Enhanced Glioma Targeting and Penetration by Dual-Targeting Liposome Co-modified with T7 and TAT

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ABSTRACT: The development of a drug delivery strategy that can not only cross the blood–brain barrier (BBB) rapidly, but also target the glioma and reach the core of glioma is essential and important for glioma treatment. To achieve this goal, we established a dual-targeting liposomal system modified with TAT (AYGRKKRRQRRR) and T7 (HAIYPRH), in which the specific ligand T7 could target BBB and brain glioma tumor and the nonspecific ligand TAT could enhance the effect of passing through BBB, and elevate the penetration into the tumor. The dual-targeting effects were evaluated by both *in vitro* and *in vivo* experiments. To identify the targeting effect, *in vitro* cellular uptake and BBB model were performed. Tumor spheroid penetration was performed to evaluate the penetration characteristics of the dual-targeting liposomes. *In vivo* pharmacokinetic studies were utilized to evaluate the influence of T7 and TAT peptides on the behavior of nanoparticle drug delivery system, and tissue distribution was further utilized to evaluate the glioma-targeting efficiency of the dual-targeting liposomes.
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INTRODUCTION

Nowadays, the treatment of glioma, one of the most malignant brain tumors, accounting for about 42% of all brain tumors,[1,2] remains a big challenge because of its highly proliferative, infiltrative, and invasive property.[3] Their surgical resection is necessarily restricted because of the important functions of the brain. Moreover, infiltration of tumor cells into normal tissues makes complete surgical resection impossible.[4] Invasive growth of brain tumors leads to a poor prognosis and frequent recurrence.[2] Therefore, chemotherapy is the most common auxiliary treatment for glioma.[5,6] However, the efficiency of drug delivery to glioma is highly limited by the nonspecific, nontargeted nature of antitumor agents, the presence of blood–brain barrier (BBB), and blood–brain tumor barrier that are extremely exclusive.[7–9] Accordingly, it is imperative to develop a targeted drug delivery system (DDS) with high BBB penetration and glioma-targeting abilities.

To overcome BBB and blood tumor barrier, dual-targeting DDSs based on receptor-mediated endocytosis were developed to deliver chemotherapeutic agent across BBB and simultaneously target brain tumor.[10,11] It has been reported that some receptors (such as transferrin receptor, TfR, insulin receptor, and low-density lipoprotein receptor, etc.) is not only overexpressed on BBB but also on glioma cancerous cells.[9] Thus, a dual-targeting strategy was developed to decorate the surface of nanocarriers with one single ligand.[12,13] Although the single peptide could enhance the targeting effect, the presence of receptor-targeting moiety alone on liposomes limited the enhanced uptake of liposomes because of the receptor saturation.[14–16] Considering the fact that an ideal tumor-targeted DDS should not only selectively deliver drugs to targeted tumor, but also deliver the drugs into the center of tumor with high efficacy, the receptor saturation need to be overcome. Therefore, another and more effective dual-targeting strategy is based on modification of nanocarriers with two kinds of ligands, one of which could target to BBB and tumor cells, the other could promote the targeting effect.[17]

The receptor of transferrin (Tf) is the TfR enriched in tumor cells, especially brain tumor cells, and brain capillary endothelial cells (BCECs).[18] Nanoparticles modified with Tf have the dual-targeting effect of targeting to BBB and brain tumor, which has been validated in many studies.[19,20] However, the application of proteins and antibodies are restricted by their instability and immunogenicity. Small molecules, such as peptides and aptamers, are better choices.[9,21] T7 is a short sequence of peptide composed of seven amino acids and can also bind with TfR. Compared with Tf, T7-modified nanoparticles have more advantages, such as no immunogenicity and better stability. In addition, the endogenous Tf may competitively inhibit the binding of Tf-modified DDSs to TfR but would not inhibit the binding of T7 to TfR. On the contrary, endogenous Tf *in vivo* can promote the uptake of T7.[22] In short, T7, as a ligand-targeting TfR, is more advantageous than Tf.

TAT, one of the cell-penetrating peptides (CPPs), is the transactivating protein of the human immunodeficiency virus type-1 and is essential for viral replication.[23] TAT contains a basic region consisting of six arginine and two lysine residues.[24] Their cationic charges facilitate interaction with the normally negatively charged cell membrane, triggering permeabilization

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of the cell membrane.[25] Liposomes modified with TAT can deliver the cargoes into cells with high efficiency via an unsaturated and receptor/transporter-independent pathway.[26]

In this study, we established a dual-targeting liposomal system modified with TAT and T7, in which the specific ligand T7 could target to BBB and brain glioma tumor and the nonspecific ligand TAT could enhance the effect of passing through BBB, and elevate the penetration efficiency into the tumor. CFPE, coumarin-6, and doxorubicin (DOX) were utilized to track the behavior of liposomes *in vitro* and *in vivo*. To identify the targeting effect, *in vitro* cellular uptake and BBB model were performed. Tumor spheroid penetration was performed to evaluate the penetration characteristics of the dual-targeting liposomes. *In vivo* pharmacokinetic studies were utilized to evaluate the influence of T7 and TAT peptides on the behavior of nanoparticle DDS and tissue distribution was further utilized to evaluate the glioma-targeting efficiency of the dual-targeting liposomes.

MATERIALS AND METHODS

Materials

Doxorubicin was a gift from Haizheng Company Ltd. (Zhejiang, China). TAT peptide with terminal cysteine (Cys) was synthesized by Chengdu KaiJie Bio-pharmaceutical Company, Ltd. (Chengdu, China). T7 with a cysteine on the N-terminal (cys-T7) was synthesized by China Peptides Company, Ltd. (Shanghai, China). Soybean phospholipid (SPC) was purchased from Shanghai Advanced Vehicle Technology L.T.D. Company (Shanghai, China), and cholesterol (CHO) was purchased from Chengdu Kelong Chemical Company (Chengdu, China). N-hydroxysuccinimide (NHS)-polyethylene glycol $(PEG)_{1000}$-maleimide (Mal) was obtained from Jenkem Technology (Beijing, China). 4′-6-Diamidino-2-pheylindole (DAPI) was purchased from Beyotime Institute Biotechnology (Haimen, China). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), DSPE-PEG_{2000}, DSPE-PEG_{2000}-Mal, and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(carboxyfluorescein) (CFPE) were purchased from Avanti Polar Lipids (Alabaster, Alabama). Plastic cell culture dishes and plates were purchased from Wuxi NEST Biotechnology Company (Wuxi, China). Other chemicals and reagents were of analytical grade.

BALB/c mice were purchased from experiment animal center of Sichuan University. All animal experiments were performed in accordance with the principles of care and use of laboratory animals and were approved by the experiment animal administrative committee of Sichuan University.

Synthesis of DSPE-PEG_{1000}-TAT and DSPE-PEG_{2000}-T7[27]

DSPE-PEG_{1000}-Mal was synthesized by conjugating NHS-PEG_{1000}-Mal with DSPE in dry chloroform at room temperature under argon and triethylamine for about 5 h. The obtained DSPE-PEG_{1000}-Mal and DSPE-PEG_{2000}-Mal were, respectively, reacted with Cys-T7 and Cys-TAT in the mixed solvent of $CHCl_3$/MeOH (v:v, 2:1) containing triethylamine for about 30 h. After the reaction completed, the organic solvent was evaporated by rotary evaporation and the residues were redissolved in chloroform and filtered to purify the products. The filtrates were evaporated by rotary evaporation and stored at −20°C until used.

Table 1. Composition of Liposomes (moL%)

	Corresponding Compositions		
Abbreviations	SPC	CHO	Functional Lipids Composition
T7-TAT-Lip	57%	33%	6%DSPE-PEG_{2000}-T7/0.5%DSPE-PEG_{1000}-TAT/3.5%DSPE-PEG_{2000}
TAT- Lip	57%	33%	0.5%DSPE-PEG_{1000}-TAT/9.5%DSPE-PEG_{2000}
T7-Lip	57%	33%	6%DSPE-PEG_{2000}-T7/4%DSPE-PEG_{2000}
PEG-Lip	57%	33%	10%DSPE-PEG_{2000}

Preparation and Characterization of Liposomes

Coumarin-6 and CFPE-loaded liposomes were prepared by the lipid hydration method.[27,28] Briefly, various amounts of lipid materials (see Table 1) and CFPE/coumarin-6 were dissolved in the mixed solvent of chloroform and ethanol (v:v, 2:1). The solvent was then removed by rotary evaporation. The thin film was hydrated in phosphate-buffered saline (PBS, pH 7.4) and then intermittently sonicated with a probe sonicator. For coumarin-6-loaded liposomes, the solution was then centrifuged at 13400 g for 10 min to remove coumarin-6 precipitates. The liposomes were stored at 4°C for later use.

DOX-loaded liposomes were prepared by remote loading using an ammonium sulfate gradient.[27] Briefly, the lipids of each composition (see Table 1) were dissolved in the mixed solvent of chloroform and ethanol (v:v, 2:1), dried into a thin film on a rotary evaporator, and then hydrated with 300 mM of ammonium sulfate solution. The free ammonium sulfate was removed by passing through a Sephadex G-50 column in PBS (pH 7.4) solution. DOX (doxorubicin hydrochloride:phospholipids = 1:20, w/w) was added, mixed, and then incubated at 45°C for 20 min. Free DOX was removed by passing through a Sephadex G-50 column. The envelopment efficiency of the liposomes was measured at Ex = 470 nm and Em = 590 nm, respectively, on a spectrofluorimeter (Shimadzu, Tokyo, Japan). The DOX-loaded liposomes were stored at 4°C for later use.

Characterization of the Nanoparticles

The mean particle sizes and zeta potentials of the liposomes were measured by Malvern Zetasizer Nano ZS90 instrument (Malvern instruments Ltd., Malvern, UK). Prior to measurement, 100 μL of the sample (lipid concentration: 3 μmol/mL) was diluted by using pure water to 1 mL. Particle morphology was detected by a transmission electron microscope (TEM) (100CX; JEOL, Tokyo, Japan) following negative staining with sodium phosphotungstate solution.

In Vitro Stability of Liposomes in Serum

Fifty microliter of different formulations of liposomes was added to 1 mL of culture medium containing 10% fetal bovine serum (FBS) and incubated at 37°C, with gentle shaking at 50 rpm. At different time points, the size change was determined by using a Malvern Zetasizer Nano ZS90 instrument (Malvern instruments Ltd., Malvern, UK) and the transmittance was measured at 750 nm by a microplate reader (Thermo Scientific Varioskan Flash, Waltham, MA).