

# Membrane Solubilization by a Hydrophobic Polyelectrolyte: Surface Activity and Membrane Binding

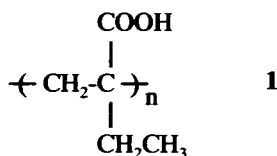
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**ABSTRACT** We have previously observed that the hydrophobic polyelectrolyte poly(2-ethylacrylic acid) solubilizes lipid membranes in a pH-dependent manner, and we have exploited this phenomenon to prepare lipid vesicles that release their contents in response to pH, light, or glucose (Thomas, J. L., and D. A. Tirrell. *Acc. Chem. Res.* 25:336-342, 1992.) The physical basis for the interaction between poly(2-ethylacrylic acid) and lipid membranes has been explored using surface tensiometry and fluorimetry. Varying the polymer concentration results in changes in surface activity and membrane binding that correlate with shifts in the critical pH for membrane solubilization. Furthermore, the binding affinity is reduced as the amount of bound polymer increases. These results are consistent with a hydrophobically driven micellization process, similar to those observed with apolipoproteins, melittin, and other amphiphilic  $\alpha$ -helix-based polypeptides. The absence of specific secondary structure in the synthetic polymer suggests that amphiphilicity, rather than structure, is the most important factor in membrane micellization by macromolecules.

## INTRODUCTION

The ability of macromolecules to control membrane structure or permeability is of central importance in biology and biomimetic chemistry. Synthetic polymers are currently used to modify membrane properties in liposomal drug delivery systems (Needham et al., 1992) and to enhance the rate of membrane permeation by hydrophobic fluorescent dyes. The hydrophobic polyacid poly(2-ethylacrylic acid) (PEAA, **1**) exhibits a pH-dependent interaction with biological membranes: it acts as a molecular switch, permeabilizing and solubilizing membranes at slightly acid pH (pH  $\sim$ 6.3), while having no effect at alkaline pH (Thomas and Tirrell, 1992). This "environmentally sensitive" membrane control has potential applications in therapeutics and biosensors.



The structures and properties of biological membranes are controlled in vivo by proteins, which are polyelectrolytes of precise sequence, molecular weight, stereochemistry, and conformation. These proteins contain structural features that allow exquisite temporal and spatial specificity of their actions on membranes by enabling them to be recognized by appropriate controlling enzymes. In contrast, PEAA is polydisperse and stereoirregular, yet it reorganizes phospholipid

vesicles in response to pH in a cooperative manner, "turning on" over a very narrow pH range (0.1 pH unit). Because the statistical nature of PEAA ensures that this behavior is not critically dependent on any specific details of macromolecular structure, study of the physical basis of the interaction of this polymer with membranes may lead to the identification of some general principles of membrane control by macromolecules.

We report herein the use of surface tension measurements to characterize changes in the surfactant capability of PEAA with pH. In addition, we have examined the changes in the critical pH for membrane solubilization with varying polymer concentration and found that the pH shift is close to that predicted from the changes in interfacial free energy per molecule. A fluorescently labeled polymer was used to confirm that the polymer shows pH-dependent membrane binding. Finally, polymer binding showed diminishing membrane affinity with increasing adsorption in a manner not consistent with polymer charge-charge repulsion, but consistent with changes in membrane surface energy.

## MATERIALS AND METHODS

PEAA was synthesized as described previously (Ferritto and Tirrell, 1992) and had a weight average molecular weight of  $\sim$ 30 kDa, determined by gel permeation chromatography (TSK6500 and TSK6300 columns) with poly(ethylene oxide) standards (Toyo Soda Co., Tokyo, Japan). Fluorescent labeling of the polymer was accomplished by reacting dansyl chloride (Aldrich, Milwaukee, WI) with ethylenediamine (Aldrich) to form *N*-dansyl ethylenediamine, and then coupling the *N*-dansyl ethylenediamine to the carboxyl groups on the polymer via an amide bond. The procedure is detailed in the reference by Devlin (1990). The fraction of carboxyl groups coupled to dansyl was 2%, determined by absorbance at 336 nm.

Dimyristoyl phosphatidylcholine (DMPC) (99+%) was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. To prepare vesicles, a solution of DMPC in  $\text{CHCl}_3$  was dried under  $\text{N}_2$  and then placed under vacuum for 20 min. The phospholipid was hydrated by

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vortexing in 100 mM phosphate buffer to produce a suspension of multilamellar vesicles (MLVs). MLVs were used in optical density assays for phospholipid micellization. Small vesicles were formed by sonicating the suspension (Branson tip sonifier, 40 W) under  $N_2$  for 24 min, while maintaining the temperature at 30–35°C with a stirring water bath. The sample was then centrifuged in an IEC (Needham Heights, MA) clinical centrifuge at 2500 rpm for 20 min to remove any remaining multilamellar vesicles and titanium particles that had been shed by the sonicator tip.

Because sonicated vesicles may contain residual membrane stress, some measurements were performed with larger vesicles formed by extrusion. The vesicle suspension was forced through a polycarbonate filter with 100-nm pores (Nuclepore, Pleasanton, CA) at least 15 times at flow rates up to ~2 ml/s. This procedure produces single-walled vesicles, as demonstrated by  $^{31}P$ -NMR and freeze-fracture electron microscopy (Hope et al., 1985).

## Surface Tension

Surface tensions of PEAA solutions in 100 mM phosphate buffer were measured by means of a Wilhelmy plate, weighed in contact with the solution surface, and then freely hanging. The plate consisted of a rectangle of filter paper (Whatman no. 1) 3 cm × 1 cm. The apparatus was closed to the atmosphere and allowed to equilibrate before the first weighing. The excess weight of the plate when in contact with the solution is approximately equal to the surface tension times twice the length of the plate. A correction for the end effects (at the edges of the plate) was made by measuring the surface tension of pure, high resistivity (18.2 M $\Omega$ -cm) water. All measurements were reproducible to within 1 dyne/cm. Water for all surface tension measurements was obtained from a Millipore (Bedford, MA) Milli-Q system.

## Fluorimetry

Fluorimetry measurements were made in a standard 1-cm quartz cuvette using a Perkin-Elmer MPF-66 fluorimeter, with 340 nm excitation, 420–640 nm emission, and 5-nm slits. A spectrum of 2 ml of a dansylated PEAA (dPEAA) solution in 100 mM phosphate buffer was taken (pH adjusted with 1.2 N HCl), and then a small amount of a vesicle stock solution (2 mg/ml for most measurements, 20 mg/ml for 400 mg/l final vesicle concentration) was added to bring the final phospholipid concentration to each stated value. A spectrum in the presence of phospholipid was taken. The difference in areas of the two spectra was found. This fluorescence increment increases linearly with the amount of membrane present, when polymer is present in excess. The fluorescence increment is normalized to the spectrum in the absence of phospholipid to correct for inner filter effects at high polymer concentrations. Scattering background was negligible. All measurements were made at  $26 \pm 1^\circ\text{C}$ . All samples in the same run were drawn from the same vesicle stock solution. Above pH 6.8, the fluorescence emission of dPEAA solutions was found to be independent of pH.

## RESULTS

The surface tensions of aqueous buffered solutions of PEAA are shown in Fig. 1. The surface tension is depressed linearly with the logarithm of the concentration at all pH values studied. This behavior would be expected for ideal solutions with saturating surface excesses, as given by the Gibbs equation:

$$\Gamma_i = -\frac{\partial \gamma}{\partial \mu_i} = -\frac{1}{RT} \frac{\partial \gamma}{\partial \ln c_i} \quad (1)$$

where  $\Gamma_i$  is the polymer surface excess,  $\gamma$  is the surface tension,  $\mu_i$  is the polymer chemical potential, and  $c_i$  is the polymer concentration. At the ionic strength used in these experiments, the Debye screening length is extremely short

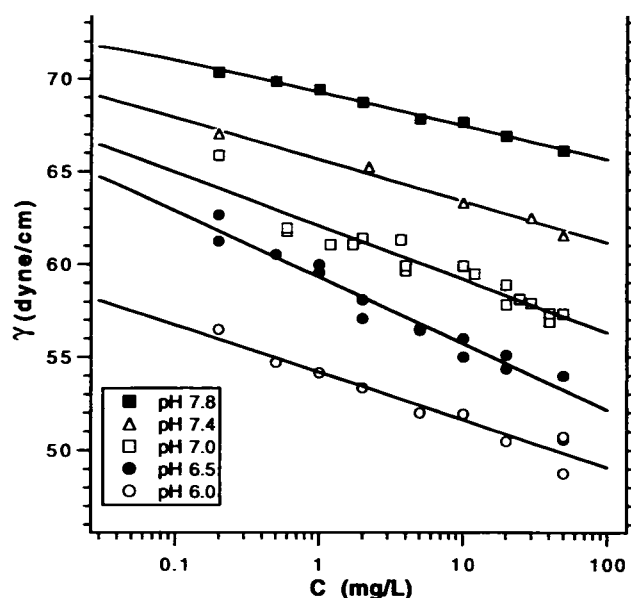


FIGURE 1 Surface tensions of dilute PEAA solutions in excess electrolyte (100 mM sodium phosphate), measured by the Wilhelmy plate method. The depression of the surface tension below that of pure buffer (~72 dyne/cm) is linear with the logarithm of the polymer concentration, indicating saturating surface excesses at these pH values.

(~0.6 nm), while the average intermolecular separation is more than two orders of magnitude greater, e.g., ~370 nm at a concentration of 1 mg/l. Therefore, in contrast to solutions of polyelectrolytes without excess simple electrolyte, the chemical potential of PEAA can be expected to behave ideally in this concentration range, and the Gibbs equation may be used to determine the surface excesses at each pH. We find that the saturating surface excess varies from 0.19 molecules/nm<sup>2</sup> at pH 7.8 to 0.37 molecules/nm<sup>2</sup> at pH 6.5 (Table 1). At pH 6.0, the surface excess actually diminishes, even though the surface activity is greater than at pH 6.5. The greater area per molecule at pH 6.0 is likely to be due to a conformational collapse of the polyelectrolyte at about pH 6.3 (Joyce and Kurucsev, 1981). Below this pH, water is a poor solvent for the polymer chain. As a consequence, the formation of loops and tails by the adsorbed polymers will be energetically less favorable (de Gennes, 1981), and a larger area of the surface will be obstructed by each adsorbed molecule, resulting in a smaller surface excess. In general, however, the surface excess of PEAA is very high at pH 7.8 or lower. The hydrodynamic radius of the polymer has been measured by quasielastic light scattering to be ~5 nm (Eum

TABLE 1 Summary of surface tension results

pH	$\Gamma$ (molecules/nm <sup>2</sup> )	$\sigma$ , area per molecule (nm <sup>2</sup> )	$\Delta \gamma \sigma^*$ (kT/molecule)
6.0	0.27	3.8	18.4
6.5	0.37	2.7	10.5
7.0	0.30	3.3	10.3
7.4	0.23	4.3	8.9
7.8	0.19	5.3	5.8

\* At 10 mg/l concentration, 100 mM phosphate buffer.

et al., 1989), which would lead to a "footprint" of size  $\pi r^2 = 75 \text{ nm}^2$ . At all pH values examined, the polymer interacts so strongly with the surface that significant molecular distortion must occur. In essence, the polymers are forced to adopt a "brush" conformation, with only a few anchorage points per molecule and long loops and tails extending into the aqueous solution.

The dominant effect of lowering pH is to decrease the surface tension (or surface energy) of PEAA solutions. Decreased surface energy is attributed to lower free energy of PEAA molecules situated at the interface, compared with PEAA molecules in solution. The energetic cost of creating new surface area is the cost of creating a pure water surface ( $\sim 72 \text{ ergs/cm}^2$ ), minus the free energy returned when PEAA molecules migrate to the new interface. The reduction in surface energy (compared with pure water) is therefore equal to the average free energy change per molecule of PEAA on migrating to the surface, divided by the surface area occupied per molecule of PEAA. The average free energy changes have been computed from the surface tension data and are given in column 4 of Table 1, in units of kT per molecule. Reducing pH dramatically increases the free energy change; in other words, reducing pH increases the energetic driving force for PEAA to migrate to the air-water interface.

The increased driving force at lower pH may be anticipated from the reduced charge on the polymer. First, charges in aqueous solution (dielectric constant  $\epsilon = 80$ ) are repelled from interfaces with media of lower dielectric constant (Onsager and Samaras, 1934). Second, the accumulation of polymers at the interface will produce a surface potential, which will tend to oppose additional adsorption of charged molecules. Because the charge on the polymer is reduced as pH is depressed, these forces that oppose adsorption will also be reduced, and the adsorption energy will be increased.

The increases in surface activity with increasing concentration suggest that, if polymer surface activity is the driving force for membrane solubilization, then the pH at which solubilization occurs should be dependent on concentration, shifting to higher pH at higher polymer concentrations. Membrane solubilization is easily observed by the reduction in light scattering that accompanies the dissolution of MLV on acidification (Fig. 2 *inset*). The pH at the onset of solubilization (as defined by a reduction in scattering 10% of the total) does change with PEAA concentration, as shown in Fig. 2. As expected, higher concentrations of PEAA act to solubilize membranes at higher pH than do lower concentrations. The shift in pH is  $\sim 0.23 \text{ pH unit}$  for each factor of 10 increase in concentration, or about  $0.10 \text{ pH unit}$  for each factor of  $e$  increase in concentration.

Increasing concentration increases the polymer chemical potential in solution, and thereby increases the driving force for migration to either the air-water interface or the membrane-water interface. As shown by the surface tension measurements, the driving force can also be increased by lowering the pH. Lowering the pH from 7.0 to 6.0 increased the driving force from  $10.3 \text{ kT/molecule}$  to  $18.4 \text{ kT/molecule}$ , as shown in Table 1. This corresponds to a pH shift

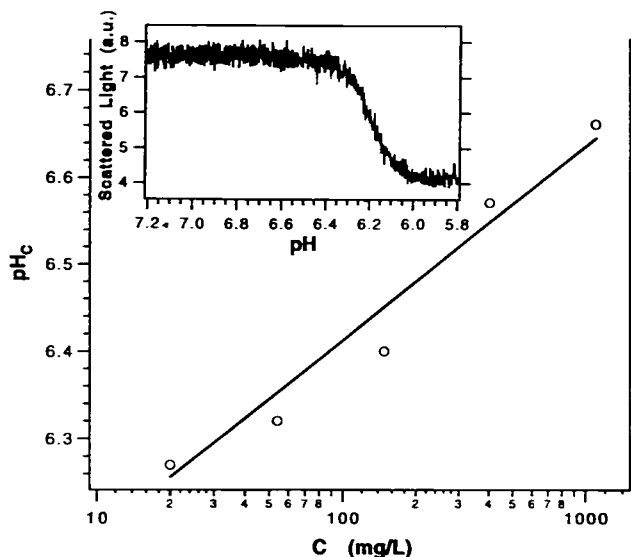


FIGURE 2 Critical pH for the onset of vesicle solubilization as a function of polymer concentration. The onset of vesicle rupture was determined from measurements of scattered light intensity, as shown in the inset. Suspensions of DMPC MLV (20 mg/l) in 100 mM sodium phosphate buffer containing several different concentrations of PEAA were acidified by the continuous addition of 1.2 N HCl at a rate of  $\sim 0.01 \text{ pH unit/s}$ . The reduction in scattered light (beginning at  $pH \sim 6.3$  in the *inset*) is due to the solubilization of the vesicles.

of  $-0.12 \text{ pH unit}$  for each increase of kT in driving force. From the surface tension data, then, we would anticipate that an increase in polymer concentration by a factor of  $e$  would result in a corresponding elevation of the critical micellization pH by roughly  $0.12 \text{ pH unit}$ : the increase in driving force provided by the higher solution polymer concentration is  $\Delta\mu = kT \ln e = kT$ , and this change in driving force is compensated by a pH shift of  $\sim 0.12 \text{ pH unit}$ . This number is in reasonable agreement with the directly measured pH shift of  $\sim 0.10 \text{ pH unit}$ . (Note: Some discrepancy between the surface tension measurements and the micellization measurements is to be expected. The surface tension measurements yield average free energies per molecule adsorbed, for all molecules adsorbed. The amount of polymer adsorbed at the membrane surface,  $\sim 0.06 \text{ molecules/nm}^2$  at rupture (determined from compositional analysis of the resultant micelles), is much lower than that adsorbed at the air-water interface. Therefore, the surface tension measurement includes driving forces for "additional" molecules which accumulate at the air-water interface, but which do not accumulate at the membrane-water interface due to the intervention of the micellization process.)

To explore more directly the pH dependent interaction of the polymer with membranes, a modified PEAA (dPEAA) with 2 mol % pendant dansyl groups was used. The fluorescence emission from the dansyl fluorophore depends strongly on environmental polarity (Waggoner and Stryer, 1970). In polar environments, the emission is centered at  $570\text{--}580 \text{ nm}$ , as observed for dPEAA in phosphate-buffered aqueous solutions at  $pH > 6.8$ , Fig. 3. In a hydrophobic

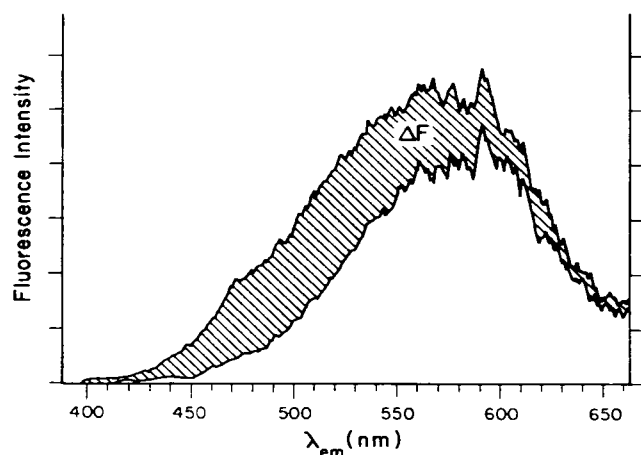


FIGURE 3 dPEAA fluorescence indicates environmental polarity. The fluorescence of a dansyl-modified polymer (340 nm excitation) in the absence (lower line) and presence (upper line) of membranes. The adsorption of dansyl fluors to the membrane results in increased fluorescence quantum yield and a blue-shifted emission. The enhancement in fluorescence on addition of membranes,  $\Delta F$ , is used as a semiquantitative measure of fluorophore adsorption.

environment, the dansyl emission is blue-shifted, and the quantum yield is much higher. In vesicular suspensions of DMPC, dPEAA shows enhanced fluorescence in the region from 430 to 570 nm, but no significant decrease in emission at 570–650 nm, indicating that there is no significant depletion of dansyl groups exposed to the aqueous environment. (Even when a significant fraction of polymers are adsorbed to membranes, a large fraction of mers need not be; except in the cases of very strong binding between each mer and the membrane, or of poor solvents, large loops and tails will be present.) In Fig. 4 A is shown the increase in fluorescence ( $\Delta F$ ) of the dansyl-labeled polymer on addition of membranes as a function of pH. The amount of polymer bound to the membrane increases substantially with decreasing pH below pH  $\sim 8.0$ , as judged by the dansyl fluorescence enhancement. As expected, the higher solution concentration of dPEAA drives more polymer to the membrane surface. Furthermore, this higher membrane concentration of dPEAA causes the reorganization of these membranes at a higher pH ( $\sim 7.3$ ) than does the lower concentration (pH  $\sim 7.0$ ) (Fig. 4 B). (The dansyl fluorophore is itself hydrophobic, and consequently shifts the critical pH for reorganization with respect to the unmodified polymer.)

It is interesting to examine the adsorption of dPEAA as a function of solution dPEAA concentration. The dansyl fluorescence enhancement is shown in Fig. 5 for pH 7.24, for preparations of small sonicated vesicles and large extruded vesicles. Both vesicle preparations show similar amounts of polymer adsorption. In both cases, the adsorption is not proportional to the solution concentration, but shows a saturation effect starting at very low levels of adsorption. At pH 7.41, there is less dansyl adsorption at low polymer concentrations (below 150 mg/l), as is expected from the greater

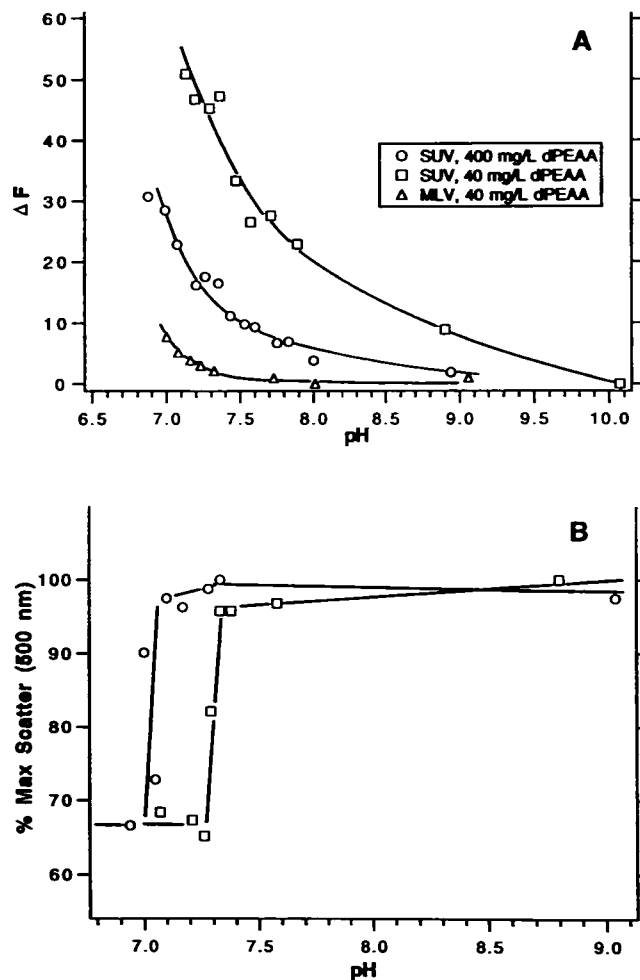


FIGURE 4 (A) The adsorption of dansylated PEAA to DMPC vesicles versus pH. The fluorescence enhancement on addition of small sonicated vesicles (20 mg/l final) to phosphate-buffered polymer solutions is plotted versus pH, for polymer concentrations of 40 mg/l ( $\circ$ ) and 400 mg/l ( $\square$ ). The adsorption increases as pH is lowered, as anticipated from the measurements of surface activity. MLV at the same concentration adsorb much less polymer at 40 mg/l ( $\Delta$ ). (B) Solubilization of small vesicles (40 mg/l) by dPEAA determined by scattered light at 500 nm. The symbols indicate the same dPEAA concentrations as in A. There is a shift in the solubilization from about pH 7.0 to pH 7.3, concomitant with the increased surface binding observed in A.

charge on the polymer at higher pH. Interestingly, higher concentrations of polymer result in as much adsorption as occurs at lower pH. (Measurements at pH 7.24 were made only up to polymer concentrations of 170 mg/l, because higher concentrations will begin to induce vesicle rupture and solubilization.) This result would not be expected if the charge on the polymer were the dominant factor in determining the amount of adsorption under these conditions, either through an image charge repulsion or through charge-charge repulsion between adsorbed polymers, because the higher pH produces a more highly charged polymer and should substantially decrease adsorption (as it does at low polymer concentrations). Instead, these results indicate that, at high levels of adsorption, other forces are dictating the

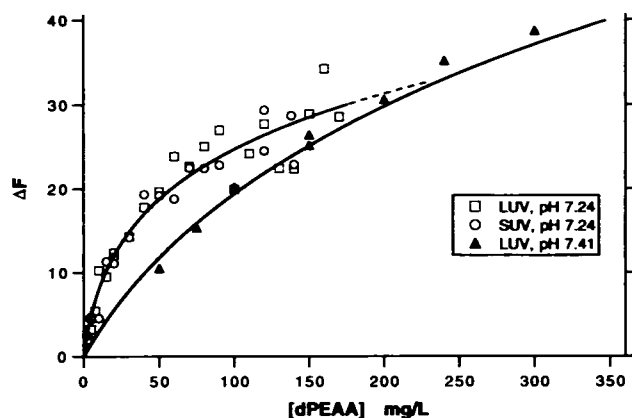


FIGURE 5 The adsorption of dansylated PEAA to DMPC vesicles versus polymer concentration. The fluorescence enhancement on addition of vesicles (20 mg/l final) to phosphate buffered polymer solutions is plotted for small sonicated vesicles (○) and large extruded vesicles (□) at pH 7.24. Also shown is the adsorption to large vesicles at pH 7.41 (▲). Polymer shows reduced affinity for membranes as the amount bound increases.

affinity of the membrane for the polymer. The polymer has been shown to significantly reduce surface tension at the air-water interface; most probably, it will also act to reduce surface tension at the membrane. This reduced surface tension will lower the affinity of the membrane for additional polymer, regardless of polymer charge. (Note that the adsorption of dansyl-modified mers need not be (and most certainly is not) proportional to the adsorption of unmodified mers. Nonetheless, the arguments presented above still hold true: the more highly charged polymer should always have fewer dansyls adsorbed to the membrane, if image charge or charge-charge repulsion is responsible for limiting adsorption at high polymer concentrations.)

## CONCLUSIONS

Titration of PEAA results in changes in surface activity and membrane binding that correlate well with changes in the capacity of the polymer to reorganize DMPC membranes into mixed micelles. These measurements support the hypothesis that increasing polymer surface activity (by reducing ionization) drives membrane adsorption. The adsorption of a charged, hydrated polymer will disrupt the phospholipid hydration. Interfacial tension in bilayers has been shown to be proportional to the square of the water density gradient (Davis and Scriven, 1982), so that disruption of the hydration layer will alter the water density gradient and dramatically affect the surface tension of the bilayer outer leaflet.

In agreement with these theoretical considerations, we find that the membrane affinity of a fluorescent reporter attached to PEAA diminishes as the extent of adsorption increases. Importantly, the dansyl reporter is able to adsorb to as high a level at pH 7.41 as at pH 7.24, which would not be true if charge effects were limiting this adsorption. Rather, these results are consistent with a polymer-induced reduction

in interfacial tension, which then reduces the driving force for further polymer association.

Changes in interfacial tension have been proposed to account for a variety of structural changes in biological membranes. The conversion of chylomicron surface components into micelles is thought to be due to a reduction in surface tension caused by the addition of apoproteins from serum (Atkinson and Small, 1986). Upsetting the balance of interfacial forces in a bilayer can cause destabilization (Oster et al., 1989) and has been proposed to drive Golgi vesicle formation and to contribute to viral budding. Thermal denaturation of spectrin in erythrocytes causes membrane fragmentation (Gallez and Coakley, 1987). This last observation is particularly germane to the actions of PEAA, since denatured proteins, like this polymer, will have a random coil structure with both hydrophobic and hydrophilic groups exposed.

Observations reported here support a model of surface activity driven micellization of DMPC membranes by PEAA. It is especially interesting, in this regard, to note that the solubilization products from DOPC membranes are structurally distinct from those of the saturated phosphatidylcholines, as observed by Eum et al. (1989): PEAA reorganizes DOPC into very small vesicles (~40 nm diameter), rather than micelles of 15 nm diameter. PEAA-induced reorganization of membranes is sensitive to the membrane composition, even though no specific molecular recognition is thought to occur. Differentiation between DOPC and DMPC must occur at a supramolecular level; i.e., the different physical properties of these two materials must play a role.

In spite of the statistical, random coil structure of PEAA, it demonstrates highly cooperative, pH-dependent molecular switching of bilayer membrane structure. The results reported herein show that this structural conversion is correlated with the surface activity and membrane binding properties of the polymer.

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## REFERENCES

- Atkinson, D., and D. M. Small. 1986. Recombinant lipoproteins: implications for structure and assembly of native lipoproteins. *Annu. Rev. Biophys. Biophys. Chem.* 15:403-456.
- Davis, H. T., and L. E. Scriven. 1982. Stress and structure in fluid interfaces. *Adv. Chem. Phys.* 49:357-454.
- de Gennes, P. G. 1981. Polymer solutions near an interface. 1. Adsorption and depletion layers. *Macromolecules.* 14:1637-1644.
- Devlin, B. 1990. Sensitization of vesicles to pH and glucose. Ph.D. Dissertation, University of Massachusetts at Amherst.
- Eum, K. M., K. H. Langley, and D. A. Tirrell. 1989. Quasi-elastic and electrophoretic light scattering studies of the reorganization of dioleoylphosphatidylcholine vesicle membranes by poly(2-ethylacrylic acid). *Macromolecules.* 22:2755-2760.

- Ferritto, M. S., and D. A. Tirrell. 1992. Poly(2-ethylacrylic acid). *Macromol. Synth.* 11:59-62.
- Gallez, D., and W. T. Coakley. 1987. Interfacial instability at cell membranes. *Prog. Biophys. Mol. Biol.* 48:155-199.
- Hope, M. J., M. B. Bally, G. Webb, and P. R. Cullis. 1985. Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume, and ability to maintain a membrane potential. *Biochim. Biophys. Acta.* 812:55-65.
- Joyce, D. E., and T. Kurucsev. 1981. Hydrogen ion equilibria in poly(methacrylic acid) and poly(ethacrylic acid) solutions. *Polymer.* 22: 415-417.
- Needham, D., K. Hristova, T. J. McIntosh, M. Dewhirst, N. Wu, and D. D. Lasic. 1992. Polymer-grafted liposomes: physical basis for the "stealth" property. *J. Liposome Res.* 2:411-430.
- Onsager, L., and N. Samaras. 1934. The surface tension of Debye-Huckel electrolytes. *J. Chem. Phys.* 2:528-536.
- Oster, G. F., L. Y. Cheng, H.-P. H. Moore, and A. S. Perelson. 1989. Vesicle formation in the Golgi apparatus. *J. Theor. Biol.* 141:463-504.
- Thomas, J. L., and D. A. Tirrell. 1992. Polyelectrolyte-sensitized phospholipid vesicles. *Acc. Chem. Res.* 25:336-342.
- Waggoner, A. S., and L. Stryer. 1970. Fluorescent probes of biological membranes. *Proc. Natl. Acad. Sci. USA.* 67:579-589.