Membrane formation from oxyethylene bearing cationic cholesterol derivatives†

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Received 26 December 2000; accepted 23 May 2001

Eight cholesterol based amphiphiles, 1a-d and 2a-d, bearing differing lengths of oxyethylene units at different locations, have been synthesized. Membrane formation from the aqueous suspensions of these compounds has been confirmed by transmission electron microscopy and dye entrapment. X-Ray diffraction and fluorescence anisotropy measurements reveal modulation of the membrane characteristics by both the length and location of the oxyethylene segment.

Cholesterol is the third major component of animal membranes after phosphoglycerides and glycolipids.1 Its polar OH group at 3-position gives it a weak amphiphilic character, while the fused rings of its hydrophobic portion provide it with greater rigidity than other lipids in membranes. Hence cholesterol is an important determinant of the membrane properties2 even though cholesterol on its own cannot aggregate in water to form membranes. One way to achieve vesicle formation from cholesterol derivatives alone would be through covalent attachment of a charged residue to the cholesteryl backbone. Indeed examples are known where polar derivatives of cholesterol have been shown to form vesicles or related aggregates3. However, so far there has been no attempt to exploit cholesterol based amphiphiles to develop membranes, the organization and thickness of which could be modulated by molecular design.

We have previously examined the behavior of electrostatically complementary amphiphiles4 and the effects of insertion of a polymethylene spacer chain between two lipid monomers5. An alternative concept in lipid molecular design is introduced herein by the incorporation of oligo-oxyethylene units either at the headgroup bearing cholesteryl or between the cholesteryl backbone and the headgroup. Herein we report the synthesis of two families of cationic cholamphiphiles, 1a-d and 2a-d containing oxyethylene units (Scheme I).

In the first series of cholesteryl derivatives la-d, the cationic headgroup is linked to the 3β-cholesterol via an ester group. In all of these derivatives, the positive charge is located at a fixed distance from the cholesteryl backbone although the headgroup hydration is continually modified with the progressive increase in the number of oxyethylene units. In order to see the difference in properties, if any, depending on the sequence in which the two moieties were attached another series of cationic cholesteryl amphiphiles 2a-d has also been synthesized. In the second series of cholesterol derivatives, the cationic NMe₃⁺ groups are linked at the 3β-OH of cholesterol via an ether link. The cationic NMe₃⁺ group is however, placed at incrementally greater distance from the cholesteryl backbone with the insertion of increasing number of oxyethylene units in 2a-d.

Cholesteryl tosylate, 3 upon refluxing in dry dioxane with the appropriate oligoethylene glycol in slight excess yielded 4a-d in good yields. 4a-d were then converted to the respective tosylates, 5a-d which upon heating to ~65 °C with 1 eq. LiBr in dry DMF furnished the corresponding bromides, 6a-d, in excellent yields. Quaternization of the bromides with the appropriate tertiary amine in a 1:1 mixture of dry acetone/EtOH yielded the amphiphiles 2a-d, in moderate to good isolated yields. Cholesteryl bromoacetate was similarly quaternized with the appropriate tertiary amine to yield 1a-d also in good yields.†

Upon dispersal in water (0.4 mM) by sonication 1a-d and 2a-d gave opalescent suspensions5. TEM examination (JEOL 200CX) of the individual, air-dried suspensions of 1a-d or 2a-d layered on carbon-formvar coated copper grids revealed the existence of polydisperse, closed aggregate structures in all the cases. Interestingly the cholesteryl derivatives 1a-c formed predominantly unilamellar, nearly spherical

†Dedicated to Prof. U. R. Ghatak on his 70th birthday.

†All the amphiphiles were characterized by IR, 1H NMR, MALDI-TOF and elemental analysis to establish their chemical purity and are consistent with their proposed structures.

‡The desired weight of amphiphile was dissolved in CHCl₃ (0.5 mL) and then dried first under a stream of N₂ and then under high vacuum for another 1.5 hr to yield a thin film of amphiphile. After this, requisite amount of water (Millipore, pH=6.8) was added and left for 10 min. Bath sonication (35 kHz) for 10 min at ~60°C yielded translucent, stable aqueous suspensions.
vesicles. In contrast, 1d and 2a-d generated mostly multilamellar vesicles (not shown). Closer scrutiny suggested that the thickness of each lamella was highly uniform in a given vesicular suspension for all the samples 1d and 2a-d. Although there was no correlation in size of the vesicles so formed for a given series of amphiphile, the size distributions ranged from ~40 nm to ~400 nm.

Having confirmed vesicle formation from these newly developed cholesteryl derivatives, dye entrapment experiments\(^\text{17}\) were performed to examine whether these vesicles comprised closed, inner aqueous compartments. For this purpose, a watersoluble dye, methylene blue (MB), which has been encapsulated in inner aqueous compartments of vesicles of other amphiphiles,\(^3\) was used. Importantly in all the cases, MB entrapped vesicles could be distinctly separated from the 'free' MB molecules confirming the presence of closed structures containing an inner aqueous compartment. The percentages of entrapment ranged from 0.5-4.5% of total dye as given in Table 1. Interestingly the percentage entrapment increased with the increase in vesicle sizes as well as their multilamellar character.

To examine the response of the temperature variation on the presently described aggregates, fluorescence anisotropy values (\(r\)) due to each vesicle doped with 1,6-diphenylhexa-1,3,5-triene (DPH) at various temperatures were then determined. Comparison of \(r\) vs. \(T\) plots (not shown) of the vesicles of 1a-d or 2a-d with that of naturally occurring dipalmitoyl phosphatidylcholine (DPPC) clearly brings out the differences in the thermal responses between membranes produced from the cholamphiphiles and that from a hydrocarbon-chain based lipid, DPPC. No pronounced break in \(r\) vs. \(T\) plots from 15-65°C with 1a-d or 2a-d indicates lack of any detectable solid gel to fluid phase transitions. Conventional lipids such as DPPC, by contrast, undergo a thermally induced conversion from an ordered, solid-like, gel phase into a liquid-crystal like, fluid phase replete with \(\beta\)-gauche conformations in its hydrocarbon chains. Absence of any detectable thermal transition most likely stems from the conformationally immobile nature of the steroidal rings. Thus upon heating only temperature induced lateral separation of monomers coupled with local disordering due to trans-to-gauche isomerization of the side chain at the C-17 position contribute to the low \(r\)-value.

The sonicated aqueous dispersions of 1-2 (5 mg/mL) were also converted to regular self-supporting films

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\(^{17}\) Vesicles 1a-d and 2a-d were generated by sonication of a dry film of a given amphiphile (5 mM) in water (pH ~6.8) containing MB (0.1 mM) for 10 min at ~60°C. Then, 2 mL of this translucent blue suspension was loaded onto a pre-equilibrated Sephadex G-50 (Pharmacia) column and gel filtration was performed using water as eluent.
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Table 1—Entrapment capacity and membrane widths of aggregates formed by 1-2

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compd</th>
<th>Entrapment capacitya (%)</th>
<th>Unit Agregate layer widthsb (Å)</th>
<th>Calcdc</th>
<th>Obsd (%)d</th>
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<tr>
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<td>42.0</td>
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<td>2</td>
<td>1b</td>
<td>0.5</td>
<td>42.0</td>
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<tr>
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<td>1c</td>
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<td>42.0</td>
<td>46(58), 28(14), 25 (28)</td>
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<tr>
<td>4</td>
<td>1d</td>
<td>4.5</td>
<td>42.0</td>
<td>28(82), 25 (18)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2a</td>
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<td>42.0</td>
<td>36.6 (100)</td>
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<tr>
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<tr>
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<td>63.0</td>
<td>57.9 (100)</td>
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</table>

aAbsorbance at 665 nm was measured for all fractions; [MB] = 0.1 mM; [amphiphile] = 5 mM; pH = 6.8. b[amphiphile] = 5 mg/mL. cLengths of two molecular layers of lipids as obtained from models. dAs obtained from the X-ray diffraction measurements of cast films. Values in parentheses indicate the percentage of a given morph.

by casting on glass slides as described. Reflection X-ray diffraction (Scintag XDS-2000) of these cast films gave the long spacings from individual lipid films as given in Table 1. The formation of regular bilayer-like arrangement is indicated with the aggregates of 1a as the layer length (42 Å) obtained from XRD is in good agreement with the sum of calculated lengths (CPK model) of two molecular layers of 1a. Slight reduction in the layer length (40 Å) of 1b aggregates could be explained on the basis of the formation of a tilted bilayer arrangement. In contrast, however, the aggregates of 1c and 1d in water showed evidences of complex polymorphism with significant reduction in the layer lengths of the predominant morphs. Such a dramatic reduction in aggregate layer width could be explained on the basis of pronounced interdigitiation and tilting. Thus in the series 1a-d, there is steady decrease in the membrane thickness upon increasing in the number of oxyethylene units (n-value) in 1a before reaching a plateau at n = 4. Under comparable conditions, the XRD of self-supporting cast films from aqueous suspensions of 2a-d were also measured. Remarkably here the results indicated an exactly opposite trend to what was observed with 1a-d. 2a formed a rather thin aggregate (~37 Å) indicating a significant interdigitigation and/or tilting in its bilayer plans. As the n-value is increased the aggregates gradually tend to adopt regular bilayer or hydrated bilayer arrangements. Examination of the r-values at 25°C for the membranes formed from the two series of cholamphiphiles as a function of their molecular structures reveals strikingly opposing trends. In the series of vesicles of 1a-d, a steady decrease in r-value was observed as the n-value increased (Figure 1A). Loss of r-value at a given temperature indicates increasing disorder in the membrane packing. In contrast, in the series of 2a-d, a monotonous increase in r-value was observed as oxyethylene units were incrementally added to the spacer between the NMe3+ headgroup and the cholesterol backbone (Figure 1A.). Thus a gradual enhancement of lipid packing is manifested with increase in n-value in the series 2a-d. These findings demonstrate the remarkably opposing trends of the bilayer widths and membrane rigidity in the two series of cholamphiphiles 1a-d and 2a-d (Figures 1A and 1B), where the sequence of attachment of the -(CH2CH2O)n- segment and the positive charge were interchanged. In 1a-d the attachment of a -(CH2CH2O)n-H segment to the cationic -NMe3+ group progressively increases the size of the head group and enhances hydration. This also leads to greater charge dispersal. When the charge is concentrated to the small -NMe3+ group in 1a, the mutual repulsion at the headgroup level is so strong that only a bilayer arrangement is possible where the charges of the monomers in the inner and outer leaflets are farthest from each other. However, as the charge becomes more dispersed over increasingly bulky headgroup, in 1b-d, repulsion between the charges of the inner and outer leaflets gets progressively reduced leading to the formation of strongly interdigitated and other non-bilayer organizations. Thus the membrane thickness in 1a-d decreases with the increase in n-value as seen in Figure 1B. At the same time, the increase in the size of the cationic headgroup increases the lateral separation between the adjacent monomers. This in turn leads to greater disorder (reflected in the loss in r-value) with increase in n-value. As a consequence of the greater lateral separation with increase in n-value, the monomers from the inner and outer leaflets come...
closer to each other in order to allow the interdigitation of the cholesteryl side chains to fill the 'voids'. The aggregate width thus decreases with greater interdigitation.

The oxyethylene group has unique ability to show both hydrophilic as well as hydrophobic character\(^7\). In the series 2a-d, the \(-(\text{CH}_2\text{CH}_2\text{O})_n\)- unit is located in between the cholesteryl backbone and the \(N^+\text{Me}_3\) headgroup. Here it appears to act like a hydrophobic spacer as an increase in the n-value in 2 results in an increase in the length of the hydrophobic portion of the lipid monomer. This in turn translates into an increase in the bilayer width with increase in n-value as evidenced from the XRD data. At the same time, since this moiety is located close to the interfacial region near the headgroup in 2a-d, it also has the propensity to get hydrated. This presumably facilitates water promoted hydrogen bonding\(^7b\) between adjacent lipid monomers drawing them closer together leading to more efficient packing and enhancing \(r\)-value\(^8\).

Thus a longer oxyethylene segment results in a longer "sticky strip" being made available for water which acts as a "glue" serving to increase membrane rigidity and order.

Acknowledgement

This work was supported in the form of a Swarnajayanti Fellowship Grant of the Department of Science and Technology, Government of India, awarded to S.B.

References


7 (a) See Gokel G W & Murillo O, in Comprehensive supramolecular chemistry, Vol. 1, Ch. 1, (Pergamon: New York), 1996, p.1; (b) ibid, Moyer, B A, Ch. 10, p. 377.