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Kinetics of membrane micellization by the hydrophobic polyelectrolyte poly(2-ethylacrylic acid)

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Abstract

Rates of pH-dependent micellization of multilamellar vesicles by the hydrophobic polyelectrolyte poly(2-ethylacrylic acid) (PEAA) have been measured turbidometrically. This polymer shows a strong pH-dependence in its affinity for phospholipid membranes, binding in increasing amounts as pH is lowered and ultimately solubilizing membranes to form mixed micelles (Tirrell, Takigawa and Seki (1985) Ann. N.Y. Acad. Sci. 446, 237). The rate of solubilization of dipalmitoylphosphatidylcholine (DPPC) vesicle suspensions by PEAA increases approximately linearly with reductions in pH below a threshold at pH 6.55. Interestingly, negatively-charged dipalmitoylphosphatidylglycerol membranes showed qualitatively similar behavior in the presence of PEAA, and incorporation of 10% or 20% dipalmitoylphosphatidic acid in DPPC membranes did not affect solubilization rates, demonstrating that membrane charge is not an important factor in determining micellization kinetics. Micellization of DPPC and dimyristoylphosphatidylcholine membranes occurs most rapidly at their respective gel-liquid crystalline transition temperatures (T_m); the rate enhancement is correlated with a peak in the temperature-dependent binding of a fluorescently-modified PEAA in slightly alkaline solutions in which no micellization is observed. The lateral compressibility of the membrane, which has a similar peak at T_m, is proposed to be an important determinant of the rate and extent of polymer adsorption, and consequently of the rate of micellization.

Keywords: Phosphatidylcholine; Phosphatidylglycerol; Polyelectrolyte; Micellization; Adsorption; Kinetics

1. Introduction

Control of biological membrane structure and permeability is a central problem in cell physiology, and is receiving growing attention in the development of vesicular drug delivery vehicles and biomimetic systems. In many instances, the proteins responsible for structural control of biological membranes are only beginning to be identified, and the mechanisms by which they operate remain elusive [1,2]. On the other hand, a number of synthetic model systems have been developed that demonstrate environmental control of membrane structure and permeability. Interest in such systems is motivated by potential applications in pharmaceutics and sensors, and by the prospect that they may shed light on mechanisms of biological membrane control. Some of these model systems have exploited the tendency of certain phospholipids to form non-lamellar phases [3–8]; others are based on interactions with exogenous macromolecules ([9] and references therein). We have been exploring the ability of a hydrophobic polyelectrolyte, poly(2-ethylacrylic acid), **1**, to bind to and rupture phospholipid vesicles in a highly pH-dependent manner.

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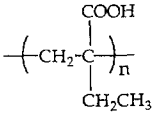

PEAA is extremely sensitive to solution pH, interacting only weakly with phosphatidylcholine vesicles at alkaline pH, but 'turning on' as pH is lowered to ca. pH 6.5 and binding to the membrane to form phospholipid-polymer micelles [10]. In this paper we report measurements of the pH and temperature dependence of the rate of this micellization process, as observed turbidometrically. These kinetic measurements have helped to elucidate the physical parameters of the membrane and of the polymer solution that are important for micellization.

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2. Materials and methods

Poly(2-ethylacrylic acid) was synthesized as described elsewhere [11]. Gel permeation chromatography through TSK 6500 PW and TSK 6300 PW columns with 50 mM aqueous tris(hydroxymethyl)aminomethane-HCl (pH 8.0) as eluent gave estimates of $M_w = 31\,000$ (degree of polymerization approx. 300), and $M_w/M_n = 1.6$, with poly(ethylene oxide) molecular weight standards. Fluorescent labeling of the polymer was accomplished by reacting dansyl chloride (Aldrich, Milwaukee, WI) with ethylene diamine (Aldrich) to form *N*-dansyl ethylene diamine, and then coupling the *N*-dansyl ethylene diamine to the carboxyl groups on the polymer via an amide bond. The procedure is detailed in the reference by Devlin [12]. The fraction of repeat units coupled to dansyl was 2%, determined by absorbance at 336 nm.

L-α-Dimyristoylphosphatidylcholine (DMPC) (99%), L-α-dipalmitoylphosphatidylcholine (DPPC) (99%), L-α-dipalmitoylphosphatidylglycerol (DPPG) (99%), and L-α-dipalmitoylphosphatidic acid (DPPA), sodium salt (98%) were purchased from Sigma (St. Louis, MO) and used without further purification.

Multilamellar vesicles (MLV) were prepared by vortexing 2 mg/ml phospholipid in 10 mM phosphate buffer, pH 7.4, 3 × 30 s, at 15°C above the gel-liquid crystalline phase transition temperature for the lipid used. For turbidometric measurements, 1 ml of a 2 mg/ml solution of PEAA in 100 mM phosphate buffer was added to 1 ml of the 2 mg/ml lipid suspension in a thermostatted sample cell in a Beckman DU-7 spectrophotometer. The final pH was determined after the turbidity measurement. Optical density was measured at 400 nm.

Measurements of the adsorption of dansyl-modified PEAA were made on extruded vesicles, in order to maximize the vesicle surface area exposed to polymer and minimize light scatter. Multilamellar vesicle suspensions were forced through a polycarbonate filter with 100 nm pores (Nuclepore, Pleasanton, CA) at least 15 times, at flow rates up to approx. 2 ml/s. This procedure produces single-walled vesicles, as demonstrated by ^{31}P nuclear magnetic resonance and freeze fracture electron microscopy [13].

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. An emission spectrum of 2 ml of 50 mg/l dansylated PEAA (dPEAA) in 100 mM phosphate buffer was taken at 8°C, pH 7.4. The emission spectrum of dPEAA does not vary with temperature between 8°C and 30°C at this pH. A 100 μl aliquot of a 2 mg/ml stock solution of extruded liposomes was added to the polymer solution. Emission spectra were then recorded as the sample was heated to 30°C, and then cooled back to 8°C. These spectra show a fluorescence enhancement due to the sequestering of the dansyl fluorophore in a hydrophobic environment. The fluorescence enhancement, as determined by the difference in areas of the spectra taken in the presence and absence of liposomes, is used as a relative estimate of the amount of polymer bound to vesicles. (The fluorescence enhancement was found to be proportional to the amount of lipid present, at low lipid concentrations, supporting this interpretation. We cannot rule out some change due to temperature-varying hydrophobicity of the membrane itself.)

3. Results

3.1. Temperature dependence

Complexation of PEAA with phosphatidylcholine membranes results in the formation of small, discoidal mixed micelles, as determined by negative stain electron microscopy [14] and Raman spectroscopy [15]. The micelles are too small (ca. 15 nm) to scatter light efficiently; consequently, reorganization of a suspension of vesicles, especially multilamellar vesicles, to form micelles results in a clearing of the suspension that can be monitored turbidometrically. The change in optical density (400 nm) of a DMPC suspension of MLVs on addition of PEAA is shown in Fig. 1 for several temperatures, at pH 6.45. At 20.5°C, micellization is extremely slow. This temperature is significantly below the gel-liquid crystal transition temperature (T_m) for DMPC, 24°C [16]. As the temperature approaches T_m, the rate of micellization increases dramatically. Above T_m, the rate again diminishes.

In a multilamellar suspension, the optical density is a

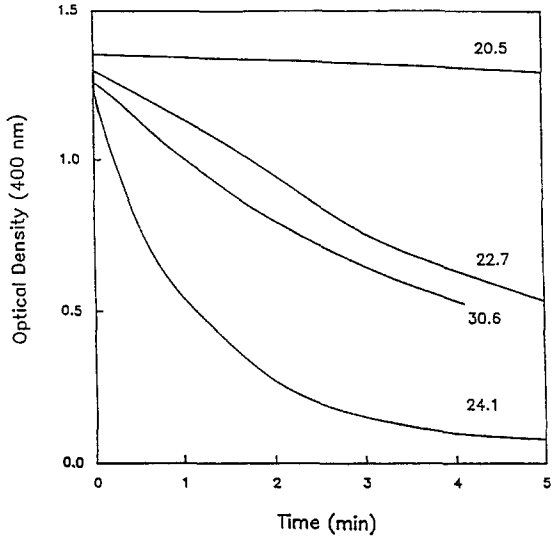

Fig. 1. Decrease in optical density on micellization of DMPC multilamellar liposomes by PEAA. A PEAA solution (2 mg/ml, 100 mM phosphate buffer pH 6.4) was mixed with a liposome suspension (2 mg/ml, 10 mM phosphate buffer pH 7.4) at temperatures indicated on each curve. The final pH was 6.45. The optical density decreases most rapidly near the phase transition temperature, T_m (24°C).

non-linear function of the vesicle size. Furthermore, the micellization may well occur initially on the exposed surface of these vesicles, 'peeling off' membrane bilayers one by one, as has been shown for micellization by bile salts [17]. Consequently, the observed decreases in optical density cannot be simply related to the extent of micellization, nor would we expect that the extent of micellization of MLVs would show exponential (first order) kinetics. We have chosen the approach used by Pownall et al. [18] in their analysis of the kinetics of DMPC micellization by apolipoprotein A-I: the rate of micellization is characterized by the reciprocal of the time to reduce the optical density by half, $1/T_{1/2}$. This rate is proportional to the underlying rate of micellization, although the proportionality constant is indeterminate.

The rates of micellization of DMPC and DPPC MLVs at pH 6.45 are shown in Fig. 2, top and bottom, respectively. Micellization is significantly faster near the main phase transition (P'_{β}-L_{α}) of each phospholipid, and the

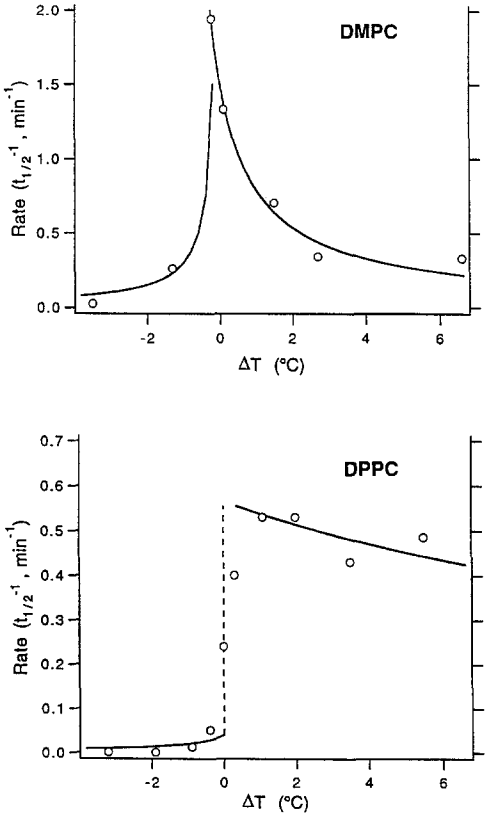

Fig. 2. Rates of micellization, as determined from the times required to reduce the optical density by half, for DMPC (top) and DPPC (bottom) multilamellar liposomes. The temperatures are given as differences from the transition temperature (24°C for DMPC, 41.4°C for DPPC). The lines drawn are simply guides to the eye, but they are of the functional form expected for bilayer permeability or compressibility κ near T_m,

$$\kappa \propto \frac{1}{|T - T_{\pm}^{*}|}$$

$T_{\pm}^{*}$ is a 'spinodal' temperature that is $< T_m$ when the transition is approached from above and $> T_m$ when the transition is approached from below. At the critical point of a second-order transition, $T_m = T_{\pm}^{*}$ [28].

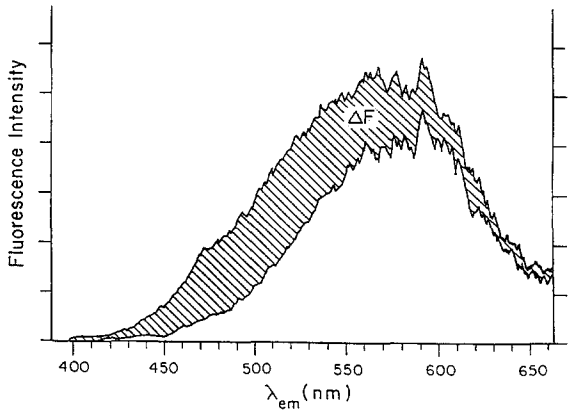

Fig. 3. Fluorescence emission enhancement of dansyl-PEAA in the presence of membranes. Lower curve, emission of a 40 mg/l solution of dansyl-PEAA in 100 mM phosphate buffer, pH 7.2. Upper curve, emission after addition of DMPC sonicated vesicles to a final concentration of 40 mg/l. $T = 25$°C. ΔF is the fluorescence enhancement indicated by the hatched area between the spectra.

enhancement is greater for DMPC than for DPPC. The increased rate of micellization near the phospholipid melting transition is similar to rate enhancements that have been observed for apolipoprotein A-I micellization of DMPC [18]. The dependence of the rate enhancement on the lipid structure is also similar for the lipoprotein and for PEAA; in each case, the apparent rate of micellization of DPPC is small in comparison with that of DMPC [18]. In fact, in Fig. 2, the maximum rate for DPPC is approximately that observed well above the phase transition, in the L_{α} phase of DMPC. The rates in the L_{α} phases of the two lipids are quite similar, but DPPC shows only a weak rate maximum at T_m.

To explore further the effects of temperature on polymer-membrane behavior, we measured the adsorption of a fluorescently-modified PEAA, dansyl-PEAA, to DMPC vesicles as a function of temperature. The dansyl tag shows an approx. 3-fold enhancement in fluorescence emission on sequestration to a hydrophobic environment, as occurs when PEAA hydrophobically collapses at low pH [12]. Fig. 3 shows fluorescence emission spectra from dansyl-PEAA in the presence and absence of DMPC vesicles. The fluorescence increase results from the adsorption of the pendant dansyl moieties into the hydrophobic interior of the vesicles; the hydrophobic nature of this adsorption allows us to use the fluorescence enhancement as an assay for PEAA-membrane binding.

Fig. 4 shows the fluorescence enhancement of a polymer solution due to adsorption onto DMPC lipid vesicles at temperatures from 8°C to 30°C. A vesicle sample (100 mg/l) was slowly heated (ca. 10°C per hour) in the presence of 50 mg/l dansyl-PEAA, at pH 7.4. This slightly alkaline pH was chosen because micellization does not occur above pH 6.55; micellization might cause a changing fluorescence emission even with no change in polymer

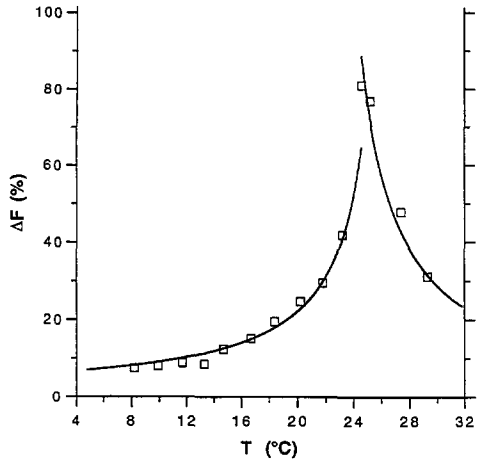

Fig. 4. Adsorption of dansylated PEAA to DMPC extruded liposomes as a function of temperature, at pH 7.4. The adsorption is quantified by the increase in fluorescence (ΔF) of the pendant dansyl group on incorporation into the hydrophobic part of the membrane.

adsorption. The fluorescence of a vesicle-free polymer solution does not change with temperature at this pH.

Binding of the polymer increased sharply as the transition temperature was approached, and actually fell as the temperature was raised significantly above T_m. On cooling, the binding again increased at T_m, but did not decrease significantly on cooling below T_m (ΔF was 72% at 10°C) which indicates that the polymer has been 'frozen' into the gel phase phospholipid. In order to independently verify that polymer adsorption does in fact decrease as the temperature is raised above T_m, we allowed dansyl-PEAA to bind to multilamellar liposomes at 25°C and at 32°C, at pH 7.4, then centrifuged the liposomes at 12000 rpm and measured the fluorescence of the pelleted lipid after dissolution in MeOH. The fluorescence of the 25°C pellet was twice that of the 32°C pellet, in agreement with the measurements shown in Fig. 4.

As can be seen by comparing Figs. 2 and 4, the temperature dependence of PEAA adsorption at a non-permissive pH is quite similar to the temperature dependence of the rate of micellization at a permissive pH. Similar temperature dependence has been seen in the permeabilities of membranes near the transition temperature [19–22], and in the lateral compressibility of DMPC bilayers [23].

3.2. pH dependence

It has been shown that the micellization of phosphatidylcholine membranes by PEAA is extremely sensitive to pH [10]. In addition, pH-dependent binding of PEAA has been observed, with the amount bound increasing as pH is lowered [24,25]. The effects of pH on the rate of membrane solubilization are presented in Fig. 5. Above a threshold pH, solubilization is essentially halted. As pH is lowered, the micellization rates increase approximately linearly. Interestingly, a negatively charged phospholipid,

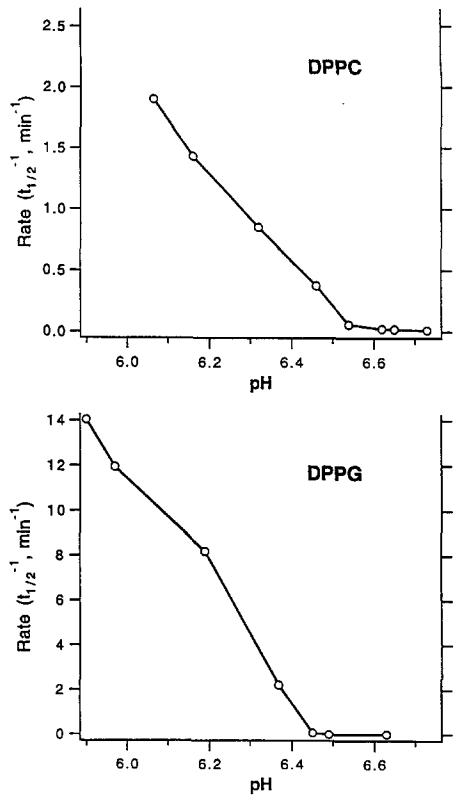

Fig. 5. Rates of micellization of DPPC and DPPG multilamellar liposomes as a function of pH at 45°C. The threshold pH for DPPG micellization is 0.1 pH unit lower than for DPPC, but the rate at lower pH is much faster for the negatively charged DPPG than for the zwitterionic DPPC.

DPPG, showed only a slight reduction in the threshold pH and significantly faster micellization at all lower pH values. The incorporation of 10% or 20% phosphatidic acid also had little effect on the rate of solubilization of dipal-

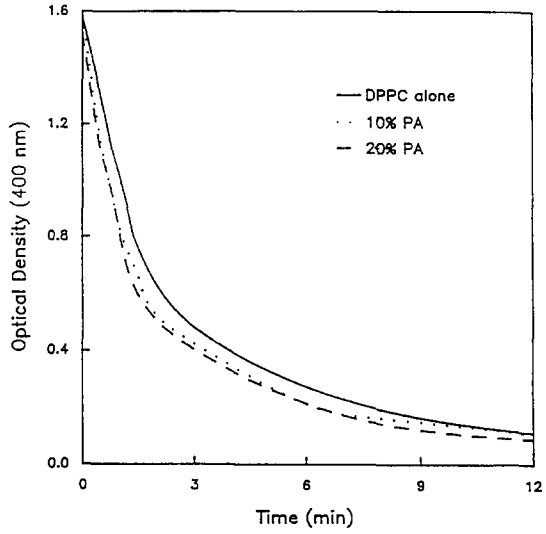

Fig. 6. Solubilization of DPPC membranes containing 0, 10, and 20 wt% phosphatidic acid (PA), at pH 6.45 and 45°C. Solid line, 0% PA; · · ·, 10% PA; – – –, 20% PA.

mitoylphosphatidylcholine membranes (Fig. 6). These results are at first sight surprising, since the polymer is highly negatively charged in solution at these pH values, with about a third of the carboxylic acids deprotonated [24,26].

Note that a direct comparison of the solubilization rate of MLVs of different phospholipids is certainly not warranted, as charged phospholipids are likely to produce MLVs with structures (i.e., number or spacing of lamella) that differ from those produced by the zwitterionic phosphatidylcholines.

4. Discussion

4.1. Temperature effects

Both the kinetics of DMPC micellization (Fig. 2) and the levels of polymer adsorption at pH 7.4 (Fig. 4) show pronounced maxima near the P'_β-L_α transition temperature T_m. The changes in kinetics and in adsorption are quite similar to anomalies in permeability and compressibility that have been previously observed, as discussed above. These effects have been attributed to gel-liquid crystalline boundaries at the transition temperature [23], but a rigorous theoretical treatment of the transition shows that domain boundaries need not be invoked to explain these anomalies [27–29]. Moreover, domain boundary models have difficulty explaining the breadths of the anomalous permeability and compressibility peaks: since the L_α-P'_β phase transition in pure lipids is first order, domains cannot coexist at any temperature other than T_m, i.e., the anomalies should be δ-functions. This problem has been overcome by assuming a limited cooperativity for the phase transition, which could arise from bilayer impurities. However, it is difficult to explain why permeability and adsorption peaks in DMPC are sharper than in DPPC on the basis of domain boundaries, whereas a Landau phase transition model makes this prediction based on differing internal lateral pressures in DMPC and DPPC membranes [29].

Regardless of the proper theoretical treatment of the bilayer chain melting transition, it is clear experimentally that the physical properties of membranes are dramatically affected near this temperature. Of special relevance to adsorption phenomena is the bilayer lateral compressibility κ_T. The adsorption of an exogenous molecule into the bilayer should depend on the compressibility; the energy required to open a 'hole' of size ΔA in a bilayer of size A is [16]

$$\Delta G = \frac{\Delta A^2}{A \cdot \kappa_T}$$

Since this energetic cost must be paid by each adsorbing unit, a more compressible membrane will lead to more extensive adsorption, other factors being equal. Our results on adsorption of the dansylated PEAA are consistent with this dependence. In addition, the compressibility energy should contribute to the total free energy barrier to adsorption, so that the rate of adsorption should also increase with increasing compressibility. The rates of membrane solubilization by PEAA might simply reflect the rate at which polymer is deposited on the membrane.

It must be pointed out that higher compressibility will also permit larger thermally-driven fluctuations in membrane density and structure, which could lead to the micellar transition. However, because compressibility clearly affects adsorption, it need not also play a rate-limiting role in micellization in order to generate the temperature dependence we have observed.

4.2. pH effects

PEAA shows qualitatively similar kinetics in the solubilization of neutral and negatively charged liposomes, and rates of solubilization of DPPC were essentially unaffected by the addition of up to 20% phosphatidic acid. These results are at first surprising, given that the polymer is about 30–40% ionized at all the pH values examined [26]. However, significant polymer adsorption precedes micellization [24]. Mixed micelles contain one-third to one-half PEAA by weight, which corresponds to a surface number density of carboxylic acids much greater than the number density of lipids, owing to the smaller molecular weight of the PEAA repeat unit. Because PEAA is negatively charged, early polymer adsorption will confer a negative charge on phosphatidylcholine vesicles. Subsequent adsorption will be unaffected by the charge of the phospholipid, because all membranes will present a surface with a high negative charge density.

Our results are consistent with a micellization process that is kinetically controlled by the rate of polymer adsorption. Membrane surface charge has little effect on the critical pH for micellization and on the rate of solubilization, as shown for the DPPC/DPPA mixtures. This result is likely due to the large amount of polymer adsorption that preceeds micellization and confers a large negative charge on the zwitterionic vesicles. We cannot rule out other explanations for the kinetic results, however. In particular, it has been predicted theoretically that incorporation of proteins or polymers will increase the amplitude of membrane structural fluctuations [28], and incorporation of synthetic peptides into bilayers has been shown to dramatically increase the compressibility [30]. It is possible that polymer adsorption saturates well before the structural transition to mixed micelles, and that the rate of that transition is controlled by the amplitude of membrane fluctuations. The pH-dependence of the micellization rate would then originate from differing saturating levels of adsorption at different pH, while the temperature dependence follows naturally from the temperature-dependent compressibility (which governs the fluctuation amplitudes).

Distinguishing these two models is difficult, since pH-induced changes in polymer deposition rates ('on-rates', or k_f) will also affect the extent of deposition ($k_{eq} = k_f/k_r$).

5. Conclusions

Measurements of the rates of membrane micellization by the hydrophobic polyelectrolyte PEAA indicate strong effects of the phospholipid gel-liquid crystalline transition and solution pH, but little effect from membrane surface charge. The observed rates are consistent with a simple model of prompt bilayer disruption upon sufficient polymer adsorption, but do not rule out more complicated mechanisms in which structural fluctuations or lateral fluctuations in polymer density are rate limiting for micellization.

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