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Utilizing the Hydrophobic Polyelectrolyte, Poly(2-Ethylacrylic Acid), for Drug Delivery

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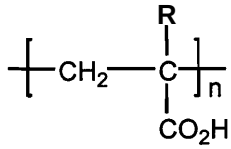
The pH-dependent conformational transition of poly(2-ethylacrylic acid) [PEAA] and its interaction with phosphotidylcholine bilayer membranes were investigated. The conformational transition was monitored with fluorescence spectroscopy using pyrene as a probe; the location and breadth of the conformational transition was shown to be dependent on the molecular weight and polydispersity of the sample, respectively. The pH-dependent destabilization and fusion of extruded large unilamellar vesicles (LUVs) by PEAA was characterized by optical density measurements, transmission electron microscopy, and lipid mixing and contents release assays. Reduction of either the chain length or the polymer concentration caused the fusion and contents release events to shift to lower pH values. Release of entrapped calcein was observed at pH values ca. 1 unit higher than those found to cause membrane fusion. Decreased levels of fusion were observed when the concentration of PEAA was lower than that of the lipid; however, quantitative release of encapsulated calcein could be effected at very low polymer concentrations (~3% w/w PEAA/lipid).

Model membrane systems that exhibit controlled fusion and permeability behavior are receiving increased attention for applications such as gene therapy,[1,2] targeted drug delivery,[3,4] and signal amplification in biochemical assays.[5,6] In biological systems, the cell orchestrates precise control of membrane-mediated events such as endocytosis and exocytosis, fusion, translocation, transport, and recognition.[7] These phenomena have prompted study of model membrane systems that respond to environmental cues.

Strategies for the design of responsive liposome systems can utilize the lipid constituents of the membrane or other molecules (most commonly macromolecules) that disrupt the membrane in response to various stimuli. The use of phospholipids that form nonlamellar phases under a certain set of conditions can be used for membrane destabilization? Diacyl lipids that are prone to cleavage of one of the acyl chains can also act as permeabilizing agents.[9,10] Furthermore, inclusion of defect forming molecules in the membrane can effect membrane permeabilization.[11]

The interactions of macromolecules with bilayer membranes have been extensively studied and one approach toward responsive systems involves synthesis of model peptides based on the putative fusion domains of known fusogenic proteins.[12] Peptides derived from the fusion protein of the influenza virus,[13] and the sperm surface proteins fertilin[14] and PH-30,[15] showed acid-induced destabilization and fusion of PC vesicles when ligated to a lipid anchor. A second macromolecular approach to responsive liposomes involves the use of non-peptide, water soluble synthetic polymers. Poly(ethylene glycol) (PEG) has been widely used to mediate cell-cell fusion and in the fusion injection of macromolecules into cultured cells fiom erythrocytes or liposomes.[16]

Furthermore, synthetic polyelectrolytes have been shown to alter membrane properties in response to changes in pH. Poly(acrylic acid) [PAA, **1a]** and poly(methacrylic acid) [PMA, **lb]** have been shown to modify PC vesicles upon acidification by increasing the gel to liquid-crystalline phase transition temperature (T_m),[17,18] by inducing aggregation,[19] and by increasing permeability of the bilayer toward small molecules.[19,20]



1 a: R=H **b:** R= CH_3 **c:** R= CH_2CH_3

The interaction of the hydrophobic polyelectrolyte, poly(2-ethylacrylic acid) [PEAA, **lc]** with PC membranes has been studied extensively in this laboratory.21 This system has been tailored to create vesicles that respond to pH,[22] temperature,[22] light intensity,[23,24] and concentration of a solute such as glucose.[25] Encapsulated material is released rapidly and quantitatively upon membrane destabilization. PEAA undergoes a conformational collapse from an expanded coil to a more compact structure upon acidification in aqueous solutions.26 This conformational collapse induces membrane leakage at low concentration (<3% w/w PEAA/lipid), while at higher concentrations (50% w/w PEAA/lipid), collapse is responsible for structural reorganization from membrane vesicles at high pH to mixed polymer-lipid micelles at low pH.[21] Although PMA has been shown to solubilize simple hydrocarbons,[27] the ability of PEAA to cause membrane reorganization distinguishes PEAA from its less hydrophobic counterparts (PAA and PMA) which show no ability to solubilize lecithin membranes at any pH.[20]

These properties make PEAA a candidate for use in the development of functionalized drug carriers for pharmaceutical applications. In this chapter, the effect of molecular weight on the conformational collapse of PEAA in aqueous solution is described with special attention paid to the role of polydispersity on the shape of the transition. Furthermore, the pH-dependent interaction of PEAA with PC vesicles is examined. Membrane destabilization and fusion are characterized by optical density measurements, by transmission electron microscopy, and by lipid mixing and contents release assays. Furthermore, we report the effects of molecular weight and concentration of PEAA on membrane fusion and contents release.

Molecular Weight Control

Our initial goal for the project was to synthesize PEAA of well defined molecular architecture. We particularly targeted PEAA of low molecular weight (<8000) with narrow molecular weight distributions. PEAA was obtained from a bulk free-radical polymerization of 2-ethylacrylic acid[28]. The polydispersity of the isolated sample was consistent with what was expected for a free radical synthesis whose termination is primarily through disproportionation (PDI–2). Solvent fractionation was used to reduce the polydispersity of a PEAA sample, and was carried out by inducing precipitation of PEAA from methanolic solutions through addition of diethyl ether, a nonsolvent for the polymer. The highest molar mass species precipitate first so that fractions of decreasing molar mass are obtained as the proportion of nonsolvent is increased. Gel permeation

chromatography shows that each of the PEAA fractions obtained by ether precipitation is of lower polydispersity (1.1-1.5) than the initial sample obtained via radical polymerization (1.9). Molecular weight data are presented in Table 1. Weight average molecular weights (estimated on the basis of poly(ethylene oxide) GPC standards) range from 5,300 to 24,900.

Table 1. Molecular Weights[a] of PEAA Samples Obtained from Solvent Fractionation

Sample[b]	Mw	Mn	PDI
Unfractionated	16,600	8,900	1.9
1:1	24,900	16,700	1.5
2:1	10,400	8,400	1.2
6:1	7,300	6,500	1.1
10:1	6,300	5,400	1.1
Soluble	5,300	4,700	1.1

[a]Determined by GPC with PEO calibration

[b]Ratios in the sample column refer to the ether:methanol ratio used to precipitate each fraction.

Solution Conformation Properties of PEAA

The effects of molecular weight and polydispersity on the conformational collapse of PEAA in aqueous solution were monitored by fluorescence spectroscopy with pyrene as a probe of environmental polarity.[29] The steady-state fluorescence of pyrene exhibits an increase in the emission intensity of peak 1 (373 nm) and peak 3 (384 nm) in nonpolar environments (e.g., in the hydrophobic interior of a more compact coil). The conformational transition was monitored for four different fractions of PEAA, and the behavior of each fraction was compared to that of the unfiactionated sample. The results are presented in Figures 1a and 1b, which show the peak 1 and peak 3 intensities vs. pH for all samples. The transition midpoint ranges from pH 6.0 for the highest molecular weight sample, to pH 5.3 for the lowest molecular weight sample.

The molecular weight effect on the conformational transition can be explained, at least in part, by the molecular weight dependence of the ionization behavior of PEAA. Although ionization of poly(acrylic acid) shows no molecular weight dependence, titration curves of PMA[30] and PEAA[31] exhibit strong molecular weight effects in the transition region, where shorter chains behave as stronger acids than their higher molecular weight counterparts. Thus, even the simplest assumption (i.e., that the conformational collapse occurs at a fixed value of the degree of ionization) would require that the solution pH must be reduced further to induce collapse of short PEAA chains.

The role of polydispersity in determining the breadth of the conformational transition was examined by fitting the data in Figures 1a and 1b to a logistic function.[32] The first derivative of the resulting function was calculated, and the full width at half maximum (FWHM) of the derivative curve was taken as a measure of the transition width. In Figures 1c and 1d, the first derivative plots for the 1:1 and unfractionated samples (which have similar number-average molecular weights) are shown. The fractionated sample exhibits reduced transition width, which we attribute to a decrease in polydispersity (PDI of 1.5 vs. 1.9). The transition widths and midpoints for each of the

fiactions are presented in Table 2. Note that for the 10:1 and soluble fiactions, the width at half maximum was obtained by doubling the pH difference between the midpoint (peak value) and the higher pH half maximum value of the first derivative plot, because these fiactions did not fully complete the transition at the minimum pH value investigated.

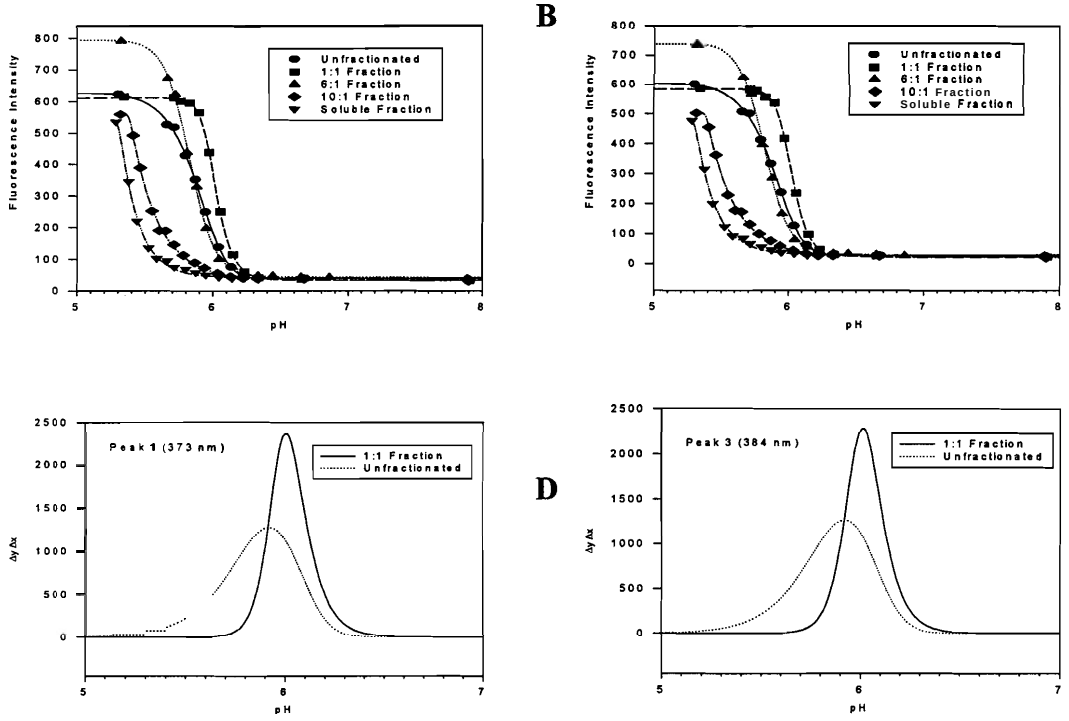


Figure 1. Fluorescence intensity at 373 nm (Peak 1,A) and 384 nm (Peak 3,B) for pyrene dissolved in phosphate-buffered solutions of PEAA of different molecular weights. First derivative plots for the 1:1 and the unfiactionated samples demonstrate the effect of polydispersity on the transition width (Peak 1,C and Peak 2,D).

Table 2: Transition Midpoints and Widths Obtained from Fluorescence Measurements

	Peak 1 (373 nm)		Peak 3 (384 nm)	
Sample	**FWHM**	**Transition pH**	**FWHM**	**Transition pH**
Unfractionated	0.33	5.93	0.41	5.93
1:1	0.22	6.01	0.21	6.02
6:1	0.34	5.83	0.33	5.82
10:1	0.28[a]	5.42	0.29[a]	5.42
Soluble Fraction	0.22[a]	5.34	0.23[a]	5.34

[a]FWHM value was obtained by doubling the pH difference between the midpoint (peak value) and the higher pH half maximum value of the first derivative plot

Characterization of PEAA's Interactions with Bilayer Memebranes

1) Optical Density Measurements and Transmission Electron Microscopy

PEAA has been shown to bind to PC membranes in a pH dependent manner, and at high enough concentrations (~50% w/w PEAA/lipid), to cause complete structural reorganization from membrane vesicles to mixed polymer-lipid micelles.[21] In our previous work, membrane reorganization was monitored by optical density measurements on MLVs (100 nm to 10 μm) produced by vortex hydration of dried lipid films. A smooth transition is observed when the pH of a suspension of MLVs in aqueous PEAA is reduced, producing an optically clear solution of polymer-lipid micelles (Figure 2).

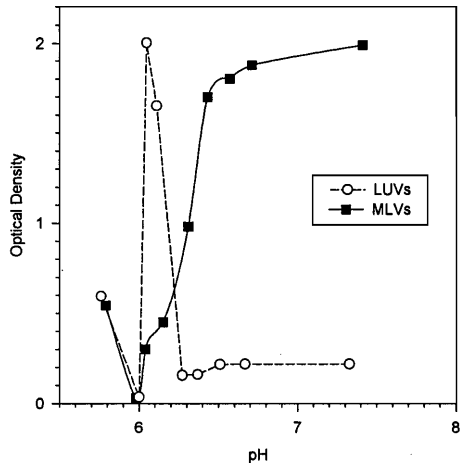


Figure 2. Turbidity measurements on mixtures of EYPC vesicles and PEAA, both at concentrations of 1 mg/mL. As the pH is reduced, MLVs undergo a smooth turbidity transition, while LUVs show a large increase in optical density prior to clarification.

When we repeated experiments of this kind using extruded LUVs, we observed an abrupt increase in the optical density prior to clarification of the suspension (Figure 2). Such a rise in optical density constitutes evidence either for vesicle aggregation or for vesicle fusion. The increase in optical density was stable over several days at room temperature, indicating that the aggregates or fused vesicles were not transient intermediates in the vesicle to micelle transition.

Transmission electron microscopy (TEM) on PEAA/EYPC mixtures obtained from optical density experiments provided clear evidence of membrane fusion. Particular attention was given to the mixture prepared at pH 6.1, which showed the highest turbidity; in this sample TEM revealed fivefold increase in the apparent diameter of the vesicles (Figure 3). Figure 3A shows that vesicles prepared in PEAA solutions at pH 8.3 are ca. 100 nm in diameter, consistent with the fact that they were extruded through 100 nm pores.[33] After vesicles are added to PEAA solution at pH 6.1, vesicle size increases with time to a final average diameter of 500 nm (Figures 3 B-F).

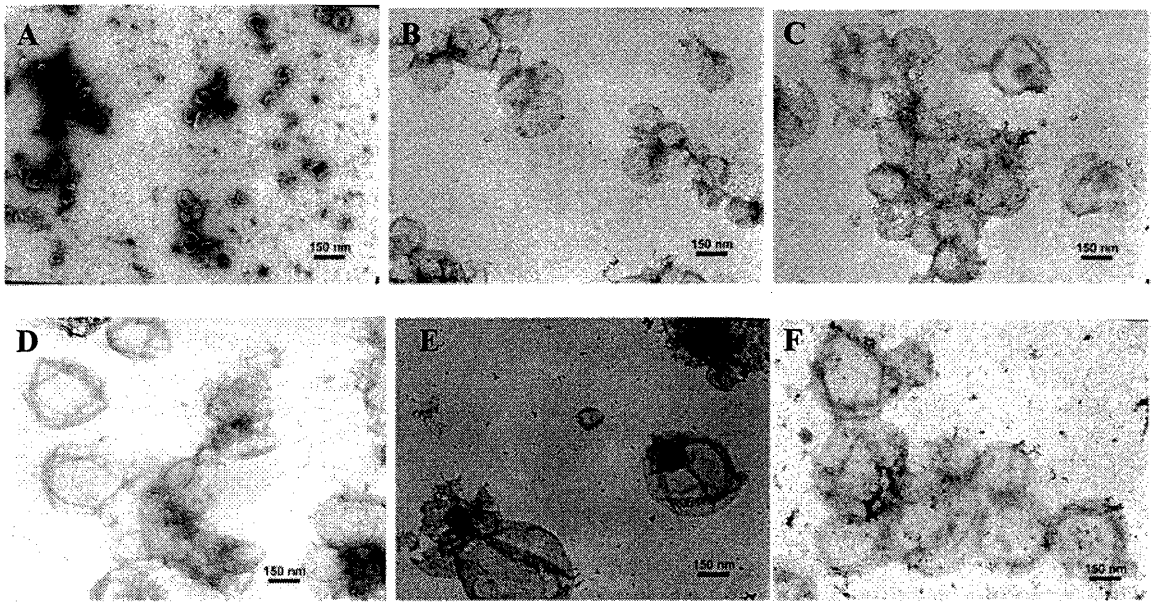


Figure 3. TEM micrographs of PEAA/EYPC vesicle mixtures stained with uranyl acetate at: B) 20 min, C) 50 min, D) 90 min, E) 180 min, and F) 1440 min after mixing at pH 6.1 where a maximum increase in optical density occurs; A) control sample prepared at pH 8.5, where the polymer-lipid interactions are weak or absent.

2) Fusion Assay and Contents Release

Membrane fusion was monitored by the decrease in resonance energy transfer (RET) resulting fiom dual probe dilution.[13] Extruded EYPC vesicles were prepared with 1 mol% each of NBD-PE and Rh-PE. Unlabelled EYPC vesicles were mixed with labeled vesicles at a ratio of 3:1 and fusion was monitored by an increase in fluorescence of the NBD-PE emission at 530 nm (excitation 468 nm). Figure 4 shows the fluorescence emission spectra obtained during a typical lipid mixing fusion assay. At pH 8.3, the fluorescence of NBD-PE at 525 nm is quenched via resonance energy transfer to Rh-PE. As the pH is lowered below 6.5, fusion is induced and more bilayer area becomes available for dilution of the chromophores. This leads to an increase in the NBD-PE emission at 525 nm and a corresponding decrease in the fluorescence of Rh-PE at 585 nm. Fifty percent fusion occurs by pH 6.25 and fusion is complete (100%) by pH 6.0.

Percent fusion was calculated for each point along the pH axis from equation *1*:

$$\% \text{ Fusion} = \frac{F_{meas} - F_0}{F_{max} - F_0} \times 100 = \frac{\Delta F}{\Delta F_{max}} \times 100 \qquad 1$$

where F_{meas} is the fluorescence intensity of NBD-PE at 530 nm, F_0 is the fluorescence intensity in the quenched state, and F_{max} is the fluorescence intensity achieved by infinite probe dilution, determined by the addition of 40 μL of 100 mM Triton X-100.

Although PEAA has long been known to cause release of vesicle contents and lipid reorganization fiom vesicles to mixed micelles, this is the first demonstration of the ability of the polymer to induce membrane fusion. This behavior is different fiom that of PAA and PMA, both of which induce vesicle aggregation upon acidification of the medium.[17,19]

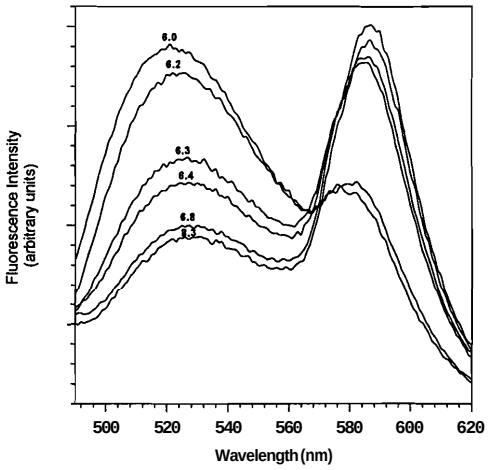


Figure 4. Fluorescence emission spectra of mixtures of PEAA and EYPC vesicles [containing 1 mol% of each NBD-PE (donor) and Rh-PE (acceptor)] at 6 pH values. At high pH, the fluorescence of NBD-PE at 525 nrn is quenched by resonance energy transfer to Rh-PE. As the pH is lowered, the fluorescent probes diffuse apart and an increase in NBD-PE fluorescence is observed, along with a decrease in the Rh-PE emission at 585 nm.

Efflux of calcein from phosphatidylcholine vesicles was observed by fluorescence spectroscopy to study the bilayers permeability to encapsulated molecules.[32] Release of calcein was monitored by an increase in fluorescence emission at 525 nm (excitation 495 nm). Percent release was calculated as $(\Delta F/\Delta F_{max})x100$, where the maximum fluorescence intensity was given by the complete release of encapsulated calcein caused by the addition of 40 μL of 100 mM Triton X-100.

In biological fusion events such as endocytosis, viral infection, or fertilization, membrane-bound proteins and glycoproteins mediate so-called "leakless" fusion. In model membrane systems created either with synthetic polymers or with biologically derived peptides, there are few reports of such leakless fusion events. Comparison of the fusion and contents-release events triggered by PEAA shows that fusion in this system is preceded (or accompanied) by essentially complete leakage of a marker dye (calcein) entrapped in the vesicular interior. The results from the fusion and contents release assays are presented in Figures 5 and 6.

Figures 5A and 5B show the effects of the molecular weight (MW) of PEAA on the location of the transition for fusion and for contents release, respectively. For fusion and contents release, the transition midpoints shift to lower pH values as the MW of the polymer is decreased. In all cases, release of encapsulated calcein occurred at pH values

0.4-1.0 pH units higher than those required for fusion.

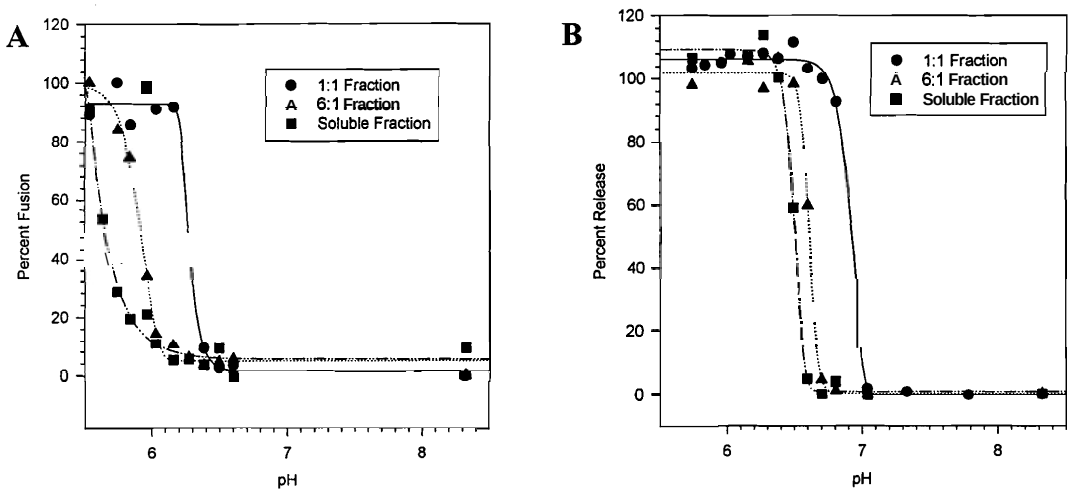


Figure 5. Effects of PEAA molecular weight on A) fusion and B) on contents release. Samples were prepared as 3.311 w/w mixtures of PEAA to lipid. Release of encapsulated material occurs at pH values 0.43-0.96 pH units higher than those required for fusion.

In Figure 6, the effects of PEAA concentration on fusion and release are shown. Decreased levels of fusion were observed when the concentration of PEAA was lower than that of the lipid (Figure 6A); however, quantitative release of encapsulated calcein could be effected at very low polymer concentrations (–3% w/w PEAA/lipid). As little as 1.65% PEAA causes significant release of calcein (–80%). Table 4 summarizes the effects of concentration of PEAA on membrane fusion and contents release.

Table 4. Concentration Dependence[a] of Transition Midpoints for Fusion and Contents Release

Concentration wt % PEAA/EYPC	Transition pH Fusion	Calcein Release	PEAA Molecule[b] Lipid Molecule	PEAA Strands[c] per Vesicle
330	6.27	6.94	1:8	10,600
98	6.25	---	1:27	3100
66	6.23	6.70	1:40	2100
33	6.10	6.68	1:80	1100
16.50	no fusion	6.67	1:160	530
8.25	no fusion	---	1:320	270
3.30	---	6.66	1:800	110
1.65	---	6.66	1:1600	55

[a]1:1 fraction was used for the concentration study
[b]Ratios based on molar masses of 760 g/mol and 20,000 g/mol for lipid and PEAA, respectively.
'Number based on assumption of above molar masses, vesicles of 100 nm in diameter, and area per lipid in a bilayer = 70 Å^2

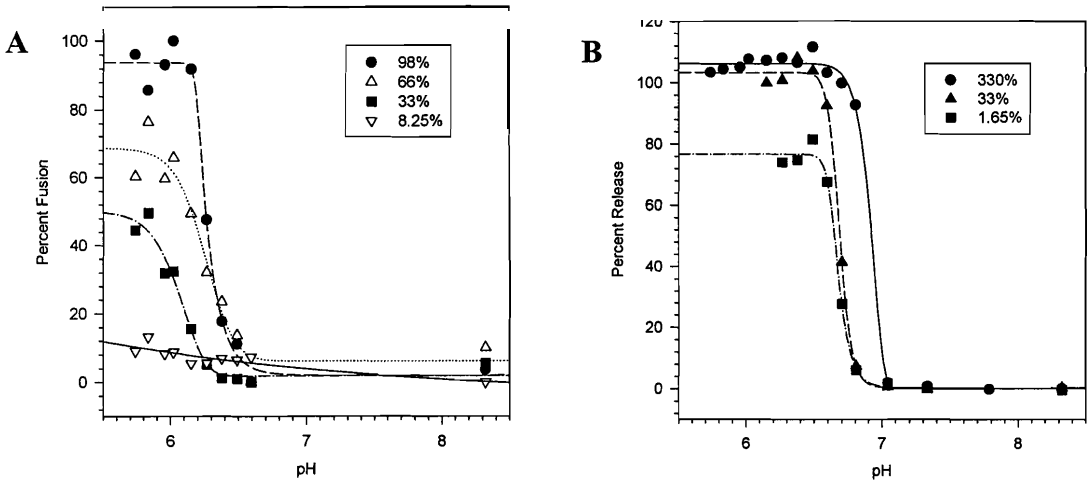


Figure 6. Effects of PEAA concentration on A) fusion and B) contents release. Percentages shown represent the weight of PEAA compared to lipid. The extent of membrane fusion is reduced as the concentration of PEAA is lowered below 100% and is not detected below concentrations of 16.5%. Release of encapsulated calcein is efficient down to low concentrations of PEAA; 80% release is observed at concentrations as low as 1.65%.

Included in Table 4 are the results of calculations that give the molar ratios of PEAA to lipid, as well as the number of PEAA strands per vesicle, at the concentrations examined. These calculations assumed vesicles of 100 nm in diameter, 70 $Å^2$ as the area per lipid molecule in the bilayer, and molar masses of 760 glmol and 20,000 glmol for EYPC and PEAA, respectively. As few as 50-100 chains of PEAA are sufficient to cause efficient release of vesicle contents upon mild acidification. This result is consistent with single channel recording experiments reported previously, which showed that even a single molecule or oligomer of PEAA can induce pore formation in artificial bilayer membranes in a pH-dependent fashion.[34]

Conclusions

The pH-dependent conformational collapse of PEAA was shown to depend upon molecular weight and polydispersity of the sample by affecting the location and breadth of the conforrnational transition, respectively. PEAA induces fusion of phosphatidylcholine LUVs upon acidification in aqueous solution. Transmission electron microscopy shows a large increase in the size of the vesicles when the pH of a PEAA/EYPC mixture is lowered to pH 6.1, which coincides with the abrupt increase in optical density. Fusion and contents release were observed to depend upon MW and concentration of PEAA; both events shifted to lower pH values with a reduction in MW or concentration. Release of contents occurred at pH values 0.4 to 1.0 pH units higher than those required for vesicle fusion.

The above properties make PEAA a candidate for use in the development of functionalized drug carriers for pharmaceutical applications. Future work will focus on

PEAA-conjugated liposomes that have been developed in this laboratory[35,36] and will examine their efficacy as intracellular delivery vehicles.

Acknowledgment

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