Impact of Membrane Cholesterol Content on the Resistance of Vesicles to Surfactant Attack†

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Vesicle leakage experiments were carried out to establish how cholesterol content regulates membrane permeability as induced by surfactant exposure. Vesicles containing up to 50% cholesterol were examined. Four different surfactants were chosen as membrane perturbants, including nonoxynol-9 which is commonly used in spermicidal formulations. As part of this study, we establish that the extrusion procedure commonly used to fabricate unilamellar vesicles does not unintentionally alter the desired composition of these model membrane systems. The kinetics of the leakage process is well characterized by a single exponential rate of release, similar to the form seen in the absence of membrane cholesterol. Our leakage experiments show that membranes become more resistant toward surfactant attack, in direct proportion with cholesterol content. This rise in resistance is surfactant specific. Above 30%, all membranes show positive deviation from the linear increase in resistance with increasing cholesterol content. Two other sterols, dihydrocholesterol and coprostanol, were also found to increase membrane resistance and behaved similarly despite a key difference in molecular structure. A peculiar leakage response was observed when membranes were exposed to the surfactant sodium dodecyl sulfate (SDS) above its critical micelle concentration. Our findings support the hypothesis that SDS micelles solubilize phospholipid molecules, creating a membrane with higher cholesterol content that is extremely resistant to perturbation.

Introduction

While perhaps best appreciated as the precursor of all known steroid hormones, as well as bile acids, cholesterol also regulates many of the biophysical properties of membranes which make this molecule so essential to animal life. For example, direct interactions between cholesterol and various membrane receptors influence affinity states, binding capacities, and signal transduction.[1−3] Interactions between cholesterol and various lipid components control membrane stability, permeability, fluidity, and even the ability of these systems to self-organize into microdomains, also known as lipid rafts.[4,5]

Membrane cholesterol contents can vary widely, from one organism to the next, within the various membranes of a given organism, or even within a particular membrane depending on its need to respond to different environments. Sperm cells, for example, continuously modulate the cholesterol contents of their plasma membranes during their journey from the epididymis through the female reproductive tract.[6,7] A distinguishing characteristic of bacterial membranes is, indeed, their lack of cholesterol. On the other hand, plasma membranes of eukaryotic cells bear some of the highest cholesterol contents (30−50%).[1,8]

All cells rely upon the many services of their finely tuned membranes, and thus, membrane perturbation schemes provide powerful opportunities for pathogen control. Nowhere is this more pressing than in the worldwide struggle to prevent sexually transmitted diseases (STDs). An ideal microbicide should be at the same time "broad-spectrum" (displaying activity against a large range of pathogens) and innocuous toward healthy and useful cells, such as the epithelial cells of the vaginal lining or the lactobacilli of the vaginal microflora. It is thus conceivable that membrane perturbation strategies could be designed to exploit the variability in membrane cholesterol content across organisms or be designed to alter the native cholesterol content of a membrane thus causing it to lose proper functioning.

Some efforts to employ synthetic compounds as broad-spectrum microbicides/spermicides have focused on the use of surfactants. These are amphiphilic molecules composed of a polar "head" group attached to a nonpolar hydrocarbon chain. Their dual nature gives rise to self-assembling tendencies (e.g., micelle formation at high enough aqueous concentrations), as well as a propensity to interact with membranes, which are themselves composed of amphiphiles, most notably phospholipids.

In this work, cholesterol-laden membranes in the form of vesicles, otherwise made of the phospholipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) or a more complex lipid mixture that mimics the sperm plasma membrane, were perturbed by surfactants. The surfactants used have current or promising application in contraception and as vaginal microbicides for STD prevention: nonoxynol-9 (N-9), C31G (an amphoteric mixture of two surface-active molecules, C_{14} alkylamine oxide and

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C_{16} alkyl betaine), benzalkonium chloride (BZK), and sodium dodecyl sulfate (SDS).

This study presents a systematic analysis of the impact of membrane cholesterol content on the permeability characteristics of the membrane as a result of vesicle exposure to surfactant molecules. The perturbation process is examined by a leakage experiment in which the extent of encapsulated fluorophores permeating across the membrane is recorded. Cholesterol is generally known to reduce the ability of membrane-active agents to induce vesicle leakage,[9−13] but a detailed study of this effect is lacking. We also compare the effect of cholesterol to two other sterols (dihydrocholesterol and coprostanol). As part of our studies, we also describe a novel method that utilizes SDS micelles for increasing the cholesterol concentration within a membrane, making it more resistant to perturbation. Finally, we establish that the vesicle preparation procedures do not alter the intended cholesterol content of these useful model membranes.

Materials and Methods

Materials. POPC, brain sulfatide, 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine, C16:0−22:6 1-alkyl-2-acyl-sn-glycero-3-phosphocholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine], egg sphingomyelin, and cholesterol (Chol) were obtained from Avanti Polar Lipids (Birmingham, AL). BZK, as well as SDS, dihydrocholesterol, coprostanol, sodium chloride (NaCl), HEPES (2-[4-(2-hydroxyethylene)-1-piperazinyl]-ethanesulfonic acid), Infinity cholesterol reagent, cholesterol calibrator, cardiolipid control-level 1, and phosphorus standard solution, were obtained from Sigma (St Louis, MO). All were at the highest purity available. 5,6-Carboxyfluorescein (CF) was obtained from Molecular Probes (Eugene, OR). C31G (equimolar mixture of C14 amine oxide and C16 alkyl betaine at purities of 88.7 and 98.2%, respectively) was obtained from Biosyn (Philadelphia, PA). N-9 was obtained from Biosyn (as Rhone−Poulenc's Igepal CO-630 Special at a purity of 95%). All surfactants were used without further purification. All solutions were prepared using Millipore water.

Methods. *Surfactant and CF Solutions Preparation.* Surfactant stock solutions (N-9, C31G, and BZK at 0.1 mM solutions; SDS at 10 mM) were prepared by dissolving the surfactant in 16.66 mM Hepes buffer containing 125 mM NaCl. The same solution was prepared in the absence of surfactant; we refer to this in particular as the 'external solution' from here on. The pH of the solutions was adjusted to pH of 7.4 with NaOH solution. CF solution (100 mM) was prepared using 16.66 mM Hepes buffer and also adjusted to pH of 7.4. Isoosmotic conditions of the surfactant, external, and CF solutions were assured by measuring osmolarity using a Fiske Micro Osmometer Model 210. The osmolarity was matched if needed to a value of 265 mOs by the addition of NaCl.

Vesicle Preparation and Characterization. Large unilamellar vesicles (diameter ca. 100 nm) were prepared following the procedure previously described in detail[14] with minor modifications. Briefly, a chloroform solution of lipid(s) and cholesterol was sonicated for 5 min. The lipid(s)/Chol solution was dried overnight in a vacuum oven. The dried mixture film was rehydrated in CF solution to yield a 10 mM lipid solution. A large multilamellar vesicle dispersion was prepared via five cycles of vortex mixing followed by freeze−thawing (liquid nitrogen). The dispersion was extruded (Lipex Biomembranes, Inc.) two times through a double-stack of 400-nm polycarbonate filters (Nucleopore Co.) followed by 10 times through double-stack of 100-nm polycarbonate filters. Vesicles were separated from unencapsulated dye by passing the solution through a Sephadex G-50 medium resin column, using the external solution as the eluant. The vesicle fraction was collected and was characterized as described below.

The phospholipid content of vesicles was determined according to the ascorbic acid spectrophotometric assay for total phosphorus,[15] in a procedure made available by Avanti Polar Lipids. The measurements were performed using a Genesys 2 spectrophotometer (Thermo Spectronic Instruments). Dynamic light scattering experiments (Brookhaven, Inc.) were performed to determine the vesicle size distribution. The size of the vesicles in all cases was found to be 100 ± 14 nm in diameter with a relatively homogeneous size distribution (polydispersity lower than 0.059).

Cholesterol Content Studies. POPC and POPC/Chol (10−50% Chol) vesicles were prepared following the procedures above with one alteration: the dried lipid film was redissolved in external solution (no probe) instead of using the CF solution. This was done because CF was found to interfere with the cholesterol assay reaction. Samples were taken at the beginning of the process, after the extrusion step, and at the end after the column separation step.

The phospholipid content of all samples was determined using the method previously described. The cholesterol content was determined using the following enzymatic cholesterol assay: Sigma Procedure No. 401 for total cholesterol based on the procedure of Roeschla et al.[16] In particular, 20 μL of the sample was incubated with 2 mL of the Infinity cholesterol reagent for 15 min at room temperature, after which the signal at 500 nm was measured using a Genesys 2 spectrophotometer (Thermo Spectronic Instruments). Cardiolipid control, level 1, was used as the assay control.

Leakage Experiments. Membrane permeability was studied by monitoring the increase in the fluorescence signal, $I(t)$, due to the release of the fluorophore, CF, across the perturbed membrane. At high concentrations as encapsulated in the vesicle, CF has negligible fluorescence due to very efficient quenching. As the CF is diluted (after leaking to the external solution), the self-quenching efficiency decreases and the observed fluorescence increases. Almost all of the fluorescence seen can be ascribed to the CF released from the vesicles.[17,18]

Fluorescence measurements were made using a Hitachi F-4500 fluorescence spectrophotometer. The samples were excited at 495 nm, and the emission was read at 519 nm. Surfactant solution was added to vesicle solution in such amounts as to yield the desired final surfactant and lipid concentrations. The resulting solution was mixed by shaking the cuvette gently for a few seconds.

The extent of vesicle leakage is determined by normalizing the intensity of the fluorescence signal:

$$\%\text{Leakage} = \left(\frac{I(t) - I_0}{I_\infty - I_0}\right) \times 100 \qquad (1)$$

where I_0 is the initial signal before adding the surfactant, and I_∞ corresponds to the fluorescence signal at $t \rightarrow \infty$, after all the dye is released. The latter is actually determined by complete vesicle lysis as accomplished by addition of 100 μL of 2% (w/w) Triton X-100. To establish the kinetics of CF release, intensity was tracked as function of time. To compare the performance of one membrane/surfactant system to the next, we report the percent of leakage recorded after 4 h. The vesicle concentration in the experiments was 4.6×10^{-3} mM. Each experiment was repeated at least three times. Repeatability was within ±5%, unless otherwise stated.

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Vesicle Recovery after SDS Perturbation. As will be described, the interaction of vesicles with SDS forms the basis of a method for increasing the membrane cholesterol content by recruiting phospholipids from the vesicles without causing lysis. This was tested by characterizing vesicles recovered after incubation with SDS. POPC/40% Chol vesicles were prepared following the procedures above but were redissolved in external solution (no probe) instead of using the CF solution. The vesicles (total vesicle concentration of 4.6×10^{-3} mM) were incubated with 0, 0.65, and 3 mM SDS for 4 h. At the end of the perturbation period, the samples were placed in Millipore Centricon centrifugal filter devices YM-100 (Billerica, MA) which are low-adsorption, hydrophilic membranes with 100 000 NMWL (nominal molecular weight limit) cutoff. Prior to use, the Centricon membranes were prerinsed by filling the devices with 2 mL of external solution and spinning it at 1000*g* in order to wash any existing glycerin and sodium azide traces. The Centricon tubes containing the perturbed vesicles samples were centrifuged at 3500*g* for 15 min using a Beckman Allegra 6 centrifuge (Beckman Coulter, Inc.) to facilitate the separation of the vesicles from free surfactant and lipid molecules. The recovered vesicles were transferred to the retentate vial by using another centrifugation step at 3500 rpm for 2 min. The phospholipid content of the retentate (at least four samples were combined to reach the minimum of the assay) was determined as described in the Vesicle Preparation and Characterization section. This protocol was repeated four times.

Results and Discussion

Cholesterol Content Studies. Vesicles are arguably the most robust and valuable model systems used in membrane research. The most common way of making large unilamellar vesicles (LUV) is through the spontaneous formation of large multilamellar vesicles in aqueous solution followed by pressurized extrusion through a narrow-pore-size filter to produce a nearly monodisperse population. As the vesicle preparation process calls for a series of steps (see in Materials and Methods section), one cannot take for granted that the final composition of vesicles formed is indeed established by the relative amounts of starting materials, particularly with regard to cholesterol content. (For example, cholesterol crystallization can occur during sample preparation,[19] which might lead to excess material loss relative to other components). To the best of our knowledge, this issue has never been addressed. We thus examined the effect on composition of every key step in this routine vesicle preparation process.

Vesicles used in this study were composed of cholesterol and the phospholipid POPC. Lipids of the phosphatidylcholine group generally constitute the largest class of phospholipids found in most plasma membranes. Thus, we chose POPC as the lipid to be mixed with Chol in these simple model membranes.

The vesicles preparation process can be divided to three main steps. The first step involves resuspending the dry lipid in aqueous solution; we refer to this as "start". The following step is extrusion of the dispersion through narrow-pore-size filters. The last step is the separation of vesicles from solutes exterior to them (This is necessary in leakage studies, where unencapsulated probe must be removed). This last step is accomplished by passing the vesicular solution through a resin column.

Figure 1 shows the influence of the preparation process on vesicle composition. Each column of the graph represents a different stage of the process and is composed of two parts to reflect the amounts of lipid and cholesterol. As is notable from Figure 1, the amount of cholesterol and lipid is decreased throughout the process of vesicle preparation. More material is lost in the separation step as compared to the extrusion step. Most importantly, however, the total percentages of cholesterol and lipid lost through the whole process are similar; thus, the starting composition is preserved (values are shown in Figure 1).

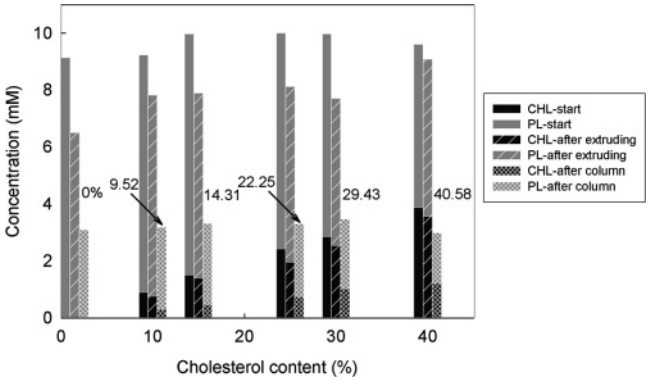

Figure 1. Yield of cholesterol (black bars) and POPC (grey bars) as a function of steps in the vesicle preparation process. Amounts measured at the start, after the extrusion step, and after the column separation step. The abscissa represents the target cholesterol content of the vesicles, i.e., the starting composition of the raw materials. The numbers shown in the graph represent the cholesterol mole percents at the end of the preparation, i.e., after the separation.

Surfactant-Induced Vesicle Leakage Experiments. The surfactants used in this study are (1) N-9 (nonionic); (2) C31G, an amphoteric mixture of two surface-active molecules, C_{14} alkylamine oxide and C_{16} alkyl betaine (zwitterionic); (3) BZK (cationic); and (4) SDS (anionic). Two different membrane systems were used, each with variable amounts of cholesterol: (1) POPC/Chol, (2) sperm mimic mixtures[20] which contain 16:0−22:6 acyl PC; 16:0−18:1 PC; 16:0−16:0 PE; egg-SPH; 16:0−18:1 PS; SGC; and cholesterol (relative fractions of noncholesterol components were kept constant as the cholesterol amount was varied).

In a previous study,[14] we examined in detail the form of the leakage response induced in single-component, POPC vesicles upon exposure to these surfactants. We showed, in particular, that vesicle leakage was continuous, obeying first-order kinetics described by the following relationship:

$$\%\text{retention} = A \exp(-k_{\text{exp}} t) \qquad (2)$$

Here, %retention is the fraction of initially encapsulated probe that remains in the vesicle interior; the factor A is an empirical constant, k_{exp} is the leakage rate constant, and t is the time elapsed since the addition of the surfactant to the system. We found that the rate constant was a function of the surfactant used, as well as its concentration.

With cholesterol-laden vesicles, we find similar leakage characteristics. Representative examples are shown in Figure 2, for the case of 30% Chol/70% POPC membranes in the presence of various amounts of the surfactant N-9. As is readily seen, the rate constant, and thus the characteristic half times, depend on the bulk surfactant concentration and change significantly over relatively narrow ranges of surfactant concentrations. For example, the characteristic half-time of the leakage process (30% membrane cholesterol content) can vary from more than

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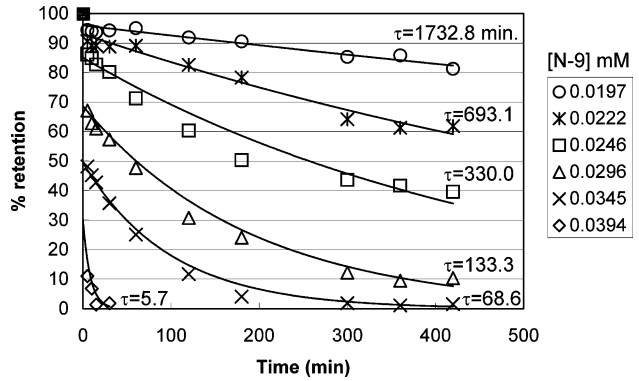

Figure 2. Time dependence of CF leakage from 70% POPC/30% Chol vesicles (vesicles concentration 4.6×10^{-3} mM) in the presence of N-9 at various overall concentrations. (All fits have R^2 higher than 0.9, with the exception of 0.55 for the curve corresponding to the lowest surfactant concentration).

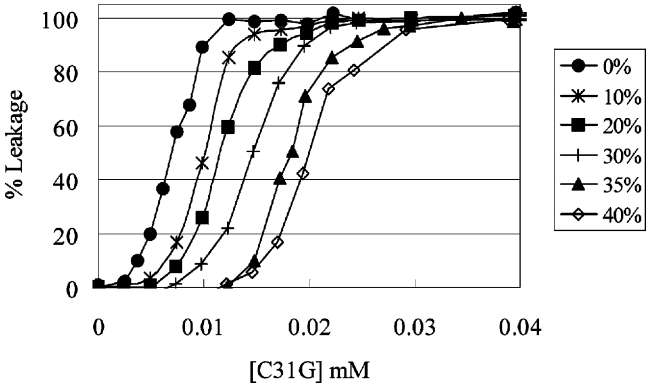

Figure 3. Leakage response (extent after 4 h) of cholesterol/POPC vesicles in the presence of C31G (vesicles concentration 4.6×10^{-3} mM). Vesicles containing 0% (●), 10% (∗), 20% (■), 30% (+), 35% (▲), and 40% Chol (◇) were examined.

a day to only few minutes as the overall concentration of N-9 increases from 0.02 to 0.04 mM.

To directly evaluate the role of cholesterol in modulating the resistance of membranes toward surfactant attack, we tracked the leakage of vesicles as measured after 4 h. (We did this in lieu of the detailed kinetic measurements described above for practical reasons, as well as to avoid excessive handling and disruption of the samples.) An example for the case of membrane perturbation by C31G is plotted in Figure 3. As is clearly seen, cholesterol increases the resistance of membranes, i.e., to achieve a given fractional release, more surfactant is needed as the cholesterol content increases. Our results are consistent with many other studies which show that cholesterol decreases membrane susceptibility toward surfactants, as well as antimicrobial peptides.[9,10,12,13] We note, however, that this behavior has not been ubiquitously observed. Some studies[11,21] report no decrease in leakage until a threshold concentration of cholesterol is reached, and others[22,23] report that leakage increases with increasing cholesterol content after a threshold value is exceeded.

Various aspects of this "toughening effect" become apparent by plotting the surfactant concentration required to induce 50% contents release (after 4 h), $\%L_{50}$, as a function of membrane cholesterol content as shown in Figure 4. Most interestingly, the membrane resistance is linear up to 30% cholesterol, regardless of surfactant type

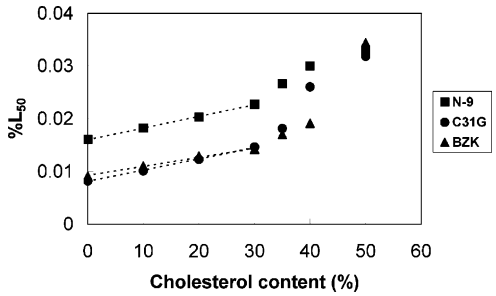

Figure 4. Surfactant concentrations (N-9 (■), C31G (●), and BZK (▲)) causing 50% leakage as a function of membrane cholesterol content in sperm mimic vesicles.

or the lipids used in the membrane (e.g., Figure 4 corresponds to the complex lipid mixture used to mimic sperm membranes; similar behavior is seen for POPC/Cholesterol membranes). Above this threshold, the resistance of the membranes increases in a nonlinear fashion. Second, the resistance to surfactant attack depends on the nature of the surfactant. For example, SDS-induced leakage up to 30% cholesterol content displays a much steeper slope than that observed for the surfactants shown in Figure 4. The slope for BZK-induced leakage is slightly different than that for N-9 and C31G. This difference is more pronounced for the simpler POPC/Cholesterol systems (data not shown). Interestingly, the slope of the leakage response is very similar for the two net-neutral surfactants, the nonionic N-9 and the zwitterionic C31G, irrespective of the membrane's lipid constitution (POPC vs sperm mimic mixture).

As our results suggest a clear change in membrane behavior upon reaching a threshold cholesterol concentration of about 30%, it is interesting to note that eukaryotic plasma membranes are generally at least this rich in cholesterol (typically 30−50%).[1,24] Meanwhile, most intracellular membranes fall well below this threshold.[1,25] For example, membranes of the endoplasmic reticulum (the site of cholesterol synthesis) contain only 10−12% cholesterol[1]. It is conceivable that the "super-toughening" effect associated with high-cholesterol content is critical to the performance and properties of the cellular envelope. We note that our experiments evaluated the behavior of membranes as the cholesterol content was increased up to only 50%. Cholesterol is not completely miscible with lipid,[26,27] and so solubility limits would undoubtedly place upper bounds on the trends we observe.

Our measurements cannot reveal the molecular basis of the trends observed, but we can speculate on their origin. Cholesterol has a well-known structural effect on the hydrocarbon chains of the other membrane constituents. Located parallel to the acyl chains of the lipid bilayer near the headgroups (with its hydroxyl group close to the phospholipid ester carbonyl group), cholesterol restricts the conformations of those parts of the chains adjacent to it. Meanwhile, chains extending beyond this region become less ordered. The ordering effect of cholesterol also leads to a condensing effect on the lipid chains above the gel to liquid-crystalline phase transition temperature (−5 °C for POPC), i.e., making the chain packing tighter, reducing

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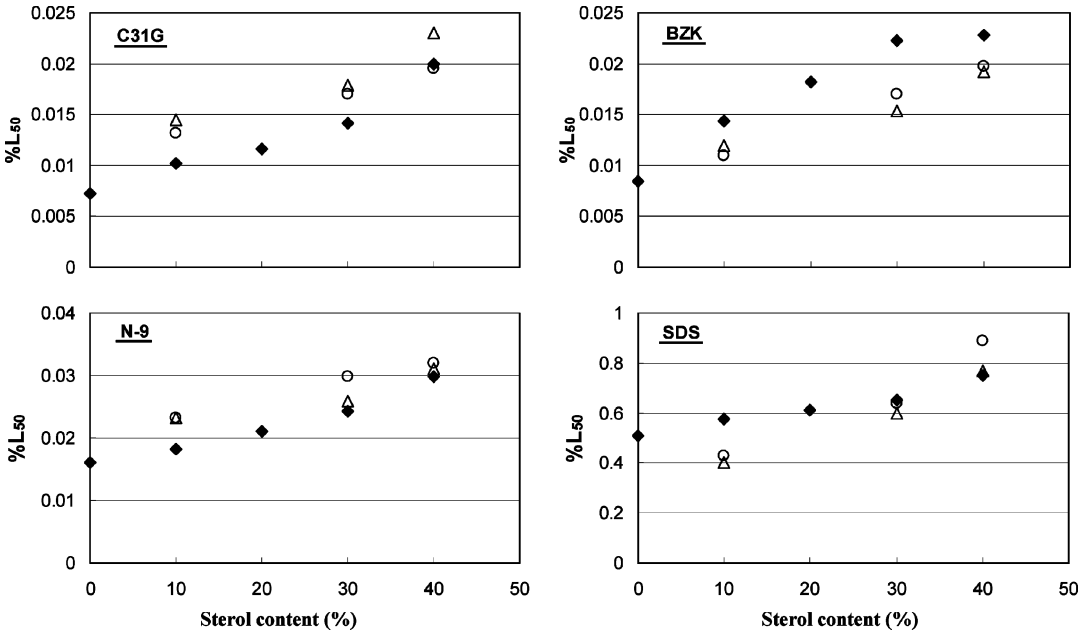

Figure 5. Surfactant concentrations causing 50% leakage as a function of membrane sterol content in POPC/sterol vesicles. Dihydrocholesterol (○)- and coprostanol (△)-laden vesicles are compared with cholesterol-laden vesicles (◆).

the surface area per lipid molecule and reducing the fluidity of the membrane.[1,28]

It is not difficult to appreciate how changes in lipid packing might relate to changes in membrane resistance toward surfactant attack. A more tightly packed membrane could well have different surfactant binding characteristics (e.g., lower partition coefficients). The packing may also affect the kinetics of mechanisms responsible for the permeation process. For example, one possible mechanism for surfactant-induced membrane permeation is transient pore formation. The curvature energy of such microstructures decreases considerably when surfactant is incorporated in the system. Pore formation would likely be a cooperative process, involving a number of surfactant molecules (and indeed our previous kinetic studies suggest a cooperative based mechanism[14]). Clearly, more investigations will be required to reveal exactly how the changes in lipid packing density are linked to changes in membrane resiliency. Of these, direct binding studies to establish the amount of surfactant incorporated into the membrane would certainly be most valuable.

The origin of the "super-toughening" effect (i.e., nonlinear resistance at cholesterol contents above 30%) is more difficult to explain, but we offer one possible rationale. Cholesterol is now well known for inducing domain formation (e.g., rafts), as well as macroscopic phase separation in certain membrane systems. Domains and/or phases rich in cholesterol are noted for their high degree of order while maintaining fluidlike lateral mobility and are referred to as "liquid-ordered". Although such states are prevalent in model systems containing cholesterol and disaturated lipids, the work of Mateo and co-workers[29] suggests that the POPC/cholesterol system also displays a phase envelope between liquid-disordered and liquid-ordered states, with the liquid-ordered phase boundary occurring at about 38% cholesterol at room temperature. One would therefore expect a linear trend in behavior as one traverses the phase coexistence regime, ending at the edge of the phase envelope, which is close to what we observe. The phase behavior of the sperm mimic lipid mixture is not known. However, recent studies on simple multicomponent membranes show transitions between liquid-disorder and liquid-ordered phases at cholesterol concentrations close to 30%; here, the transition may be continuous.[30] It is therefore conceivable that the nonlinear behavior exhibited by the sperm mimic membrane is also related to a transition to a liquid-ordered state.

Although cholesterol is the major sterol present in biological membranes, other sterols are also found.[27] We examined the surfactant-induced leakage properties of membranes formed with two other sterols: dihydrocholesterol (5α-cholestane-3β-ol) and coprostanol. Cholesterol is composed of three parts: a planar steroid ring system, a polar 3β-OH group that gives it an amphipathic character, and a hydrophobic chain. Dihydrocholesterol lacks the double bond in the ring system, while coprostanol, its A/B-cis isomer, is a nonplanar sterol. The double bond in the ring system has been shown to have little effect on membrane properties,[27,31] and so one might anticipate similar results for dihyrocholesterol and cholesterol. On the other hand, one might expect the nonplanar coprostanol to exhibit behavior different from the other two sterols. This was seen by Demel and de Kruyff[27] who found that cholesterol and dihydrocholesterol changed the flux of [^{14}C]erythritol through the cell membrane of *Acheloplasma laidlawii* while coprostanol did not. Xu and London[31] found that domain formation was inhibited in mixtures of saturated lipid(s) and coprostanol while detergent solubility increased in the presence of coprostanol; mixtures of the same lipid(s) with cholesterol or dihydrocholesterol showed the opposite effects.

Membranes composed of mixtures of POPC and 10, 30, and 40 mol% sterol were prepared as discussed in the Materials and Methods section. The membranes were incubated with surfactant for 4 h, and the extent of leakage of CF was measured as function of surfactant concentration. As can be seen from Figure 5, the presence of all sterols increases the resistance of the membrane to surfactant attack. On the other hand, the increase in

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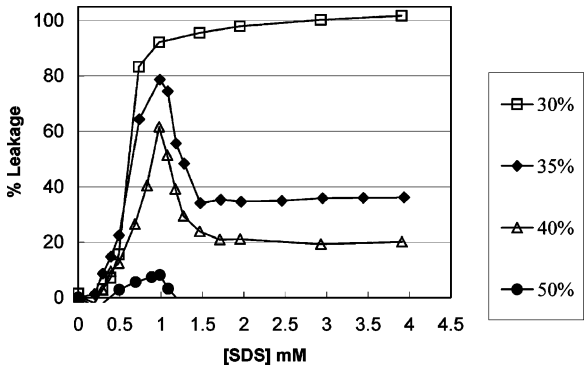

Figure 6. Leakage response (extent after 4 h) of cholesterol/POPC vesicles in the presence of SDS (vesicles concentration 4.6×10^{-3} mM). Vesicles containing 30% (□), 35% (◆), 40% (△), and 50% Chol (●) were examined.

resistance is not linear, as is the case for cholesterol. Also, a membrane can be more, or less, resistant as compared to the cholesterol system, depending on the nature of the surfactant. Interestingly, the two isomers behave similarly, and coprostanol did not stand out. While we cannot explain the behavior observed, we note that sterol−lipid interactions are complex[32] and depend on the specific sterol and lipid. The specifics of our systems and experimental conditions could contribute to the propensities observed.

Micellar SDS Interactions with Cholesterol-Rich Vesicles. A peculiar phenomenon takes place when membranes are perturbed by SDS. As Figure 6 reveals, higher concentrations of surfactant do not necessarily induce greater leakage. In particular, the leakage extent for membranes containing more than 30% cholestertol increases with surfactant concentration as expected, up to a maximum around the CMC of SDS (1 mM), and then mysteriously drops to a lower plateau. The same behavior was also seen in the case of the sperm mimic membranes.

Various tests were performed in an attempt to identify the origin of this behavior. These tests not only ruled out possible causes but also revealed some interesting findings. First, the purity of the SDS solution was verified to test for dodecanol contamination. The surfactant was not contaminated as determined by tracking the tension-reducing properties of SDS (which display a characteristic dip before the CMC if dodecanol is present). In addition, the perturbing nature of dodecanol was explicitly examined. Dodecanol solutions did not cause membrane leakage up to concentrations of about 2.5 mM; also, the leakage curves for pure SDS and those with 1 and 5 mol% dodecanol added overlapped each other. These studies revealed that dodecanol is not a potent membrane perturbant.

We also tested directly for the existence and associated sizes of vesicles using dynamic light scattering. Vesicle radii were measured after 4 h of surfactant exposure (corresponding to the leakage data). For membranes containing 30% cholesterol or less, vesicles were actually solubilized by SDS at a concentration around or below the CMC. (We note that none of the other surfactants caused membrane solubilization under conditions where leakage data were obtained.) In contrast, vesicles containing 35 or 50 mol% Chol continued to exist even at 3 mM SDS, though their size decreased from their original radius of 55 nm to 45 nm.

The peculiar maximum seen in the leakage curves (see Figure 6) occurs around the CMC value of SDS and suggests that micelles may be responsible for the remarkable resistance of vesicles containing more than 30% cholesterol. We believe that SDS micelles recruit POPC from these vesicles, thus shifting the membranes toward higher cholesterol concentrations. The vesicles remain intact (as confirmed by light scattering) but become even more resistant to surfactant attack. Vesicles with lesser cholesterol content do not undergo this in-situ modification of membrane cholesterol content; instead, the vesicles are solubilized. At SDS concentrations below the CMC, the leakage behavior is as expected, following the trends discussed previously.

To prove this hypothesis, we performed the following experiment. Vesicles with an original cholesterol fraction of 40% were incubated for 4 h with SDS at three concentrations: 0, 0.65 (below the CMC), and 3 mM (above the CMC). The vesicles were then separated from the surfactant molecules and any other free molecules in the solution using centrifugation steps. (We used a cut-off filter chosen to retain the vesicles but not the smaller SDS micelles.) The phosphorus content of the separated vesicles was characterized. For all samples, the phosphorus content was a bit lower than would be expected probably because some vesicles degrade as a result of the centrifugation. Nonetheless, the lipid content of the vesicles that were incubated with 3 mM SDS was 28% lower on average compared to the samples that were exposed to the monomeric form of SDS or no SDS at all. We tried to also measure directly for membrane cholesterol content, but our samples were not of sufficient size to achieve a reasonable signal-to-noise ratio. The loss of phospholipid above, but not below, the CMC, combined with the decrease in vesicle size determined by light scattering, provide strong evidence of the hypothesis proposed.

So, for example, a 40% (as prepared) vesicle when exposed to SDS far above the CMC will initially leak with a rate similar to membranes exposed to monomeric SDS at the CMC. During the leakage process, however, these membranes are becoming richer in cholesterol due to phospholipid removal by the SDS micelles. Correspondingly, the kinetic constant corresponding to the leakage rate decreases. Eventually, the membranes become rich enough in cholesterol that the rate of leakage is essentially zero. This explains why the leakage data exhibit a plateau at higher SDS concentrations—one reaches a point where the concentration of micelles is sufficient to "shut-off" leakage from all the vesicles present in the system.

These studies thus present a new method for easily modifying vesicles so as to be extremely rich in cholesterol and, correspondingly, very resistant to surfactant attack. More interestingly, the same process could be used to modify in-vivo cellular membranes for various useful purposes. Indeed, a similar cholesterol modification scheme is directly involved gallstone pathogenesis where bile salt micelles selectively solubilize lecithin over cholesterol to yield parent vesicles supersaturated in cholesterol.[33]

Conclusions

Given the variety of cells implicated in STD prevention (e.g., positive and negative strains of bacteria, sperm, epithelial cells, and vaginal microflora), it is valuable to understand the impact of cholesterol on surfactant-induced membrane perturbation, a common approach to pathogen control. We have undertaken such a systematic study, with particular focus on surfactants with current or potential use as microbicides/spermicides in STD

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prevention. First, we found that the vesicle preparation procedures, which are those commonly used in most vesicle-based membrane research, do not alter the proposed cholesterol content of these model membranes. We show that cholesterol addition to the membrane does not alter the fundamental form of the leakage response (which goes to completion following first-order kinetics); however, for a given surfactant concentration, the rate constant associated with the leakage process decreases as membrane cholesterol content increases. This increase is linear up to cholesterol contents of about 30 mol%. Other sterols similar to cholesterol also serve to increase membrane resistance to surfactant attack. Surprisingly, the nonplanar coprostanol exhibited behavior similar to the other sterols studied. We also present a novel way of creating extremely resistant membranes using SDS micelles to increase membrane cholesterol content (via removal of phospholipids). As eukaryotic cell membranes contain cholesterol while bacterial cells do not, our results may have significance in pathogenic drug targeting.

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