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PAPER

Self-assembled micelles of multi-functional amphiphilic fusion (MFAF) peptide for targeted cancer therapy

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A new multi-functional amphiphilic fusion (MFAF) peptide comprised of a multi-functional fusion peptide sequence (GFLGR$_8$GDS) and a hydrophobic polycaprolactone (PCL) tail was designed and prepared. In aqueous solution, through the strong hydrophobic interaction among the PCL tails, this MFAF peptide can self-assemble into core-shell micelles at a low concentration with the anti-tumor drug doxorubicin (DOX) loaded in the core and multi-functional fusion peptide sequence located on the shell. When incubating the DOX-loaded micelles with tumor and normal cells, the micelles can use the RGD and membrane-penetrating peptide (eight continuous arginine residues, R$_8$) sequences to target tumor cells and penetrate cell membranes. Subsequently, cathepsin B, an enzyme over-expressed in late endosomes and lysosomes of tumor cells that can specifically hydrolyze the GFLG sequence, can break the micellar structure and lead to a rapid release and escape of loaded DOX from endosomes, resulting in the apoptosis of tumor cells. The MFAF peptide presents a great potential as a new drug delivery platform for targeted cancer chemotherapy.

Introduction

Since the first FDA approval of nano-sized drug delivery system (DDS), Liposomal amphotericin B, in 1990, numerous other organic and inorganic nano-sized DDSs have been developed for disease treatment.[1-3] In comparison with free drugs, the administration of nano-sized DDSs can enhance the drug stability, prolong the drug circulation time in blood and lower the side effect to normal tissues.[4] To date, nano-sized DDSs have achieved encouraging results and there are over two dozen nano-sized DDSs that have been approved for clinical use.[5] Core-shell micelles made from amphiphilic polymers are a class of very important nano-sized DDSs in the clinic, including the marketed Genexol-PM (paclitaxel loaded PLA-PEG micelles).[6] Because micelles can encapsulate hydrophobic drugs in the core and stabilize the structure by the hydrophilic shell, this nano-size DDS is capable of avoiding chemical modification of the loaded drugs and maintain their pharmaceutical bioactivity. However, the inherent shortcomings especially lack of bioactivity to overcome various extra- and intracellular barriers significantly limits the wide medical applications of traditional micelles. Although conjugation bioactive factors such as aptamers[7-9] and functional peptides[10-12] could endow the resulted micelles with bioactivity, it is extremely difficult to precisely control the degree of conjugation, leading to batch-to-batch variation of the amount of the bioactive factors. In aiming to solve these issues, amphiphilic peptides have been considered as effective building blocks to construct bioactive micelle-based DDSs.[13-15]

Arising from the abundant examples of protein self-assembly existing in nature, peptide-based building blocks have been extensively explored to construct self-assembled materials, such as α-helical peptide bundles,[16,17] β-sheet peptide fibers or tubes[18-20] and cylindrical or spherical micelles.[21-23] These self-assembled materials are particularly attractive for biomedical applications in drug delivery, tissue engineering and gene therapy due to their inherent biodegradability and good biocompatibility.[24-28] Amphiphilic peptides, a class of very important peptide-based building blocks, are widely used to construct cylindrical or spherical micelles to deliver therapeutic drugs for disease treatment.[14,15,24] In particular, compared to the traditional amphiphilic polymers, it is more convenient to design and prepare bioactive amphiphilic peptides since there are numerous well-demonstrated bioactive peptide sequences. For example, incorporation of the tumor-targeting arginine-glycine-aspartic acid (RGD) sequence and membrane-penetrating peptide sequence into one amphiphilic peptide can confer the resulted micelles with tumor-targeting and membrane-penetrating functions.[15,29] It is known there are twenty kinds of natural amino acids with different physiochemical properties. Through rational molecular design and diverse synthesis techniques, one can therefore integrate different functions into one peptide sequence to obtain a multi-functional fusion peptide. Unfortunately, the fusion of different functional peptide sequences generally enhances the overall hydrophilicity of the resulting amphiphilic peptides, leading to a significant increase in the critical micelle concentration (CMC) and sometimes the failure of micelle formation. We have previously demonstrated that the amphiphilic peptide containing tumor-targeting and membrane-penetrating peptide sequences can not self-assemble into micelles with only one hydrophobic tail.[15,29,30] Although increasing the number of

hydrophobic tail can strengthen the aggregation ability, it will simultaneously increase the synthesis difficulty and the amount of impurities.

In an attempt to address the aforementioned issues, we designed and prepared a new amphiphilic fusion peptide with tumor-targeting (RGD), membrane-penetrating (eight continuous arginine residues, R_8) and endosome-escaping (GFLG) functions. As shown in Fig. 1, in order to endow this multi-functional amphiphilic fusion peptide with a strong aggregation ability, polycaprolactone (PCL), a classic polyester with strong hydrophobicity, was incorporated as a hydrophobic tail. This multi-functional amphiphilic fusion (MFAF) peptide can self-assemble into core-shell micelles, in which the anti-tumor drug doxorubicin (DOX) is loaded in the core and the multi-functional fusion peptide sequence is positioned on the shell. When incubating the DOX-loaded micelles with tumor and normal cells, the micelles can target tumor cells via the specific interaction between RGD and integrins over-expressed on tumor cells[31] and then use membrane-penetrating function to penetrate cell membrane. After cellular uptake, cathepsin B, a late endosomal and lysosomal protease over-expressed in many types of tumor cells that can specifically hydrolyze the GFLG sequence,[32-35] breaks the micellar structure and lead to a rapid release and escape of loaded DOX, ultimately resulting in the apoptosis of tumor cells.

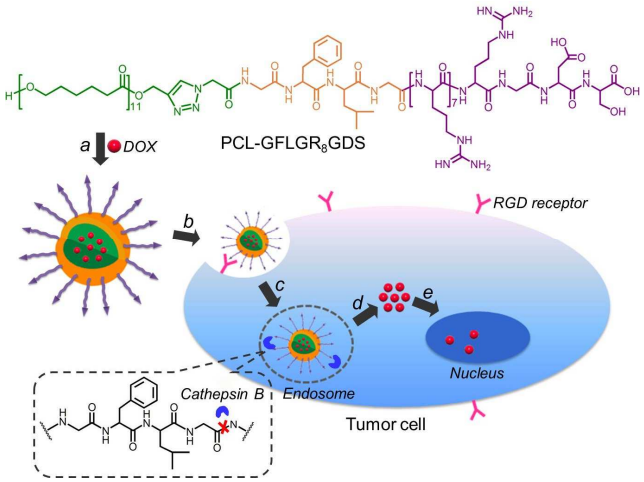


Fig. 1. Schematic illustration of the self-assembly of the MFAF peptide (PCL-GFLGR_8GDS) to load the anti-tumor drug DOX for targeted cancer therapy. Through the self-assembly of the MFAF peptide, DOX can be loaded into the hydrophobic core of the formed micelles and the multi-functional fusion peptide sequence is thus positioned on the shell (*a*). After RGD-mediated cellular uptake (*b*), cathepsin B over-expressed in late endosomes and lysosomes of tumor cells can specifically hydrolyze the GFLG sequence (*c*), leading to a rapid release and escape of loaded DOX from endosomes (*d*) and ultimate apoptosis of tumor cells (*e*).

Experimental

Materials

N-Fluorenyl-9-methoxycarbonyl (FMOC) protected L-amino acids (FMOC-Gly-OH, FMOC-Asp(OtBu)-OH, FMOC-Ser(tBu)-OH, FMOC-Arg(Pbf)-OH, FMOC-Leu-OH and FMOC-Phe-OH) and 2-chlorotrityl chloride resin (100-200 mesh, loading: 0.5 mmol/g) were purchased from GL Biochem (Shanghai) Ltd. (China) and used as received. ε-Caprolactone (ε-CL, Sigma-Aldrich) was dried and distilled over calcium hydride. *N*-Hydroxybenzotriazole (HOBt), phenol, 1,2-ethanedithiol (EDT), trifluoroacetic acid (TFA), *o*-benzotriazole-*N,N,N',N'*-tetramethyluroniumhexafluorophosphate (HBTU), bromoacetic acid, sodium azide (NaN_3), triisopropylsilane (TIS), dimethylsulfoxide (DMSO) and piperidine were provided by Shanghai Reagent Chemical Co. (China) and used directly. Dimethylformamide (DMF), stannous 2-ethyl hexanoate ($SnOct_2$), propargyl alcohol and diisopropylethylamine (DiEA) were obtained from Shanghai Reagent Chemical Co. (China) and distilled prior to use. Doxorubicin (DOX) was purchased from Zhejiang Hisun Pharmaceutical Co. (China). Molecular probe (Hoechst 33258) and LysoTracker Green were purchased from Invitrogen (CA, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT), trypsin and penicillinestreptomycin were purchased from Invitrogen Corp. All other reagents and solvents are of analytical grade and used directly.

Synthesis of azidoacetic acid

The azidoacetic acid was synthesized according to our previous study.[36] In brief, NaN_3 (13 g, 0.2 mol) was dissolved in 50 mL of distilled water. In an ice-water bath, bromoacetic acid (13.9 g, 0.1 mol) dispersed in 20 mL of distilled water was added dropwise. After stirring at room temperature for 24 h, the solution was acidified to a pH of 2 with dilute HCl and then extracted with diethyl ether (3 × 40 mL). The organic phase was combined and dried over anhydrous $MgSO_4$. After filtration, the solvent was removed in vacuum, giving 7.2 g of azidoacetic acid (yield ~71.3%) as pale yellow oil.

Synthesis of azide-terminated peptide

The azide-terminated peptide was synthesized manually in 0.5 mmol scale on the 2-chlorotrityl chloride resin employing a standard FMOC solid phase peptide synthesis (SPPS) technique. The resin was washed with CH_2Cl_2 (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 protected amino acid (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% piperidine/DMF (V/V) solution was added 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 protected amino acid (4 equiv), HBTU (4 equiv), HOBt (4 equiv) and DiEA (6 equiv) was added. After shaking for 1.5 h at room temperature, 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 repetition of the deprotection and acylation reaction to obtain the required amino acid sequence, a DMF solution of the mixture of azidoacetic acid (2 equiv), HBTU (2 equiv), HOBt (2 equiv) and DiEA (3 equiv) was added. After shaking for 1.5 h at room temperature, the reaction solution was drained off and the resin

was finally washed with DMF (three times) and DCM (three times). Cleavage of the azide-terminated peptide and the removal of the protected groups were performed using a mixture of TFA, TIS, distilled water, phenol and EDT in the ratio of 82.5:4.5:4.5:6:2.5. After 2 h shaking at room temperature, the cleavage mixture and three subsequent TFA washing were collected. The combined solution was concentrated and cold ether was then added to precipitate the product. After washing with cold ether (five times) to remove TFA residue, the precipitate was dissolved in distilled water and then dialyzed against distilled water in dialysis tube (MWCO 1000 Da) to remove small molecular impurities. After freeze-drying under vacuum, the azide-terminated peptide was collected as white powder. MALDI-TOF-MS, 1985.7 $[M+H]^+$.

Synthesis of alkyne-terminated PCL

The alkyne-terminated PCL was synthesized via ring-opening polymerization. ε-CL (2 g, 17.5 mmol), $SnOct_2$ (4 mg, 0.2-wt% with respect to CL) and propargyl alcohol (84 mg, 1.5 mmol) were placed in a glass ampule. Subsequently, the ampule was sealed under vacuum and then immersed in an oil bath at 120 °C for 24 h. The product was collected by dissolving the mixture in DCM and precipitating thrice in 10-fold cold ether.

Nuclear magnetic resonance (NMR)

The ^{1}HNMR spectrum of the alkyne-terminated PCL was recorded on a Mercury VX-300 spectrometer at 300 MHz (Varian, USA) by using $CDCl_3$ as a solvent and TMS as an internal standard.

Synthesis of MFAF peptide

Azide-terminated peptide (0.2 g, 0.1 mmol) and alkyne-terminated PCL (0.19 g, 0.15 mmol) were dissolved in 4 mL of DMF/isopropanol (v/v = 1/1). Under N_2 protection, PMDETA (21 μL, 0.1 mmol) and CuBr (14.4 mg, 0.1 mmol) were added. The mixture was stirred at room temperature for 24 h. Subsequently, the solution was transferred to a dialysis tube (MWCO 1000) and dialyzed against the EDTA aqueous solution to remove CuBr. After further dialyzing against distilled water for 3 days, the product was freeze-dried and collected.

Gel permeation chromatographic (GPC)

Number- (M_n) and weight-average molecular weights (M_w) of PCL and MFAF peptide were determined by a GPC system equipped with Waters 2690D separations module, Waters 2410 refractive index detector. DMF was used as the eluent at a flow rate of 0.3 mL/min. Waters millennium module software was used to calculate molecular weight on the basis of a universal calibration curve generated by polystyrene standard of narrow molecular weight distribution.

Determination of critical micelle concentration (CMC)

The CMC of the MFAF peptide was determined by using pyrene as a hydrophobic fluorescent probe. In brief, aliquots of pyrene solution (6×10^{-6} M in acetone, 0.1 mL) were added to tubes. After the complete evaporation of acetone from the tubes, 1 mL aqueous solution of the MFAF 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 360 nm. Based on the excitation spectra of pyrene, 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 MFAF peptide. The corresponding 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.

Micelle formation and size distribution

The micelles were prepared by directly dissolving the MFAF peptide in distilled water (pH 7.0) at a concentration higher than CMC. The average size and size distribution of the self-assembled micelles were determined using dynamic light scattering (DLS) techniques with a Nano-ZS ZEN3600 instrument (MALVERN Instruments).

Transmission electron microscopy (TEM)

The morphology of the self-assembled micelles of the MFAF peptide was observed on a transmission electron microscope (Tecnai G20 S-TWIN). Before the observation, the aqueous solution of the self-assembled micelles was applied to a copper grid and stained by a 0.2% (w/v) phosphotungstic acid solution.

Circular dichroism (CD)

The aqueous solution of the self-assembled micelles of the MFAF peptide was fixed in a 0.5mm quartz cell and analyzed on a Jasco J-810 spectropolarimeter with 4 s accumulations every 1 nm and averaged over three acquisitions.

Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectrum of the self-assembled micelles of the MFAF peptide was collected on a Perkin-Elmer spectrophotometer. Before the analysis, the sample was freeze-dried and then pressed into KBr pellets.

***In vitro* drug release**

The anti-tumor drug DOX was used as a model drug to examine the drug release behavior of the self-assembled micelles of the MFAF peptide. The DOX loaded micelles were prepared as follows: MFAF peptide (8 mg) and DOX (2 mg) were dissolved in 5 mL of DMSO. The solution was transferred to a dialysis tube (MWCO 1000 Da) and then subjected to dialysis against 2000 mL of distilled water for 24 h. During this period, the distilled water was replaced every 4 h to remove DMSO and unloaded DOX. After drug loading, the in vitro drug release behavior was carried at 37 °C by directly immersing the dialysis tube into 10 mL of PBS buffer (pH 7.4). At a predetermined time interval, the total PBS buffer was withdrawn and another 10 mL of fresh PBS buffer was added after each sampling. The amount of DOX released from the micelles was measured by using a UV spectrophotometer (Perkin-Elmer Lambda Bio 40 UV/VIS spectrometer, USA) at 497 nm. The average value of three independent experiments was collected and the cumulative DOX release was calculated as follow:

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

where M_t is the amount of DOX released from the micelles and

M_∞ is the amount of DOX loaded in the micelles.

The method used to calculate M_∞ is described as follows: The micelles after drug loading were freeze-dried and then dissolved in 1 mL of DMSO. The amount of loaded DOX was determined through the UV absorbance at 497 nm. The DOX encapsulation efficiency (EE) and DOX loading level (LL) was calculated as follows:

EE = (mass of loaded DOX/mass of feed DOX) × 100%

LL = (mass of loaded DOX/mass of DOX loaded micelles) × 100%

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.

Confocal laser scanning microscope (CLSM)

HeLa and COS7 cells were respectively seeded in 6-well plates and incubated in 2 mL of DMEM containing 10% FBS for 24 h. Subsequently, DOX loaded self-assembled micelles dispersed in DMEM (1 mg/mL) were added and the cells were allowed to incubate for another 4 h. Then the nuclei were stained by blue molecular probe Hoechst 33258 for 15 min. After removing the medium and subsequently washing with PBS buffer thrice, the stained cells were viewed under a laser-scanning confocal microscope (Nikon C1-si TE2000, Japan) with the excitation at 408 nm for blue molecular probe and 488 nm for DOX. To evaluate the endosome escape ability, the DOX loaded self-assembled micelles were incubated with HeLa cells for 2 h. Before CLSM observation, the nuclei and endosomes were respectively stained by Hoechst 33258 and LysoTracker Green.

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. DOX loaded self-assembled micelles 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 FACSAria^{TM} III, USA).

In vitro cytotoxicity assay

HeLa cells were seeded in a 96-well plate with a density of 5000 cells/well. After the incubation in 100 μL of DMEM containing 10% FBS for 24 h, a fixed amount of DOX loaded self-assembled micelles 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 four independent experiments was collected and the cell viability was calculated as follows:

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

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

Results and discussion

Synthesis of the MFAF peptide

The synthesis of the MFAF peptide is shown in Fig. 2. We used propargyl alcohol to initiate the ring opening polymerization of ε-CL to obtain alkyne-terminated PCL. The ^{1}H-NMR spectrum of this alkyne-terminated PCL is presented in Fig. 3*A*. Based on the integration ratio between the methylene protons (-OCH_2C≡CH, signal *a*) of the terminal alkyne group and the methylene protons (-OCH_2-, signal *f*) of CL repeating units, the molecular weight of the alkyne-terminated PCL can be calculated as ~1300 g/mol (M_w = 1800 g/mol determined by GPC analysis, Fig. 3*B*). Through the click reaction between alkyne-terminated PCL and azide-terminated multi-functional fusion peptide (N_3-GFLGR$_8$GDS, M_w = 1984 g/mol, Fig. 3*C*), the expected MFAF peptide (M_w = 4300 g/mol determined by GPC analysis, Fig. 3*B*) was obtained and the corresponding MALDI-TOF-MS spectrum is shown in Fig. 3*C*. Like the conventional PCL-based polymers, the MFAF peptide presents a distribution of molecular weight. The molecular weight difference between two adjacent peaks is ~114, which is the molecular weight of CL repeating unit, indicating the success of the click reaction between alkyne-terminated PCL and azide-terminated multi-functional fusion peptide.

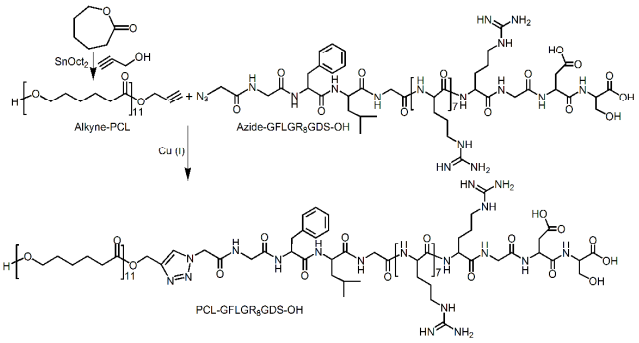


Fig. 2. Synthesis of the MFAF peptide, PCL-GFLGR$_8$GDS.

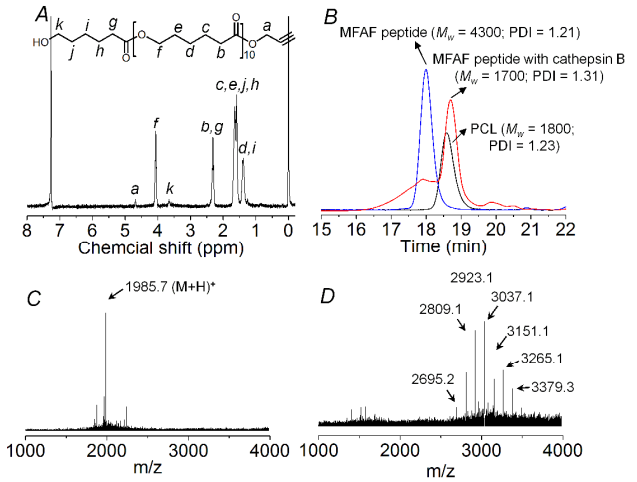


Fig. 3. ^{1}HNMR spectrum of alkyne-terminated PCL in $CDCl_3$ (*A*), GPC profiles of PCL, MFAF peptide and the one incubated with cathepsin B (*B*), and MALDI-TOF-MS spectra of the azide-terminated multi-functional fusion peptide (*C*) and the MFAF peptide (*D*).

Micelle formation and characterization

To investigate the micellization behavior of the MFAF peptide, we dissolved the peptide in distilled water at a fixed concentration and the corresponding micellization behavior was examined by using pyrene as a hydrophobic fluorescent probe. The fluorescence intensity of pyrene in the excitation spectra increases with the elevated concentration of the MFAF peptide and a clear red shift of peak from 338 nm (I_1) to 342 nm (I_3) can be observed (Fig. S1*A*), indicating the formation of core-shell micelles and transfer of pyrene from the hydrophilic outer medium into the hydrophobic inner core. From the tangent to the curve at the inflection intersected with the horizontal tangent, the point of intersection at low concentration is defined as the critical micelle concentration (CMC) and the corresponding CMC value of is calculated as 19.9 mg/L (Fig. S1*B*), which is much lower that the CMC value of our reported amphiphilic peptide (338.8 mg/L) with two hydrophobic tails.[29] This result strongly demonstrates that the incorporation of PCL as a hydrophobic tail can significantly strengthen the hydrophobic aggregation ability of the MFAF peptide.

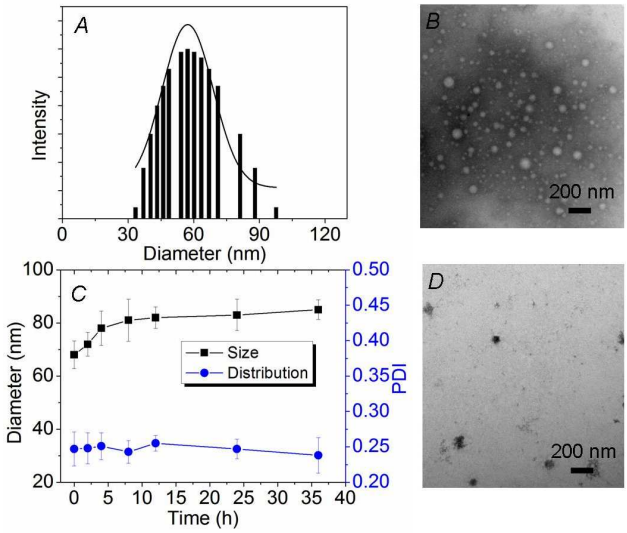


Fig. 4. (*A*, *B*) Size distribution (*A*) and TEM image (*B*) of the self-assembled micelles of the MFAF peptide (1 mg/mL) in PBS buffer (pH 7.4); (*C*) Size distribution change of the self-assembled micelles of the MFAF peptide (1 mg/mL) in the PBS buffer (pH 7.4) with 10% FBS; (*D*) TEM image of the self-assembled micelles of the MFAF peptide (1 mg/mL) after 2 h incubation in PBS buffer (pH 7.4) with cathepsin B (200 U).

The size of the self-assembled micelles was examined by DLS. As shown in Fig. 4*A*, the hydrodynamic diameter of the micelles is around 59 nm (PDI = 0.247). The morphology of the self-assembled micelles is observed by TEM (Fig. 4*B*). It can be found that the self-assembly of the MFAF peptide leads to the formation of well-dispersed spherical micelles with an average size of ~50 nm, which is close to the result of DLS analysis. This small difference in the size of micelles between dried (TEM) and solvated (DLS) states once demonstrates that the strong hydrophobic interaction among PCL tails can stabilize the micellar structure of the MFAF peptide. The good stability of the self-assembled micelles is further proven by the size change in PBS buffer with 10% FBS. From the data in Fig. 4*C*, there is a slight increase in the size and distribution of the self-assembled micelles incubated in the FBS containing solution. And after 4 h incubation, the micelles tend to stabilize and there is no apparent change in the size. We then evaluated the sensitivity of the self-assembled micelles of the MFAF peptide to cathepsin B. As displayed in Fig. 4*D*, after 2 h incubation in PBS buffer with cathepsin B, the self-assembled micelles disappear and only a small amount of random aggregates can be observed. The PBS buffer was freeze-dried and GPC was used to analyze the molecular weight change. From the GPC profile in Fig. 3*B*, with the addition of cathepsin B, the molecular weight of the MFAF peptide decreases from 4300 to 1700, which is close to the molecular weight of PCL. All these results indicate that the cleavage of GFLG sequence by cathepsin B leads to the removal of hydrophobic PCL from the MFAF peptide and ultimate disassembly of the micelles.

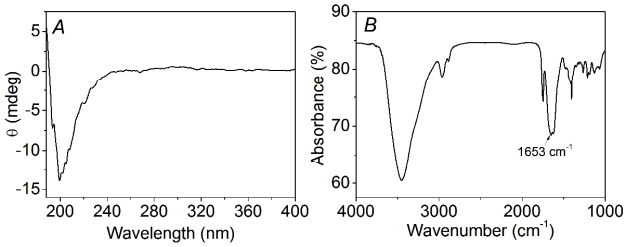


Fig. 5 CD (*A*) and FT-IR (*B*) spectra of the self-assembled micelles of the MFAF peptide.

Secondary structure

The functions of many bioactive peptides are significantly dependent on the conformation of the peptide backbones or the activities of the specific chemical groups. However, the driving forces of peptide self-assembly, especially inter- or intramolecular hydrogen bonding interaction, will possibly alter the conformation of the peptide backbones or restrain the freedom of the peptide chains, thus leading to the loss of bioactivity. Taking the membrane-penetrating peptide for example, this bioactive peptide is generally composed of 5 to 11 positively charged arginine residues and its membrane-penetrating function is built on the hydrogen bonding interaction between the guanidinium groups of arginine residues and the carboxyl, phosphoryl or sulfuryl groups of the cell surface carbohydrates and phospholipids.[37-39] If there is hydrogen bonding interaction among arginine residues after self-assembly, the cell-penetrating function will be weakened and even lost. To examine whether there is conformation transition after the self-assembly of the MFAF peptide, CD and FT-IR were employed to study the secondary structure of the self-assembled micelles. From the CD spectrum revealed in Fig. 5*A*, the negative band located at ~200 nm is a typical CD signal of polypeptide with a random-coil conformation,[29,40-42] implying that the $GFLGR_8GDS$ sequence of the MFAF peptide adopts a random-coil conformation in the self-assembled micelles. The result of FT-IR experiment is similar as that of CD analysis. As shown in Fig. 5*B*, the absorbance of amide I located at ~1653 cm^{-1} also indicates a random-coil conformation adopted by the $GFLGR_8GDS$ sequence in the self-assembled micelles.[43] It is known that the CPP generally adopts a random-coil structure in aqueous solution due to the presence of electrostatic repulsion interaction. The results

of CD and FT-IR analysis indicate that the self-assembly of the MFAF peptide is mainly driven by the hydrophobic interaction among the PCL tails and the multi-functions of the GFLGR$_8$GDS sequence will not be affected since there is no conformation transition after self-assembly.

In vitro drug release

After evaluating the micellization behavior and determining the conformation of the peptide sequence, we then examined the multi-functions of the self-assembled micelles of the MFAF peptide. The anti-tumor drug DOX was chosen as a model drug to first evaluate the cathepsin B responsive release behavior of the self-assembled micelles. As shown in Fig. 6*A* and 6*B*, due to the encapsulation of DOX into the hydrophobic cores of the micelles, the size of micelles increases from ~59 nm (Fig. 4*A*) to ~78 nm (PDI = 0.294). In current work, the EE and LL of the self-assembled micelles are 28.7% and 26.1%, respectively. This result indicates that there is over 75% loss of the feed MFAF peptide during the DOX loading process. The main reason is the lower molecular weight and smaller size of the MFAF peptide compared to the traditional amphiphilic polymers. Although using the dialysis tube with the MWCO higher than that of MFAF peptide, most of the peptide molecules can still diffuse out within the solvent exchange period and thus induce a significant loss of the MFAF peptide, which is usually encountered during the drug loading of the amphiphilic peptides.[15,23,29] The drug release behavior of the DOX loaded micelles is shown in Fig. 6*C*. In the absence of cathepsin B, the self-assembled micelles present a sustained and retarded DOX release behavior. Around of 31.7% of loaded DOX is released from the micelles within the first 8 h and the cumulative release reaches to ~64.8% 72 h later. In comparison, the DOX release rate is much faster when incubating the self-assembled micelles with cathepsin B. Around of 70.1% of loaded DOX is released from the micelles within the first 8 h and the cumulative release reaches to ~96.7% 72 h later. This increased drug release rate upon the addition of cathepsin B indicates that the GFLG sequence in the MFAF peptide can be cleaved by cathepsin B and thus leads to the rupture of the drug-loaded micelles, which has been proven by the molecular weight decrease in Fig. 3*B* and morphology change in Fig. 4*D*.

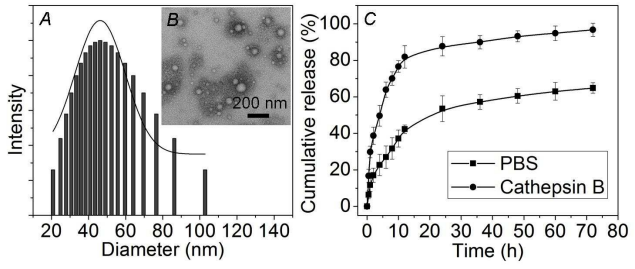


Fig. 6. (*A, B*) Size distribution and TEM image (*B*) of the DOX loaded micelles of the MFAF peptide in PBS buffer (pH 7.4; (*C*) Cumulative drug release of the DOX loaded micelles of the MFAF peptide in PBS buffer (pH 7.4) with cathepsin B (200 U).

Endosome esacpe and cellular uptake

Cathepsin B is a late endosomal and lysosomal protease over-expressed in many types of tumors.[32-35] To examine whether the self-assembled micelles can employ cathepsin B sensitivity to improve the endosome escape ability of the loaded DOX, HeLa cells were chosen to incubate with the self-assembled micelles. From the CLSM images revealed in Fig. 7, by staining the endosomes with green fluorescence (Fig. 7A_3), it can be found that a majority of the loaded DOX can escape from the endosomes (Fig. 7A_4). In contrast, if the cathepsin B sensitive GFLG sequence is changed to a GGGG sequence (Fig. S2 and S3), the endosome escape ability is significantly weakened, which is demonstrated by the many overlapped regions of red and green fluorescence in Fig. 7B_4. This result strongly demonstrates that the self-assembled micelles of the MFAF peptide can utilize cathepsin B sensitivity to enhance the endosome escape ability of the loaded DOX. In other words, due to the cleavage of GFLG sequence from the MFAF peptide by cathepsin B, the loaded DOX could be rapidly released from the self-assembled micelles (Fig. 6*C*) and accumulate in endosome at a high concentration, which thus facilitate the rapid diffusion and escape of small molecule DOX from endosomes.

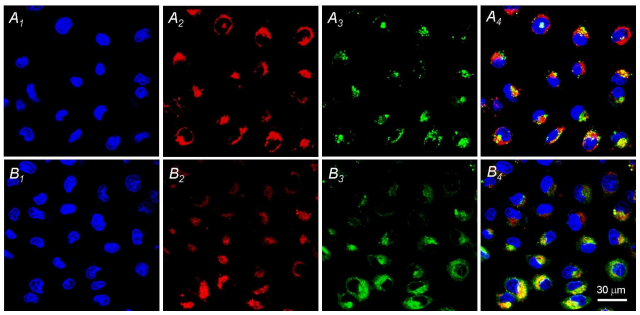


Fig. 7. CLSM images of HeLa cells respectively incubated with the DOX loaded self-assembled micelles of the MFAF peptide (A_1-A_4) and the MFAF peptide derivative without GFLG sequence (B_1-B_4) for 2h. (*1*) Nuclei stained by blue fluorescence; (*2*) DOX with red fluorescence; (*3*) Endosomes stained by green fluorescence; (*4*) Overlap of (*1*), (*2*) and (*3*).

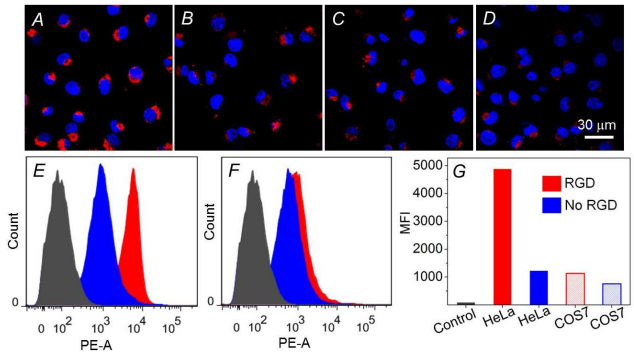


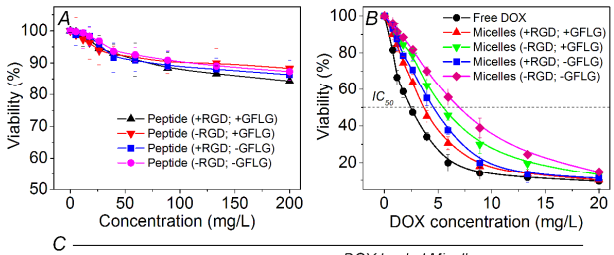
Fig. 8. (*A-D*) CLSM images of HeLa (*A*, *B*) and COS7 (*C*, *D*) cells respectively incubated with the DOX loaded self-assembled micelles of the MFAF peptide (*A*, *C*) and the MFAF peptide derivative without RGD sequence (*B*, *D*) for 4 h; The nuclei were stained by blue fluorescence. (*E-G*) Flow cytometry profiles (*E*, *F*) and MFI (*G*) of HeLa (*E*) and COS7 (*F*) cells respectively incubated with the DOX loaded self-assembled micelles of the MFAF peptide (red) and the MFAF peptide derivative without RGD sequence (blue) for 4 h.

After evaluating the function of GFLG sequence, we then investigated the tumor-targeting and membrane-penetrating functions of the self-assembled micelles of the MFAF peptide. HeLa and COS7 cells were respectively incubated with the self-assembled micelles for 4 h and the corresponding cellular uptake was examined by CLSM and flow cytometry. As shown in Fig.

8*A*, the bright red fluorescence indicates that HeLa cells with over-expressed integrins show a strong ability to uptake the self-assembled micelles of the MFAF peptide.[31] If removing the RGD sequence from the MFAF peptide (Fig. 8*B*), due to the absence of the specific interaction between RGD and integrins, the cellular uptake efficiency turns weak although there is a membrane-penetrating sequence on the self-assembled micelles. This result implies that the tumor-targeting RGD sequence dominates the cellular uptake efficiency and membrane-penetrating sequence co-operate with RGD sequence to enhance the cellular uptake efficiency, which has been found in our previous studies.[15,29] As expected, due to the low expression of integrins on COS7 cells, there is no significant difference in the cellular uptake for the cells incubated with the self-assembled micelles with (Fig. 8*C*) or without (Fig. 8*D*) RGD sequence. The result of flow cytometry is consistent with that of CLSM observation. In the presence of RGD sequence, the uptake of HeLa cells is very high (Fig. 8*E*). The corresponding mean fluorescence intensity (MFI, Fig. 8*G*) is more than 4-fold higher than the cells incubated with the self-assembled micelles without RGD sequence. However, there is no significant difference in the cellular uptake (Fig. 8*F*) and MFI (Fig. 8*G*) for COS7 cells incubated with the self-assembled micelles with or without RGD sequence.

***In vitro* cytotoxicity**

Combining the results of *in vitro* drug release, endosome escape and cellular uptake, the self-assembled micelles of the MFAF peptide can use their multi-functions to target tumor cells, penetrate cell membranes and escape from endosomes, presenting great potential as a new platform for cancer chemotherapy. To evaluate the anti-tumor efficacy, the DOX loaded micelles were incubated with HeLa cells and the cytotoxicity was then examined via MTT assay. We first examined the cytotoxicity of the different functional amphiphilic peptides against HeLa cells. As shown in Fig. 9*A*, all the functional amphiphilic peptides with or without RGD or GFLG sequence exhibit low cytotoxicity against HeLa cells. The cytotoxicity increases slightly with the increased concentration of the functional amphiphilic peptides, which is attributed to the increased concentration of cationic charges from arginine residues. The cytotoxicity of the DOX-loaded self-assembled micelles is presented in Fig. 9*B*. Regardless of the presence or absence of GFLG sequence, the self-assembled micelles with RGD sequence show a much higher toxicity than that of the micelles with no RGD sequence. From IC50 values summarized in Fig. 9*C*, the IC_{50} of the self-assembled micelles of the RGD-containing amphiphilic peptide with (MFAF peptide) or without GFLG sequence is ~3.68 or 4.58 mg/L, which is lower than that of the micelles without RGD sequence (~5.51 mg/L for the self-assembled micelles of the amphiphilic peptide with GLGF sequence but no RGD sequence and ~6.96 mg/L for the self-assembled micelles of the amphiphilic peptide without RGD and GLGF sequences, Fig.S4). Since the cathepsin B sensitive GFLG sequence is in favor of endosome escape, the self-assembled micelles with GFLG sequence show a relatively higher toxicity than that of the self-assembled micelles without GFLG sequence. For example, the IC_{50} value of the self-assembled micelles of the MFAF peptide is ~3.68 mg/L, which is lower than that of the micelles self-assembled from the RGD-containing amphiphilic peptide without GFLG sequence (4.58 mg/L, Fig.S4). The results of *in vitro* cytotoxicity assay indicate that the self-assembled micelles of the MFAF peptide can sufficiently use their tumor-targeting, membrane-penetrating and endosome-escaping functions to deliver DOX into tumor cells to induce the apoptosis.



C

	Free DOX	DOX loaded Micelles			
		+RGD +GFLG	-RGD +GFLG	+RGD -GFLG	-RGD -GFLG
IC_{50} (mg/L)	2.47	3.68	5.51	4.58	6.96

Fig. 9. Cytotoxicity of the different amphiphilic peptides (*A*) and DOX loaded self-assembled micelles (*B*) against HeLa cells, and IC50 values (*C*) of the DOX loaded self-assembled micelles. *(*+RGD, +GFLG) MFAF peptide with RGD and GFLG sequences; (-RGD, +GFLG) MFAF peptide derivative with GFLG sequence but no RGD sequence; (+RGD, -GFLG) MFAF peptide derivative with RGD sequence but no GFLG sequence; (-RGD, -GFLG) MFAF peptide derivative without RGD and GFLG sequences.

Conclusions

A new MFAF peptide comprised of a hydrophobic PCL tail and a multi-functional $GFLGR_8GDS$ sequence was designed and prepared. In aqueous solution, the strong hydrophobic interaction among the PCL tails leads to the self-assembly of this MFAF peptide into core-shell micelles without altering the conformation of the multi-functional peptide sequence. When employing self-assembled micelles to load the anti-tumor drug DOX and then incubating with tumor cells, the micelles can efficiently use their tumor-targeting, membrane-penetrating and endosome-escaping functions to targetedly deliver DOX into tumor cells, leading to apoptosis of tumor cells. The MFAF peptide reported in this study presents great potential as a new platform for targeted cancer chemotherapy.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [structures and MALDI-TOF-MS spectra]. See DOI: 10.1039/b000000x/

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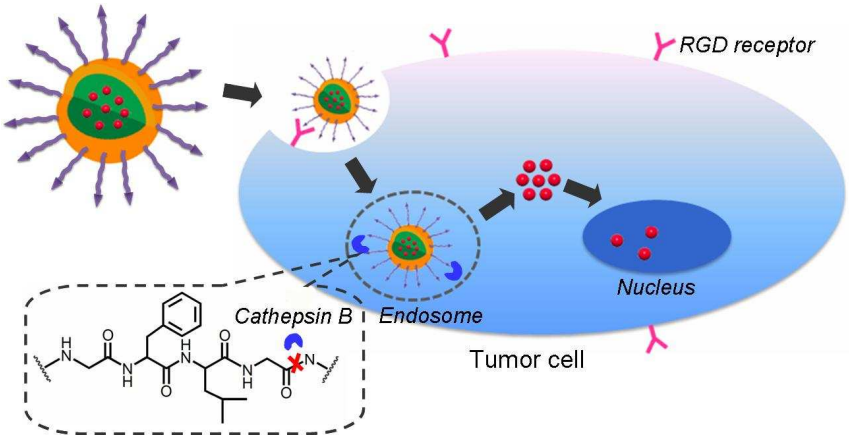
Graphical Table Content

Self-assembled micelles of multi-functional amphiphilic fusion (MFAF) peptide for targeted cancer therapy

By Yin-Jia Cheng, Hong Cheng, Xiao-Ding Xu, Ren-Xi Zhuo and Feng He

Keywords: Multi-functional amphiphilic fusion (MFAF) peptide, Self-assembly, Micelles.



A new MFAF peptide comprised of a hydrophobic PCL tail and a multi-functional GFLGR$_8$GDS sequence was designed and prepared. In aqueous solution, the strong hydrophobic interaction among the PCL tails leads to the self-assembly of this MFAF peptide into core-shell micelles. When incubating the anti-tumor drug loaded micelles with tumor cells, the micelles can efficiently use their tumor-targeting, membrane-penetrating and endosome-escaping functions to targetedly deliver the drug into tumor cells, leading to the apoptosis of tumor cells.