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Biotin and glucose dual-targeting, ligand-modified liposomes promote breast tumor-specific drug delivery

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ABSTRACT

Breast cancer is the second leading cause of cancer-related deaths in women. Ligand-modified liposomes are used for breast tumor-specific drug delivery to improve the efficacy and reduce the side effects of chemotherapy; however, only a few liposomes with high targeting efficiency have been developed because the mono-targeting, ligand-modified liposomes are generally unable to deliver an adequate therapeutic dose. In this study, we designed biotin-glucose branched ligand-modified, dual-targeting liposomes (Bio-Glu-Lip) and evaluated their potential as a targeted chemotherapy delivery system *in vitro* and *in vivo*. When compared with the non-targeting liposome (Lip), Bio-Lip, and Glu-Lip, Bio-Glu-Lip had the highest cell uptake in 4T1 cells (3.00-fold, 1.60-fold, and 1.95-fold higher, respectively) and in MCF-7 cells (2.63-fold, 1.63-fold, and 1.85-fold higher, respectively). The subsequent cytotoxicity and *in vivo* assays further supported the dual-targeting liposome is a promising drug delivery carrier for the treatment of breast cancer.

Breast cancer is the second leading cause of cancer-related deaths worldwide and the most common cancer in women.[1] WHO reported that 17.5 million breast cancer-related deaths are expected to occur each year by 2050.[2] Treatments for breast cancer include surgery, radiotherapy, immunotherapy, and chemotherapy, with greater focus on survival rate improvement by chemotherapy.[1] However, the clinical applications of chemotherapy are limited, mainly due to its high toxicity and non-selectivity.[3] A new drug delivery system with tumor-specific targeting has been gaining interest in recent research. The liposome-based drug delivery systems are of particular interest due to their biocompatibility, non-toxic and biodegradable features, particularly with breast tumor-specific liposome as the preferred vector for chemotherapy for the treatment of breast cancer.[4–6]

Cancer cells need more essential vitamins and glucose to grow and replicate rapidly.[7–9] Biotin (vitamin B_7, vitamin H) is a water-soluble vitamin that plays an essential role in cell proliferation, signal transduction, and many other cellular functions.[7,10] The sodium-dependent multivitamin transporter (SMVT) is a key transporter for biotin found on the cell surface and over-expressed in breast cancer cells such as 4T1 and MCF-7, and the biotinylated complexes and liposomes have been developed to improve the efficacy of cancer diagnosis and therapy.[11,12] The glucose transporter 1 ($GLUT_1$) is known to be overexpressed in various types of cancer cells due to the Warburg effect, an insufficient glycolysis pathway to generate adenosine triphosphate,[9,13] making glucose a suitable targeting ligand for drug delivery.[14,15]

Although the mono-targeting ligand can promote the binding and internalization of liposomes into the cancer cells, only a few liposomes with high targeting efficiency have been developed so far, because the traditional mono-branched ligand modified liposomes generally fail to deliver an adequate therapeutic payload.[16,17] We previously designed and developed the double-branched biotin modified liposome with increased breast cancer cell targeting and cell uptake.[18] The use of the dual-targeting ligands (biotin and glucose) can potentially promote the SMVT and $GLUT_1$-mediated recognition and transportation. Therefore, in this present study, we designed and synthesized a series of biotin- and/or glucose-modified cholesterols, prepared the liposomes with different targeting ligands, and assessed the tumor targeting and *in vivo* distribution of these liposomes. We concluded that the dual-targeting liposomes modified with biotin-glucose cholesterol is a breast tumor-specific targeting drug delivery system.

The biotin and glucose co-modified cholesterol (Fig. 1) was synthesized by the synthetic routes (Scheme 1). Briefly, *N*-Boc-*N′*-Fmoc-*L*-

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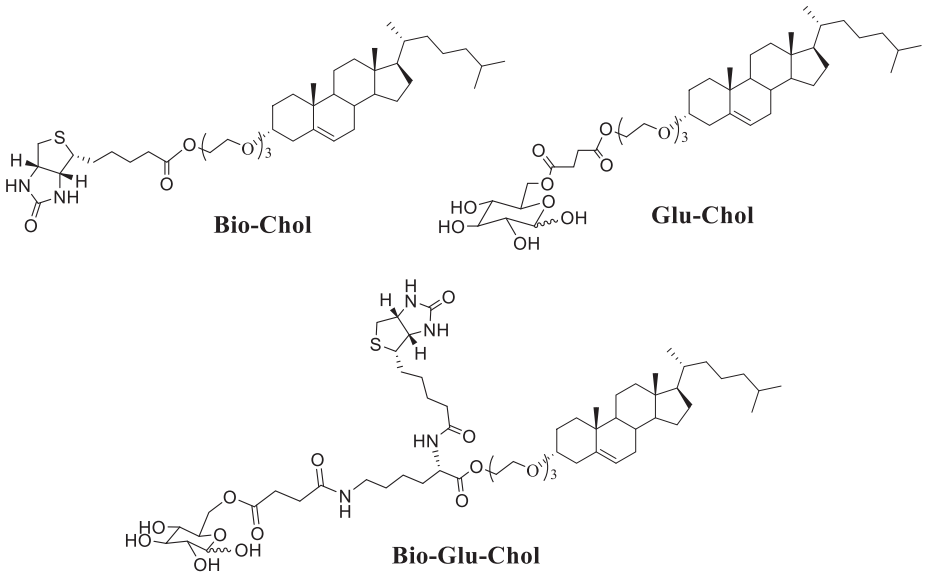
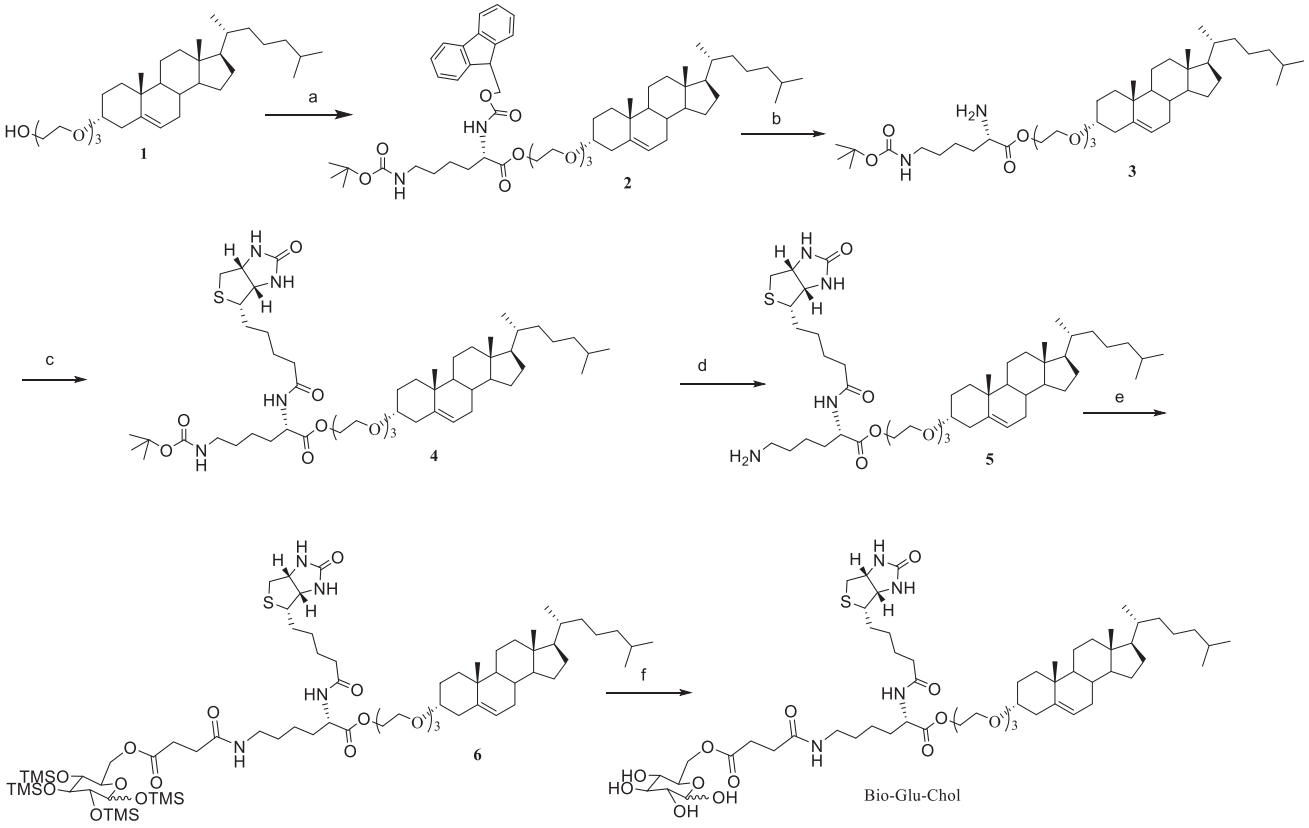


Fig. 1. Structures of biotin- and/or glucose-modified cholesterols.



Scheme 1. The synthetic route of ligand Bio-Glu-Chol. (a) *N*-Boc-*N'*-Fmoc-*L*-Lysine, DCC, DMAP, r.t., 4 h, 93%; (b) DBU, DCM, r.t., 0.5 h, 93%; (c) Biotin, HATU, DIPEA, DCM, r.t., 4 h, 90%; (d) CF_3COOH, DCM, r.t., 0.5 h, 80%; (e) compound **13**, IBCF, NMM, DCM, r.t., 6 h, 53%; (f) CF_3COOH, DCM, r.t., 0.5 h, 46%.

lysine was used as the branched bridge to conjugate biotin, glucose derivatives, and cholesterol derivatives. First, a glucose derivative containing 1, 2, 3, 4-OH protected by trimethylsilyl (TMS) groups and 6-OH extended by succinic acid (compound **13**, Scheme S1) was generated by a multi-step reaction.[19] Second, the cholesterol derivative (compound **1**) was condensed with *N*-Boc-*N'*-Fmoc-*L*-lysine, followed by a selective removal of the Fmoc protecting group to free an amine group to derive compound **3**, on the basis of our previous study.[20] Then, compound **3** was linked with biotin under HATU/DIPEA to obtain compound **4**. Similarly, the Boc protection of compound **4** was removed under CF_3COOH and connected with the glucose derivative (compound **13**; Scheme S1) in the presence of IBCF/NMM to derive compound **6**. Finally, after deprotecting the TMS groups, the desired Bio-Glu-Chol was obtained. The ligands Bio-Chol and Glu-Chol were synthesized according to our previous studies.[18,19] All the titled compounds and important intermediates were characterized by ^{1}H nuclear magnetic resonance (^{1}H-NMR) or mass spectrometry (MS).

Liposomes were prepared by the thin film hydration method

Table 1
Particle sizes, zeta potential, and the PTX encapsulation efficiency of different liposomes (mean ± SD, n = 5 for each liposome).[a]

Liposomes	Size (nm)	PDI	Zeta potential (mV)	EE (%)
PTX-Lip	105.5 ± 4.7	0.203 ± 0.024	−3.07 ± 0.14	86.65 ± 1.53
PTX-Bio-Lip	107.9 ± 3.7	0.207 ± 0.026	−4.91 ± 0.19	89.29 ± 2.63
PTX-Glu-Lip	109.9 ± 3.3	0.205 ± 0.037	−4.66 ± 0.11	82.96 ± 1.99
PTX-Bio-Glu-Lip	114.2 ± 4.5	0.202 ± 0.023	−4.82 ± 0.16	89.66 ± 2.63

[a] Measurements were performed in PBS buffer unless otherwise noted.

described previously.[18] The average particle sizes of all liposomes were approximately 110 nm, and the values of polymer dispersity index were close to 0.20 (Table 1), which indicated the liposomes exhibited a uniform spherical shape. The encapsulation efficiencies of PTX in each type of liposomes were all greater than 82%, and the liposomes were all negatively charged with the zeta potentials from −3.0 mV to −4.9 mV. These results indicated the liposomes met the requirements for *in vitro* and *in vivo* assays.

The stability of liposomes in the biological conditions is important for the therapeutic agent. To examine their stability, the transmittance of these liposomes was assessed in the presence of 50% FBS. All of the transmittance were above 90% and remained similar after 48 h incubation in 50% FBS (Fig. 2A), indicating these liposomes were able to prevent their interactions with serum proteins and likely have long blood half-lives *in vivo*.[21]

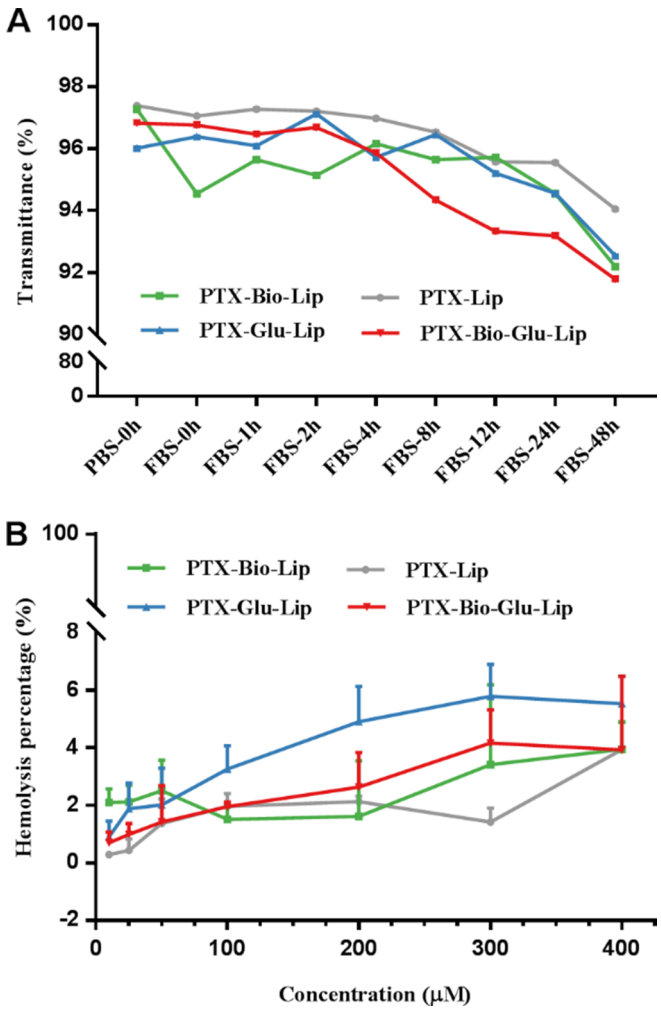


Fig. 2. Transmittance and hemolysis of various modified liposomes. (A) Transmittance of liposomes in 50% FBS (n = 3 for each liposome, mean ± SD). (B) Hemolysis percentage of liposomes (n = 3 for each liposome, mean ± SD).

The hemocompatibility testing was conducted to evaluate the safety of these ligand-modified liposomes during the blood circulation. As shown in Fig. 2B, the hemoglobin release of all liposomes did not increase significantly and were < 7%, even when the concentration of phospholipids was increased to 400 μM, indicating the non-toxicity of the lipid material.[22]

The release property is crucial for a liposome delivery system. In this study, the PTX release property of the various liposomes was evaluated in PBS containing 0.1% Tween 80. The PTX-loaded liposomes exhibited sustained release behaviors, with the cumulative PTX released from liposomes < 70% after 48 h (Fig. 3A). No significant difference on the PTX release property was observed among PTX-Lip, PTX-Bio-Lip, PTX-Glu-Lip, and PTX-Bio-Glu-Lip, and none of these PTX-loaded liposomes displayed initial burst release patterns (Fig. 3A). In contrast, the free PTX was released rapidly, with more than 86% of the drug released into the media within 24 h (Fig. 3A).

The MTT assay was performed to assess the cytotoxicity of PTX-loaded liposomes and blank liposomes in 4T1 and MCF-7 breast cancer cell lines (method is further described in Supplementary Material). As shown in Fig. 3B and C, the free PTX had higher cytotoxicity than PTX-loaded liposomes, consistent with the release property of free PTX and PTX-loaded liposomes (Fig. 3A), because the free drugs could be transported into the cells directly by passive diffusion, without a drug release process. Furthermore, the PTX-Bio-Glu-Lip had the highest inhibition among the four PTX-loaded liposomes (Fig. 3B and C). All four blank liposomes also exhibited no significant cytotoxicity (Figs. S1A and B), demonstrating the safety of the liposome drug delivery system for *in vivo* use.

Cell uptake of CFPE-labeled Lip, Bio-Lip, Glu-Lip, and Bio-Glu-Lip was analyzed to determine whether the dual-targeting liposomes, Bio-Glu-Lip, was able to deliver more drugs into 4T1 and MCF-7 cells compared with the mono-ligand modified or non-targeting liposomes. The uptake of ligand-modified liposomes was shown to be significantly higher than that of the conventional liposomes (Lip). Furthermore, the relative fluorescence of Bio-Lip, Glu-Lip, and Bio-Glu-Lip in 4T1 cells was 1.87-fold, 1.53-fold, and 3.00-fold higher compared with the non-targeting liposome (Lip group), respectively. The cell uptake of Bio-Glu-Lip was the highest when compared with Lip, Bio-Lip, and Glu-Lip (3.00-fold, 1.60-fold, and 1.95-fold higher, respectively) in 4T1 cells (Fig. 4). Similar results were also shown for the uptake of Bio-Glu-Lip in MCF-7 cells, which was 2.63-fold, 1.63-fold, and 1.85-fold higher when compared with Lip, Bio-Lip, and Glu-Lip, respectively (Fig 4). These results indicated the dual-targeting ligands promoted the cell uptake of liposomes in the 4T1 and MCF-7 breast cancer cell lines.

To further investigate the penetrability of Bio-Glu-Lip, the uptake mechanism was evaluated by incubating the 4T1 and MCF-7 cells with endocytosis inhibitors and analyzing the inhibition rate. Chlorpromazine, filipin and amiloride were selected to block the clathrin-mediated endocytosis, caveolae-mediated endocytosis, and micropinocytosis, respectively.[23–25] The temperature of 4 °C and NaN_3 (oxidative phosphorylation inhibitor) were selected to assess the effect of energy.[26] The pre-incubation of both the 4T1 cells and MCF-7 cells with free biotin or free glucose inhibited the uptake of Bio-Glu-Lip competitively (Fig. 5), which confirmed the biotin or glucose ligand

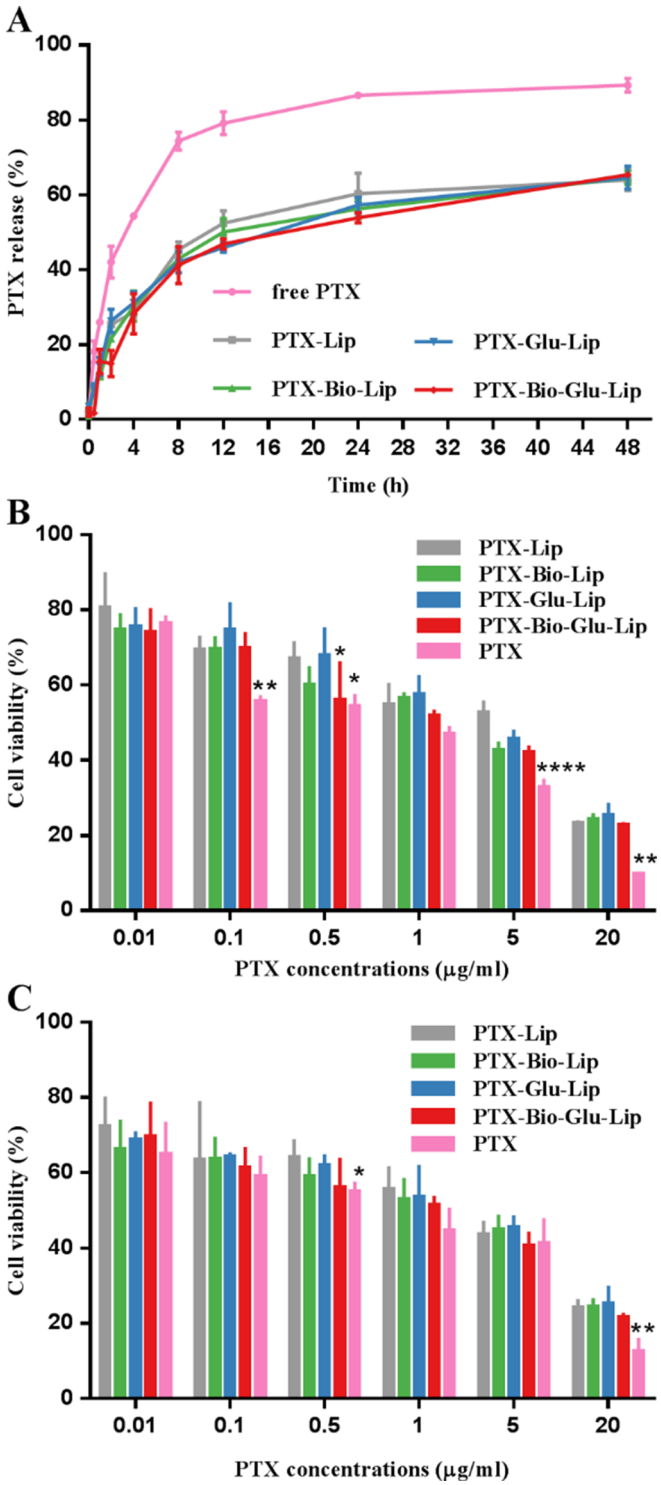


Fig. 3. The PTX release profiles and cytotoxicity of free PTX and PTX-loaded liposomes (mean ± SD, n = 3 for each). (A) The PTX release profiles of free PTX, PTX-Lip, PTX-Bio-Lip, PTX-Glu-Lip, and PTX-Bio-Glu-Lip in PBS (pH 7.4) containing 0.1% Tween 80 over 48 h (n = 3 for each, mean ± SD). The cytotoxicity in 4T1 cells (B) and MCF-7 cells (C) after treatment with various concentrations of PTX-Lip, PTX-Bio-Lip, PTX-Glu-Lip, PTX-Bio-Glu-Lip, and free PTX for 24 h (n = 3 for each, mean ± SD). *, **, and *** represent $p < 0.05$, $p < 0.01$, and $p < 0.001$ versus PTX-Lip group, respectively.

increased the cell internalization by specifically binding to SMVT and $GLUT_1$, respectively. The endocytosis inhibitors chlorpromazine, filipin, and amiloride were shown to inhibit the cell internalization (Fig. 5). Low temperature (4 °C) also had a strong inhibitory effect on cellular

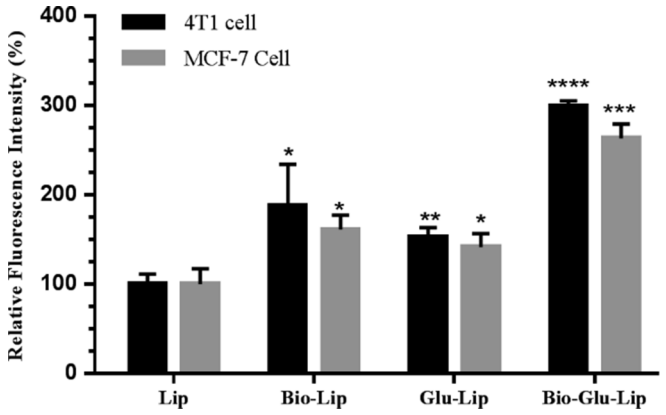


Fig. 4. Cellular uptake of CFPE-labeled liposomes in 4T1 and MCF-7 cell lines. The relative fluorescence intensity of different liposomes was measured by a flow cytometer and compared with the Lip group (mean ± SD, n = 3 each). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ versus control (Lip group).

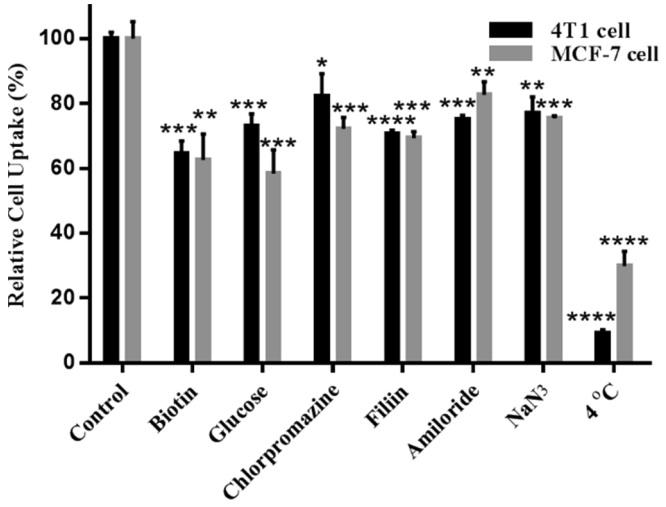


Fig. 5. Uptake mechanism assessment of Bio-Glu-Lip in 4T1 cells or MCF-7 cells after treatment with different inhibition conditions, as determined by flow cytometer (mean ± SD, n = 3 each). *, **, and *** represent $p < 0.05$, $p < 0.01$, and $p < 0.001$ versus control, respectively.

uptake (approximately 90% and 70% in 4T1 and MCF-7 cells, respectively; Fig. 5), suggesting the energy-dependent properties of endocytosis. These data indicate the recognition of Bio-Glu-Lip by the biotin transporter SMVT and the glucose transporter $GLUT_1$ on the outer membrane of cells via the residues on the liposome surface, which leads to the energy-dependent internalization of Bio-Glu-Lip via a synthetic endocytic pathway, including clathrin-mediated, caveolae-mediated, and micropinocytosis-mediated endocytosis.

To analyze the *in vivo* targeting delivery and the accumulation in the breast tumor site, DID-loaded liposomes were injected to 4T1 breast tumor-bearing BALB/c mice *via* the caudal vein. The DID fluorescence signal (E_x 620 nm, E_m 670 nm) was imaged by the IVIS Lumina Series III imaging system (Perkin-Elmer, USA) at 2 h, 6 h, 12 h, 16 h, and 24 h after injection. Female BALB/c mice (5–6 weeks, 18–22 g) were purchased from Dashuo Biotechnology Co., Ltd. (Chengdu, China), and all animal experiments were performed in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978), and in accordance with the experimental guidelines of the Animal Experimentation Ethics Committee of Sichuan University. As shown in Fig. 6A, DID-Bio-Glu-Lip had the highest fluorescence intensity *in vivo* and the highest

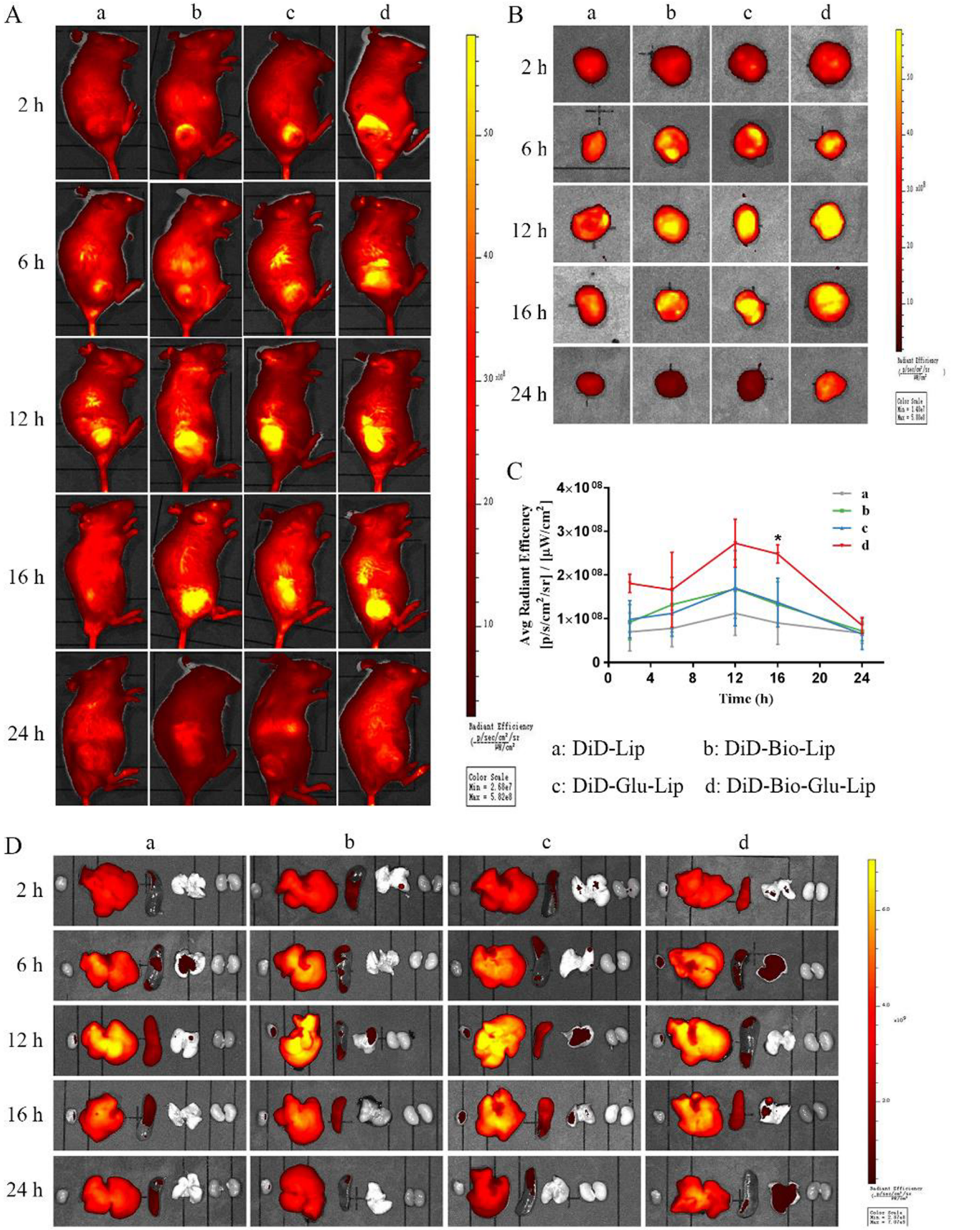


Fig. 6. Biodistribution of liposomes. (A) *In vivo* imaging of 4T1 breast cancer-bearing mice at different time points after the systemic administration of DID-loaded liposomes. (B) *Ex vivo* imaging of tumor tissues at different time points. (C) Quantification of fluorescence intensity in *ex vivo* tumor tissues, shown by the average radiant efficiency in the tissues versus time. * represent $p < 0.05$. (D) *Ex vivo* imaging of isolated organs (from left to right: heart, liver, spleen, lungs, and kidneys) at different time points after the systemic administration of DID-loaded liposomes.

accumulation in tumor region from 2 h to 16 h after a systemic administration. The fluorescence signal of all liposomes was time dependent, showing weak intensity at tumor site at 2 h after injection, the maximum intensity at 12 h post injection, and decreasing intensity from 16 h to 24 h after injection (Fig. 6). The mice were sacrificed at predetermined time points, and the tumor tissues, hearts, livers, spleens, lungs, and kidneys were harvested and imaged. *Ex vivo* images of the tumor tissues and other organs were shown in Fig. 6B and D, respectively. The fluorescence intensity semi-quantification in *ex vivo* tumor tissues was shown in Fig. 6C, with the highest intensity by DID-Bio-Glu-Lip, 2.23-fold higher than that of Lip at 12 h after injection. From 2 h to 24 h after injection, strong fluorescence signal of different liposomes was shown in the liver and in part of the spleen, with no apparent signal in hearts, lungs, and kidneys. These *in vivo* imaging results suggested that biotin and glucose on the surface of liposomes equip the liposomes with the breast tumor dual-targeting delivery ability. These findings were consistent with those of *in vitro* cellular uptake. As the compounds encapsulated within liposomes would not change the targeting properties of liposomes, it is reasonably to believe that the targeting and time related accumulation in the breast tumor of other compounds loaded in Bio-Glu-Lip are similar to DID loaded in the same liposome vectors. Furthermore, we had analyzed the pharmacokinetic parameters of free PTX, PTX-Lip, PTX-Glu-Lip, and PTX-Bio-Lip in our previous studies[18,19]; the plasma PTX concentration–time profiles were shown and the pharmacokinetic parameters of PTX from different formulations were summarized previously. The results showed that the ligands modified liposomes could significantly enhance the concentration–time profile ($AUC_{0\text{-}t}$) of paclitaxel within 24 h, the $AUC_{0\text{-}t}$ of PTX-Glu-Lip was 2.2-fold that of free PTX, 1.2-fold that of PTX-Lip[19]; the $AUC_{0\text{-}t}$ of PTX-Bio-Lip was 3.0-fold that of free PTX, 1.7-fold that of PTX-Lip[18]; and the ligands modified liposomes could significantly extend the elimination half-life ($t_{1/2}$) of PTX compared with the free PTX and PTX-Lip. Thus, it is reasonable to believe that the PTX-Bio-Glu-Lip has qualified pharmacokinetic parameters, so we had not repeated a pharmacokinetic experiment in this work.

In summary, to improve the targeting delivery of liposomes to breast cancer cells, we have developed a dual-targeting drug delivery system with the use of biotin and glucose as the targeting ligands. We have designed and synthesized a novel biotin-glucose cholesterol as a bidentate ligand and prepared a series of PTX-loaded liposomes. Bio-Glu-Lip was recognized by the biotin transporter SMVT and the glucose transporter $GLUT_1$ on the cell membrane via the residues on the liposome surface and was energy-dependently internalized *via* a synthetic endocytic pathway, including clathrin-mediated, caveolae-mediated, and micropinocytosis-mediated endocytosis. In addition, the dual-targeting ligands on one liposome particle exhibited positive cooperativity to promote the recognition and internalization of the liposomes into breast cancer cells, which is why Bio-Glu-Lip had higher cytotoxicity than other liposomes. Bio-Glu-Lip also significantly increased the accumulation of liposomes in the breast tumor sites, with some distribution in the liver and part of the spleen and limited distribution in the hearts, lungs, and kidneys. Collectively, these results indicate that the biotin and glucose dual-targeting ligands can modify the liposome to significantly enhance its tumor-targeting ability. This supports the use of a modified cholesterol with multi-branched ligands for the design of tumor-specific diagnostic reagents and an active targeting drug delivery system for cancer treatment.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127151.

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