Surfactants as Microbicidal Contraceptives: A Calorimetric Study of Partitioning and Translocation in Model Membrane Systems

Meirav Apel-Paz,[†] Gustavo F. Doncel,[‡] and T. Kyle Vanderlick*,[†]

Department of Chemical Engineering, Princeton University, Princeton, New Jersey 08544, and CONRAD Program, Department of Obstetrics and Gynecology, Eastern Virginia Medical School, 601 Colley Avenue, Norfolk, Virginia 33507

Surfactant partitioning into lipid vesicles was studied using isothermal titration calorimetry (ITC), comparing the behavior 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), and sodium dodecyl sulfate (SDS). Membranes varied in composition from a single-component system, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, to a complex lipid mixture that models the sperm plasma membrane. The partitioning of N-9 into the membranes was found to be endothermic in contrast to the other surfactants studied. For all four surfactants, the partition coefficient decreased as the membrane cholesterol content increased. Surfactant translocation across the membrane leaflets was also determined, with SDS being the only surfactant of the four not to exhibit "flip-flop" on experimental time scales. The results of these studies shed light on the process of surfactant-induced membrane permeabilization.

Introduction

The amphiphilicity of surfactants leads not only to self-assembly in solution (such as the formation of micelles) but also to co-assembly with microstructures such as lipid membranes. A large portion of the literature that has focused on surfactant−membrane interactions is dedicated to the membrane solubilization process. This proceeds through several stages.[1,2] In the low surfactant concentration regime, the surfactants incorporate into the membrane without much change in the general structure of the bilayer. Above a certain surfactant concentration, mixed surfactant−phospholipid micelles are formed, and further addition of surfactant results in complete disassembly of the membrane.

The incorporation of surfactant molecules into the lipid membrane in the sub-lytic regime can alter the physical properties of the bilayer, leading, for example, to changes in stability, permeability, and fusogenicity. Several biological and biotechnological processes exploit surfactant−membrane interactions. For example, one of the simplest vaginal contraceptive strategies involves the exposure of sperm cells to surfactants, such as nonoxynol-9 (N-9), which is used in various commercial spermicides.[3] Although the exact mechanism is not known, evidence strongly suggests that sperm cells are inactivated as a result of surfactant incorporation into the sperm plasma membrane, with associated harmful consequences. Surfactant-based disruption of membranes is also a method of choice for attacking pathogens involved in different sexually transmitted diseases (STDs).[4,5] A complete understanding of the surfactant−membrane interactions is still lacking and continues to be of fundamental and clinical interest.

It is generally accepted that the incorporation of surfactant in the membrane obeys an equilibrium partitioning in which the surfactant distributes between the lipid bilayer and the aqueous medium. The transfer of surfactant molecules between these phases involves the consumption or release of heat and can thus be followed by calorimetry. Isothermal titration calorimetry (ITC) is a label-free technique that is widely employed for affinity studies (binding or partitioning).[6,7] This relatively simple method allows one to determine the partition coefficient as well as other thermodynamic parameters for the system. Moreover, through clever experimental design,[8] ITC can also be used to study surfactant translocation between the leaflets of the membrane bilayer. In particular, one can determine if surfactant "flip-flop" occurs during experimental time scales.

In this work, we used ITC to study the membrane partitioning of surfactants with current or promising applications in contraception and as vaginal microbicides for STD prevention. Four surfactants were considered: (1) nonoxynol-9 (N-9), a non-ionic surfactant already widely used as a spermicidal agent;[9] (2) C31G, an amphoteric mixture of two surface-active molecules, C14 alkylamine oxide and C16 alkyl betaine;[10] (3) benzalkonium chloride (BZK), a cationic vaginal spermicide[11] used worldwide; and (4) sodium dodecyl sulfate (SDS), an anionic surfactant with protein denaturing and antiviral activities.[5] We performed a comprehensive and comparative study of the partitioning of these surfactants into a single-component phospholipid membrane and a multicomponent lipid membrane designed to model the plasma membrane of the sperm cell. As cholesterol is known to influence membrane properties,[12] we also studied the impact of the membrane cholesterol content on surfactant partitioning. Finally, we combined results from these studies with our prior work on surfactant-induced membrane leakage[13,14] to elucidate key features of the membrane perturbation process.

Materials and Methods

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), brain sulfatide (SGC), 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (16:0−22:6 di-ester PC),1,2-di-palmitoyl-*sn*-glycero-3-phosphoethanolamine (16:0−16:0 PE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (16:0−18:1 PS), egg-sphingomyelin (egg-SPH), and cholesterol (Chol) were obtained from Avanti Polar Lipids (Birmingham, AL). BZK, SDS, sodium chloride (NaCl), and HEPES (2-[4-(2-

* To whom correspondence should be addressed. Tel.: (609) 258-4891. Fax: (609) 258-0211. E-mail: vandertk@princeton.edu.
[†] Princeton University.
[‡] Eastern Virginia Medical School.

10.1021/ie071105e CCC: $40.75
Published on Web 01/26/2008

hydroxyethylene)-1-piperazinyl]-ethanesulphonic acid) were obtained from Sigma (St. Louis, MO). All were at the highest purity available. N-9 was a gift from Biosyn Corp., Philadelphia PA (as Rhone-Poulenc's Igepal CO-630 Special at a purity of 95%). C31G (equimolar mixture of C14 amine oxide and C16 alkyl betaine at purities of 88.7 and 98.2%, respectively) was also obtained from Biosyn (Philadelphia, PA). All surfactants were used without further purification. All solutions were prepared using Millipore water.

Surfactant and Buffer Solutions. Surfactant stock solutions (N-9, C31G, and BZK at 0.1 mM solutions and SDS at 10 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 hereon. The pH of the solutions was adjusted to 7.4 with 2 M NaOH. Isoosmotic conditions of all 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 (LUVs) were prepared using the following procedure. A chloroform solution of lipid(s) was transferred to a 25 mL round-bottomed flask. The lipid solution was dried overnight in a vacuum oven. In those cases where membranes with more than one component were created, the mixture was sonicated for 5 min before it was dried. The dried lipid film was redissolved in buffer solution to yield a 30 mM lipid solution. To make surfactant-loaded vesicles, the lipid film was redissolved in surfactant solution instead of in buffer. A multilamellar vesicle dispersion was prepared via five cycles of vortex mixing followed by freeze−thawing (liquid nitrogen). The dispersion was extruded (Lipex Biomembranes, Inc., Vancouver, BC, Canada) 2 times through a 400 nm polycarbonate filter (Nucleopore Co., Pleasanton, CA) followed by 10 times through a 100-nm polycarbonate filter.

The phospholipid content of the vesicles was determined according to the ascorbic acid spectrophotometric method for total phosphorus assay,[15] in a procedure made available by Avanti Polar Lipids. The measurements were performed using a Genesys 2 spectrophotometer (Thermo Spectronic Instruments, Waltham, MA).

The average vesicle diameter was determined to be 100 ± 16 nm using dynamic light scattering (Brookhaven, Inc., San Antonio, TX, 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 size distribution was reasonably homogeneous (polydispersity lower than 0.06).

ITC. ITC measurements were performed using a MicroCal MCS isothermal titration calorimeter (MicroCal, Inc., Northampton, MA). The sample cell (cell volume of 1.3626 mL) was filled with surfactant solution or external solution depending on the type of experiment as discussed next. The reference cell was filled with the external solution. The injection syringe (250 μL) was loaded with the vesicle solution, and a series of aliquots (4−10 μL) was injected into the sample cell. The syringe was rotated at constant speed (400 rpm) throughout the experiment. All solutions were degassed under vacuum before use to avoid air bubbles. Experiments were performed at 25 °C. Each titration was repeated at least three times.

At each injection during an ITC titration, surfactant molecules were incorporated into the membrane, leading to a characteristic heat signal. The heat of reaction for each injection was calculated by integration of the peaks in the heat flow curve as determined using MicroCal Origin software.

Two types of experiments were performed: uptake and release. For the uptake experiment, a highly concentrated solution of vesicles (typically 15 mM) was serially injected into the surfactant solution at a concentration below its critical micelle concentration (cmc). Heats of dilution were obtained by injecting the vesicles into the surfactant-free external solution. The idea of a release experiment is to begin with surfactant-loaded vesicles and monitor heat effects as the surfactant leaves the membrane when the vesicles are injected into the buffer.[8] The injection syringe was loaded with vesicles that were prepared by resuspending dry lipid film (typically 15 mM) in a surfactant solution instead of in a buffer. This procedure ensures that the surfactant is incorporated into both membrane leaflets.[8] The surfactant/lipid ratio in the syringe, R_{syr}, is such that practically all surfactant molecules are incorporated within the vesicles.

Partitioning Model. Schurtenberger et al.[16] and others[6,17−19] showed that surfactant partitioning into lipid membranes generally obeys a simple empirical relationship relating the surfactant concentration in solution, C_S^w, to the ratio of surfactant to lipid in the membrane, R_b

$$K = \frac{R_b}{C_S^w} \tag{1}$$

Here, K is the so-called partition coefficient, and R_b is given by

$$R_b = \frac{n_S^b}{n_L} \tag{2}$$

where n_S^b is the amount of surfactant incorporated in the lipid bilayer, and n_L is the number of moles of lipid or, more generally, all constituents that make up the membrane (e.g., cholesterol is included).

If the surfactant is charged, this simple partitioning model must be adapted to account for electrostatic effects. Partitioning of charged surfactant molecules into the lipid membrane will be accompanied by the formation of a charged membrane surface (with charge density, σ), which gives rise to a surface potential, ψ_0. As a result, the surfactant concentration in the vicinity of the surface of the membrane, C_S^m, which is in equilibrium with the membrane-bound surfactant, will differ from the surfactant bulk concentration, C_S^w. These two concentrations are related by Boltzmann's law

$$C_S^m = C_S^w \exp\left(\frac{-zF_0\psi_0}{RT}\right) \tag{3}$$

where z denotes the valence, F_0 is Faraday's constant, R is the gas constant, and T is the temperature. The partition coefficient is then properly defined as

$$K = \frac{R_b}{C_S^m} = \frac{R_b}{C_S^w} \exp\left(\frac{+zF_0\psi_0}{RT}\right) \tag{4}$$

We note that if electrostatics is not taken into account, the partition coefficient defined as in eq 1 would decrease significantly as the surfactant concentration increases. This behavior results from the build-up of surface charge, which provides resistance to the adsorption process.

Well-known Guoy-Chapman theory[20,21] can be used to relate surface potential to surface charge density, σ, and the details need not be repeated here. As described by Tan and co-workers,[18] the set of working equations is completed by relating the surface charge density to the concentration of surfactant in the membrane

$$\sigma = \frac{zR_b}{A_L + R_b A_S} e_0 \quad (5)$$

Here, e_0 is the elementary electronic charge (1.6×10^{-19} C), A_L denotes the average surface area of the lipids ($A_{L,POPC} = 68$ Å^2),[22,23] and A_S is the surface area of a surfactant molecule. For SDS, we set $A_S = 30$ Å^2.[18] We used the same value for the other charged surfactant used in this study, BZK, and note that the partition coefficients determined from the analysis were not sensitive to the value of A_S in the range of 20−40 Å^2.

The heat measured after the injection of lipid vesicles into surfactant solution, q, is proportional to the change in n_S^b

$$q = \Delta H^\circ \Delta n_S^b + q_{dil} \quad (6)$$

where ΔH° is the molar enthalpy of surfactant incorporation into the membrane, and q_{dil} is the heat of dilution. The heat of dilution is small and can either be treated as a fitting parameter or estimated directly by running an additional experiment in which vesicles are injected into a buffer. We performed both, and the results were consistent.

Keller and co-workers[24] have developed a general fit function for both uptake and release assays that is derived by coupling the previous energy equation with the mass balances associated with each injection. Fits to the data yield the partition coefficient and the enthalpy of adsorption. The analysis takes into account the ability of surfactant molecules to translocate between the inner and the outer bilayer leaflets and also takes into account electrostatic effects due to incorporation of charged surfactants. As we used their method exactly (including a least-squares nonlinear fitting program supplied by Keller), the reader is simply referred to the literature for all details.[24] At least two initial guesses were always attempted to test for multiple solutions. In most cases, fits converged to one unique solution (with excellent agreement between theory and experiment), and the results were consistent across runs for a given surfactant−membrane pair. In rare cases where different initial guesses generated different solutions, we not only chose the one with the best χ^2 quality of fit but also retested runs from the same surfactant−membrane system to ensure that the original fit remained valid. We note that we also fit the data for uncharged surfactants using Stata, a statistical and data management program, as based on operating equations derived by Heerklotz.[25] Results obtained by the two programs varied by at most 1% where comparisons were available.

Thermodynamic variables were determined from partition coefficients according to the following:

$$\Delta G^\circ = -RT \ln(55.5 \text{ M K}) \quad (7)$$

$$T\Delta S^\circ = \Delta H^\circ - \Delta G^\circ \quad (8)$$

Results and Discussion

Two types of experiments were performed in this study (as detailed in the previous section): uptake and release. The first tracks the transfer of surfactant from solution into the membrane, and the second tracks the transfer of surfactant from the membrane to solution. Results will be divided into three sections.

Table 1. Systems Studied[a]

System	C_L (mM)	C_S (mM)	R_{syr}
Nonoxynol-9 (N-9) (cmc = 0.022 ± 0.004 mM)	C_9H_{19}–⬡–$(OCH_2CH_2)_9OH$		
POPC	15	0.005, 0.0145, 0.018	–
	30	0.018	–
	10	0.018	–
Sperm mimic	15	0.0145, 0.018	–
POPC/N-9	15	–	0.08
C31G (1:1 equimolar solution of C_{14} amine oxide & C_{16} betaine) (cmc = 0.02 ± 0.001 mM)	$C_{14}H_{29}N^+(CH_3)_2O^-$, $C_{16}H_{33}N^+(CH_3)_2COO^-$		
POPC	15	0.005, 0.0145, 0.018	–
Sperm mimic	15	0.018	–
POPC/C31G	15	–	0.08
Benzalkonium chloride (BZK) (cmc = 0.25 ± 0.01 mM)	⬡–$CH_2N^+(CH_3)_2R$ Cl^- R=C_8H_{17} to $C_{18}H_{37}$; Predominantly $C_{12}H_{25}$		
POPC	15	0.005, 0.0145, 0.018	–
Sperm mimic	15	0.018	–
POPC/BZK	15	–	0.15
Sodium dodecyl sulfate (SDS) (cmc = 1.00 ± 0.05 mM)	$CH_3(CH_2)_{11}OSO_3^-Na^+$		
POPC	15	0.05, 0.1, 0.2, 0.4, 0.6	–
70% POPC / 30% cholesterol	15	0.05, 0.1, 0.2, 0.4, 0.6	–
60% POPC / 40% cholesterol	15	0.2, 0.4	–
Sperm mimic	15	0.4	–
POPC/SDS	15	–	0.3, 0.43
70% POPC / 30% cholesterol / SDS	10, 15	–	0.3

[a] Listed under each surfactant are the vesicle compositions as prepared for uptake experiments and as prepared for release experiments (where R_{syr} is the molar ratio of surfactant/lipid as injected into buffer).

The first section centers on the main findings related to surfactant partitioning. The second section focuses on the issue of surfactant flip-flop. The last section compares and combines the results of this study with our previous work on surfactant-induced membrane permeability.

Surfactant Partitioning. Uptake experiments were performed for a variety of surfactants, surfactant concentrations, membrane compositions, and vesicle concentrations as elaborated in Table 1. Lipid membranes varied from single-component to multi-component vesicles. As lipids of the phosphatidylcholine group generally constitute the largest class of phospholipids found in most plasma membranes, we chose POPC as our single-component system. Our multicomponent systems were composed either of POPC/cholesterol mixtures or of a more complex lipid mixture that was found to represent both the composition and the behavior of the natural plasma membrane of sperm cells.[14] These sperm-mimic vesicles were made of the following components (numbers represent mol %): 16:0−22:6 di-ester PC (28%); cholesterol (28%); egg-SPH (15%); 16:0−16:0 PE (12%); POPC (10%); SGC (5%); and 16:0−18:1 PS (2%).

Figure 1A shows the data obtained in a typical uptake experiment (in this case, the titration of 0.05 mM SDS with 15 mM POPC vesicles). Each peak represents the heat transfer associated with surfactant incorporation into the membrane. As the amount of vesicles in the sample cell increases with recurrent injections, the amount of surfactant available for partitioning is reduced, and the heat of reaction decreases until it reaches a small constant value, the heat of dilution. All experiments were performed at surfactant concentrations below the cmc so that

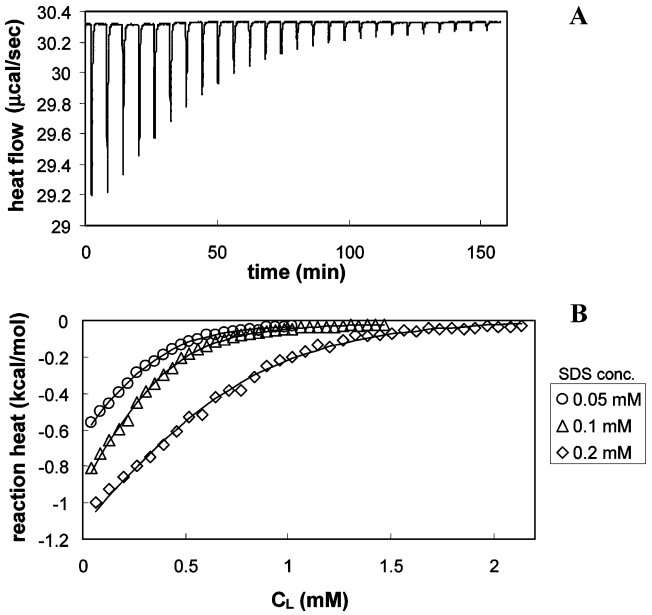

Figure 1. ITC uptake experiment: (A) heat flow (raw data) recorded upon titration of 100 nm POPC vesicles (15 mM) into SDS (0.05 mM). Aliquots of 4 μL of the vesicles solution were injected into the surfactant solution at 6 min intervals. (B) Reaction heats per mol of lipid injected as integrated from the data shown in panel A (circles). The heat flows of two other experiments are also shown: 0.1 mM (△) and 0.2 mM (◇) SDS titrated with 15 mM POPC vesicles. The solid lines correspond to theoretical fits using eq 1. The fit parameters were $K = 7.96 \times 10^4$ M^{-1} and $\Delta H = -3.7$ kcal/mol.

surfactant demicellization would not have to be considered. The cmcs were previously determined in our laboratory[13] using a method based on the spectrum change of pyrene that occurs when it associates with micelles.

The heat of reaction for each injection can be calculated by integration of the peaks in the heat flow curve. Figure 1B shows the reaction heats for the example presented in Figure 1A along with heats associated with higher concentrations of surfactant (titrations of 0.1 and 0.2 mM SDS with 15 mM POPC). The surfactant partition coefficient, K, as well as the partition enthalpy, $\Delta H°$, can be determined from a fit to these isotherms (shown as solid lines in Figure 1) as detailed by Keller et al.[24] Partition coefficients for all systems studied, as well as derived thermodynamic parameters, are summarized in Table 2.

As Figure 1B shows, the closeness of the fits to the experimental data as taken across a range of different concentrations establishes the validity of the mol ratio partitioning model for the systems studied herein. For a given surfactant−lipid system, the variation in K between individual isotherms (corresponding to different surfactant and/or lipid concentrations) is similar in magnitude (ca. 15%) to that which is observed just by repeating experiments. The variation in $\Delta H°$ is about 9% at most.

We find that the enthalpy of surfactant partitioning at room temperature follows trends reported in the literature. The partitioning of N-9 is endothermic, consistent with the behavior of many non-ionic surfactants.[26] The partition enthalpy of zwitterionic molecules is often small but exothermic,[27] and the partitioning of charged surfactants is usually energetically favorable.[18,28] N-9, like most non-ionic surfactants, has a bulky headgroup. The other three surfactants have smaller headgroups, and their cross-sectional areas are of the same order as that of their hydrocarbon chains. The larger headgroup of N-9 probably inhibits efficient packing of the surfactant in the membrane, thus eliminating the energetic driving force for incorporation

Table 2. Partition Coefficients and Other Thermodynamic Data for Surfactant−Lipid Membrane Systems Studied[a]

surfactant	K (M^{-1})	K^* (M^{-1})	$\Delta H°$ (kcal/mol)	$\Delta G°$ (kcal/mol)	$T\Delta S°$ *(kcal/mol)*
		POPC Vesicles			
N-9	4.85×10^3	4.85×10^3	2.8	−7.4	10.2
C31G	1.16×10^4	1.16×10^4	−0.5	−7.9	7.4
BZK	1.63×10^4	1.63×10^4	−1.3	−8.1	6.8
SDS	7.96×10^4	7.96×10^4	−3.7	−9.1	5.4
		Sperm-Mimic Vesicles			
N-9	1.04×10^3	1.45×10^3	6.4	−6.7	13.1
C31G	6.97×10^3	9.68×10^3	−2.7	−7.8	5.0
BZK	1.01×10^3	1.40×10^3	−3.8	−6.7	2.8
SDS	5.43×10^4	7.55×10^4	−2.8	−9	6.2
		70% POPC/30% Cholesterol Vesicles			
SDS	5.13×10^4	7.33×10^4	−2.9	−9	6.0
		60% POPC/40% Cholesterol Vesicles			
SDS	4.22×10^4	7.03×10^4	−2.4	−9	6.6

[a] K, K^*, and $\Delta H°$ reported are based on the average of all uptake and release experiments for a particular surfactant−membrane system. We note that $\Delta H°$, $\Delta G°$, and $T\Delta S°$ are based on membrane phospholipid content only (i.e., are derived from K^*).

that is present in the other surfactant systems. Clearly, favorable van der Waals interactions are strong enough to more than compensate for the unfavorable electrostatic interactions between charged surfactants.

Of course, the hydrophobic effect[29] (leading to positive entropy changes) provides a natural driving force for all surfactants to partition into the membrane. The anomalously large binding entropy for N-9 may arise from both the hydrophobic effect as well as an increase in membrane disorder. Comparing partition coefficients, that for N-9 is the smallest and that for SDS is the largest. As seen in Table 2, the variations in $\Delta G°$ observed across the different surfactants are driven by the variations in $\Delta H°$ that overtake opposing variations in $\Delta S°$ (i.e., SDS has the most favorable free energy of partitioning despite the lowest gain in system entropy).

As discussed previously, partitioning of charged surfactants into the lipid membrane requires the inclusion of electrostatic effects in the analysis. Figure 2 shows the behavior of the cationic surfactant BZK. In accordance with the partitioning model (see eq 4), the amount of bound surfactant, R_b, is proportional to the surface membrane concentration of the charged surfactant (see Figure 2A) and is a non-linear function of the bulk surfactant concentration (Figure 2B). The incorporation of the cationic surfactant into the membrane creates a positive surface charge density; the resulting surface potentials are shown in Figure 2C. Our analysis assumes that the membranes originally bear no surface potential of their own, and we note that this is not strictly true. Electrokinetic data[30] show that even simple POPC membranes can have non-negligible negative surface potentials (our sperm-mimic membranes contain a small amount of anion lipids and likely carry a small negative charge). While we did not take into account these details, doing so would likely decrease the surface potentials derived for BZK-laden membranes and increase the surface potentials for SDS-laden membranes.

Among the systems studied herein, to the best of our knowledge, the partition behavior of SDS into POPC is the only behavior that was studied previously. Comparing our results to the work of Tan et al.[18] (who used a different buffer and a slightly different temperature), we find that our partition coefficient is slightly higher, by about a factor of 4 (our enthalpy of partitioning is lower, and our entropy is higher). Our partition coefficient is about 50 times lower than the value determined

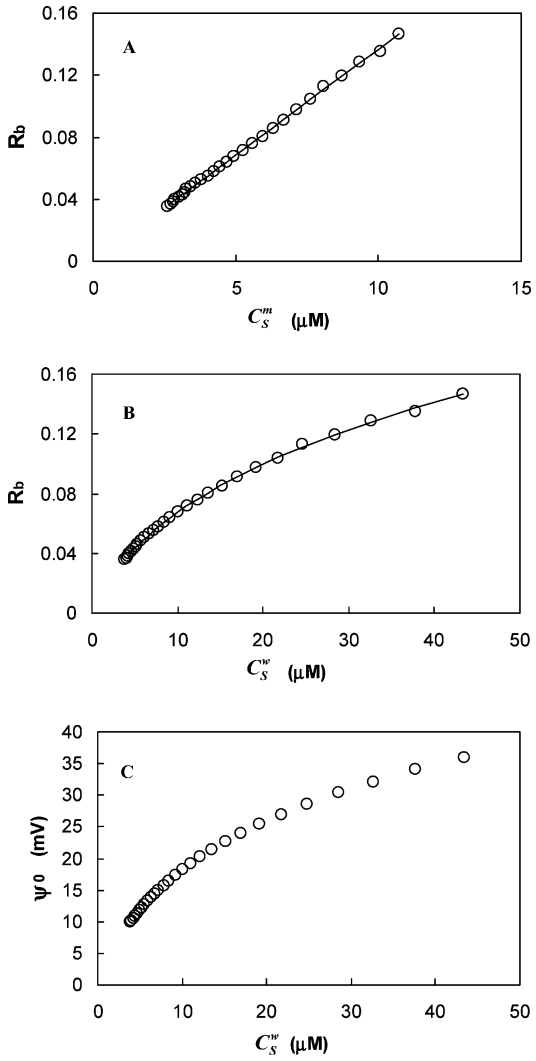

Figure 2. Partition isotherms and membrane potentials correspond to the titration of 100 nm POPC vesicles (15 mM) into BZK (0.05 mM). (A) Mol ratio of surfactants bound, R_b, as a function of BZK surface concentration, C_S^m. (B) R_b as a function of BZK concentration in the aqueous solution C_S^w. (C) Membrane surface potential, ψ_0, as a function of C_S^w. The solid lines correspond to theoretical fits using eq 4.

by Keller et al.[24] (who used a different buffer but same temperature; our enthalpy of partitioning is lower, and our entropy is higher).

We now compare the partitioning of all surfactants into the more complex membranes that are designed to mimic the sperm plasma membrane. As described previously, these membranes contain approximately 30% cholesterol, as well as a large fraction of sphingomyelin (15%). As shown in Table 2, in comparison to those for the simple POPC membranes, the partition coefficients are significantly lower. This is true for all four surfactants studied.

We have recently characterized membrane lipid order (with and without surfactant incorporation) using time-resolved fluorescence analysis with the membrane probe diphenylhexatriene.[31] These studies showed that these sperm-mimic membranes display a higher degree of order than either simple POPC membranes or POPC/30% Chol membranes (effective DPH lipid order parameters spanned from 0.15 for POPC membranes, to 0.56 for POPC/Chol membranes, to 0.7 for the sperm-mimic membranes). The more efficient lipid packing renders the membranes less receptive to surfactant incorporation, consistent with the lower partition coefficients reported here. The fractional reduction of the partition coefficients is greatest for BZK. This behavior may well reflect specific interactions with certain components of the membrane and also surfactant inhomogeneities as BZK includes chains of varying lengths. More work is needed to resolve the origin of these differences.

Last, we also examined the specific effect of cholesterol on surfactant partitioning, focusing our attention on one surfactant, SDS, comparing its partitioning into cholesterol-laden vesicles: POPC/30% Chol, POPC/40% Chol, and sperm-mimic vesicles that contain ca. 30% Chol. Cholesterol is the major sterol component of the plasma membrane and affects molecular packing and various membrane properties such as permeability, stability, and fusogenicity.[12,14] Table 2 shows that the partition coefficients of SDS in the cholesterol-laden membranes are lower than for the cholesterol-free (POPC) membrane. If, however, the partition coefficient is calculated based on phospholipid content only, K^*, we see that the partition coefficients are quite similar (only a small decrease with increasing cholesterol content). A similar phenomenon was reported for the partitioning of octyl-β-D-glucopyranoside into membranes with varying cholesterol contents.[17]

Although the amount of cholesterol seems not to dramatically affect the partitioning of SDS (in terms of K^*, that is), the thermodynamic variables are impacted. Increasing the amount of cholesterol reduces the energetic driving force and increases the entropic driving force, leaving the free energy nearly constant (see Table 2). Our recent fluorescence anisotropy studies showed that SDS incorporation into lipid membranes containing cholesterol led to a significant decrease in lipid order; in contrast, there was little change in lipid order when SDS was incorporated into simple POPC membranes.[31] These findings are consistent with results reported here that show that incorporation of SDS into cholesterol-laden vesicles causes a more pronounced decrease in membrane order (leading to an increased ΔS value) relative to a cholesterol-free system. While SDS partitioning into membranes is relatively similar when expressed on a cholesterol-free basis (in terms of K^*, that is), this behavior is not as closely followed by the other surfactants. In these other three systems, K^* is noticeably lower for the complex sperm-mimic membranes than for the simple POPC membranes. As discussed previously, both cholesterol and sphingomyelin impart significant lipid ordering to the sperm-mimic membranes, and this is reflected in the diminished thermodynamic driving force for surfactant adsorption.

Flip-Flop. When surfactants partition into a lipid bilayer, the first step is incorporation into the outer leaflet followed by flip-flop into the inner leaflet. On experimental time scales (minutes), the first step was found to be fast,[13] but the translocation step can be fast or very slow depending on the system of study.[32−35] Previous studies have shown that SDS does not flip-flop in experimental time scales at room temperature[18] but does so at temperatures above ~60 °C.[24] Flip-flop of the other surfactants used in this study has not been previously determined.

Heerklotz et al.[8] developed a simple and elegant protocol for determining the fraction of lipid accessible to the surfactant, γ, by combining standard surfactant uptake experiments with additional release experiments. In the release assay, the surfactant is loaded into the lipid bilayers during vesicle preparation. These vesicles are then injected into buffer, and this dilution process is accompanied by partitioning of surfactant out of the lipid membrane into solution. All surfactant molecules will be released to the aqueous medium in the case of fast flip-flop. Only those originally in the outer leaflet will be released if the

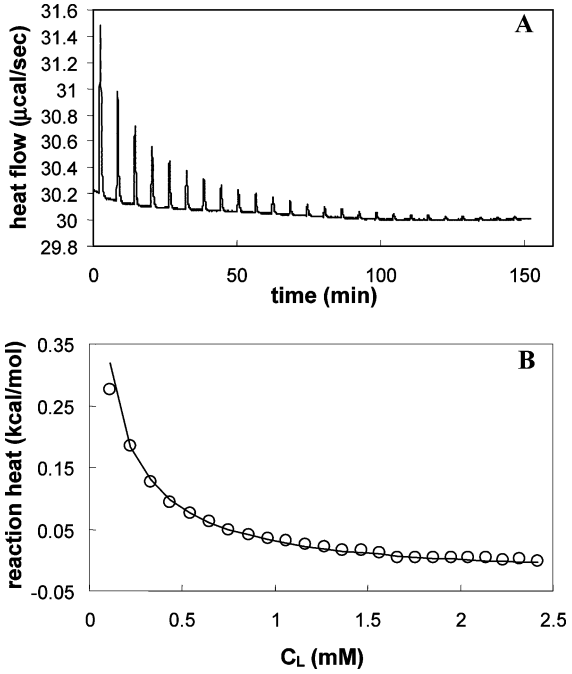

Figure 3. Surfactant release experiment: heat flow (A) and reaction heat per mol of lipid injected (B) observed upon 10 μL injections of SDS-laden POPC vesicles (15 mM lipid concentration, $R_{syr} = 0.3$) into external solution. The solid line is the fit to the data with $K = 7.96 \times 10^4$ M^{-1}, $\Delta H = -3.7$ kcal/mol, and $\gamma = 0.48$.

flip-flop is slow. Simultaneous fitting of uptake and release data yields K, $\Delta H°$, and γ (see Keller et al. for details[24]). The value of γ is equal to 1 if flip-flop is fast and equal to 0.5 if flip-flop is not occurring.

Figure 3 presents an example of release data for the case of SDS/POPC vesicles. The process is endothermic as expected (the corresponding exothermic update experiment was shown in Figure 1A). The simultaneous fitting of uptake and release data yield the following parameters: $K = 7.96 \times 10^4$ M^{-1}, $\Delta H° = -3.7$ kcal/mol, and $\gamma = 0.48$. The negligible flip-flop of SDS we observe is consistent with two other translocation studies using an equilibrium dialysis-based method[34] or fluorescence spectroscopy[32] as well as being consistent with the calorimetric study of Keller and co-workers.[24]

All the other surfactants studied exhibited flip-flop ($\gamma = 1$), regardless of membrane composition. The fast flip-flop of N-9 was expected as similar molecules (alkyl ethoxy-ethers) were reported to transfer rapidly across the bilayer.[8,34] The zwitterionic C31G surfactant also exhibits fast flip-flop. The general tendency is that uncharged surfactants are amenable to flip-flop, consistent with our results, but we do note that one can find examples in the literature of neutral surfactants that exhibit slow flip-flop.[36] As intuitively expected, the general tendency is for charged surfactants not to flip-flop (consistent with SDS behavior at room temperature). Hence, we were surprised to find that the cationic BZK translocates readily. As discussed next, surfactant flip-flop appears to be an important parameter governing membrane perturbation.

Surfactant-Induced Membrane Perturbation: Comparison of Permeability and Partitioning Trends. We have previously studied the process of surfactant-induced membrane permeability.[13,14] The behavior of the different membranes was examined using vesicle leakage assays in which encapsulated 5(6)-carboxyfluorescein (CF) permeated across the membrane as a result of vesicle exposure to surfactant. A leakage response curve reflects, generally speaking, the resistance of membranes

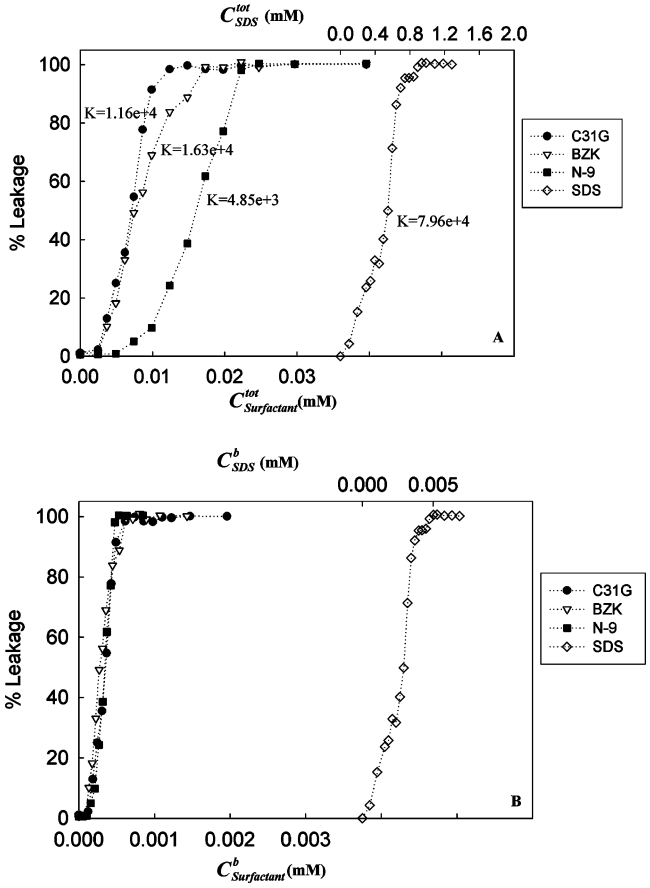

Figure 4. Leakage response of POPC vesicles (lipid concentration 4.6×10^{-3} mM) in the presence of different surfactants (N-9, C31G, BZK, and SDS). Extent of leakage after 4 h is plotted versus (A) overall surfactant concentration and (B) bound surfactant concentration. The abscissa for SDS is located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

to surfactant attack as the bulk concentration of surfactant is increased. The data from these experiments, however, do not expose how leakage correlates with surfactant concentration in the membrane but rather just with overall surfactant concentration. de la Maza and co-workers have shown that it is possible to infer bound surfactant concentrations by carrying out a series of leakage experiments at different overall concentrations,[37] but calorimetric experiments such as the ones conducted here provide direct information on surfactant partitioning and can be readily combined with simple leakage experiments to reveal the connection between membrane perturbation and amount of surfactant incorporation.

Figure 4a compares the performance of the four surfactants in perturbing a POPC membrane. The extent of leakage (fraction of encapsulated probe released from the vesicle interior) observed after 4 h of vesicle incubation with surfactant solutions at various concentrations is shown as a function of the total surfactant concentration. By comparing the leakage responses shown in Figure 4a with the partition coefficients determined in this work, we see no obvious correlation between partitioning and membrane perturbation. On the other hand, knowledge of the partition coefficient allows one to recast the permeability data into the form shown in Figure 4B, which plots extent of leakage as a function of the amount of membrane-bound surfactant. Interestingly, the data for three surfactants (N-9, C31G, and BZK) collapse onto one curve, and the leakage response for SDS remains distinct from that of the others. Notably, SDS is the only surfactant that does not translocate across the membrane. An in vitro study comparing the sper-

micidal and virucidal activities of SDS, N-9, and BZK shows SDS displaying the lowest potency;[38] indeed, SDS is almost 10-fold less spermicidal than N-9. Although it may just be a coincidence, our work suggests that spermicial activity may well be regulated by the ability of the surfactant to translocate to the inner leaflet of the sperm membrane.

It makes sense that surfactant flip-flop is a critical step in the membrane permeabilization process. The cooperation of several surfactant molecules between the two bilayer leaflets seems to be required for loss of barrier function. There are several studies in the literature that suggest that pores or channels are created by the surfactants, facilitating diffusion of solute through the bilayer.[39−41] Such transmembrane pores are stabilized by the presence of surfactant that is ideally shaped for high curvature microstructures. Surfactant availability in both leaflets would be critical to mechanisms of pore formation, and this may explain as to why our leakage data divide into two groups (SDS vs others). Interestingly, our previous studies on membrane leakage[13] revealed a nonlinear dependence of the kinetic rate constant on surfactant concentration consistent with a highly cooperative mechanism that might be associated with pore formation. Meanwhile, the mechanism for SDS-induced leakage may simply be related to membrane strain as a result of the incorporation of surfactant (indeed a significant amount, as much as 1:1 surfactant/lipid ratio) in only the outer leaflet of the membrane. Clearly, more work is required to test the hypothesis that flip-flop is a critical determinant of spermicidal activity, but our results suggest that this is worthy of study.

What our studies do directly show is that the membrane permeability induced by the supposed pore forming surfactants (N-9, C31G, and BZK) is mainly dictated by the amount of bound surfactant and is only slightly sensitive to specific chemical interactions. In the case of a more complex membrane system (sperm-mimic vesicles), the leakage curves as a function of bound surfactant do not overlap as perfectly as those for POPC, but they are reasonably close together (data not shown). Our studies therefore suggest that most uncharged surfactants are good candidates for membrane permeabilization applications (since most translocate), and one can choose simply by considering the balance between price and single fundamental variable, namely, the partition coefficient.

Conclusion

We have obtained membrane partitioning data for four surfactants with current or potential application in contraception and the prevention of STDs. Both energetic and entropic driving forces are at play in determining the free energy of partitioning; while there is always an entropic driving force for surfactant incorporation, the energetic contribution is not always favorable (exothermic). For example, the partitioning of the non-ionic surfactant N-9 is considerably endothermic and can be related to molecular packing effects. Meanwhile, energies of partitioning can be quite favorable for charged surfactants (e.g., SDS) in spite of charge repulsion. Indeed, of the four surfactants examined, SDS exhibited the highest partition coefficient. As compared to simple POPC membranes, we find that more complex sperm-mimic membranes adsorb less surfactant (i.e., lower K values), even when renormalized to a cholesterol-free basis (K*). This decrease is most likely related to the pronounced effect cholesterol has on lipid packing, although other molecular interactions can also contribute. We also used calorimetry to determine the ability of these surfactants to "flip-flop". Consistent with many other uncharged surfactants, N-9 and C31G exhibited flip-flop. Surprisingly, the cationic surfactant BZK also exhibited flip-flop, leaving SDS as the only surfactant that did not readily translocate. Finally, by bringing together partitioning data with previous studies on surfactant-induced membrane permeabilization, we find that surfactant flip-flop is critical to the loss of barrier function. The results of our study can provide useful guidelines for the design of microbicidal and spermicidal surface-active agents.

Acknowledgment

We thank Dr. Heerklotz for helpful comments. We are indebted to Sandro Keller for sharing his fitting program with us, for endless discussions, and for his assistance with the data analysis. We are also grateful to Giovanni Oppenheim for his help with the use of the Stata fitting program. Support for this subproject (GMP-01-25) was provided by the Global Microbicide Project (GMP) of the CONRAD Program, Eastern Virginia Medical School. The views expressed by the authors do not necessarily reflect the views of CONRAD or GMP.

Literature Cited

(1) Lichtenberg, D. Characterization of the Solubilization of Lipid Bilayers by Surfactants. *Biochim. Biophys. Acta* **1985**, *821*, 470.

(2) Helenius, A.; Simons, K. Solubilization of Membranes by Detergents. *Biochim. Biophys. Acta* **1975**, *415*, 29.

(3) Mauck, C.; Doncel, G. F. Spermicides. *Infertility Reprod. Med. Clinics North Am.* **2000**, *11*, 657.

(4) Wyrick, P. B.; Knight, S. T.; Gerbig, D. G.; Raulston, J. E.; Davis, C. H.; Paul, T. R.; Malamud, D. The Microbicidal Agent C31G Inhibits Chlamydia Trachomatis Infectivity In Vitro. *Antimicrob. Agents Chemother.* **1997**, *41*, 1335.

(5) Howett, M. K.; Neely, E. B.; Christensen, N. D.; Wigdahl, B.; Krebs, F. C.; Malamud, D.; Patrick, S. D.; Pickel, M. D.; Welsh, P. A.; Reed, C. A.; Ward, M. G.; Budgeon, L. R.; Kreider, J. W. A Broad-Spectrum Microbicide with Virucidal Activity against Sexually Transmitted Viruses. *Antimicrob. Agents Chemother.* **1999**, *43*, 314.

(6) Heerklotz, H.; Seelig, J. Titration Calorimetry of Surfactant−Membrane Partitioning and Membrane Solubilization. *Biochim. Biophys. Acta* **2000**, *1508*, 69.

(7) Wiseman, T.; Williston, S.; Brandts, J. F.; Lin, L. N. Rapid Measurement of Binding Constants and Heats of Binding Using a New Titration Calorimeter. *Anal. Biochem.* **1989**, *179*, 131.

(8) Heerklotz, H.; Binder, H.; Epand, R. M. A "Release" Protocol for Isothermal Titration Calorimetry. *Biophys. J.* **1999**, *76*, 2606.

(9) Hillier, S. L.; Moench, T.; Shattock, R.; Black, R.; Reichelderfer, P.; Veronese, F. In Vitro and in Vivo: The Story of Nonoxynol 9. *J. Acquired Immune Defic. Syndr.* **2005**, *39*, 1.

(10) Corner, A. M.; Dolan, M. M.; Yankell, S. L.; Malamud, D. C31g, a New Agent for Oral Use with Potent Antimicrobial and Antiadherence Properties. *Antimicrob. Agents Chemother.* **1988**, *32*, 350.

(11) Mendez, F.; Castro, A.; Ortega, A. Use Effectiveness of a Spermicidal Suppository Containing Benzalkonium Chloride. *Contraception* **1986**, *34*, 353.

(12) Yeagle, P. L. Cholesterol and the Cell Membrane. *Biochim. Biophys. Acta* **1985**, *822*, 267.

(13) Apel-Paz, M.; Doncel, G. F.; Vanderlick, T. K. Membrane Perturbation by Surfactant Candidtaes for STD Prevention. *Langmuir* **2003**, *19*, 591.

(14) Apel-Paz, M.; Vanderlick, T. K.; Chandra, N.; Doncel, G. F. A Hierarchy of Lipid Constructs for the Sperm Plasma Membrane. *Biochem. Biophys. Res. Commun.* **2003**, *309*, 724.

(15) Chen, P. S.; Toribara, T. Y.; Warner, H. Microdetermination of Phosphorus. *Anal. Chem.* **1956**, *28*, 1756.

(16) Schurtenberger, P.; Mazer, N.; Kanzig, W. Micelle to Vesicle Transition in Aqueous Solutions of Bile Salt and Lecithin. *J. Phys. Chem.* **1985**, *89*, 1042.

(17) Wenk, M. R.; Alt, T.; Seelig, A.; Seelig, J. Octyl-β-D-Glucopyranoside Partitioning into Lipid Bilayers: Thermodynamics of Binding and Structural Changes of the Bilayer. *Biophys. J.* **1997**, *72*, 1719.

(18) Tan, A.; Ziegler, A.; Steinbauer, B.; Seelig, J. Thermodynamics of Sodium Dodecyl Sulfate Partitioning into Lipid Membranes. *Biophys. J.* **2002**, *83*, 1547.

(19) Almog, S.; Litman, B. J.; Wimley, W.; Cohen, J.; Wachtel, E. J.; Barenholz, Y.; Benshaul, A.; Lichtenberg, D. States of Aggregation and

Phase Transformations in Mixtures of Phosphatidylcholine and Octyl Glucoside. *Biochemistry* **1990**, *29*, 4582.

(20) McLaughlin, S. Electrostatic Potentials at Membrane−Solution Interfaces. In *Current Topics in Membranes and Transport*; Bronner, F., Kleinzeller, A., Eds.; Academic Press: San Diego, 1977; Vol. 9.

(21) Grahame, D. C. The Electrical Double Layer and the Theory of Electrocapillarity. *Chem. Rev.* **1947**, *41*, 441.

(22) Altenbach, C.; Seelig, J. Ca^{2+} Binding to Phosphatidylcholine Bilayers as Studied by Deuterium Magnetic Resonance. Evidence for the Formation of a Ca^{2+} Complex with Two Phospholipid Molecules. *Biochemistry* **1984**, *23*, 3913.

(23) Schwartz, G.; Wackerbauer, G.; Taylor, S. E. Partitioning of a Nearly Insoluble Lipid Monolayer into Its Aqueous Subphase. *Colloids Surf., A* **1996**, *111*, 39.

(24) Keller, S.; Heerklotz, H.; Blume, A. Monitoring Lipid Membrane Translocation of Sodium Dodecyl Sulfate by Isothermal Titration Calorimetry. *J. Am. Chem. Soc.* **2006**, *128*, 1279.

(25) Heerklotz, H. The Microcalorimetry of Lipid Membranes. *J. Phys.: Condens. Matter* **2004**, *16*, 441.

(26) Heerklotz, H.; Seelig, J. Correlation of Membrane/Water Partition Coefficients of Detergents with the Critical Micelle Concentration. *Biophys. J.* **2000**, *78*, 2435.

(27) Høyrup, P.; Davidsen, J.; Jørgensen, K. Lipid Membrane Partitioning of Lysolipids and Fatty Acids: Effects of Membrane Phase Structure and Detergent Chain Length. *J. Phys. Chem.* **2001**, *105*, 2649.

(28) Heerklotz, H. Lipid−Surfactant Interactions. In *The Phospholipids Handbook*, 2nd ed.; Cevc, G., Ed.; Marcel Dekker: New York, 2004.

(29) Tanford, C. *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*; Krieger Publishing Company: Malabar, FL, 1991.

(30) Cummings, J. E.; Satchell, D. P.; Shirafuji, Y.; Ouellette, A. J.; Vanderlick, T. K. Electrostatically Controlled Interactions of Mouse Paneth Cell a: Defensins with Phospholipid Membranes. *Aust. J. Chem.* **2003**, *56*, 1031.

(31) Troup, G. M.; Wrenn, S. P.; Apel-Paz, M.; Doncel, G. F.; Vanderlick, T. K. A Time-Resolved Fluorescence Diphenylhexatriene (DPH) Anisotropy Characterization of a Series of Model Lipid Constructs for the Sperm Plasma Membrane. *Ind. Eng. Chem. Res.* **2006**, *45*, 6939.

(32) Cocera, M.; Lopez, O.; Estelrich, J.; Parra, J. L.; de la Maza, A. Transbilayer Movment of Sodium Dodecyl Sulfate in Large Unilamellar Phospholipid Vesicles. *Langmuir* **1999**, *15*, 6609.

(33) Lecuyer, H.; Dervichi, D. Structure of Aqueous Mixtures of Lecithin and Cholesterol. *J. Mol. Biol.* **1969**, *45*, 39.

(34) Kragh-Hansen, U.; le Maire, M.; Moller, J. V. The Mechanism of Detergent Solubilization of Liposomes and Protein-Containing Membranes. *Biophys. J.* **1998**, *75*, 2932.

(35) Pantaler, E.; Kamp, D.; Haest, C. W. Acceleration of Phospholipid Flip-Flop in the Erythrocyte Membrane by Detergents Differing in Polar Head Group and Alkyl Chain Length. *Biochim. Biophys. Acta* **2000**, *1509*, 397.

(36) Heerklotz, H. Membrane Stress and Permeabilization Induced by Asymmetric Incorporation of Compounds. *Biophys. J.* **2001**, *81*, 184.

(37) de la Maza, A.; Lopez, O.; Cocera, M.; Coderch, L.; Parra, J. L. Influence of the Level of Ceramides in the Permeability of Stratum Corneum Lipid Liposomes caused by Sodium Dodecyl Sulfate. *Chem. Phys. Lipids* **1998**, *94*, 181.

(38) Fichorova, R. N.; Bajpai, M.; Chandra, N.; Hsiu, J. G.; Spangler, M.; Ratnam, V.; Doncel, G. B. Interleukins (IL)-1, IL-6, and IL-8 Predict Mucosal Toxicity of Vaginal Microbicidal Contraceptives. *Biol. Reprod.* **2004**, *71*, 761.

(39) Edwards, K.; Almgren, M. Surfactant-Induced Leakage and Structural-Change of Lecithin Vesicles: Effect of Surfactant Headgroup Size. *Langmuir* **1992**, *8*, 824.

(40) Lesieur, S.; Grabielle-Madelmont, C.; Menager, C.; Cabuil, V.; Dadhi, D.; Pierrot, P.; Edwards, K. Evidence of Surfactant-Induced Formation of Transient Pores in Lipid Bilayers by Using Magnetic-Fluid-Loaded Liposomes. *J. Am. Chem. Soc.* **2003**, *125*, 5266.

(41) Nagawa, Y.; Regen, S. L. Surfactant-Induced Release from Phosphatidylcholine Vesicles: Regulation of Rupture and Leakage Pathways by Membrane Packing. *J. Am. Chem. Soc.* **1992**, *114*, 1668.

Received for review August 13, 2007
Revised manuscript received October 29, 2007
Accepted November 1, 2007

IE071105E