Quaternary Ammonium-Based Surfactants That Can Recognize Cholesterol-Rich Membranes and Proton-Ionizable Analogs That Cannot[1]

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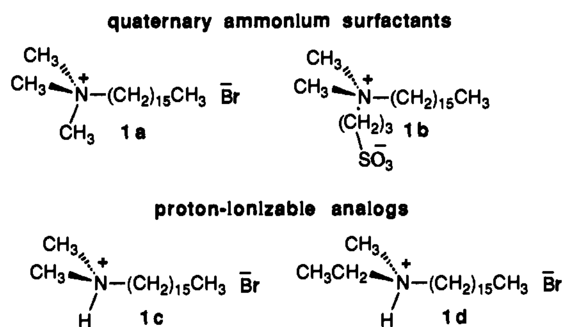
Abstract: This paper documents the discovery that simple quaternary ammonium-based surfactants can recognize cholesterol-rich phospholipid membranes and that proton-ionizable analogs cannot. Specifically, hexadecyltrimethylammonium bromide (**1a**) and 3-(hexadecyldimethylammonio)propane-1-sulfonate (**1b**) have been shown to be effective in disrupting cholesterol-poor but not cholesterol-rich bilayers of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). In striking contract, hexadecyldimethylamine hydrogen bromide (**1c**) and hexadecylethylmethylamine hydrogen bromide (**1d**) were found to be very active against both types of targets. These findings reveal major differences between membrane-disrupting surfactants that bear a fixed positive charge and those that can eliminate such charge via deprotonation; they also indicate that *repulsive forces between pendant ammonium groups and the hydrophobic interior of lipid bilayers can be used to modulate membrane disruption.* The implications of these findings for the rational design of membrane-disrupting drugs are briefly discussed.

Introduction

The required presence of positively charged nitrogen groups for antibiotic activity within certain classes of compound has been known for more than 50 years.[2] Some common examples of such agents, which are of considerable current importance, include cetylpyridinium chloride (antibacterial),[2] amphotericin B (antifungal),[3] polymyxin B (antibacterial),[4] and doxorubicin (anticancer).[5] Although many of these antibiotics are believed to act at the membrane level, the precise role that the nitrogen group plays in promoting cytotoxicity remains unclear. Several mechanistic studies that have focused on simple model systems have demonstrated that electrostatic *attractive* forces with negatively charged phospholipids can enhance a drug's affinity toward a lipid bilayer.[6-8] Only recently, however, have similar effects been confirmed in more complex and more biologically relevant membranes.[9] To date, no clear distinction has been made between proton-ionizable versus quaternary nitrogen groups, in terms of their fundamental interactions with lipid bilayers.[3] Moreover, the potential importance of *repulsive* forces between pendant ammonium groups and the hydrophobic components of lipid bilayers has been largely ignored.

During the course of our studies involving membrane disruption, we have discovered that simple quaternary ammonium-based surfactants can recognize cholesterol-rich membranes and that proton-ionizable analogs cannot.[10-12] Specifically, we have found that hexadecyltrimethylammonium bromide (**1a**) and 3-(hexadecyldimethylammonio)propane-1-sulfonate (**1b**) are effective in disrupting cholesterol-poor but not cholesterol-rich phosphatidylcholine bilayers and that hexadecyldimethylamine hydrogen bromide (**1c**) and hexadecylethylmethylamine hydrogen bromide (**1d**) are very active against both types of targets. Our principal results, which are reported herein, reveal major differences between membrane-disrupting surfactants that bear a fixed positive charge and those that can eliminate such charge via deprotonation; they also indicate that *repulsive* forces between pendant ammonium groups and the hydrophobic interior of lipid bilayers can be used to modulate membrane disruption.



Experimental Section

General Methods. Unless stated otherwise all reagents and chemicals were obtained from Aldrich Chemical Co. and used without further purification. Chloroform that was used for vesicle formation was HPLC-grade (Burdick & Jackson). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was obtained from Avanti Polar Lipids (Birmingham, AC) as a chloroform solution and used directly. 5(6)-Carboxyfluorescein (CF) was obtained from Eastman Kodak and purified according to literature methods.[13] Cholesterol was purchased from Fluka and was recrystallized prior to use. House-deionized water was purified using a Millipore Milli-Q-filtering system containing one carbon and two ion-exchange stages. All fluorescence measurements were made using a

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Perkin-Elmer LS-50 luminescence spectrometer, equipped with polarizers. Excitation of CF was at 491 nm; the observed emission was measured at 521 nm. Critical micelle concentrations were determined in a 10 mM borate buffer (pH 7.4, 140 mM NaCl, 2 mM NaN_3) by dye methods;[14] nearly identical values were obtained by standard surface tension measurements (Nima Model ST tensiometer, Coventry, U.K.). This buffer, which is isotonic with respect to 79 mM CF, was used in all release experiments, except for those in which the pH was adjusted to 8.4. Phosphorus analyses were performed using methods previously described.[15] Hexadecyltrimethylammonium bromide (**1a**) (Aldrich Chem) was purified by recrystallization from acetone/water. Hexadecyldimethylamine was converted into its sultaine derivative (**1b**) by direct alkylation with 1,3-propane sultone;[16] both **1b** and the hydrogen bromide salt of the parent amine (**1c**) were recrystallized from acetone. Hexadecylethylmethylamine hydrogen bromide (**1d**) was prepared via literature methods and recrystallized from acetone/methanol.[17]

Surfactant-Induced Release of Liposome-Encapsulated CF. Large unilamellar vesicles (1000 Å diameter), containing 5(6)-carboxyfluorescein (CF), were prepared from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) or POPC/cholesterol mixtures, using standard extrusion procedures.[18,19] Typically, 1 mL of a chloroform solution, containing 10 mg (0.013 mmol) of POPC, was placed in a test tube (13 × 100 mm), and the chloroform was evaporated under a stream of nitrogen. Cholesterol (4.1 mg, 0.011 mmol) was added directly to this tube, and the mixture was redissolved in 1 mL of chloroform. The chloroform was then removed under a stream of nitrogen. After further drying (12 h, 23 °C, 0.3 mmHg), the resulting film was dispersed in 0.45 mL of a 79 mM solution of CF (corresponding to 269 mOsM) via vortex mixing. The resulting multilamellar vesicle dispersion was allowed to equilibrate for 0.5 h, subjected to five freeze–thaw cycles (liquid nitrogen), and passed through a 0.1 μm polycarbonate filter (Nuclepore) 15 times. Nonentrapped CF was removed *via* gel filtration on a Sephadex G-50 column (1.2 × 20 cm), using an isotonic pH 7.4 borate buffer (10 mM borate, 140 mM NaCl, and 2 mM NaN_3) as the eluant, followed by dialysis against 1.5 L of borate buffer for 10 h at 5 °C. Vesicle fractions were collected, and the final volume was adjusted to 2 mL by adding additional buffer.

An aliquot (20 μL) of a given dispersion was diluted with 2 mL of 10 mM borate buffer (pH 7.4, 140 mM NaCl, 2 mM NaN_3). The dispersion was then incubated for 1 h at 25 ± 1 °C. Aliquots (60 μL) were then added to each of a series of test tubes (13 × 100 mm), which contained 540 μL of varying concentrations of a given surfactant in 269 mOsM borate buffer, followed by vortex mixing for 10 s. Each tube was then mechanically shaken at 70 strokes/min. In all cases, the final phospholipid concentration was 3.5 μM. After allowing the vesicle–surfactant mixture to incubate for 0.5 h at 25 ± 1 °C, 45 μL-aliquots were withdrawn and diluted with 4-mL borate buffer, and then measured for fluorescence. A blank value was determined in every case by treating 60-μL vesicle aliquots with 540 μL of borate buffer, in the absence of detergent. A total fluorescence value was determined by complete disruption of the vesicles, using 54 μL of an aqueous solution that was 80 mM in Triton X-100. The percentage of released CF was calculated according to $I(\%) = 100[I_a - I_b]/[I_x - I_b]$, where I_x is the 100% fluorescence intensity determined using an excess of Triton X-100; I_a and I_b are the fluorescence intensities after incubation with and without surfactant, respectively.[13,20] Values of R_{50} respresent the ratio of phospholipid/surfactant that is needed to release 50% of the entrapped CF from a 3.5 μM dispersion of liposomes after 30 min. Kinetic experiments were carried out in a similar manner except that the extent of release was monitored as a function of time.

Fluorescence Polarization Experiments. Vesicles (1000 Å diameter) were prepared from POPC/Ch (55/45) or pure POPC, using methods similar to those described above, except that the CF solution was replaced by borate buffer. In each case, the stock dispersion was 10 mM in phospholipid. A aliquot of the dispersion (0.40 mL) was mixed with 1.0 μL of a 2.0 mM dimethyl sulfoxide solution of 1,6-diphenyl-1,3,5-hexatriene (DPH), and the mixture then added directly to 3.6 mL of surfactant solution (**1a** or **1c**) in 10 mM borate buffer (final concentration of surfactant, phospholipid, and DPH were 0.375 mM, 1.0 mM and 0.5 μM, respectively). The dispersion was incubated for 0.5 h at 25 °C and then analyzed for fluorescence polarization, where the excitation and emission wavelengths were 363 and 428 nm, respectively. The fluorescence polarization, P, was calculated according to $P = [I_{VV} - I_{VH}G]/[I_{VV} + I_{VH}G]$, where I_{VV} and I_{VH} are the emission intensities that are detected through an analyzer that is oriented parallel and perpendicular to the direction of the vertical polarization of the exciting beam, respectively; G is a factor that is used to correct for the inability of the instrument to transmit differently polarized light equally.

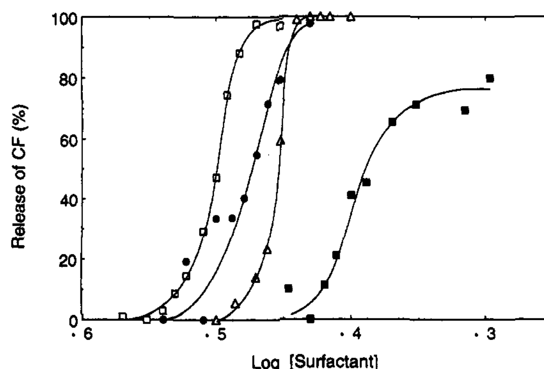

Figure 1. Percent release of CF from 3.5 μM liposomal targets made from POPC as a function of surfactant concentration **1a** (■), **1b** (△), **1c** (●), and **1d** (□) after 30 min at 25 °C.

Results and Discussion

Membrane Targets and Membrane-Disrupting Activity. Large unilamellar vesicles (1000 Å diameter) that were used in this study were prepared from varying mixtures of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and cholesterol (Ch). In order to detect membrane disruption, we have monitored the release of encapsulated 5(6)-carboxyfluorescein (CF).[13,20] At high internal vesicular concentrations (e.g., 79 mM), CF exhibits negligible fluorescence due to efficient self-quenching. As the fluorophore is released into the external bulk phase, it becomes diluted and strongly fluorescent. The detailed protocols that we have used for vesicle preparation, surfactant-induced membrane disruption, and fluorescence analysis in the present work were similar to those previously described.[11] In brief, incubation of 3.5 μM liposomal dispersions with varying concentrations of surfactant for 30 min at 25 °C generate release profiles of the type that are shown in Figure 1. For purposes of comparison, we define membrane-disrupting activity as R_{50} values, where R_{50} represents the ratio of phospholipid/surfactant that is needed to induce the release of 50% of the entrapped CF.[11] The percentage of released CF was calculated according to $I(\%) = 100[I_a - I_b]/[I_x - I_b]$, where I_x is the 100% fluorescence intensity determined using an excess of Triton X-100; I_a and I_b are the fluorescence intensities after incubation with and without surfactant, respectively.[13,20]

Table 1 summarizes the principal results that have been obtained with **1a–d**. In the absence of cholesterol, both quaternary ammonium surfactants **1a** and **1b** as well as the proton-ionizable analogs (**1c** and **1d**) were effective in inducing the release of vesicle-encapsulated CF. Similar results were obtained with membrane targets that were composed of a 9/1 molar ratio of POPC/Ch. However, when the sterol content was increased 33 and 45 mol %, both quaternary ammonium-based surfactants were *completely inactive*. In striking contrast, the activities of the proton-ionizable analogs were almost unchanged. Similar surfactant-induced release experiments that were carried out at pH 8.2 (instead of 7.4) showed a significant decrease in activity for **1c**, a modest increase for **1a**, and no significant effect on **1b**

Although clear differences in the *permeabilizing* effects between a quaternary ammonium surfactant and a proton-ionizable analog

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Table 1. Membrane Disruption by Ammonium-Based Surfactants

target membrane	surfactant R_{50}[a]			
	1a, 50 μM[b]	**1b**, 20 μM[b]	**1c**, 55 μM[b]	**1d**, 85 μM[b]
POPC	0.028 ± 0.000 (130)[c]	0.12 ± 0.000 (30)	0.20 ± 0.00 (18)	0.35 ± 0.00 (10)
POPC/Ch, 9/1	0.033 ± 0.000 (110)	0.10 ± 0.01 (35)	0.24 ± 0.01 (15)	
POPC/Ch, 2/1	<0.0001 (35 000)	<0.0001 (35 000)	0.19 ± 0.01 (18)	
POPC/Ch, 55/45	<0.0001 (35 000)	<0.0001 (35 000)	0.15 ± 0.00 (23)	0.19 ± 0.01 (18)
POPC[d]	0.056 ± 0.004 (63)	0.12 ± 0.01 (29)	0.030 ± 0.011 (120)	

[a] R_{50} is defined in the text; molar ratios of POPC/Ch are shown following the target membrane designation. All release curves that were used to calculate R_{50} values were derived from a minimum of five independent experiments, using a concentration of phospholipid that was 3.5 μM and a buffer that was pH 7.4. Values reported are averages of duplicate release curves ±1 SD. [b] Critical micelle concentration of the disruptive surfactant. [c] Numbers in parentheses refer to the surfactant concentration (μM) that was required for inducing 50% release. [d] pH 8.2.

were observed with POPC/Ch (55/45) membranes, the fluidizing effect of each surfactant proved to be similar as judged by fluorescence polarization measurements. Thus, the observed fluorescence polarization, P, of 1,6-diphenyl-1,3,5-hexatriene (DPH) that was incorporated into POPC/Ch (55/45) vesicle membranes was 0.401 ± 0.043.[21,22] Subsequent incubation with **1a** resulted in increased fluidity, as indicated by a depolarization of the observed fluorescence (P = 0.295 ± 0.014); incubation of a similar target with the same concentration of **1c** afforded a polarization that was equal to 0.289 ± 0.015. Thus, both surfactants had similar fluidizing effects. It is also noteworthy that the fluidity within these membranes is considerably less than that found in bilayers made from pure POPC, where P = 0.131 ± 0.035.

Membrane Rupture versus Membrane Leakage. Recent mechanistic studies that we have carried out, involving the perturbation of cholesterol-rich POPC bilayers by Triton X-100 and by sodium deoxycholate, have revealed that the aggregation state of attacking surfactant can determine whether the bilayer becomes "leaky" or whether the loss of membrane integrity is more catastrophic in nature.[12] Specifically, we showed that when these cholesterol-rich membranes were "hit" by surfactant monomers, CF was slowly released from all of the vesicles. In contrast, similar "impact" by a micellar form of the surfactant resulted in the rapid and complete release of CF from some, but not all, of the vesicles. We further demonstrated that loosely packed phospholipid membranes (i.e., POPC targets that were devoid of cholesterol) were susceptible only toward leakage events, regardless of whether the attack came from monomers or from micelles. In essence, we established that *it is the combination of membrane packing and the supramolecular state of the attacking agent that controls rupture/leakage pathways.*

Examination of the efflux kinetics from vesicles that were prepared from POPC and POPC/Ch (55/45), using concentrations of surfactant that were required for R_{50}-release, showed that the release of the fluorophore was *continuous* in all cases (Figure 2). This finding indicates that leakage is the primary pathway through which *CF* escapes from the vesicles. Since loosely packed POPC vesicles would be expected to favor leakage, regardless of the supramolecular state of **1a–d**, these results are fully consistent with our previous findings. Similarly, since only submicellar concentrations of **1c** and **1d** were required for R_{50}-release, for vesicles composed of POPC/Ch (55/45), the observation of dominant leakage by both surfactants is also the expected result. A new and intriguing finding, however, is that POPC/Ch (55/45) is highly robust toward rupture as well as leakage by **1a** and **1b**. This result is significant because it reveals, for the first time, that *rupture events also depend upon the specific structure of the monomeric component of an attacking micelle.*

Repulsive Forces between Target and Attacking Surfactant Are Implicated in Membrane Recognition. Why are membrane-disrupting, quaternary ammonium surfactants sensitive toward the presence of cholesterol while proton-ionizable analogs are

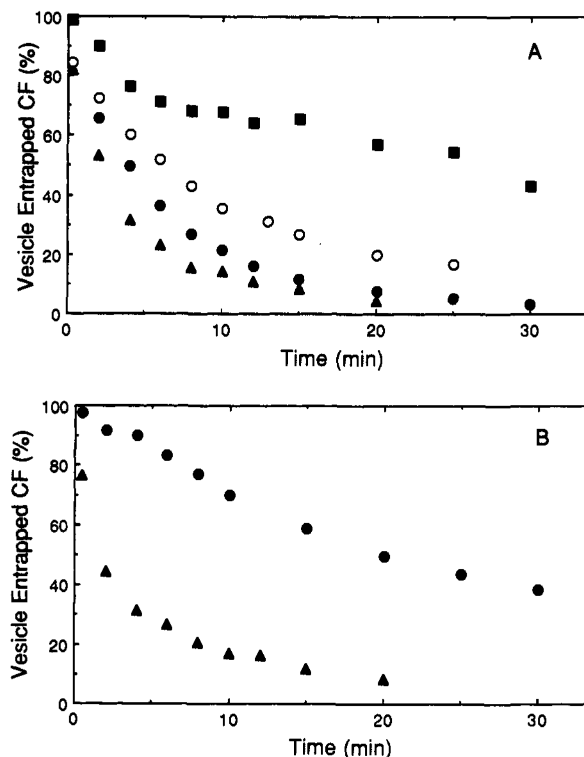

Figure 2. Percentage of CF that remains entrapped within the interior of (A) POPC and (B) POPC/Ch (55/45) vesicles (3.5 μM) as a function of time. The concentrations of **1a** (○), **1b** (■), **1c** (●), and **1d** (▲) that were used against POPC vesicles were 200, 30, 17, 11 μM, respectively (Figure A); concentrations of **1c** and **1d** used against POPC/Ch (55/45) vesicles were 23 (●) and 20 (▲) μM, respectively (Figure B).

not? The answer, we believe, lies in *strong repulsive forces between hydrated ammonium groups (plus counterion) and compact, cholesterol-rich bilayers.*[23] In order for **CF** to escape from the vesicle interior, a contiguous channel (or defect) must be created that spans the membrane. Thus, disruptive surfactants should be present on *both* sides of the bilayer. The ineffectiveness of **1a** and **1b** in inducing the release of **CF** from cholesterol-rich vesicles can be readily accounted for in terms of their inability to reach the inner monolayer leaflet due to repulsive interactions between the hydrated, ionic head groups and the densely packed hydrocarbon core of the bilayer. In contrast, proton-ionizable analogs **1c** and **1d**, which can avoid such repulsive forces by "shedding" their positive charge (proton), their counterion, and their water of hydration, should be better able to penetrate the bilayer. The fact that the activity of **1c** is significantly reduced as the pH of the dispersion is raised from 7.4 to 8.2, further indicates that it is the protonated form of the surfactant that is primarily responsible for its membrane disrupting properties (Figure 3). Presumably, the charged head group, occupying a relatively large area at the membrane surface, confers a more conical shape to the surfactant, which results in a greater geometric

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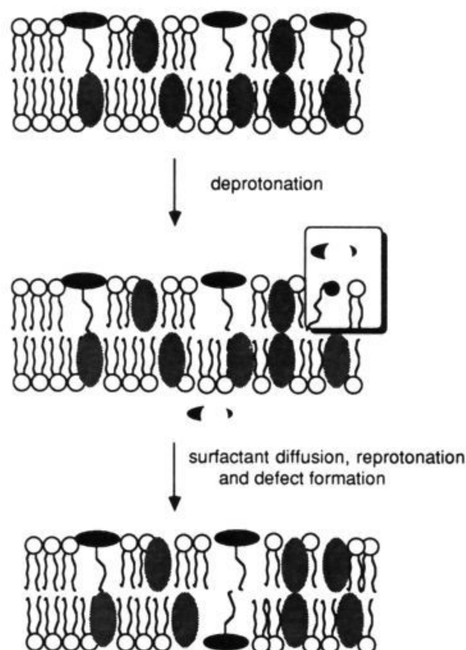

Figure 3. Styilized illustration of proton-ionizable surfactant penetrating a cholesterol-rich bilayer via successive deprotonation, diffusion, and reprotonation. The ellipsoidal structure within the membrane represents cholesterol.

mismatch with the cylindrical-shaped phospholipids.[24] The fact that **1c** and **1d** exhibit similar activities toward cholesterol-rich and cholesterol-deficient membranes also indicate that steric factors and the hydrophobic content of the disruptive surfactant are not of primary importance for the recognition of choleserol-rich phospholipid membranes.

The observation that **1a** and **1b** display similar activities, together with the fact that the **1b** is zwitterionic, provide a compelling argument that both surfactants induce the release of **CF** via channel (or defect) formation and not by a carrier mechanism, since a carrier process for **1b** is not possible.[25] Such channels must, of course, be larger in size than ion channels that are created by naturally occurring ionophores; e.g., those that are created by gramicidin.[26] In particular, the effective diameter of each channel that is produced from **1a–d** must be at least as large as that of the **CF** molecule itself. Although the **CF** molecule has a hydrophobic component, the lack of significant efflux from fluid-phase POPC vesicles (in the absence of a disruptive surfactant) indicates that solubility contributions toward the release of **CF** are negligible. The primary role of **1a–d** must be, therefore, to create gaps within the membrane. Although we loosely refer to these gaps as "channels", their precise geometry and size remains to be defined. In this context, we note the poignant remarks that have been made by other researchers regarding the use of the term, *channel*: "the structure of gramicidin channels makes one associate a tunnel shape with the term 'channel'. The gramicidin channel is an excellent channel, but it is not the only one and should be regarded as an example rather than a definition. The present channel may be flatter and more like, as the dictionary says, a 'groove' or a 'trench'".[27]

Implications for Drug Design. The fact that mammalian cells are rich in cholesterol and bacteria and fungi are devoid of cholesterol (fungi does contain varying levels of ergosterol) adds special meaning to the present findings from a drug-design standpoint.[26] It leads us to propose that the presence of a quaternary ammonium group may enhance the cytotoxicity of a membrane-disrupting agent toward bacteria and fungi relative to mammalian cells. Preliminary biological studies provide some support for this hypothesis. In particular, preliminary in vitro experiments that have been carried out with **1b**, at concentrations that show modest toxicity toward human H9 cells (i.e., 10 μg/mL) have shown that it effectively inhibits the growth of Cryptococcus neoformans (a fungi that is commonly found in AIDS patients).[28] However, it did not inhibit the growth of two other fungi (Candida albicans and Aspergillus fumigatus) as well as a variety of bacteria: E. coli (ATCC 25922), Pseudomonas aeruginosa (27853), Staphylococcus aureus (29213), Streptococcus faecalis (29212), Proteum vulgaris (13315), Serratia marcescens (8100), and Streptococcus pyogenes (MSK 211X). Although the activity of **1b** against Cryptococcus neoformans is of interest, its mechanism of action remains to be established. Further studies aimed at exploring and exploiting quaternary ammonium-based surfactants, from an antibiotic standpoint, are in progress.

Finally, in a broader context, the present findings lend strong support for our assertion that surfactant-lipid bilayer interactions are much richer in complexity and more exploitable than has previously been considered.[12]

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