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Synthesis of transferrin (Tf) conjugated liposomes via Staudinger ligation

Songlin Xu[b,1], Ying Liu[a,b,1], Heng-Chiat Tai[b], Jing Zhu[b], Hong Ding[a,**], Robert J. Lee[b,c,*]

[a] Department of Pharmacy, Wuhan University, Wuhan, Hubei 430072, China
[b] Division of Pharmaceutics, College of Pharmacy, The Ohio State University, Columbus, OH, USA
[c] OSU Comprehensive Cancer Center (CCC) and Center for Affordable Nanoengineering of Polymeric Biomedical Devices (CANPBD), The Ohio State University, Columbus, OH, USA

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ABSTRACT

Staudinger ligation was evaluated as a strategy for synthesizing receptor targeted liposomes. First, an activated lipid derivative was synthesized by reacting dioleoyl phosphatidylethanolamine (DOPE) and 2-(diphenylphosphino) terephthalic acid 1-methyl 4-penta-fluorophenyldiester. Second, transferrin (Tf) was activated with p-azidophenyl isothiocyanate. Third, liposomes containing the activated lipid were prepared and then coupled to the activated Tf via the Staudinger reaction. These liposomes were evaluated in KB cells for cellular uptake and cytotoxicity, and in mice for pharmacokinetic properties. Tf-derivatized liposomes encapsulating calcein prepared by this conjugation method effectively targeted Tf receptor expressing KB cells. In addition, the Tf-targeted liposomes entrapping doxorubicin showed greatly enhanced in vitro cytotoxicity relative to non-targeted control liposomes. Pharmacokinetic parameters indicated that these liposomes retained long circulating properties relative to the free drug. In summary, Staudinger ligation is an effective method for the synthesis of receptor targeted liposomes.

1. Introduction

Synthesis of targeted liposomes typically requires chemical conjugation of a targeting ligand to the surface of the liposomes. A common method used is to couple a thiolated protein ligand with a maleimide-derivatized lipid in the micellar form, followed by "post-insertion" of the conjugate into liposomes (Drummond et al., 2005; Zheng et al., 2010; Pan et al., 2007; Mendonca et al., 2010). A potential drawback of this strategy is that the maleimide moiety has only limited hydrolytic stability and specificity for thiols. In contrast, chemical ligation reactions, exemplified by the "click chemistry" and the Staudinger reaction, are known to be highly efficient and specific (Hong et al., 2002; Chiu et al., 2006; Li et al., 2009). These approaches have only recently been incorporated into synthesis of bioconjugates for targeted drug delivery (Fatouma et al., 2006). For example, Staudinger ligation, in which an azide and a triphosphine selectively react to form an amide, has been used for chemoselective modification of recombinant proteins under mild conditions (Kiick et al., 2002). In contrast to the "click chemistry", this reaction occurs at room temperature without the need for a catalyst (Zhang et al., 2009). In this study, we synthesized Tf-conjugated liposomes using a similar reaction. Tf receptor (TfR) is frequently overexpressed on tumor cells (Ponka and Lok, 1999; Li and Qian, 2002; Visser et al., 2005; Daniels et al., 2006a,b). Tf, an 80 kDa glycoprotein, is the natural ligand for TfR that undergoes internalization through TfR-mediated endocytic pathway (Yang et al., 2009). Tf-liposomes loaded with calcein or doxorubicin were synthesized using Staudinger ligation and evaluated for tumor cell targeting efficiency and pharmacokinetic properties.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (egg PC) and dioleoyl phosphatidylethanolamine (DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol, doxorubicin (Dox), p-azidophenyl isothiocyanate (azido-ITC), 2-(diphenylphosphino) terephthalic acid 1-methyl-4-pentafluorophenyldiester (DPPTPA), holo-human Tf, Sepharose CL-4B chromatography resin, calcein, chloroform and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Monomethoxy polyethylene glycol 2000-distearoyl phosphatidylethanolamine (mPEG-DSPE) was purchased from Genzyme Pharmaceuticals (Cambridge, MA). Bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Scientific (Rockford, IL). Phosphate buffered saline (PBS) was purchased from Fisher Scientific (Pittsburgh, PA).

* Corresponding author at: College of Pharmacy, The Ohio State University, 542 LM Parks Hall, 500 W. 12th Ave., Columbus, OH 43210, USA.
Tel.: +1 614 292 4172; fax: +1 614 292 7766.
** Corresponding author. Tel.: +86 27 6875 9306; fax: +86 27 6875 9306.
E-mail addresses: dinghong1365@yahoo.com.cn (H. Ding), lee.1339@osu.edu (R.J. Lee).
[1] These authors contributed equally to this work.

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2.2. Cell culture

KB cells (a human carcinoma cell line identified as a subline derived from HeLa cervical cancer cells) were cultured in RPMI-1640 media (Gibco, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA), 100 units/ml penicillin and 100 μg/ml streptomycin, and maintained in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Synthesis of DOPE-DPPTPA

DPPTPA (0.04 mmol, dissolved in 10 ml chloroform) was added dropwise to a solution of DOPE (0.04 mmol, dissolved in 10 ml chloroform) over 30 min. The reaction proceeded for 6 h under argon at room temperature. Then, the reaction mixture was concentrated on a rotovap and purified by flash chromatography on silica gel (9/1, $CHCl_3$/MeOH). The product fractions were concentrated and dried on a rotovap and then placed under vacuum overnight to yield a light yellow solid product DOPE-DPPTPA.

2.4. Preparation of Tf-azide conjugate

Azido-ITC (17.6 μl of 10 mg/ml solution in DMSO) was added to 0.5 ml of Tf (10 mg/ml in PBS). The reaction mixture was incubated under stirring for 1 h at room temperature in the dark. The Tf-azide product was purified on a PD-10 desalting column and collected in the void volume fractions. The protein concentration of the product was then determined by the BCA assay (Smith et al., 1985).

2.5. Preparation of Tf-liposomes encapsulating calcein

Calcein is a water soluble fluorescent dye. Liposomes encapsulating calcein were prepared by thin film hydration of lipids followed by polycarbonate membrane extrusion. Briefly, egg PC/cholesterol/DOPE-DPPTPA at the molar ratio of 10/5/1 were dissolved in ethanol in a round bottom flask. After solvent removal on a rotary evaporator, the resultant thin film was dried further under vacuum and then hydrated in 50 mM calcein. The resulting suspension was subjected to five cycles of freeze–thaw and then extruded three times at room temperature through a 100-nm polycarbonate membrane. The calcein liposomes were then purified on a Sepharose CL-4B column to remove the unentrapped calcein. The conjugation of Tf-azide to the purified calcein liposomes (containing DOPE-DPPTPA) was carried out at the ratio of 1/1000 (mole/mole Tf/total lipid) at room temperature for 6 h. The final product was again purified on a Sepharose CL-4B column with PBS as the elution buffer and was store at 4 °C.

2.6. In vitro characterization of calcein loaded Tf-liposomes

For the in vitro cellular uptake experiment, KB cells were seeded in a 6-well plate at a density of 5×10^4 cell per well and were cul-

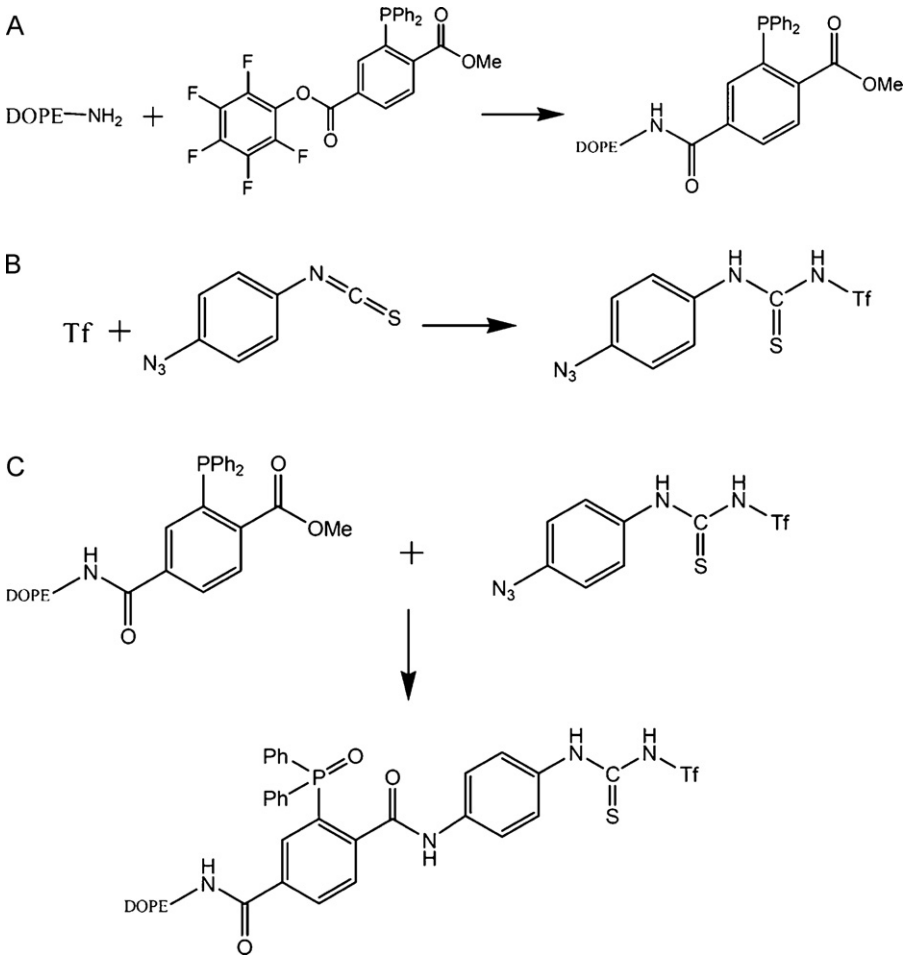

Fig. 1. Synthesis of Tf-liposomes by Staudinger ligation. Reaction schemes are shown for the synthesis of DOPE derivative DOPE-DPPTPA (A), Tf-azide conjugate (B), and the Tf-DOPE conjugate (C).

tured for 24 h. For the cellular uptake study, the treatment groups were: (1) PBS, (2) unmodified calcein liposomes (L-calcein), (3) Tf-conjugated calcein liposomes (Tf-L-calcein), and (4) Tf-L-calcein with TfR pre-block by excess free Tf. The lipid concentrations of all groups were set at 87.5 μg/ml. The cells were incubated for 4 h at 37 °C, washed three times with PBS, and then analyzed by fluorescence microscopy and by flow cytometry.

2.7. Preparation of Tf-targeted Dox liposomes (Tf-L-Dox)

Dox liposomes were prepared by the same method as described above, followed by remote-loading of the drug. The lipid composition used was egg PC/cholesterol/DOPE-DPPTPA/mPEG-DSPE at a molar ratio of 54/40/5/1. The lipids were dissolved in ethanol and dried to a thin film in a round-bottom flask. The lipid film was hydrated with 300 mM citrate buffer (pH 4.0), subjected to 5 cycles of freeze–thaw, and then the suspension was extruded through a 100-nm polycarbonate membrane. The product was then passed through a Sepharose CL-4B column to exchange the external buffer with PBS pH 7.4. For Tf conjugation, Tf-azide was added to the liposomes at a molar ratio of 1/1000 (mole/mole Tf/total lipid) and the mixture was incubated at room temperature for 6 h. Dox was then loaded into liposomes at a Dox/lipid weight ratio of 1/20 by drug addition to the liposomes followed by incubation at 37 °C for 30 min. The liposomes were purified on the Sepharose CL-4B column and the drug loading efficiency was determined by comparing the drug concentration in the liposomal fractions and the total eluted drug in all fractions collected. The zeta potential and particle size of the liposome were measured by Zeta PALS (Zeta Potential Analyzer, Brookhaven Instruments Corporation, NY) and 370 Nicomp Submicron Particle Sizer (Particle Sizing Systems, Santa Barbara, CA), respectively.

2.8. Assay for cytotoxicity of Tf-L-Dox

Cytotoxicity of the free drug (Dox), non-targeted liposomal drug (L-Dox) and targeted liposomal drug (Tf-L-Dox) were determined by the MTT assay. This assay is based on the mitochondrial conversion of a water-soluble tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT] to the water-insoluble purple formazan product (Marks et al., 1992). Cells were transferred to a 96-well tissue culture plates at 2×10^4 cells per well 24 h prior to drug treatment. The culture medium was then replaced with 200 μl of medium containing serial dilutions of various drug-containing formulations. Following 4 h incubation under 5% CO_2 at 37 °C, the cells were washed twice with PBS and cultured in fresh medium for another 48 h until untreated control wells reached above 90% confluence. Twenty μl of MTT stock solution (5 mg/ml in PBS) was then added to each well, and the plate was incubated for 2 h at 37 °C. The culture medium was then removed, and formazan crystal formed in each well was dissolved in 200 μl of DMSO. Cell viability was determined by measuring the absorbance of each well at the wavelength of 490 nm on an automated plate reader.

2.9. Determination of pharmacokinetic properties of Tf-L-Dox

Female imprinting control region (ICR) mice were randomly divided into 3 groups and were treated with either free Dox, L-Dox or Tf-L-Dox liposomes by intravenous injection. All groups were given a single dose of 10 mg Dox per kg body weight. Blood samples were then collected from the treated animals in heparin-pretreated centrifuge tubes at the time points of 0.25, 0.5, 1, 2, 4, 8, 16, 24 and 48 h. Plasma was isolated by centrifugation at 5000 rpm for 10 min at 4 °C and were stored at −20 °C.

Table 1
Mean particle size, zeta potential and loading efficiency of various liposome formulations ($n = 3$).

Formulation	Mean particle size (nm)	Zeta potential (mV)	Loading efficiency
Empty liposome	89.4 ± 8.5	−8.93 ± 1.23	
L-Dox	107.7 ± 15.3	−7.93 ± 3.46	97.9%
Tf-empty liposome	105.6 ± 10.2	−14.47 ± 0.53	
Tf-L-Dox	118.3 ± 12.7	−16.64 ± 0.57	98.6%

Plasma extract was prepared by mixing 200 μl of plasma and 800 μl of methanol, followed by centrifugation at 12,000 rpm for 20 min. The collected supernatant was dried under nitrogen, redissolved in 100 μl of mobile phase and centrifuged at 12,000 rpm for 10 min. Twenty μl of the resultant sample was then analyzed by HPLC, under following conditions: a Hypersil ODS column (5 μm, 4.6 mm × 200 mm) was used for the analysis; the mobile phase was acetonitrile/10 mM of SDS and 0.1% phosphoric acid/methanol (1/1/0.12 vol/vol), at a flow rate of 1.0 ml/min at room temperature; the wavelength of detection was 232 nm. The pharmacokinetic

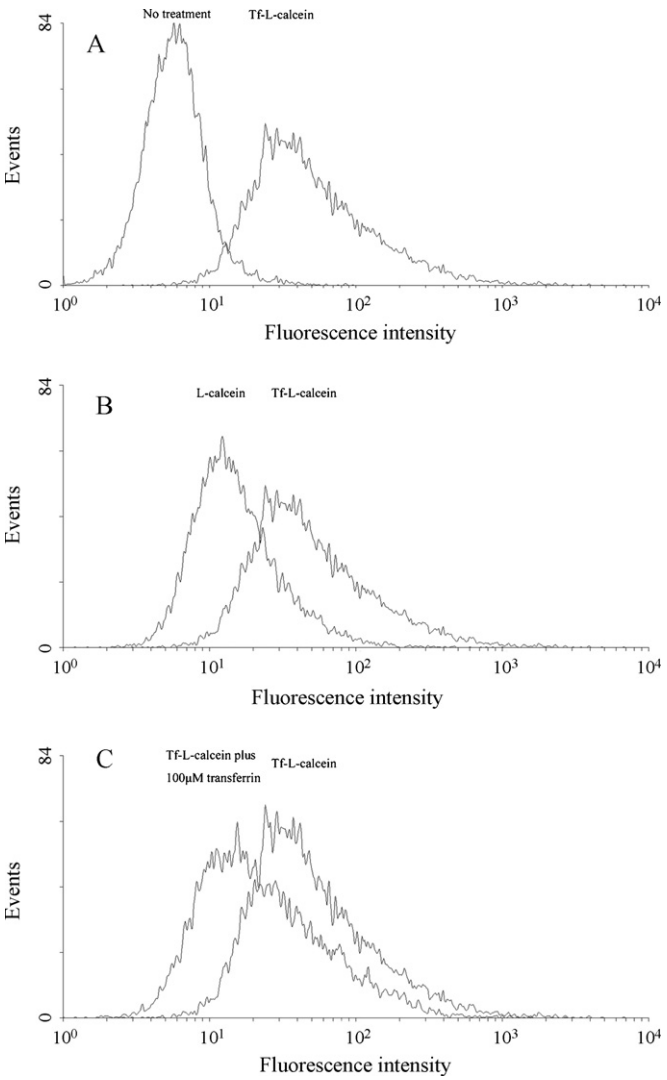

Fig. 2. Cellular uptake of Tf-L-calcein by KB cells. Cells were treated with liposomal calcein and cellular fluorescence was measured by flow cytometry. *X*-axis indicate cellular fluorescence intensity and the *Y*-axis indicate cell count. (A) Cells treated with Tf-L-calcein or PBS, (B) cells treated with Tf-L-calcein or L-calcein and (C) cells treated with Tf-L-calcein in a culture medium with or without 100 μM of Tf.

parameters were obtained by data analysis using the Winnonlin software.

3. Results and discussion

3.1. Synthesis of an activated DOPE derivative (DOPE-DPPTPA) and an activated Tf derivative (Tf-azide)

The reactions were carried out as described in Section 2 and the schemes are shown in Fig. 1. ^{1}H NMR analysis of DOPE-DPPTPA (300 MHz, $CDCl_3$) showed principal peaks of the DOPE moiety [5.33–5.39 (m, 4H), 4.40 (dd, J = 3.0 Hz, 12.0 Hz, 1H), 4.07–4.21 (m, 3H), 3.97 (t, J = 6.0 Hz, 2H), 3.14–3.23 (br, 2H), 2.31 (q, J = 6.0 Hz, 9.0 Hz, 4H), 1.98–2.09 (m, 8H), 1.87–1.95 (m, 8H), 1.25–1.40 (m, 36H), 0.86–0.94 (m, 6H)] and of 2-(diphenylphosphino) terephthalic acid 1-methyl 4-pentafluorophenyldiester moiety [8.48 (br, 2H), 8.18–8.20 (m, 2H), 7.72–7.75 (m, 1H), 7.29–7.41 (m, 10H), 3.78 (s, 3H)]. DOPE was selected because the availability of its nucleophilic primary amine group for easy derivatization. In this reaction strategy, all procedures were performed under mild conditions and the yield was about 85%, which is close to the value obtained in a previous report (Chang et al., 2007). For activation of Tf, isothiocyanate group was reacted to the primary amines on the protein. This conjugation scheme is widely used in biochemical research for protein or antibody fluorescence labeling (Heckl et al., 2008).

3.2. Characterization of Tf-L-Dox

The particle size, zeta potential and encapsulation efficiency of empty liposomes, L-Dox and Tf-L-Dox are presented in Table 1. The average particle sizes of the liposomes were 90–120 nm, which should enable extravasation at the site of the tumor for thera-

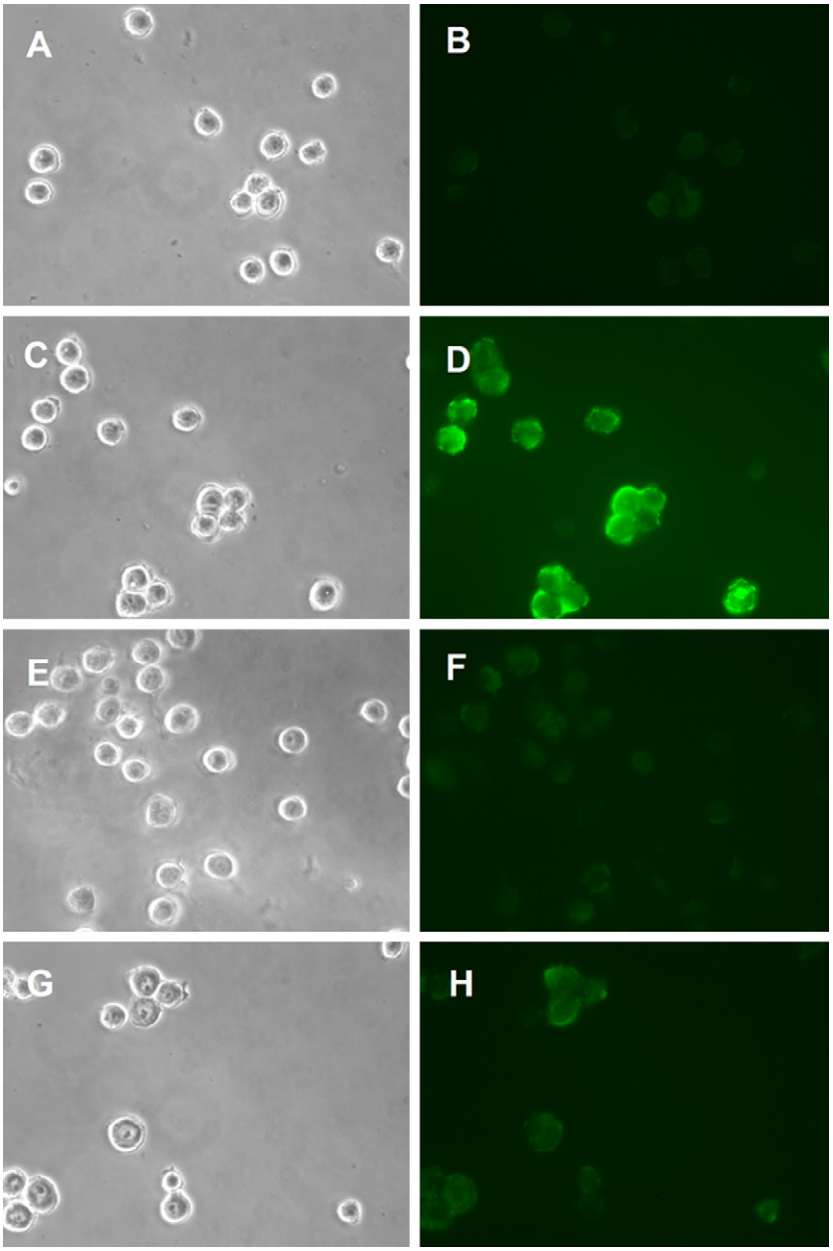

Fig. 3. Cellular uptake of Tf-L-calcein by KB cells visualized by fluorescence microscopy. KB cells were treated with Tf-L-calcein, Tf-L-calcein plus 100 μM of Tf or L-calcein. Right panels indicate cells visualized with fluorescent microscopy and left ones are the same fields in the phase-contrast mode. (A and B) cells treated with PBS, (C and D) cells treated with Tf-L-calcein, (E and F) cells treated with L-calcein, (G and H) cells treated with Tf-L-calcein plus 100 μM of Tf.

Table 2
IC_{50} of different Dox formulations in KB cells.

Group	IC_{50} (μM)
Free Dox	14.38
L-Dox	43.74
Tf-L-Dox	13.91
Tf-L-Dox + Tf	46.86

peutic applications. There was not a significant change in particle size after the conjugation of Tf with L-Dox. The zeta potential data showed negative values in all liposome preparations. This may be due to the presence of mPEG-DSPE, which is an anionic lipid, among the liposomal components (Xiang et al., 2008). The Tf-liposomes carried a greater negative surface charge than the unconjugated liposomes, presumably due to the negative charge on the Tf. The loading efficiency of Dox was ~97% by a pH gradient-based remote loading method, which was consistent with previous reports (Wang et al., 2000). The liposomes showed excellent stability and could be stored at 4 °C for >2 weeks without significant drug loss or change in particle size.

3.3. Cellular uptake studies of Tf-L-calcein on KB cells

KB cells have been reported to express a high level of TfR (Fritzer et al., 1996). In this study, KB cells were selected to investigate the cellular uptake of liposomes by flow cytometry and by fluorescence microscopy. The data showed that the fluorescence intensity of Tf-L-calcein treated cells was significantly higher than that of non-targeted liposomes (Fig. 2B) and that of Tf-L-calcein in the presence of 100 μM free Tf in the culture medium (Fig. 2C). This indicated that the Tf-liposomes synthesized were able to efficiently target TfR expressing tumor cells.

Fluorescence microscopy was used to observe the cellular uptake of the Tf-L-calcein liposomes. As shown in Fig. 3, cells incubated with Tf-L-calcein displayed more intense fluorescence than those in the L-calcein group. In addition, the targeting effect could be blocked by the addition of 100 μM free Tf, thus confirming the selectivity of liposomal cellular uptake for TfR.

3.4. Cytotoxicity of Dox liposomes

Cytotoxicity was evaluated by the MTT method using KB cells. IC_{50} values are shown in Table 2. As expected, L-Dox showed greatly reduced cytotoxicity compared to the free drug. In contrast, Tf-L-Dox showed higher cytotoxicity, presumably due to TfR mediated uptake and endocytosis. Meanwhile, the cytotoxicity found in the free Tf preblock group was significantly lower than that of the group without free Tf, suggesting the targeting effect was due to TfR, which correlated well with the result of cellular uptake experiment described above. These data showed that Tf-L-Dox synthesized by Staudinger ligation was fully functional and could efficiently and selectively deliver Dox into the targeted cells.

3.5. Pharmacokinetic parameters of Tf-L-Dox

The plasma Dox concentration-time profiles of different groups of formulations in ICR mice are shown in Fig. 4. Pharmacokinetic parameters were calculated and are summarized in Table 3. Consistent with previous studies, free Dox was cleared from the plasma rapidly (Li et al., 2009). Both liposomal formulations, including L-Dox and Tf-L-Dox, substantially prolonged the systemic circulation time of the drug due to the size of the liposomes which precludes renal clearance. However, the clearance of Tf-L-Dox was more rapid than that of L-Dox. This might be due to the expression of TfR on macrophages (Tartle and Honeysett, 1988; Li et al., 2008), which partly constitute the reticuloendothelial system that is responsible for liposome clearance.

Table 3
Pharmacokinetic parameters of Tf-L-Dox in ICR mice.

	AUC (μg h/ml)	$t_{1/2\beta}$ (h)	Cl (ml/h)	MRT (h)
Free Dox	14.8	3.09	22.43	4.46
L-Dox	363.7	9.16	0.90	11.88
Tf-L-Dox	219.9	5.45	1.51	7.39

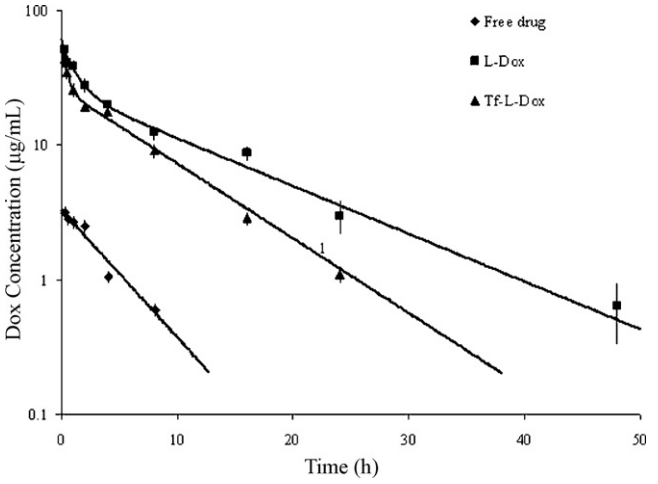

Fig. 4. Plasma clearance of Tf-L-Dox. ICR mice were injected with10 mg/kg Dox in different formulations. Data represent the mean ± standard deviation ($n = 3$). (♦) Free Dox, (■) L-Dox and (▲) Tf-L-Dox.

4. Conclusion

Staudinger ligation is a relatively novel strategy for the synthesis of bioconjugates, especially for liposomes. All reactions involved proceed under mild reaction conditions and do not require an additional catalyst, as is the case with the classic "click" reaction. In this study, this facile method was used to synthesize Tf-conjugated liposomes, which were loaded with calcein or Dox. The data obtained confirmed that the resulting targeted liposomes were highly functional both in terms of cellular uptake and cytotoxicity. Compared with alternative conjugation methods, Staudinger ligation is simple to perform, the activated reaction intermediates are relatively stable, and the conjugation reaction is highly specific. Therefore, this synthetic method holds great promise for future utility in the development of targeted drug delivery systems.

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