Peptide-22 and Cyclic RGD Functionalized Liposomes for Glioma Targeting Drug Delivery Overcoming BBB and BBTB

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ABSTRACT: Chemotherapy outcomes for the treatment of glioma remain unsatisfactory due to the inefficient drug transport across BBB/BBTB and poor drug accumulation in the tumor site. Nanocarriers functionalized with different targeting ligands are considered as one of the most promising alternatives. However, few studies were reported to compare the targeting efficiency of the ligands and develop nanoparticles to realize BBB/BBTB crossing and brain tumor targeting simultaneously. In this study, six peptide-based ligands (Angiopep-2, T7, Peptide-22, c(RGDfK), D-SP5 and Pep-1), widely used for brain delivery, were selected to decorate liposomes, respectively, so as to compare their targeting ability to BBB or BBTB. Based on the in vitro cellular uptake results on BCECs and HUVECs, Peptide-22 and c(RGDfK) were picked to construct a BBB/BBTB dual-crossing, glioma-targeting liposomal drug delivery system c(RGDfK)/Pep-22-DOX-LP. In vitro cellular uptake demonstrated that the synergetic effect of c(RGDfK) and Peptide-22 could significantly increase the internalization of liposomes on U87 cells. In vivo imaging further verified that c(RGDfK)/Pep-22-LP exhibited higher brain tumor distribution than single ligand modified liposomes. The median survival time of glioma-bearing mice treated with c(RGDfK)/Pep-22-DOX-LP (39.5 days) was significantly prolonged than those treated with free doxorubicin or other controls. In conclusion, the c(RGDfK) and Peptide-22 dual-modified liposome was constructed based on the targeting ability screening of various ligands. The system could effectively overcome BBB/BBTB barriers, target to tumor cells and inhibit the growth of glioma, which proved its potential for improving the efficacy of chemotherapeutics for glioma therapy.

KEYWORDS: glioma, blood–brain barrier, blood–brain tumor barrier, liposome, c(RGDfK), Peptide-22

1. INTRODUCTION

Glioma is one of the most common and aggressive intracranial malignancies with high morbidity and mortality. The median overall survival of glioma patients is 12–18 months and the 5-year survival rate is less than 10%.1,2 Because of the invasive growth and an undefined tumor edge, surgery can hardly remove the tumor completely and the recurrence rate is very high.3,4 Once relapse, the average survival duration is only 1.5 years.1 Systemic chemotherapy has been widely used in the treatment of glioma.5–7 Unfortunately, clinical outcomes remain unsatisfactory due to the unfavorable pharmacokinetics, severe side effects and poor drug accumulation in the brain parenchyma.5,8 Recently, the exploitation of nanocarriers for drug delivery has emerged as a promising alternative to conquer these problems.9 However, the effective transport of nanocarriers to the glioma site is still limited.10,11

It is essential to deliver anticancer drug loaded nanocarriers across the blood–brain barrier (BBB) and blood–brain tumor barrier (BBTB) simultaneously in order to treat glioma. The BBB, an endothelial cell monolayer associated with pericytes and astrocytes, prevents approximately 98% small molecules and nearly 100% large molecules from transporting into the brain.12 Meanwhile, it behaves as the main obstacle for nanocarriers to enter the brain parenchyma in the early stage of glioma.13 With the gradual progression of glioma, the integrity of BBB is compromised to some extent while the BBTB emerges. The BBTB exists between the brain tumor tissues and capillary vessels, which also impedes the effective transport of nanocarriers into tumor.14,15 Many efforts have been made to overcome BBB or BBTB separately. However, in most cases, BBB and BBTB exist simultaneously when glioma is diagnosed.16 Therefore, it is significant to develop a novel drug delivery system possessing BBB and BBTB dual-targeting ability.
As is reported previously, receptor-mediated transcytosis (RMT) is one of the most common strategies for nanocarriers to penetrate BBB/BBTB.\textsuperscript{17} Studies have found that brain capillary endothelial cells and endothelial cells of tumor angiogenic vessels express numerous different receptors.\textsuperscript{14,18} Nanocarriers decorated with specific ligands can efficiently bind to corresponding receptors overexpressed on BBB/BBTB and then initiate the RMT process, resulting in the enhancement of drug penetration across the barriers and accumulation in the glioma site.\textsuperscript{19} Various ligands have been demonstrated to possess high binding affinities to specific receptors and exhibit obvious brain tumor targeting properties when conjugated to nanoparticles. There are thousands of reports about the modification with targeting ligands on nanocarriers. Almost all the research results are positive and prove that the application of ligands could significantly improve the targeting efficiency of the nanosystems. However, it is difficult to compare the results and determine which ligand is more effective for brain delivery, because the important factors influencing the targeting efficiency, such as particle type, formulation parameters and the ligand density, were quite different in various studies.\textsuperscript{20–22} Therefore, it is essential to evaluate the BBB/BBTB targeting ability of different ligands in the same nanocarrier, which will pick out the most efficient ligand for the establishment of brain targeting drug delivery system.

Angiopep-2,\textsuperscript{23} T7\textsuperscript{24} and Peptide-22\textsuperscript{25} are widely used as specific ligands for low-density lipoprotein receptor-related protein-1 (LRP1), transferrin receptor (TfR) and low-density lipoprotein receptor (LDLR), respectively, which are overexpressed on BBB/BBTB and then initiate the RMT process, resulting in the enhancement of drug penetration across the barriers and accumulation in the glioma site.\textsuperscript{19} Various ligands have been demonstrated to significantly improve the targeting efficiency of the nanosystems. However, it is difficult to compare the results and determine which ligand is more effective for brain delivery, because the important factors influencing the targeting efficiency, such as particle type, formulation parameters and the ligand density, were quite different in various studies.\textsuperscript{20–22} Therefore, it is essential to evaluate the BBB/BBTB targeting ability of different ligands in the same nanocarrier, which will pick out the most efficient ligand for the establishment of brain targeting drug delivery system.

In this study, we try to develop a glioma-targeted delivery system modified with ligands facilitating across not only BBB but also BBTB, and targeting tumor cells and neovasculature as well. Liposomes were chosen as the delivery vehicle because of the nontoxic and nonimmunogenic characteristics, the high drug loading ability and the possibility to be decorated with different ligands. Six peptide-based ligands with different density were screened and compared the targeting efficiency. We divided these functionalized liposomes into two groups: one for the evaluation of BBB targeting capacity (Angiopep-2-LP, T7-LP, Peptide-22-LP) and the other for the investigation of BBTB targeting ability (c(RGDfK)-LP, Pep-1-LP, D-SP5-LP). On the basis of the comparison results, we selected the optimal ligand from each group and combined them to develop a BBB/BBTB dual-crossing, glioma-targeting liposomal drug delivery system (c(RGDfK)/Pep-22-DOX-LP). To verify further the brain tumor targeting efficacy, in vitro cellular uptake and in vivo imaging study were performed. Eventually, therapeutic effects of different formulations were evaluated on intracranial glioma-bearing mice model, choosing doxorubicin (DOX) as the model drug.
were seeded into a 12-well plate at a density of 5 × 10⁵ cells/well and cultured for 24 h at 37 °C. Different formulations of doxorubicin loaded liposomes were diluted to predetermined concentrations with complete medium and then were added into each well. Forty-eight hours later, the medium was removed and the cells were washed twice with PBS, then 100 μL of MTT solution (0.5 mg/mL) was added. After 4 h incubation at 37 °C, MTT solution was replaced with 150 μL of DMSO, followed by gentle shaking to dissolve the formazan crystals. The absorbance was measured via a microplate reader (spectrum TM 2, BioTek, USA) at 490 nm. The cells without treatment were served as control.

2.8. In Vivo Antiglial Efficacy. In vivo antitumor efficacy was performed in intracranial glioma-bearing mice. Seven days after tumor inoculation, the mice were randomly divided into six groups (n = 6). The mice in blank control group were intravenously injected with saline and the other five treatment groups were administered with free DOX, DOX-LP, c(RGDfK)-DOX-LP, Pep-22-DOX-LP and c-(RGDfK)/Pep-22-DOX-LP via tail vein at day 7, 14 and 21 with 5 mg DOX/kg, respectively. Body weights were monitored every other day. Survival time was recorded and analyzed by GraphPad Prism 5 (GraphPad Software Inc., California, USA).

2.9. Cardiotoxicity Evaluation. Thirty-six intracranial gloma-bearing mice were divided into 6 groups randomly and given injections of saline, free DOX, DOX-LP, c(RGDfK)-DOX-LP, Pep-22-DOX-LP and c-(RGDfK)/Pep-22-DOX-LP, respectively, at a dose of 5 mg DOX/kg at day 7, 14 and 21. Two days after the last administration, the mice were sacrificed. The sections of heart were stained with hematoxylin and eosin to evaluate the cardiotoxicity of DOX.

2.10. Statistical Analysis. All data were analyzed using GraphPad Prism 5 software. Statistical comparisons were performed by one-way ANOVA. Data were expressed as mean ± SD. Statistical significance was defined as *p < 0.05 and extreme significance was defined as **p < 0.01.

3. RESULTS AND DISCUSSION

3.1. Synthesis and Characterization of Functionalized Phospholipid. We conjugated T7, D-SP5, Pep-1 and Angiopep-2 to the distal end of DSPE-PEG₄₀₀-Mal by Michael addition of cysteine residue and maleimide, respectively. Similarly, c(RGDfK) and Peptide-22 were conjugated to the distal end of DSPE-PEG₄₀₀-NHS, respectively, by the reaction of amino groups with active groups NHS. The results of MALDI-TOF mass spectrometry (see Supporting Information) confirmed the successful synthesis of all the six products, with the observed molecular weight around 5099 Da (DSPE-PEG₄₀₀-T7), 5689 Da (DSPE-PEG₄₀₀-D-SP5), 5648 Da (DSPE-PEG₄₀₀-Pep-1), 6797 Da (DSPE-PEG₄₀₀-Angiopep-2), 5180 Da (DSPE-PEG₄₀₀-Peptide-22) and 5096 Da (DSPE-PEG₄₀₀-c(RGDfK)), which were close to the theoretical molecular weights.

3.2. Evaluation of BBB/BTB Targeting Ability of Different Ligands Modified Liposomes. We successfully prepared different ligands modified FAM loaded liposomes with 1%, 3% and 5% DSPE-PEG₄₀₀-ligand. The mean particle sizes of various formulations ranged from 100 to 125 nm, PDI were less than 0.3 and ζ-potentials were between −15 and −30 mV. The BBB targeting ability of Angiopep-2-LP, T7-LP and Pep-22-LP was evaluated on BCECs and then initiated RMT process. However, fluorescence intensity of different ligands decorated liposomes differed from each other. We
speculated that it was correlated with the expression level of corresponding receptor on BECs, the ligand–receptor binding affinity and the cellular uptake mechanism. Herein, the cellular uptake of Pep-22-LP was significantly higher than that of Angiopep-2-LP and T7-LP (p < 0.001), indicating the strongest potency of Pep-22 in transporting liposomes across BBB. Pep-22, generated by phage display biopanning, is a cyclic peptide with properties of good stability, easy modification, and ability to pass the BBB. Peptide-22, generated by phage display biopanning, showed the strongest potency of Peptide-22 in transporting liposomes across BBB. Pep-22-LP displayed a peptide density-dependent manner. With the increased amount of DSPE-PEG3400-Peptide-22 in the lipid constitution, the cellular uptake was improved as well. Compared with 1% modification ratio, the cellular uptake of 3% DSPE-PEG3400-Peptide-22 modified liposomes was significantly higher (p < 0.001). Nevertheless, there was no obvious difference in fluorescence intensity between 3% and 5% modification ratio of Pep-22, which might be due to the receptor saturation. Considering that large amount of DSPE-PEG3400-ligand would influence the integrity and the stability of liposomes in serum, 3% DSPE-PEG3400-Peptide-22 was opted for the following studies.

Similarly, the BBB targeting capacity of c(RGDfK)-LP, DS-PS-LP and Pep-1-LP was evaluated on human umbilical vascular endothelial cells (HUVECs), which was the commonly used model for mimicking BBB. As shown in Figure 1B, compared with unmodified liposomes, the cellular uptake of ligand decorated liposomes was enhanced, among which c(RGDfK)-LP was significantly higher than those of DSP-5-LP and Pep-1-LP (p < 0.001). c(RGDfK) is the specific ligand of integrin αvβ3/αvβ5, which is overexpressed on tumor neovascularature (BBTB). Thus, c(RGDfK) could mediate endocytosis to improve the cellular uptake of neovascular endothelial cells. Analogously, Pep-1 could also increase cellular internalization through interleukin 13 receptor α2 (IL-13Rα2) mediated endocytosis. However, it has been found that the expression level of IL-13Rα2 was lower than that of αvβ3 on HUVECs, DS-PS is a novel retro-inverso analogue of L-SP5. Although the binding mechanism of D-SP5 is not much clear, it has been reported that the targeting efficiency of D-SP5 might be lower on inactivated HUVECs than VEGF stimulated HUVECs. Moreover, from the fluorescence intensity of different density of DSPE-PEG3400-c(RGDfK) modified liposomes, we could find that 3% modification ratio showed significant increase than 1% (p < 0.001), but there was no difference between 3% and 5%. Therefore, 3% DSPE-PEG3400-c(RGDfK) was chosen for the following studies.

3.3. Preparation and Characterization of Dual Ligands-Modified Liposomes. Conventional chemotherapy of glioma has been hampered due to ineffective drug delivery across BBB/BBTB. In most cases, BBB and BBTB exist simultaneously when glioma is diagnosed. Based on the cellular uptake results on BECs and HUVECs (Figure 1AB), 3% c(RGDfK) and 3% Peptide-22 were selected to construct a dual-modified liposomal drug delivery system, c(RGDfK)/Pep-22-DOX-LP, in which Pep-22 and c(RGDfK) could enhance the targeting and permeation of the liposomes of the BBB and the BBTB, respectively. Moreover, both of the ligands could bind with the specific receptors on the tumor cells further and then target the liposomes to glioma, which was of great significance for the specific tumor distribution of the system in the brain.

Appropriate particle size and uniform distribution of nanoparticles were required for BBB/BBTB permeation and brain tumor targeting. The size distribution graph of c(RGDfK)/Pep-22-DOX-LP is shown in Figure 2A. The mean particle sizes, ζ-potentials, DOX encapsulation efficiency and loading efficiency of different formulations are listed in Table 1. For all the liposomes with or without ligand modification, the mean particle sizes ranged from 95 to 105 nm, PDI’s were less than 0.1 and the DOX encapsulation efficiency and loading efficiency were about 95% and 5.5%, respectively. The results indicated that the incorporation of DSPE-PEG3400-c(RGDfK) and DSPE-PEG3400-Peptide-22 did not affect the physical properties of liposomes significantly. The morphology of c(RGDfK)/Pep-22-DOX-LP obtained by TEM revealed that the liposomes were homogeneously spheroids with moderate dispersivity (Figure 2B). No significant change of particle size and PDI of different DOX loaded liposomes was observed in 10% FBS-contained medium over 24 h, suggesting good stability of liposomes in serum-contained medium (see Supporting Information Figure S2). The average size of different formulations maintained approximately 110 nm with a narrow distribution within 14 days, revealing good stability under the storage condition (Data not shown).

3.4. In Vitro DOX Release. The in vitro release of DOX from liposomes was performed in PBS (0.01 M, pH 7.4). As shown in Figure 3, compared with the rapid release of free
DOX, all the DOX loaded liposomes exhibited sustained release behaviors that the cumulative DOX release from liposomes was less than 40% within 48 h and no burst initial release was detected. Similar release patterns were observed among DOX-LP, c(RGDfK)-DOX-LP, Pep-22-DOX-LP and c(RGDfK)/Pep-22-DOX-LP, implying that the modification of ligands did not affect the release behavior of DOX obviously.

### 3.5. Cellular Uptake on U87 cells.

The targeting effect of FAM loaded dual-modified liposomes was investigated by the cellular uptake on U87 cells through fluorescent microscopy (Figure 4A) and flow cytometry (Figure 4B) in vitro. As shown in Figure 4A, compared with unmodified liposomes (LP), c(RGDfK)-LP and Pep-22-LP exhibited stronger green fluorescence signals inside U87 cells, which indicated that both c(RGDfK) and Peptide-22 modification could effectively increase the uptake of liposomes by U87 cells. This result was consistent with previous reports. For instance, Kim et al. demonstrated that the cellular uptake of DOX in cRGD-modified liposomes into U87MG was 8-fold higher than that of unmodified liposomes.\(^{44}\) Zhang et al. found that the fluorescence intensity of C6 cells treated with coumarin-6-labeled peptide-22-decorated nanoparticles were obviously higher than those treated with unmodified NPs.\(^{39}\) Furthermore, the liposomes modified with c(RGDfK) and Peptide-22 simultaneously resulted in the strongest fluorescence signal among four groups, indicating that the synergetic recognition of integrin \(\alpha_\text{v}\beta_3\) and LDLR on U87 cells could significantly improve the internalization of liposomes.

Similar results were obtained from flow cytometry (Figure 4B). Compared with LP, the cellular uptake of liposomes modified with ligands was significantly increased on U87 cells. Moreover, the fluorescence intensity of c(RGDfK)/Pep-22-LP was 7.57- and 1.58-fold higher than those of c(RGDfK)-LP and Pep-22-LP. These data illustrated that dual-modification could not only combine the transcytosis capability of c(RGDfK) and Peptide-22 themselves, but also exerted a synergetic effect on tumor cell uptake. The synergetic effect was realized by expanding the number of the recognized receptors (binding sites) and strengthening the binding affinity via conjugations of two different ligands to the corresponding receptors on the same cell surface, which made the process of internalization more easily.

#### 3.6. In Vivo Imaging Study.

During the occurrence and development of glioma, BBB is gradually impaired and BBTB emerges as the new obstacle for the transport of nanoparticles into tumor.\(^{45}\) Therefore, it is essential to construct a novel system to realize BBB/BBTB dual-crossing and brain tumor targeting simultaneously. In our study, we evaluated the in vivo glioma targeting efficiency of different liposomes containing near-infrared dye DiR in nude mice bearing intracranial glioma. The liposomes were administrated 15 days after the inoculation of tumor cells, at the time the BBB and the BBTB exist simultaneously. A real-time distribution of liposomes was

### Table 1. Characterization of Different Doxorubicin Liposomes

<table>
<thead>
<tr>
<th>liposomes</th>
<th>particle size (nm)</th>
<th>PDI</th>
<th>(\zeta)-potential (mV)</th>
<th>encapsulation efficiency of DOX (%)</th>
<th>loading efficiency of DOX (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX-LP</td>
<td>97.56 ± 0.71</td>
<td>0.058 ± 0.007</td>
<td>-28.57 ± 0.75</td>
<td>97.54 ± 1.13</td>
<td>5.68 ± 0.21</td>
</tr>
<tr>
<td>c(RGDfK)-DOX-LP</td>
<td>99.59 ± 0.87</td>
<td>0.094 ± 0.005</td>
<td>-17.45 ± 1.25</td>
<td>95.26 ± 3.19</td>
<td>5.54 ± 0.32</td>
</tr>
<tr>
<td>Pep-22-DOX-LP</td>
<td>101.33 ± 0.40</td>
<td>0.091 ± 0.008</td>
<td>-16.57 ± 0.61</td>
<td>94.04 ± 0.70</td>
<td>5.16 ± 0.15</td>
</tr>
<tr>
<td>c(RGDfK)/Pep-22-DOX-LP</td>
<td>103.43 ± 0.92</td>
<td>0.098 ± 0.010</td>
<td>-17.40 ± 1.15</td>
<td>96.65 ± 3.13</td>
<td>5.39 ± 0.27</td>
</tr>
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*Data represented mean ± SD (n = 3).*
observed under IVIS Spectrum system. As shown in Figure 5A, the c(RGDfK)/Pep-22-LP group exhibited the strongest fluorescence signal in the glioma region at all-time points when compared with that of unmodified or single-modified groups, indicating that dual-modification of Peptide-22 and c(RGDfK) could significantly facilitate liposomes to traverse the BBB/BBTB and enhance the accumulation of liposomes in the tumor site via the synergetic effect of integrin $\alpha_v\beta_3$ and LDLR mediated endocytosis. The efficient BBB/BBTB transporting capability and precise glioma targeting ability of c(RGDfK)/Pep-22-LP were further verified by $ex$ $vivo$ imaging of the brains 24 h postinjection (Figure 5C). Excised organs were also harvested and imaged (Figure 5B). An Intense fluorescence signal was observed in liver and spleen, corresponding to the main clearance route of liposomes. Moreover, no perspicuously difference in fluorescence signal of peripheric organs was observed among groups, suggesting that c(RGDfK) and Peptide-22 modification would not change the nonspecific distribution behavior of liposomes.

3.7. In Vitro Cytotoxicity Study. In vitro cytotoxicity of free DOX and different DOX loaded liposomes on U87 cells was measured by MTT assay (Figure 6 and Table 2). It could be concluded from the results that both free DOX and DOX loaded liposomes could inhibit the proliferation of U87 cells in a concentration-dependent manner, proving the anticancer effect of DOX on such kind of brain tumors. As shown in Table 2, free DOX (IC$_{50}$ = 0.325 $\mu$g/mL) exhibited stronger inhibitory effect to the proliferation of U87 cells than DOX-LP (IC$_{50}$ = 0.747 $\mu$g/mL), c(RGDfK)-DOX-LP (IC$_{50}$ = 0.504 $\mu$g/mL) and Pep-22-DOX-LP (IC$_{50}$ = 0.389 $\mu$g/mL). This phenomenon was also observed in other reports, which was because free DOX could be quickly transported into cells directly via passive diffusion without the drug release process, while liposomes were internalized through endocytosis which cost time and energy, and then probably underwent a
sustained-release process. Moreover, the PEGylation of liposomes might retard the interaction between liposomes and cells, resulting in the lower cytotoxicity of unmodified or single-modified liposomes. However, c(RGDfK)/Pep-22-DOX-LP displayed the strongest cytostatic activity among the groups, whose IC50 value (IC50 = 0.164 μg/mL) was only half of the free DOX. The effect was ascribed to the tremendously enhanced cellular uptake mediated by the specific binding between c(RGDfK)/αβ3 and Peptide-22/LDLR. Targeting more than one receptors in the same cell surface could increase the number of binding sites, strengthen the binding affinity, and subsequently deliver more anticancer drugs into tumor cells, thus leading to the enhanced cytotoxicity.

3.8. In Vivo Antiglioma Effect. To investigate the in vivo antiglioma effect of functionalized liposomes, intracranial glioma-bearing mice were injected with different DOX formulations on day 7, 14 and 21. Figure 7A shows the body weight changes of mice during the experiment. Compared with other groups, the mice treated with free DOX exhibited a rapidest loss in body weight, which was caused by the severe systemic toxicity of free DOX. The gentler loss of body weight was observed in the groups administrated with DOX-loaded liposomes, indicating the reduction of systemic side effect by liposome encapsulation. As shown in Figure 7B, the modification of c(RGDfK) or Peptide-22 could improve the antglioma effect of DOX-loaded liposomes, the median survival time of mice were notably prolonged. Among all the groups, the mice treated with c(RGDfK)/Pep-22-DOX-LP, i.e., 39.5 days, was significantly longer than that of mice treated with saline (27 days, p < 0.001), free DOX (30.5 days, p < 0.001), DOX-LP (31.5 days, p < 0.001), c(RGDfK)-DOX-LP (34.5 days, p < 0.01) and Pep-22-DOX-LP (36 days, p < 0.05), respectively. The results demonstrated that c(RGDfK) and Peptide-22 dual-modified liposomes exhibited a significant improvement in antglioma effect than those decorated with any of the single ligand, indicating the synergetic effect of two ligands in treating brain tumors. Nanocarries functionalized with targeting ligands were frequently reported for overcoming BBB and targeting the tumor cells in the treatment of glioma.17,47,50 But there were only some reports about modifying ligands to the surface of nanoparticles to realize simultaneous BBB/BBTB crossing and brain tumor targeting. This study was the first one to construct c(RGDfK) and Peptide-22 comodified liposomes loaded with antitumor drugs, which could cross the BBB/BBTB simultaneously and deliver drugs into the tumor.

3.9. Cardiotoxicity Evaluation. DOX, one of the most effective chemotherapeutic agents, is used as a first-line drug in numerous types of cancer. However, it exhibits serious adverse effects, especially lethal cardiotoxicity.51 To evaluate further the safety of different formulations, sections of heart were stained with hematoxylin and eosin. As shown in Figure 8, myocardial hypertrophy was obviously observed in the heart section of free DOX treated group. However, there was no injury found in the samples of all the liposome treated groups, which indicated that the liposomes could decrease the cardiac toxicity of DOX and had good in vivo safety.

4. CONCLUSIONS

In this study, we investigated the BBB targeting capacity of Angiopep-2-LP, T7-LP and Peptide-22-LP and the BBTB targeting ability of c(RGDfK)-LP, D-SP5-LP and Pep-1-LP, respectively. Based on the cellular uptake results on BCECs and HUVECs, Peptide-22 and c(RGDfK) were picked to develop a BBB/BBTB dual-crossing, glioma targeting liposomal drug delivery system, c(RGDfK)/Pep-22-DOX-LP. Compared with the modification of a single peptide, the co-operative effect of integrin αβ3 and LDLR mediated recognition significantly improved the internalization and cytotoxicity of DOX loaded liposomes on U87 cells. In vivo biodistribution and antglioma...
effects further verified that c(RGDfK)/Peptide-22-DOX-LP could efficiently transport across BBB/BBTB and specifically accumulate in the glioma site, and then prolong the survival time of intracranial glioma-bearing mice. Taking these together, we claimed c(RGDfK) and Peptide-22 dual-modified liposomes could serve as a promising platform for glioma chemotherapy in different progression stages.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.6b15831.

MALDI-TOF mass spectrum of DSPE-PEG\textsubscript{3400}-T7, DSPE-PEG\textsubscript{3400}-D-SP5, DSPE-PEG\textsubscript{3400}-Pep-1, DSPE-PEG\textsubscript{3400}-Angiopep-2, DSPE-PEG\textsubscript{3400}-Peptide-22 and DSPE-PEG\textsubscript{3400}-c(RGDfK); serum stability of different DOX-loaded liposomes over 24 h (PDF)

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Notes

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