Synthesis and vesicle formation from novel pseudoglyceryl dimeric lipids. Evidence of formation of widely different membrane organizations with exceptional thermotropic properties

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Eight new bis-cationic dimeric lipids 2a-h have been synthesized; TEM of their aqueous dispersions confirmed the vesicle formation and from the thermal, spectroscopic, DLS and XRD studies it has been revealed that they form three different kinds of membranous aggregate depending on the *m*-value.

While the properties of vesicular membranes derived from a large number of 'monomeric' natural lipids¹ as well as their synthetic analogues² have been examined in great detail, there is no report in the literature that examines the properties of the membranes prepared from 'multimeric' lipids. This is despite the fact that several 'dimeric' and 'multimeric' lipid systems occur naturally and have important biological functions. Thus, cardiolipins (glycerol bridged dimeric phosphatidate lipids) constitute a class of complex phospholipids occurring mainly in the heart and skeletal muscles and show high metabolic activity.³ Similarly, glycolipid A2 (composed of two phosphate headgroups and seven hydrophobic chains) serves as immunomodulator in mediating selective recognition of toxins, bacteria or viruses at the cell surfaces.⁴

Although dimeric (gemini) amphiphiles^{5,6} that aggregate to form micelles are known, no analogous gemini form of lipids has been studied. Since cationic liposomes by themselves are receiving enormous current attention as effective non-viral gene transfection agents,7 the resulting aggregates of dimeric lipids if cationic might be potentially useful. Along the lines of the above considerations, here we report the first synthesis of a family of bis-cationic, gemini lipids 2a-h and their unusual membrane-forming properties on the basis of a combination of thermal, spectroscopic, dynamic light scattering (DLS), transmission electron microscopic (TEM) and reflection X-ray diffraction (XRD) methods supported by energy-minimization studies. To place the experimental data on these novel gemini membranes into appropriate perspective, we also include herein the related results from the vesicles of the corresponding 'monomeric' (control) lipid 1.

Altogether eight new dimeric lipid systems $2\mathbf{a}-\mathbf{h}$ were synthesized (Scheme 1). First the CH₂OH group of the building



Scheme 1 Reagents and conditions: i, BnCl, KOH, C_6H_6 , reflux, 87%; ii, aq. HCl-MeOH, reflux, 84%; *n*- $C_{16}H_{33}Br$, KOH, C_6H_6 , reflux, 58%; iii, Pd-C/H₂, 60%; iv, CBr₄, PPh₃, CH₂Cl₂, 90%; v, Me₂NH–EtOH, heat, pressure tube, 95%; vi, Br(CH₂)_mBr, EtOH, reflux, 60–75% (exact yields depend on *m*); vii, Me₃N–EtOH, heat, pressure tube, 80%

block, 1,2-isopropylideneglycerol, **3** was protected in the form of a benzyl ether **4**. The isopropylidene group was then removed by treatment with HCl in aq. MeOH and the diol obtained upon preparative column chromatography over silica gel was alkylated with n-C₁₆H₃₃Br (2.2 equiv.) to give **5** which upon hydrogenolysis afforded **6** in ~ 60% yield. Compound **6** was then converted to the corresponding bromide **7** by reaction with PPh₃-CBr₄; **7** was quaternized in the presence of Me₃N in EtOH to give **1** in 80% yield. The bisquaternary systems **2a**-**h** were obtained from the corresponding dimethylamino derivative **8** upon refluxing with individual alkanediyl α, ω -dibromides in 60–75% yields.

Dispersion of lipids 1, 2a–h in water using probe or bath sonication afforded liposomes with a unimodal distribution of particle diameters as revealed by DLS (Table 1). Examination of DLS data on bath sonicated vesicles shows that vesicles from dimeric lipids were invariably larger. Within the vesicles among the dimeric lipid series, however, the hydrodynamic diameter variation as a function of *m*-value was very complex. The vesicular sizes were found to be the largest with lipids, 2, of m = 3 and then decreased with increase in *m*-value up to m = 12 and then went up again with m > 12. Vesicle sizes were also found to strongly depend on the lipid concentration, thermal history and method of vesicle preparation. Thus while vortexing afforded mostly open lamellae and sonication gave small spherical, closed microstructures, reverse phase evaporation (REV) gave multilamellar vesicles (not shown).

Table 1 The hydrodynamic diameters of vesicular aggregates, main phasetransition temperatures of vesicular 1, 2a–h and the unit-layer thicknesses of their corresponding self-supporting cast films

		Main transition ^b (T/°C)		Unit bilayer thickness/Å	
Lipid (<i>m</i> -value)	DLS ^a size/nm	Microcal	Fluor. Pol.	Obs ^c	Calc. ^d
0	15	45.2	45	45.9	49
3	93	57 <i>°</i>	57	45	49
4	52	48.3	48	45.9	49
5	29	44.2	43	45.9	49
6	29	43.9	43	44.2	49
12	30	46.5	45	31.3	49, 16.8, f 31 g
16	37	40.8	41	29.6	49, 21.9, f 31 g
20	ND	69.1	62	29.8	49, 27 ^f
22	52	66.3	53	30.8	49, 29 ^{<i>f</i>}

^{*a*} Dynamic light scattering was examined with the vesicles prepared under identical conditions. All the vesicles $(1 \times 10^{-3} \text{ M})$ were prepared by bath sonication (Julabo USR3) for 10 min, at 70 °C and at 35 KHz. ^{*b*} Average deviation is ±0.5 °C for microcalorimetry; ±1.0 °C for fluorescence polarization method. ^{*c*} As obtained from reflection X-ray diffraction of cast films. ^{*d*} Lengths of two molecular layers of lipids as obtained from models. ^{*e*} Additional minor peaks at 66.1 and 71.6 °C were also observed. ^{*f*} Lengths of unit 'mono'layer-bilayer plans of lipids as obtained from models ^{*g*} Lengths of unit interdigitated and tilted bilayer plans of lipids as obtained from models.



Fig. 1 Schematic representation of possible bilayer forming motifs with gemini lipids as a function of *m*-value

The sonicated dispersions of different lipids (7 mM) were then converted to regular self-supporting films by casting on glass slides as described.⁸ Reflection X-ray diffraction (Scintag XDS-2000) of these cast films gave the long spacings from individual lipid films as given in Table 1. Comparison of the corresponding molecular lengths of the gemini lipid units as determined from CPK models suggests the formation of slightly tilted bilayer arrangement [Fig. 1(*b*)] with **1** and with gemini lipids of *m*-values 3, 4, 5 and 6. But with **2e**, m = 12, the experimentally determined XRD data on the unit layer thicknesses cannot be explained by tilting alone suggesting the presence of additional interdigitations [Fig. 1(*d*)]. The alternative plan (*e*) is energetically unfavorable with the lipophilic chains protruding into the polar headgroup regions. To explain the reflection XRD data of **2g** and **2h**, we suggest a kind of monolayer–bilayer hybrid organization as shown in Fig. 1(*f*).

Independent examination of the thermotropic properties of the vesicles of 1 and 2a-h by DSC, microcalorimetry and fluorescence methods showed that the changes in *m*-value in 2 profoundly influence their gel-to-liquid crystalline phasetransition temperatures (Table 1). Vortexing of 60 mM of either of 1 and 2a-h in water afforded lameller gels which gave wellresolved, nearly reversible peaks as melting transitions (T_m) (Perkin Elmer DSC 7). Microcalorimetry (MC-2) of 1.0-2.5 mm sonicated vesicular specimens of either of the lipids reproduced the peaks due to $T_{\rm m}$. Relative to 1, there is a substantial increase in the gel-to-liquid crystalline transition temperature of the membranes derived from dimeric 2a. In addition, unlike the membranes that are assembled from 1, which show a sharp endothermic main transition at 45 °C, those made from 2a exhibit a more complex melting pattern. For lipids with $m \ge 16$, the melting transition profiles appeared remarkably broadened (less cooperative), while for lipids with $m \leq 12$, the main transitions were quite sharp. Thus although the cooperativities of melting transitions with 2 (m = 20-22) were severely reduced, alternative membrane organizational features allow broad but higher melting temperatures (see below). The latter could be a consequence of perturbation within the headgroup and/or direct interaction of the spacer chain with the hydrocarbon chain regions.

The fluorescence polarizations (*P*) due to the vesicle doped 1,6-diphenylhexa-1,3,5,-triene at various temperatures were then determined. The temperatures related to the break in *P* vs. *T* plot for the vesicles with m = 0-16 were similar to the $T_{\rm m}$ values obtained by calorimetric methods (Table 1). But the

lipids with m = 20 and 22 gave broader *P vs. T* variation, the mid-points of which were lower than the $T_{\rm m}$ values obtained using microcalorimetry. Notably again the microviscosities (*ca.* 3.5–4.0 poise) of the vesicular aggregates of lipids with m = 20-22 in their gel states were considerably lower than that of the lipids with lower *m*-values (*ca.* 10–11 poise) again suggesting gross differences in their suprastructural organizations.

Molecular mechanics calculation of the energy-minimized conformations of the dimeric lipids using DISCOVER (IN-SIGHT II) suggested that the packing in 2a and 2b, where Me₂N⁺ headgroups are separated by three and four CH₂ units respectively, is much tighter than that in lipids with higher *m*-values. This should lead to a progressive decrease in $T_{\rm m}$ as m-value increases from 3 to 10. As the m-value in lipid, 2, reaches ≥ 12 , this situation changes when the (CH₂)₁₂ connector in 2e 'loops' into the vesicle interior to avoid 'undesirable' contacts with the bulk water. At m = 16, the spacer chain should adopt an even more folded, wicket-like conformation further impairing the bilayer packing.[‡] The greater extent of spacer chain looping into the membrane interior should disturb 'intra'gemini lipid packing at m = 16 even further [Fig. 1(d)]. This is consistent with the observation of lower $T_{\rm m}$ value with vesicular 2f than that with 2e. As the *m*-value surpasses 16 in 2, the suprastructural organizations in the membranes change again. This is because at $m \ge 20$, the length of spacer may now span as a 'monolayer' giving rise to 'mono'layer-bilayer hybrid type of suprastructures. Thus, strikingly, when *m*-value reaches ≥ 20 , spacer chains probably hyperextend to produce (absolutely unrelated to the ones with lower *m*-values) 'monolayer' type organizations reminiscent of the assemblies seen in the bolaamphiphilic membranes [Fig. 1(f)].⁹

Since the freedom of motion of four independent hydrocarbon chains is critical to their properties at the membrane level, the present study clearly demonstrates that the interconnection of the 'monomeric' lipid by a polymethylene segment into a 'dimeric' lipid brings about ramifications far exceeding the seemingly trivial structural modification at the lipid level. These findings further emphasize the need for newer designs of synthetic lipid structures to expand the understanding of their behaviour upon membrane formation.

Footnotes and References

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[‡] This is consistent with the observations of E. Alami, G. Beinert, P. Marie and R. Zana, *Langmuir*, 1993, **9**, 1465.

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