Tuning the Response of a pH-Sensitive Membrane Switch

James L. Thomas,* Hong You, and David A. Tirrell*

Department of Polymer Science and Engineering, University of Massachusetts Amherst, Massachusetts 01003

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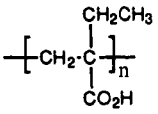
We have shown that the hydrophobic polyelectrolyte poly(2-ethylacrylic acid) (PEAA, **1**) causes destabilization of lipid bilayer membranes at pH 6.55 or lower and that this pH-sensitive



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response can be used to trigger release of hydrophilic compounds entrapped in liposomes.[1,2] Responsive membrane systems are finding increasingly important applications in drug delivery,[3−5] signal amplification in biochemical assays,[6−9] and molecular recognition.

To extend the versatility of the PEAA system, we have modified the critical pH for the onset of membrane destabilization. This has been achieved by copolymerizing 2-ethylacrylic acid (EAA) with methacrylic acid (MAA) to obtain copolymers of varying composition. That these copolymers would show behavior distinct from that of PEAA was anticipated on the basis of earlier work on poly(methacrylic acid), which shows no ability to solubilize lecithin membranes at any pH.[10] In fact, increasing MAA mole fraction leads to progressive reduction in the critical pH for membrane solubilization, which can be observed turbidometrically. This allows for "tuning" of the copolymer to any desired critical pH, from pH 5.7 to 6.5. Such tuning is useful in biological applications that require targeting to different intracellular destinations, which may maintain different degrees of acidity.[11,12] It is interesting to note that a variety of viral fusion proteins exhibit similar variations in their pH sensitivity, which may relate to a preferred point in the endocytic pathway for cytoplasmic entry.[13]

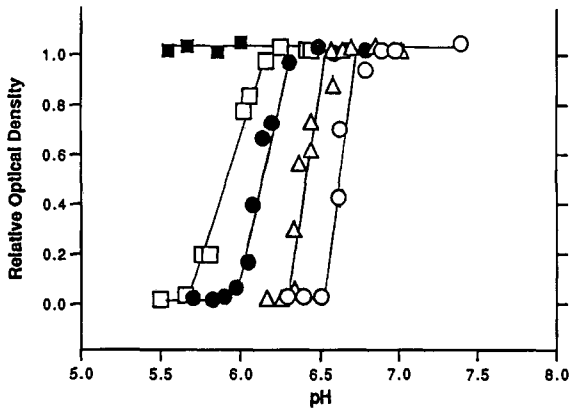

Figure 1. Optical densities (relative to optical density at pH 7.0) at λ = 500 nm of 1:1.5 (w/w) DPPC/copolymer mixtures in 50 mM phosphate buffer. Symbols ○, △, ●, □, and ■ refer to 100, 73, 58, 49, and 0 mol % EAA in the copolymer, respectively.

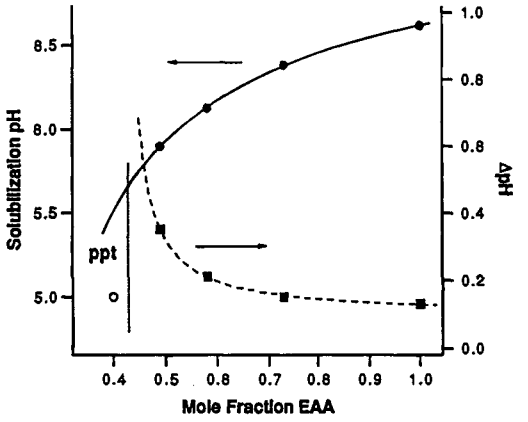

Figure 2. Shift in critical pH for vesicle solubilization with mole fraction EAA in copolymer. The critical pH is taken as the midpoint of the optical density transition. Precipitation and aggregation were observed with polymers containing 40 mol % or less EAA at reduced pH. Also plotted is the apparent width of the turbidometric transition, ΔpH, dashed line.

Figure 1 shows the relative optical densities (λ = 500 nm) of multilamellar preparations of DPPC suspended in aqueous solutions of PEAA or EAA/MAA copolymers, as functions of pH.[14,15] PEAA shows clarification of the DPPC suspension at pH 6.5 ± 0.1. Increasing the MAA content of the copolymers leads to a progressive reduction in the pH at which vesicle solubilization occurs and to an apparent increase in the breadth of the transition, Figure 2.

* Authors to whom correspondence should be addressed.

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(14) The syntheses of PEAA (Ferritto, M. S.; Tirrell, D. A. *Macromol. Synth.* **1992**, *11*, 59−62) and copolymers of EAA and MAA (You, H.; Tirrell, D. A. *J. Polym. Sci. Polym. Chem. Ed.* **1990**, *28*, 3155−3163) have been been described elsewhere. Polymers used in this work were prepared by radical polymerization in bulk. Molecular weights of the polymers were determined relative to poly(ethylene oxide) by gel permeation chromatography with three columns (TSK 3000 PW, TSK 5000 PW, and TSK 6000 PW) and a differential refractometer. Copolymer molecular weights were in the range 26 × 10^4 to ~47 × 10^4, the larger molecular weights being found with copolymers of greater MAA content.
(15) L-α-Dipalmitoylphosphatidylcholine (99%) (DPPC) and L-α-dimyristoylphosphatidylcholine (99+%) (DMPC) were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. For standard turbidity measurements, multilamellar vesicles (MLVs) of DPPC were first prepared by repeated vortexing of 2.5 mg/mL suspensions of DPPC in 50 mM aqueous sodium phosphate buffer, at 54×a1°C. Aliquots of 0.8 mL of the suspension were then mixed with 0.8 mL aliquots of a 3.75 mg/mL polymer solution in 50 mM phosphate buffer, and the pH was adjusted to stated values with 0.1 N HCl. Samples were held at 54 °C for 3 h. (Membrane solubilization by PEAA is known to occur at convenient rates only above the T_m of the phospholipid.[1]) Optical densities of the resulting suspensions were then measured at 28 °C on a Beckman DU-7 UV/vis spectrophotometer at λ = 500 nm.

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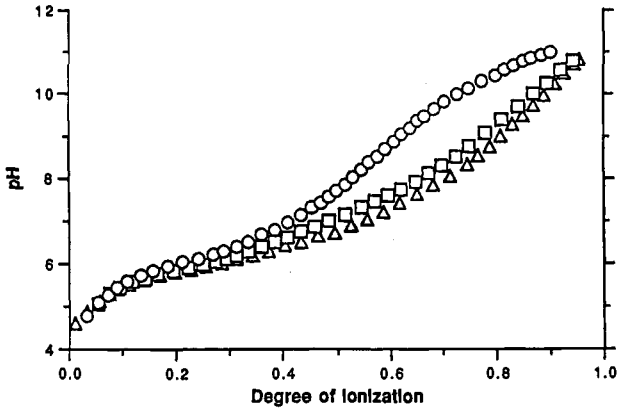

Figure 3. pH vs degree of ionization in polymer solutions (1 mg/mL, 90 mM NaCl) at 25 °C. ○, PEAA; □, 65 mol % EAA copolymer; and △, 49 mol % EAA copolymer.

To investigate the role of polymer ionization in determining the critical pH, titrations of several copolymers were examined.[16] From the titration measurements, the polymer ionization was calculated from the charge neutrality condition:

$$\alpha = \frac{[Na^+] + [H^+] - [OH^-] - [Cl^-]}{[mers]}$$

where [mers] is the concentration of the polymer repeating units (mers). The ionization behavior of PEAA and of the 65 mol % EAA and 49 mol % EAA copolymers is shown in Figure 3. There are clear differences in the ionization behavior of these polymers. The larger the MAA content of the copolymer, the greater the molecular charge at a given pH. This effect is especially pronounced for pH > 6. It is probable that the greater hydrophobicity of the EAA leads to a more compact conformation for PEAA than is characteristic of the MAA copolymers. A more compact conformation would enhance charge–charge interactions, resulting in suppression of the molecular charge at a given pH. Charge–charge interactions will also be enhanced by the lower dielectric constant of PEAA, compared with that of MAA copolymers. Lastly, the "intrinsic" pK_a of the methacrylic acid mer will likely be somewhat lower than that of the ethacrylic acid mer, due to local hydration and dielectric effects.

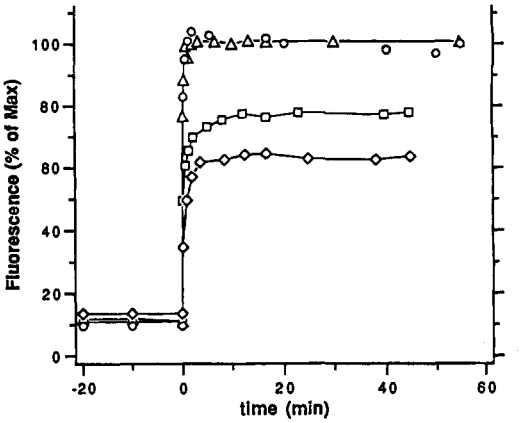

Figure 4. Release of calcein by copolymers. Fluorescence intensity is plotted as a percentage of the maximum intensity, obtained by vesicle solubilization with Triton X-100. Each sample was acidified from pH 7.7 at $t = 20$ min. PEAA at pH 6.7 effected complete release of calcein (○), as did the 49 mol % EAA copolymer at pH 5.9 (△). Copolymers lower in EAA content effected only partial dye release: (□) 34 mol % EAA at pH 5.55 and (◇) 19 mol % EAA at pH 5.45.

The differences in the ionization curves are most pronounced at pHs higher than the critical pHs for membrane dissolution. Consequently, these differences alone are not likely to be responsible for the shifts in critical pH with copolymer composition. From Figure 3, it can be seen that the degree of ionization at the critical pH varies with composition. At the critical pH (6.55) for PEAA, α is about 0.34. At the critical pH for the 49 mol % EAA copolymer (pH 5.8), α is only 0.15. Therefore, some factor other than simply molecular ionization or acidity is important in establishing the critical pH for membrane dissolution. The principal distinction between these monomers is hydrophobicity: apparently, the more hydrophobic EAA monomer promotes membrane dissolution at higher degrees of ionization than does MAA.

Figure 4 shows release of calcein entrapped in unilamellar liposomes by PEAA and several copolymers with MAA.[17] Polymer concentrations in this experiment were 0.2 mg/mL. Acidification of the PEAA solution to pH 6.7 resulted in quantitative release of calcein. This pH is slightly higher than the critical pH for membrane solubilization. Quantitative release was also obtained with the 49 mol % EAA copolymer at pH 5.9. In addition, significant (though not quantitative) release was obtained with copolymers with 34 and 19 mol % EAA, at pH 5.55 and 5.45, respectively. The latter copolymers did not solubilize membranes at any pH, but aggregation is observed in these samples and precludes a clear identification of the nature of the polymer–phospholipid aggregates. In fact, the formation of aggregates may be responsible for the observed partial dye release: aggregation may serve to protect some vesicles from attack by polymer.

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(16) For potentiometric titrations, polymer samples were first neutralized with 1 equiv of 0.100 N standardized NaOH (Fisher Scientific, Boston, MA), and then diluted into 100 mM NaCl solutions to final concentrations of 1 mg/mL polymer (sodium salt), 90 mM NaCl. Each sample was thermostated to 25 °C and deoxygenated by bubbling with argon gas for 10 min. During titration, an argon blanket was maintained over the sample. Titration was carried out by the injection of 5, 10, 20, or 30 μL aliquots of 0.100 N standardized HCl solution into the stirring sample. After mixing, stirring was stopped for 10 min, at which time the pH of the sample was measured with a Corning 155 ion analyzer with a ultrathin Hg/Hg_2Cl_2 pH combination microelectrode (Aldrich Chemical Co., Milwaukee, WI). The pH meter was calibrated before titration with standardized buffers at pH 4, 7, and 10.

(17) To study the polymer-mediated release of entrapped fluorescent dye from vesicles, unilamellar vesicles of egg phosphatidylcholine (EPC) (Sigma) were prepared according to the procedure of Huang and Thompson (*Methods Enzymol.* **1974**, *32*, 485–489.) EPC (40 mg) was dried from $CHCl_3$ solution on the bottom of a round-bottom flask and hydrated with 2.7 mL of a buffer solution containing 200 mM calcein, 10 mM Tris-HCl, and 100 mM NaCl, pH 10. The sample was first vortexed to produce multilamellar liposomes and then sonicated for 10 min at 35 W with a Branson Model 185 cell disruptor equipped with a $^1/_8$ in. diameter titanium microtip. The suspension was kept in an ice-water bath to prevent excessive heating. Residual MLVs and titanium particles shed by the sonicator tip were removed by centrifugation for 30 min in an IEC clinical centrifuge at ~2500 rpm. Small vesicles in the supernatant were applied to a gel filtration column (Sepharose CL-4B 300, 2 × 25 cm), and vesicles of elution volume 18–31 mL were collected and stored at 5 °C. To measure the release of calcein from the vesicles, polymer solutions in the above buffer solution were added to vesicle suspensions to give final concentrations of 0.2 mg/mL polymer, 0.04 mg/mL EPC vesicles. Fluorescence (495 nm excitation, 520 nm emission) was measured on a Perkin-Elmer MPF-66 fluorometer on samples held at 25 °C. Maximum fluorescence, corresponding to complete release of entrapped dye, was determined at the end of each run by addition of 50 μL of 15% Triton X-100 detergent.