(28) White, G. K.; Smith, T. F.; Birch, J. A. *J. Chem. Phys.* **1976**, *65*, 554.
(29) Dainton, F. S.; Evans, D. M.; Hoare, F. E.; Melia, T. P. *Polymer* **1962**, *3*, 263.
(30) Chiba, A.; Hasegawa, A.; Hikichi, K.; Furuichi, J. *J. Phys. Soc. Jpn.* **1966**, *21*, 1777.
(31) Kobayashi, M.; Morishita, H.; Shimomura, M.; Iguchi, M. *Macromolecules* **1987**, *20*, 2453.

Structural Reorganization of Phosphatidylcholine Vesicle Membranes by Poly(2-ethylacrylic acid). Influence of the Molecular Weight of the Polymer

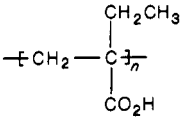
Ulrich K. O. Schroeder and David A. Tirrell*

Department of Polymer Science and Engineering, University of Massachusetts, Amherst, Massachusetts 01003. *Received March 22, 1988; Revised Manuscript Received March 25, 1988*

ABSTRACT: Three samples of poly(2-ethylacrylic acid) (PEAA) of different molecular weights ($\bar{M}_w$ = 164 000, 43 000, and 12 000) were prepared by radical polymerization of 2-ethylacrylic acid in bulk. The conformational properties of the samples were determined and correlated with the pH-dependent structural reorganization observed in phosphatidylcholine vesicles suspended in the respective polymer solutions. In all experiments the sample of lowest molecular weight behaved differently from the other two: The cooperativity of the conformational transition was reduced and the transition midpoint was shifted to lower pH as compared to the samples of higher molecular weight. These effects of polymer molecular weight were reflected in the behavior of the vesicle-to-micelle transition observed upon acidification of aqueous mixtures of PEAA and dipalmitoylphosphatidylcholine.

Introduction

The pH-dependent conformational transition of poly(2-ethylacrylic acid) (PEAA, **1**) has been used to sensitize

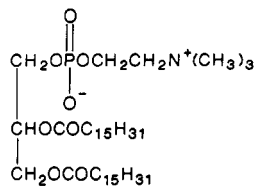


1

synthetic bilayer membranes to changes in pH,[1] temperature,[2] light intensity,[3] and solute (e.g., glucose) concentration.[4] The mechanism of the membrane response has been shown to consist of a structural reorganization of the membrane lipid from a vesicular form at high pH to a mixed polymer–lipid micelle at low pH, the reorganization being driven by collapse of the polymer chain from an extended, hydrophilic form to a compact, hydrophobic coil upon acidification.[5]

The use of macromolecules to effect molecular switching in synthetic bilayer membranes offers substantial advantages in membrane design. One would anticipate, for example, that the pH-dependent conformational transition of PEAA[6-8] would be subject to modulation by variation in polymer chain structure (e.g., in tacticity or molecular weight) and that one might exploit such variations to adjust either the "critical" pH for membrane regorganization[1] or the cooperativity of the structural transition.

In our previous work on the interaction of PEAA with dipalmitoylphosphatidylcholine (DPPC, **2**), we prepared



2

polymer samples that spanned a range of tacticities from 91% isotactic triads to 88% syndiotactic triads, and we found that there were indeed useful shifts in critical pH with changes in chain configuration.[1] But the preparation of these samples required the use of a variety of polymerization methods, and variations in tacticity were accompanied by large differences in polymer molecular weight. Furthermore, the cooperativity of the conformational transition should be molecular weight dependent, so that the shape of the polymer-driven vesicle-to-micelle transition might be subject to control. We describe herein an investigation of the effects of polymer molecular weight on the structural reorganization of phosphatidylcholine vesicle membranes by poly(2-ethylacrylic acid).

Table I
Radical Polymerization of 2-Ethylacrylic Acid

sample	[AIBN], mol %	temp, °C	time, h	conv, %	$\bar{M}_w$	$\bar{M}_n$	$\bar{M}_w/\bar{M}_n$
P164	0.05	50	96	22	164 000	84 000	1.96
P43	0.50	60	22	24	43 000	22 000	1.96
P12	5.00	70	4.75	25	12 000	6 500	1.85

Experimental Section

Materials. Diethyl ethylmalonate, formaldehyde solution (37% w/w in water), diethylamine, and pyrene were obtained from Aldrich Chemical Co. and used without further purification. Azobis(isobutyronitrile) (AIBN) was purchased from Aldrich and recrystallized from methanol. Synthetic L-α-dipalmitoylphosphatidylcholine (approximately 99%) was obtained from Sigma Chemical Co. and used without further purification. Cellulose dialysis tubing (Spectra/Por 6, molecular weight cutoff 1000) was purchased from Fisher Scientific Co.

Polymerization. 2-Ethylacrylic acid (EAA) was synthesized from diethyl ethylmalonate by the method described earlier.[9] The monomer was distilled twice (bp = 48 °C (0.8 mm Hg)) and transferred to ampules [10 g (0.1 mol) per ampule]. After adding AIBN each reaction mixture was degassed by three freeze–thaw cycles and each ampule was sealed under vacuum. Various polymerization temperatures and AIBN concentrations were chosen (cf. Table I) in order to obtain samples of different molecular weights. The reaction mixtures were then diluted with diethyl ether and filtered, and the recovered polymer was washed with ether and dried under vacuum. In order to remove residual monomer the polymer samples were suspended in water and

0024-9297/89/2222-0765$01.50/0

dialyzed against water for 4 days in cellulose dialysis tubes with a molecular weight cutoff of 1000.

Molecular Weight Determination. Molecular weights were determined relative to poly(ethylene oxide) (PEO) by gel permeation chromatography (GPC) with a set of two TSK columns (TSK 3000 PW, TSK 5000 PW) and a differential refractometer. Calibration was done using nine PEO samples of narrow molecular weight distribution (Toyo Soda Mfg. Co.) with average molecular weights in the range from monomer up to 10^6. The polymer concentration was about 0.2 wt % in a phosphate buffer solution (0.034 M, pH 8) that contained 0.3 M NaCl. This solvent was used in order to suppress coil expansion.[10] A similar solvent system with a pH of 11 gave the same results.

Vesicle Preparation. A solution of 75 mg of DPPC in 10 mL of $CHCl_3$ was evaporated to dryness on a rotary evaporator. After drying under vacuum the DPPC film was hydrated in 15 mL of deionized H_2O and vortexed at 50 °C for about 10 min.

Measurements. Calorimetric scans were recorded on a Microcal MC-1 scanning calorimeter at a heating rate of 15 °C/h. Solutions of PEAA in phosphate buffer and suspensions of DPPC (hydrated as described above) were mixed so that the final concentrations of PEAA and DPPC were 1 mg/mL each. The mixture was then heated to 50 °C for 10–15 min. Ionic strength (I) was the same for the solutions at pH 6 and higher (I = 0.16 M); for the samples of pH 5.9 and 5.75 I was 0.18 and 0.20 M, respectively. Reheating of DSC samples afforded thermograms nearly identical with those obtained on the first run.

Optical densities (OD) of samples that contained phospholipid were measured on a Beckman DU-7 spectrophotometer at λ = 600 nm. Samples were prepared in the manner described above for calorimetric experiments.

Samples for fluorescence measurements were prepared as described above for turbidimetric and calorimetric experiments. Measurements were done on lipid-free polymer solutions as well as on samples containing lipid. Pyrene (5×10^{-6} M in the sample) was used as probe; it was introduced subsequent to sample preparation as a 10^{-3} M solution in acetone. Emission spectra were recorded on a Perkin-Elmer MPF-66 fluorescence spectrophotometer at room temperature (excitation at λ = 337 nm, excitation and emission slit widths were 1 and 2 nm, respectively). Intensities of emission peaks I (at 373 nm) and III (at 384 nm) were determined.

Results and Discussion

Sample Preparation. Radical bulk polymerization of 2-ethylacrylic acid (EAA) was carried out under a variety of reaction conditions in order to obtain a set of PEAA samples of $\bar{M}_w$ in the range 12 000–164 000. The results of these polymerization experiments are summarized in Table I, and the molecular weight distributions of the three polymer samples used in this work are shown in Figure 1. Despite the polydispersities of the samples ($\bar{M}_w/\bar{M}_n$ = 1.8–2.0), these materials provided us a useful range of molecular weights, as illustrated in the following sections.

Several alternatives to simple thermal polymerization in bulk were explored. Attempted solution polymerizations of EAA, either in methanol or in 2-propanol at 60 °C with AIBN, failed to provide polymer. Polymerization in bulk at 1.5 °C with initiation by photoinduced decomposition of AIBN (0.25–1.0 mol %) did not provide higher molecular weights than those attained by thermal initiation; furthermore, polymer yields were reduced and the molecular weight distributions of the products were bimodal.

The ethyl ester of the acid failed to polymerize with radical initiation either in toluene solution (60 °C, AIBN) or in bulk (−78 °C, AIBN, UV light). This behavior is probably a result of a low ceiling temperature (T_c) for ethyl 2-ethylacrylate; with increasing length of both the ester and α-alkyl groups, the equilibrium monomer concentration may be raised sufficiently to preclude reasonable polymer yields at moderately low polymerization temperatures. This kind of ceiling temperature depression is well-known and is illustrated, for example, by the monomer pairs styrene/α-methylstyrene and methyl methacrylate/ethyl methacrylate. In the former pair, α-substitution depresses T_c by about 250 °C;[11] for the latter, an estimate of ceiling temperatures based on the heats and entropies of polymerization[12] suggests that extension of the ester side chain should reduce T_c by approximately 20 °C. Methyl 2-ethylacrylate appears to suffer from a low ceiling temperature, as indicated by the need to carry out anionic polymerizations at temperatures below 0 °C and the low yields of polymers from those reactions.[1,13] Additional depression of T_c for the ethyl ester seems likely.

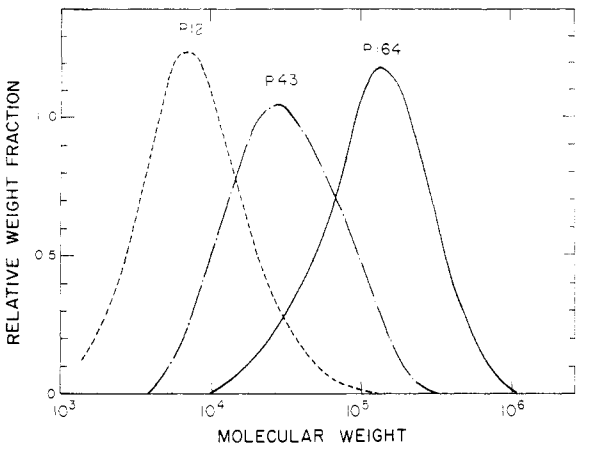


Figure 1. Molecular weight distributions of PEAA samples P12, P43, and P164 (areas under the curves are normalized).

Steady-State Fluorescence. The pH-dependent conformational transition of PEAA is readily monitored by observation of the steady-state fluorescence of codissolved pyrene.[5] Pyrene is commonly used as a probe of environmental polarity, because its emission intensity and vibronic band structure are sensitive to solvation.[14] Chen and Thomas have demonstrated the use of pyrene to examine the conformational states of poly(methacrylic acid) in water,[15] and our own results for PEAA, reported previously,[15] are consistent with theirs. In each case, the transition from an expanded, hydrophilic coil at high pH to a compact, globular structure in acidic solutions is manifested by increases in the total emission intensity and in the ratio of intensities emitted at 384 nm (commonly designated as peak III) and 373 nm (peak I). Figure 2 shows typical emission spectra for pyrene dissolved in a series of PEAA solutions of pH 5.75–7.00.

In Figure 3 are plotted the intensities of peaks I and III and the III/I intensity ratio as functions of pH, for aqueous solutions of three samples of PEAA of different molecular weights. In each sample, the emission intensity rises sharply as the pH is depressed; the inflection points in the intensity curves lie at pH 6.00 ± 0.02 for the sample of $\bar{M}_w$ = 12 000 (P12) and at pH 6.08 ± 0.02 for the samples of higher molecular weight (P43 and P164). There is little separation between the intensity curves for the latter samples. When the III/I intensity ratio is plotted as a function of pH (Figure 3c), the transition midpoints are shifted slightly to higher pH (as would be expected if each of the intensity transitions shows a cosh dependence on pH) and some separation of the behavior of P43 and P164 is realized. Regardless of the form in which the transition is represented, a small increase in cooperativity with increasing molecular weight is apparent.

After addition of DPPC, the emission intensity curves look quite similar (Figure 4a,b), though in each case, the transition is shifted by about 0.5 units to higher pH by addition of the lipid. This observation is in excellent agreement with our previous work on the interaction of

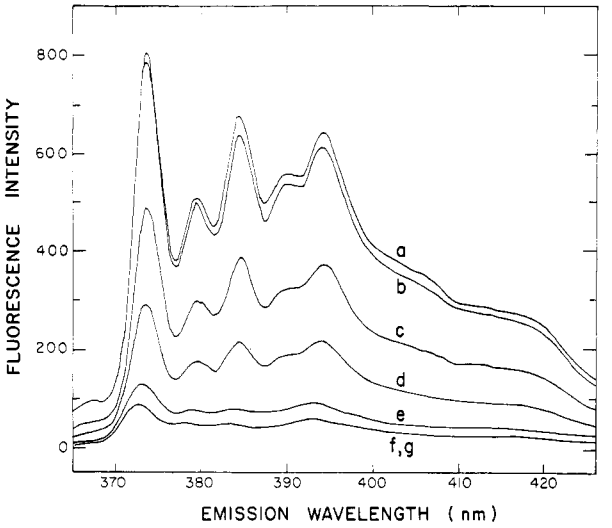


Figure 2. Fluorescence emission spectra of pyrene in presence of PEAA sample P164 in phosphate buffers of pH 5.75 (a), 5.99 (b), 6.07 (c), 6.15 (d), 6.26 (e), 6.46 (f), and 7.01 (g) (I = 0.20 M in (a), 0.16 M otherwise).

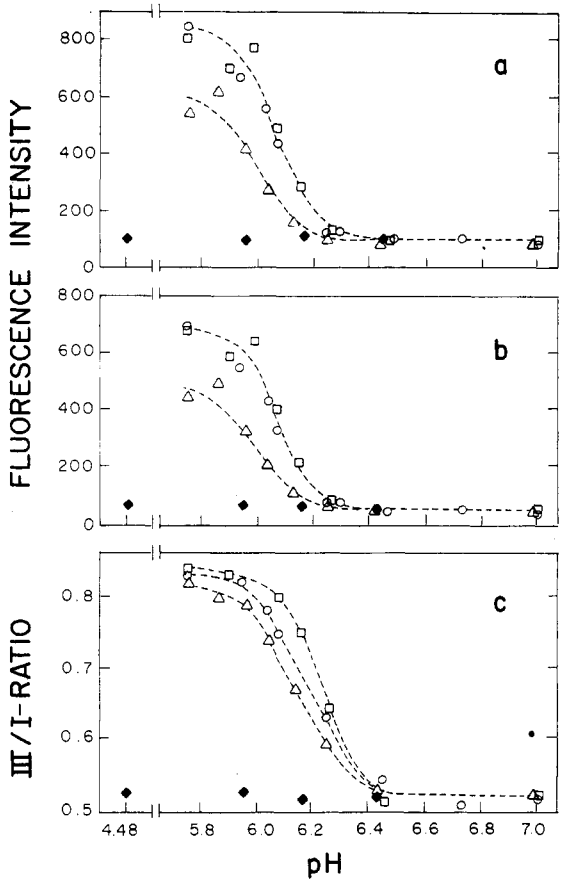


Figure 3. Fluorescence intensities at 373 nm (peak I, a) and at 384 nm (peak III, b) from pyrene dissolved in phosphate-buffered solutions of PEAA. (c) The ratio of intensities emitted at 384 and 373 nm: (□) P164; (○) P43; (△) P12; (◆) polymer-free control. Estimated errors in intensities are ≤10%, and arise largely from uncertainties in pyrene concentration. Errors in the III/I ratio are estimated to be of the order of the size of the symbols.

PEAA and DPPC as reported by a *polymer-bound* pyrene chromophore,[5] and the correspondence of results obtained with polymer-bound and exogenous probes lends credibility to each experiment. Our interpretation of the pH shift is discussed in the following section.

Addition of DPPC causes marked changes in the pH dependence of the III/I ratio (Figure 4c). At high pH, the

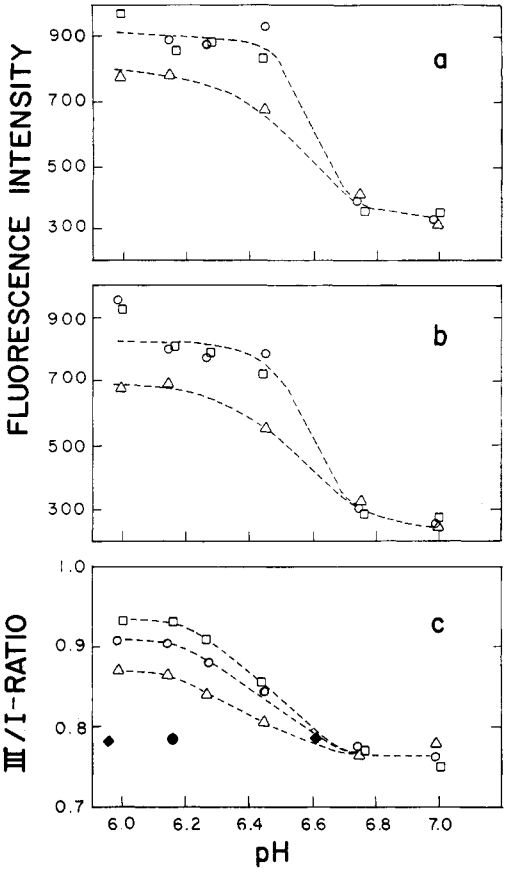


Figure 4. Fluorescence intensities at 373 nm (peak I, a) and at 384 nm (peak III, b) from pyrene dissolved in phosphate-buffered solutions of PEAA and DPPC. (c) The ratio of the intensities emitted at 384 and 373 nm: (□) P164; (○) P43; (△) P12; (◆) polymer-free control. Estimated errors are ±10% in the intensities and ±0.01 in the III/I ratio.

III/I ratio is considerably larger than that observed in lipid-free solutions of PEAA and is in fact rather similar to that characteristic of polymer-free DPPC suspensions. The ratio of peak intensities is independent of the molecular weight of the added PEAA at high pH. The most logical interpretation is that the pyrene probe is solubilized within the lipid membrane under conditions where the polymer chain is ionized and well solvated in the aqueous medium. As the pH is reduced to below 6.6, there is a modest but significant increase in III/I, which reaches a final value in the range 0.88–0.92. This final III/I ratio shows a mild dependence on polymer molecular weight but is generally in the range characteristic of pyrene solubilized in micellar surfactants.[14] The rise in III/I upon acidification of DPPC/PEAA mixtures signals a decrease in the polarity of the environment sampled by the pyrene probe, and the molecular weight dependence of the final III/I ratio suggests that chains of high molecular weight form mixed aggregates with DPPC that exclude water more effectively than those formed from shorter chains.

Turbidity. The reorganization of DPPC from vesicular aggregates into mixed polymer–lipid micelles is accompanied by large changes in turbidity, as a result of the decrease in average aggregate size.[5] Figure 5 shows the pH dependences of the turbidities of 1:1 (by weight) aqueous mixtures of DPPC with each of the three samples of PEAA examined in this work. Polymer-free DPPC suspensions are uniformly turbid over the pH range 6.0–7.1. In contrast, each of the polymer–lipid mixtures clarifies upon acidification, with transition midpoints found at pH 6.66 ± 0.02 for P164, pH 6.63 ± 0.02 for P43, and pH 6.47 ±

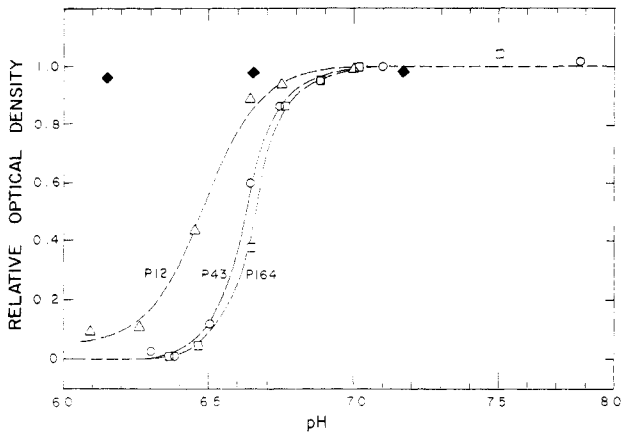


Figure 5. Optical density (relative to optical density at pH 7.0) of preheated 1:1 DPPC/PEAA mixtures in aqueous phosphate buffer solutions: (□) P164; (○) P43; (△) P12; (◆) polymer-free control. Estimated errors are about ±0.02 in relative OD and in pH.

0.02 for P12. Similar experiments on samples that contained 5×10^{-6} M pyrene gave nearly identical results, although in each case the transition midpoint was shifted by about 0.1 unit to lower pH.

The combined results of fluorescence and turbidimetric measurements provide a consistent picture of the polyelectrolyte-induced vesicle-to-micelle transition. The transition midpoints determined by the two techniques are nearly identical and suggest that the overall change in aggregate morphology is accompanied by an abrupt change in solvation (and probably conformation) of the polymer chain. The fact that these transitions occur at higher pH than the collapse of the free chain (ca. pH 6.6 versus pH 6.1) argues that adsorption must precede conformational collapse in a pseudoequilibrium experiment in which acidification is infinitely slow. As chain ionization is depressed further, desolvation of the polymer causes the bilayer aggregate to become unstable with respect to a mixed micelle, in which water–polymer (and water–pyrene) contacts are reduced. It is not a simple matter to deduce the chain conformation in the micellar aggregate.

Fluorescence and turbidity measurements are also consistent regarding the influence of polymer molecular weight on the position and character of the structural transition. Decreasing molecular weight shifts both the conformational transition and the structural reorganization to lower pH and spreads each transition along the pH axis. These effects are small in comparisons of the samples of $\bar{M}_w$ = 164 000 and $\bar{M}_w$ = 43 000 but readily apparent in the behavior of the polymer of lowest molecular weight ($\bar{M}_w$ = 12 000). Collapse of these shorter chains appears to be accompanied by diminished dehydration, as reported by smaller increments in pyrene fluorescence intensity at low pH (Figure 3), by smaller increases in the III/I ratio upon acidification (Figure 4), and by the incomplete clarification of acidic aqueous mixtures of DPPC and P12 (Figure 5).

Differential Scanning Calorimetry. The thermal phase transition observed in hydrated DPPC provides a sensitive probe of the state of order within the hydrocarbon core of the bilayer.[16] Figure 6 shows a series of calorimetric scans of DPPC–P43 mixtures hydrated in phosphate buffers of pH 6.30–7.10. At pH 7.10, the normal $L_{\beta'} \rightarrow P_{\beta'}$ and $P_{\beta'} \rightarrow L_\alpha$ transitions of DPPC are observed at 34.7 and 41 °C, respectively.[17] As the pH is depressed, a second endotherm emerges on the high-temperature side of the main melting peak, and at pH 6.74, the latter endotherm is clearly resolved at T = 42.5 °C. Further acidification is accompanied by growth of the high-T transition and

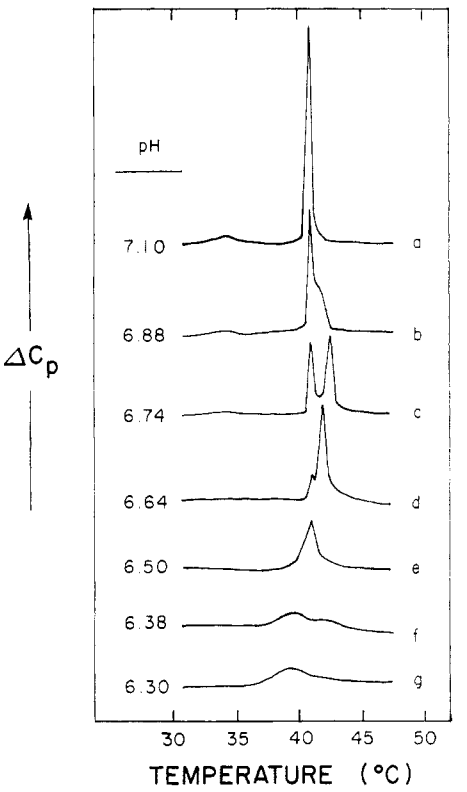


Figure 6. Calorimetric scans of 1:1 DPPC/PEAA (P43) mixtures in aqueous phosphate buffer solutions.

finally by its shift to lower temperature. Below pH 6.50, only a single, broad endotherm is observed. Although each of the scans shown in Figure 6 was recorded on a first heating of the sample, second runs were identical, with the exception of small increases in transition widths.

The occurrence of the second melting peak is a particularly interesting result. This transition was consistently observed in samples of pH ca. 6.75 and was found in suspensions prepared by a variety of protocols. We believe it to be real and to reflect the presence of two lipid populations—one (of T_m = 41 °C) unaffected in its melting behavior by PEAA and a second (of T_m = 42.5 °C) in which the ordered lipid phase is stabilized by polymer adsorption. Since it is known that dehydration of phosphatidylcholines increases T_m,[18] we suggest that the latter, i.e., higher melting, population is dehydrated by surface-bound PEAA. It is interesting to note that the dual melting behavior is observed under conditions where the turbidity of the sample is reduced only slightly, if at all (Figure 5). Further acidification then causes a loss of the cooperative thermal phase transition as the lipid is converted to mixed micellar form.

The general features shown in Figure 6 are common to the three polymer samples examined in this work. Figure 7 shows the temperatures of the peak maxima (T_m) and the transition widths at half-maximum ΔC_p ($\Delta T_{1/2}$), for each sample over the pH range 6.0–7.8. The calorimetric results are consistent with those of the fluorescence and turbidimetric experiments discussed previously; decreasing molecular weight within this series of samples shifts the structural transition to lower pH and spreads the transition along the pH axis. As before, the influence of molecular weight is most apparent in a comparison of the behavior of samples of $\bar{M}_w$ = 43 000 and $\bar{M}_w$ = 12 000.

Conclusions

The pH-dependent structural reorganization of phosphatidylcholine vesicle membranes suspended in aqueous

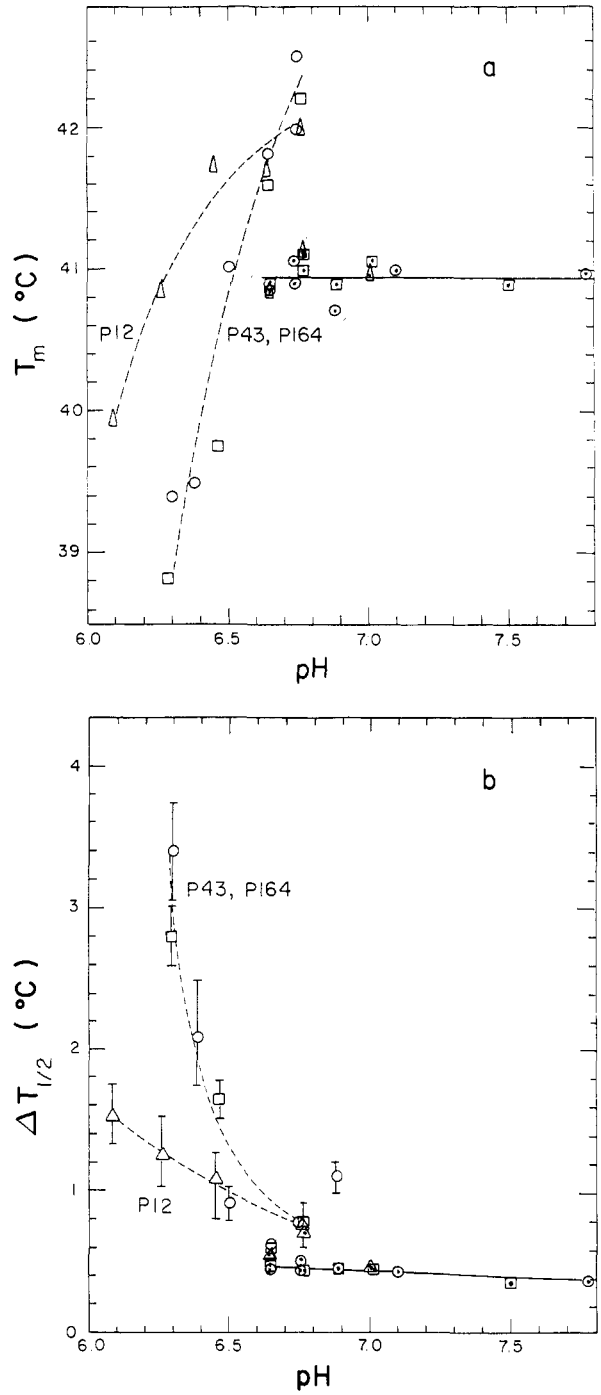


Figure 7. Melting temperature T_m (a) and transition half-width $\Delta T_{1/2}$ (b) of DPPC in aqueous phosphate buffer solutions in the presence of PEAA: (⊡, □) P164; (⊙, ○) P43; (▲, △) P12. Dotted symbols represent T_m for that portion of the thermogram that corresponds to the unshifted melting of DPPC. Estimated error in T_m is ±0.1 °C at pH >6.5 and slightly larger at lower pH because of peak breadth. Error bars on $\Delta T_{1/2}$ are given in the figure.

solutions of poly(2-ethylacrylic acid) is sensitive to the molecular weight of the polymer. Polymer samples of $\bar{M}_w$ = 164 000 and $\bar{M}_w$ = 43 000 behave nearly identically, in that each induces a sharp vesicle-to-micelle transition with a transition midpoint at pH 6.6. The polymer of $\bar{M}_w$ = 12 000 causes a broader structural transition centered at pH 6.5. Similar behavior is observed with regard to the conformational transition of PEAA in aqueous solution: The longer chains appear to collaspe in a more highly cooperative fashion and to produce globular coils at low pH that are more effective in excluding water from the portions of the chain interior that are sampled by a co-dissolved pyrene probe. Differential scanning calorimetry reveals a splitting of the lipid phase transition into two endothermic events at about pH 6.75 in aqueous PEAA solutions and suggests that polymer adsorption is accompanied by some degree of headgroup dehydration.

A final note concerns the origin of the apparently reduced cooperativity of the structural and conformational transitions observed in samples prepared from the PEAA sample of $\bar{M}_w$ = 12 000. Because our samples are not monodisperse, we cannot be certain that the reduced cooperativity is a result of low molecular weight per se. Although the polydispersities of our samples are similar, our results suggest that the effects of this dispersity should be most apparent in short chain lengths; thus the conformational and structural transitions may be spread along the pH axis at least in part as a result of the mixture of chain lengths that comprise sample P12. Nevertheless, the results reported herein provide a clear and consistent description of the influence of polymer molecular weight on the polyelectrolyte-induced structural reorganization of phosphatidylcholine vesicle membranes.

Acknowledgment. This work was supported by a grant from the U.S. Army Research Office (DAAL03-88-K-0038) and by a Presidential Young Investigator Award of the National Science Foundation. Financial support by the Deutsche Forschungsgemeinschaft for one of the authors (U.K.O.S.) is gratefully acknowledged.

Registry No. 1, 62607-09-4; 2, 2644-64-6.

References and Notes

(1) Seki, K.; Tirrell, D. A. *Macromolecules* **1984**, *17*, 1692.
(2) Tirrell, D. A.; Takigawa, D. Y.; Seki, K. *Ann. N. Y. Acad. Sci.* **1985**, *446*, 237.
(3) Ferritto, M. S.; Tirrell, D. A. *Polym. Prepr. (Am. Chem. Soc., Div. Polym. Chem.)* **1987**, *28*(1), 189.
(4) Devlin, B. P.; Tirrell, D. A. *Macromolecules* **1986**, *19*, 2465.
(5) Borden, K. A.; Eum, K. M.; Langley, K. H.; Tirrell, D. A. *Macromolecules* **1987**, *20*, 454.
(6) Sugai, S.; Nitta, K.; Ohno, N.; Nakano, H. *Colloid Polym. Sci.* **1983**, *261*, 159.
(7) Joyce, D. E.; Kuruscev, T. *Polymer* **1981**, *22*, 415.
(8) Fichtner, F.; Schönert, H. *Colloid Polym. Sci.* **1977**, *255*, 230.
(9) Ferritto, M. S.; Tirrell, D. A. *Macromol. Synth.*, in press.
(10) Kato, K.; Matsuda, T.; Hashimoto, T. *J. Chromatogr.* **1985**, *332*, 39.
(11) Lenz, R. W. *Organic Chemistry of Synthetic High Polymers*; Wiley: New York, 1967; p 329.
(12) *Polymer Handbook*, 2nd Ed.; Brandrup, J., Immergut, E. H., Eds; Wiley: New York, 1975; p II-421.
(13) Hatada, K.; Kokan, S.; Niinomi, T.; Miyaji, K.; Yuki, H. *J. Polym. Sci., Polym. Chem. Ed.* **1975**, *13*, 2117.
(14) Kalyanasundaram, K.; Thomas, J. K. *J. Am. Chem. Soc.* **1977**, *99*, 2039.
(15) Chen, T. S.; Thomas, J. K. *J. Polym. Sci., Polym. Chem. Ed.* **1979**, *17*, 1103.
(16) Mabrey, S.; Sturtevant, J. In *Methods in Membrane Biology*; Korn, E. D., Ed.; Plenum: New York, 1978; Vol. 9, p 237.
(17) We note that the $L_{\beta'} \rightarrow P_{\beta'}$ transition (T_p) in DPPC/PEAA mixtures appears to be shifted slightly to higher temperature even at pH 7.1 (i.e., T_p = 34.7 °C for the polymer–lipid mixture versus 34.2 °C for pure DPPC) even though the main melting temperature (T_m) is not yet shifted at all. Thus it may be that T_p is actually a more sensitive measure of polymer adsorption than is T_m. But because the small size and rather large width of the pretransition make accurate measurements of T_p difficult, we have chosen to focus on polyelectrolyte-induced changes in the main melting transition.
(18) Chapman, D.; Williams, R. M.; Ladbrooke, B. D. *Chem. Phys. Lipids* **1967**, *1*, 445.