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Enhanced tumor accumulation and cellular uptake of liposomes modified with ether-bond linked cholesterol derivatives

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Novel liposomal formulations based on cholesterol modification had been designed previously by our lab, but we found them with a poor stability and short half-life, especially in blood circulation. The results might be attributed to the hydrolysis of ester linked cholesterol derivatives by esterase in plasma. Thus, in this study, we newly synthesized ether-linked cholesterol derivatives and compared them to other preparations. A comparison with the substrate cholesterol-PEG_{2000}-maleimide showed that ether-linked cholesterol-PEG_{2000}-maleimide could relatively maintain integrity in serum, with only a small mottle emerging on TLC (thin-layer chromatography) plates through the experiment. Then a cell-penetrating peptide TAT was attached to the distal end of CHO-$PEG_{2000}$to prepare liposomes and to further evaluate the two cholesterol derivatives. Optimized liposomes (65:35, lipid/cholesterol, molar ratio) composed of 3% CHO-PEG_{2000} and 3% CHO-PEG_{2000}-TAT showed good stability in 50%FBS (fetal bovine serum). *In vitro* experiments showed that as incubation time prolonged, ether-linked-TAT LIP showed a 3.67-fold higher uptake amount than ester-linked-TAT LIP. *In vivo*, ether-linked-TAT LIP accumulated better in tumors and had a 40% higher cellular uptake amount. Altogether, we could conclude that our newly ether-linked cholesterol derivatives possessed better stability especially in blood circulation which led to increased tumor cellular uptake *in vitro* and *in vivo*. Our study may offer a better way in cholesterol modification to prepare functionalized liposomes.

1. Introduction

In novel drug delivery systems, nanocarriers are used to improve drug properties, enhance drug water-solubility, reduce side-effects and even to improve drug targeting ability. The commonly used nanocarriers for drug delivery include liposomes, nanoparticles, nanotubes and micelles.

Liposomal drug delivery systems have been the subject of intensive investigations and frequently studied nanocarriers (Chen et al. 2010). Their composition from natural biological lipids and structural resemblance to cell membranes suggests metabolic compatibility, low toxicity, overall expectations of biocompatibility, and lack of a strong immune response (Martin C. 1995). Additionally, their capability of carrying both hydrophilic and hydrophobic drugs makes liposomes superior among all kinds of nanocarriers. However, drug delivery systems based on unmodified liposomes always suffer from short blood circulation time and instability *in vivo*.

To increase stability and circulation time of nanocarriers, surface coating of a layer of hydrophilic polymer like PEG (poly ethylene glycol) is becoming more and more popular since PEG was first used to modify nanocarriers in the 1990's (Klibanov et al. 1990; Allen et al. 1991; Allen et al. 1995). The coating of liposomes with PEG polymer provides them stability and thus prolongs their circulation time at an effective drug concentration *in vivo* (Lasic and Martin 1995; Gabizon et al. 1994). As the inert hydrophilic PEG at the surface of liposomes could create a steric barrier to avoid or at least intensively reduce the adsorption of opsonising proteins, liposomes thus evade being uptaken by the reticuloendothelial system (RES), making them the so-called "stealth" liposomes (Heald et al. 2002; Kenausis et al. 2000). Moreover, PEGylation could affect the pharmacokinetic behavior of nanocarriers, apparently turning them more likely to accumulate in tumor tissue through enhanced permeability and retention (EPR) effect than plain nanocarriers. What's more, the physicochemical properties of PEG allow further attachment of a series of bioactive molecules such as antibodies, bioactive proteins or peptides to its distal end to fabricate functionalized nanocarriers (Hansen et al. 1995).

Therefore, several attempts have been made to connect lipids and PEG and incorporate them into liposome bilayer, examples including phosphatidylethanolamine (Zalipskyet al. 1995), ceramides (Webb et al. 1998), diglycerides (Shimada et al. 1997) and cholesterol (Bradley et al. 1998; Janzen et al. 1996). Among all, distearoylphosphatidylethanolamine/methoxypoly(ethylene glycol) (DSPE–PEG) and its derivatives are most widely used to construct long circulating and several functionalized liposomes. However, despite their extraordinary properties, the expense of phosphatidylethanolamine derivatives and the difficulty in industrialized production make a clinical application of DSPE-PEG containing drug carriers impractical.

Hence, cholesterol derivatives are alternative candidate, since it confers cohesion on lipid bilayers by increasing the half-life of liposomes *in vivo* (Semple et al. 1996) and PEGylated choles-

Table 1: Composition of different liposomes (n = 3)

Liposomes	SPC	Cholesterol	CHO-PEG_{2000}	CHO-PEG_{2000}-TAT
Ether-linked-TAT LIP	65%	29%	3% CHO-ether-PEG_{2000}	3% CHO-ether-PEG_{2000}-TAT
Ester-linked-TAT LIP	65%	29%	3% CHO-ester-PEG_{2000}	3% CHO-ester-PEG_{2000}-TAT

terol molecules can be easily synthesized in large amounts on an industrial scale. In our previous study, a prevalent ester bond linked cholesterol-PEG was used for further modification to construct functionalized liposomal nanocarriers, and they did exhibit desirable characteristics *in vitro*. However, *in vivo* studies showed that they manifested poor stability and short half-life.
Taking all possible reasons into consideration, esterase in the plasma may mainly be responsible for the short half-life. Esterase belongs to the plasma functionality enzymes that include carboxylesterase and cholinesterase (Munger et al. 1991). Esterase is suspected to be produced by lysosome and secreted to plasma, and it efficiently catalyzes the breakage of ester bonds or thioester bonds to free acids from endogenous or exogenous materials (Maxwell et al. 1987), which is a major factor of the cleavage of ester linked cholesterol derivatives. Therefore, a series of more stable bond linked cholesterol derivatives were designed, such as ether bonds or amide bonds (James et al. 2006).
In this study, we newly synthesized ether bond linked cholesterol-PEG derivatives and a TAT peptide segment was subsequently attached to the distal end of both ester and ether linked cholesterol-PEG to test which could exhibit better properties as a functionalized ligand especially *in vivo* studies. TAT peptide is one of the most frequently used CPP (cell penetrating peptide) that had the ability to translocate across the plasma membrane and deliver the payload intracellularly. TAT peptide is derived from the transcriptional activator protein encoded by human immunodeficiency virus type 1 (HIV-1) (Jeang et al. 1999). The most commonly used TAT is a small (9 amino acids) stretch of basic amino acids, with the sequence RKKRRQRRR (Ruben et al. 1989), which contains six arginines (R), thus making TAT peptide positively charged. And this positive charge in the transduction domain is believed to be responsible for the transduction ability of TAT peptide. So TAT peptide is widely used to modify nanocarriers for enhancing cellular uptake. We also use TAT to modify our liposome to enhance cellular uptake, TAT has another role, since TAT was attached to cholesterol bearer through different chemical bonds (ester and ether bond) and incorporated to fabricate functionalized liposomes, the uptake efficiency of liposomes here is closely related to the density of TAT ligand on the surface of liposomes (Romberget al. 2008), so the uptake efficiency of liposomes could reflect the stability of our cholesterol bearer and that is what our work is focused on. This study may offer a relatively better way of cholesterol modification to fabricate superior liposomal nanocarriers as well as make the most use of the essential advantages of cholesterol.

2. Investigations and results

2.1. Synthesis of CHO-ether-PEG_{2000}-OMe

TLC (DCM: MeOH=8:1) showed a yield of more than 90% and a purity of more than 95%. CHO-ether-PEG_{2000}-OMe was verified by ^{1}H-NMR (400 MHz, CDCl3, δppm): 7.08 (t, 2H), 5.17 (d, 1H), 3.81(s, 1H), 3.65–3.28 (br, m, PEG protons, ∼170 H), 3.19 (s, 2H), 3.02–3.96 (m, 1H), 2.68 (t, 1H), 1.85–0.67 (m, Chol protons), with 0.50 (s, 3H), 0.68 (d, 3H), 0.69 (d, 3H), 0.74 (s, 3H), 0.81 (s, 3H).

2.2. Synthesis of CHO-ether-PEG_{2000}-TAT

To prepare the TAT modified cholesterol derivates (CHO-PEG_{2000}-TAT), we first synthesized CHO-PEG_{2000}-MAL by conjugating the PEG with a maleimide group (-MAL) to compound 2, then linked TAT to the distal end of CHO-PEG_{2000}-MAL *via* a thioether bond. The structure of CHO-ether-PEG_{2000}-TAT was verified by ^{1}H-NMR (400 MHz, CDCl3, δppm): 7.27 (s, 2H), 5.35 (d, 1H), 3.82–3.45 (br, m, PEG protons, ∼181H), 2.80(d, 2H), 1.88–0.83 (m, Chol protons), with 0.675 (s, 3H), 0.89 (d, 6H), 0.91 (d, 3H,), 1.02 (s, 3H).
TOF MS ES+ confirmed the formation ofCHO-ether-PEG_{2000}-TAT (Mw calculated = 4255 Da, Mw observed = 4356 Da).

2.3. Stability comparison of CHO-ether-PEG_{2000}-MAL and CHO-ester-PEG_{2000}-MAL in mouse serum

To test if any of the two cholesterol derivatives may exhibit better stability, 50% fresh mouse serum was used to simulate *in vivo* environment. We found that a mottle emerged below ester linked CHO-MAL just 1 h after incubation and became bigger at 8 h, which lasted to the end of the experiment while ether linked CHO-MAL did not show any small mottle until 24 h, which remained quite small through the whole experiment (TLC data not shown). The mottle formation signified the cleavage of the materials, and a relatively low cleavage of ether linked CHO-MAL reflected a better stability in the mimicked *in vivo* environment.

2.4. Characteristics of liposomes

Liposomes prescription was optimized in previous studies which mainly contained soybean phospholipids (SPC) and cholesterol. The size of both liposomes used in the following experiments was around 115 nm, with a PDI (polydispersity index) about 0.2, and because of the incorporation of positively-charged TAT, ζ-potential rose up to about +22 mV (Tables 1 and 2).

2.5. Liposomes stability in FBS

As TAT segment is positively charged, liposomes may gather and form precipitation through charge-charge interaction, especially in the blood circulation. Good stability in serum could ensure efficient liposome accumulation in tumor tissue. The transmit-

Table 2: Size and ζ-potential of liposomes with different linkages (n = 3)

Liposomes	Size(nm)	PDI	ζ-Potential(mV)
Ether-linked-TAT LIP	113.8 ± 6.736	0.227 ± 0.029	22.367 ± 3.855
Ester-linked-TAT LIP	114.5 ± 12.308	0.207 ± 0.039	22.3 ± 1.4

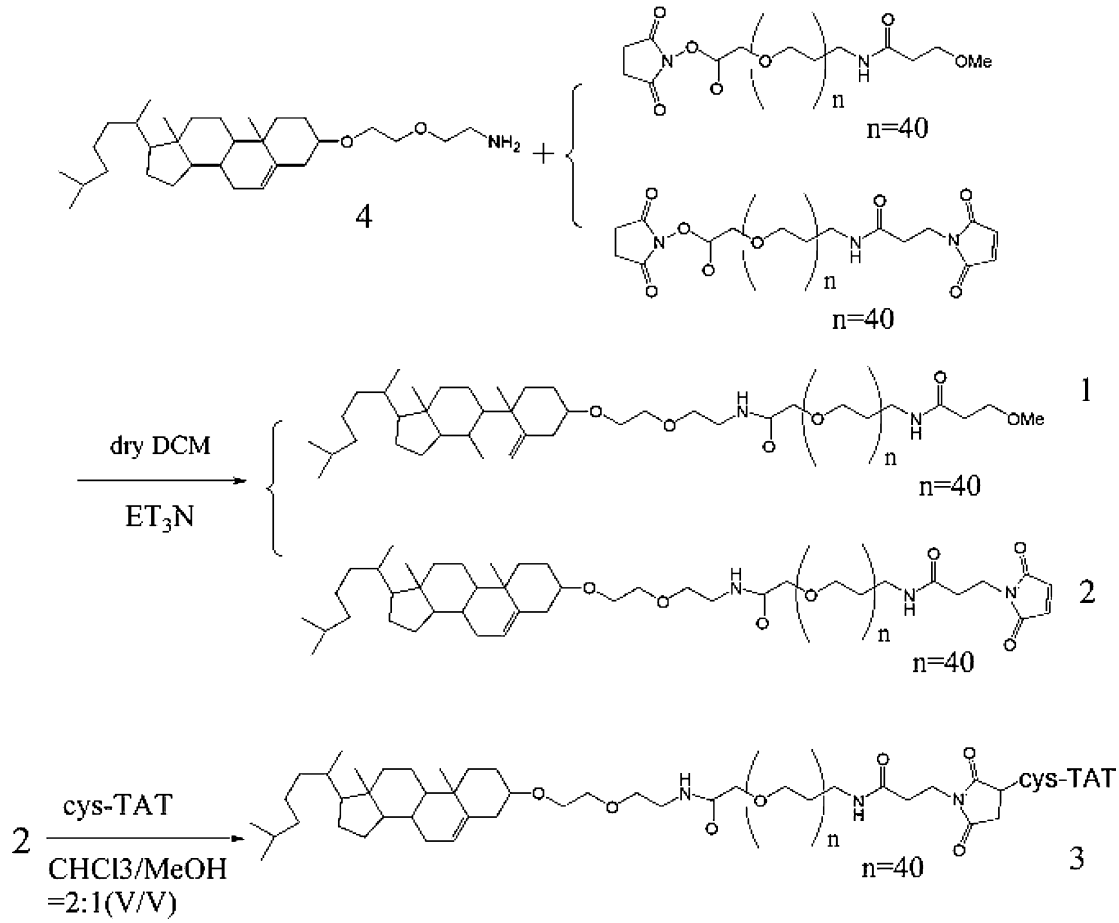


Fig. 1: Synthetic route of cholesterol-ether-linked derivatives. Compound 1 stands for Cho-ether-PEG_{2000}-OMe, compound 3 stands for Cho-ether-PEG_{2000}-OMe.

tancy variation of different liposomes in 50% FBS showed that they formed no precipitation even up to 24 h (Fig. 2a). The mean diameter of liposomes may have a slightly be increased after one hour of incubation with 50% FBS and remained stable at about 140 nm up to 48 h (Fig. 2b). This suggested a good stability of both ether and ester linked CHO-TAT liposomes.

2.6. Cellular uptake characterization in vitro

In vitro cellular uptake characterization was investigated over different liposome concentrations and incubation times. As liposome concentration rose or time prolonged, both ether and ester linked CHO-TAT modified liposomes showed gradually increased cellular uptake efficiency, demonstrating that these liposomes entered cells through both liposome concentration- and time-dependent manners. However, ether-linked-TAT LIP always had better cellular uptake compared to that of ester-linked-TAT LIP

Fluorescence showed that the uptake amount of ether-linked-TAT LIP were 1.66, 1.5 and 3.48-fold higher than that of ester-linked-TAT LIP at liposome concentrations of 0.12 $\mu mol \cdot ml^{-1}$, 0.18 $\mu mol \cdot ml^{-1}$ and 0.24 $\mu mol \cdot ml^{-1}$, respectively (Fig. 3a). FACS also showed that the cellular uptake of ether-linked-TAT LIP was significantly higher than ester-linked-TAT LIP (1.3, 1.43 and 2.36-fold higher at liposome concentrations of 0.12 $\mu mol \cdot ml^{-1}$, 0.18 $\mu mol \cdot ml^{-1}$ and 0.24 $\mu mol \cdot ml^{-1}$, respectively) (Fig. 3b).

Fluorescence showed that the uptake amount of ether-linked-TAT LIP were 1.58, 3.67 and 1.77-fold higher than that of ester-linked-TAT LIP at 1 h, 4 h, and 8 h after incubation, respectively (Fig. 4a) while FACS analysis was in accordance with fluorescence, the uptake amount of ether-linked-TAT LIP were 1.39, 2.4 and 2-fold higher than that of ester-linked-TAT LIP at 1 h, 4 h, and 8 h after incubation, respectively (Fig. 4b).

2.7. In vivo tumor accumulation and cellular uptake

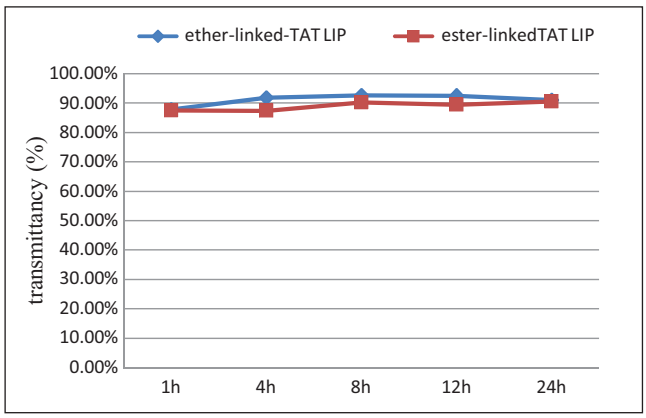
Qualitative image showed that ether-linked-TAT LIP had higher fluorescence intensity than ester-linked-TAT LIP (Fig. 5). In fact, the fluorescence intensity 30% higher than that of the ester-linked-TAT LIP (data not shown), the results illustrated that ether-linked-TAT LIP had better tumor accumulation than ester-linked-TAT LIP.

The cytometric data allowed quantitative analysis of the results obtained from treated C26 tumor-bearing mice. The fluorescent intensity represented the cellular uptake amount of different liposomes in tumor cells. The fluorescent intensity of the group receiving ether-linked-TAT LIP was about 40% higher than that of the group receiving ester-linked-TAT LIP (Fig. 6).

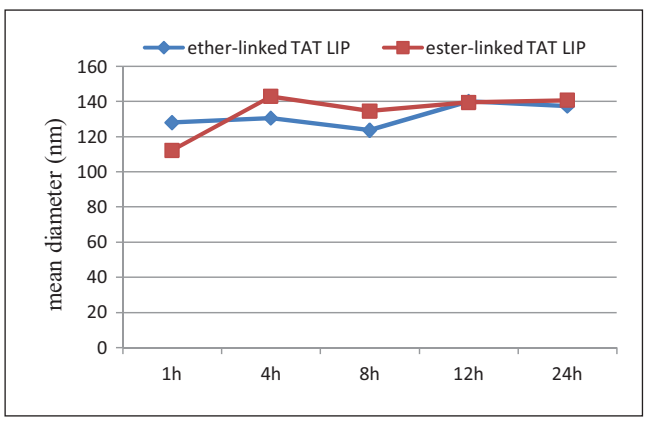
All *in vivo* study results supported the point that ether-linked-TAT LIP had better delivery characteristics in blood circulation which resulted in both increased tumor accumulation and cellular uptake.

3. Discussion

This study aimed to design and synthesize ether bond-linked cholesterol derivatives which could be more stable than previous ester bond-linked ones. A series of studies demonstrated that a liposomal drug delivery system modified by our newly synthesized ether bond-linked cholesterol derivatives showed enhanced tumor accumulation and cellular uptake both *in vitro* and in *vivo*.TAT peptide was terminated with cysteine in order

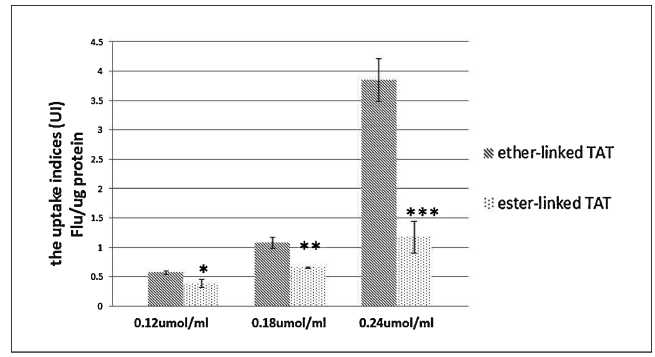


(a)

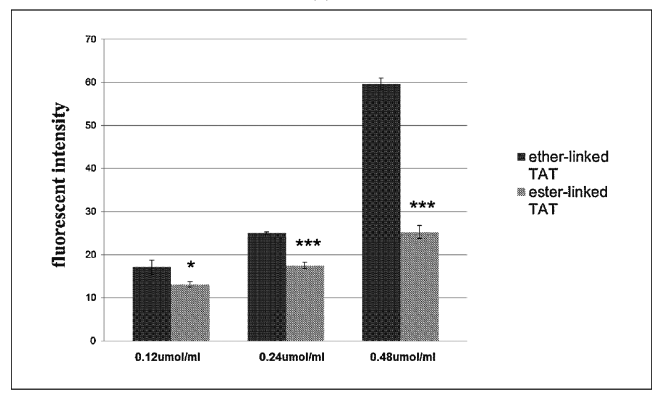


(b)

Fig. 2: Liposome stability evaluation in 50% FBS at different time points (1 h, 4 h, 8 h, 12 h, 24 h). **(a)**The transmittancy variation of different liposomes in 50%FBS at different time point (1 h, 4 h, 8 h, 12 h, 24 h). **(b)**The mean diameter variation of different liposomes in 50% FBS at different time points (1 h, 4 h, 8 h, 12 h, 24 h).



(a)



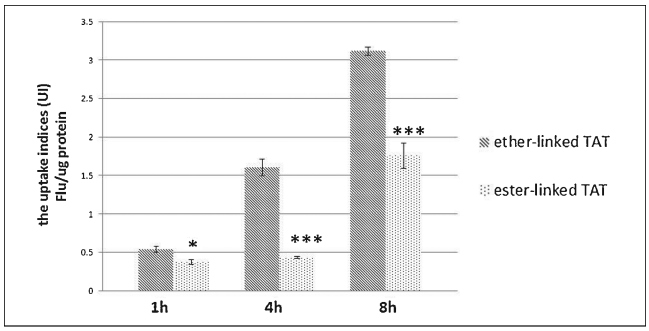
(b)

Fig. 3: Lipid concentration dependent cellular uptake analysis (0.12 μmol/ml, 0.24 μmol/ml, 0.48 μmol/ml) at 1 h. **(a)**Absorbance reading corrected by cellular proteins in different lipid concentrations using rhodamine as a fluorescence probe, the uptake efficiency was corrected by the amount of cellular proteins. **(b)**Cytometric quantitation of cellular uptake in different lipid concentrations using rhodamine as a fluorescence probe, data was corrected by blank group. Data represented the mean ± SD, n = 3. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus ether-linked TAT.

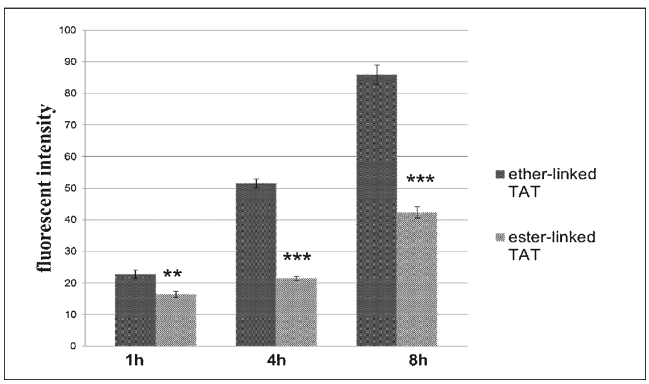
to introduce free sulfhydryl (-SH), and then TAT peptide could be conjugated to CHO-ether-PEG_{2000}-MAL via the sulfhydryl-maleimide reaction, which allowed TAT to be conjugatedat a specific site (-SH).

We first tested the integrity of ether or ester linked cholesterol-PEG_{2000}-maleimide substrates in 50% fresh mouse serum. For TAT segment was attached to cholesterol-PEG_{2000} through a maleimide linker, the stability of cholesterol-PEG_{2000}-maleimide might reflect the stability of our functional cholesterol-PEG_{2000}-TAT. We had investigated originally the biodegration behavior in 50% FBS, but we found no difference, both CHO-ester-MAL and CHO-ether-MAL were stable in 50% FBS up to 72 h. As esterase played the major role in the cleavage of CHO-ester-MAL, we later supposed that esterase amount in FBS was insufficent or its activity was suppressed or lost after long time freezing. So, we compared the stability of CHO-ether-MAL and CHO-ester-MAL in fresh mouse serum and the difference was obvious, CHO-ether-MAL still stayed integral while CHO-ester-MAL showed cleavage behavior which in turn proved our conjecture. More integral cholesterol-ether-PEG2000-maleimide reflected that ether bond was more stable. As it was reported that a PEG_{2000} density higher than 2% could endow the liposomes with long circulating properties *in vivo* (Dos et al. 2007), so 3% molar ratio PEG_{2000} was chosen as our long circulation PEG. Due to our previous studies, 1% TAT was practically enough to achieve a good cell-penetrating effect, but in this study, 3%TAT was chosen to maximize this effect. As we know, TAT is a positively-charged peptide, and a high density of TAT at the surface of liposomes may result in aggregation, so liposome stability studies were necessary. Stability study was done in 50% FBS, although FBS could not provide a real environment encountered by the liposomes in the blood (in FBS, the effect of blood cells was not considered), this method has already been widely used to test the aggregation characteristics of liposomes (Maeda et al. 2004), because not the cells but various chemicals (such as proteins) may have affected the aggregation of liposomes. We use both turbidity and size distribution to measure the aggregation characteristic. But we tended that the PCS method was not so accurate since the chaotic particles in FBS would interfere with assaying the mean diameter to some extent. If any of aggregations occurs in the presence of 50% FBS, the turbidity would increase, and the absorbance at 750 nm would also increase, leading to the decrease of the transmittancy (T%), The transmittance of blank PBS or 50%FBS was set at 100%, and the T% of liposomal suspension was normalized by the T% of blank PBS or FBS: A ratio of approximately 100%, indicated there was no aggregation; if a ratio far from 100%, indicated aggregation occurred. Fig. 2a showed the transmittancy variation of different liposomes was approximate 100% at different time points in 50%FBS, and we conclude that both ester and ether CHO-TAT modified liposomes did not form aggregation with each other, which verified that 3% exposed TAT did not alter the stability of liposomes. This experiment might lay a foundation for our next studies *in vitro* and *in vivo*. So finally, 3% molar ratio of both PEG and PEG-TAT with a PEG chain length of 2000 were incorporated to fabricate our liposomes,which could remain stable (did not form aggregation) in blood circulation and maintain enough cell-penetrating capability.

For the two cholesterol derivatives modified liposomes studied in this paper, they possessed similar cellular uptake behavior *in vitro* in a short time. But as the incubation time prolonged,



(a)



(b)

Fig. 4: Time dependent cellular uptake study at different time points (1 h, 4 h, 8 h) at a lipid concentration of 0.18 μmol/ml. (a) Absorbance reading corrected by cellular proteins at different time points using rhodamine as a fluorescence probe, the uptake efficiency was corrected by the amount of cellular proteins. (b) Cytometric quantitation of cellular uptake at different time points using rhodamine as a fluorescence probe, data was corrected by blank group. Data represented the mean ± SD, n = 3. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus ether-linked TAT.

the difference became obvious. Fluorescence showed at 4 h, a 3.67-fold increase of ether-linked-TAT LIP than that of ester-linked-TAT LIP reflected a stable existence of TAT of ether linked liposomes and FACS results had the same tendency. A higher uptake efficiency of ether-linked-TAT LIP indicated a better stability property as a nanocarrier. As TAT induced cellular uptake was highly depended on the TAT density at the surface of liposomes, low cellular uptake amount reflected a loss of TAT segment originally attached at the surface of liposomes. Thus the esterase secreted by the cells might push forward the cleavage of the ester bond between cholesterol and the "functional molecular" TAT, which, at last, lessened the uptake of liposomes. As Tables 1 and 2 shows, both ether linked CHO-PEG_{2000} and CHO-PEG_{2000}-TAT were chosen to fabricate ether-linked-TAT LIP while both ester linked CHO-PEG_{2000} and CHO-PEG_{2000}-TAT were chosen to fabricate ester-linked-TAT LIP to keep consistency. Thus, in blood circulation, ester-linked-TAT LIP might have a loss of both PEG_{2000} and PEG_{2000}-TAT. We used DiD as a fluorescence probe. DiD was an analogue of DiR (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indotricarbocyanine iodide), they both were always used for *in vivo* imaging. DiD could be stably encapsulated into liposomes easily. The excitation and emission wavelength of DiD was 644 nm and 665 nm, belonging to the near-infrared (NIR) spectrum, within which light could penetrate deeply into tissue, and background from tissue autofluorescence and absorption from intrinsic chromophore were low (Zhang et al. 2010). In addition, the uptake characteristics of DiD loaded liposomes were parallel with Rh-PE labeled liposomes (Kuai et al. 2011). In this study, the functionalized liposomes might accumulate in tumor tissue mainly through EPR effects, thus, for ether-linked-TAT LIP, stable presence of both PEG_{2000} and PEG_{2000}-TAT resulted in good tumor accumulation and cellular uptake. While on the contrary, the loss of PEG might decrease the half-life of ester-linked-TAT LIP, weaken the EPR effects and result in poor tumor accumulation and additionally, the loss of functionalized segment PEG_{2000}-TAT further lessened the uptake of ester-linked-TAT LIP. That was why the tumor accumulation of ether-linked-TAT LIP was 30% higher than ester-linked-TAT LIP but cellular uptake of ether-linked-TAT LIP was 40% higher than that of ester-linked-TAT LIP. Finally, better tumor accumulation and cellular uptake strongly indicated the superiority of ether-linked-TAT LIP than ester-linked-TAT LIP as a nanocarrier.

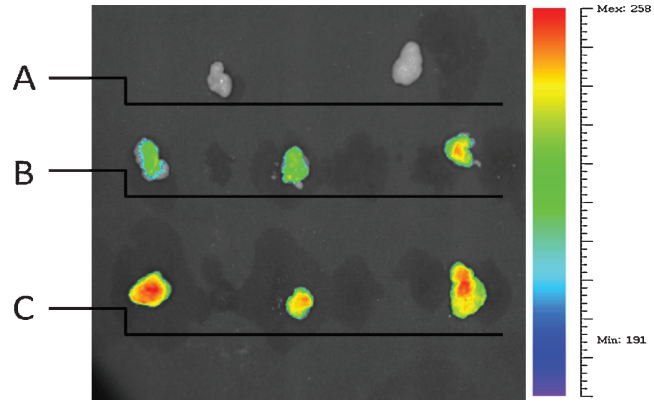


Fig. 5: Fluorescence intensity of excised tumor tissue using DiD as a fluorescence probe. A, B, C represent mice received blank PBS (phosphate buffered saline), ester-linked-TAT LIP and ether-linked-TAT LIP, respectively. For A, n = 2, for B and C, n = 3.

We have designed and synthesized ether-linked CHO-PEG_{2000}, and further attached a functional molecular TAT to the distal end of it. From *in vitro* and *in vivo* aspects, we have demonstrated that ether linked cholesterol derivatives could formulate well into functional liposomes as our previous ester linked ones, and these liposomes showed better stability especially in blood circulation, leading to both enhanced tumor accumulating and cellular uptake.

4. Experimental

4.1. Materials and animals

Soybean phospholipids (SPC) and cholesterol (Cho) were purchased from Bio Life Science & Technology Co., Ltd. Shanghai, PR China. 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-*N*-(lissaminerhodamine B sulfonyl) (ammonium salt) (Rh-PE) were purchased from Avanti Lipids. 1,1-Dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine (DiD) was purchased from Biotium (USA). Collagenase type IV and DNase I were purchased from Biosharp. NHS-PEG_{2000}-MAL and mPEG_{2000}-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 Bio-pharmaceutical Co., Ltd. (Chengdu, China). Ester linked CHO-PEG_{2000}-MAL,CHO-PEG_{2000}, CHO-PEG_{2000}-TAT and compound 4 (CHO-ether-diglycolamine) (Fig. 1) were synthesized previously by our lab (Qin and He 2011ab). Other chemicals and reagents were of analytical grade and used without further purification.

Male BALB/c mice (about 20 g) were purchased from the Experimental Animal Center of Sichuan University (P.R. China). All of 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.

4.2. Synthesis of compound 1 (CHO-ether-PEG_{2000}-OMe)

Compound 4 was reacted with mPEG_{2000}-NHS (molar ratio = 2:1) in 5 ml dry dichloromethane (DCM) at room temperature under argon in the presence of 10 μl triethylamine for about 24 h. Excess 4 was removed by adding 5 mL of acetonitrile for precipitating, and the mixture was kept at 4 °C overnight. Then it was centrifuged at 5000 rpm for 10 min. After that, the

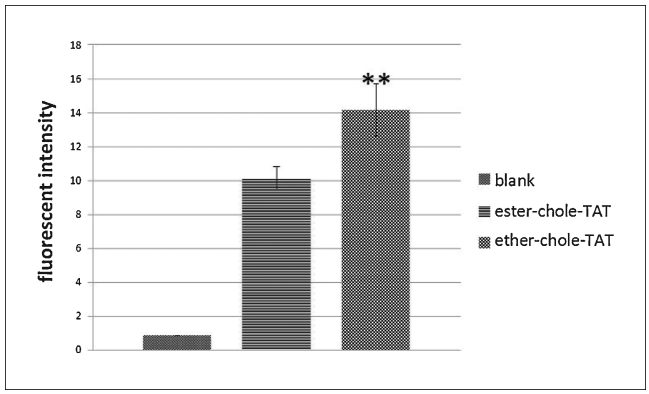


Fig. 6: Cytometric quantitation of cell suspensions from C26 tumor-bearing mice receiving different formulations of DiD loaded liposomes. Data represented the mean ± SD. For blank, n = 2, for ester-chole-TAT and ether-chole-TAT, n = 3 *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, versus ester-chole-TAT.

supernatant was collected and evaporated again under vacuum to get the dry compound 1 (CHO-ether-PEG_{2000}-OMe).

4.3. Synthesis of compound 3 (CHO-ether-PEG_{2000}-TAT)

Compound 4 was reacted with NHS-PEG_{2000}-MAL (molar ratio = 2:1) in 5 ml dry dichloromethane (DCM) at room temperature under argon in the presence of 10ul triethylamine for about 4 h. After the thin layer chromatography (TLC) showed the disappearance of NHS-PEG_{2000}-Mal, the reaction mixture was filtered and evaporated under vacuum. Followed by the same purification method with compound 1 (CHO-ether-PEG_{2000}-OMe) to get compound 2 (CHO-ether-PEG_{2000}-MAL).
The compound 2 (CHO-PEG_{2000}-MAL) and Cys-TAT (molar ratio = 1:1.5) were reacted in 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 CHO-ether-PEG_{2000}-MAL, the mixture was evaporated under vacuum, the slight excess of Cys-TAT was removed by slowly adding a small volume of $CHCl_3$until no more precipitation occurred.The insoluble material was filtered, and the supernatant was evaporated again under vacuum to afford compound 3 (Fig. 1).

4.4. Stability comparison of CHO-ether-MAL and CHO-ester-MAL in mouse serum

The stability of CHO-ether-MAL and CHO-ester-MAL in mouse serum was conducted according to our previous study with some modifications (Kuai and He 2010). Briefly, 12 mg of material cholesterol-ether-PEG_{2000}-maleimide (CHO-ether-MAL) and cholesterol-ester-PEG_{2000}-maleimide (CHO-ester-MAL) were dissolved in chloroform, respectively, dried under reduced pressure and stored in vacuum over night to remove residual chloroform. 3 ml of 5% glucose solution (ph 7.4) was added before incubation for hydration, and micelles were formed after subsequent probe sonication. Micelles were then incubated with fresh serum from normal mice for 72 h (1:1,voulume), at different time points (0 h, 1 h, 8 h, 24 h, 48 h, 72 h), TLC assay was used to measure if our substrates material were decomposed.

4.5. Preparation of liposomes

Both ether and ester linked TAT modified liposomes were prepared by thin film hydration methods (Maeda and Fujimoto 2006). Briefly, SPC, cholesterol, CHO-ether-PEG_{2000} or CHO-ester-$PEG_{2000}$and CHO-ether-PEG_{2000}-TAT or CHO-ester-PEG_{2000}-TAT were dissolved in chloroform (total molar ratio of phospholipid and cholesterol derivatives was 2:1). Chloroform was then evaporated by rotary evaporation and residual organic solvent was removed in vacuum over night.
For the liposomes used in the *in vitro* experiment, rhodamine labeled lipid (Rho-PE) was incorporated within total lipids as a fluorescent probe (Cryanet al. 2006). Then the thin film was hydrated in phosphate-buffered saline (PBS, pH 7.4) for 1 h at 37 °C, followed by an intermittently probe sonication for 50 s at 100W.
For the liposomes used in the *in vivo* experiment, the procedure was the same than with the liposomes used in the *in vitro* experiment except that fluorescent probe was replaced by the DiD and that hydration solution was 5% glucose (PH 7.2) instead.

4.6. Liposome characterization

The size and ζ-potential of the liposomes were determined using a Malvern Zetasizer Nano ZS90 instrument (Malvern instruments Ltd.,U.K.). Prior to measurement, 100 μL of the sample (lipid concentration 2.1 mg/mL) was diluted to 1 mL using PBS.

4.7. Stability of liposomes in the presence of FBS

Both two kinds of liposomes were incubated in phosphate buffer with 50% FBS for 24 h. Stability was determined by measuring their relative turbidity and mean diameter change. Briefly, transmittance was measured in 750 nm at different time points (1 h, 4 h, 8 h, 12 h, 24 h) and relative turbidity was calculated by the ratio of the transmittance in phosphate buffer with or without 50% FBS. Mean diameter was assayed by using Zetasizer Nano ZS90 instrument and prior to measurement, 100 μL of the sample were diluted to 1 mL using PBS.

4.8. In vitro cellular uptake

Murine colon carcinoma cells (C26, 1×10^5cells/well) were seeded in 24 well plates and 6 well plates for fluorescence measurement and FASC analysis, respectively. Cells were cultured in RPMI-1640 medium (GIBCO, supplemented with 10 FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin) and were maintained at 37 °C in a humidified incubator with 5% CO_2. After 48 h, culture medium was removed and ether-linked-TAT LIP and ester-linked-TAT LIP were added, respectively.
To a liposome concentration dependent uptake, fresh RPMI-1640 medium was added to adjust the final liposome concentration to 0.12 μmol·ml^{-1}, 0.18 μmol·ml^{-1} and 0.24 μmol·ml^{-1}, respectively. After incubation for another 1 h, medium were discarded and cells were collected, washed three times with cold PBS. For fluorescence measurement, cellular uptake efficiency was determined by a 560 nm absorbance reading afterlysis with 1% TritonX 100 for 20 min, and as well, the amount of proteins in the samples was determined by bicinchoninic acid (BCA) protein assay, the uptake efficiency was corrected by the amount of cellular proteins. For FACS analysis, washed cells were resuspended in 0.5 ml PBS for flow cytometry measurement.
To a time dependent uptake, fresh RPMI-1640 medium was add to adjust the final liposome concentration to 0.18 μmol·ml^{-1}, and cells were collected at different time points (1 h, 4 h and 8 h), then measurements were taken as described above.

4.9. In vivo tumor uptake

Tumor-bearing mice were established as described previously (Chang et al. 2010). Briefly, about 2×10^6 tumor cells were subcutaneously injected in the left flank of 5-week-old male BALB/c mice. *In vivo* experiments were performed at day 13 after tumor implantation. Size-matched C26 bearing mice (for blank, n = 2; and for others, n = 3) were injected with the DiD-labeled liposomes via tail veins, mice injected with PBS only served as blank. 24 hours after injection, the mice were sacrificed and tumors were excised and imaged using in-vivo FX professional imaging system (Kodak, America) with a wavelength set at $E_x = 655$ nm, $E_m = 715$ nm and an exposure time of 0.05 s. After photographing, tumor tissues were cut into small pieces, then the dissociation solution [Collagenase type IV (1 mg/ml) and DNase I (30 μg/ml)] was added at 37 °C for incubation for 1 h, following the process of sieving (70-μm mesh), centrifugation and washing with PBS for three times. Finally, cells were resuspended in 0.5 ml PBS for flow cytometry measurement.

4.10. Statistical analysis

Analysis of variance (ANOVA) was used to check the variance of the whole values in each group. Statistical significance was evaluated by using Student's t-test for the comparisons of experimental groups.

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