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Doxorubicin-loaded micelles with high drug-loading capacity and stability based on zwitterionic oligopeptides

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Results

Synthesis of methacrylohydrazide (MAH)

To demonstrate methacrylohydrazide (MAH) were synthesized successfully, hydrogen composition of MAH was characterized by ^{1}H NMR. Fig.S1 showed the ^{1}H NMR spectra of MAH, and the solvent was D_2O. The two absorption peaks (a and b) at chemical shift 5.52 and 5.72 ppm suggested the product had double bond (CH_2=CH_2). In addition, the absorption peak (c) at chemical shift 1.8 ppm derived from methylic hydrogen atoms. The ratio of integral area of a, b and c was 1:1:3, which proved the synthesized product was MAH. Except for the characteristic absorption peaks of a, b and c and the solvent peak, no other impurity peaks appeared in the ^{1}H NMR spectra of the MAH. Therefore, the purity of synthesized MAH was very high.

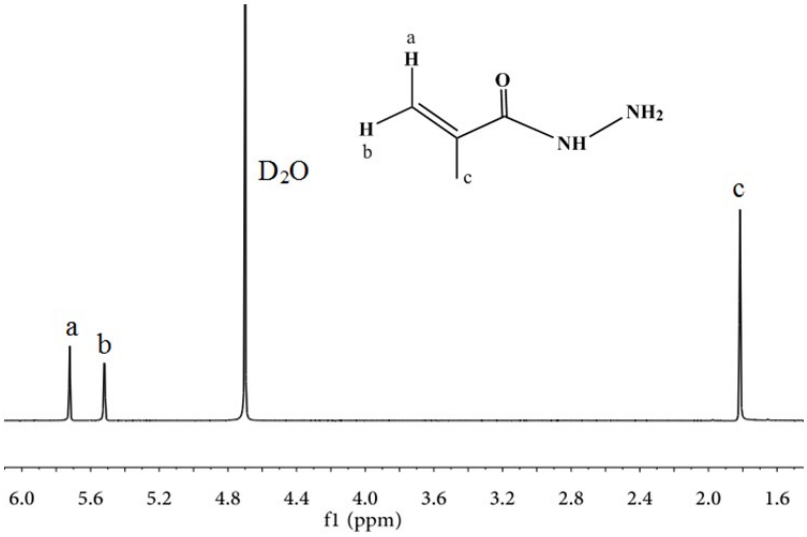

Fig.S1 ^{1}H NMR spectra of MAH in D_2O.

Synthesis of EKCEK-MAH

Fig.S2 showed ^{1}H NMR characterization result of synthesized EKCEK-MAH. The absorption peaks at chemical shifts 4.0, 4.25 and 4.35 ppm (a, b and c) were respectively corresponding to α-C hydrogen atoms of amino acid residues E, K and C. The absorption peaks at chemical shifts 2.5 and 2.8 ppm were respectively

corresponding to hydrogen atoms (c+h and j+k+l) of EKCEK-MAH. Due to similar chemical situations, the absorption peaks overlapped at chemical shifts 2.5 and 2.8 ppm. The absorption peak at chemical shifts 1.25 ppm (m) derived from methylic hydrogen atoms of EKCEK-MAH. In addition, the absorption peaks at chemical shifts 2.05, 2.15, 1.55 and 1.35 ppm were respectively corresponding to hydrogen atoms (b, e, g and f) of EKCEK-MAH. The red arrow pointing to chemical shifts 5.52 and 5.72 ppm represented the characteristic absorption peaks of double bond, implying a little bit unreacted MAH still existed. Although a little bit unreacted MAH existed, the drug-loaded micelles could form at the presence of a little bit impurity.

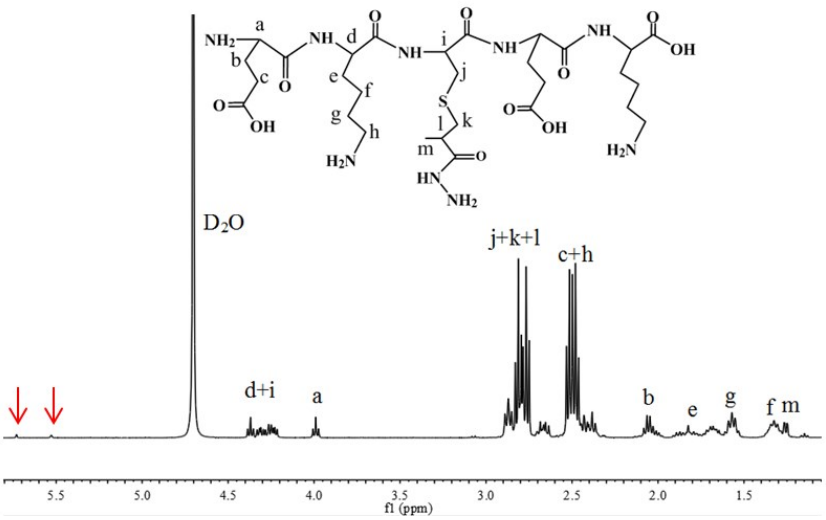

Fig.S2 ^{1}H NMR spectra of EKCEK-MAH in D_2O.

Synthesis of EKCEK-Dox

Fig.S3 showed ^{1}H NMR characterization result of synthesized EKCEK-Dox. The absorption peaks at chemical shifts 2.3 and 2.4 ppm (a and b) represented characteristic hydrogen absorption (CH_2) of side chain of E, and absorption peaks at chemical shifts 1.8, 1.3, 2.1 and 3.6 ppm (c, d, e and f) represented characteristic hydrogen absorption (CH_2) of side chain of K. The absorption peaks at chemical shifts 1.2 and 2.8 ppm (h and i+g) respectively represented characteristic hydrogen absorption (CH_3, CH and CH_2) of the small molecule MAH. In addition, the absorption peaks at chemical shifts 1.5, 3.75, 5.35, 7.2 and 7.5 ppm were respectively corresponding to characteristic hydrogen atoms (x, y, j+k, n and l+m) of drug molecule Dox. Therefore, it can be demonstrated that EKCEK-MAH was successfully

conjugated with the drug molecule Dox to form EKCEK-Dox.

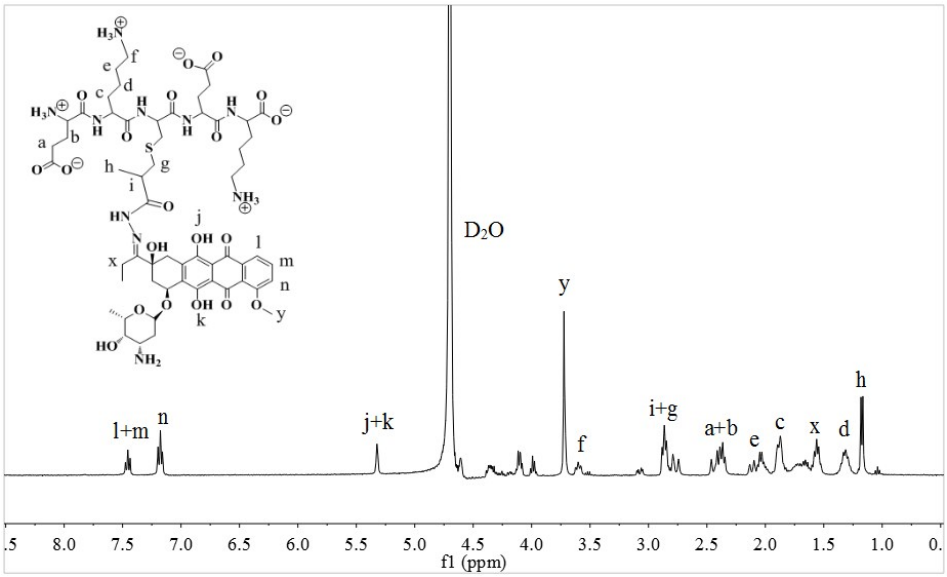

Fig.S3 ^{1}H NMR spectra of EKCEK-Dox in D_2O.

Methods

Synthesis of methacrylohydrazide hydrobromide (MAH•HBr)

MAH•HBr was synthesized through two-step reactions, and the synthesis procedure was shown in Fig.S4. Firstly, methacryloyl chloride reacted with tert-butyl carbazate to form methacrylohydrazide hydrobromide with tert-butoxycarbonyl (Boc) protection. Specifically, 19.8 g of tert-butyl carbazate (150 mmol) was dissolved in 20 mL of anhydrous THF, and 28 mL of TEA were added. The solution was placed in ice-bath conditions and stirred to react with nitrogen. 10.2 mL of methacryloyl chloride in 20 mL of anhydrous THF were added dropwise into the above mixture by a constant pressure funnel, and the reaction continued for 1 h in ice-bath conditions and for another 2 h at room temperature. After the reaction finished, the reaction mixture was filtered to obtain filtrate, which was then concentrated by reduced pressure rotary evaporation. The concentrated solution was dissolved in 150 mL EA, and then the mixture was washed by saturated sodium chloride and saturated sodium carbonate for 2 times respectively and then washed by saturated sodium chloride for another one time. The organic phase was collected, dried by anhydrous sodium sulfate, and then concentrated. The solid precipitated after the concentrated solution was added into anhydrous ether. The precipitation was collected by centrifuge and dried

for one night. Finally, methacrylohydrazide hydrobromide with Boc protection was obtained, and the yield was about 90%.

Secondly, the product obtained from the above reaction was deprotected to remove Boc. 5 g of methacrylohydrazide hydrobromide with Boc protection were dissolved in 50 mL of EA solution and stirred in ice-bath conditions. 5 mL of HBr/HOAc (33wt.%) solution were added into the above solution dropwise, and after that the mixture was stirred to react for half an hour. After the reaction finished, the mixture was filtered, and the residue was washed by 50 mL of anhydrous ether and dried for one night. Eventually, methacrylohydrazide hydrobromide without Boc protection was obtained, and the yield was about 95%.

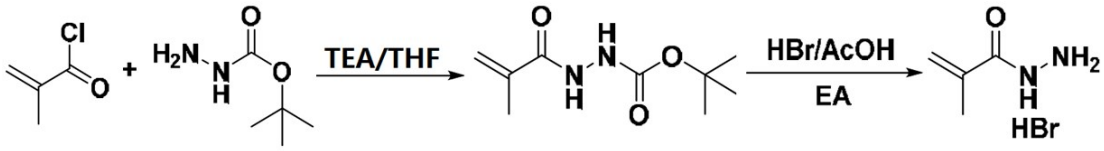

Fig.S4 Synthesis procedure of MAH•HBr

Preparation of drug-loaded micelles based on EKCEK

Firstly, MAH was reacted with EKCEK through Michael addition reaction between free thiol group and the double bond, and the product was further conjugated with Dox molecules by forming hydrazone bond between carbonyl group of Dox and hydrazide group of MAH. EKCEK, as zwitterionic oligopeptides, had strong hydrophilic effect, and Dox molecules had hydrophobic effect. As a result, the complex EKCEK-Dox self-assembled to form nano-micelles in water.

200 mg of EKCEK lyophilized powders and 67 mg of MAH were dissolved in 20 mL of deionized water and stirred to react for 4 h at room temperature. After that, the solution was freeze-dried to obtain lyophilized powders. 50 mg of the above lyophilized powder and 42.8 mg of Dox•HCl were dissolved in anhydrous methanol, one drop of glacial acetic acid and appropriate amount of anhydrous sodium sulfate were added, and then the mixture was stirred to react for 48 h in dark at room temperature. After the reaction finished, the mixture was filtered to remove anhydrous sodium sulfate, and the filtrate was collected. A small amount of TEA was added into

the filtrate to remove HCl of Dox•HCl so that Dox molecule could show hydrophobic effect. The above solution was added dropwise into deionized water and stirred for 4 h in dark. At last, the solution was dialyzed to remove free Dox and freeze-dried to obtain the drug-loaded micelles lyophilized powders (EKCEK-Dox).