Modulation of Mobilities of Fluorescent Membrane Probes by Adsorption of a Hydrophobic Polyelectrolyte

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ABSTRACT: We have used three different fluorescence methods to examine the effect of the pH-dependent adsorption of a hydrophobic polyelectrolyte, poly(2-ethylacrylic acid), on the mobility of fluorescent probes in phosphatidylcholine membranes. Measurements of lateral diffusion, made by either pyrene excimer formation or fluorescence photobleaching recovery, show reduced probe mobility on polymer adsorption; formation of intramolecular excimers by 1,1′-propylenebis(pyrene) is also reduced. Fluorescence depolarization measurements, however, show increased rotational freedom for diphenylhexatriene and other depolarization probes. Extensive polymer adsorption solubilizes membranes into micelles, but mobilities do not exhibit sharp transitions upon membrane reorganization. Polymer adsorption appears to affect probe mobilities locally; the global structure of the membrane does not play a direct role.

Introduction

The mobilities of molecules embedded in phospholipid membranes can affect their chemical activities and thus modulate membrane-related chemical and biological processes. Many enzymes have activities that depend on membrane "fluidity", including adenylate cyclase,[1] phospholipase A_2,[2,3] rhodopsin (in its ability to activate many G protein molecules),[4] microsomal enzymes,[5] and redox enzymes in mitochondria.[6] Membrane treatments that modulate these reaction rates may be important; for example, fluidization of biomembranes by anesthetics may be responsible for some of their actions. In addition, some receptors (e.g., Mg^{2+}-ATPase) desensitize to omnipresent ligands if they are mobile but can be inhibited from desensitization by agglutinizing lectins. In these cases, desensitization may occur by a diffusion-mediated interaction between the activated receptor and a membrane-bound desensitizing agent.[7] In contrast, some receptors become desensitized when cell surface constituents are immobilized,[8,9] as would be expected when signal transduction requires an additional membrane-bound component to interact with the liganded receptor.

Modulation of membrane "fluidity" is usually accomplished by the incorporation of small, amphipathic molecules, such as cholesterol, short chain alcohols, or phospholipids containing unsaturated fatty acids. Larger molecules, such as proteins or polypeptides, have received less attention with respect to their effects on membrane fluidity. There is some evidence that intrinsic membrane proteins (specifically a Ca^{2+}-ATPase) can fluidize membranes in an interfacial layer between ordered boundary lipid and bulk lipid,[10] as determined by diphenylhexatriene (DPH) fluorescence anisotropy. Annexins (and polylysine), which bind ionically to phosphatidylserine membranes, reduced the formation of excimers of pyrene phosphatidylcholine probes,[11] which is interpreted as a *decrease* in membrane fluidity. These contrary fluidity effects could be due to the different localization of these macromolecules in the bilayer or might simply be a consequence of the different methods used to assess fluidity in each system.

To further elucidate potential effects of macromolecules on the mobilities of membrane molecules, we have studied the effects of a hydrophobic polyelectrolyte, poly(2-ethylacrylic acid) (PEAA), on mobility of several fluorescent probes in phosphatidylcholine membranes. We chose this molecule for several reasons. First, the surface activity and membrane association of PEAA can be varied with pH.[12] Second, sufficiently high concentrations of adsorbed PEAA lead to structural reorganization of lamellar phosphatidylcholine membranes into mixed polymer−phospholipid micelles with consequent pH-dependent release of liposomal contents.[13] We were interested in changes in membrane fluidity that might accompany these events. Finally, the fact that this synthetic polymer has a random-coil conformation (rather than a more compact and organized folding typical of proteins) suggests that the membrane interactions of PEAA may be quite "generic", i.e., dependent only on the balance of hydrophobic and dielectric forces, and not critically dependent on the presence of specific amphiphiles in the membrane.

Three different methods were used to assess "fluidity": formation of inter- and intramolecular excimers, fluorescence depolarization, and fluorescence photobleaching recovery (FPR). These three methods did *not* give identical results. While both FPR and excimer experiments report a decrease in membrane fluidity with increasing polymer adsorption, fluorescence depolarization reports an increase. Clearly, "fluidity" of membranes is very much a phenomenological parameter and is highly dependent on the probe and the process used to measure it. The probes we have used are known to localize at different depths in the membrane. Consequently, our results are most easily interpreted as indicating differential effects of polymer at different depths in the bilayer.

Materials and Methods

Materials. Pyrene (99+%), **1**, was obtained from Aldrich Chemical Co., Milwaukee, WI. 1,1′-Propylenebis(pyrene) (bispyrene), **2**, 1,1′-didodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (diI), **3**, 4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoic acid (bodipy-C5), **4**, 3-palmitoyl-2-(3-(diphenylhexatrienyl)propanoyl)-L-α-phosphatidylcholine (DPH-PC), **5**, and 3-palmitoyl-2-(1-pyrenyldecanoyl)-α-phosphatidylcholine (Py-PC)< **6**, were obtained from Molecular Probes, Eugene, OR. 1,6-Diphenylhexatriene (DPH)

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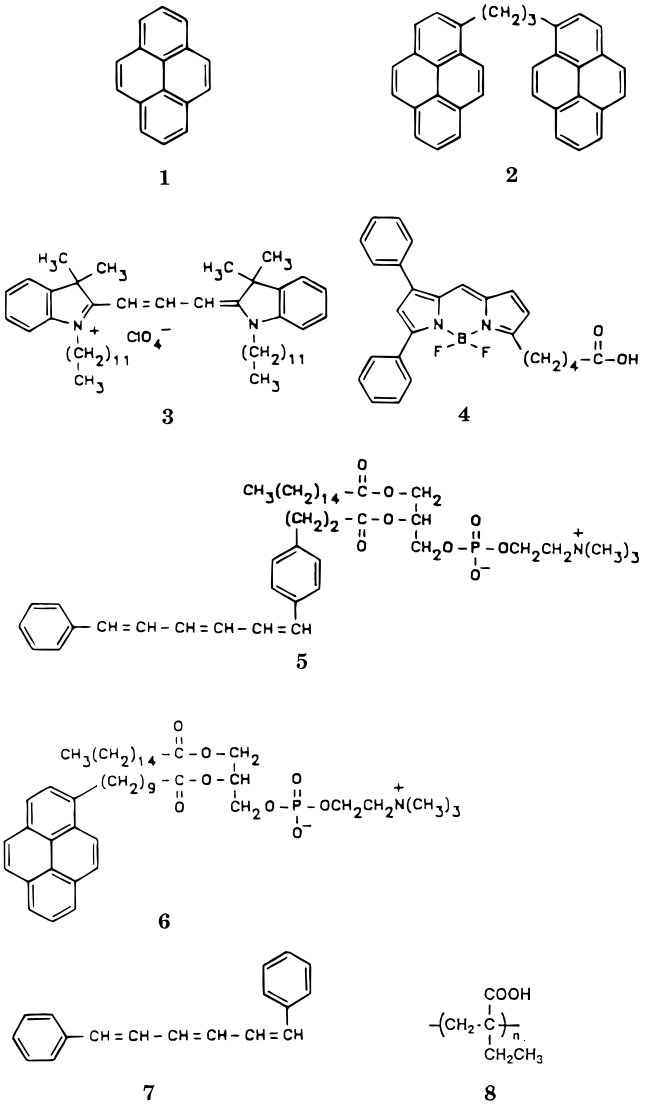
(98%), **7**, was obtained from Aldrich Chemical Co. and recrystallized from 50/50 hexane/acetone. L-α-Dimyristoylphosphatidylcholine (DMPC) (99+%) and L-α-dipalmitoylphosphatidylcholine (DPPC) (99%) were obtained from Sigma Chemical Co., St. Louis, MO.

Poly(2-ethylacrylic acid), **8**, was synthesized as described elsewhere.[14] Gel permeation chromatography through TSK 6500 PW and TSK 6300 PW columns with 50 mM aqueous tris(hydroxymethyl)aminomethane hydrochloride (pH 8.0) as eluent gave estimates of $M_w = 31$ kDa (ca. 300 mers/chain), $M_w/M_n = 1.6$, with poly(ethylene oxide) molecular weight standards.



Excimer Formation. Vesicles were prepard by drying a DMPC/$CHCl_3$ solution containing 0.1 wt % bis-pyrene, first under N_2 and then in vacuum for 20 min. The phospholipid was hydrated with 100 mM sodium phosphate buffer at pH 7.2 to a concentration of 12 mg/mL, vortexed, and then forced through a 100 nm polycarbonate filter (Nuclepore, Pleasanton, CA) at least 15 times, at flow rates up to ca. 2 mL/s. This procedure produces single-walled vesicles, as demonstrated by ^{31}P-NMR spectroscopy and freeze fracture electron microscopy.[15] Extruded vesicles were chosen for excimer experiments because sonicated vesicles yield Arrhenius plots with broad phase transitions and poorly defined activation energies.[16] An individual sample was made by first cooling a 3 mL aliquot of PEAA/phosphate buffer (1 mg/mL) in ice water and then adding 260 μL of the vesicle suspension. Each sample was degassed by bubbling with argon for 20 min and then cannulated into an argon-purged quartz cuvette. The cuvette was placed in the fluorimeter (Perkin-Elmer MPF-66) and a slight overpressure of argon was maintained in the cuvette at all times by piercing the cuvette septum with an argon line. The overpressure of argon was found to be necessary to prevent oxygenation of the samples on repetitive heating and cooling cycles. Fluorescence was excited at 331 nm, and emission was measured at 378 nm (monomer) and 488 nm (excimer), with 5 nm slits. A neutral density filter (1.0) was used to reduce the illumination intensity and eliminate potential bleaching artifacts. Scattered light was found to be negligible compared to either fluorescence signal. On the Arrhenius plots, the leakage of the monomer emission into the excimer wavelength was corrected by subtracting a small amount (2%) of the monomer signal from the excimer signal. The appropriate correction was determined by examining the emission at both wavelengths from a dilute pyrene/EtOH control.

Pyrene excimer measurements were made with an identical protocol, except that the probe concentration was higher, 1 wt %, to generate significant intermolecular excimer formation.

Pyrene Vibronic Bands. DMPC-extruded vesicles were prepared as described above, containing 0.125 wt % pyrene. Vesicles were added to a PEAA solution (1 mg/mL) in 100 mM sodium phosphate buffer to a final concentration of 1 mg/mL. The addition was made at 4 °C. Fluorescence spectra were taken with 331 nm excitation (5 nm slit) and scanned emission from 369 to 399 nm (1 nm slit). The heights of the vibronic bands (peak III, 383 nm, and peak I, 372 nm) were measured on the emission spectra.

Fluorescence Anisotropy. Fluorescence anisotropies were measured on sonicated suspensions of DPPC. Multilamellar suspensions of DPPC in phosphate buffer were sonicated for 10−15 min at 30 W with a Branson Model 185 cell disruptor with a $^1/_8$ in. diameter titanium microtip at 50−60 °C. The vesicles were centrifuged in an IEC Model CL clinical centrifuge at 2500 rpm for 30 min to sediment titanium particles and any residual multilamellar vesicles. Samples of 0.25 mg/mL PEAA and 0.25 mg/mL DPPC were labeled to a lipid/dye mole ratio of 1000 from stock solutions of one of three probes: DPH/acetone, 10^{-3} M; DPH-PC/EtOH, 6×10^{-4} M; Py-PC/EtOH, 6×10^{-4} M. Samples were equilibrated overnight at 50−60 °C. Steady-state fluorescence polarization was measured on deaerated (argon-flushed) samples at 23 °C. Excitation/emission: DPH, 360 nm/430 nm; DPH-PC, 363 nm/430 nm; Py-PC, 347 nm/398 nm; emission slits, 10 nm; excitation slits, 5 nm for DPH, 4 nm for others. The anisotropy (r) is found as

$$r = \frac{k-1}{k+2} \quad \text{where } k = \frac{I_{VV} \cdot I_{HH}}{I_{VH} \cdot I_{HV}}$$

which corrects for instrumental effects.[17] I_{HV} is the fluorescence intensity with horizontally polarized excitation and a vertical emission polarizer; similar notation indicates other combinations of excitation and emission polarizations.

Fluorescence Photobleaching Recovery. The effect of adsorbed PEAA on long-range lateral mobility of fluorescent probes in supported bilayers was measured by fluorescence photobleaching recovery (FPR). Supported bilayers were deposited on 22 mm square glass coverslips from a Langmuir trough to 20−22 dynes/cm lateral pressure, as described by McConnell et al.[18] Coverslips were cleaned by boiling in a solution of Alconox detergent for 1 h, rinsing with high-purity water, and then immersing in NoChroMix/sulfuric acid overnight followed by rinses with high-purity water, $NaOH/H_2O$ solution, and a final water rinse. A monolayer of DMPC (with 1 wt % diI or bodipy-C5) in 90:10 hexane:EtOH was spread on the surface of a 100 mM phosphate buffer subphase at pH 7.7. Coverslips were withdrawn from the subphase at ca. 4 mm/min. The second leaflet of the bilayer was deposited by horizontal reimmersion of the coverslip. The coverslip was mounted in a chamber so that the buffer could be changed and examined by epifluorescence microscopy, using a Zeiss Universal upright microscope. Fluorescence of the films appeared uniform. The 514 nm line from an argon ion laser (Innova 90, Coherent, Inc.) was used for photobleaching, as described elsewhere,[19,20] using 40× and 100× objectives. The sizes of the bleaching spots were estimated from spot images on thin

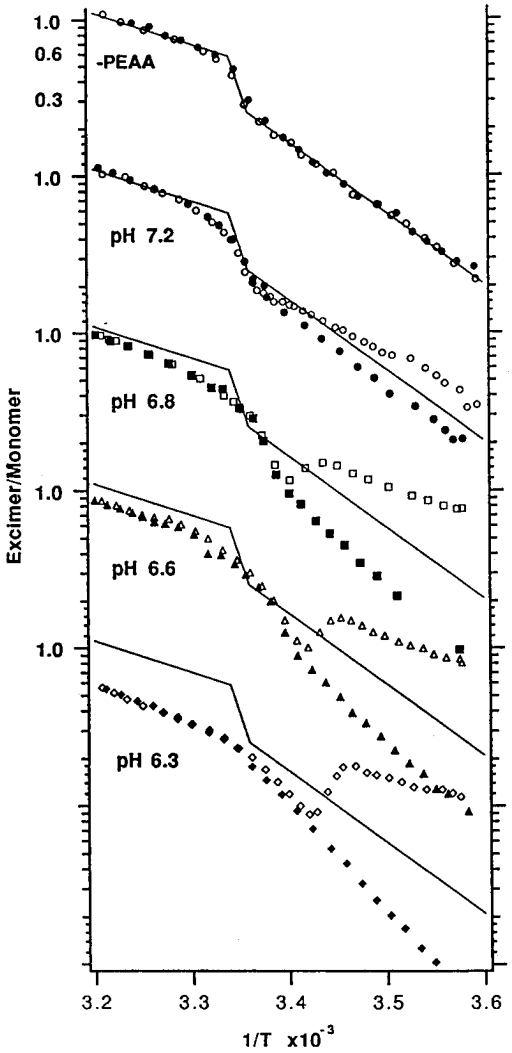


Figure 1. Effect of PEAA adsorption on bis-pyrene excimer formation in deoxygenated, extruded membrane suspensions. The ratio of excimer emission to monomer emission is plotted against inverse temperature. For clarity, each sample is offset by 1 decade. The first run with each sample is marked with open symbols and the second run with filled symbols. Top, control: DMPC vesicles (1 mg/mL) in phosphate buffer at pH 7.2, with no PEAA. The line shows fits to the data in the gel and liquid crystal phases. The lower curves show runs in the presence of polymer (1 mg/mL) at the indicated pH's, with the control line replotted for each sample as a visual reference.

formvar/diI films. Fluorescence recoveries were fit as described by Ygeurabide et al.[21]

Results

Titration of poly(2-ethylacrylic acid) changes the polymer charge density and consequently its surface activity and biomembrane-binding properties.[12] Upon acidification to ca. pH 6.5, the polymer induces a dramatic reorganization of dimyristoyl- or dipalmitoylphosphatidylcholine bilayers into mixed phospholipid−polymer micelles, similar to membrane micelles induced by apolipoproteins[22] or by lactalbumin at acidic pH.[23] PEAA binding to membranes begins at a much higher pH, ca. pH 7.8, and increases as pH is lowered.[24] In this paper, we report observations on the effects of PEAA binding on the mobility of several lipophilic fluorescence probes, using the techniques of excimer formation, depolarization, and photobleaching recovery.

Excimer Formation. Figure 1 shows the temperature and pH dependence of the formation of intramolecular excimers of bis-pyrene in extruded membranes of DMPC (1 mg/mL) in the presence of PEAA (1 mg/mL). Polymer and membranes were mixed at 6 °C. The excimer and monomer emissions were measured as each sample was heated to 40 °C, shown with open symbols. Each sample was then cooled, and a second heating run was made, shown with filled symbols. In the absence of polymer, the runs superimpose and yield activation energies of about 38 kJ/mol above T_m and 86 kJ/mol below T_m, Table 1. These activation energies are close to those previously reported in DMPC, 42 and 74 kJ/mol above and below T_m, respectively.[25] The presence of PEAA alters the excimer/monomer ratio in all samples. Below the gel−liquid crystalline phase transition (T_m), the polymer enhances the formation of excimers and reduces the apparent activation energy for their formation. As the phase transition is approached, there is a reduction in excimer formation. Above T_m, excimer formation is reduced (relative to pure DMPC membranes) but with little change in activation energy. On the second run, excimer formation is inhibited in both gel and liquid crystal phases; the gel phase also shows an increased activation energy, compared with pure membranes (Table 1).

Table 1. Activation Energies for Bis-pyrene Excimer Formation, Computed from the Slopes of the Curves in Figure 1[a]

	E_A (kJ/mol)		
		$T < T_m$	
	$T > T_m$	run 1	run 2
control (no PEAA)	38	86	86
pH 7.2	43	61	88
pH 6.8	45	44	113
pH 6.6	38	44	113
pH 6.3	47	37	131

[a] Typical uncertainty is ±4 kJ/mol.

These measurements show that irreversible complexation between polymer and membrane occurs slightly below T_m. This can been seen in the changes in the vibronic bands of the fluorescence emission of (monomeric) pyrene incorporated into DMPC vesicles, shown in Figure 2. The ratio of the intensity of band III, at 383 nm, to that of band I (at 372 nm) depends on solvent polarity (more precisely, solvent dipole moment),[26] a higher ratio indicating a less polar probe environment (lower dipole moment). At low temperatures (<15 °C), the pyrene probe experiences an environment of similar polarity either in the presence (■) or absence (○) of PEAA, at pH 6.4. In a solution containing only polymer and pyrene probe (i.e., in the absence of lipid), the III/I ratio is much lower, at this pH: ca. 0.74. Therefore, the pyrene is not associating with free polymer but remains in the membrane; the bis-pyrene probe should be similarly situated in the membrane. Consequently, the enhancement of excimer formation at low temperatures reported in Figure 1 and Table 1 is indicative of a change in the membrane environment of bis-pyrene and does not result simply from bis-pyrene associating with free polymer.

As the DMPC/PEAA sample is heated above 15 °C, a transition occurs in the pyrene environment: The pyrene probe reports a less polar environment, Figure 2 (■). The environmental change is coincident with the reduction in bis-pyrene excimer formation and, at this pH, with a conversion of the membrane to mixed micelles. The environmental change is irreversible on cooling: During a second heating run (□), only the less polar environment is observed. Importantly, the environmental change of pyrene probes occurs even at pH's

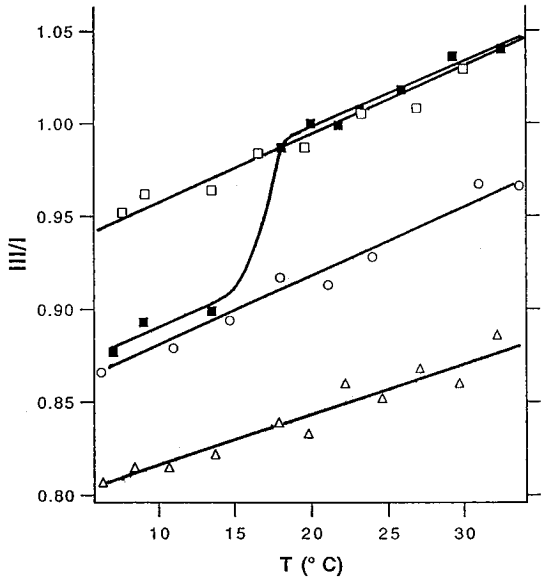


Figure 2. Environmental polarity of pyrene in DMPC (1 mg/mL) vesicles, as determined by the III/I vibronic band ratio. A higher ratio indicates a less polar environment. In the absence of polymer (○), apparent polarity varies smoothly from 5 to 35 °C. In the presence of 1 mg/mL PEAA at pH 6.38, the pyrene environment polarity decreases dramatically between 15 and 20 °C (■). The polarity change is irreversible: On a second run (□), the pyrene environment remains less polar at all temperatures. Pyrene in ethanol (△) shows a gradual decrease in environmental polarity with increasing temperature, similar to that of the control vesicles.

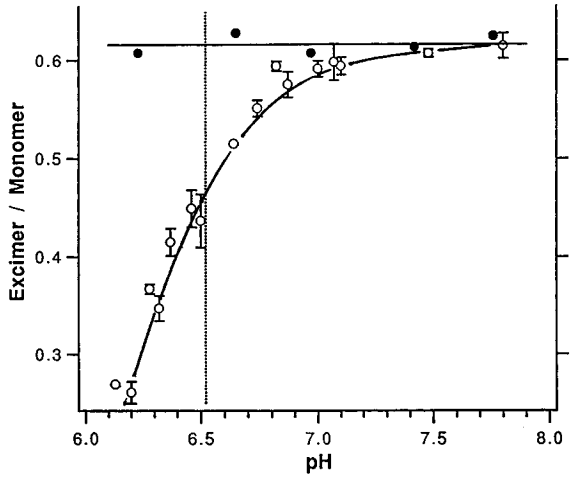


Figure 3. pH dependence of the bis-pyrene excimer/monomer ratio in deoxygenated, extruded DMPC membranes (1 mg/mL) with adsorbed PEAA (1 mg/mL), at 30 °C. Error bars indicate variation in repeated measurements of the same sample. In the absence of PEAA (●), there is no pH-dependent variation of excimer formation in this pH range. The vertical dotted line indicates the approximate pH of micellization.

well above pH 6.5, which do not permit conversion of the membrane to mixed micelles.[24]

In Figure 1, it is apparent that significant changes in intramolecular excimer formation occur even when the pH is too high to allow conversion of the membrane to mixed micelles (i.e., >pH 6.5). Figure 3 shows that, at temperatures above T_m, inhibition of excimer formation begins at pH 7 and increases continuously with increasing acidification, through the vesicle−micelle transition at pH 6.5.

When incorporated into the membrane at a sufficiently high concentration, pyrene can form intermolecular excimers by lateral diffusion during the excited-state lifetime. PEAA adsorption inhibits the

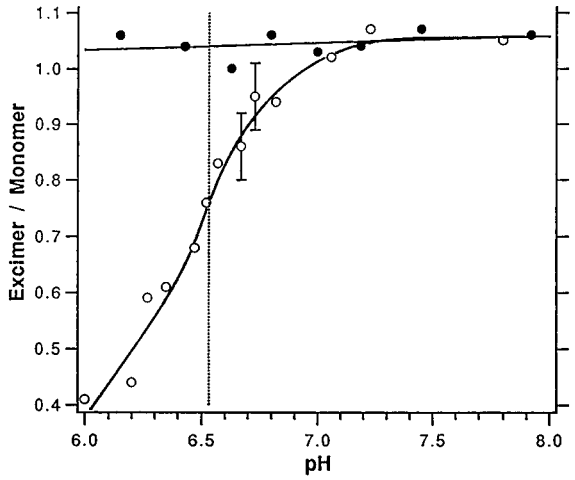


Figure 4. pH dependence of the formation of intermolecular pyrene excimers in deoxygenerated, extruded DMPC membranes (1 mg/mL) with adsorbed PEAA (1 mg/mL), at 30 °C. In the absence of PEAA (●), there is no pH-dependent variation of excimer formation in this pH range. The vertical dotted line indicates the approximate pH of micellization.

formation of intermolecular pyrene excimers with a pH dependence similar to that of the inhibition of bis-pyrene intramolecular excimers, Figure 4. Again, there is a monotonic decrease in the efficiency of excimer formation, beginning significantly above the vesicle−micelle transition and continuing through it. The micelles that result from membrane disruption are discoidal aggregates of about 8 nm diameter,[27] and each therefore contains several hundred molecules of phospholipid. Consequently, these micelles will each contain several pyrene probes: Excimer formation is not being inhibited simply by the sequestration of individual pyrene molecules into separate micelles.

Fluorescence Photobleaching Recovery. It is well established that, in membranes that contain protein, intermolecular pyrene excimer formation need *not* reflect the rate of lateral diffusion of the probe,[28] due to inhibition of excimer formation by protein contact. In addition, diffusion in membranes can be differentially modulated at different length scales.[29] To study the effects of PEAA adsorption on long-range lateral diffusion, we have employed FPR on DMPC bilayers deposited on glass coverslips. Bilayers were formed by a modified Langmuir−Blodgett deposition technique, as described in Materials and Methods. A fluorescent probe was incorporated into the bilayer, and lateral diffusion of the probe was measured by photobleaching a small spot and observing the recovery of fluorescence in the bleached region. Results are shown in Figure 5, for the probe diI. Laterial diffusion of this probe is hindered by polymer adsorption, beginning at about pH 7. Above pH 7, the measured diffusion coefficient was 1.8×10^{-9} cm^2/s and essentially unaffected by the addition of PEAA ($(1-2.5) \times 10^{-9}$ cm^2/s). (This diffusion coefficient is somewhat lower than is generally observed for diI in multilamellar lipid vesicles, ca. 10^{-8} cm^2/s,[30−32] but was reproducible from sample to sample. Some reduction in diffusion coefficient may be due to frictional coupling to the glass surface or to the use of a rather high diI concentration (1 wt %) in our experiments.) Interestingly, acidifications to pH 6.5 did not result in complete dissolution of the deposited bilayer; instead, some decrease in fluorescence intensity was observed, but the film that remained was photobleachable and showed fluorescence recovery. In all measurements on diI-labeled membranes, the fluorescence recovery was essentially complete. While the polymer hinders diffu-

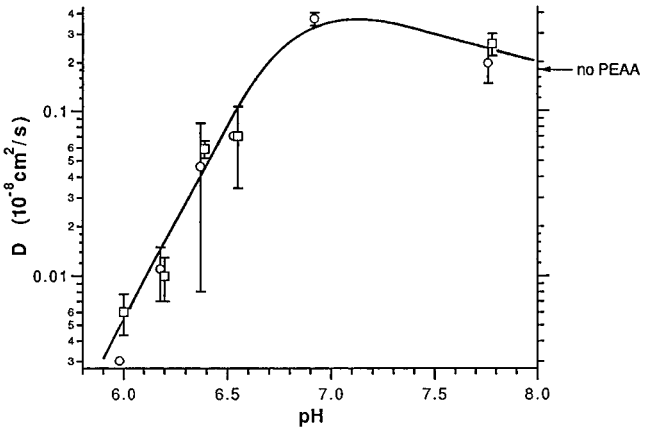


Figure 5. Fluorescence photobleaching recovery measurements of the long-range lateral mobility of diI in supported DMPC bilayers in the presence of PEAA (0.1 mg/mL). DiI diffusion was measured with a 3.1 μm laser spot (○) and a 1.7 μm spot (□). In the absence of polymer, the dye diffusion was relatively rapid, ca. 2×10^{-9} cm^2/s. Measurements were performed at room temperature (25 °C).

Table 2. Diffusion of Bodipy-C5 in DMPC Bilayers

pH		D ($\times 10^8$ cm^2/s)	mobile %
7.7		2.8 ± 1.2	91
6.0		2.5 ± 0.8	96
7.7	+0.1 mg/mL PEAA	2.4 ± 0.8	101
6.3	+0.1 mg/mL PEAA	0.6 ± 0.1	85
6.0	+0.1 mg/mL PEAA	0.4 ± 0.1	72
5.7	+0.1 mg/mL PEAA	0.3 ± 0.2	46

sion, it does not create domains or "corrals" from which diI cannot escape. Furthermore, no difference was found between measurements made with a 40× objective, which yielded a bleaching spot of 3.1 μm measured diameter, and a 100× objective, which gave a spot of 1.7 μm diameter.

Finally, a negatively charged probe, bodipy-C5, also showed hindered mobility in the presence of adsorbed polymer (Table 2), suggesting that a charge interaction between diI and PEAA is not responsible for the changes in mobility. Interestingly, the bodipy-C5 probe, which is essentially a single-chain amphiphilic probe, showed much more rapid diffusion than the double-chain diI. PEAA adsorption induced both a decrease in the diffusion of bodipy-C5 and the appearance of a nondiffusing fraction. It should be noted, however, that, a "nondiffusing fraction" may be merely differing at a very slow rate, e.g., 1 order of magnitude or more slower than the "diffusing fraction".

Fluorescence Anisotropy. Lastly, we report measurements of the steady-state fluorescence anisotropy of three different probes, Figure 6. Diphenylhexatriene, DPH-phosphatidylcholine, and pyrene-PC were incorporated into DPPC membranes. The fluorescence anisotropy of each of these probes shows a biphasic response to acidification in the presence of PEAA, first increasing slightly as the pH is lowered to ca. pH 7 and then decreasing as the pH is lowered further. These results indicate greater probe (rotational) mobilities at pH below 7, in contrast to the excimer and FPR measurements, which indicated lesser probe mobilities under those conditions.

Discussion

Inhibition of the formation of pyrene excimers has been observed in many membranes with integral or adsorbed proteins, including α-lactalbumin,[23] Ca^{2+}- and Mg^{2+}-ATPase,[28] annexins and polylysine,[11] the cytochrome b_5.[33] Reduction in intermolecular excimer formation does *not* require changes in lateral mobility of the probe, unless it can be demonstrated that excimer formation is diffusion-limited in all probe environments. For the Ca^{2+}- and Mg^{2+}-ATPase, Jones and Lee demonstrated that excimer formation was inhibited in so-called "annular" sites, independent of changes in probe lateral diffusion.[28] In our study, the parallel reductions in intramolecular bis-pyrene excimer formation and intermolecular pyrene excimer formation suggest that changes in lateral diffusion need not be invoked to account for reduced excimer formation. Instead, probe molecules that are in contact with the adsorbed polymer may be unable to form excimers.

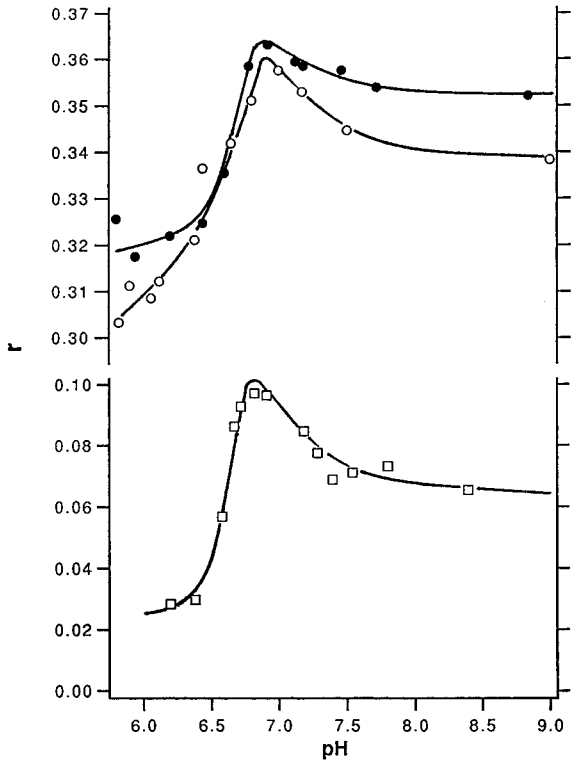


Figure 6. Fluorescence anisotropy of DPH (○), DPH-PC (●), and Py-PC (□) in DPPC membranes with adsorbed polymer. Samples were prepared and measured as described in Materials and Methods. In the absence of polymer, measured anisotropies were the same as those in the presence of polymer at pH >8.0.

The formation of bis-pyrene excimers in the gel phase shows unusual behavior: Before the first heating, excimer formation is actually enhanced by the addition of PEAA and the apparent activation energy is decreased. After heating, excimer formation is reduced, and the apparent activation energy is increased. These changes result from an irreversible change in the polymer−membrane complex that occurs on heating through T_m, as confirmed by observing the relative intensity changes of the vibronic bands of pyrene, Figure 2. The changes in the pyrene III/I ratio indicate that the pyrene molecules experience a more hydrophobic environment after polymer−membrane complexation at T_m.

The changes in excimer formation in the gel phase are most likely, as with the liquid crystal phase, to be due to local interactions with the polymer. However, the significantly increased activation energy in the polymer−complexed gel membranes implies that excimer formation occurs predominantly in the vicinity of the polymer *and not in the bulk lipid*. This will occur if the bis-pyrene tends to localize to the polymer-

disrupted regions of the membrane. This hypothesis is quite reasonable: In the gel phase, the bis-pyrene is itself a defect and will prevent proper local crystallization of the DMPC acyl chains. Exclusion of both bis-pyrene and PEAA to domain boundaries will permit more energetically favorable van der Waals contact between the lipids.

The enhancement of excimer formation on initial introduction of polymer at low temperature is perhaps the most intriguing observation. Under these conditions, no change in the pyrene probe environment is evident in the vibronic band structure, yet the dynamics of the bis-pyrene probe are significantly altered. We speculate that these changes are due to superficial adsorption of polymer, especially adsorption to defects that must be created by the bis-pyrene. (That bis-pyrene significantly pertubs the local environment in the gel phase has been deduced by Zachariasse, Kühnle, and Weller from estimates of the enthalpy of the T_m transition in the presence of the probe.[16]) Such superficial PEAA adsorption must be qualitatively different from adsorption above T_m, in order to promote, rather than suppress, excimer formation. The difference may be that the polymer is less able to penetrate into the hydrophobic region of the bilayer below T_m: PEAA modified with a pendant dansyl fluorophore does not report a hydrophobic environment for the adsorbed polymer until the temperature is raised to near T_m.

It is interesting that the structural transition from a lamellar phase to a mixed micelle does not, in itself, alter probe mobility. The formation of excimers is inhibited (after a first heating of the sample) beginning at pH 7, well above the critical pH for the lamellar to micellar conversion, pH 6.5. Excimer formation decreases continuously as pH is lowered throughout the transition region. The decrease is not due to the reorganization of some fraction of the vesicles, since both turbidity measurements and quasi-elastic light-scattering measurements indicate a sharp (<0.1 pH unit) transition from vesicle to micelle.[34,35] These results emphasize that the formation of excimers, even intermolecular excimers, is a *local* phenomenon on the length scales we are considering: The mixed micelle is large enough to contain several pyrene molecules.

In summary, the reduction of excimer formation by the adsorption of PEAA may simply reflect inhibition in the immediate vicinity of the polymer. While local polymer−probe interactions may also hinder probe diffusion, we cannot draw that conclusion from the excimer data alone. Lastly, PEAA shows distinctly different effects on bis-pyrene excimer formation before and after cycling through T_m, first *increasing* the excimer yield but then decreasing it after a melting of the bilayer permits greater hydrophobic penetration by the polymer.

Long-Range Diffusion. While changes in "microviscosity" are difficult to ascertain with the excimer-forming probes, changes in long-range lateral diffusion of fluorescent probes are clearly seen with the fluorescence photobleaching technique. PEAA impedes the long-range diffusion of the lipophilic probes diI and bodipy-C5. Because both a cationic (diI) and an anionic (bodipy-C5) probe showed retarded diffusion, a charge interaction is not likely to be responsible for these effects. A reduction in long-range lateral diffusion rates can be anticipated simply from the presence of the polymer as an "obstacle" in the membrane. If PEAA acts as an impenetrable obstacle, then long-range diffusion requires that the probe molecules diffusively circumnavigate the polymer chain. Even if probe molecules can diffuse across polymeric chains, the free energy of a probe in contact with a chain is certainly different from that of a probe in the lipid "bulk", owing to differing enthalpic and entropic interactions with the polymer versus with lipids. This potential (regardless of whether it is attractive or repulsive) will hinder diffusion. Similar reductions in diffusion have been observed with monomeric tracers in partially polymerized membranes and have been analyzed with Monte Carlo methods.[36]

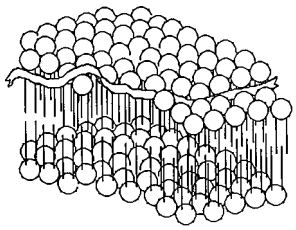


Figure 7. Hypothetical localization of adsorbed polymer near the glycerol-backbone region of the membrane. This localization would hinder the long-range diffusion of amphiphilic probes and could inhibit pyrene excimer formation while expanding and fluidizing the more hydrophobic part of the bilayer.

Fluorescence Anisotropy. In contrast to the excimer experiments, fluorescence anisotropy measurements show substantial fluidization of gel phase membranes on adsorption of PEAA below pH 7. While some of the changes in anisotropy may be due to variation in fluorescence lifetimes, this factor is unlikely to significantly modify our conclusions. First, and most importantly, time-resolved studies have shown that the rotational correlation times of these probes in membranes are typically smaller than the probe lifetimes (even for DPH), so that steady-state fluorescence depolarization is primarily a measure of the accessible probe orientations.[37] In other words, fluorescence depolarization in membranes measures the *range* and not the *rate* of the rotational motion of the probe. In light of this fact, it is not surprising that we have found qualitatively similar results with DPH, which has a typical lifetime of <10 ns, and pyrene-PC, which has a lifetime of about 200 ns. Finally, a measurement of the fluorescence lifetime of PyPC in polymer-treated vesicles showed <20% variation over the entire pH range of our measurements,[24] which could not account for depolarization changes of a factor of 4, even in an isotropic system.

Several proteins have also been observed to have a disorganizing or fluidizing effect on membranes, as measured by fluorescence anisotropy.[10,38] The discrepancy between excimer formation and anisotropy measurements may be due in part to differences in probe localization in the membrane. The anisotropy probes, especially DPH and DPH-PC, tend to localize to the most hydrophobic part of the membrane (as determined by energy transfer experiments[39]), while the pyrene probes prefer an environment near the glycerol backbone of the phospholipids, estimated from the vibronic band intensities.[28] The small amphipathic mers of PEAA will be most favorably situated when they are near the steepest polarity gradient, i.e., near the glycerol backbone. This localization will promote the direct interaction of the polymer with the pyrene probes. In addition, polymer may crowd this section of the membrane while expanding and fluidizing the deeper, more hydrophobic layers, Figure 7.

Changes in pyrene vibronic structure are consistent with preferential polymer adsorption at the glycerol-

backbone layer of the membrane. The more hydrophobic environment of pyrene on PEAA adsorption may be due to a displacement of water from the glycerol-backbone region by adsorbed polymer or the displacement of some pyrene to deeper, more hydrophobic sites in the membrane.

Similar results have been obtained with biological membranes. Oleic and linoleic fatty acids, when added exogenously to lymphocytes, fluidize the plasma membranes, as judged by DPH depolarization. These compounds do not increase fluidity measured by either pyrene excimer formation or FPR.[40] The drug tamoxifen increases DPH depolarization in gel phase lipids but inhibits bis-pyrene excimer formation in the liquid crystal phase and has no effect on excimer formation in the gel phase.[41]

Conclusions

The results presented in this paper indicate that the adsorption of a hydrophobic polyelectrolyte alters the mobility of lipophilic fluorescent probes incorporporated into the bilayer. Amphipathic probes report hindered long-range lateral mobility, and excimer-forming probes report an increased effective local viscosity, probably due to direct interactions with adsorbed polymer chains. These probes all sample the more hydrophilic molecular domains of the phospholipid bilayer. Probes of the most hydrophobic regions of the bilayer, such as DPH, DPH-PC, and Py-PC, show enhanced rotational mobility on polymer adsorption. The different effects on these probes illustrate the lack of correlation between fluorescence depolarization and lateral diffusion, previously noted by Kleinfeld et al.[40] Moreover, the different localization of the probes suggests that the cause of that lack of correlation may be due to differential effects on different layers or regions in the membrane. It must be noted, however, that because fluorescence depolarization in membranes principally measures the accessible angles through which the fluor can rotate (rather than a tumbling rate, as in isotropic solutions), it remains primarily a static measure of membrane order. The differences observed between fluorescence depolarization and lateral diffusion measurements may simply reflect the differences between static and dynamic measurements of membrane order. A definitive test of these ideas will require simultaneous measurement of diffusion and depolarization of the same probe molecule.

Lastly, the mobility changes we have observed begin at pH's significantly higher than required for membrane micellization and vary continuously throughout the vesicle to micelle transition. Consequently, these changes must be due to interactions between polymer and probe and not to any specific properties of the larger structure of which those molecules are a part. Modulating these local molecular motions could provide a mechanism for reaction rate control in synthetic and natural membrane systems.

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References and Notes

(1) Galla, H.-J.; Hartmann, W. *Chem. Phys. Lipids* **1980**, *27*, 199−219.
(2) Grainger, D. W.; Reichert, A.; Ringsdorf, H.; Salesse, C. *FEBS Lett.* **1989**, *252*, 73−82.
(3) Scott, D. L.; White, S. P.; Otwinowski, Z.; Yuan, W.; Gelb, M. H.; Sigler, P. B. *Science* **1990**, *250*, 1541−1546.
(4) Salesse, C.; Lamarche, F.; Leblanc, R. M. *Biochem. Cell Biol.* **1989**, *67*, 422−427.
(5) Garda, H. A.; Brenner, R. R. *Biochim. Biophys. Acta* **1984**, *769*, 160−170.
(6) Schneider, H.; Hoechli, M.; Hackenbrock, C. R. *J. Cell Biol.* **1982**, *94*, 387−393.
(7) Beeler, T. J.; Gable, K. S.; Keffer, J. M. *Biochim. Biophys. Acta* **1983**, *734*, 221−234.
(8) Robertson, D.; Holowka, D.; Baird, B. *J. Immunol.* **1986**, *136*, 4565−4572.
(9) Fajer, P.; Knowles, P. F., Marsh, D. *Biochemistry* **1989**, *28*, 5634−5643.
(10) Lentz, B. R.; Moore, B. m.; Kirkman, C.; Meissner, G. *Biophys. J.* **1982**, *37*, 30−32.
(11) Meers, P.; Daleke, D.; Hong, K.; Papahadjopoulos, D. *Biochemistry* **1991**, *30*, 2903−2308.
(12) Thomas, J. L.; Barton, S. W.; Tirrell, D. A. *Biophys. J.* **1994**, *67*, 1101−1106.
(13) Tirrell, D. A.; Takigawa, D. Y.; Seki, K. *Ann. N. Y. Acad. Sci.* **1985**, *446*, 237−248.
(14) Ferritto, M. S.; Tirrell, D. A. *Macromol. Synth.* **1992**, *11*, 59−62.
(15) Hope, M. J.; Bally, M. B.; Webb, G.; Cullis, P. R. *Biochim. Biophys. Acta* **1985**, *812*, 55−65.
(16) Zachariasse, K. A.; Kuhnle, W.; Wheeler, A. *Chem. Phys. Lett.* **1980**, *73*, 6−11.
(17) Lakowicz, J. R. *Principles of fluorescence spectroscopy*; Plenum: New York, 1983; pp 496.
(18) McConnell, H. M.; Watts, T. H.; Weis, R. M.; Brian, A. A. *Biochim. Biophys. Acta* **1986**, *864*, 95−106.
(19) Axelrod, D.; Koppel, D.; Schlessinger, J.; Elson, E.; Webb, W. *Biophys. J.* **1976**, *16*, 1055−1069.
(20) Thomas, J. L.; Webb, W. W. In *Noninvasive techniques in cell biology*; Foskett, J. K., Grinstein, S., Eds.; Wiley-Liss: New York, 1990; pp 129−152.
(21) Yguerabide, J.; Schmidt, J.; Yguerabide, E. *Biophys. J.* **1982**, *39*, 69−75.
(22) Atkinson, D.; Small, D. M. *Annu. Rev. Biophys. Biophys. Chem.* **1986**, *15*, 403−456.
(23) Dangreau, H.; Joniau, M.; De Cuyper, M.; Hanssens, I. *Biochemistry* **1982**, *21*, 3594−3598.
(24) Borden, K. A. Ph.D. Dissertation Thesis, University of Massachusetts at Amherst, MA, 1989.
(25) Melnick, R. L.; Haspel, H. C.; Goldenberg, M.; Greenbaum, L. M.; Weinstein, S. *Biophys. J.* **1981**, *34*, 499−515.
(26) Kalyanasundaram, K.; Thomas, J. K. *J. Am. Chem. Soc.* **1977**, *99*, 2039−2044.
(27) Borden, K. A.; Eum, K. M.; Langley, K. H.; Tan, J. S.; Tirrell, D. A.; Voycheck, C. L. *Macromolecules* **1988**, *21*, 2649−2651.
(28) Jones, O. T.; Lee, A. G. *Biochemistry* **1985**, *24*, 2195−2202.
(29) Yechiel, E.; Edidin, M. *J. Cell Biol.* **1987**, *105*, 755−760.
(30) Fahey, P. F.; Webb, W. W. *Biochemistry* **1978**, *17*, 3046−3053.
(31) Derzko, Z.; Jacobson, K. *Biochemistry* **1980**, *19*, 6050−6057.
(32) Klausner, R. D.; Wolf, D. E. *Biochemistry* **1980**, *19*, 6199−6203.
(33) Freire, E.; Markello, T.; Rigell, C.; Holloway, P. W. *Biochemistry* **1983**, *22*, 1675−1680.
(34) Borden, K.; Eum, K. M.; Langley, K. H.; Tirrell, D. A. *Macromolecules* **1987**, *20*, 454−456.
(35) Eum, K. M. Ph.D. Thesis, University of Massachusetts, Amherst, MA, 1988.
(36) Eggl, P.; Pink, D.; Quinn, B.; Ringsdorf, H.; Sackmann, E. *Macromolecules* **1990**, *23*, 3472−3480.
(37) van Langen, H.; van Ginkel, G.; Levine, Y. K. *Chem. Phys.* **1989**, *130*, 271−278.
(38) Kunz, B. C.; Rehorek, M.; Hauser, H.; Winterhalter, K. H.; Richter, C. *Biochemistry* **1985**, *24*, 2889−2895.
(39) Davenport, L.; Dale, R. E.; Bisby, R. H.; Cundall, R. B. *Biochemistry* **1985**, *24*, 4097−4108.
(40) Kleinfeld, A. M.; Dragsten, P.; Klausner, R. D.; Pjura, W. J.; Matayoshi, E. D. *Biochim. Biophys. Acta* **1981**, *649*, 471−480.
(41) Custodio, J. B.; Almeida, L. M.; Madeira, V. M. *Biochim. Biophys. Acta* **1993**, *1150*, 123−129.

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