Drug Nanocarriers

DOI: 10.1002/anie.200900111

Disterolphospholipids: Nonexchangeable Lipids and Their Application to Liposomal Drug Delivery**

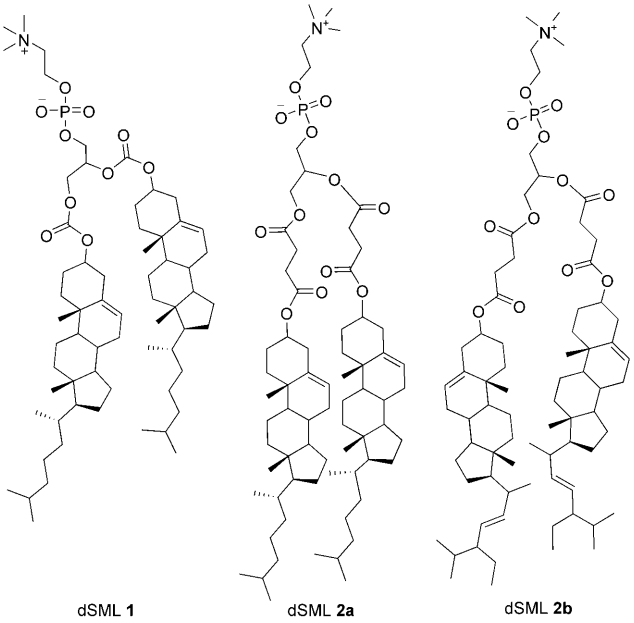
*Zhaohua Huang, Mahmoud Reza Jaafari, and Francis C. Szoka, Jr.**

Vesicles composed of lipids (liposomes) are widely used models of biomembranes and successful drug carriers.[1,2] Retention of the encapsulated drug in the liposome is controlled by the lipid composition;[3] the two important lipid types are phospholipids and cholesterol. Cholesterol is important, because its inclusion at 30 mol% eliminates the phase transition of synthetic phospholipids,[4,5] reduces membrane permeability,[6] and inhibits protein insertion into the bilayer.[7,8] All these factors control liposome stability in blood.

However, liposomes can not be formed from pure cholesterol; in fact, above 50 mol%, a cholesterol phase separates from the bilayer into crystals. Cholesterol can also be transferred rapidly between biomembranes, lipoproteins, and lipid vesicles.[9] Cholesterol transfer from the bilayer results in a decrease in liposome stability and a subsequent loss of encapsulated contents from the liposome.[3,10] Therefore, to obtain membranes with the desired properties, it is important to regulate the sterol concentration in the liposome bilayer.

Chemistry has played a major role in the field of liposome research. Synthetic efforts have focused on pegylated lipids,[11] cationic lipids,[12] fluorinated lipids,[13] lipid prodrugs,[14] molecular umbrellas,[15] and, to a lesser extent, nonionic amphiphiles.[16] Recently, we reported a new category of lipids: sterol-modified phospholipids (SMLs)[17], in which the sterol is covalently linked to the lysophospholipid at either the *sn*-1 or the *sn*-2 position. SMLs can form liposomes and do not transfer from the bilayer. We extend this concept to disterol-modified phospholipids (dSMLs), in which sterols are covalently attached to both the *sn*-1 and *sn*-2 positions of glycerophosphocholine (Scheme 1).

The synthesis of dSMLs is illustrated in Scheme 2 (see the Supporting Information for a detailed procedure). Glycerophosphocholine (GPC) was used as the starting material for the direct coupling of sterol derivatives, such as cholesteryl chloroformate, cholesterol hemisuccinate, and stigmasterol hemisuccinate. The use of GPC in the semisynthesis of phospholipids generally requires a transition-metal ion to increase the solubility of GPC in organic solvents. We used tetraphenyl borate as the organic counterion of GPC to generate a complex that is soluble in anhydrous pyridine. This procedure provided compounds **2** and **2a** in about 50% yield, and compound **1** in lower yield (17%). The low yield of **1** may be due to steric hindrance between the closely apposed sterols at the glycerol moiety.



Scheme 1. Structures of disterol-modified phospholipids.

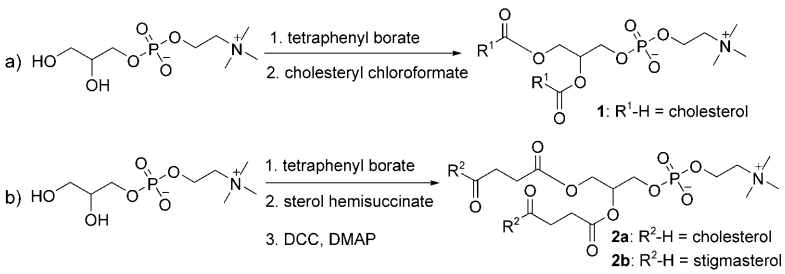
To investigate whether dSMLs can form liposomes with diacyl lipids, we formulated 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) with dSMLs in a 2:1 molar ratio (equivalent to 50% free cholesterol in conventional liposomes) either in HEPES-buffered saline (pH 7.4; HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) or in 100 mM carboxyfluorescein (CF). Sonicated DPPC/**2a** as a suspension had a particle diameter of around 80 nm, as measured by dynamic light scattering. Additionally, CF could be encapsulated and retained in the DPPC/dSML liposomes. The formation of DPPC/**2a** vesicles was confirmed further by freeze–fracture electron microscopy: Clear evidence of convex (shadow behind vesicles) and concave shapes (shadow in front of vesicles) of liposome spheres was observed (Figure 1). Together the results suggest that dSMLs can form liposomes with diacyl phospholipids. Lip-

[*] Dr. Z. Huang, Dr. M. R. Jaafari,[+] Prof. F. C. Szoka, Jr.
Department of Bioengineering and Therapeutic Sciences
University of California at San Francisco
San Francisco, CA 94143-0912 (USA)
Fax: (+1) 415-476-0688
E-mail: szoka@cgl.ucsf.edu

[+] Current address: School of Pharmacy
Mashhad University of Medical Sciences
P.O. Box 91775-1365, Mashhad (Iran)

[**] This research was supported by NIH EB003008, GM61851, and CA119343. We thank Nichole Macaraeg for the animal study, and Katherine Jerger for the leakage study.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200900111.



Scheme 2. Synthesis of disterol-modified phospholipids. DCC = *N*,*N*′-dicyclohexylcarbodiimide, DMAP = 4-dimethylaminopyridine.

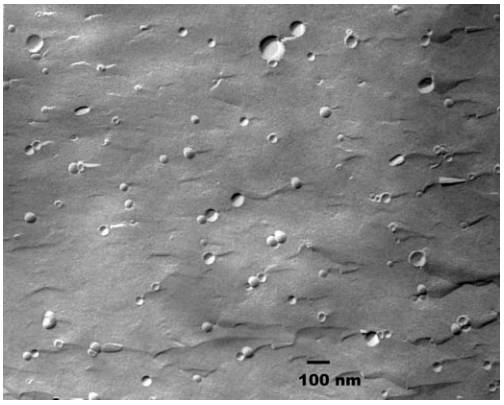


Figure 1. Freeze–fracture electron microscopy image of the sonicated liposome DPPC/**2a** (2:1).

osomes can also be formed from the single component dSML **2a**, but dispersions of the single component dSML **1** or **2b** aggregate upon hydration.

We investigated the thermotropic phase behavior of DPPC/dSML liposomes of various DPPC–sterol mole ratios by using differential scanning calorimetry (DSC). The inclusion of all three dSMLs in a DPPC dispersion influenced the transition of DPPC in a similar way to unmodified cholesterol: A broadening of the transition peaks, a lowering of the midpoint of the transition temperature, and a complete elimination of the transition at about 40% cholesterol were observed (Figure 2). The incorporation of dSMLs **1** and **2b** in the bilayer led to a small transition observed at around 20°C in the DSC traces of dSML **1** and **2b** (see the Supporting Information) when the sterol content was equivalent to more than 20 mol% cholesterol. In the case of dSML **1**, the transition appeared at a progressively lower temperature as the relative amount of **1** in the liposome increased.

One of the major challenges for in vivo liposome drug delivery is the propensity of serum proteins and biological membranes to extract free cholesterol from the liposome bilayer. In nature, cholesterol exists as a free molecule, as the 3-sulfate, or as the 3-ester of a fatty acid. These compounds can not form bilayers by themselves. Synthetic cholesterol derivatives with a hydrophilic head group at the 3-position, such as a phosphate,[18] phosphatidylcholine, hemisuccinate, or ethylene oxide, assemble into bilayers but can transfer between bilayers.[19] The transfer rate of dSMLs from the liposome bilayer was measured in a cholesterol-exchange experiment. In this experiment, neutral liposomes of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were used in 10-fold excess as the acceptor, and the donor liposomes consisted of 50% DPPC, 40% cholesterol (or the equivalent amount of a dSML), and 10% 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylglycerol (DPPG), a negatively charged lipid. The donor and acceptor liposomes were mixed and incubated at 37°C for various lengths of time, then separated on an anion-exchange column. The amount of cholesterol transferred to the acceptor was determined by a cholesterol assay[20] (see the Supporting Information). Free cholesterol exchanged with a half-life of 2 h, but there was no detectable transfer of dSML **1** from the donor liposome over the 8 h duration of the experiment (Figure 3a). The resistance of dSMLs to the extraction (similar behavior was observed for **2a** and **2b**) may be the result of the increased hydrophobicity of the disterol derivative as well as a strong interaction between the dSML and other phospholipids in the bilayer. Such a mechanism has been suggested previously for hydrophilic cholesterol derivatives.[19]

We studied the leakage of CF from liposomes consisting of dSML **2a** and diacyl phosphocholines of different chain lengths in 30% fetal bovine serum at 37°C (Figure 3b; see the Supporting Information for the detailed procedure). Conventional liposomes composed of a diacyl lipid and free cholesterol were used as controls. All liposome formulations contained 45 mol% cholesterol or the equivalent amount in dSML **2a**. Both dSML lip-

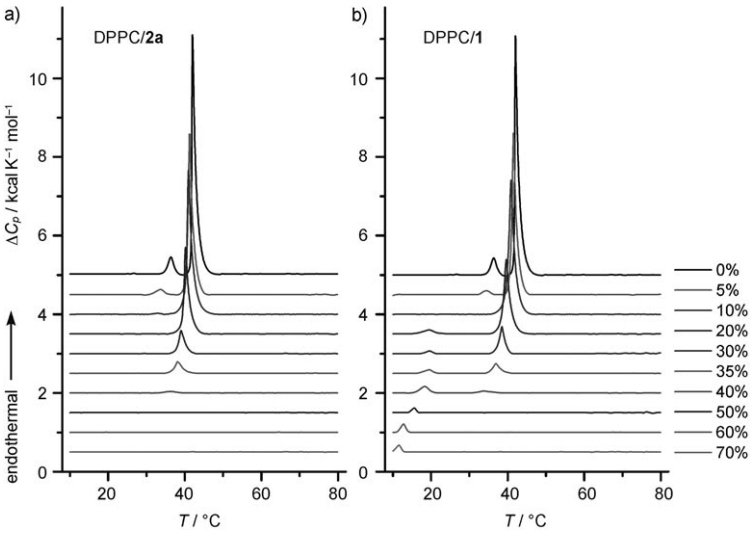


Figure 2. Differential scanning calorimetry thermograms of lipid dispersions. a) DPPC/dSML **2a**, b) DPPC/dSML **1**. The mole percentage of cholesterol in the liposome is indicated in the key on the right. Stacked spectra are shifted with respect to the vertical axis.

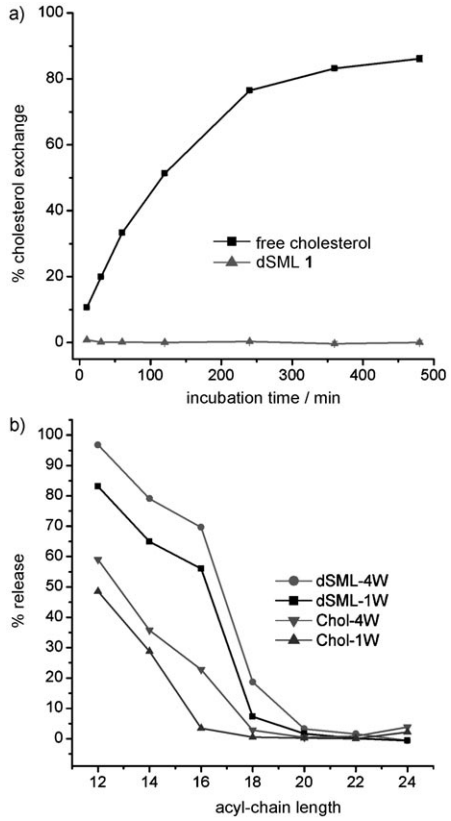


Figure 3. a) Rate of cholesterol exchange from the donor liposome to the acceptor liposome (present in 10-fold excess) at 37 °C. b) Leakage of carboxyfluorescein (CF) from liposomes in fetal bovine serum (30 vol %) in HEPES-buffered saline (pH 7.4) at 37 °C. The release of CF from liposomes was monitored by measuring the change in the fluorescence intensity at different incubation times. The percentage of CF released from the liposome on day 7 (1W) and day 28 (4W) was calculated and plotted against the acyl-chain length in the liposome formulations. Each data point is the average of three measurements with a standard deviation less than 5 %.

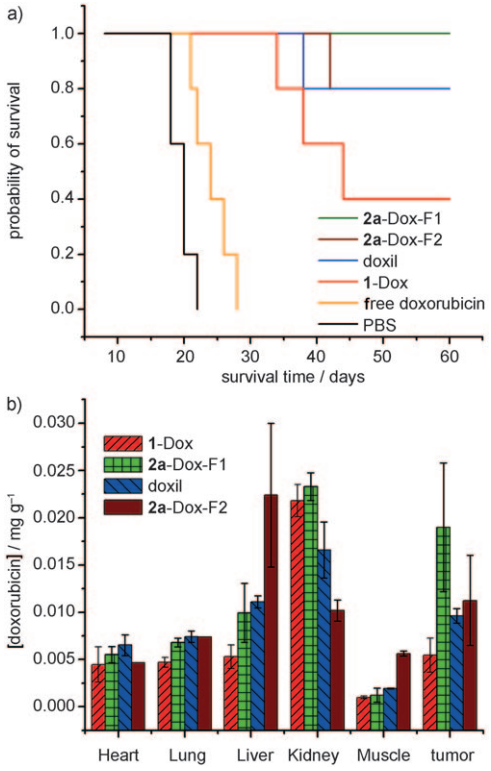


Figure 4. Survival and biodistribution data for doxorubicin-loaded dSML liposomes in BALB/C mice with a C26 tumor. A single dose of liposomal doxorubicin (15 mg kg^{-1}) or free doxorubicin (10 mg kg^{-1}) was administered intravenously on day 8 after tumor inoculation. a) Survival curves. **1**-Dox: dSML **1**/DSPC/DSPE–PEG2000/α-tocopherol (33.0/61.8/5.0/0.2; PEG = poly(ethylene glycol)); **2a**-Dox-F1: dSML **2a**/DSPC/DSPE–PEG2000/α-tocopherol (33.0/61.8/5.0/0.2); **2a**-Dox-F2: dSML **2a**/DSPE–PEG2000/α-tocopherol (94.8/5.0/0.2). Log-rank analysis of the paired survival data: $p = 0.0019$ for **1**-Dox, **2a**-Dox-F1, **2a**-Dox-F2, and doxil versus phosphate-buffered saline (PBS); $p = 0.0018$ for **1**-Dox, **2a**-Dox-F1, **2a**-Dox-F2, and doxil versus free doxorubicin; no significant difference between **1**-Dox, **2a**-Dox-F1, **2a**-Dox-F2, and doxil. b) Biodistribution of liposomal doxorubicin 48 h after i.v. injection of a 15 mg kg^{-1} dose.

osomes and conventional liposomes showed chain-length-dependent leakage profiles, whereby higher stability was observed for lipids with longer acyl chains. This trend is true for leakage measured at both day 7 and day 28. The conventional liposome composition with free cholesterol was more stable up to an acyl-chain length of 18 carbon atoms; thereafter, liposomes containing the dSML were as stable as vesicles containing free cholesterol.

On the basis of the above properties of dSMLs, we chose to compare the drug-carrier properties of liposome formulations with dSMLs to those of liposome formulations with free cholesterol for the delivery of the antitumor drug doxorubicin. Doxorubicin was encapsulated in the liposomes by the remote loading method,[21] and the antitumor effect was evaluated in a C26 murine adenocarcinoma model[22] (see the Supporting Information). With a single intravenous (i.v.) dose of doxorubicin (15 mg kg^{-1}) on day 8 after tumor inoculation, all the mice ($n = 5$) treated with **2a**-Dox-F1, a formulation similar to doxil (FDA-approved pegylated liposomal doxorubicin), were cured (with no palpable tumor) without evident toxicity (Figure 4a; see the Supporting Information for tumor growth curves). Notably, the therapeutic effect of **2a**-Dox-F2, a formulation with 94.8 mol % **2a** and no 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC), was comparable to that of **2a**-Dox-F1. The antitumor effects of both formulations were equivalent to that of doxil, and all formulations were superior to free doxorubicin. Liposomes composed of dSML **1**–doxorubicin had a weaker antitumor effect, and the accumulation of the drug in the tumor was lower (Figure 4b). Formulation **2a**-Dox-F2 has one component fewer (DSPC) than all other formulations. This difference could be an advantage for manufacture and quality control.

In summary, we have created a new class of sterol-modified lipids, dSMLs, by linking sterols to both hydroxy groups of glycerophosphocholine in a one-pot synthesis. It will be possible to synthesize a variety of dSMLs by varying the combination of sterols, the linkages, and the head groups. dSML lipids preserve the condensing effect on lipid-chain packing but are not transferred. They extend the range of lipid compositions that can be employed to control contents

release from liposomes, and dSML **2a** can be used by itself to form a lipid vesicle. Doxorubicin encapsulated in dSML liposomes accumulated in murine C26 tumor and showed a therapeutic effect equivalent to that of doxil. The promising results of this study open the way for further therapeutic studies with water-soluble drugs, such as gemcitabine, erlotinib, vinorelbine, topotecan, floxuridine, vincristine, and platinum compounds, for which no therapeutically successful formulations have yet been produced with traditional liposome compositions.[1–3]

Received: January 7, 2009
Published online: May 7, 2009

Keywords: antitumor agents · cholesterol · drug delivery · liposome formulation · phospholipids

[1] T. Lammers, W. E. Hennink, G. Storm, *Br. J. Cancer* **2008**, *99*, 392.
[2] V. P. Torchilin, *Nat. Rev. Drug Discovery* **2005**, *4*, 145.
[3] D. C. Drummond, C. O. Noble, M. E. Hayes, J. W. Park, D. B. Kirpotin, *J. Pharm. Sci.* **2008**, *97*, 4696.
[4] T. P. McMullen, R. N. Lewis, R. N. McElhaney, *Biochemistry* **1993**, *32*, 516.
[5] M. Z. Lai, N. Duzgunes, F. C. Szoka, *Biochemistry* **1985**, *24*, 1646.
[6] D. Papahadjopoulos, S. Nir, S. Oki, *Biochim. Biophys. Acta Biomembr.* **1972**, *266*, 561.
[7] J. Senior, G. Gregoriadis, *Life Sci.* **1982**, *30*, 2123.
[8] S. C. Semple, A. Chonn, P. R. Cullis, *Biochemistry* **1996**, *35*, 2521.
[9] M. C. Phillips, W. J. Johnson, G. H. Rothblat, *Biochim. Biophys. Acta Rev. Biomembr.* **1987**, *906*, 223.
[10] V. P. Torchilin, V. Weissig, *Liposomes: A Practical Approach*, 2nd ed., Oxford University Press, Oxford, New York, **2003**.
[11] D. D. Lasic, *Angew. Chem.* **1994**, *106*, 1765; *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 1685.
[12] B. Martin, M. Sainlos, A. Aissaoui, N. Oudrhiri, M. Hauchecorne, J. P. Vigneron, J. M. Lehn, P. Lehn, *Curr. Pharm. Des.* **2005**, *11*, 375.
[13] M. P. Krafft, J. G. Riess, *Biochimie* **1998**, *80*, 489.
[14] L. Linderoth, G. H. Peters, R. Madsen, T. L. Andresen, *Angew. Chem.* **2009**, *121*, 1855; *Angew. Chem. Int. Ed.* **2009**, *48*, 1823.
[15] V. Janout, S. L. Regen, *Bioconjugate Chem.* **2009**, *20*, 183.
[16] A. Taubert, A. Napoli, W. Meier, *Curr. Opin. Chem. Biol.* **2004**, *8*, 598.
[17] Z. Huang, F. C. Szoka, Jr., *J. Am. Chem. Soc.* **2008**, *130*, 15702.
[18] S. C. Davis, F. C. Szoka, Jr., *Bioconjug. Chem.* **1998**, *9*, 783.
[19] C. C. Kan, J. Yan, R. Bittman, *Biochemistry* **1992**, *31*, 1866.
[20] D. R. Wybenga, V. J. Pileggi, P. H. Dirstine, J. Di Giorgio, *Clin. Chem.* **1970**, *16*, 980.
[21] E. M. Bolotin, R. Cohen, L. K. Bar, N. Emanuel, S. Ninio, D. D. Lasic, Y. Barenholz, *J. Liposome Res.* **1994**, *4*, 455.
[22] S. K. Huang, E. Mayhew, S. Gilani, D. D. Lasic, F. J. Martin, D. Papahadjopoulos, *Cancer Res.* **1992**, *52*, 6774.

