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Tumor-specific pH-responsive peptide-modified pH-sensitive liposomes containing doxorubicin for enhancing glioma targeting and anti-tumor activity

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ABSTRACT

The pH environment in gliomas is acidic. Therefore, in the present research, we selected our previously reported tumor-specific pH-responsive peptide $H_7K(R_2)_2$ as a targeting ligand, which could respond to the acidic pH environment in gliomas, possessing CPP characteristics. The pH-sensitive liposomes were selected as carriers which could also respond to the acidic pH environment in gliomas triggering encapsulated drug release from these pH-sensitive liposomes. The $H_7K(R_2)_2$-modified pH-sensitive liposomes containing doxorubicin (DOX-PSL-$H_7K(R_2)_2$) were designed and prepared in order to evaluate their potential targeting of glioma tumor cells and their anti-tumor activity in mice with glioma tumor cells. DOX-PSL-$H_7K(R_2)_2$ was prepared by the thin-film hydration method followed by remote loading using an ammonium sulfate gradient method. The *in vitro* release of DOX from pH-sensitive liposomes was tested and the *in vitro* targeting characteristics of $H_7K(R_2)_2$-modified liposomes regarding C6 (rat C6 glioma cells) and U87-MG (human glioblastoma cells) were evaluated. The *in vivo* anti-tumor activity of DOX-PSL-$H_7K(R_2)_2$ was also investigated in C6 tumor-bearing mice and in U87-MG orthotopic tumor-bearing nude mice. A specific targeting effect triggered by an acidic pH was observed in our *in vitro* experiments in C6 and U87-MG glioma cells. The pH-triggered DOX release from the pH-sensitive liposomes under acidic conditions was also confirmed in our *in vitro* experiment. Anti-tumor activity of DOX-PSL-$H_7K(R_2)_2$ was found in C6 tumor-bearing mice and U87-MG orthotopic tumor-bearing nude mice in *in vivo* experiments. The antiangiogenic activity of DOX-PSL-$H_7K(R_2)_2$ was confirmed in C6 tumor-bearing mice in the *in vivo* experiment. These $H_7K(R_2)_2$-modified pH-sensitive liposomes containing anti-tumor drugs developed in this study are a promising delivery system involving the response stimuli at the acidic pH in the glioma tumor microenvironment and are suitable for anti-tumor therapy.

1. Introduction

Malignant gliomas are the most common and deadly brain tumors [1]. Nanoparticles have been reported to be a potentially useful tool for the treatment of brain tumors due to their capacity of exploiting some biological pathways to achieve specific delivery to cellular and intracellular targets, including transport across the blood–brain barrier (BBB), which many anti-tumor drugs cannot achieve on their own [2]. In addition, a ligand-modified nanoparticle strategy has been developed to improve the delivery efficiency by "decorating" with specific ligands targeting specific receptors in brain tumor cells or the tumor microenvironment [3–4].

Cell-penetrating peptides (CPPs) are relatively short cationic or amphipathic peptides which can control entry into cells by their intrinsic pathway [5]. Since there is a high concentration of negative charges in the BBB, the positively charged CPPs can attach themselves to the BBB and then trigger the transport pathway across the BBB [6]. It has been reported that CPP-modified liposomes can be transported through brain capillary endothelial cells and accumulate in the brain [7].

In order to overcome the effect of the CPPs on non-specific binding, the development of a dual targeting nanoparticle delivery system co-modified with CPP and other ligands has been reported [8–10]. In addition, pH-responsive CPPs for tumor-targeting drug delivery systems have been designed to respond to the acidic pH environment of tumors, with CPP characteristics at a low pH in tumor tissues and non-CPP characteristic at a normal pH in normal tissues [11–13].

In a previous study we designed a tumor-specific pH-responsive peptide $H_7K(R_2)_2$ which could respond to the acidic pH in tumor tissues [14]. This peptide contains the CPP sequence $(R_2)_2$ and the pH trigger

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sequence H_7. It has been reported that arginine(R)-rich peptides have the ability to cross cell membranes in a seemingly energy-independent manner. The activity of branched R-rich peptides is stronger than that of those with a linear structure. PolyHis has the ability to respond to the acidic pH in a tumor microenvironment due to the ionization of polyHis switching from hydrophobic to hydrophilic under acid conditions. The specific targeting effect of $H_7K(R_2)_2$-modified polymeric micelles containing paclitaxel triggered at an acidic pH has been confirmed in tumor cells [14].

The pH-sensitive liposomes rapidly become destabilized in the acidic pH environment of the tumor tissues [15] and, therefore, pH-sensitive liposomes can be used for delivering anti-cancer drugs to specific cancer cells, enhancing cellular internalization and rapid intracellular drug release. In order to increase the targeting activity, ligand-modified pH-sensitive liposomes have been used for tumor targeting [16].

Doxorubicin (DOX) is widely used clinically because of its broad-spectrum anti-tumor activity and is known to bind to DNA-associated enzymes, intercalate with DNA base pairs, and target multiple molecular targets to produce a range of cytotoxic effects [17]. However, the anti-tumor activity was hampered by the poor targeting efficiency. Therefore in this study, DOX was used as a model drug.

Considering the acidic pH environment in tumor tissues as well as pH-responsive targeting and triggering release, in the present research, we designed and prepared tumor-specific pH-responsive peptide $H_7K(R_2)_2$ modified pH-sensitive liposomes containing doxorubicin (DOX-PSL-$H_7K(R_2)_2$). The anti-tumor activity of DOX-PSL-$H_7K(R_2)_2$ in brain tumor was investigated *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (methoxy(polyethylene glycol)-2000) (DSPE-PEG) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-n-[poly(ethyleneglycol)]-hydroxy succinamide (DSPE-PEG-NHS) were provided by NOF Co. (Tokyo, Japan). Dioleoylphosphatidylethanolamine (DOPE), cholesteryl hemisuccinate (CHEMS), cholesterol, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), ammonium sulfate, Trizma-base, and Tris base were obtained from Sigma Aldrich (St, Louis, MO, USA). Egg phosphatidylcholine (EPC) was purchased from Lipoid GmbH (Ludwigshafen, Germany). The Ac-RRK(HHHHHHH)RR-NH_2 peptide ($H_7K(R_2)_2$) was synthesized by Beijing Scilight Biotechnology Co., Ltd. (Beijing, China). Doxorubicin hydrochloride (DOX) was supplied by Hisun Pharmaceutical Co. Ltd. (Zhejiang, China). Coumarin-6, Hoechst 33258 and FITC goat anti-rabbit secondary antibodies (1:1000) were supplied by Molecular Probes Inc. (Eugene, OR, USA). Rabbit polyclonal antibody to CD31 (10 mg/ml) was obtained from Abcam Inc. (Cambridge, Massachusetts, USA).

Cell culture medium F10 and MEM, penicillin–streptomycin, fetal bovine serum (FBS), and donor equine serum were obtained from GIBCO., Invitrogen Co. (Carlsbad, USA). All other reagents were of analytical or HPLC grade.

2.2. Animals

Female BALB/C nude mice weighing 18–22 g (5–6 weeks) were obtained from the Experimental Animal Center of Peking University Health Science Center. All care and handling of the animals were performed with the approval of the Institutional Authority for Laboratory Animal Care of Peking University.

2.3. Cell culture

Rat C6 glioma cells (the Cell Resource Center, Peking Union Medical College, Beijing, China) were used and maintained in Ham's F10 medium supplemented with 2.5% FBS, 15% donor equine serum, and 1% penicillin–streptomycin solution. The cells were incubated at 37 °C in a humidified environment of 5% CO_2.

Human glioblastoma U87 MG cells (the Cell Resource Center, Peking Union Medical College, Beijing, China) were cultured in MEM supplemented with 1% non-essential amino acids, 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin. The cells were incubated at 37 °C in a humidified environment of 5% CO_2.

2.4. Synthesis of DSPE-PEG-$H_7K(R_2)_2$

The DSPE-PEG-$H_7K(R_2)_2$ was synthesized by our laboratory according to a previously reported method [14]. $H_7K(R_2)_2$ was conjugated with DSPE-PEG through the NHS group. Briefly, DSPE-PEG-NHS was added with $H_7K(R_2)_2$ at a 2:1 M ratio (DSPE-PEG-NHS:$H_7K(R_2)_2$) in DMF, and the pH was adjusted to 7.0 with N-methyl morpholine. The reaction was maintained for 24 h at room temperature under moderate stirring. The resulting reaction mixture was placed in a dialysis bag (molecular weight cutoff = 3500 Da) and dialyzed against deionized water for 72 h to remove the unconjugated peptides. The final solution in the dialysis bag was lyophilized and stored at −20 °C until required.

2.5. Preparation of DOX-PSL-$H_7K(R_2)_2$

The $H_7K(R_2)_2$-modified pH-sensitive liposomes containing DOX (DOX-PSL-$H_7K(R_2)_2$) were prepared by the thin-film hydration method, as described previously [18]. Briefly, a mixture of DOPE, CHEMS, DSPE-PEG and DSPE-PEG-$H_7K(R_2)_2$ was dissolved in chloroform. Then the solvent was evaporated using an RE52 rotary evaporator (Shanghai Yarong Biochemistry Instrument Company, China) in a round-bottomed flask at 45 °C for about 40 min to obtain a solid film. This film was then flushed with nitrogen gas for 30 min and stored overnight in a desiccator to remove any traces of chloroform.

DOX was encapsulated into the liposomes by remote loading using an ammonium sulfate gradient method [19] with minor modification [20]. Briefly, the thin-film was hydrated in a 250 mM ammonium sulfate solution at pH 8.5 by sonication in water bath for 10 min to produce a suspension of liposomes. The liposome suspension was extruded 10 times through a polycarbonate membrane (Millipore, Bedford, MA, USA) with a pore size of 100 nm. Then, the external buffer was exchanged by elution through a Sephadex G-50 (Sigma-Aldrich, St. Louis, MO, USA) column equilibrated with 10% sucrose, using a 25 mM Trizma base (Sigma Chemical, MO, USA), at pH 10.0. The DOX solution was added to the liposomes at a DOX/lipid (*w*/*w*) ratio of 1:20, then, the mixture was incubated for 1.5 h at 37 °C. The free DOX was separated from the liposome-encapsulated DOX using the Sephadex G-50 column by eluting with HEPES buffered saline (pH 7.4).

For the preparation of pH-sensitive liposomes containing DOX (DOX-PSL), a similar procedure was carried out except that the equivalent molar DSPE-PEG-$H_7K(R_2)_2$ was replaced by DSPE-PEG.

The sterically stabilized liposomes containing DOX (DOX-SSL) were prepared according to our previous report [21–22].

The preparation of liposomes loaded with coumarin-6 (coumarin-6-PSL-$H_7K(R_2)_2$, coumarin-6-PSL and coumarin-6-SSL) was carried out using the thin-film hydration method, as described previously [22]. For preparation of coumarin-6-PSL-$H_7K(R_2)_2$, coumarin-6-PSL and coumarin-6-SSL, the lipids and coumarin-6 were dissolved in chloroform and dried until a thin film was obtained. The dried lipid film was then hydrated with HEPES, sonicated in a bath-type sonicator, extruded using a mini-extruder, and then passed through the Sephadex G-50 column to remove the unentrapped coumarin-6 with HEPES.