Intra- and Interliposomal Effects of Cholesterol on Pseudoglyceryl Cationic Liposomes

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The importance of cholesterol as a component of animal cell membranes has fostered many studies of its properties in biological and model membranes and liposomes.[1,2] Of particular interest are the *intraliposomal* effects of the sterol on the properties of the constituent lipids and their liposomal aggregates[1-3] and the *interliposomal* transport of cholesterol between donor and acceptor liposomes.[4,5] The latter topic intersects the important area of drug delivery via liposomes.[6]

Our development of methodology for the chemical differentiation of lipid head groups in the outer (exo) and inner (endo) leaflets of synthetic bilayer liposomes,[7] together with a protocol for following the subsequent endo-to-exo trans-bilayer migration (flip-flop) of the differentiated lipids, allowed us to study the connection between lipid molecular structure and intraliposomal dynamics,[8] and recently, we began an investigation of the effects of extrinsic modulators (*e.g.*, bound polymers) on intraliposomal dynamics.[9]

Here, we describe the effects of cholesterol on the intraliposomal dynamics of the model liposomal membrane constructed from the cationic, pseudoglyceryl lipids **1**-F and **1**-NF.[8a] Not only do the results reveal a pronounced sensitivity of trans-bilayer lipid migration to cholesterol content, but they also form the basis of a remarkably simple assay of interliposomal cholesterol transfer that requires neither the separation of donor and acceptor liposomes nor the use of radiolabeling or fluorescence methodologies. The latter procedures significantly complicate the tracking of cholesterol transfer.[4]

Intraliposomal Effects. Small, unilamellar coliposomes of 1:7 functional (**1**-F) and nonfunctional (**1**-NF) pseudoglyceryl ammonium ion lipids,[8a] containing variable quantities of cholesterol, were created from $CHCl_3$-cast films of the ternary mixtures in 10 mM aqueous KCl–HCl solutions at pH 3.9. Solutions were preincubated and purged with nitrogen at 25 °C for 10 min and then sonicated (3 min, 55 °C, 60 W, immersion probe), cooled to 25 °C, and filtered through 0.8-μm Millex filters. Final lipid concentrations were 5×10^{-5} M **1**-F and 3.5×10^{-4} M **1**-NF

Coliposomes prepared in this way and containing 0–20 mol % of cholesterol were 37–40 nm in diameter by dynamic light scattering[7] and exhibited a gel-to-liquid crystalline phase transition (T_c) at 43–44 °C, as determined from the temperature dependence

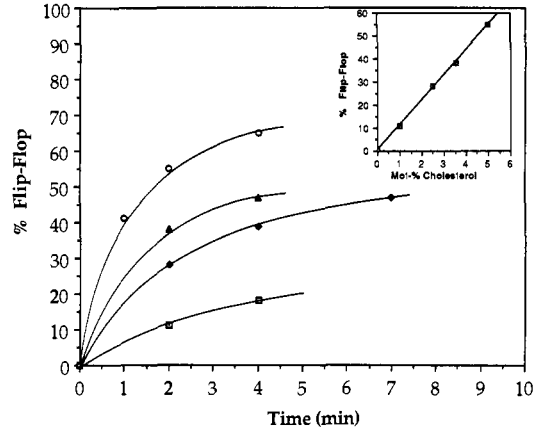
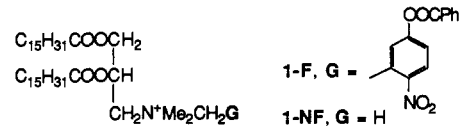


Figure 1. Percent flip-flop of surface-differentiated coliposomes of 1:7 **1**-F/**1**-NF as a function of incubation time at 40 °C. From bottom to top, the curves represent coliposomes containing 1, 2.5, 3.5, or 5 mol % of cholesterol. Inset: linear relationship ($r = 1.000$) between % flip-flop and mol % cholesterol in the **1**-F/**1**-NF coliposomes after 2 min of incubation at 40 °C.



of the fluorescence polarization of added *trans*-1,6-diphenyl-1,3,5-hexatriene.[10]

The cholesterol-loaded coliposomes of 1:7 **1**-F/**1**-NF were surface differentiated by brief exposure to 1×10^{-4} M glutathione at 25 °C in 0.005 M Tris buffer, pH 8, $\mu = 0.01$ (KCl).[7,8a-c] Rapid ($k = 0.09\ s^{-1}$) cleavage of the exoliposomal *p*-nitrophenyl benzoate esters of **1**-F afforded *p*-nitrophenylate residues that were readily monitored by UV spectroscopy at 400 nm. Exoliposomal cleavage was complete in 1.5 min,[11] and the flip-flop protocol[7,8] was then initiated. The external pH was lowered to 2.5 with HCl; the liposomes were incubated for several minutes at 40 °C to induce flip-flop and then recooled to 25 °C. Readjustment to pH 8 with NaOH then generated a new, rapidly-formed *p*-nitrophenylate absorbance that represented the esterolysis of formerly endoliposomal **1**-F lipids that had flipped to exo sites during incubation. Residual endoliposomal **1**-F was then determined by hydrolysis at pH 8 (70 °C, 10 min.).

The extent of flip-flop, expressed as a percentage of the 22–25% endoliposomal **1**-F remaining after the initial surface differentiation, was strongly dependent on the cholesterol content of the coliposomes. The data appear in Figure 1, where % flip-flop is plotted against incubation time at 40 °C. The curves represent the time course of flip-flop for coliposomes containing 1, 2.5, 3.5, and 5 mol % of cholesterol.[12] At 40 °C, below the T_c, 1–5 mol % of cholesterol *enhances* the flip-flop mobility of **1**-F, in accord with previous observations concering its effect on phospholipid mobility in gel-phase liposomes.[13]

Most interestingly, the extent of flip-flop for the coliposomes with cholesterol loadings of 1–5 mol % is found to be *linear* in cholesterol when we examine data for 2-min (or 4-min) incubations at 40 °C (see inset, Figure 1). This correlation time implies that

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(2) Yeagle, P., Ed.; *The Structure of Biological Membranes*; CRC Press: Boca Raton, FL, 1992.
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(4) Bittman, R. In ref 1, pp 45–65.
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(6) Ostro, M. J., Ed. *Liposomes: From Biophysics to Therapeutics*; Dekker: New York, 1987.
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(8) (a) Moss, R. A.; Ganguli, S.; Okumura, Y.; Fujita, T. *J. Am. Chem. Soc.* **1990**, *112*, 6392. (b) Moss, R. A.; Fujita, T.; Okumura, Y. *Langmuir* **1991**, *7*, 440. (c) Moss, R. A.; Bhattacharya, S. *J. Phys. Org. Chem.* **1992**, *5*, 467. (d) Moss, R. A.; Okumura, Y. *J. Am. Chem. Soc.* **1992**, *114*, 1750. (e) Moss, R. A.; Li, J.-M. *Ibid.* **1992**, *114*, 9227. Corrections were made to take account of OH^- permeation.
(9) Bhattacharya, S.; Moss, R. A.; Ringsdorf, H.; Simon, J. *J. Am. Chem. Soc.* **1993**, *115*, 3812.
(10) Andrich, M. P.; Vanderkooi, J. M. *Biochemistry* **1976**, *15*, 1257. Moss, R. A.; Swarup, S. *J. Org. Chem.* **1988**, *53*, 5860. At high cholesterol content (≥20 mol %), the phase transition (*i.e.*, the discontinuity in the polarization vs temperature) became indistinct. Cholesterol suppresses phase transitions; *cf.*, Lewis, N. A. H.; McElhaney, R. N. In ref 2, pp 123f.
(11) The exo/endo **1**-F distributions of these coliposomes ranged from 75:25 to 78:22.
(12) In the *absence* of cholesterol, flip-flop is insignificant after 10 min at 40 °C; see ref 8a.
(13) Finean, J. B. *Chem. Phys. Lipids* **1990**, *54*, 147. Yeagle, P. L. *Biochim. Biophys. Acta*, **1985**, *822*, 267.

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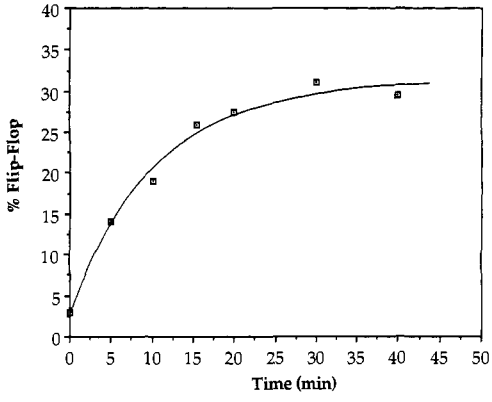

Figure 2. Results of donor-to-acceptor coliposome cholesterol transfer at 40 °C. Percent flip-flop in acceptor 1:7 **1**-F/**1**-NF coliposomes as a function of incubation time with 33 mol % cholesterol loaded donor coliposomes at 40 °C, pH 4. The donor/acceptor ratio was 5:95, and the subsequent flip-flop protocol involved a 2-min incubation at 40 °C. Longer incubation times of 60 or 120 min did not significantly alter the % flip-flop from that observed at 30 or 40 min.

a determination of % flip-flop for a **1**-F/**1**-NF coliposome containg x mol % of cholesterol ($x \leq 5$) will define the cholesterol content. A simple procedure for tracking cholesterol transfer between coliposomes can be based upon these observations.

Interliposomal Cholesterol Transfer. Aqueous solutions of "donor" 1:7 **1**-F/**1**-NF coliposomes, containing 33 mol % cholesterol, were mixed and incubated at pH 4 and 40 °C with solutions of similar "acceptor" coliposomes that contained no cholesterol. The donor/acceptor ratio was 5:95, so that if all of the cholesterol equilibrated, the acceptors would contain ~2.5 mol % of the sterol.[14,15] At various times, aliquots were removed, stored at 0 °C, and then subjected to the flip-flop protocol.[16] The observed % flip-flop (after 2 min of incubation at 40 °C) was a function of the time of the initial donor acceptor incubation at 40 °C, *cf.* Figure 2.

Based on the results contained in Figure 1, we interpret Figure 2 to mean that cholesterol is transferred from the donor to acceptor liposomes at 40 °C, thus enabling flip-flop when the coliposomes are subsequently subjected to the appropriate protocol.[12,17] Moreover, using the % flip-flop vs % cholesterol relation of the inset in Figure 1, the % flip-flop data of Figure 2 can be converted to a correlation of the mol % of cholesterol transferred as a function of time. A first-order kinetics treatment then affords $k = 1.8 \times 10^{-3}$ s^{-1} for cholesterol transfer at 40 °C.

We also obtained donor-to-acceptor cholesterol transfer data at 43 °C ($\sim T_c$) and 50 °C ($> T_c$). Analysis as above gave $k = 2.6 \times 10^{-3}$ s^{-1} (43 °C) and $k = 7.9 \times 10^{-3}$ s^{-1} (50 °C). Although the temperature interval is too small for a reliable Arrhenius treatment, we estimate E_a for cholesterol desorption from the donor coliposomes at ~30 kcal/mol and the half-time for the transfer process at 25 °C at ~1.3 h. The activation energy seems too large; the E_a for cholesterol transfer between phosphatidylcholine liposomes is only ~21 kcal/mol.[15] However, the transfer half-time seems reasonable,[4] especially in view of the small size and high curvature of our liposomes. These factors result in more facile desorption of cholesterol and faster transfer.[18] Of course, a major difference between our liposomes and those of previous cholesterol exchange experiments resides in the lipid head groups: cationic ammonium ions vs zwitterionic phosphocholines. The effects of this alteration are still unclear.

Cholesterol transfer from liposomes that contain sphingomyelin is significantly slowed, possibly due to "greater lateral packing density in the lipid–water interface when sphingomyelin is present."[4,19] Indeed, incorporation of *N*-palmitoyl sphingomyelin into the **1**-F/**1**-NF donor coliposomes[20] did slow cholesterol transfer to acceptor coliposomes: at 40 °C, $k = 8.4 \times 10^{-4}$ s^{-1} in the presence of sphingomyelin and 1.8×10^{-3} s^{-1} in its absence.

The novel method of following interliposomal cholesterol transfer described here is distinguished by its simplicity; analysis of acceptor cholesterol content based on lipid flip-flop does not require separation of the donor and acceptor liposomes, nor is it necessary to use labeled or fluorescent sterols.[15,18,21] This new methodology should be applicable whenever the transferred molecules stimulate lipid flip-flop in the acceptor lipsome. Applications to drug delivery[6] are obvious; for example, experiments with amphotericin B are now in progress in our laboratory and will be reported in due course.

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(14) Simple cholesterol transfer between phospholipid liposomes occurs by (rate-limiting) desorption from the donor liposome, followed by transit through solution to the acceptor.[4,5,15] The transfer of cholesterol is faster than lipid exchange between the liposomes,[5,15] and that sould also be true in our example, which features mutually repulsive cationic liposomes and lipids.

(15) McLean, L. R.; Phillips, M. C. *Biochemistry* **1981**, *20*, 2893. McLean, L. R.; Phillips, M. C. *Ibid.* **1982**, *21*, 4053.

(16) Apparent liposome size did not significantly increase during donor–acceptor incubation, mitigating against liposome fusion as a cholesterol transfer process. Controls indicated that significant cholesterol transfer did not occur over 30 min at 0 °C.

(17) The 19-fold excess of acceptor over donor coliposomes insures that the observed flip-flop is essentially all from the acceptors.

(18) McLean, L. R.; Phillips, M. C. *Biochim. Biophys. Acta* **1984**, *776*, 21. Thomas, P. D.; Poznarsky, M. J. *Biochem. J.* **1988**, *254*, 155.

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(20) The molar ratio of **1**-F/**1**-NF/sphingomylin was 1:3.5:3.5; cholesterol was 33 mol % of the total lipids.

(21) Examples of radiolabel methodology include: Wattenberg, B. W.; Silbert, D. F. *J. Biol. Chem.* **1983**, *258*, 2284. Bruckdorfer, K. R.; Sherry, M. K. *Biochim. Biophys. Acta* **1984**, *769*, 187. Fluorescence methodology is illustrated by: Nemecz, G.; Fontaine, R. N.; Schroeder, F.; *Biochim. Biophys. Acta* **1988**, *943*, 511. Hapala, I.; Butko, P.; Schroeder, F. *Chem. Phys. Lipids* **1990**, *56*, 37.