

Membrane Perturbation by Surfactant Candidates for STD Prevention

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We studied surfactant-induced leakage of vesicles, comparing the efficacy of four surfactants with current or potential application in contraception and the prevention of sexually transmitted diseases: nonoxynol-9 (N-9); the amphoteric mixture known as C31G; benzalkonium chloride (BZK); sodium dodecyl sulfate (SDS). Several features are generic to the membrane leakage process independent of the surfactant used. The kinetics of the leakage process is well-characterized by a single exponential rate of release, and the rate constant increases with surfactant concentration according to a power-law dependence. Notably, however, the formation of surfactant micelles serves to enhance the rate of vesicle leakage. A method for quickly changing the effective surfactant concentration in situ, and thus the rate of vesicle leakage, is presented. Specifically, methyl- β -cyclodextrin is added to the external aqueous solution and forms inclusion complexes with surfactant, effectively reducing the concentration of soluble surfactant. These studies show that the partitioning of surfactant between the aqueous solution and the lipid membrane is quick and certainly not a rate-limiting step in the membrane leakage process. We also show that the distribution of surfactant between the membrane and solution does not strictly follow a simple partitioning model. In comparing the membrane perturbing capabilities of the four surfactants, we find that C31G > BZK > N-9 \gg SDS. Finally, our studies clearly show that the potencies of the surfactants toward membrane attack do not scale with the magnitudes of their critical micelle concentrations.

Introduction

Sexually transmitted diseases (STDs), such as acquired immunodeficiency syndrome, chlamydial infection, gonorrhea, and syphilis, are among the most common and persistent infectious diseases in the United States, and their worldwide occurrence is growing dramatically.¹ The short-term and long-term consequences of STDs include pelvic inflammatory disease, infertility, ectopic pregnancy, preterm delivery, low birth weight, several types of cancer, and even death.^{1,2} The variety of different pathogens implicated in STDs make the development of preventative broad-spectrum vaginal microbicides a healthcare priority.

To guarantee potency against a wide range of pathogens, the basis of attack must be relatively fundamental and generic. Membrane perturbation is one obvious strategy, as the proper functioning of cells and enveloped viruses rely upon the many services of their finely tuned membranes. Moreover, it is a strategy that is not easily thwarted by pathogen adaptability, thus reducing the notorious potential of microbicidal resistance. It should be appreciated, however, that the advantage of broad-spectrum activity is offset by the need to reduce the attack on healthy or useful cells, such as vaginal epithelial cells or the lactobacilli of the vaginal flora.

It appears that many, if not most, invertebrates and vertebrates (including humans) have indeed adopted a membrane perturbation strategy as a part of their innate defense system. A key regiment in this mode of pathogen warfare is a class of membrane-lytic peptides known as antimicrobial peptides (AMPs). The majority of AMPs are short amphiphilic cationic polypeptides composed of 20–

60 amino acids. Some are linear, mostly helical, while others contain one or more disulfide bonds allowing β -sheet formation.³ More than 400 different AMPs have been isolated and characterized, among them the well-studied melittin,⁴ magainin,⁵ and defensins.⁶ Available evidence supports the hypothesis that the principal mode of action of AMPs involves perturbation and associated permeabilization of pathogen membranes.^{4,5,7,8}

Efforts to employ synthetic compounds as broad-spectrum microbicides have focused on the use of surfactants. These are amphiphilic molecules composed of a polar “head” group (zwitterionic, anionic, cationic, or nonionic) 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.

Attempts to understand the membrane perturbation mechanisms and properties of AMPs, surfactants, and other membrane-active agents have often taken advantage of model membrane systems for fundamental studies. Insoluble monolayers⁹ and planar black lipid membranes have proven useful,¹⁰ but the most robust and configurationally realistic model membranes are lipid bilayers in the form of unilamellar vesicles (for examples see refs 11–13).

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Vesicles, for example, have been extensively used to establish the mechanisms of AMP activity. Such studies have revealed two different modes of membrane permeation: (1) One mode is a "carpet mechanism"^{14,15} in which molecules bind to the membrane to cover it partially or fully in a carpet-like manner. The permeation process occurs only after threshold coverage has been reached. Higher peptide concentrations lead to disintegration of the membrane, followed by withdrawal of peptide-lipid assemblies. (2) Another mode is a "barrel-stave mechanism"¹⁶ in which aggregates of peptide monomers insert into the hydrophobic core of the membrane to form pores. Certainly the ability of various peptides to associate and form transient or long-lived pores continues to capture the attention of many researchers.^{5,17,18}

Most studies that have focused on surfactant/membrane interactions have been motivated by the use of surfactants in membrane solubilization and protein isolation applications. A large portion of the relevant literature is dedicated to the surfactant saturation and membrane solubilization limits, as well as mechanism of the solubilization process.^{19,20} It is now fairly well established that the latter occurs via a three-stage process in which the first step is the disruption of the membrane by monomeric surfactant molecules. Above a certain surfactant concentration, mixed surfactant-phospholipid micelles are formed.²¹ Further addition of surfactant to the system results in solubilization of the membrane. At subsolubilizing concentrations, it is generally accepted that the incorporation of surfactant in the membrane obeys an equilibrium partitioning of the surfactant between the lipid bilayer and the aqueous medium.²² Given the primary motivation for these investigations, the most studied surfactants have been those generally used to solubilize membranes, such the Triton-X series, poly(oxyethylene nonylphenols), and bile salts.

In this paper we perform a comprehensive and comparative study of lipid membrane perturbation as caused by surfactants with current or promising application in contraception and as vaginal microbicides for STD prevention. Four surfactants are considered: (1) nonoxynol-9 (N-9), a nonionic surfactant already widely used as spermicidal agent;²³ (2) C31G, an amphoteric mixture of two surface-active molecules, C₁₄ alkylamine oxide and C₁₆ alkyl betaine;^{24,25} (3) benzalkonium chloride (BZK), a vaginal spermicide²⁶ used worldwide; (4) sodium dodecyl sulfate (SDS), an anionic surfactant with protein denaturing activity, which was found to be much less toxic than either N-9 or C31G and is effective against HIV-1 and Herpes simplex viruses.^{25,27} For this study, the membrane is composed of the phospholipid POPC.

Our studies center on one of the simplest and most common experiments that can be performed with vesicles—a leakage experiment, in which encapsulated fluorophores permeate across the membrane as a result of vesicle exposure to a membrane-active agent. We establish and quantify the time dependence of the leakage response as induced by these four surfactants. We also show how the leakage response can be systematically altered by the addition of a cyclodextrin to the external aqueous solution. The ability of cyclodextrin to form inclusion complexes with surfactant allows us to regulate the amount of surfactant in the membrane and, thus, the rate of leakage.

Materials and Methods

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was obtained from Avanti Polar Lipids (Birmingham, AL). Methyl- β -cyclodextrin (methyl- β -CD) as well as benzalkonium chloride (BZK), sodium dodecyl sulfate (SDS), sodium chloride (NaCl), HEPES (2-[4-(2-hydroxyethylene)-1-piperazinyl]ethanesulfonic acid), phosphorus standard solution, and pyrene 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). Nonoxynol-9 (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 20 mM) were prepared by dissolving the surfactant in 16.66 mM Hepes buffer containing 125 mM NaCl. The same buffer solution including salt 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 2 M NaOH. A 100 mM 5,6-carboxyfluorecin (CF) solution was prepared using 16.66 mM Hepes buffer and also adjusted to a 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 were prepared using the following procedure. A chloroform solution of POPC was transferred to a 25 mL round-bottom flask. The lipid solution was dried overnight in a vacuum oven. The dried lipid film was redissolved in CF solution to yield a 10 mM lipid solution. A multilamellar vesicle dispersion was prepared via 5 cycles of vortex mixing followed by freeze-thawing (liquid nitrogen). The dispersion was extruded (Lipex biomembranes Inc) 2 times through a 400-nm polycarbonate filter (Nucleopore Co.) followed by 10 times through a 100-nm polycarbonate filter. 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 method for total phosphorus assay,²⁸ in a procedure made available by Avanti Polar Lipids. The measurements were performed using Genesys 2 spectrophotometer (Thermo Spectronic Instruments). Dynamic light scattering experiments were performed to determine the vesicle size distribution resulting from the extrusion procedure. The measurements were done using a commercial apparatus [Brookhaven, Inc., BI-200SM laser light scattering goniometer equipped with solid-state laser ($\lambda = 532.5$ nm) and an ALV-5000 correlator]. All measurements were performed at 25 °C under a scattering angle of 90°. The experimental autocorrelation function was fitted by second-order cumulant data analysis and ALV

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nonlinear data analysis. Measurements on three separate vesicle solutions confirmed a relatively homogeneous size distribution (polydispersity lower than 0.054) having a mean diameter of 100 ± 11 nm.

Leakage Experiments. The membrane permeability was studied by monitoring the increase in the fluorescence signal, $I(t)$, due to the release of CF molecule 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 bulk 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.^{29,30}

Fluorescence measurements were made using a Hitachi F-4500 fluorescence spectrophotometer. The samples were excited at 490 nm, and an emission maximum 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 few seconds. We note that vortexing in lieu of mild shaking gave inconsistent and unreasonable results.

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

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

Here 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. Intensity was tracked as function of time to establish the kinetics of CF release. Each experiment was repeated at least 3 times. Repeatability was within $\pm 5\%$, unless otherwise stated.

The importance of particle scattering in the leakage measurements was investigated. Vesicles were prepared by following the procedures above with one alteration: the dried lipid film was redissolved in external solution (no probe) instead of using the CF solution. Vesicles were incubated for 4 h with the four surfactants used for this study. The same range of surfactant concentrations used for leakage experiments was used to perturb the vesicles sans probe. To examine the extent of vesicle scattering and its influence on the measured fluorescence signal, all samples were excited at a wavelength of 490 nm and the emission was read at 490, 519, and 650 nm. In addition, a subset of samples (corresponding to low, medium, and highest surfactant concentrations used) and the unperturbed vesicle solution, as well as pure surfactant solutions above their respective micellar concentrations, were all subject to a 3D scan (scanned at excitation wavelengths between 300 and 700 nm and emission wavelengths between 300 and 700 nm). In all cases, the relevant signal (i.e., excitation wavelength 490 nm and emission wavelength 519 nm corresponding to conditions probed in fluorescent leakage experiments) was lower than 1% of our leakage experiment baseline signal (I_0) and can even be attributed to machine error. Thus, the fluorescence signal measured during leakage experiment results from the release of CF molecules and any contribution as a result of particle scattering can be neglected.

The possibility of leakage as a result of vesicle aggregation and not only perturbation was examined. Dynamic light scattering (DLS) measurements were performed on samples that were perturbed by different surfactants, at different surfactant concentrations after incubation of 4 h as described before. The intensity of scattered light remains relatively constant (within 10% difference). An increase of up to 10% in vesicle size was recorded in virtually all cases (the one exception is for the highest concentrations of SDS studied corresponding to 100% leakage; here the light scattering revealed a complete annihilation of vesicles). According to Edwards and Almgren¹¹ the stage of slight swelling in vesicle size precedes the stage at which higher surfactant concentrations induce the formation of significantly

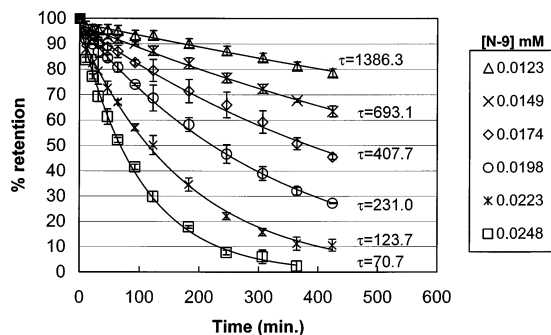


Figure 1. Time dependence of CF leakage from POPC vesicles (lipid concentration 0.01 mM) in the presence of N-9 at various overall concentrations.

larger aggregates. Thus, it seems that the fluorescence signal we observe results solely from leakage of CF molecules from the original vesicles.

Cyclodextrin Experiments. The CF leakage behavior of POPC vesicles in the presence of N-9 was changed, in situ, by adding different amounts of methyl- β -CD to the solution. Vesicle leakage was initiated by adding N-9 (0.0247 mM final concentration) to a vesicle solution (4.6×10^{-3} mM final lipid concentration). The kinetics of the leakage response was tracked for 10 min. At $t = 15$ min, a given amount of methyl- β -CD (from 0 to 3.125×10^{-2} mM final concentration) was introduced into the vesicle solution, and the new time-dependent fluorescence response was tracked for a total duration of 240 min.

Cmc Measurements. The critical micelle concentrations (cmc) of surfactants in the presence of the same buffer and the salt employed in the membrane leakage experiments were determined using the procedure of Kalyanasundaram and Thomas.³¹ The method is based on the change in the spectrum of pyrene as the molecule associates with micelles. As the solubility of pyrene in water is only 2–3 μM , we prepared our pyrene solution by mixing excess pyrene with external solution for several hours, after which the solution was filtered to eliminate undissolved material. Different surfactant concentrations were mixed with equal volumes of pyrene solution. The solution was excited at 324 nm, and the emission signal was scanned between 350 and 450 nm using a Hitachi F-4500 spectrofluorometer.

Results and Discussion

The results of this study will be divided into two sections. The first centers on the features that were found to be generic to the membrane leakage process. The second concentrates on the specific characteristics of the leakage process associated with the four surfactants chosen for study: nonionic N-9; zwitterionic C31G; cationic BZK; anionic SDS.

Generic Characteristics of the Surfactant-Induced Vesicle Leakage Process. *Kinetics of the Leakage Process.* Regardless of the surfactant used, its concentration, or the phospholipid concentrations we employed (2.4×10^{-3} –0.1 mM), the observed leakage process is continuous and can be well described by a single-exponential decay:

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

Here % retention is defined as 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. Representative examples are shown in Figure 1 for a fixed lipid concentration (0.01

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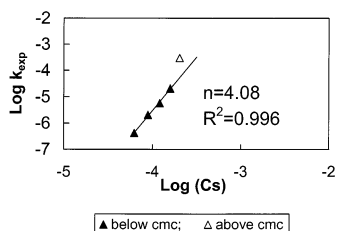


Figure 2. Dependence of leakage rate constant, k_{exp} , on N-9 concentration in the case of POPC vesicles (lipid concentration 4.6×10^{-3} mM).

mM) in the presence of various amounts of the surfactant N-9.

Figure 1 also clearly shows that the rate constant increases with overall surfactant concentration, a result consistent with the expectation that leakage is promoted with increasing concentration of the membrane perturbant. What is more interesting is the range of rate constants obtained over relatively narrow ranges of surfactant concentrations. For example the characteristic half-time of the leakage process can vary from more than 1 day to less than 1 min as the overall concentration of N-9 increases from 0.01 to 0.02 mM (this for the case of a lipid concentration of 2.4×10^{-3} mM; we note that the sharpness of this effect does broaden as overall lipid concentration is raised).

The extreme sensitivity of the leakage kinetics to changes in overall surfactant concentration, C_s , is reflected in Figure 2, which shows that $\ln(k_{\text{exp}})$ varies linearly with $\ln(C_s)$, revealing a power-law dependence on surfactant concentration. The slope of this line is specific to the particular surfactant used; it is also slightly sensitive to the overall phospholipid concentration.

One must appreciate that surfactants can form micelles when the solution concentration exceeds the critical micelle concentration (cmc). How their presence changes the leakage response is not well established. In our experiments, the actual aqueous surfactant concentration is not equal to the overall concentration as a result of surfactant partitioning into the membrane. In the absence of methods to detect the presence of micelles, it is not immediately obvious what surfactant concentrations are required for micellization. We believe, however, that the leakage response does indeed reflect the appearance of micelles, as noted by the data point in Figure 2, which lies above the linear fitting. An equilibrium partitioning analysis (described below) can be used to estimate the concentration of surfactant in the membrane for a given rate of leakage, i.e., for a given k_{exp} . A mass balance on surfactant can then be used to estimate the concentration of surfactant in the external solution; this value can be compared to the cmc. For the data set shown in Figure 1, such an analysis predicts that the most concentrated surfactant system does indeed meet the conditions for micelle formation. Similar analysis for all the other systems we studied (i.e., for different total concentrations of phospholipid and/or for different surfactants used) consistently showed that when conditions are met for micellization, the rate constant is greater than that predicted from the linear power-law extrapolation.

Our results concerning the generic features of the membrane leakage process can be compared to related studies in the literature. Others have also reported exponential release kinetics.^{11,32,33} Some investigations, however, have noted a biphasic behavior: very fast initial

release of marker over seconds, followed by a slower exponential decay.^{17,34} We did not observe such a dual-time constant response; however our first measurements commenced at 5 min, and so we cannot rule out the existence of such behavior.

Although we did not track leakage beyond 7 h, we also have no reason to expect that the form of the leakage response would change at even larger times; in other words, it is likely that the vesicles continue to follow exponential release kinetics until there is no concentration gradient of probe across the membrane (indeed at high enough surfactant concentrations, the leakage proceeds to 100%). This is in agreement with other studies.^{11,35} Our results do not support the notion proposed by others^{36–38} of a partial leakage process, in which the release actually stops within the experimental time scale (usually less than 1 h). Incomplete leakage was also observed by Kragh-Hansen and co-workers,³⁹ who examined vesicle leakage over a 24 h time period.

Finally, a nonlinear dependence of rate on perturbant concentration has also been reported in some previous surfactant studies^{11,40} as well as in studies with antimicrobial peptides.^{5,41} This behavior is often ascribed to a “cooperative effect” of the perturbant molecules (e.g., as implicated in pore formation), but the actual mechanism of surfactant-induced leakage is very much an open question.

Modulating the Leakage Kinetics via Cyclodextrin Addition. Cyclodextrin (CD) is a torous-shaped, cyclic oligosaccharide consisting of 6, 7, or 8 (α , β , γ , respectively) glucopyranose units. The circular molecule has a polar surface and a relatively nonpolar cavity. This structure allows the CD molecule to accommodate a variety of compounds in its cavity such as organic molecules, ions, and gases.⁴² Cyclodextrins can also be modified to tune their physical properties; methyl- β -CD is a popular one due to its increased water solubility. Given their ability to form host–guest complexes, CDs have been exploited in many studies to modulate membrane cholesterol content.^{43–45} For example, Wojcik and co-workers⁴⁴ treated spermatozoa with methyl- β -CD before exposure to maganin-2 amide and found that this increased the potency of the antimicrobial peptide as a result of a reduction in the membrane cholesterol content.

In contrast to peptide-induced membrane leakage, surfactant-induced perturbation can be suppressed by the presence of CD as the surfactant binds more strongly than cholesterol.⁴⁶ It should be noted that the β -CD molecule

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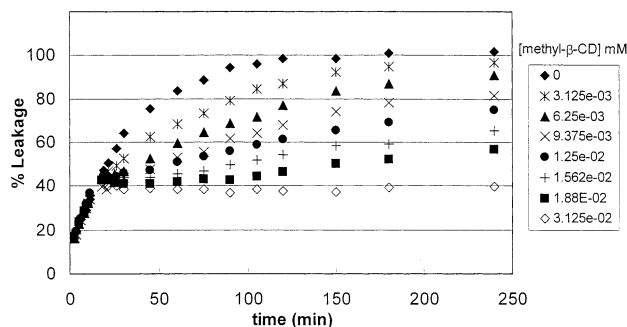


Figure 3. Leakage behavior of POPC vesicles in the presence of N-9 before and after the addition of methyl- β -cyclodextrin at $t = 15$ min. The surfactant concentration was 0.0247 mM, the lipid concentration 4.6×10^{-3} mM, and the methyl- β -cyclodextrin concentration ranged from 0 to 3.125×10^{-2} mM.

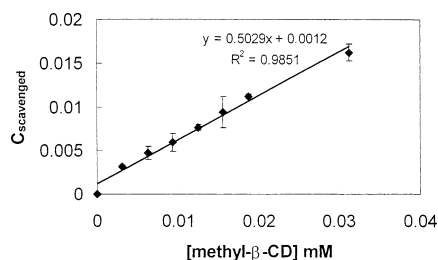


Figure 4. Concentration of surfactant scavenged by methyl- β -cyclodextrin as a function of the methyl- β -cyclodextrin concentration.

is not itself surface active.⁴⁵ We take advantage of the strong association of CD with surfactants to directly modulate, in situ, the surfactant-induced leakage response of vesicles. The idea is to commence the leakage process by exposing vesicles to a given amount of surfactant. After a prescribed duration, but well before complete loss of vesicle contents, a given amount of methyl- β -CD is added to the system. After addition of methyl- β -CD, the vesicles undergo a new rate of leakage, as shown in Figure 3.

As is readily seen in Figure 3, the leakage rate is systematically reduced with increasing concentrations of the methyl- β -CD. The new rate of leakage is well described by exponential kinetics, with a reduced rate constant as compared to the original. The methyl- β -CD locks up a fraction of the surfactant and, thus, serves to decrease the overall surfactant concentration. The lower rate of release is consistent with exposure to a decreased concentration of surfactant. At a high enough concentration and beyond (e.g., 0.03125 mM in Figure 3), the leakage is essentially halted. We note that this ability to literally “shut off” the leakage process may provide a useful experimental tool in future studies.

One of the most interesting results is that the new rate of leakage commences within minutes, if not seconds, of CD addition. Clearly the distribution of surfactant between the aqueous solution and the lipid membrane adjusts quickly to the new effective overall surfactant concentration. These experiments thus provide convincing evidence that surfactant partitioning is quick and certainly not a rate-limiting step in the surfactant-induced vesicle leakage process.

One can now also say with confidence that the leakage process is also not limited by transport of surfactant to the membrane, i.e., is not diffusion-controlled. Given that the partitioning is quick, transport of surfactant to the membrane is as high as diffusion and/or convection will

allow, i.e., corresponding to total depletion of surfactant near the membrane surface. The time scales observed for the leakage process are much slower than those needed to transport surfactant to the membrane under these conditions. Hence, while the mechanism of surfactant-induced vesicle leakage is still not fully understood, it is clear that the rate-limiting step involves motion and/or organization of the molecules in the membrane.

Finally, we can also use these experiments to establish the nature of the surfactant-CD inclusion complex. Such complexes have been the separate focus of several studies. While a 1:1 inclusion complex is commonly suggested, growing evidence for various other kinds of inclusion complexes, such as 1:2, 2:1, 2:2, or 1:3 (guest:host), has been reported in the literature for different surfactants (e.g., SDS and CTAB).^{46–48}

To analyze our experiments we exploit the fact that a given rate of leakage corresponds to a given overall surfactant concentration, with the relation following a power-law dependence such as that shown in Figure 1. Hence, after addition of methyl- β -CD, the reduced rate constant can be used to identify a new effective overall surfactant concentration. The difference between the original surfactant concentration and new effective surfactant concentration is the concentration of surfactant scavenged by the added amount of cyclodextrin.

Figure 4 shows the relationship between the amount of methyl- β -CD added to the system and the amount of surfactant scavenged. Our results clearly support the formation of 1:2 (guest:host) complex between the surfactant and the methyl- β -CD. As the length of the hydrophobic part of N-9 (~ 32 Å) is much longer than the cavity height of methyl- β -CD (7 Å⁴⁹), it is quite reasonable to imagine that more than one CD molecule is involved in the association complex.

Surfactant Partitioning. Our work confirms that the partitioning of surfactant between the aqueous solution and the lipid membrane is fast, and hence, it is reasonable to assume that the surfactant distribution is in equilibrium over the experimental time scales considered.

Lichtenberger et al.,²² Almog et al.,⁵⁰ and Schurtenberger et al.⁵¹ suggested that this equilibrium can be described in terms of a partition coefficient, K , defined as follows:

$$K = \frac{x_m}{C_{\text{aqueous}}} \quad (3)$$

Here C_{aqueous} is the concentration of surfactant in solution and x_m is the mole fraction of surfactant in the membrane.

This equation can be combined with a mass balance on the surfactant to yield a useful expression that relates two key experimental variables, namely the total phospholipid concentration (PL) and the overall surfactant concentration (C_s):

$$C_s = \frac{x_m}{1 - x_m} \text{PL} + \frac{x_m}{K} \quad (4)$$

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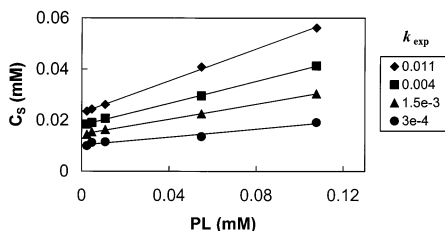


Figure 5. Dependence of the overall surfactant concentration on lipid concentration for specific values of k_{exp} . The linear dependence predicted by eq 4 is observed.

In lieu of direct binding studies, this expression provides a route for estimating the partition coefficient, an approach popularized by the group of de la Maza and co-workers.³⁶ As suggested by the form of eq 4, the appropriate methodology is to track different phospholipid and overall surfactant concentrations *over conditions where the membrane surfactant mole fraction, x_m , is constant*. The well-characterized kinetic response of surfactant-induced vesicle leakage provides a means to establish such conditions. In particular, we assume that a specific rate of vesicle leakage corresponds to a unique membrane surfactant mole fraction: $k_{exp} \leftrightarrow x_m$.

Hence, a convenient experiment approach is to perform a series of leakage experiments at a fixed phospholipid concentration to yield a power-law correlation of rate constant with overall surfactant concentration, such as that shown in Figure 1. This process is repeated over a set of different phospholipid concentrations. Then, eq 4 can be fit to the appropriate concentration variables (PL, C_s) which correspond to a specific rate of leakage. As shown in Figure 5, we find these concentration variables obey, without failure, the linear dependence predicted.

The slope and intercept of the plot in Figure 5 yield the partition coefficient and the surfactant mole fraction for this specific rate of leakage. While the latter is expected to vary with the rate of leakage, the former is not. However, we found that the partition coefficient determined in this manner does indeed vary from 7.24 to 11.07 mM^{-1} (for k_{exp} varying from 3×10^{-4} to $3 \times 10^{-2} \text{ min}^{-1}$, respectively).

Others, most notably de la Maza, have followed this basic approach. Instead of using experimentally determined rate constants as a criterion for identifying membranes with a unique surfactant concentration, they consider in this regard those systems that display 50% fractional release of contents after a fixed length of time. Following their approach, but now across different degrees of fractional release, we again see a variation in partition coefficients.

Clearly the simple equilibrium partitioning relationship does not describe the system behavior perfectly. This relationship is founded upon ideal solution assumptions, and so nonideal mixing effects (especially in the membrane) may well be one source of the discrepancy. On the other hand, we find it disconcerting that the partition coefficient does not appear to reach an asymptotic value at low rates of leakage, i.e., at low concentrations of surfactant. Direct measurements of surfactant concentration in and/or out of the membrane will ultimately be required to resolve this issue.

With due regard for the lack of thermodynamic consistency of the simple partitioning model, it is still worth using it to estimate the fraction of surfactant in the membrane as a function of rate of leakage. We find that x_m ranges from 0.075 up to 0.31 for rates of leakage varying from 3×10^{-4} to $3 \times 10^{-2} \text{ min}^{-1}$. We have used these estimates to evaluate the possibility of micelle formation

(i.e., to estimate the actual surfactant concentration for a given overall surfactant concentration). Indeed, as stated previously, we find that such predictions of micelle formation always correspond to experimentally observed enhanced leakage rates (beyond that extrapolated from the power-law surfactant dependence).

Finally, while the amount of surfactant loaded into the membrane seems substantial under certain conditions (e.g., 30% for large rates of induced leakage), we note that Ueno³³ reports that membranes can include surfactant up to 80% mol fraction before solubilization occurs.

Surfactant Specific Characteristics of the Leakage Process. A large number of studies have focused on the surfactant-induced perturbation of cellular and model membranes. Surfactants commonly studied include those in the Triton-X family, alkyl sulfates, sodium cholate, octyl glucoside, poly(ethylene glycol) *n*-dodecyl monoethers, alkyltrimethylammonium, Tween series, and oxyethylated nonylphenol.^{11,12,32,34,37,40,50}

Unfortunately, it is difficult to make meaningful comparisons between one study and the next as many other parameters beyond surfactant structure (e.g., membrane composition, solution conditions, mixing effects) can influence surfactant/membrane interactions.^{13,22,32} A dramatic example of this is to compare our experiments with the SDS-induced vesicle leakage studies of Ruiz and co-workers.¹² These investigators report that half of the encapsulated carboxyfluorescein is released after 30 min when that ratio of surfactant to phospholipid is 1.2. Their studies were based on sonicated egg-PC vesicles in 10 mM Tris HCl, pH = 7.4. Our studies are based on extruded POPC vesicles in a different buffer (see Materials and Methods), and we find it takes a surfactant-to-phospholipid ratio of 207 to achieve the same fractional release.

Thus, a main goal of this work is to compare head-to-head the membrane-perturbing capabilities of leading surfactants with current or potential application in STD prevention. To make these fundamental studies as relevant as possible, we performed experiments not in water or mild buffer but in salt solution that better approximates physiological conditions. Since the critical micelle concentration is generally sensitive to the presence of salt, we also measured the cmc of each surfactant in these solution conditions.

Cmc's were determined using the pyrene fluorescence method. This fluorophore associates with micelles once they are formed, resulting in a marked change in the probe's fluorescent spectrum (see Materials and Methods for details). In particular, the ratio of the third spectral peak to the first spectral peak is constant below the cmc and increases sharply to a higher value after micelles are formed. The change in spectrum occurs over a narrow but finite surfactant concentration range, and we take the onset of this change as the cmc. We note that the spectral change occurred very sharply for the surfactants C31G, BZK, and SDS, which made the cmc determination straightforward. On the other hand, the spectral signals for N-9 were much more scattered below the cmc, and so the onset of micellation could not be determined with as well a precision. N-9 is a mixture of oligomers which probably explains this behavior. The cmc's obtained are summarized in Table 1 (as based on at least three separate experiments for each surfactant). SDS is the only surfactant among the four that has been studied in various salt solutions. Our cmc is in agreement with findings in the literature.⁵²

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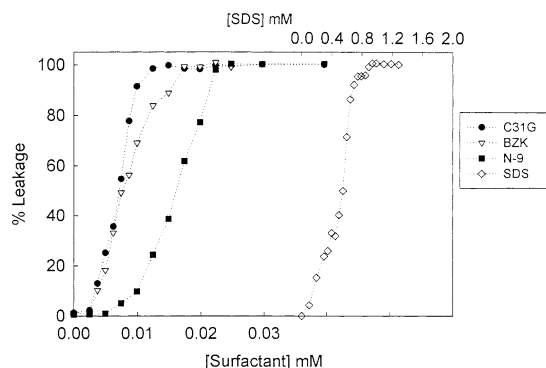


Figure 6. Leakage response of POPC vesicles (lipid concentration 4.6×10^{-3} mM) in the presence of different surfactants (N-9, C31G, BZK, and SDS). Extent of leakage after 4 h is plotted versus overall surfactant concentration. The abscissa for SDS is located at the top.

Table 1. Surfactants Used in This Study and Their Critical Micelle Concentrations (Cmc's) in the Salt Solution Used for Vesicle Leakage Experiments

Surfactant	Type	Structure	CMC (mM)
Sodium dodecyl sulfate (SDS)	Anionic	$\text{CH}_3(\text{CH}_2)_{11}\text{OSO}_3^- \text{Na}^+$	1.00 ± 0.05
Nonoxonyl-9 (N-9)	Non-ionic	$\text{C}_9\text{H}_{19}-\text{C}_6\text{H}_4-(\text{OCH}_2\text{CH}_2)_9\text{OH}$	0.022 ± 0.004
C31G (1:1 equimolar solution of C14 amine oxide & C16 betaine)	Zwitterionic	$\text{C}_{14}\text{H}_{29}\text{N}^+(\text{CH}_3)_2\text{O}^-$ $\text{C}_{16}\text{H}_{33}\text{N}^+(\text{CH}_3)_2\text{COO}^-$	0.02 ± 0.001
Benzalkonium chloride (BZK)	Cationic	$\text{C}_6\text{H}_4(\text{NO}_2)-\text{CH}_2\text{N}^+(\text{CH}_3)_2\text{R} \text{Cl}^-$ R = C_8H_{17} to $\text{C}_{18}\text{H}_{37}$	0.25 ± 0.01

To compare the performance of the four perturbants, we examined as a function of surfactant concentrations the extent of leakage (i.e., fractional release) observed after 4 h. The result is shown in Figure 6. As the concentrations of SDS were comparatively so much higher, a separate abscissa at the top of the graph is employed. The effectiveness of the surfactants in causing vesicle leakage is clearly the following: C31G > BZK > N-9 >> SDS. At the low concentration range, C31G and BZK display about the same performance. These results are in qualitative agreement with studies of the permeabilization of sperm cells by C31G, N-9, and SDS.^{53,54} Complete sperm immobilization (in 30 min) was also studied in one of our laboratories (Doncel, unpublished data). The efficacy order was found to be C31G > N-9 > BZK >> SDS.

Differences between surfactants are also observed in the power-law dependencies of the leakage rate constants (such as that shown in Figure 2 for the case of N-9). We find, for example, that $n = 2.2$ for SDS, 3.3 for C31G, and 4.1 for N-9 (in the case of lipid concentration of 4.6×10^{-3} mM). However, we did not study this effect in any further detail. As noted previously, the power-law observed is sensitive not only to the surfactant but also to the membrane cholesterol content (unpublished data) and even slightly to the total phospholipid concentration.

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Finally, there is debate in the literature regarding the relevance of the cmc to the ability of a surfactant to disrupt a membrane. Some studies have found a direct relation between the cmc and various perturbation metrics,^{55–57} while other studies confirm there is no such relationship.^{12,21,58,59} Our experiments clearly show the membrane perturbing capabilities of these surfactants does not scale with the magnitudes of their cmc's. For example, BZK has a much larger cmc than the nonionic N-9, yet it causes comparatively more leakage. At a basic level, one can presume that the efficacy of a surfactant correlates with its ability to intercalate within the membrane; this of course relies on intermolecular forces mainly between the surfactant and membrane constituents. Self-assembly of surfactant into micelles is governed by forces between like molecules. So, for example, charged surfactants have comparatively high cmc's relative to net uncharged surfactants because of electrostatic repulsions between surfactant headgroups. Meanwhile, these unfavorable interactions between membrane-intercalated surfactants would only be important at high surfactant loads. Clearly a variety of subtle forces conspire to regulate the binding of surfactant to membranes (e.g. hydrophobic forces, dipole–dipole interactions, etc.). It is conceivable, for example, that the dipolar headgroups of the zwitterionic C31G surfactants interact favorably with the zwitterionic headgroups of POPC; the same might help drive the adsorption of BZK (which bears a nitrobenzyl group) into the membrane. Direct binding studies of surfactant to membranes will be needed to gain further insight.

Conclusions

In this study we examined the leakage behavior of POPC vesicles as induced by several surfactants with current or potential application in contraception and STD prevention. We show that the process follows first-order kinetics and that the rate constant is dependent on the bulk surfactant concentration in a nonlinear manner. The kinetics of the perturbation can be regulated, in situ, by addition of cyclodextrin to the system. This method can be used to stop the leakage almost instantaneously, if desired. We also show that the distribution of surfactant between the membrane and solution does not strictly follow a simple partitioning model. The efficacy of membrane perturbation depends on the surfactant used. To cause the same extent of leakage, C31G requires the lowest concentrations followed by BZK, N-9, and SDS, with no correlation to the cmc of these surfactants. The results of this study may also give some important and useful guidelines for choosing the active ingredients in formulations intended for STD prevention.

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