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KINETIC EVIDENCE FOR INTERDIGITATION IN MODEL LIPID BILAYERS

ROBERT A. MOSS* AND SOUMENDU BHATTACHARYA

Department of Chemistry, Rutgers, The State University of New Jersey, New Brunswick, New Jersey 08903, USA

Pseudoglyceryl ammonium ion coliposomes constructed from an arachidoyl probe lipid and either stearoyl or palmitoyl host lipids display unusually slow transverse bilayer migration ('flip-flop') of the arachidoyl probe molecule compared with the behavior of shorter probe lipids in similar colipsomes. This phenomenon is probably associated with interdigitation of the arachidoyl lipid across the bilayer's midplane.

INTRODUCTION

Liposomal bilayers are usually depicted as opposing monolayer leaflets with the acyl chain termini meeting in a well defined bilayer midplane. However, depending on structural factors, such as overall chain length and chain length asymmetry, one or both of the lipids' hydrocarbon chains might penetrate the opposing monolayer, affording interdigitated chains.[1] With glycerol-based phospholipid or glycosphingolipid bilayers, grossly mismatched chains that are longer than 16 carbon atoms can interdigitate among the chains of the opposing bilayer, leading to enhanced local chain order, decreased fluidity and better packing.[2,3]

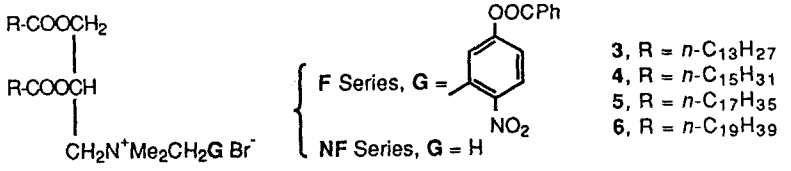
However, kinetic and dynamic studies of simple tetraalkylammonium ion vesicles or covesicles, constructed of surfactants **1** or **2** (with R and R′ varied from n-$C_{16}H_{33}$ to n-$C_{22}H_{45}$), provided no cogent evidence for chain interdigitation or enhanced bilayer packing or stability.[4] We now report on related experiments with liposomes derived from the pseudoglyceryl ammonium ion lipids **3**–**6**. Using the functional arachidoyl (eicosanoyl) lipid, **6-F**, as a probe, we find that liposomes constructed of **6-F**/**4-NF** and **6-F**/**5-NF** manifest unusual dynamic stability at 55 °C that may well be associated with chain interdigitation.

$R_2N^+MeAr,\ Br^-$ (1) $\qquad$ $RR'N^+MeAr,\ Br^-$ (2)

RESULTS

Synthesis

Lipids **4-F**, **4-NF**, **5-F** and **5-NF** were available from previous studies.[5,6] The functional and non-functional lipids of structures **3** and **6** were prepared as shown in Scheme 1. Thus, in the myristoyl series (**3**), *rac*-3-*N*,*N*-dimethylamino-1,2-propanediol (**7**) was acylated with myristoyl chloride (Et_3N, Et_2O, 0–25 °C, 48 h) affording *tert*-amine (**8**) in 89% yield. The latter was quaternized with MeBr (saturated in Et_2O, sealed tube, 25 °C, 5 days, 89%) to give **3-NF**, or with 3-(bromomethyl)-4-nitrophenyl benzoate[7] (Et_2O, 25 °C, 48 h, 68%) to yield **3-F**. The quaternary salts were purified either by recrystallization from acetone (**3-NF**) or by trituration with diethyl ether, followed by hot



* Author for correspondence.

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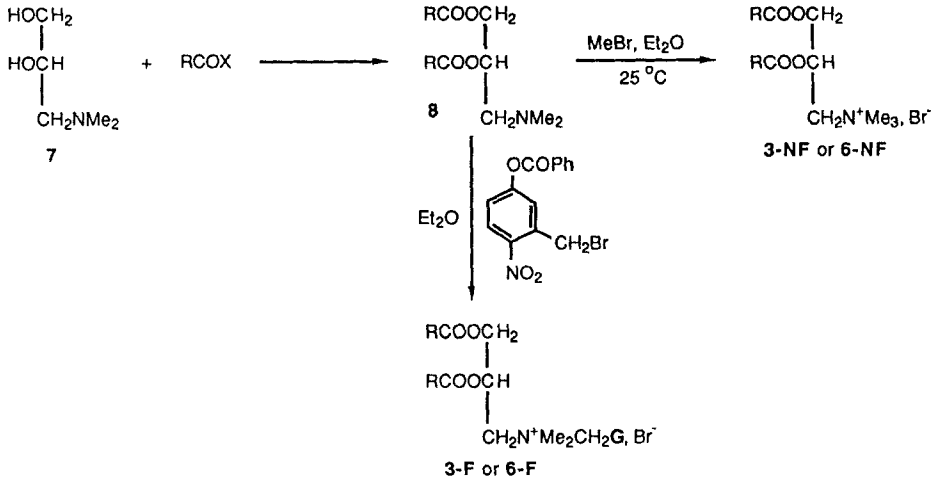

Scheme 1

acetone (**3-F**). Structures were established by 200 MHz ^{1}H NMR spectroscopy and by appropriate C,H,N microanalyses.

In the arachidoyl series (**6**), amine **7** was directly acylated with arachidic (eicosanoic) acid, using Ph_3PBr_2 as the coupling agent[8] (CH_2Cl_2, $NaHCO_3$, 25 °C, 12 h). The acylated product, **8**, was obtained in 55% yield, after elution through silica gel to remove Ph_3PO; it was used without further purification. Quaternization with MeBr (saturated in Et_2O, sealed tube, 25 °C, 12 days, 90%) afforded **6-NF** (recrystallized from $CHCl_3$), whereas reaction of **8** with 3-(bromomethyl)-4-nitrophenyl benzoate (acetone, 45 °C, 2 days, 66%) gave **6-F** (recrystallized from acetone). Both **6-F** and **6-NF** afforded appropriate ^{1}H NMR spectra and C,H,N microanalyses. Details of synthetic procedures, melting points, TLC conditions, and spectroscopic and analytical characterizations are available on request.

Kinetics and dynamics

Coliposomes of 1:7 blends of functional (F) and nonfunctional (NF) lipids were created by sonication (probe-type sonicator, 60 W, 3 min) of $CHCl_3$-cast lipid films in 0·01 M aqueous KCl solution that had been adjusted to pH 3·9 with HCl. The sonication temperature was maintained at or above the gel to liquid crystalline phase transition temperature (T_c) of the particular coliposome (see below).

The T_c values of the coliposomes were determined from temperature-dependent discontinuities in the fluorescence polarization of covesicallized 1,6-diphenylhexa-1,3,5-triene;[7,9] they are collected in Table 1. The T_c values increase with increasing acyl chain length in an appropriate manner. It is interesting that the T_c values of the heteroacyl (1:7) 20/16 and 20/18 coliposomes were only 2–3 °C lower than those of the corresponding homoacyl 16/16 and 18/18 systems.

Hydrodynamic diameters (d) of the coliposomes were obtained from dynamic light-scattering measurements (argon laser, 488 nm), and are also given in Table 1. Details of this procedure were given in a previous paper.[7] The coliposomes all had d values between 41 and 45 nm.

The coliposomes of 1:7 F/NF lipid pairs, prepared at pH 3·9, were surfaced differentiated[7] by brief exposure to 1×10^{-4} M glutathione in 0·005 M Tris buffer (pH 8), $\mu = 0{\cdot}01$ (KCl). The final lipid concentrations were $[F] = 5 \times 10^{-5}$ M and $[NF] = 3{\cdot}5 \times 10^{-4}$ M.

The exoliposomal p-nitrophenyl benzoate esters of the F lipids were rapidly cleaved ($k_f = 0{\cdot}1$–$0{\cdot}2\ s^{-1}$, Table 1), affording p-nitrophenylate residues that were monitored spectrophotometrically at 400 nm. Endoliposomal esterolyses occurred at slower rates (cf. the k_s values in Table 1), limited by counterion-coupled H^+/OH^- permeation across the liposomal membranes, and driven by the imposed pH 8/3·9 exo/endo gradient.[10] All cleavage processes were clearly biphasic; the division between exoliposomal and endolipsomal cleavage averaged *ca* 72:28% (cf. Table 1).

We note that k_s decreased with increasing acyl chain length of the liposomes, from $1{\cdot}3 \times 10^{-3}\ s^{-1}$ for the 14/14 system to $1{\cdot}7 \times 10^{-5}\ s^{-1}$ with the 20/20 coliposome. This expected phenomenon[10] reflects the increasing barrier to H^+/OH^- permeation afforded by the increasing hydrophobic membrane barriers created by the longer acyl chains.

Lipid dynamics within surface differentiated coliposomes could be studied according to our normal protocol.[5–7] Immediately following the cleavage of the exoliposomal benzoate groups, the bulk pH was

Table 1. Dynamics of liposomes

System[a]	Liposome[a]	d[b](nm)	T_c[c](°C)	k_f[d](s^{-1})	$10^4 k_s$[d](s^{-1})	%f : %s[e]	$t_{1/2}$[f](min)
14/14	**3-F/3-NF**	45	25	0.2[g,h]	13[g]	70 : 30	<0.5[g]
16/16	**4-F/4-NF**	41	44[i]	0.15	1.4	76 : 24	4
18/18	**5-F/5-NF**	44	59[j]	0.13	0.6	75 : 25	5
20/20	**6-F/6-NF**	45	70	0.10	0.17	67 : 33	>30
20/16	**6-F/4-NF**	45	42	0.17	0.94	69 : 31	>30
20/18	**6-F/5-NF**	44	56	0.15	0.40	77 : 23	18

[a] See text for structures; F/NF = 1 : 7. The entries under 'System' refer to the acyl chain lengths of the F and NF lipids (respectively) in the coliposome.
[b] Diameters from dynamic light scattering at pH 3·9, 0·01 M KCl.
[c] Temperature of gel to liquid crystal transition.
[d] *Rate constants* (±10%) for glutathione cleavage of exoliposomal (k_f) and endoliposomal (k_s) *p*-nitrophenyl benzoate moieties; see text. Reaction temperature is 25 °C, unless stated otherwise.
[e] Ratio (±10%) of fast to slow kinetic phases.
[f] Approximate (±20%) half-time for decay of surface differentation at 55 °C; see Refs 5 and 6.
[g] At 20 °C.
[h] [Glutathione] = 6×10^{-4} M; this concentration is 1×10^{-4} M in the other cases.
[i] Ref. 5.
[j] Ref. 6.

lowered to 3·9 with HCl, quenching further esterolysis. The liposomes were then incubated at 55 °C for periods of 2, 3, 5, 10, 25 or 30 min, fostering transverse lipid migration between the endo- and exoliposomal bilayer leaflets (i.e. lipid 'flip-flop'). Cooling to 25 °C (20 °C for 14/14) and readjustment to pH 8 with NaOH then generated a new fast (k_f) *p*-nitrophenylate absorbance that was taken to represent the rapid esterolysis of formerly endolipsomal G residues that had flipped with their lipid molecules to exoliposomal sites during incubation.[5-7] Residual endoliposomal G groups were detected in subsequent, slow (k_s) esterolysis reactions. The sum of all observed *p*-nitrophenylate absorbances (initial k_f, post-incubation k_f and k_s) came to within 10% of the stoichiometric value.

The observed partition between the new, post-incubation k_f and the residual k_s esterolyses gives the extent of flip-flop equilibration induced in the surface differentiated liposomes by the incubation process. The approximate equilibration half-times ($t_{1/2}$) estimated from four incubation experiments at 55 °C (with incubation times chosen from those cited above) are given in Table 1 for the six coliposomal systems that were studied.

DISCUSSION

It is clear that, at 55 °C, reequilibrations of the exo/endo differentiated coliposomes are relatively fast ($t_{1/2} < 5$ min) for the 14/14, 16/16 and 18/18 systems, but considerably slower for the 20/16, 20/18 and 20/20 systems, where the functional arachidoyl lipid, **6-F**, is employed as the flip-flop probe. With the 20/20 coliposomes, very slow flip-flop is expected at 55 °C because these aggregates are in their less fluid gel phase ($T_c = 70$ °C). In contrast, the T_c values of 18/18 and 20/18, and also those of 16/16 and 20/16, are closely matched, with the heteroacyl liposomes possessing a slightly lower T_c in each case. Nevertheless, the 20/18 and 20/16 coliposomes are much more resistant to flip-flop than are their 18/18 and 16/16 analogues.

We attribute these behavioral differences to interdigitation of the 20 (**6-F**) arachidoyl probe lipid molecules across the bilayer midplanes of their coliposomes with the nonfunctional 18 (**5-NF**) or 16 (**4-NF**) host lipids. Thus, extension of the C_{20} acyl chains of **6-F** into the C_{18} or C_{16} acyl chains of the host **5-NF** or **4-NF** lipids in the opposing leaflet of the bilayer would enhance the resistance of the **6-F** probe lipids to thermally driven flip-flop.

We note that it is not simply the sum of the acyl chain lengths in the two bilayer leaflets that is important. The homoacyl 18/18 systems (**5-F/5-NF**), acyl carbon sum = 36, has $t_{1/2} \approx 5$ min at 55 °C, whereas the heteroacyl 20/16 (**6-F/4-NF**) coliposome, with the same acyl carbon sum, has $t_{1/2} > 30$ min, and is much more resistant to flip-flop. Apparantly, a mismatch in acyl chain length is important to chain interdigitation.[1-3] In this respect, the arachidoyl pseudoglyceryl ammonium ion probe lipid **6-F** appears to resemble certain long-chain interdigitating phospholipids.[1-3]

One may question whether the effects reported here might in some way be attributable to domain formation within the heteroacyl coliposomes. This seems unlikely because all of the coliposome systems display similar sizes and comparable kinetic phasings (i.e., %f:%s), in addition to T_c values and k_s rate constants that vary rationally with acyl chain length. There are no behavioral discontinuities that might signal domain formation.

We are intrigued by the apparently greater resistance to flip-flop of the 20/16 coliposomes vs the 20/18 system

($t_{1/2} > 30$ min vs $t_{1/2} \approx 18$ min). Perhaps there is greater (and more effective) interdigitation when there is a larger mismatch in the chain lengths of the probe and host lipids. In a very simple model, where the bilayer's host lipids do not interdigitate, and all lipid head groups align at the membrane surfaces, the C_{20} probe lipid would insert two carbons into an opposing C_{18} lipid leaflet, but it would intrude to a depth of four carbon atoms into an opposing C_{16} lipid leaflet.

The effects, however, must be delicately balanced. If the incubations of surface differentiated 20/16 and 20/18 coliposomes are carried out at 60 °C, rather than 55 °C, the $t_{1/2}$ values for both systems decrease to about 5 min; differential and overall dynamic stabilities are undermined together.

Finally, we note that these apparent effects of interdigitation, although observable with liposomes derived from the better packed[5] pseudoglyceryl ammonium ion lipids of general structure **3–6**, are not seen with liposomes constructed from the simple ammonium ion surfactants **1** or **2**.[4]

CONCLUSION

The arachidoyl pseudoglyceryl ammonium ion probe lipid **6-F** exhibits low transverse bilayer migration ('flip-flop') in coliposomes with stearoyl (**5-NF**) or palmitoyl (**4-NF**) host lipids. This behavior is most likely associated with interdigitation of the arachidoyl lipid.

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