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Dual-active targeting liposomes drug delivery system for bone metastatic breast cancer: Synthesis and biological evaluation

Ze Zhao[a,*], Yi Zhao[b,*], Changwei Xie[a], Changqing Chen[a], Dong Lin[a], Sheng Wang[a], Dong Lin[a], Xinhua Cui[a], Zhongshuai Guo[a], Junfeng Zhou[a]

[a] Department of Orthopedics, the First Affiliated Hospital of Henan Polytechnic University (the Second People's Hospital of Jiaozuo City), No.17 Minzhu South Road, Jiaozuo, 454001, China
[b] Translational Medicine Center, the First Affiliated Hospital of Zhengzhou University, No.1 Jianshe East Road, Zhengzhou, 450052, China

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ABSTRACT

Bone is the most common organ affected by metastatic breast cancer. Targeting cancers within the bone remains a great challenge due to the inefficient delivery of therapeutic to bone. In order to increase the distribution of paclitaxel (PTX) in bone metastases, in this study, a novel bone-targeted glutamic oligopeptides-RGD peptide (Glu_6-RGD) derivative was designed and synthesized as liposome ligand for preparing liposome to effectively deliver PTX to bone metastases. The liposome was prepared and its particle size, zeta potential, encapsulation efficiency, release profile, stability, hemolysis and cytotoxicity were also characterized. What's more, the Glu_6-RGD-Lip showed superior targeting ability *in vitro* and *in vivo* evaluation as compared to naked PTX, non-coated, singly-modified and co-modified by physical blending liposomes. All the results suggested that Glu_6-RGD-modified liposome showed excellent targeting activity to metastatic bone cancer. This study may be conducive to the field of bone-targeting drugs delivery.

1. Introduction

Bone metastasis is a frequent complication in advanced stage of many cancers, especially breast cancer and prostate cancer [Zhao et al., 2017]. This is because the physiological microenvironment of bone is very suitable for tumor cells to support tumor inoculation and progress [Wang et al., 2014]. The patients with bone metastases suffer from bone pain, hypercalcemia and pathologic fractures, which lead to the decrease of life quality [Anada et al., 2009; Liao and Xu, 2017]. At present, metastatic bone cancer is mainly treated by surgery, radiotherapy and chemotherapy. Therefore, strategies that can effectively deliver drugs, such as paclitaxel (PTX), to the site of metastasis are of great interests for the treatment of metastatic bone cancer due to lack of target specificity of anticancer drugs [Liu et al., 2017].

Desired accumulation at tumor sites is the principal purpose of anticancer drug delivery systems. The inorganic compound hydroxyapatite (HAP), which is the main component of bone, is a promising target for selective drug delivery to bone [Duan et al., 2009]. In our previous study, we have found that glutamic acid oligopeptides has an excellent bone-targeted ability [Zhao et al., 2015]. The mechanisms of the targeting ability to bone was showed by acidic oligopeptides: the ionic interaction with Ca^{2+} in the mineral component of bone was built by the peptides bearing negative charges [Sarig, 2004]. Moreover, the ability of glutamic acid oligopeptides targeting to bone was depended on the number of exposed carboxyl residues [Sarig, 2004].

What's more, it is well known that RGD peptide is one of the most frequently-used neoplastic targeting ligands in drug delivery system [Wang et al., 2014]. The RGD sequence, an arginine-glycine-aspartic (Arg-Gly-Asp) tripeptide, has been identified as a high affinity $\alpha_v\beta_3$ selective ligand [Fu et al., 2019]. The $\alpha_v\beta_3$, one of the intergrin receptor family, is proved to be over-expressed in the most malignant tumors. As a results, a lot of interesting researches had been done on the nano-carriers modified by RGD to target drug to the tumors [Fu et al., 2019; Xiao et al., 2018]. In addition, the $\alpha_v\beta_3$ is also highly expressed in bone metastatic cells and osteoclasts, so that the $\alpha_v\beta_3$ integrin is also an attractive target for bone [Jiang et al., 2012]. There the RGD peptide may also have the property of bone targeting.

Over the years, we has done a lot of researches on bone targeting and cancer targeting moieties specially based on RGD or peptides by active process [Fu et al., 2019; Zhao et al., 2015]. The advantageous property of peptides such as biocompatibility, non-cytotoxicity, and enzymatic degradation can make the drug more biocompatible and no unexpected long-term effects. And we are still exploring how to further improve the carrier's bone targeting ability. As parts of our continuing

* Corresponding authors.
E-mail addresses: zezhao0910@163.com (Z. Zhao), zhaoyi0910@163.com (Y. Zhao).

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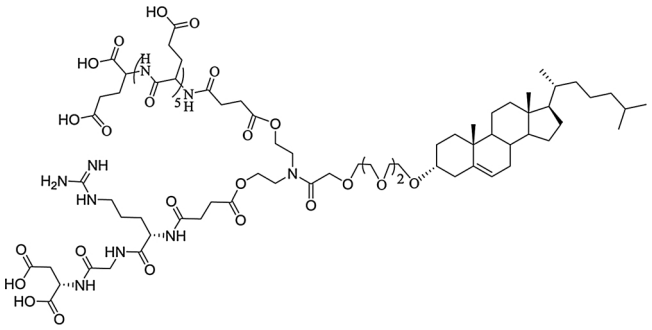


Fig. 1. The structure of the ligand Glu_6-RGD-Chol.

efforts, we focused on utilizing glutamic oligopeptide and RGD peptide to selectively deliver drug to bone metastases. Herein, we developed a PTX-loaded liposome co-modified with glutamic oligopeptide and RGD peptide. Such delivery system had a potential bone-targeted affinity through the Glu_6 and RGD exposed on the surface of the liposome, and facilitated bone metastases cellular uptake by the modification of RGD. To investigate the further bone targeting, the *in vitro* bone targeting was evaluated through hydroxyapatite (HAP) binding assay. What's more, the Glu_6-RGD modified (Fig. 1), non-coated, singly-modified and co-modified by physical blending liposomes (Glu_6 + RGD) were prepared by the lipid film hydration-ultrasound method, and targeting ability *in vivo* were also investigated.

2. Materials and methods

2.1. Materials

All liquid reagents were distilled before use. All unspecified reagents were from commercial resources. TLC was performed using precoated silica gel GF254 (0.2 mm), while column chromatography was performed using silica gel (100–200 mesh). The melting point was measured on a YRT-3 melting point apparatus (Shantou Keyi instrument & Equipment Co. Ltd, Shantou, China). Elemental analyses were performed by Atlantic Microlab (Atlanta, GA, USA). NMR spectra were taken on a Varian INOVA 400 MHz (Varian, PaloAlto, CA, USA). Mass spectra were recorded on an Agilent 1946B ESI-MS instrument (Agilent, PaloAlto, CA, USA). Reversed-phase chromatography performed on C18 chromatographic analysis was carried out using the HPLC system (Alltech, IL, USA) consisted of a RF-530 fluorescence detector (Shimadzu, Japan) and Allchorom plus data operator, respectively. A Diamonsil column (200 × 4.6 mm, 5 μm) was used. Soybean phospholipids (SPC) were purchased from Shanghai Taiwei Chemical Company (Shanghai, China). Paclitaxel (PTX) was purchased from AP Pharmaceutical Co. Ltd. (Chongqing, China). 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Beyotime Institute Biotechnology (Haimen, China).

2.2. Methods

2.2.1. Chemistry

*2.2.1.1. Synthesis of compound **3-17**.* The synthesis of compound **3-17** was reported in our previous work [Fu et al., 2019; Xiao et al., 2019; Zhao et al., 2014, 2015; Zhao et al., 2018].

*2.2.1.2. Synthesis of compound **18**.* To a solution of compound **11** (0.10 g, 0.15 mmol) in CH_2Cl_2 (5 mL) were added dicyclohexylcarbodiimide (DCC, 37 mg, 0.18 mmol) and cat. dimethylaminopyridine (DMAP), then the mixture was stirred at −5 °C for 30 min. Compound **17** (0.11 g, 0.17 mmol) in CH_2Cl_2 (5 mL) was added to the above reaction mixture slowly. After stirring for 10 h at room temperature, the reaction was terminated and then filtered. The filtrate was concentrated, and the residue was purified by flash chromatography to give **18** (128 mg, 65%) as white solid. HRMS: (ESI+) calculated for $C_{69}H_{104}N_8O_{17}Na$ $[M + Na]^+$ 1339.7417, found 1339.7414. Elemental Analysis: C, 62.90; H, 7.96; N, 8.50, found C, 62.76; H, 8.11; N, 8.39.

*2.2.1.3. Synthesis of compound **19**.* To a solution of compound **5** (0.27 g, 0.18 mmol) in CH_2Cl_2 (10 mL) were added DCC (55 mg, 0.27 mmol) and cat. DMAP, then the mixture was stirred at −5 °C for 30 min. Compound **18** (0.24 g, 0.18 mmol) in CH_2Cl_2 (5 mL) was added to the above reaction mixture slowly. After stirring for 20 h at room temperature, the reaction was terminated and then filtered. The filtrate was concentrated, and the residue was purified by flash chromatography to give **19** (0.18 g, 36%) as white waxy solid. HRMS: (ESI+) calculated for $C_{152}H_{192}N_{14}O_{38}Na$ $[M + Na]^+$ 2845.3453, found 2845.3450. Elemental Analysis: C, 64.67; H, 6.86; N, 6.95, found C, 64.79; H, 6.96; N, 6.77.

2.2.1.4. Synthesis of ligand Glu6-RGD-Chol. To a solution of compound **19** (50 mg, 0.018 mmol) in CH_3OH (5 mL), Pd/C (20 mg, 10%) was added. Then, the mixture was stirred in hydrogen atmosphere at 50 °C for 40 h. Pd/C was filtered, and the filtrate was concentrated to give ligand Glu_6-RGD-Chol (18 mg, 52%) as white waxy solid. HRMS: (ESI+) calculated for $C_{89}H_{139}N_{13}O_{36}Na$ $[M + Na]^+$ 1988.9343, found 1988.9347. Elemental Analysis: C, 54.34; H, 7.12; N, 9.26, found C, 54.22; H, 7.29; N, 9.37.

*2.2.1.5. Synthesis of compound **20**.* To a solution of compound **16** (1.00 g, 1.73 mmol) in CH_2Cl_2 (25 mL) was added N-methylmorpholine (NMM, 0.29 mL, 2.60 mmol) and isobutyl chlorocarbonate (IBCF, 0.26 mL, 2.08 mmol), and the reaction was stirred at −10 °C for 15 min. Then compound **4** (2.22 g, 1.56 mmol) in CH_2Cl_2 (10 mL) was added slowly. After stirring for another 10 h at r.t., the mixture was washed with 1 mol/L HCl, saturated $NaHCO_3$ and saturated NaCl. The organic layer was dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography to afford **20** (1.75 g, 56%) as white waxy solid. HRMS: (ESI+) calculated for $C_{114}H_{144}N_6O_{25}Na$ $[M + Na]^+$ 2021.0112, found 2021.0115. Elemental Analysis: C, 68.52; H, 7.26; N, 4.21, found C, 68.37; H, 7.39; N, 4.28.

2.2.1.6. Synthesis of ligand Glu6-Chol. To a solution of compound **20** (0.10 g, 0.050 mmol) in CH_3OH (5 mL), Pd/C (20 mg, 10%) was added. Then, the mixture was stirred in hydrogen atmosphere at r.t. for 20 h. Pd/C was filtered, and the filtrate was concentrated to give ligand Glu_6-Chol (46 mg, 68%) as white waxy solid. HRMS: (ESI+) calculated for $C_{65}H_{102}N_6O_{25}Na$ $[M + Na]^+$ 1389.6792, found 1389.6790. Elemental Analysis: C, 57.09; H, 7.52; N, 6.15, found C, 57.22; H, 7.35 N, 6.01.

2.2.1.7. Synthesis of ligand RGD-Chol. The synthesis of RGD-Chol was reported in our previous work [Fu et al., 2019].

2.2.2. Preparation and characterization of liposomes

Liposomes were prepared by thin film hydration method as our previous reported [Fu et al., 2019; Peng et al., 2018; Xiao et al., 2019]. Lipid compositions of the prepared liposomes were as follows: (1) conventional liposomes (Lip), SPC/cholesterol/ (molar ratio = 62: 33); (2) Ligand Glu_6-Chol modified liposomes (Glu_6-Lip), SPC/cholesterol/ ligand Glu_6-Chol (molar ratio = 62: 33: 3); (3) Ligand RGD-Chol modified liposomes (RGD-Lip), SPC/cholesterol/Ligand RGD-Chol (molar ratio = 62: 33: 3); (4) Ligand Glu_6-Chol and RGD-Chol co-modified liposomes (Glu_6 + RGD-Lip), SPC/cholesterol/Ligand Glu_6-Chol/RGD-Chol (molar ratio = 62: 33: 3: 3); (5) Ligand Glu_6-RGD-Chol modified liposomes (Glu_6-RGD-Lip), SPC/cholesterol/Ligand Glu_6-RGD-Chol (molar ratio = 62: 33: 3). All lipid materials were dissolved in the chloroform/methanol (v/v = 2:1), and then the organic solvent

was removed by rotary evaporation to get a lipid film, which was further dried in vacuum overnight. Next, the film was hydrated in PBS (pH 7.4) for 0.5 h at 20 °C. Finally, it was further sonicated intermittently by a probe sonicator at 80 W for 80 s to form liposomes. PTX-loaded liposomes were prepared with paclitaxel added to the lipid organic solution prior to the solvent evaporation. The entrapment efficiency of paclitaxel was determined by HPLC. The mean size and zeta potential of Lip, Glu_6-Lip, RGD-Lip, Glu_6 + RGD-Lip and Glu_6-RGD-Lip were detected by Malvern Zeta sizer Nano ZS90 (Malvern Instruments Ltd., UK).

2.2.3. Release of free PTX in vitro

In vitro PTX release study was performed using dialysis method. Each PTX-loaded liposomes (0.4 mL) or free PTX were placed into dialysis tube (MWCO = 8 000–14 000 Da) and tightly sealed. Then the dialysis tubes were placed into 40 mL PBS containing 0.1% (v/v) Tween 80 and incubated under 37 °C for 48 h with gentle oscillating at 50 rpm. At predetermined time points (0 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h and 48 h), 0.1 mL release medium was taken out and replaced with equal volume of fresh release medium. Then the samples were diluted with acetonitrile and the concentrations of PTX were determined at the wavelength of 225 nm by HPLC.

2.2.4. In vitro stability of liposomes in serum

Turbidity variations were measured to demonstrate the serum stability of liposomes in the presence of fetal bovine serum (FBS). Briefly, liposomes were mixed with equal volume of FBS under 37 °C with moderate shaking at 45 rpm. The transmittance of the mixture was measured at predetermined time points (0 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h and 48 h) at 750 nm by a microplate reader (Thermo Scientific Varioskan Flash, USA).

2.2.5. Hemolysis assays

To evaluate the safety of ligand-modified liposomes during body circulation, hemolysis assay was performed. Fresh mouse blood was collected in heparin sodium-containing tubes. The red blood cells (RBCs) were separated and collected by centrifugation at 5×10^3 rpm for 5 min and washed several times with PBS until the supernatant became colorless. After the last wash, the RBCs were diluted with PBS to a concentration of 2% (v/v). Various concentrations of liposomes were incubated with equal volume of 2% RBCs solutions for 1 h at 37 °C with gentle shaking, followed by centrifugation at 1×10^4 rpm for 10 min. Absorbance of hemoglobin was measured using a microplate reader (Thermo Scientific Varioskan Flash) at 540 nm. The values for 0% and 100% hemolysis were determined by incubating erythrocytes with PBS or 1% (v/v) Triton X-100. The hemolysis percentage was calculated using the following equation:

$$\text{The percent hemolysis} = \frac{A_{Sample} - A_{Negative}}{A_{postive} - _{Negative}} \times 100\%$$

where A is the ultraviolet absorbance of hemoglobin.

2.2.6. Cytotoxicity assay

MDA-MB-231 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL streptomycin and 100 U/mL penicillin at 37 °C in a humidified incubator containing 5% CO_2 (Thermo Scientific, USA). The cytotoxicity of PTX-loaded liposomes was measured with MTT assay. Generally, the cells were seeded in a 96-well tissue culture plate at a density of 5×10^3 cells/well and cultured for 24 h at 37 °C. PTX-loaded liposomes and free PTX were diluted to predetermined concentrations with PBS and added into each well. The final concentrations of PTX were in the range of 0.1–20 μg/mL. After 24 h incubation, 20 μL MTT solution (5.0 mg/mL) was added to each well and incubated for 4 h at 37 °C. After removal of the culture medium, the reduced MTT dye was solubi1ized by DMSO (150 μL) and the absorbance was read at 490 nm wavelength on an automatic microplate spectrophotometer. Cell viability was calculated using the following equation: A_{test} / $A_{control}$ × 100%, where A_{test} and $A_{control}$ represent the absorbance of treated cells and control cells, respectively.

2.2.7. HAP binding assay

An *in vitro* HAP binding assay was set up using the method described by our previous report to evaluate the binding of Glu_6-RGD modified liposomes to HAP [Zhao et al., 2015]. Briefly, 0.6 mL PTX or liposomes (calculated as PTX) were mixed with 0.3 mL HAP suspension (20 mg/mL) or 0.3 mL PBS as a control, followed by gentle shaking for 2 h at 37 °C. After centrifugation at 5000 rpm for 3 min, the HAP precipitate was separated from the unbound liposome in the supernatant. Unbound liposome were quantified through HPLC to measure PTX encapsulated in the liposome. The degree of HAP binding was calculated according to the following formula:

$$\text{HAP binding rate}(\%) = \frac{M_C - M_E}{M_C} \times 100\%$$

where M_C is the amount of PTX in the control group and M_E is the amount of PTX in the experimental groups.

2.2.8. Targeting metastatic bone in vivo

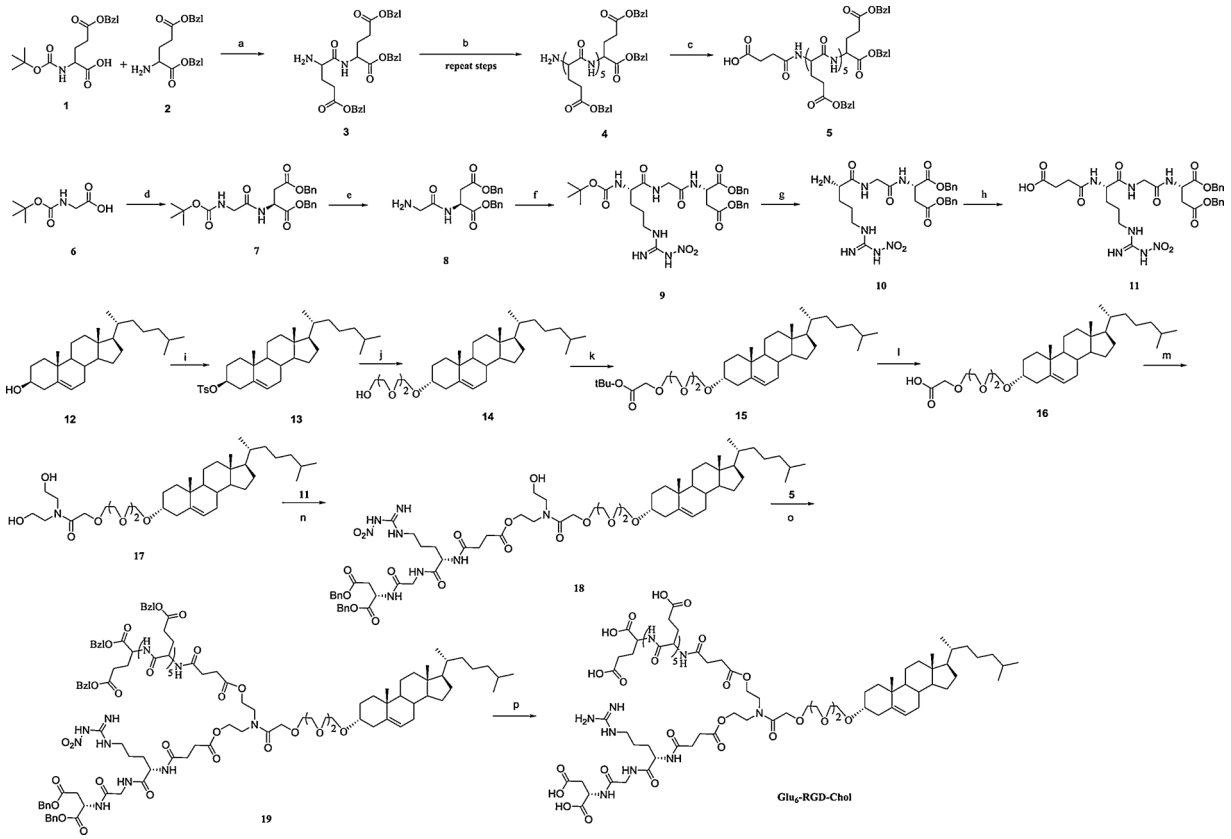
Six-week-old female Balb/c nu mice bearing MDA-MB-231 tumors in the tibia were divided into six groups with five mice in each group. PTX-Lip, PTX-Glu_6-Lip, PTX-RGD-Lip, PTX-Glu_6 + RGD-Lip, PTX-Glu_6-RGD-Lip and paclitaxel were given to the mice *via* the tail vein and each was equivalent to the administration dose of 10 mg/kg PTX. At 0.5 h, 1 h, 2 h, 4 h and 8 h after injection, the mice were killed by cervical dislocation. The bone metastatic lesions and normal bone lesions were excised, rinsed with 0.9% NaCl, dried over filter paper and weighed. The bone tissues were homogenized with twice amount of saline. An aliquot of 10 μL of internal standard (docetaxel, 100 μg/mL) was added into 100 μL bone homogenate, and extracted with 200 μL ether. The mixture was vortexed for 5 min, and centrifuged at 10 000 rpm for 10 min. The supernatant was transferred to another centrifuge tube, and dried under air stream at room temperature. The dry residue was reconstituted with 100 μL of methanol. The solution was centrifuged at 10 000 rpm for 10 min, and then 20 μL of the supernatant was injected into the HPLC system for analysis.

3. Results and discussion

3.1. Chemistry

The synthetic pathway of liposome ligand Glu_6-RGD-Chol was outlined in Scheme 1. Briefly, bone-targeting peptide **5** with carboxyl groups protected by benzyl groups was synthesized by a conventional liquid-phase peptide synthetic method which was described in our previous work [Zhao et al., 2015]. The synthesis of RGD intermediate **11** started from the available material Boc-Gly, Asp(OBzl)-OBzl and Boc-Arg(NO_2)-OH by a series of segment condensation which were described in our previous work [Fu et al., 2019]. Cholesterol **12** underwent five steps to generate compound **17** [Fu et al., 2019]. Subsequently, condensation of compound **17** and RGD derivative **11** in the presence of dicyclohexyl carbodiimide (DCC) and 4-dimethylamiopyridine (DMAP) gave **18**, which was then coupled with Glu_6 peptide intermediate **5** to generate **19**. Finally, debenzylation of the benzyl groups with 10% Pd/C reached the desired ligand Glu_6-RGD-Chol.

What's more, compound **16** was conjugated with peptide **4** in the presence of IBCF and NMM to give compound **20**, which was underwent a deprotection reaction to get the target ligand Glu_6-Chol (Scheme 2). The ligand RGD-Chol was synthesized according to our previous report [Fu et al., 2019].



Scheme 1. Synthesis of ligand Glu_6-RGD-Chol. Reagents and conditions: (a) i) IBCF, NMM, THF, −15 °C - r.t., 20 h; ii) TFA, CH_2Cl_2, r.t., 1 h. (b) Repeat steps as Step (a) for four times. (c) succinic anhydride, CH_2Cl_2, 50 °C, 2 h. (d) Asp(OBzl)-OBzl, IBCF, NMM, CH_2Cl_2, −10 °C - r.t., 2 h. (e) HCl, THF, r.t., 2 h. (f) Boc-Arg(NO_2)-OH, IBCF, NMM, DMF, −10 °C - r.t., 2 h. (g) TFA, CH_2Cl_2, r.t., 4 h. (h) succinic anhydride, dioxane, 80 °C, 0.5 h. (i) TsCl, pyridine, 50 °C, 5 h. (j) Triethylene glycol, dioxane, reflux, 6 h. (k) *t*-butyl bromoacetate, *n*-$Bu_4N^+HSO_4$, 50% NaOH, toluene, r.t., 16 h. (l) TsOH, tolunene, reflux, 8 h. (m) diethanol amine, IBCF, NMM, CH_2Cl_2, −10 °C - r.t., 5 h. (n) **11**, DCC, DMAP, CH_2Cl_2, −5 °C - r.t., 10 h. (o) **5**, DCC, DMAP, CH_2Cl_2, −5 °C - r.t., 20 h. (p) H_2, Pd/C, CH_3OH, 50 °C, 40 h.

3.2. Preparation and characterization of liposomes

One of the requirment for liposome to target bone is that they should have proper sizes and uniform distribution. The particle sizes and zeta potentials of different liposomes in this study were listed in Table 1. The encapsulation efficiencies (EE) of PTX were greater than 88%. The average particle sizes of all liposomes were close to or less than 120 nm, and the values of PDI were close to 0.2. To our knowledge, the particle size and zeta potential of liposomes were crucial to *in vitro* and *in vivo* study [Shi et al., 2015]. What's more, modification of the liposome with Glu_6 significantly increased the net negative charge due to the high density negative charge of glutamic oligopeptides [Zhang et al., 2017].

3.3. Release of free PTX in vitro

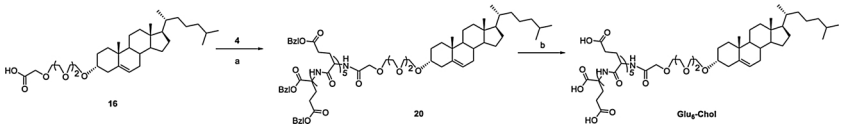
PTX release properties were evaluated in PBS containing 0.1% Tween 80. As shown in Fig. 2, free PTX exhibited a rapid release, with over 80% of the drug released into the media within 12 h incubation. On the other hand, PTX-loaded liposomes achieved sustained release behaviors that the cumulative PTX release of drug loaded liposomes was about 60% after 48 h incubation in PBS. No significant difference on release properties was observed between PTX-Lip, PTX-Glu_6-Lip, PTX-RGD-Lip, PTX-Glu_6 + RGD-Lip and PTX-Glu_6-RGD-Lip, and none of the PTX-loaded liposomes displayed burst initial release patterns.

3.4. In vitro stability of liposomes in serum

It is important for liposome to have superior stability in biological conditions, which is closely related to governing the activity of the associated therapeutic agent. Transmittance of different liposomes were monitored in the presence of 50% FBS. As shown in Fig. 3, the transmittance of the liposomes were above 90% and hardly changed after 48 h incubation with 50% FBS. This indicated that the liposomes were stable enough to prevent the interaction between liposomes and serum protein, which is important for a long blood half-life *in vivo*.

3.5. Hemolysis assays

Hemocompatibility is a key point for *in vivo* applications of liposomes. As shown in Fig. 4, hemolysis assay of PTX-loaded liposomes demonstrated that the five types of liposomes did not show any significant increase in the hemoglobin release with up to 600 nmoles of phospholipids. PTX-Glu_6-RGD-Lip did not display concentration-dependent increase in hemolysis as well and less than 10% hemolysis was always regarded as non-toxic, suggesting that it had a good biosecurity.



Scheme 2. Synthesis of ligand Glu_6-Chol. Reagents and conditions: (a) **4**, IBCF, NMM, THF, −15 °C -r.t., 10 h. (b) H_2, Pd/C, CH_3OH, r.t., 20 h.

Table 1
The characterization of different PTX-loaded liposomes (n = 3).

Liposomes	PTX-Lip	PTX- Glu_6-Lip	PTX-RGD-Lip	PTX-Glu_6 + RGD -Lip	PTX-Glu_6-RGD -Lip
Size(nm)	103.5 ± 4.2	119.3 ± 3.9	108.2 ± 2.8	115.3 ± 3.4	121.9 ± 4.7
PDI	0.185 ± 0.019	0.193 ± 0.009	0.190 ± 0.022	0.194 ± 0.038	0.197 ± 0.034
EE (%)	91.85 ± 2.78	89.21 ± 5.04	90.77 ± 4.31	89.57 ± 3.54	88.62 ± 6.26
Zeta potential (mV)	−2.39 ± 0.22	−15.69 ± 2.34	−4.52 ± 0.57	−15.11 ± 2.68	−14.37 ± 4.85

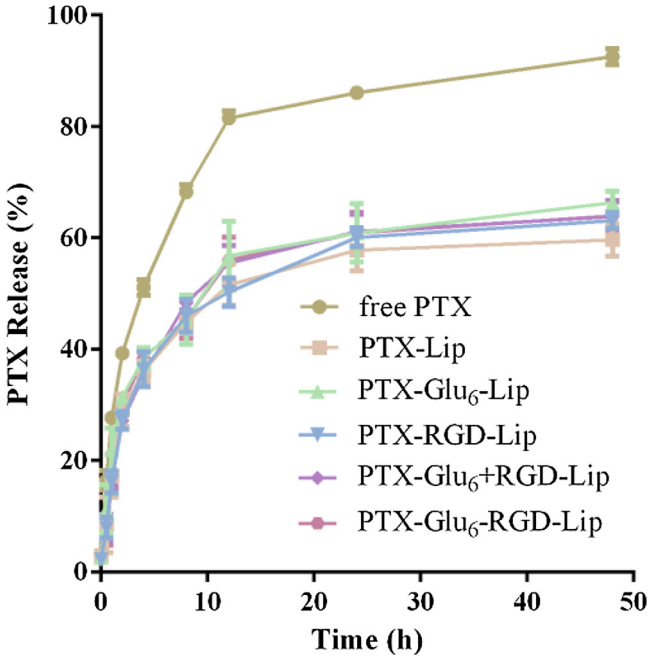


Fig. 2. The PTX release profiles of free PTX, PTX-Lip, PTX-Glu_6-Lip, PTX-RGD-Lip, PTX-Glu_6 + RGD-Lip and PTX-Glu_6-RGD-Lip in PBS (pH 7.4) containing 0.1% Tween 80 over 48 h (n = 3, mean ± SD).

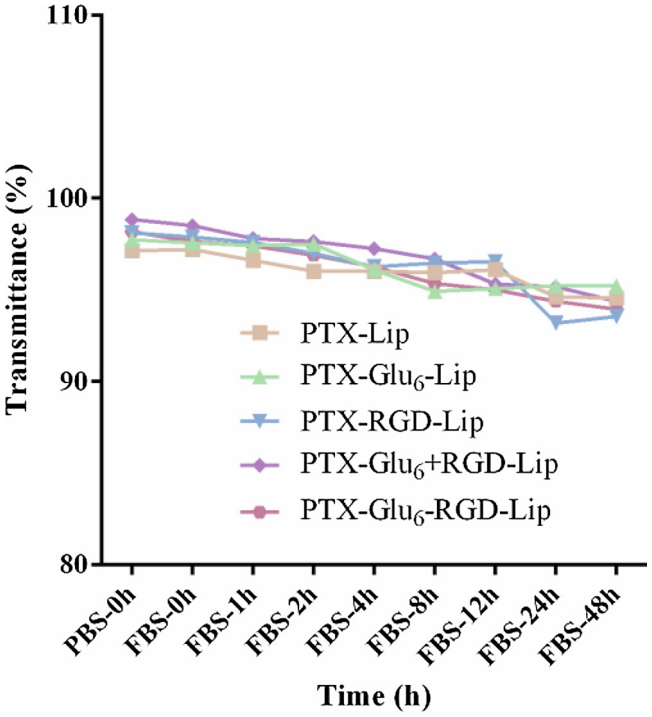


Fig. 3. The variations of transmittance of different modified liposomes in 50% FBS (n = 3, mean ± SD).

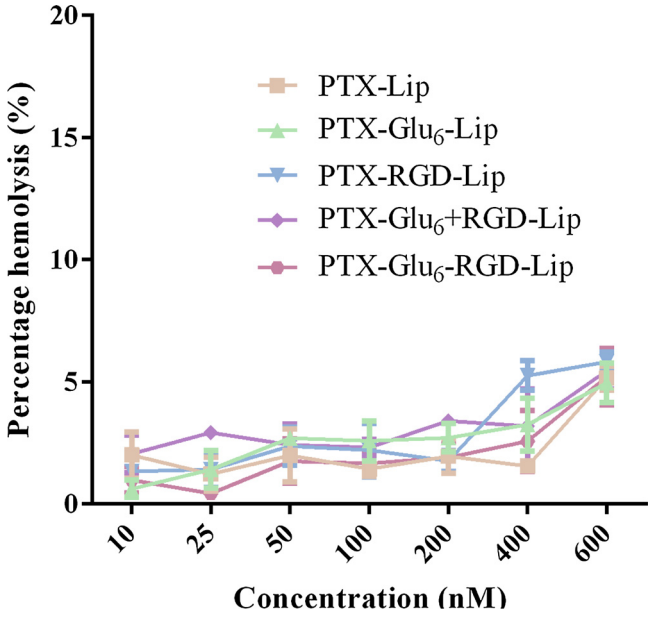


Fig. 4. Hemolysis percentage of different liposomes (n = 3, mean ± SD).

3.6. Cytotoxicity

The cytotoxicity of different liposomes on MDA-MB-231 cells was evaluated using MTT assay. As shown in Fig. 5, free PTX showed higher inhibition rate than PTX-loaded liposomes, because free drugs could be transported into the cells directly by passive diffusion, without a drug release process. Furthermore, the cytotoxicity of blank liposomes was also measured, and it was shown that all the kinds of blank liposomes had no significant cytotoxicity. Hence, our liposomal drug delivery system was safe and non-toxic to be further used *in vivo*.

3.7. HAP binding assay

HAP is a main component in bone tissue, investigation of the HAP affinity has been widely used to evaluate the potential bone targeting of nano-sized drug delivery system. In this experiment, we compared the HAP binding efficiency between PTX, PTX-Lip, PTX-Glu_6-Lip, PTX-RGD-Lip, PTX-Glu_6 + RGD-Lip and PTX-Glu_6-RGD-Lip. As shown in Fig. 6, compared with PTX, PTX-Lip and PTX-RGD-Lip, PTX-Glu_6-Lip, PTX-Glu_6 + RGD-Lip and PTX-Glu_6-RGD-Lip exhibited a significantly higher HAP binding efficiency with hydroxyapatite power. These results demonstrated that Glu_6-modified liposome had good bone-targeting ability, and facilitated the next *in vivo* experiments.

3.8. Targeting metastatic bone in vivo

To determine whether the liposomes PTX-Glu_6-RGD-Lip could promote the accumulation of paclitaxel in the bone metastatic lesions, a bone metastasis model was established by injecting MDA-MB-231 cancer cells into the tibia of Balb/c nu mice. The PTX concentration in bone metastatic lesions and normal bone was detected at 0.5 h, 1 h, 2 h, 4 h and 8 h after intravenous administration of different formulations. It

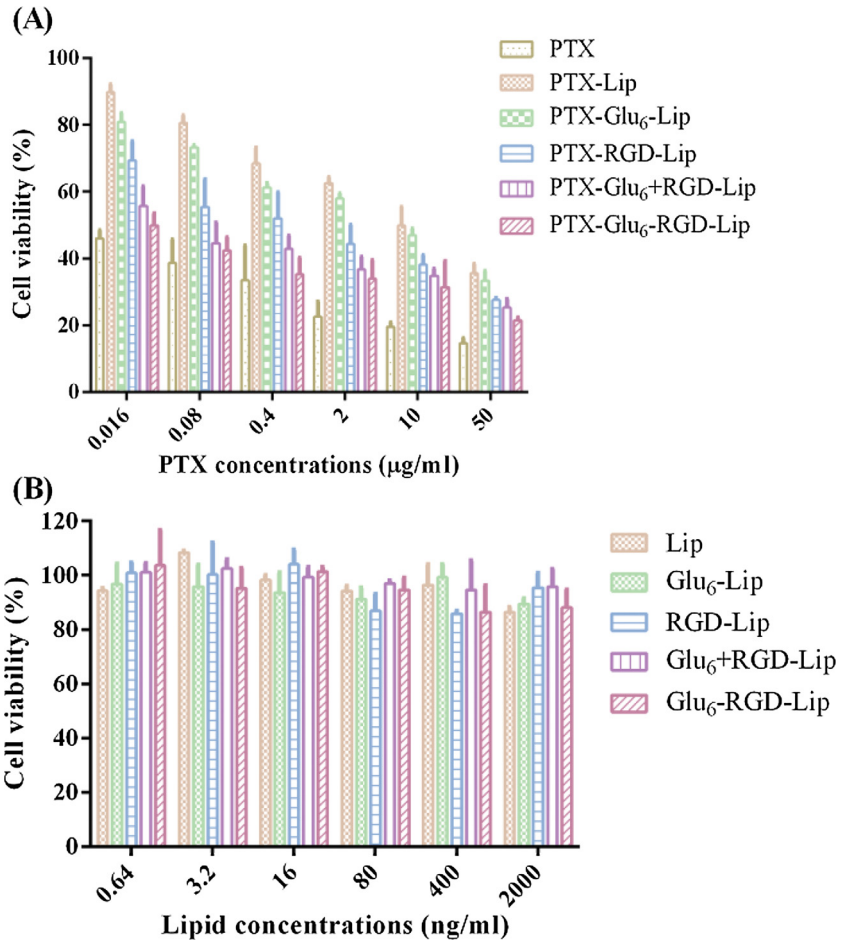


Fig. 5. (A) The cytotoxicity study of PTX-loaded liposomes and free PTX against MDA-MB-231 cells. (B) The cytotoxicity study of blank liposomes against MDA-MB-231 cells (n = 3, mean ± SD).

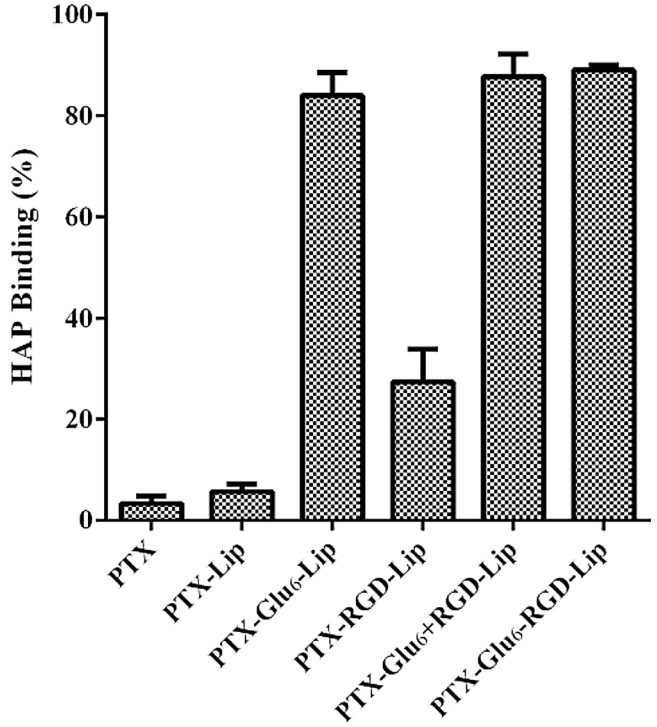


Fig. 6. HAP binding ratio of different types of PTX-loaded liposomes and PTX after incubation with HAP for 2 h (n = 3, mean ± SD).

was obviously found that all PTX-loaded liposomes had a higher concentration than that of PTX injection. As shown in Fig. 7A–B, the PTX concentration both in the metastatic bone and normal bone lesions of Glu_6-modified liposomes groups was obviously increased compared with PTX-Lip and PTX-RGD-Lip attributing to the bone targeting ability of glutamic oligopeptides, which was consistent with the result of the HAP binding assay. What's more, the results showed that it had an obviously increased accumulation in bone metastatic lesions than in normal bone. Furthermore, PTX-Glu_6-RGD-Lip had an increased concentration compared to PTX-Glu_6-Lip and PTX-Glu_6 + RGD-Lip groups due to the synergistic effect of the dual-mediated endocytosis. As shown in Fig. 7B, the PTX in metastatic bones following the injection of PTX-Glu_6-RGD-Lip was 5–8 times higher than that following PTX injected, 3–5 times higher than that following PTX-Lip injected, and 1–3 times higher than that following PTX-Glu_6-Lip, PTX-RGD-Lip and PTX-Glu_6 + RGD-Lip. These results indicated the targeting effect of PTX-Glu_6-RGD-Lip for metastatic bones, which could lead to the improved efficacy in bone metastasis and reduced toxicity to other tissues.

4. Conclusion

In this study, we have developed Glu_6-RGD-modified liposomal drug delivery system. The liposome formulation was capable of bone affinity through glutamic oligopeptides, and specific tumor recognition by RGD modification on the surface of the particles. PTX-Glu_6-RGD-Lip possess good stability, high HAP binding efficiency, as well as improved cytotoxicity. More importantly, the *in vivo* targeting metastatic bone study suggested that PTX-Glu_6-RGD-Lip favored accumulation of loaded

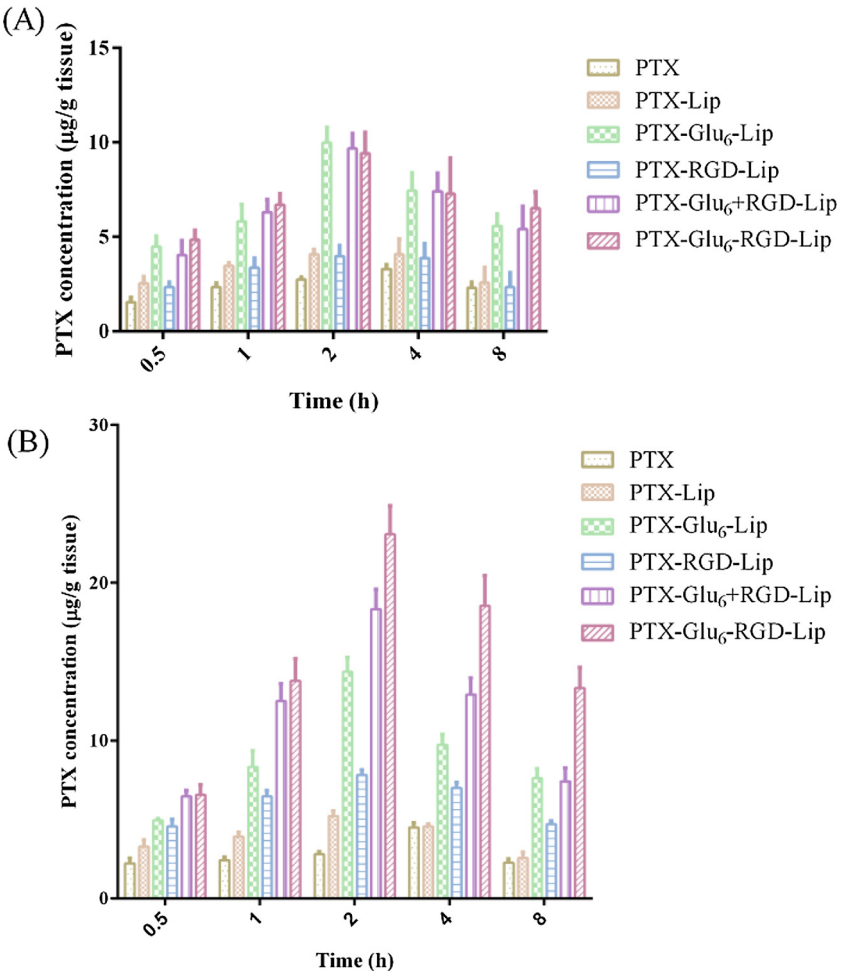


Fig. 7. PTX concentration 0.5 h, 1 h, 2 h, 4 h and 8 h after injection of free PTX, PTX-Lip, PTX-Glu$_6$-Lip, PTX-RGD-Lip, PTX-Glu$_6$ + RGD-Lip and PTX-Glu$_6$-RGD-Lip in the normal bone (A) and bone metastatic lesions (B). (Five mice in each group, mean ± SD).

paclitaxel in the metastatic bones. In general, this work has a promising application in bone metastasis targeting delivery systems and more work is in progress.

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