Conventional and Gemini Surfactants Embedded within Bilayer Membranes: Contrasting Behavior

Alexander A. Yaroslavov,*[a] Oleg Yu. Udalykh,[a] Nickolay S. Melik-Nubarov,[a] Viktor A. Kabanov,[a] Yuri A. Ermakov,[b] Vladimir A. Azov,[c] and Fredric M. Menger*[c]

Abstract: Laser microelectrophoresis (coupled with conductance, fluorescence, and dynamic light scattering) is shown to be a highly instructive tool in comparing the dynamics of conventional and gemini surfactants embedded within vesicle bilayers. The following can be listed among the more important observations and conclusions: a) Cationic conventional surfactant, added to a "solid" (gel) lipid vesicle containing an anionic phospholipid, charge-neutralizes only half the anionic charge. With a "liquid" (liquid crystalline) vesicle, however, the entire negative charge is neutralized. Thus, the cationic conventional surfactant can "flip-flop" readily only in the liquid membrane. b) A cationic gemini surfactant charge-neutralizes only the anionic lipid in the outer membrane leaflet of either solid or liquid membranes, thus indicating an inability to flip-flop regardless of the phase-state of the bilayer. c) Mixed population experiments show that surfactants can hop from one vesicle to another in liquid but not solid membranes. d) In liquid, but not solid, bilayers, a surface-adsorbed cationic polymer can electrostatically "drag" anionic surfactant from the inner leaflet to the outer leaflet where the polymer resides. e) Peripheral fluorescence quenching experiments show that a cationic polymer, adhered to anionic vesicles, can be forced to dissociate in the presence of high concentrations of salt or an anionic polymer. f) Adsorbed polymer, of opposite charge to that imparted to vesicles by a gemini surfactant, is unable to dislocate surfactant even in a liquid membrane. g) In our systems, ionic polymers will not bind to neutral vesicles made solely of zwitterionic phospholipid. On the other hand, ionic polymers bind to neutral vesicles if charge neutrality is obtained by virtue of the membrane containing equimolar amounts of cationic and anionic surfactant. This is attributable to surfactant segregation within the bilayer. h) Experiments prove that polymer migration can occur among a population of neutral ternary vesicles.

Keywords: gemini · laser microelectrophoresis · membranes · polyelectrolytes · vesicles

Introduction

Bilayer vesicles (hollow spheres composed of native or synthetic lipids) have potential use in drug targeting,[1–4] genetic transformation of cells,[5–9] cancer chemotherapy,[10–15] and in antibacterial and antiviral regimens.[16–20] Efficiency of vesicular preparations depends upon, among other parameters, the clearance rate of the vesicles from the bloodstream. The rates, in turn, are governed by the vesicle size and surface charge. Thus, it was shown that large vesicles clear faster than small ones,[10, 21] and that hepatic uptake occurs more readily with negative vesicles than with positive or neutral structures.[22, 23] Having been impressed by the medical relevance of vesicles, and having had past experience not only with vesicles[24] but with surfactants[25] and polyelectrolytes,[26] we decided to investigate the effect of these latter materials on the size, charge, and dynamics of vesicular systems. The hope was to acquire basic information on how lipid, surfactant, and polymer interact with each other at the molecular level and, thereby, to control critical physical/chemical/biological properties of the vesicles.

Various preparative methods have been developed that allow vesicle size to be varied from "small" (20–100 nm) to "large" (100–200 nm) to "giant" (5–100 μm).[27] For example, sonication of phospholipid films usually gives small

[a] Prof. A. A. Yaroslavov, Dr. O. Yu. Udalykh, Dr. N. S. Melik-Nubarov, Prof. V. A. Kabanov
Polymer Department, School of Chemistry
Lomonosov Moscow State University
Leninskie Gory, 119899 Moscow (Russia)
Fax: (+7) 095-939-0174
E-mail: yaroslav@genebee.msu.su

[b] Dr. Yu. A. Ermakov
Frumkin Research Institue of Electrochemistry
Russian Academy of Science
Leninsky Prospekt 32, 117234 Moscow (Russia)

[c] Prof. F. M. Menger, V. A. Azov
Department of Chemistry, Emory University
Atlanta, Georgia 30322 (USA)
Fax: (+1) 404-727-6586
E-mail: menger@emory.edu

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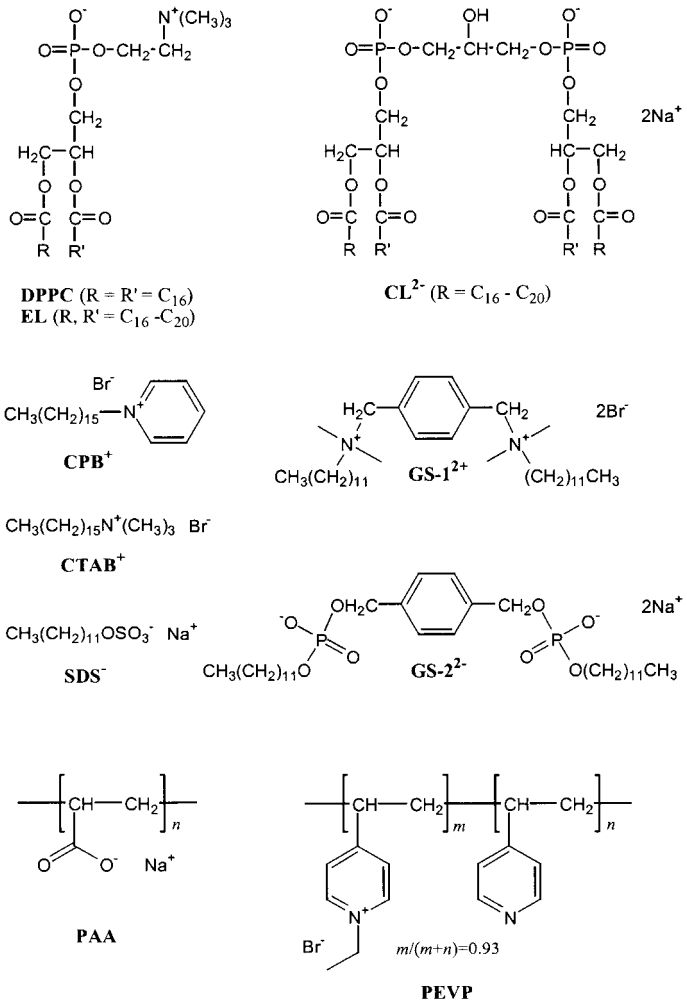
vesicles, whereas so-called "electroformation" is the method of choice for giant vesicles.[28] With regard to imparting a desirable charge on vesicles, two main approaches have been taken in the past. The first consists of incorporating charged additives into bilayer membranes composed of neutral phospholipids.[27] The second consists of coating a vesicle surface with a charged macromolecule.[29–40] Both of these approaches, individually and simultaneously, were adopted in the present manuscript.

Scheme 1 shows the structures and corresponding acronyms of the main players in the story about to unfold. Since we will always refer to compounds via their acronyms, it might be necessary to periodically consult the Scheme. The following remarks should be helpful by way of introducing our compounds:

1) Dipalmitoylphosphatidylcholine (DPPC), egg lecithin (EL), and cardiolipin(CL^{2-}) are the lipids from which we constructed the vesicles. DPPC is neutral, while CL^{2-} has two anionic charges. At 20 °C, where all the experiments were performed, DPPC is in the "solid" (or gel) state, whereas EL is in the more disordered "liquid" (or liquid crystalline) state. These distinctions are important as they provided an avenue for manipulating the charge and phase-state of the vesicular membranes.
2) Conventional surfactants, incorporated into the membranes at levels of 10–20 mol %, included 1-cetylpyridinium bromide (CPB^+), cetyltrimethylammonium bromide ($CTAB^+$), and sodium dodecyl sulfate (SDS^-). Since electrical charge imparted to the vesicles by the surfactants was a key element of our work, and we have included the surfactant charge as a superscript of the acronym.
3) Two-tailed gemini surfactants of opposite charge (GS-1^{2+} and GS-2^{2-}) were also added to the bilayer systems. Gemini surfactants have recently drawn worldwide attention owing to their unique colloidal properties (e.g. unusually high surface activity, ability to transport DNA, and capacity to induce porosity in solids).[41]
4) Finally, a cationic and an anionic polymer were used to coat the membranes: *N*-ethyl-4-vinylpyridinium/4-vinylpyridine (93/7) with a 1100 degree of polymerization (PEVP) and polyacrylic acid with a 70 degree of polymerization (PAA).

With the above materials in hand, we were able to ask a variety of intriguing questions such as: Do surfactant molecules, present in both leaflets of a vesicular bilayer, redistribute themselves when a polymer of opposite charge binds to the outer surface of the vesicle? How do the dynamics of membrane-bound conventional and gemini surfactants compare? If a polymer-induced inner-to-outer surfactant migration occurs, how does this phenomenon depend upon the phase-state of the membrane? Can a polymer adsorbed onto a surfactant-modified vesicular surface migrate among vesicles,



Scheme 1. Substances and corresponding acronyms.

Abstract in Russian:

Исследовано поведение двух типов бислойных везикул, полученных добавлением к фосфолипидному бислою (1) традиционных поверхностно-активные веществ с одной полярной группой и одним алкльным радикалом (ПАВ1) и (2) поверхностно-активных веществ с двумя полярными группами и двумя алкильными радикалами (ПАВ2). Для сравнительного изучения динамики обоих типов ПАВ в везикулярной мембране использован метод лазерного микроэлектрофореза в сочетании с фотонной корреляционной спектроскопией, флуоресценцией и кондуктометрией. Основные результаты состоят в следующем. (а) Добавление катионного ПАВ1 к твердым отрицательно заряженным фосфолипидным везикулам приводит к нейтрализации заряда анионного липида, расположенного на внешней стороне везикулярной мембраны. Добавление того же ПАВ к жидким отрицательно заряженным везикулам сопровождается переходом части адсорбированного ПАВ с внешней стороны мембраны на внутреннюю (флип-флопом) и нейтрализацией заряда всех анионных фосфолипидных молекул. (б) Катионный ПАВ2 нейтрализует только внешний заряд твердых и жидких отрицательно заряженных фосфолипидных везикул, что указывает на отсутствие флип-флопа катионного ПАВ2. (в) Молекулы ПАВ способны мигрировать между жидкими везикулами. (г) Адсорбция синтетического катионного полимера на поверхности жидких отрицательно заряженных смешанных везикул индуцирует переход анионного ПАВ1 с внутренней стороны мембраны на внешнюю. (д) Результаты экспериментов с флуоресцентно меченными везикулами показывают, что адсорбированный поликатион может быть удален с поверхности везикул при увеличении ионной силы раствора или в присутствии избытка полианиона. (е) Полиионы не взаимодействуют с нейтральными везикулами, приготовленными из цвиттер-ионных липидов. Однако полиионы адсорбируются на поверхности везикул, приготовленных из смеси цвиттер-ионнного липида и эквимольных количеств катионного и анионного ПАВ. (ж) Адсорбированные полиионы способны мигрировать между трехкомпонентными нейтральными везикулами.

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and does the polymer stabilize vesicles against aggregation? These and other questions are addressed herein.

Our chief means on investigation involved the powerful electrophoretic mobility (EPM) method.[42] Thus, vesicles and their complexes with ionic surfactants and polymers were subjected to laser microelectrophoresis in a thermostated cell. A known field was applied while the sample was illuminated by cross-focused He/Ne laser beams. Vesicles moving through the cell scattered light, the intensity of which fluctuated (due to the Doppler effect) with a frequency proportional to the velocity of the particles. The velocity, derived from the measured frequency, was expressed as "EPM units" after dividing the velocity by the applied field. Although the EPM values varied from -4 to $+4$ (depending upon the sign and magnitude of the vesicular charge), by far the most important situation existed when the EPM $= 0$, that is when the vesicles had zero charge and did not migrate in the applied field. The EPM $= 0$ state told us exactly when the ionic surfactant and polymeric additives were charge-neutralizing each other at the outer surface of the vesicles. As will be shown, this is very useful information.

We must admit to a certain "multicomponent" complexity in our experiments. This, of course, will be an on-going trend as colloid chemists move ever closer to modeling (and perhaps ultimately absorbing!) biology. The hope is, however, to portray here the experiments in as simple and palatable manner as possible.

Results and Discussion

Aside from a few conductance, dynamic light scattering, and fluorescence experiments, we confined ourselves to the laser microelectrophoresis method. It is, therefore, worthwhile to explain certain details about the method as applied to our systems. Consider a vesicle which is, for example, negatively charged owing to the presence of anionic lipid. Now anionic lipid within the inner leaflet is charge-neutralized by an equivalent amount of counterion (bound or otherwise). Since counterion in the vesicular "water pool" cannot escape when the vesicle migrates in an applied field, the inner leaflet plays no role in the mobility measurements. This is not true for the outer leaflet with its "loose" counterions which are free to migrate oppositely to the vesicle itself. If, however, a cationic polymer sticks to the anionic vesicle, the vesicle will cease to migrate only when there is a precise charge-neutralization. Thus a plot of electrophoretic mobility (EPM) versus polymer concentration (using a polymer of known charge density) reveals, at zero mobility, the anionic charge-content of the outer leaflet. This is a highly useful piece of information. We can tell, for example, if anionic lipid originally in the inner leaflet remains at that site, or whether it flip-flops to the outer leaflet under the influence of a cationic polymer.

As will be seen, we keep things simple and reliable by utilizing only the "zero-charge/zero-mobility" point on our plots. Other points on the plots are far more difficult to interpret because mobility of a charged particle in an electrical field is a complex function of many variables in addition to charge (including size and surface roughness as influenced by an adsorbed polymer). Thus, we were content to define a single but critically important property of vesicles: the electrical charge on their outer surface. Other methods that are commonly applied to vesicles (TEM, DSC, etc.) do not yield such information, explaining why we are enthusiastic proponents of EPM.

In order to organize our data and facilitate its assimilation, we have categorized the experiments into three groups of increasing complexity: 1) vesicle/surfactant systems; 2) polyion-coated binary vesicles which had been electrically charged with a cationic or anionic surfactant; 3) polyion-coated ternary vesicles that were electrically neutral by virtue of containing equimolar amounts of cationic and anionic surfactant. And, as the title of the paper indicates, attention was given throughout the study to behavioral differences between conventional and gemini surfactants absorbed within the membranes.

Recall that we are dealing here with "small" unilamellar vesicles in the 50–70 nm size range formed by sonication of solid DPPC or liquid EL lipids.

Vesicle/Surfactant systems

Conventional surfactants: Electrophoretic mobility, determined by the laser microelectrophoresis method, is a useful parameter in that it reveals the degree of charge neutralization within a membrane. Zero mobility, for example, indicates total charge neutralization. In Figure 1 (curves 1 and 2), negative surfactant SDS^- was added in various ratios to a constant level (0.07 mM) of negative lipid CL^{2-} incorporated within the membrane. The "structural lipid" (i.e., the main lipid component of the bilayers) were the "solid" DPPC (curve 1) or the "liquid" EL (curve 2). It is seen that, as expected, the SDS^- renders the CL^{2-}-bearing vesicles even more negative. When, however, cationic surfactant CPB^+ was added to the CL^{2-} bearing vesicles (curves 3 and 4), the negative electrical charge was first neutralized (mobility $= 0$) prior to becoming positive. Note that concentrations of surfactants were, by design, an order of magnitude more dilute than is normally necessary to disrupt membranes.

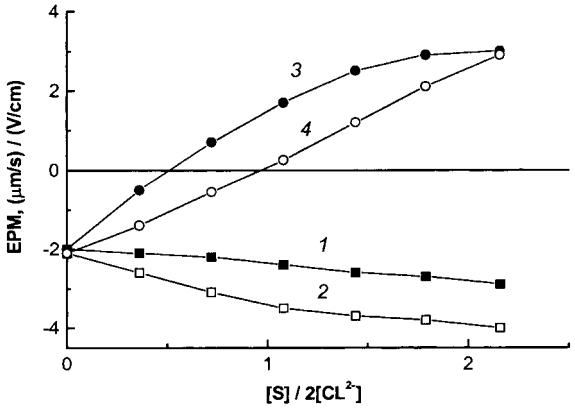

Figure 1. EPM of vesicles in the presence of conventional surfactants. DPPC/CL^{2-} vesicles + SDS^- (1); EL/CL^{2-} vesicles + SDS^- (2); DPPC/CL^{2-} vesicles + CPB^+ (3) and EL/CL^{2-} vesicles + CPB^+ (4). CL^{2-} headgroup concentration: $2[CL^{2-}] = 1.4 \times 10^{-4}$ M.

Now curve 3 of Figure 1 shows that charge neutralization in DPPC vesicles occurs when the CPB^+-to-2 CL^{2-} ratio on the *X* axis is 0.5. Clearly, it would require a ratio of one (with a molar CPB^+-to-CL^{2-} ratio of two) for the CPB^+ to neutralize *all* the CL^{2-} molecules distributed roughly equally on both sides of the bilayer.[47, 48] Thus, the vesicles assume a neutral surface charge when CPB^+ neutralizes only half of the CL^{2-} molecules. Logically these are the CL^{2-} molecules that reside at the outer leaflet of the bilayer and that are initially exposed to the externally added surfactant. We can conclude that trans-leaflet migration of CPB^+ to the inner leaflet, or trans-leaflet migration of CL^{2-} to the outer leaflet, cannot occur within the solid membrane. Otherwise full neutralization would have required double the observed amount of CPB^+ at zero mobility. One can see from this experiment how simple electrophoretic mobility data can provide highly useful information on membrane dynamics in complicated systems.

Dynamic light scattering data (not shown) indicate rapid aggregation to micron-sized particles upon addition of CPB^+ to the CL^{2-} ladened vesicles until complete neutralization is reached, after which the size decreases. This phenomenon is best explained electrostatically; vesicle aggregation reaches its maximum when the vesicles bear no charge.

Liquid EL membranes show quite different behavior than solid DPPC membranes. Thus in curve 4 of Figure 1, neutralization of CL^{2-} within EL vesicles by CPB^+ has a neutrality point of unity. This implies that CPB^+ has neutralized *all* the CL^{2-} molecules within the bilayer. This could occur in one of three ways: a) CPB^+ adsorbed from solution relocates from the outer to the inner leaflet, thereby permitting total CL^{2-} neutralization; b) CL^{2-}, originally distributed more-or-less uniformly between the two leaflets, migrates to the outer leaflet where it encounters adsorbed CPB^+; c) a combination of a) and b). Since trans-leaflet "flip-flop" of lipids, especially lipids with four chains such as CL^{2-}, should be a slow process,[49] it seems likely that it is the CPB^+ surfactant which is doing the migrating within the liquid membrane. As a result, a uniform distribution of charged molecules is established rapidly (within the experimental measurement time of a few minutes).

There is a second explanation for curve 4 in Figure 1 that we could not a priori discount: Addition of surfactant might be destroying the vesicles. To test this possiblity, EL/CL^{2-} vesicles were loaded with 1M NaCl, an amount which would lead to a large conductivity increase if the salt were released into the water. Since no increase in conductivity was observed when the vesicles were subjected to CPB^+, we can conclude that the integrity of the EL/CL^{2-} vesicles remains in tact when the surfactant is present. Curiously, in contrast to the solid DPPC vesicles, CPB^{2+} adsorbed into the liquid EL vesicles produced no aggregation.

Gemini surfactants: Experiments identical to those carried out with conventional surfactants were performed with gemini surfactants GS-1^{2+} and GS-2^{2-} (Figure 2). As before, GS-2^{2-} enhances the negative charge of vesicles composed of DPPC/CL^{2-} and EL/CL^{2-} (curves 1 and 2, respectively). Cationic gemini GS-1^{2+}, however, now manifests a different effect from that of conventional surfactant CPB^+. The plots

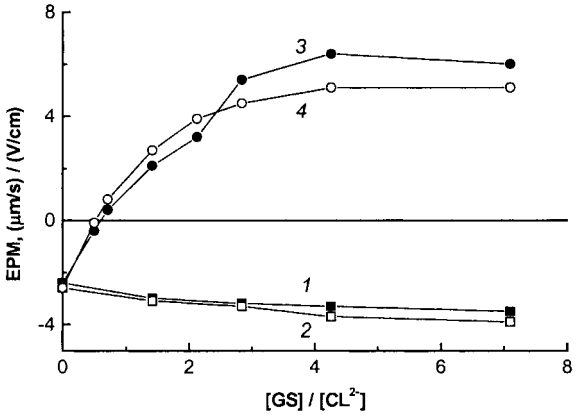

Figure 2. EPM of vesicles in the presence of gemini surfactants. DPPC/CL^{2-} vesicles + GS-2^{2-} (1); EL/CL^{2-} vesicles + GS-2^{2-} (2); DPPC/CL^{2-} vesicles + GS-1^{2+} (3) and EL/CL^{2-} vesicles + GS-1^{2+} (4). CL^{2-} headgroup concentration: $2[CL^{2-}] = 1.4 \times 10^{-4}$ M.

for solid and liquid lipids are identical, and *both* vesicle types are neutralized when there are two CL^{2-} molecules for each GS-1^{2+} (curves 3 and 4). In other words, GS-1^{2+} neutralizes only the CL^{2-} in the outer leaflet even with the liquid EL vesicles (in which, as we have just seen, conventional surfactant travel with ease). Note that lack of leaflet-to-leaflet migration of GS-1^{2+} in EL vesicles persists at the neutrality point for at least an hour. Thus, there exists a serious impediment to gemini "flip-flop". In this regard, the double-chained gemini behaves more like a phospholipid than a surfactant (one of many unique features of geminis).

Intervesicular transport: Not only can mobility measurements detect interleaflet transport, they can provide evidence for surfactant transport among different vesicles. This constitutes a powerful capability since such evidence is difficult to obtain by other means. Consider the following experiment: Negative EL/SDS^- vesicles with an electrophoretic mobility of -1.9 (μm/s)/(V/cm) were prepared (Figure 3a) and mixed with an equal number of positive EL/$CTAB^+$ vesicles with a mobility of $+1.9$ (μm/s)/(V/cm) (Figure 3b). Both types of vesicles contained inside a 1M NaCl solution. Within five minutes after mixing the two populations, only one type of vesicle, with a mobility close to zero, was found in the system (Figure 3c). According to dynamic light scattering, no change in vesicle size had occurred. And since the conductivity did not increase, no vesicle destruction had occurred. These results prove that surfactant molecules, incorporated into the liquid vesicular membrane, can hop from one vesicle to another and rapidly establish a uniform distribution.

Solid DPPC vesicles are a different story. Mixing negative DPPC/SDS^- (Figure 3d) with positive DPPC/$CTAB^+$ (Figure 3e) resulted in the appearance of large particles with a wide distribution of mobilities (Figure 3f) which did not change with time over >1 h. Conductivity experiments with NaCl-loaded vesicles plus dynamic light scattering data showed that the vesicles leaked and grew in size; this suggests the formation of defects and/or membrane fusion and disruption.

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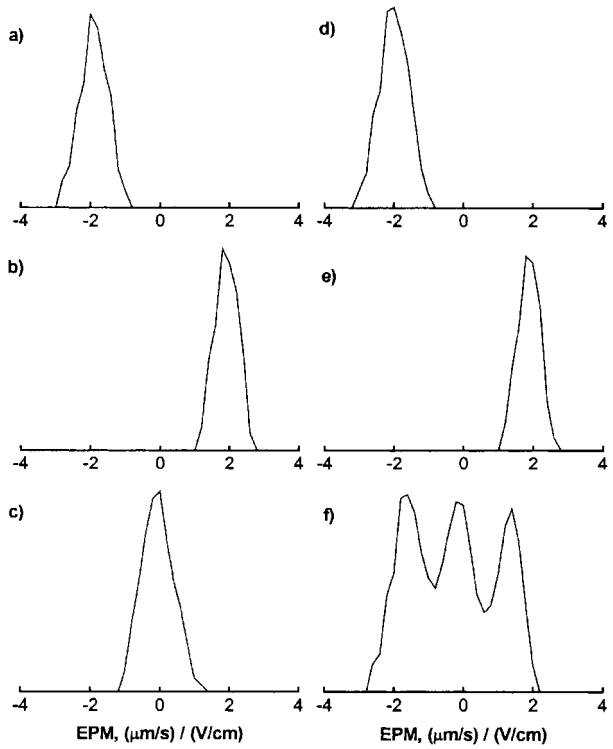

Figure 3. EPM change of oppositely charged liposomes after their mixing. EL/SDS$^-$ vesicles (a); EL/CPB$^+$ vesicles (b); (a) + (b), 5 min after mixing (c); DPPC/SDS$^-$ vesicles (d); DPPC/CPB$^+$ vesicles (e); (d) + (e), 5 min after mixing (f). Lipid concentration 1 mg mL^{-1}.

Interaction of a cationic polymer with anionic vesicles

Conventional surfactants: In this section, we ask an intriguing question: What happens to the distribution of a negative surfactant, embedded within a vesicle bilayer, when a cationic polymer is adsorbed to the outer surface of that vesicle? To address this question, we added a cationic polymer, PEVP, to vesicular suspensions of solid DPPC/SDS$^-$ and liquid EL/SDS$^-$. The concentration of PEVP was always reckoned in terms of the concentration of cationic pyridinium units. As seen in Figure 4a, addition of polymer to vesicular DPPC/SDS$^-$ (curve 1) and to vesicular EL/SDS$^-$ (curve 2) neutralizes the negative charge and, ultimately, imparts to the vesicles a positive charge. Concurrently, the vesicles grow enormously in size (Figure 4b) within the experimental time-frame (a few minutes). There is no question that the PEVP fully binds to the vesicles up to neutrality because UV analysis of the supernatant, formed upon removing the PEVP-coated vesicles by centrifugation, was devoid of polymer. NaCl-leakage experiments showed no PEVP-induced vesicle destruction.

Zero mobility was achieved at a PEVP/SDS$^-$ ratio of 0.5 for the DPPC/SDS$^-$ vesicles, indicating that surface-adsorbed PEVP forms ionic contacts with only half the SDS$^-$ molecules, namely those located at the outer membrane leaflet. On the other hand, a PEVP/SDS$^-$ ratio of unity was found at the neutrality point of the EL/SDS$^-$ vesicles. In the latter case, therefore, the cationic polymer succeeds, within the experimental time-frame of a few minutes, to electrostatically "drag" SDS$^-$ molecules (uniformly distributed in the bilayer prior to polymer addition) from the inner leaflet to the outer

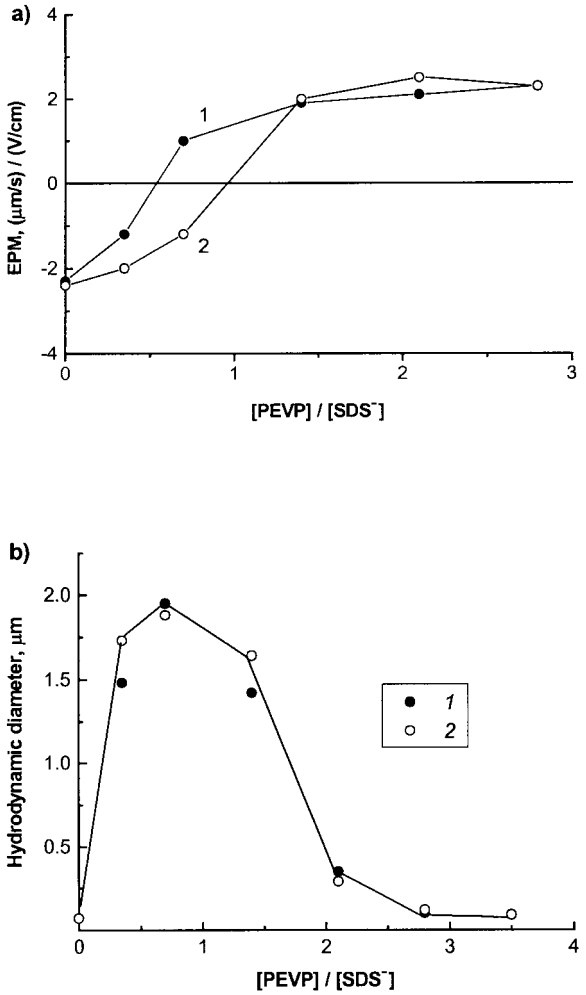

Figure 4. EPM (a) and size (b) of vesicles in the presence of PEVP. DPPC/SDS$^-$ + PEVP (1), and EL/SDS$^-$ + PEVP (2). [SDS$^-$] = 1.4×10^{-4} M.

leaflet. Such a charge-promoted vacating of the inner leaflet has apparently not been reported previously. It has obvious relevance to biological systems where natural ionic polymers (proteins) assist materials in their crossing cell membranes.

We should mention a lingering uncertainty in our polymer experiments. At present we do not know if polymers induce a phase change in the bilayer upon adsorption. This point is presently being investigated calorimetrically, and results will be reported later.

Experiments with fluourescent lipid FDPPE prove that vesicle binding of PEVP, an effective fluourescence quencher, is reversible. As seen in Figure 5 (curve 1), adsorption of PEVP quencher on EL/SDS$^-$ vesicles containing FDPPE is accompanied by a marked decrease in the fluorescence of the label. Addition of 0.3 M NaCl to the system, however, restores the fluorescence (Figure 5, curve 2). Addition of excess anionic polymer PAA has the same effect (Figure 5, curve 3). Thus, PEVP-vesicle complexes dissociate in the presence of salt owing, presumably, to electrostatic shielding of the anionic vesicles by sodium counterions. And anionic PAA must form a complex with cationic PEVP, thereby removing PEVP from the vesicle surface. It seems reasonable that the vesicle, with only a modest negative charge from 10 mol %

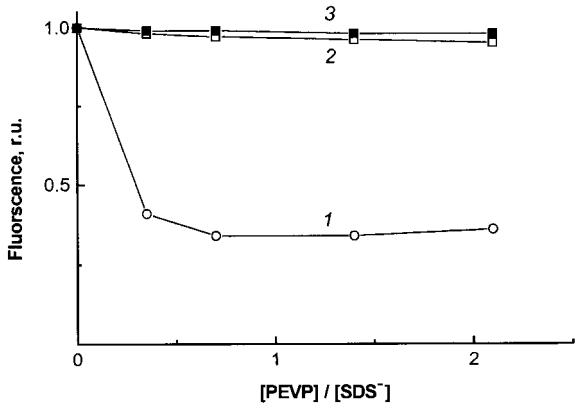

Figure 5. Relative fluorescence intensity of labeled EL/SDS$^-$ vesicles in the presence of PEVP (1). To obtain curves 2 and 3, vesicle-PEVP complexes with different vesicle/PEVP ratios were first prepared, and then 0.3 M NaCl solution (2) or PAA (3) were added to each of them. [PAA]/[PEVP] = 3; [SDS$^-$] = 1.4×10^{-4} M.

SDS$^-$, cannot compete for the attentions of PEVP with the highly anionic PAA.

Gemini surfactants: The behavior of gemini-containing vesicles in contast with PEVP was characterized by several peculiarities in comparison with the conventional systems just described:

First, solid DPPC/GS-2^{2-} vesicles, loaded with NaCl, were permeable toward the salt whether the vesicles were coated with PEVP or not. On the other hand, liquid EL/GS-2^{2-} vesicles (as with SDS$^-$-containing vesicles just discussed) were salt-impermeable in both the PEVP-uncoated and -coated state. We surmise without firm conviction that the anionic gemini creates defects in the solid bilayer that permit the passage of NaCl; such defects within liquid bilayers heal readily if they form at all.

Second, complete neutralization of the surface charge of both solid DPPC/GS-2^{2-} and liquid EL/GS-2^{2-} was achieved with [PEVP]/2[GS-2^{2-}] = 0.9 (Figure 6, curves 1 and 2, respectively). This signifies that PEVP manages to neutralize nearly *all* the GS^{2-} in the bilayer. Note the unusual sigmoidal shape of the plots in Figure 6. Since mobility can depend on factors

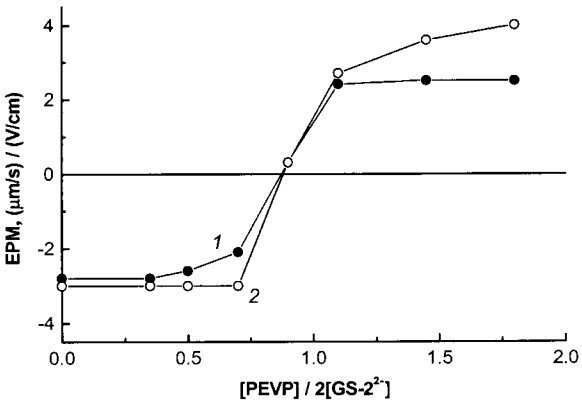

Figure 6. EPM of solid DPPC/GS-2^{2-} (1) and liquid EL/GS-2^{2-} vesicles (2) in the presence of PEVP. GS-2^{2-} headgroup concentration: 2[GS-2^{2-}] = 2.8×10^{-4} M.

other than charge (vesicle size etc.), we have said little about curve shape. Instead, attention is focused exclusively on the most informative parameter generated by the curves: the neutralization point. It is the neutralization point that provides the molar ratio of two oppositely charged species adsorbed into or onto the vesicles.

Third, as with the conventional SDS$^-$ system, adsorption of PEVP led to particle growth in both solid and liquid gemini vesicles. Dislodging the PEVP, which could again be accomplished by simple addition of salt, did not cause the vesicles to revert to their original smaller size, suggesting the presence of fusion processes.

How might these results be explained? Recall that cationic gemini GS-1^{2+}, when added to CL^{2-}-bearing vesicles (solid or liquid), neutralizes only the CL^{2-} molecules in the outer leaflet even with liquid EL vesicles. GS-1^{2+} in the outer leaflet cannot, therefore, "flip-flop" to the inner leaflet in the minute time-regime as can conventional surfactants. Our adsorbed polymer studies unveil another interesting property of the geminis. When GS-2^{2-} is co-sonicated with excess phospholipid, 90% of the gemini resides in the outer leaflet. Such marked an asymmetry is unusual, and we attribute it to the large head-group of the gemini which, apparently, much prefers the more spacious outer leaflet.[50] As a consequence, two equivalents of surface-bound PEVP per GS-2^{2-} are required for charge neutralization. The observed particle growth can then be ascribed to the eradication of electrostatic repulsion and, possibly, to hydrophobic contacts provided by the adsorbed polymer, both of which would promote vesicle aggregation.

Interaction of polyions with electrically neutral vesicles

Conventional and gemini surfactants: Polyions PEVP and PAA were found not to affect the mobility of vesicles made exclusively from zwitterionic phospholipids, indicating negligible polymer adsorption on the surfaces of such neutral vesicles. But there is another way of preparing charge-neutral vesicles: mix equimolar amounts of anionic and cationic surfactants to the membranes. It is this type of vesicle that merits our attention in the third and final section of the paper. That useful conclusions can be drawn from complex four-component systems, such as EL/SDS$^-$/CTAB$^+$/PEVP, testifies to the high information-content of mobility data.

Addition of PEVP or PAA to liquid EL/SDS$^-$/CTAB$^+$ (60/20/20) was accompanied by the appearance of positive and negative charge, respectively (Figure 7). Clearly, the polymers bind to the vesicle surface despite its net electrical neutrality. The exact mechanism for the interaction is not clear at the present time. Surfactant segregation within the bilayer is the likeliest possibility. Perhaps in the case of PEVP, for example, the SDS$^-$ migrates from the inner leaflet to the outer leaflet to partially accommodate the additional cationic charge imparted by the polymer. By this means the additional positive charge provided by the PEVP is distributed between the two leaflets rather than confined to the outer one. Alternatively, or perhaps concurrently, SDS$^-$ in the outer leaflet might form two-dimensional clusters that serve as adsorption sites for oppositely charged polymer.

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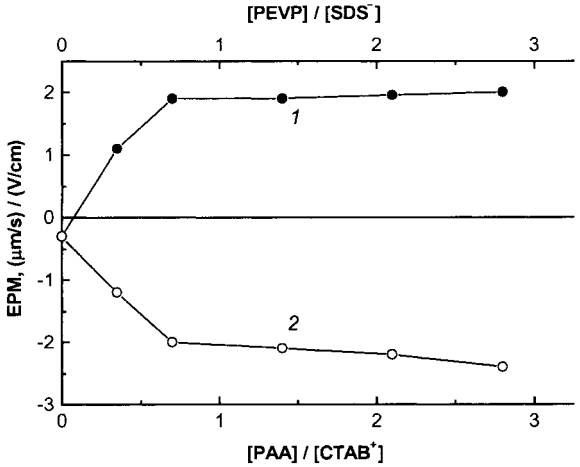

Figure 7. EPM of neutral vesicles in the presence of PEVP and PAA. EL/SDS^-/$CTAB^+$ vesicles + PEVP (1); EL/SDS^-/$CTAB^+$ vesicles + PAA (2). $[SDS^-] = [CTAB^+] = 1.4 \times 10^{-4}$ M.

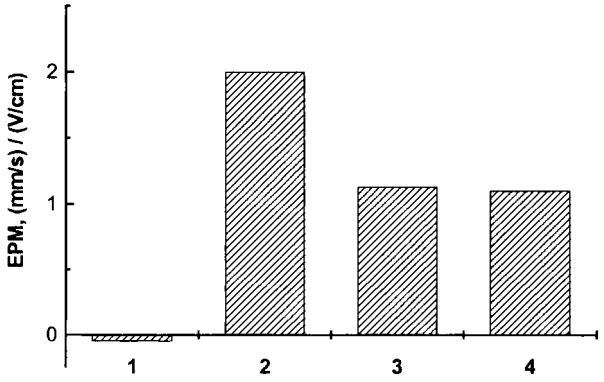

Figure 8. EPM of: EL/SDS^-/$CTAB^+$ vesicles (lipid concentration 1 mg mL^{-1}) (1); their complex with 1×10^{-4} M PEVP (2); (1) + (2) (3); and complex of vesicles (lipid concentration 2 mg mL^{-1}) with 1×10^{-4} M PEVP (4). $[SDS^-] = [CTAB^+] = 1.4 \times 10^{-4}$ M.

Ternary neutral vesicles were also prepared by admixing equal molar amounts of oppositely charged geminis GS-1^{2+} and GS-2^{2-} (20 parts each) to the EL (60 parts). These vesicles were, as with the conventional surfactant systems, able to bind both PEVP and PAA. Adsorbed PEVP could be removed from the vesicle surface by adding equimolar amounts of PAA (and vice versa). The process was accompanied by restoration of the initial zero vesicular charge. In contrast to the effect of polyions on vesicles of cationic or anionic charge, no aggregation was observed when PEVP and PAA were adsorbed onto neutral ternary vesicles. This makes intuitive sense because in the latter case the vesicles have outer shells whose net electrical charge creates an intervesicular repulsion.

Vesicle-to-vesicle migration of polymers: The question of intervesicular migration of adsorbed polymers was an intriguing one. When our two ionic polymers bind to vesicles containing only a cationic or an anionic surfactant, they do so strongly and do not migrate in the absence of large amounts of salt. One the other hand, vesicle-to-vesicle migration of polymer was in fact observed with neutral ternary vesicles. This was proven by using again the laser microelectrophoresis method that has constituted the basis of this entire paper. The experiment was carried out as follows: a) A 1 mg mL^{-1} suspension of ternary EL/SDS^-/$CTAB^+$ vesicles was prepared with the expected mobility close to zero (Figure 8, column 1). b) Next, a 0.1 mM solution of PEVP was added to the preparation to give a vesicle/PEVP complex with a mobility of +2.1 (μm/s)/(V/cm) as seen in Figure 8, column 2. c) An amount of uncoated vesicles, equal to that prepared in a), was then added. After 10 minutes, the mobility corresponded to uniform vesicles with a mobility of 1.1 (μm/s)/(V/cm) (Figure 8, column 3). No vesicle growth was observed. d) When the same 0.1 mM solution of PEVP was added to double the vesicle concentration from that in b), a mobility of 1.1 (μm/s)/(V/cm) was also observed (Figure 8, column 4). It is clear from these results that adsorbed PEVP is able to rapidly redistribute itself among a population of coated and uncoated vesicles. This is either accomplished by vesicle/vesicle collisions or by transient entry of the polymer into the solution prior to vesicle re-adsorption.

Conclusion

Laser microelectrophoresis is shown to be a high-information-content tool for comparing the dynamics of conventional and gemini surfactants embedded within vesicle bilayers. The following can be listed among the more important observations and conclusions: a) Cationic conventional surfactant, added to a "solid" (gel) lipid vesicle containing an anionic phospholipid, charge-neutralizes only half the anionic charge. With a "liquid" (liquid crystalline) vesicle, however, the entire negative charge is neutralized. Thus, the cationic conventional surfactant can "flip-flop" readily only in the liquid membrane. b) A cationic gemini surfactant charge-neutralizes only the anionic lipid in the outer membrane leaflet of either solid or liquid membranes, thus indicating an inability to flip-flop regardless of the phase-state of the bilayer. c) Mixed population experiments show that surfactants can hop from one vesicle to another in liquid but not solid membranes. d) In liquid, but not solid, bilayers, a surface-adsorbed cationic polymer can electrostatically "drag" anionic surfactant from the inner leaflet to the outer leaflet where the polymer resides. e) Peripheral fluorescence quenching experiments show that a cationic polymer, adhered to anionic vesicles, can be forced to dissociate in the presence of high concentrations of salt or an anionic polymer. f) Adsorbed polymer, of opposite charge to that imparted to vesicles by a gemini surfactant, is unable to dislocate surfactant even in a liquid membrane. g) Ionic polymers will not bind to neutral vesicles made solely of zwitterionic phospholipid. On the other hand, ionic polymers bind to neutral vesicles if charge neutrality is obtained by virtue of the membrane containing equimolar amounts of cationic and anionic surfactant. This is attributable to surfactant segregation within the bilayer. h) Experiments prove that polymer migration can occur among a population of neutral ternary vesicles.

Experimental Section

Materials: DPPC, EL, CPB^+, $CTAB^+$, and SDS^- were obtained from Sigma and used as received. JSC Biolek (Ukraine) supplied the CL^{2-}. Gemini surfactants GS-1^{2+} and GS-2^{2-} were synthesized and purified as published previously;[43] polyelectrolyte PEVP has also been previously described.[44, 45] PAA came from Aldrich.

Vesicle preparation: Small vesicles (50 – 70 nm according to dynamic light scattering) were prepared by drying under reduced pressure methanolic solutions of DPPC or EL mixed with one or two charged additives such as the surfactants or CL^{2-}. The resulting lipid films were dispersed in a 1 mM borate buffer (pH 9.2) and sonicated, while cooling in an ice bath (EL vesicles) or heating at 55 °C (DPPC vesicles), with a Cole-Palmer 4700 ultrasonic homogenizer. Vesicle samples thus obtained were separated from titanium dust (produced by the sonicator probe) by centrifugation and used within one day. It is common knowledge that vesicles prepared in this manner are unilamellar. The following binary and ternary vesicles were prepared and examined (the molar ratios of components being given in brackets): EL/CL^{2-} (90/10); EL/SDS^- (90/10); EL/CPB^+ (90/10); EL/GS-1^{2+} (80/20); EL/GS-2^{2-} (80/20); DPPC/CL^{2-} (90/10); DPPC/SDS^- (90/10); DPPC/CPB^+ (90/10); DPPC/GS-1^{2+} (80/20); DPPC/GS-2^{2-} (80/20); EL/SDS^-/$CTAB^+$ (60/20/20); and DPPC/SDS^-/$CTAB^+$ (60/20/20). Polymers were always added externally to vesicle dispersions.

Preparing vesicles loaded with 1 M NaCl involved suspending and sonicating vesicle films in 1 M NaCl/1 mM borate buffer. Non-incorporated NaCl was then removed by passing the suspension through a Sephadex G-50 column or by dialyzing against a 1 mM borate buffer. In both cases, the purified vesicles retained their normal size.

Fluorescent-labeled vesicles were obtained by adding 0.1 wt% of *N*-fluorescein-isothiocyanyl dipalmitoylphosphatidylethanolamine (FDPPE), purchased from Sigma, to the lipid mixtures prior to sonication of the films.

Methods: Mean hydrodynamic diameters of the vesicles and their complexes with polyelectrolytes were determined by photon correlation spectroscopy (dynamic light scattering) with a fixed 90° scattering angle using a Malvern Autosizer IIc instrument equipped with a He/Ne laser and a Malvern K7032N autocorrelator. Vesicle diameters were averaged from ten consecutive measurements.

Electrophoretic mobilities (EPM) of vesicles and their complexes with polyelectrolytes were measured by laser microelectrophoresis in a thermostated cell using a Malvern Zetasizer IIc equipped with a He/Ne laser. Software supplied by the manufacturer provided the electrophoretic mobility values directly. Reproducibility was excellent when tested with identical vesicle systems that had been prepared separately.

Fluorescence intensities of FDPPE-labeled vesicles were obtained with the aid of a Hitachi F-4000 fluorescence spectrophotometer at $\lambda_{em} = 525$ nm ($\lambda_{ex} = 495$ nm). A Radiometer PHM83 potentiometer supplied the pH values, while conductivity data were obtained with a Radiometer CDM83 conductometer.[46]

In experiments involving addition of polyelectrolyte PEVP to the vesicles, unbound polymer (and, by difference, the bound polymer) could be assayed as follows: PEVP/vesicle mixtures were spun for 50 min at 18000 rpm using a Beckman J-1 centrifuge. The absorbances at 257 nm were then determined from the clear supernatant using a Hitachi 150/20 UV/Vis spectrophotometer. Concentrations of unbound polymer were read from a corresponding standard plot of absorbance versus concentration.

All experiments were performed in Russia at 20 °C in a 1 mM borate buffer, pH 9.2, prepared with water which had been double-distilled and passed through a Milli-Q system.

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