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# Efficient Delivery of Payload into Tumor Cells in a Controlled Manner by TAT and Thiolytic Cleavable PEG Co-Modified Liposomes

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**Abstract:** Recently, PEGylation has been extensively employed to increase the circulation time of liposomes and enhance their accumulation in tumor tissue via the enhanced permeability and retention (EPR) effect; however, poly(ethylene glycol) (PEG) is unfavorable for the uptake of liposomes by tumor cells because of its steric hindrance. In this study, thiolytic cleavable PEG modified liposomes were used to solve this dilemma. Before arrival at the tumor tissue, PEG presents on the surface of liposomes, which is useful for passive accumulation in tumor tissue. Upon reaching the tumor tissues, the PEG chain could be removed by a safe cleaving reagent L-cysteine (L-Cys), and thus, the steric hindrance of PEG could be overcome conveniently. To further improve the uptake of liposomes, a "functional molecule" cell-penetrating peptide TAT was attached to the distal end of a shorter PEG spacer anchored to the surface of the liposomes, which could be shielded by cleavable PEG during circulation; upon arriving at tumor tissue, PEG was removed and thus the "functional molecule" TAT was exposed, and then TAT could mediate the uptake of the liposomes with high efficiency. In this study, thiolytic cleavable PEG was synthesized via a disulfide bridge, DOPE-$PEG_{1600}$-TAT was synthesized by sulfhydryl-maleimide reaction, and then Rh-PE labeled liposomes composed of 2% DOPE-$PEG_{1600}$-TAT and various amounts of cleavable $PEG_{5000}$ (2%, 4%, and 8%) were prepared, with particle size around 100 nm and slightly negative charge. These liposomes showed good stability in the presence of 10% serum. Their uptake by tumor cells HepG2 *in vitro* was assessed qualitatively and quantitatively. Liposomes modified with 2% DOPE-$PEG_{1600}$-TAT and 8% DOPE-S-S-$mPEG_{5000}$ were regarded as the optimal formulation. In this preparation, nearly no uptake could be observed before addition of L-Cys, which meant undesired uptake during circulation could be avoided, while the uptake upon addition of L-Cys was 4 times as high as that in the absence of L-Cys. For the uptake *in vivo*, calcein loaded and Rh-PE labeled 8% cleavable PEG + 2% TAT modified liposomes were injected intratumorally into H22 tumor bearing mice. Confocal laser scanning microscopy (CLSM) showed that the uptake of 8% cleavable PEG + 2% TAT modified liposomes was much higher than that of 8% noncleavable PEG + 2% TAT modified liposomes in the presence of L-Cys. Thus, tumor targeted delivery could be achieved efficiently by the liposomal drug delivery system developed here in a controlled manner.

**Keywords:** Liposome; thiolytic cleavable PEG; TAT; EPR effect; uptake

## 1. Introduction

An ideal tumor targeted drug delivery system should not only accumulate drugs in tumor tissue after systemic administration selectively[1] but also deliver the drugs into tumor cells efficiently, therefore minimizing the toxic side effects of antitumor drugs by reduced delivery to nontarget organs or tissue while the therapeutic effects are maximized.[2] Many drug carriers have been developed to achieve targeted delivery to tumor tissues, and liposomes are one of the hottest

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drug carriers that have been extensively studied because of their natural chemical composition, good protection for encapsulated drugs, controllable pharmacokinetics, and other desirable properties by various modification.[3,4]

PEGylation has been widely used to extend the circulation time of liposomes, and thus, they have more chance to accumulate in tumor via the EPR effect.[5−7] Nevertheless, PEGylation seriously hinders the uptake of the liposomes by tumor cells after they arrive at tumor tissues.[1] To overcome the dilemma, one ideal strategy is to keep the PEG on the surface of liposomes during circulation but dissociate PEG from liposomes after accumulation in tumor tissue; thus, the hindrance effect of PEG on liposome uptake can be avoided without sacrifice of long circulating properties. Several ways have been reported for the cleavage of PEG extracellularly after arrival at tumor tissue, including the conjugation of PEG onto the surface of liposomes via pH sensitive,[8,9] MMP sensitive,[10−13] esterase sensitive,[14] or reductive potential sensitive[2,3] chemical bonds; among these approaches, reductive potential sensitive chemical bonds such as disulfide are easy to construct and can be cleaved precisely by a safe cleaving agent L-cysteine (L-Cys). It is reported that the concentration of reductive chemicals in the blood is rather low, 10 $\mu$M, which is not high enough to cleave PEG anchored to the liposomes by disulfide.[2] After accumulation of liposomes in tumor tissue, PEG can be cleaved by exogenous L-Cys. To further enhance the uptake of liposomes, a "functional molecule" TAT peptide (AYGRKKRRQRRR) is attached to the distal end of a shorter PEG spacer ($PEG_{1600}$) anchored to the surface of liposomes. TAT peptide belongs to the family of cell penetrating peptide (CPP),[15] which is the basic region of the transactivating transcriptional activator protein (TAT) from HIV-1, and it was proposed as a potent CPP, capable of transporting different molecules and even 200 nm liposomes into various cell lines.[16−19] TAT can be shielded by cleavable PEG during circulation and exposed to function after dissociation of PEG from the liposome surface, and then it can mediate uptake of liposomes by tumor cells with high efficiency.

Moreover, TAT is a nonspecific functional molecule, which can penetrate any cells upon encountering then; this drawback limited the use of TAT in systemic administration. Thanks to the cleavable PEG with disulfide as a linker, TAT can be shielded by the steric hindrance of PEG during circulation, cationic TAT could not interact with the anionic charges of the cells of the blood vessels anymore, and therefore nonspecific binding can be avoided.[20] As TAT is shielded by PEG during circulation, it is not so easy for liposomes to bind with opsonin and other proteins in blood, which is beneficial for RES evasion and stability of liposomes. The combination of cleavable PEG and "functional

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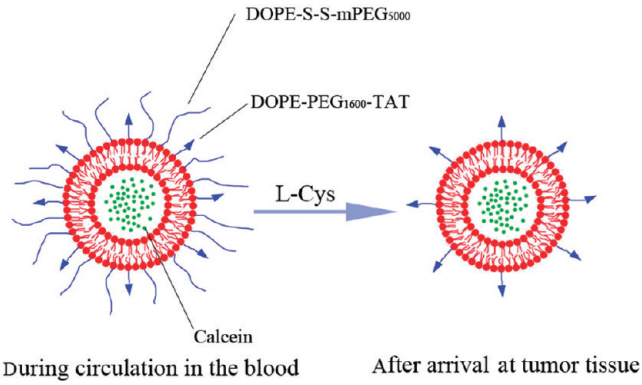


***Figure 1.*** Schematic illustration of TAT and cleavable PEG co-modified liposomal drug delivery system.

molecule" TAT on liposomes provides an indirect but more specific delivery system which works in a controlled manner.

In this study, a liposomal drug delivery system simultaneously modified with TAT and thiolytic cleavable PEG was developed (see Figure 1), whose properties were also assessed *in vitro* on HepG2 cells both qualitively and quantitatively. For the *in vivo* study, we mainly focus on the fate of liposomes after arrival at tumor tissue. Thus, calcein loaded and Rh-PE labeled liposomes were injected intratumorly to H22 tumor bearing mice to simulate the accumulation in tumor tissue, and the uptake results are presented qualitatively via confocal laser scanning microscopy.

## 2. Materials and Methods

**2.1. Materials.** 1,2-Dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rh-PE) and DOPE were purchased from Avanti lipids. NHS-$PEG_{1600}$-Mal, $mPEG_{5000}$-SH, and $mPEG_{5000}$-NHS were all purchased from JENKEM TECHNOLOGY (Beijing, China). TAT peptide with terminal cysteine (Cys-AYGRKKRRQRRR) was synthesized according to the standard solid phase peptide synthesis by Chengdu KaiJie Biopharmaceutical Co., Ltd. (Chengdu, China). *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) was purchased from Sigma (China, mainland). Other chemicals and reagents were of analytical grade and obtained commercially.

**2.2. Synthesis of DOPE-$PEG_{1600}$-TAT.** DOPE-$PEG_{1600}$-TAT was synthesized as described previously[8,21−23] with some modification (see Figure 2). Brifely, 22 mg of DOPE was reacted with 20 mg of NHS-$PEG_{1600}$-Mal in dry dichloromethane (DCM) at room temperature under argon in the presence of 6 $\mu$L of triethylamine for about 5 h. After thin-layer chromatography (TLC) (DCM/MeOH/$H_2O$ = 3:0.5:0.001) showed the disappearance of NHS-$PEG_{1600}$-Mal, the reaction mixture was filtered and the filtrate was evaporated under vacuum. Excess DOPE was removed by adding 5 mL of acetonitrile to precipitate it, and the mixture was kept at 4 °C overnight. Then it was centrifuged at 5000 rpm for 10 min. After that, the supernatant was collected and evaporated again under vacuum. The obtained DOPE-$PEG_{1600}$-Mal was used for the next reaction.

DOPE-$PEG_{1600}$-Mal (20 mg) and Cys-TAT (22 mg) were reacted in 9 mL of the mixture of $CHCl_3$/MeOH (V:$V$ = 2:1) with gentle stirring at room temperature for about 30 h. After TLC (DCM/MeOH/$H_2O$ = 3:0.75:0.12) showed the disappearance of DOPE-$PEG_{1600}$-Mal, the mixture was evaporated under vacuum, the slight excess of Cys-TAT was removed by adding a small volume of $CHCl_3$, the insoluble material was filtered, and the supernatant was evaporated again under vacuum and stored at −20 °C until use.

**2.3. Synthesis of DOPE-S-S-$mPEG_{5000}$.** DOPE-S-S-$mPEG_{5000}$ was synthesized as described previously[2,3] with some modification (see Figure 3). Briefly, 63 mg of DOPE and 25 mg of SPDP were dissolved in 4 mL of dry DCM, and then 60 $\mu$L of triethylamine was added. The mixture was reacted under argon in dark for about 5 h, and the reaction progress was momitored by TLC under UV. After TLC (DCM/MeOH/ $H_2O$ = 65:25:4) showed the disappearance of the starting material and the appearance of a faster running spot with UV absorbance, DCM in the reaction mixture was removed by rotary evaporation. The product was purified on the silica gel column, which was eluted by the following DCM/MeOH mixtures: 4:0.25, 4:0.5, and 4:1 (V/V). The obtained DOPE-PDP was then used for the next reaction.

DOPE-PDP (61 mg) and $mPEG_{5000}$-SH (180 mg) were dissolved in 6 mL of dry DCM, and then 5 $\mu$L of triethylamine was added. The mixture was allowed to react in dark under argon at room temperature for about 72 h. After TLC (DCM/MeOH = 2:0.3) showed the reaction was completed, the mixture was filtered and the supernatant was evaporated under vacuum. The residue was recrystallized twice from diethyl ether and then purified on silica column, which was eluted by the following DCM/MeOH mixtures: 50:0.5, 50:1, 50:1.5, 50:2, 50:15 (V/V). The chemical structure was verified by $^1$H NMR.

**2.4. Synthesis of DOPE-$mPEG_{5000}$.** DOPE (23 mg) and $mPEG_{5000}$-NHS (100 mg) were dissolved in 4 mL of dry DCM, and then 5.6 $\mu$L of triethylamine was added. The mixture was allowed to react in dark under argon at room temperature for about 8 h. After TLC (eluent DCM/MeOH/$H_2O$ = 5:0.5:0.03) showed the disappearance of $mPEG_{5000}$-NHS, the mixture was filtered and the filtrate was dried by rotary evaporation. Then 14 mL acetonitrile was added, the obtained suspension was centrifuged at 10000 rpm for 5 min, and the supernatant was condensed. The chemical structure was verified by $^1$H NMR.

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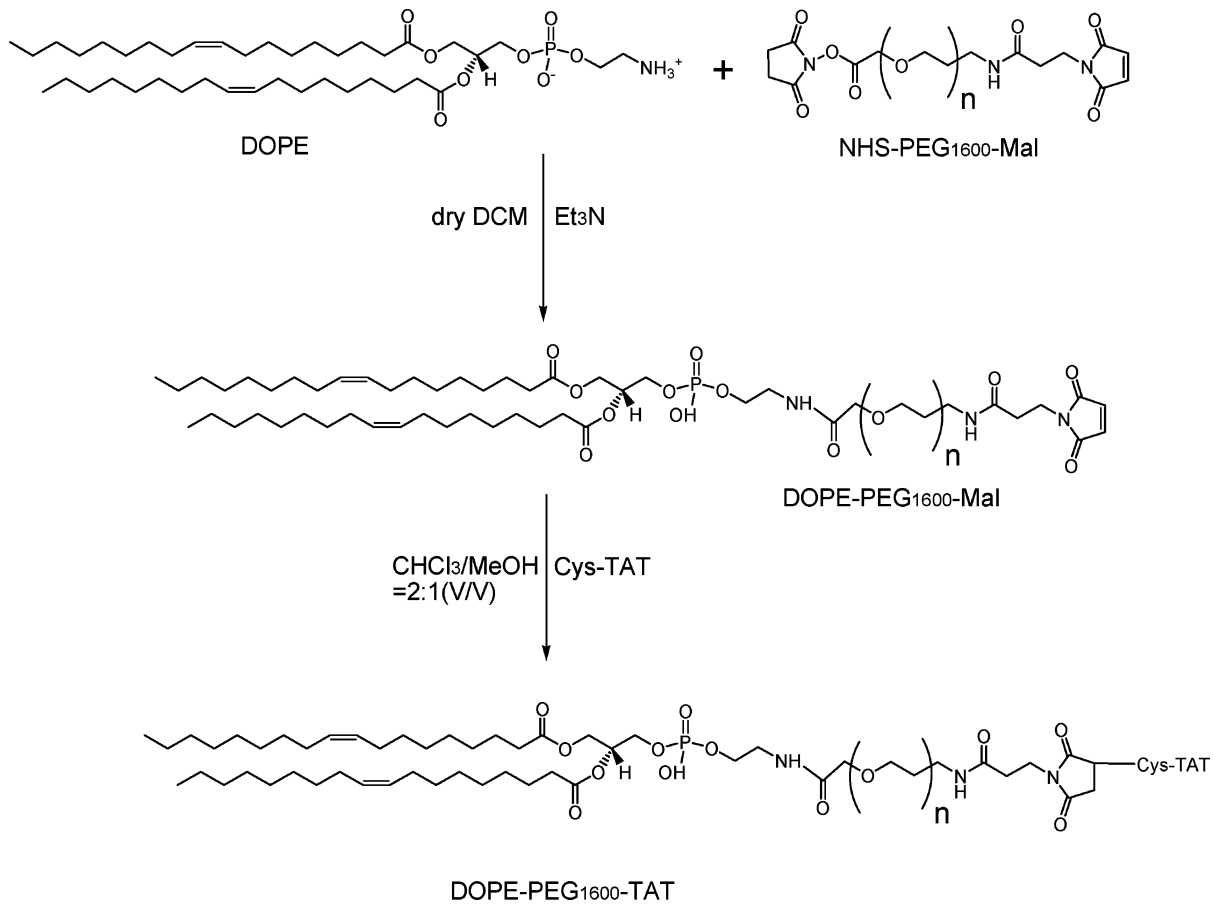


***Figure 2.*** Schematic of the synthesis of DOPE-$PEG_{1600}$-TAT. The synthesis was confirmed by $^1H$ NMR, mass spectroscopy, and thin-layer chromatography.

**2.5. Cleavage of DOPE-S-S-$mPEG_{5000}$ *in Vitro*.** The cleavage assay of DOPE-S-S-$mPEG_{5000}$ was conducted as described previously.[3] Briefly, 5 mg of DOPE-S-S-$mPEG_{5000}$ was dissolved in $CHCl_3$ and then dried to a thin film by rotary evaporation. Then 1 mL of phosphate-buffered saline (PBS, pH 7.4) was added and the mixture dispersed in a bath-type sonicator for 1 min. It was further homogenized by using a probe sonicator at 60 W for 75 s. The obtained micelle was divided into two parts: one was added PBS containing 30 mM L-Cys, the other was added blank PBS and then incubated at 37 °C. At different time points, the degree of the cleavage was monitored by TLC.

**2.6. Preparation of Liposomes.** Liposomes were prepared by the thin film hydration methods as described previously.[2] Various amounts of SPC/Cho/DOPE-$PEG_{1600}$-TAT/DOPE-S-S-$mPEG_{5000}$/DOPE-$PEG_{5000}$/Rh-PE (see Table 1) were dissolved in chloroform, and chloroform was then removed by rotary evaporation. The obtained thin film was kept in vacuum for over 6 h to completely remove the residual organic solvent. The thin film was hydrated in Hepes buffer (20 mM Hepes/140 mM NaCl, pH 7.4) in a bath-type sonicator for about 2 min. Then it was further intermittently sonicated by using a probe sonicator at 60 W for 75 s. To prepare calcein loaded and Rh-PE labeled liposomes, 80 mM calcein in Hepes buffer (pH7.4) was used to hydrate the thin film.[24−28] After being sonicated in a bath-type sonicator and with a probe sonicator as described above, it was frozen in liquid nitrogen (−196 °C) for 3 min and then thawed in a 45 °C water bath. After five cycles of freezing and thawing, the liposome suspension was extruded through 400, 200, and 100 nm pore size polycarbonate membranes sequentially using an AVESTIN extruder. The unencapsulated dye was removed by passing through a Sephadex G50 (Pharmacia) column, equilibrated with Hepes buffer (20 mM Hepes/140 mM NaCl, pH7.4). To verify the amount of calcein encap-

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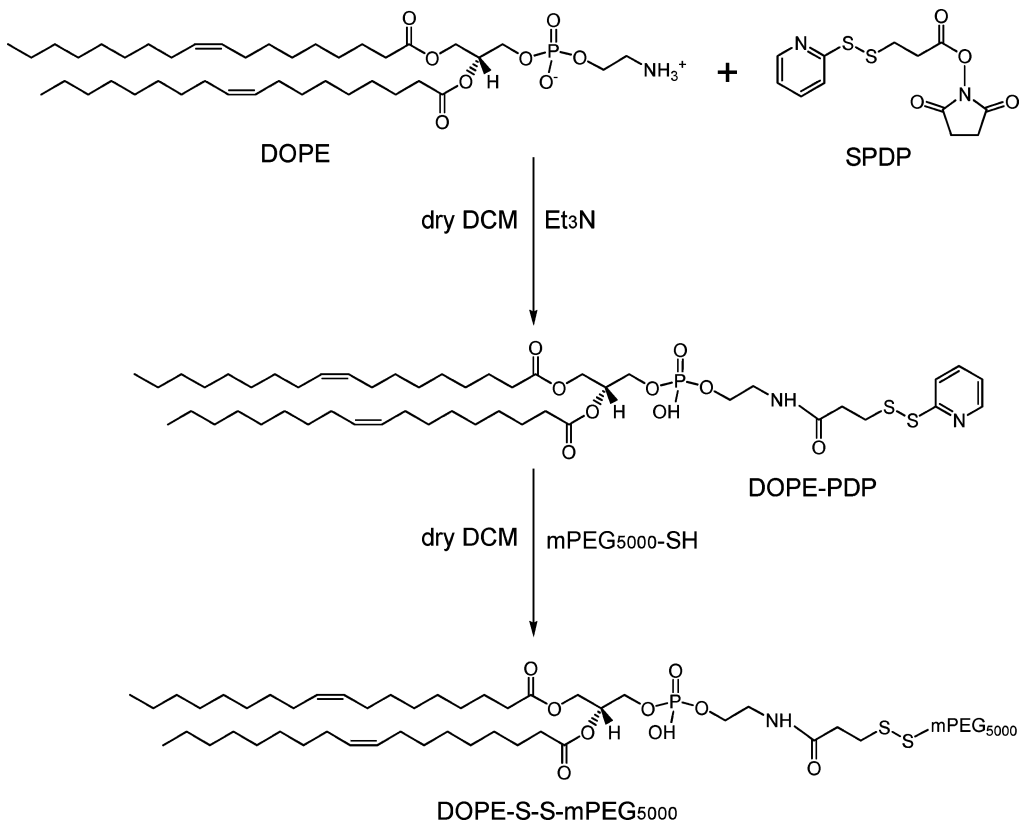


***Figure 3.*** Schematic of the synthesis of DOPE-S-S-mPEG$_{5000}$. The product was confirmed by $^1$H NMR, and thin-layer chromatography.

***Table 1.*** Various Formulations of Liposomes

| types of liposomes | SPC | Cho | DOPE-PEG$_{1600}$-TAT | DOPE-S-S-mPEG$_{5000}$ | DOPE-mPEG$_{5000}$ | Rh-PE | calcein |
|---|---|---|---|---|---|---|---|
| 2% cleavable PEG + 2% TAT | 6.60 | 3.00 | 0.20 | 0.20 | 0.00 | 0.03 | 0 |
| 4% cleavable PEG + 2% TAT | 6.40 | 3.00 | 0.20 | 0.40 | 0.00 | 0.03 | 0 |
| 8% cleavable PEG + 2% TAT | 6.00 | 3.00 | 0.20 | 0.80 | 0.00 | 0.03 | 0 |
| 8% cleavable PEG | 6.20 | 3.00 | 0.00 | 0.80 | 0.00 | 0.03 | 0 |
| 8% noncleavable PEG + 2% TAT | 6.00 | 3.00 | 0.20 | 0.00 | 0.80 | 0.03 | 0 |
| 8% cleavable PEG + 2% TAT + calcein | 6.20 | 3.00 | 0.20 | 0.80 | 0.00 | 0.03 | 80 mM |
| 8% noncleavable PEG + 2% TAT + calcein | 6.00 | 3.00 | 0.20 | 0.00 | 0.80 | 0.03 | 80 mM |

sulated into the liposomes, calcein loaded liposomes were added with 1% Triton-X 100 and then the fluorescence intensity was determined at Ex = 490, Em = 520 on a spectrofluorimeter (RF-5301 fluorospectrophotometry, Shimadzu, Japan).

**2.7. Size and Zeta Potential Measurements.** The size and zeta potential of the liposomes were determined by using a Malvern Zetasizer Nano ZS90 instrument (Malvern instruments Ltd., U.K.). Prior to measurement, 100 $\mu$L of the sample (lipid concentration 2.1 mg/mL) was diluted by using the same buffer to 1 mL.

**2.8. Stability of Liposome in the Presence of Fetal Bovine Serum.** Rh-PE labeled liposomes were prepared as described above. A total of 50 $\mu$L of different formulations of liposomes was added to 1 mL of culture medium containing 10% fetal bovine serum (FBS) and incubated at 37 °C, 5% $CO_2$ at different time points. The size change was determined by using a Malvern Zetasizer Nano ZS90 instrument (Malvern instruments Ltd., U.K.) (Figure 4).

**2.9. Cell Culture.** HepG2 cells were grown in RPMI-1640 medium (GIBCO) containg 10% FBS, 100 $\mu$g/mL streptomycin, and 100U/mL penicillin. The cells were maintained at 37 °C in a humidified incubator with 5% $CO_2$.

**2.10. Effects of PEG Density and L-Cys Concentration on the Cellular Binding and Uptake of Rh-PE Labeled Liposomes by HepG2 Cells.** HepG2 cells were plated on 24-well culture plates at a density of $1 \times 10^5$cells/well. After 24 h cultivation, different formulations of liposomes were added to the plates at 50 $\mu$L/well. Each formulation of liposome was treated with different concentrations of L-cys in Hepes buffer (pH 7.4), and the final concentration of L-cys in each well was 0, 5, 10, and 20

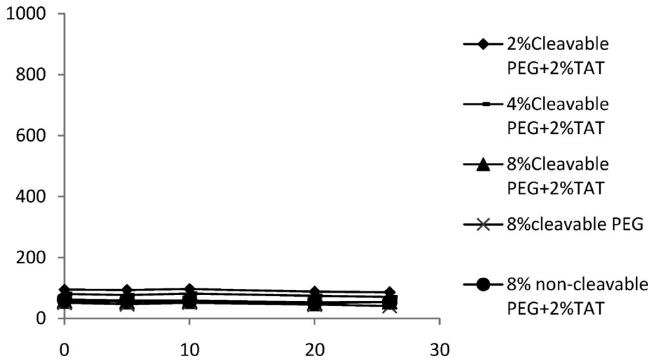


***Figure 4.*** Stability of different formulations of liposomes in the presence of 10% FBS. The particle size of each formulation was measured at each time point by using a Malvern Zetasizer Nano ZS90 instrument.

mM, respectively. After incubation at 37 °C under 5% $CO_2$ for 4 h, the cells were washed three times with cold PBS and observed under fluorescence microscopy (Axiovert 40 CFL, Carl Zeiss Shanghai Co. Ltd.). Then the cells were incubated with 500 $\mu$L/well 1% Triton-X 100 at 4 °C over 2 h, and 100 $\mu$L of the lysate was used for determining the total protein of cells using the BCA assay kit (Pierce).[29] The rest of the lysate was added with 2.4 mL of 1% Triton-X 100 and used to determine the cell-associated fluorescence at Ex = 560 nm, Em = 578 nm on a spectrofluorimeter (RF-5301 fluorospectrophotometry, Shimadzu, Japan). The results were expressed as fluorescence/mg of protein.

**2.11. Confocal Laser Scanning Microscopy (CLSM).** In order to determine whether the liposomal delivery system can be used to deliver drugs into tumor cells efficiently in a controlled manner, a membrane impermeable and water-soluble dye, calcein,[30] was used as a model compound for hydrophilic drugs to be encapsulated into the liposomes. Calcein loaded and Rh-PE labeled liposomes were prepared as described above. HepG2 cells were seeded on coverslips at a density of $2.5 \times 10^5$ cells/well in 6-well plates. After 24 h cultivation, cells were incubated with calcein loaded and Rh-PE labeled liposomes at 37 °C under 5% $CO_2$ for 4 h. Then the cells were washed three times with cold PBS (pH 7.4) and fixed using 4% paraformaldehyde. Coverslips were mounted cell-side down with slides and viewed using a Leica TCS SP5 AOBS confocal microscopy system (Leica, Germany).

**2.12. Tumor Models.** Kuming mice weighing 20−25 g were purchased from Experiment Animal Center of Sichuan University (P.R.C.). All the animal experiments adhered to the principles of care and use of laboratory animals and were approved by the Experiment Animal Administrative Committee of Sichuan University.

Mice bearing H22 tumor cells were established as described previously[31] with some modification. Briefly, H22 (conserved by our laboratory) was taken out from liquid nitrogen and subjected to a water bath at 37 °C. The cells were mixed with 3 mL of prewarmed 1640 culture medium and centrifuged for 5 min at 1500 rpm. The cell pellet was then washed once with sterile PBS and suspended in PBS. The female Kuming mice were inoculated with 0.2 mL ($1 \times 10^6$ cells/mL) of H22 cell suspension intraperitoneally to form ascites. A few days later, ascites were drawn and diluted with PBS and then reinjected intraperitoneally to new Kuming mice. Subcultivating in this way for about four times, ascites were drawn and diluted with sterile PBS and then injected subcutaneously into the left armpit of the female Kuming mice. Tumors were allowed to grow to an average size of about 10 mm in diameter before treatment.

**2.13. Tumor Cell Uptake *in Vivo*.** When the average diameter of the tumors reached about 10 mm, cleavable PEG and noncleavable PEG modified liposomes containing TAT with calcein encapsulated as a membrane impermeable hydrophilic drug were administrated via intratumor injection, and Hepes buffer containg L-cys (pH 7.4) was administrated intratumorly too. Mice were killed 4 h later by cervical dislocation, and excised tumors were put in liquid nitrogen immediately. Then tumors were frozen sectioned (4 $\mu$m in thickness). Sections were stained with DAPI (2 $\mu$g/mL) for 5 min, washed three times with cold PBS, and then observed via CLSM.

**2.14. Statistical Analysis.** Student's $t$ test was used to compare differences, and a $p$ value < 0.001 was considered to be indicative of statistical significance.

## 3. Results

**3.1. Synthesis of DOPE-$PEG_{1600}$-TAT/DOPE-S-S-$mPEG_{5000}$/DOPE-$mPEG_{5000}$.** To construct the liposomal drug delivery system, we first conjugated TAT to the distal end of $PEG_{1600}$ anchored to DOPE. TLC showed the purity of DOPE-$PEG_{1600}$-TAT as >75%, and TOF MS ES+ confirmed the formation of DOPE-$PEG_{1600}$-TAT ($M_w$ observed = 3923, $M_w$ calculated = 3941). Thiolytic cleavable PEG was synthesized as described above via a disulfide bridge. TLC showed the purity of DOPE-S-S-$mPEG_{5000}$ as >95%, and the chemical structure was verified by $^1$H NMR: $\delta$ 0.87 (t, $CH_3$, 6H), 3.37 (s, 3H, $CH_3O-$), 3.65 (s, PEG backbone 498H), 5.4 (m, 4H, double bond of DOPE). Noncleavable PEG DOPE-$mPEG_{5000}$ with a purity of >95% was confirmed by $^1$H NMR too (see the Supporting Information).

**3.2. Cleavage of DOPE-S-S-$mPEG_{5000}$ *in Vitro*.** The cleavage of DOPE-S-S-$mPEG_{5000}$ was confirmed by TLC. The DOPE-S-S-$mPEG_{5000}$ spot on the TLC plate decomposed when PBS with L-Cys was added, but the DOPE-S-S-$mPEG_{5000}$ spot remained the same when PBS without L-Cys

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***Table 2.*** Size and Zeta Potential of Different Formulations of Liposomes[a]

| type of liposomes | size (nm) | PDI | zeta potential (mV) |
|---|---|---|---|
| 2% cleavable PEG + 2% TAT | 129.8 ± 20.9 | 0.223 ± 0.052 | −2.2 ± 2.0 |
| 4% cleavable PEG + 2% TAT | 113.2 ± 11.5 | 0.226 ± 0.036 | −0.2 ± 1.9 |
| 8% cleavable PEG + 2% TAT | 83.1 ± 7.0 | 0.266 ± 0.050 | −1.4 ± 4.2 |
| 8% cleavable PEG | 77.1 ± 3.2 | 0.232 ± 0.042 | −1.1 ± 4.1 |
| 8% noncleavable PEG + 2% TAT | 85.4 ± 5.4 | 0.186 ± 0.012 | −1.3 ± 4.2 |
| 8% cleavable PEG + 2% TAT + calcein | 103.1 ± 11.3 | 0.169 ± 0.018 | −4.9 ± 1.3 |
| 8% noncleavable PEG + 2% TAT + calcein | 100.9 ± 18.2 | 0.187 ± 0.013 | −4.8 ± 3.7 |

[a] The data indicate the mean ± SD.

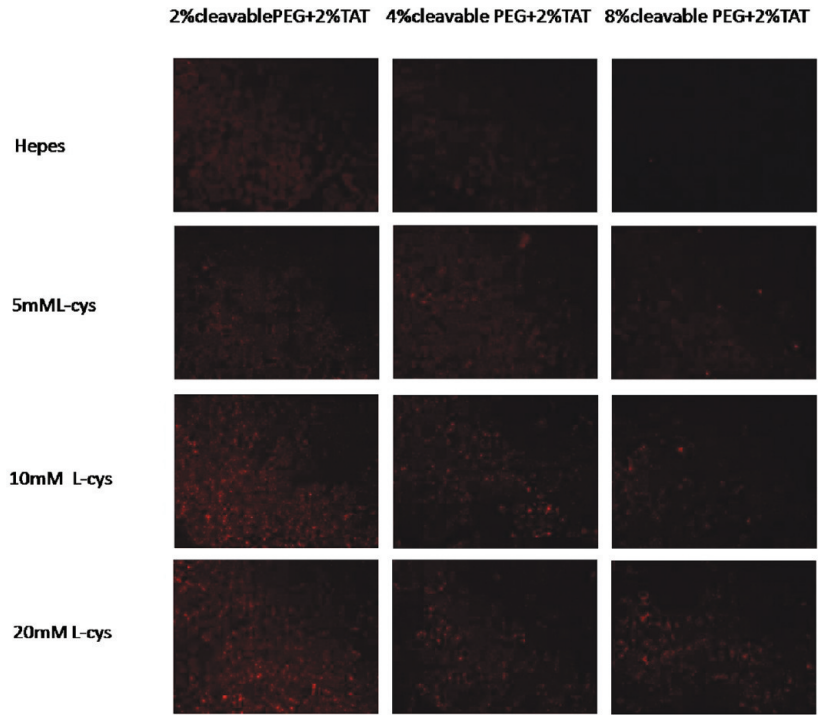


***Figure 5.*** Qualitative observation of the effect of different amounts of PEG (from left to right 2%, 4%, 8%) and different concentrations of L-Cys (from the first line 0 mM to the last line 20 mM) on the uptake of Rh-PE labeled liposomes containing 2% TAT by HepG2 cells under fluorescence microscopy.

was added (see the Supporting Information), which clearly showed the cleavage of DOPE-S-S-mPEG$_{5000}$ in the presence of L-Cys.

**3.3. Characteristics of Liposomes.** Liposome size measurements showed that the liposomes were mainly about 100 nm; the size decreased with the increase of amount of PEG in the liposome formulation. The zeta potential was slightly negative (see Table 2). The amount of calcein encapsulated into the 8% cleavable PEG + 2% TAT or 8% noncleavable PEG + 2% TAT liposomes was comparable (data not shown), which was determined by the fluorescence intensity determined via spectrofluorimeter after adding 1% Triton-X 100.

All formulations of liposomes were quite stable in the presence of 10% FBS even after 24 h (see Figure 1), which may be attributed to slightly negative charge and the steric stabilization of PEG.

**3.4. Effect of PEG Density and L-Cys Concentration on the Cellular Association and Uptake of Rh-PE Labeled Liposomes by HepG2 Cells.** The cell association and uptake of 2% TAT with various amounts of cleavable PEG DOPE-S-S-mPEG$_{5000}$ modified liposomes were assessed on HepG2 cells. On one hand, when the density of PEG in liposomes increased from 2% to 8%, the cellular association and uptake decreased drastically (Figure 6), which was caused by the steric hindrance effect of PEG, as is reported elsewhere.[11] When the PEG density reached 8%, the cellular uptake was significantly inhibited and nearly no liposomes could be seen in HepG2 cells (Figure 5), which showed at this PEG density (8%) that the "functional molecule" TAT could be highly shielded.

On the other hand, at the same PEG density, the cellular association and uptake increased significantly with the increase of concentration of L-cys from 0 to 20 mM, which might be attributed to the increased degree of cleavage of PEG from the surface of liposomes, and thus the steric hindrance effect of PEG was weakened and the "functional molecule" TAT was exposed, allowing the liposomes to enter tumor cells with high efficiency. Even when the density of PEG reached 8%, L-cys could still cleave the disulfide bridge

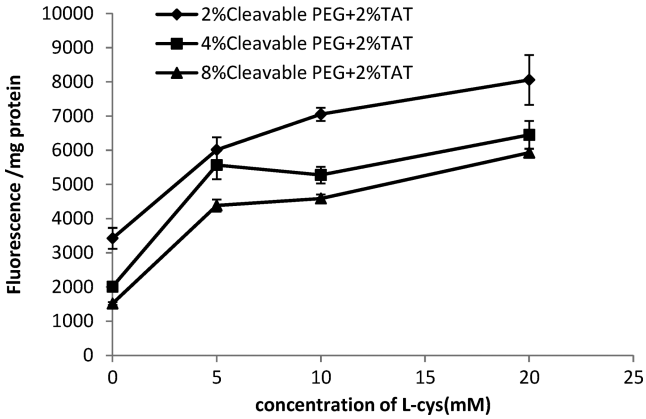


***Figure 6.*** Quantitave determination of uptake after incubation of 2% cleavable PEG + 2% TAT, 4% cleavable PEG + 2% TAT, and 8% cleavable PEG + 2% TAT modified liposomes with HepG2 cells for 4 h in the presence of different concentrations (0, 5, 10, 20 mM) of L-Cys ($n = 3$, mean ± SD).

and expose "functional molecule" TAT to help liposomes enter tumor cells efficiently. In other tumor cells such as A2780, similar results were observed (see the Supporting Information).

The cellular uptake of various amounts (2%, 4%, 8%) of cleavable PEG + 2% TAT modified liposomes before and after the addition of L-Cys was also compared with the control of 2% TAT functionalized liposomes to learn the extent of TAT being exposed indirectly. There was no difference between the uptake efficiency of 2% TAT liposomes in the absence or presence of L-Cys; to make the comparison easier, the uptake efficiency of 2% TAT liposomes (without L-Cys) was set at 100%, and the uptake efficiency of others was divided by that of 2% TAT liposomes (without L-Cys). Thus, the percentage obtained could reflect the extent of TAT being exposed. The results showed that L-Cys had great effect on the uptake of cleavable PEG and TAT co-modified liposomes, and that upon the addition of L-Cys the percentage of TAT exposed was about 99.74%, 75.16%, and 58.88% for 2%, 4%, and 8% DOPE-S-S-mPEG$_{5000}$, respectively, in terms of the cellular uptake (see Figure 7).

The 8% cleavable PEG + 2% TAT modified liposomes were used for the following experiments because this formulation exhibited the least cellular uptake in the absence of L-Cys, but the uptake upon addition of L-Cys was 4 times as high as that in the absence of L-Cys ($p < 0.001$) (see Figure 8). Thus, the behavior of liposomes could be easily controlled by L-Cys.

To further confirm whether the 8% cleavable PEG + 2% TAT modified liposomes in the presence of 20 mM exogenous L-Cys could enter tumor cells efficiently was caused simultaneously by the cleavage of PEG and the exposure of "functional molecule" TAT, we prepared 8% cleavable PEG without TAT and 8% noncleavable PEG + 2% TAT modified liposomes as control. As a result, 8% cleavable PEG without TAT modified liposomes exhibited only about one

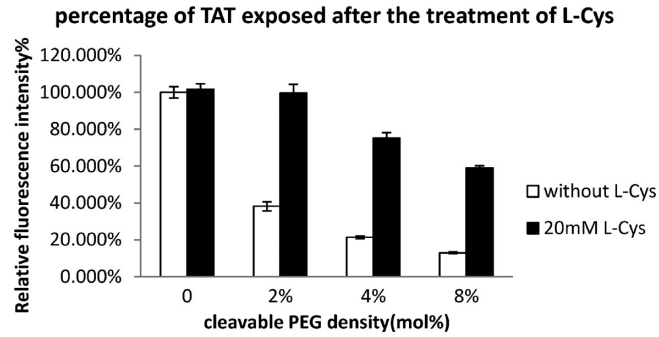


***Figure 7.*** Cellular uptake of 2% TAT + 2%, 4%, and 8% cleavable PEG modified liposome by HepG2 before and after the treatment of L-Cys; 2% TAT modified liposome was used as control. Relative fluorescence intensity against 2% TAT liposomes (without L-Cys) is shown with SD bars. ($n = 3$, mean ± SD).

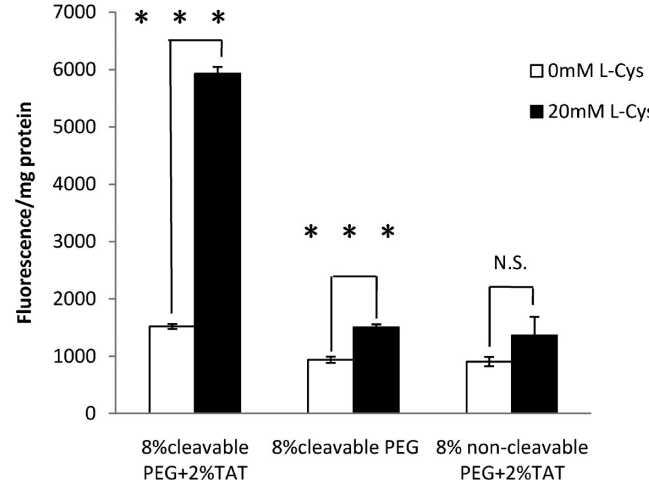


***Figure 8.*** Quantitative determination of uptake after incubation of 8% cleavable PEG + 2% TAT, 8% cleavable PEG without 2% TAT, and 8% noncleavable PEG + 2% TAT modified liposomes with HepG2 cells for 4 h in the absence or presence of 20 mM L-Cys ($n = 3$, mean ± SD). * * *: Statistically significant difference ($P < 0.001$). N.S.: No significant difference.

time higher ($p < 0.001$) uptake efficiency after addition of L-Cys, as compared with the uptake in the absence of L-Cys, far less than that of 8% cleavable PEG + 2% TAT modified liposomes upon addition of L-Cys, which was 4 times as high as that in the absence of L-Cys. While for 8% noncleavable PEG + 2% TAT modified liposomes no significant difference could be seen after addition of 20 mM L-Cys (see Figure 8), which clearly showed that the cleavage of PEG and exposure of TAT occurred simultaneously upon addition of L-cys, TAT could increase the uptake of liposomes efficiently.

**3.5. Confocal Laser Scanning Microscopy.** In order to ascertain whether the liposomal drug delivery system developed here could deliver drugs into tumor cells, calcein loaded and Rh-PE labeled 8% cleavable PEG + 2% TAT modified liposomes were incubated with HepG2 cells, using 8% noncleavable PEG + 2% TAT modified liposomes as

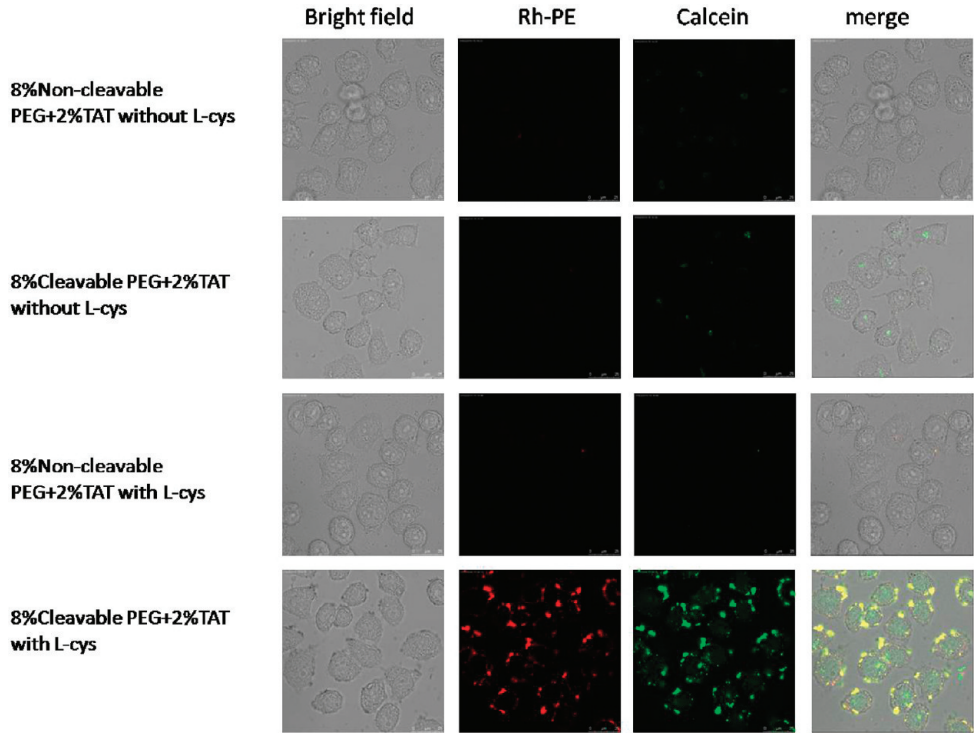


***Figure 9.*** Incubation of calcein loaded and Rh-PE labeled 8% noncleavable PEG + 2% TAT or 8% cleavable PEG + 2% TAT modified liposomes with HepG2 cells for 4 h in the absence or presence of 20 mM L-Cys. In each set from left to right: bright field, Rh-PE, calcein, merge.

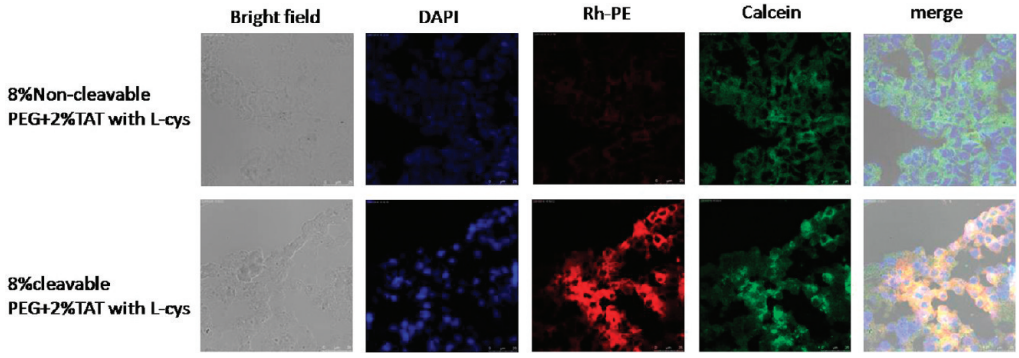


***Figure 10.*** CLSM images of the H22 tumor sections from the tumors injected with calcein loaded and Rh-PE labeled 8% noncleavable PEG + 2% TAT or 8% cleavable PEG + 2% TAT modified liposomes. In each set from left to right: bright field, DAPI stained nucleus, Rh-PE, calcein, merge.

control. The encapsulation efficiency of calcein in each formulation was comparable as described above.

As a result, almost no green fluorescence or red fluorescence (the fluorescence of calcein and Rh-PE, respectively) could be seen when 8% cleavable PEG + 2% TAT modified liposomes or 8% noncleavable PEG + 2% TAT modified liposomes were incubated with HepG2 cells in the absence of L-Cys (see Figure 9). There was still no fluorescence when 8% noncleavable PEG + 2% TAT modified liposomes were added to HepG2 cells in the presence of L-Cys. However, when 8% cleavable PEG + 2% TAT modified liposomes were incubated with HepG2 cells in the presence of L-Cys, both green and red fluorescence could be seen, and green fluorescence was mainly colocalized with red fluorescence, which showed calcein was mainly delivered by the liposomes into the tumor cells with addition of L-Cys.

**3.6. Tumor Cell Uptake *in Vivo*.** To assess the behavior of the liposomal drug delivery system *in vivo*, calcein loaded and Rh-PE labeled 8% cleavable PEG + 2% TAT or 8% noncleavable PEG + 2% TAT modified liposomes were administrated via intratumor injection to allow liposomes to accumulate in the tumor tissue in advance, with exogenous L-Cys administrated intratumorally too. The uptake of liposomes by the tumor cells *in vivo* was shown in Figure 10. The 8% Noncleavable PEG + 2% TAT modified liposomes showed weak red or green fluorescence in the tumor section even in the presence of L-Cys, while 8% cleavable PEG + 2% TAT liposomes showed strong red and

green fluorescence in the tumor section and they were colocalized in the tumor cells, indicating calcein was delivered into the tumor cells efficiently by liposomes after addition of L-Cys, which was consistent with the *in vitro* result. This difference between two liposome formulations could be explained by the steric hindrance effect of PEG on the "functional molecule" TAT. The cleavage of PEG from the liposomes could be achieved in 8% cleavable PEG + 2% TAT liposomes but not in 8% noncleavable PEG + 2% TAT modified liposomes by using L-Cys, and thus, the "functional molecule" TAT could be exposed and mediated efficient uptake of liposomes and payload.

## 4. Discussion

This study aimed to develop a liposomal drug delivery system that can highly accumulate encapsulated drugs in tumor tissue and then deliver drugs into tumor cells efficiently. It is reported the biodistribution of liposomes *in vivo* mainly depends on the EPR effect.[32] PEGylation forms an aqueous layer on the surface of liposomes, which can reduce the protein interactions with the surface of liposomes and prevent their binding to opsonins, and thus, *in vivo* circulation time of liposomes can be increased, so PEGylation provides more chance for liposomes to accumulate in tumor tissue. However, the PEGylated surface can prevent not only the interaction between the liposomes and opsonins but also that between the liposomes and cell surface.[1] For example, the stealth liposomes (Doxil) show little cellular uptake when incubated with tumor cells for 24 h. So PEG is a double-edged sword, which is favorable for accumulation of liposomes in tumor tissue but unfavorable for the uptake of liposomes by tumor cells. To solve the dilemma of PEG, a thiolytic cleavable PEG here was used to modify liposomes, which remained present on the surface of liposomes during circulation while dissociating from the surface after the arrival at tumor tissue by exogenous L-Cys. To further improve the uptake efficiency, a "functional molecule" TAT peptide was installed on the surface of the liposomes too. To make sure TAT can only work after removal of cleavable PEG, TAT was attached to the distal end of a shorter PEG spacer ($PEG_{1600}$) than cleavable PEG ($PEG_{5000}$), which could be shielded before arrival at tumor tissue.[33]

All the functional materials used were presynthesized, and thus, the liposomal system could be constructed in one step via the thin film hydration methods conveniently as described before. The TAT peptide was terminated with cysteine in order to introduce free sulfhydryl (−SH), and then TAT could be conjugated to DOPE-$PEG_{1600}$-Mal via the sulfhydryl-maleimide reaction, which allowed TAT to be conjugated at a specific site (−SH). Once TAT was conjugated to DOPE-$PEG_{1600}$-Mal, the stable thiol ether was formed and the free sulfhydryl which could cleave disulfide bonds no longer existed, so the cleavable DOPE-S-S-$mPEG_{5000}$ would not be affected.

Exogenous L-Cys mediated cleavage of DOPE-S-S-$mPEG_{5000}$ used for the preparation of liposomes is the primary step for success of the liposomal drug delivery system developed here. We first confirmed the cleavage of DOPE-S-S-$mPEG_{5000}$ *in vitro* by TLC assay, which clearly showed the cleavage of DOPE-S-S-$mPEG_{5000}$ in the presence of L-Cys, while it remained unchanged in the absence of L-Cys (see the Supporting Information).

The PEG density which provides maximum shielding effect on TAT and the optimal concentration of L-Cys used for the cleavage of DOPE-S-S-$mPEG_{5000}$ were confirmed by the uptake efficiency *in vitro* both qualitatively and quantitatively. Liposomes with various amounts of cleavable PEG (2%, 4%, 8% DOPE-S-S-$mPEG_{5000}$) + 2% TAT were prepared. After incubation with HepG2 cells for 4 h with different concentrations of L-Cys, 8% cleavable PEG + 2% TAT modified liposomes exhibited the lowest uptake in the absence of L-Cys compared with 2% and 4% cleavable PEG + 2% TAT modified liposomes, which indicated 8% PEG could provide the highest mask effect on TAT, and a higher PEG density than 8% was not used to modify liposomes here for the failure in formation of stable liposomes as reported[34] previously. TAT at 2% was used because this percentage is widely used in TAT modified liposomes.[16] The uptake efficiency was enhanced with the increase of L-Cys concentration, which indicated the cleavage of DOPE-S-S-$mPEG_{5000}$ indirectly, and the cleavage occurred in a L-Cys dependent manner. A concentration of 20 mM L-Cys was used later, as this concertration could cleave PEG quite efficiently in a controlled manner. A L-Cys concentration higher than 20 mM was not suitable for this study because some potential toxic side effects might be caused at higher concentration. After the addition of 20 mM L-Cys, the percentage of TAT exposed was about 99.74%, 75.16%, and 58.88% for 2%, 4%, and 8% DOPE-S-S-$mPEG_{5000}$, respectively.

Whether the "functional molecule" TAT could work after removal of PEG is also crucial for the success of this specific liposomal system. To confirm this, 8% cleavable PEG + 2% TAT and 8% cleavable PEG without 2% TAT modified liposomes were incubated with HepG2 cells in the presence or absence of 20 mM L-Cys. The uptake of 8% cleavable PEG + 2% TAT modified liposomes was significantly increased ($P < 0.001$) after the addition of L-Cys, which was 4 times as high as that in the absence of L-Cys. While the uptake of 8% cleavable PEG without 2% TAT modified liposomes was only slightly increased compared with that

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of 8% cleavable PEG + 2% TAT, only about 1 time higher in uptake efficiency was observed. The result showed the "functional molecule" could work as expected after the removal of PEG as the difference of uptake efficiency could only be caused by TAT (all components were the same except for TAT). The results also implied the liposome fomulation process didn't affect the stability of TAT. Besides, 8% noncleavable PEG + 2% TAT modified liposomes exhibited little uptake, no matter with the addition of L-Cys or not, indicating TAT could only work after the removal of PEG.

After the cleavable PEG and "functional molecule" of the liposomal system was confirmed to work properly, its ability to deliver drugs into cells was assessed. A membrane impermeable and water-soluble dye, calcein, was encapsulated into the inner aqueous phase of Rh-PE labeled 8% cleavable PEG + 2% TAT or Rh-PE labeled 8% noncleavable PEG + 2% TAT modified liposomes. Little red or green fluorescence could be seen when 8% noncleavable PEG + 2% TAT was incubated with HepG2 cells no matter with the addition of L-Cys or not. Incubation of 8% cleavable PEG + 2% TAT modified liposomes with HepG2 cells in the absence of L-Cys also exhibited little red or green fluorescence. Both red and green fluorescence could be seen obviously when calcein loaded Rh-PE labeled 8% cleavable PEG + 2% TAT modified liposomes were incubated with HepG2 cells in the presence of L-Cys, and most of the red and green fluorescence regions were overlapped, indicating that hydrophilic drugs such as calcein mainly might be delivered into tumor cells by the liposomal system under the control of L-Cys.

As far as we know, the reducing source in the extracellular space of tumors is as poor as that of other tissues in the body, and PEG would not disassociate from liposomes itself upon arriving at the tumor site.[2,3,35−37] However, disassociation of PEG could be precisely controlled by administration of exogenous innocuous L-Cys at the desired time (after liposomes accumulated in tumor tissue, i.e. about 24−48 h post administration of liposomes).[2,37]

In fact, despite that gradual reduction of disulfide bonds does occur gradually during the systemic circulation, the concentration of reducing agents such as cysteine (~8 $\mu$M in humans) and glutathione (~2 $\mu$M in humans) is quite low, and most of the PEG would be present on the surface of liposomes especially when 8% cleavable PEG was used. It was reported that the half-life of 8% thiolytic cleavable PEG modified liposomes *in vivo* was over 27 h,[2] which affords sufficient time for liposomes to accumulate at the tumor site. Then exogenous innocuous L-Cys could be administrated intravenously to cleave PEG from liposomes that have accumulated at the tumor site.[35−37]

In this study, for the uptake by tumor cells *in vivo*, we mainly focused on the fate of liposomes after the arrival at tumor tissue; 8% cleavable PEG + 2% TAT or Rh-PE labeled 8% noncleavable PEG + 2% TAT modified liposomes were injected intratumoraly to simulate the accumulation of liposomes in tumor tissue, and L-Cys was injected intratumoraly too. As a result, 4 h after intratumor injection, 8% cleavable PEG + 2% TAT modified liposomes exhibited stronger red and green fluorescence compared with 8% noncleavable PEG + 2% TAT modified liposomes, indicating the liposomal system developed here could deliver drugs into tumor cells efficiently under the control of L-Cys even in the *in vivo* environment. Our further investigation would focus on the systemic use of cleavable PEG and TAT comodified liposomes.

In conclusion, cleavable PEG and TAT comodified liposomes developed here could deliver drugs into tumor cells with high efficiency under the control of L-Cys. The combination of cleavable PEG and "functional molecule" TAT could overcome the dilemma of PEGylation, and it is an alternative to overcome the nonspecificity of TAT, which is quite promising in the application of tumor targeted drug delivery systems in the future.

## Abbreviations Used

PEG, poly(ethylene glycol); SPC, soy phosphatidylcholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; PDI, polydispersiy index; FBS, fetal bovine serum; L-Cys, L-cysteine; Mal, maleimide; RES, reticuloendothelial system; EPR, enhanced permeability and retention; Rh-PE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt); CLSM, confocal laser scanning microscopy.

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**Supporting Information Available:** $^1$H NMR of DOPE-PEG$_{1600}$-MAL, DOPE-S-S-mPEG$_{5000}$, and DOPE-mPEG$_{5000}$; TOF MS ES+ of DOPE-PEG$_{1600}$-TAT; and TLC results and cellular uptake of A2780 and related figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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