX

5 loaded in the nanofibers.

13. Stability of the self-assembled amphiphilic peptide/MTX complex

To evaluate the stability of the self-assembled amphiphilic peptide/MTX complex in serum-containing medium, the MTX loaded nanorods were dispersed in cell culture medium containing 10% FBS. After

10 incubation for 1 h, TEM was employed to observe whether there is aggregation or morphology change for the nanorods. As shown in Fig. S10, there are only little amount of aggregations and the self-assembled amphiphilic peptide/MTX complex still maintains the nanorod morphology, implying the good stability of the self-assembled complex in serum-containing medium.



15 **Fig. S10.** TEM image of the MTX loaded nanorods after 1 h incubation in cell culture medium.

14. Cell culture

Human cervix carcinoma (HeLa) and African green monkey kidney (COS7) cells were respectively incubated in DMEM medium with 10% FBS and 1% antibiotics (penicillinestreptomycin, 10,000

U/mL) at 37 °C in a humidified atmosphere containing 5% CO_2.

15. Confocal laser scanning microscope

HeLa and COS7 cells were respectively seeded in 6-well plates and incubated in 2 mL of DMEM
5 containing 10% FBS for 24 h. Subsequently, MTX loaded nanorods dispersed in DMEM (1 mg/mL) were added and the cells were allowed to incubate for another 4 h. After removing the medium and subsequently washing with PBS buffer thrice, the cells were viewed under a laser-scanning confocal microscope (Nikon C1-si TE2000, Japan) with the excitation at 405 nm for MTX.

10 **16. Flow cytometry**

HeLa and COS7 cells were respectively seeded in 6-well plates and incubated in 2 mL of DMEM containing 10% FBS for 24 h. MTX loaded nanorods dispersed in DMEM (1 mg/mL) were then added and the cells were allowed to incubate for another 4 h. After removing the medium and then washing with PBS buffer thrice, the cells were collected for flow cytometry quantitative analysis (BD
15 FACSAria™ III, USA).

17. *In vitro* cytotoxicity assay

HeLa and COS7 cells were chosen as cell lines to investigate the cytotoxicity of MTX loaded nanorods by using MTT assay. The cells were incubated in DMEM medium with 10% FBS and 1% antibiotics
20 (penicillinestreptomycin, 10,000 U/mL) at 37 °C in a humidified atmosphere containing 5% CO_2. Thereafter, cells were harvested and seeded in a 96-well plate with a density of 6000 cells/well. After the incubation in 100 μL of DMEM containing 10% FBS for 24 h, a fixed amount of MTX loaded nanorods dispersed in 200 μL of DMEM was added and the cells were allowed to incubate for another 48 h. After replacing the medium with 200 μL of fresh DMEM, 20 μL of MTT (5 mg/mL in PBS)

solution was added to each well and the cells were further incubated for 4 h. Subsequently, the medium was removed and 150 μL of DMSO was added. After shaking at room temperature for several minutes, the optical density (OD) was measured at 570 nm with a microplate reader model 550 (BIO-RAD, USA). The average value of three independent experiments was collected and the cell viable rate
5 was calculated as follows:

$$\text{Viable rate (\%)} = (OD_{treated} / OD_{control}) \times 100$$

where $OD_{control}$ is obtained in the absence of MTX loaded nanorods and $OD_{treated}$ is obtained in the presence of MTX loaded nanorods.

As a control, the cytotoxicity of the amphiphilic peptide against HeLa cells was also investigated
10 and the corresponding result is shown Fig. S11.

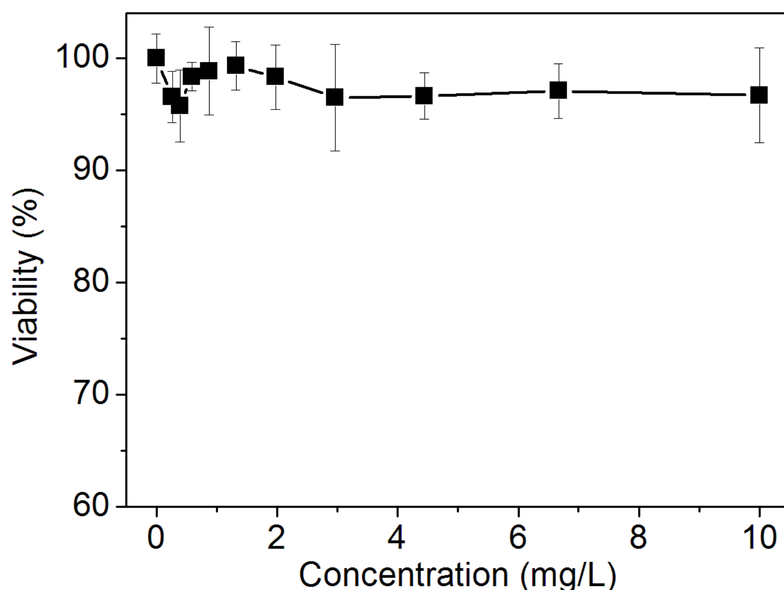


Fig. S11. Cytotoxicity of the amphiphilic peptide against HeLa cells.

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