Selective H^+-Dependent Release of Contents from Thymine-Labeled Phospholipid Vesicles by an Adenine-Labeled Polyelectrolyte

Inmaculada Molina Pinilla, Manuel Bueno Martinez, and David A. Tirrell*

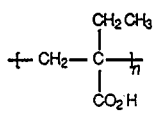
Department of Polymer Science and Engineering, University of Massachusetts, Amherst, Massachusetts 01003

Received December 21, 1993; Revised Manuscript Received February 15, 1994®

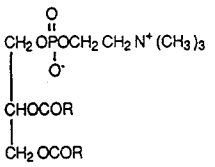
ABSTRACT: Copolymers bearing 5.6, 9, or 11.7 mol % of adenine residues were prepared by radical copolymerization of 2-ethylacrylic acid (EAA) and 9-[[2-(methacryloyloxy)ethoxy]methyl]adenine (MAAd). A double-chain surfactant with thymine in the headgroup was synthesized and used in mixtures with egg yolk phosphatidylcholine (EYPC) to prepare thymine-labeled unilamellar lipid vesicles. Analysis of the pH-dependent behavior of the copolymers revealed that release of contents could be effected from labeled vesicles in competitive experiments in which unlabeled vesicles remained intact. Selectivity was greatest for the 9 mol % copolymer and was reduced at either lower or higher levels of adenine labeling. The selective recognition of the labeled membranes is ascribed to interactions between polymer-bound adenine and surface-bound thymine functional groups.

Introduction

Poly(2-ethylacrylic acid) (PEAA, 1) undergoes a cooperative conformational transition from an expanded, hydrophilic coil at high pH to a compact, globular structure upon acidification of its aqueous solutions.[1-3] The globular polymer binds strongly to bilayer vesicles prepared from phosphatidylglycerols and phosphatidylcholines (2), and the resulting membrane reorganization leads to large and useful changes in membrane permeability.[4,5] We have exploited these phenomena to prepare suspensions of phosphatidylcholine vesicles that release their contents rapidly and quantitatively in response to small changes in pH,[5] temperature,[5] glucose concentration,[6] or light intensity.[7]



1



2

The observation that PEAA can be used to open membranes in response to specific signals raises a further question: Can the polymer be rendered capable of distinguishing one membrane from another? If so, it should be possible to design delivery systems (e.g., for therapeutic or imaging agents) that are not only signal-sensitive but site-specific as well.

The present paper describes the synthesis of PEAA derivatives that recognize phosphatidylcholine membranes bearing surface-bound thymine residues. The strategy used to prepare these derivatives involves copolymerization of 2-ethylacrylic acid with 9-[[2-(methacryloyloxy)ethoxy]-methyl]adenine (MAAd). Incubation of the resulting adenine-labeled copolymers with lipid vesicles containing a fluorescent dye results in loss of vesicle contents following mild acidification. The fact that thymine-bearing membranes are more sensitive to acidification than are unmodified membranes suggests that base-pairing interactions at the surface contribute to selective membrane recognition. Unmodified PEAA shows no such selectivity.[8]

Experimental Section

Materials. Dimyristoylphosphatidylethanolamine (DMPE), egg yolk phosphatidylcholine (EYPC), and calcein were used as received from Sigma Chemical Co. Azobis(isobutyronitrile) (AIBN; Aldrich) was recrystallized twice from methanol and dried at room temperature under reduced pressure for 48 h. Triton X-100 was purchased from Fisher Scientific Co. and used without purification. 2-Ethylacrylic acid (EAA) and poly(2-ethylacrylic acid) were prepared from diethyl ethylmalonate by the method described previously.[9] EAA was fractionally distilled (bp 46 °C/2.8 mmHg) before use. Methacrylic anhydride was fractionally distilled (bp 65 °C/2.0 mmHg) from hydroquinone immediately before use. Sepharose CL-4B was purchased from Pharmacia, Inc.

General Methods. Proton nuclear magnetic resonance (NMR) spectra were recorded at 80 and 200 MHz on Bruker NR-80 and AC-200 spectrometers. Chemical shifts are reported as ppm downfield from tetramethylsilane. Infrared spectra were recorded on a Perkin-Elmer 1320 spectrometer. All melting points were recorded on a Fisher-Johns melting point apparatus and are uncorrected. Absorbance measurements were obtained on a Hitachi U2000 spectrophotometer using quartz cuvettes with a path length of 1 cm. Solution viscosity measurements were performed with a Cannon Model 50/M765 viscometer placed in a water bath with the temperature maintained by a Lauda MT constant-temperature circulator. Elemental analyses were determined in the Microanalysis Laboratory at the University of Massachusetts at Amherst. Solution pH was measured with a Corning 155 pH/ion analyzer equipped with a Corning combination semimicroelectrode. The pH meter was calibrated with pH 4 and pH 10 standardized buffers. Fluorescence measurements were performed on a Perkin-Elmer MPF-66 fluorescence spectrophotometer. Pyrene fluorescence measurements were done on lipid-free polymer solutions (1 mg/mL, 10 mM Tris·HCl/100 mM NaCl). The pyrene probe (5×10^{-6} M in the sample) was introduced subsequent to sample preparation as a 10^{-3} M solution in acetone. Intensities of emission peaks I (at 373 nm) and III (at 384 nm) were determined at 24.6 ± 0.2°C with excitation at 337 nm.

9-[[2-(Methacryloyloxy)ethoxy]methyl]adenine. A mixture of 9-[(2-hydroxyethoxy)methyl]adenine[10] (50.0 mg, 0.239 mmol), 4-(dimethylamino)pyridine (1.0 mg), and methacrylic anhydride (68.1 mg, 0.44 mmol) in dry pyridine (5.0 mL) was stirred at room temperature for 24 h. After evaporation of the solvent under reduced pressure, the residue was dissolved in 10:1 $CHCl_3/CH_3OH$ and chromatographed on silica gel preparative

® Abstract published in *Advance ACS Abstracts,* April 1, 1994.

0024-9297/94/2227-2671$04.50/0

Table 1. Radical Copolymerization[a]

EAA (g)	MAAd (g)	AIBN (mol %)	yield (%)	adenine[b] (%)	η_{inh}[c] (dL/g)
0.402	0.040	0.040	63	5.6	0.41
0.399	0.089	0.040	60	9	0.55
0.384	0.172	0.040	64	11.7	1.46

[a] Reactions were run for 24 h at 60 °C in bulk. [b] Determined from the ultraviolet absorption spectrum of the copolymer. [c] Determined in 10 mM Tris·HCl/100 mM NaCl, pH 7.70 at 28 °C.

plates (eluent 10:1 $CHCl_3/CH_3OH$). Extraction with absolute ethanol (3 × 50 mL) and concentration of the solvent gave 49 mg (74%) of the title compound as a solid: mp 178–180 °C (after one recrystallization from benzene); λ_{max} = 259.6 nm, ϵ = 12 700 M^{-1} cm^{-1} (in 10 mM Tris·HCl/100 mM NaCl, pH 7.70); IR (KBr) 3300–3100, 1725, 1670, 1590, 1570 cm^{-1}; ^1H NMR (DMSO-d_6, 80 MHz) δ 1.80 (s, 3H, CH_3), 3.76 (m, 2H CH_2O), 4.16 (m, 2H, CH_2O), 5.57 (s, 2H, NCH_2O), 5.61 (s, 1H, $=CH_2$), 5.90 (s, 1H, $=CH_2$), 7.21 (s, 2H, $-NH_2$), 8.16 (s, 1H, purine CH), 8.25 (s, 1H, purine CH). Anal. Calcd for $C_{12}H_{15}N_5O_3$: C, 51.98; H, 5.45; N, 25.25. Found: C, 51.85; H, 5.59; N, 25.07.

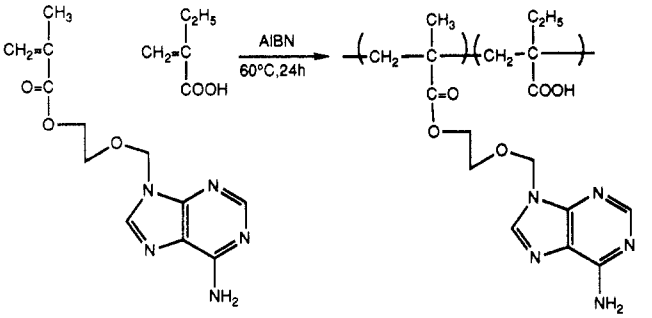
Polymerization. A glass ampule was charged with 2-ethylacrylic acid, AIBN, and 9-[[2-(methacryloyloxy)ethoxy]methyl]-adenine. The ampule was attached to a vacuum line, subjected to four freeze–degas–thaw cycles, and finally sealed under vacuum. Various monomer feed ratios were chosen (cf. Table 1) in order to obtain samples bearing different amounts of adenine. The copolymerization was carried out by heating the ampule in a bath maintained at 60 ± 1 °C; agitation was provided by a magnetic stirring bar. The polymer began to precipitate immediately on warming. After 24 h, the reaction mixture was dissolved in dimethyl sulfoxide and poured into excess ethyl acetate with vigorous stirring to precipitate the copolymer. After separation of the polymer on a fritted glass filter, the precipitation was repeated, and finally the copolymer was rinsed with ethyl acetate. The copolymer was dried at room temperature, ground to a fine powder, and dried in vacuo at 45 °C.

Dimyristoyl-*N*-[[2-(thymin-1-yl)ethyl]carbonyl]phosphatidylethanolamine (DMPE-Thy). Triethylamine (20.0 mg, 0.2 mmol) was added to a suspension of DMPE (127 mg, 0.20 mmol) and 1-(*p*-nitrophenyl-2-carboxyethyl)thymine[11] (86.5 mg, 0.27 mmol) in dry dichloromethane (10 mL). The reaction mixture was stirred at room temperature for 24 h and then concentrated to dryness. The residue was dissolved in chloroform and chromatographed on a 2 × 20 cm silica gel column using as eluent a gradient of methanol in chloroform ($CHCl_3$; 10:1 $CHCl_3/CH_3OH$). Concentration of the fractions containing the title compound gave 107 mg (65%) as a solid; thin layer chromatography (silica gel, 10:1 $CHCl_3/CH_3OH$) produced a single spot. The ^1H NMR spectrum (200 MHz) in $CDCl_3$ showed two singlets at 1.84 and 7.16 ppm which were assigned to $CH_3C(5)$ and HC(6) of the thymine ring, respectively; λ_{max} = 269.8 nm, ϵ = 7625 M^{-1} cm^{-1} (in 10 mM Tris·HCl/100 mM NaCl, pH = 7.6); IR (film) 1600–1760 cm^{-1}.

Thymine-Labeled Vesicle Preparations. Vesicles were prepared from a 9:1 mixture of EYPC and DMPE-Thy in chloroform. The solvent was removed under reduced pressure to leave a thin lipid film. The lipid film (28 mg) was hydrated with 2 mL of 10 mM Tris·HCl/100 mM NaCl by vortex agitation at room temperature for 3 min. The suspension was sonicated in an ice bath with a Branson Sonifier 185 at a setting of 30 W for 30 min. The sample was centrifuged in an Eppendorf Centrifuge Model 5402 at 14 000 rpm and 8 °C for 40 min. The top 1.5 mL of the sample was placed on a Sepharose CL-4B column (1.6 × 20 cm) and eluted at 5 °C with Tris·HCl/NaCl buffer, pH 7.6; 1.0 mL fractions were collected. Unilamellar vesicles were identified by monitoring of the eluent by optical density measurements at 250 nm. Unlabeled vesicles were also prepared from pure EYPC following this procedure.

Calcein-loaded vesicles were prepared as described above, except that the lipids were hydrated in a 200 mM calcein solution in 100 mM Tris·HCl containing 100 mM NaCl, pH 7.6. Lipid concentration was determined by the method of Charles and Stewart.[12]

Scheme 1



Samples for the determination of dye release rates were prepared by using a 1:1 mixture of thymine-labeled and unlabeled vesicles, one of which contained the entrapped dye. The total lipid concentration was 0.118 mg/mL. The suspension was adjusted to pH 7.6, and the polymer was added to a concentration of 8 × 10^{-3} mg/mL. After incubation at 10 °C for 24 h, the fluorescence intensity of this suspension at 530 nm was monitored at 25 °C under steady-state excitation at 495 nm. The pH of the suspension was adjusted by addition of aqueous HCl, and the total fluorescence of the sample was determined after addition of 100 μL of 10% aqueous Triton X-100 to effect complete release of entrapped calcein.

Results and Discussion

Preparation of Copolymers. Solution copolymerizations of EAA and MAAd in DMSO at 60 °C using AIBN as initiator failed to provide copolymers in acceptable yield probably due to a low ceiling temperature for EAA polymerization. In contrast, radical bulk copolymerization (Scheme 1) afforded satisfactory yields of copolymers bearing 5–12 mol % of the adenine label as shown in Table 1. Because the EAA–MAAd copolymers were insoluble in the monomer mixtures from which they were prepared, precipitation was observed in every case. After two precipitations from DMSO into ethyl acetate, the purity of the polymers was checked by TLC on silica gel plates (eluent: methanol); only a single spot at the origin was observed.

Pyrene Fluorescence in Lipid-Free Polymer Solutions. Pyrene is commonly used as a probe of environmental polarity, because its emission intensity and vibronic band structure are sensitive to solvation.[13] We[14,15] and others[16–18] have shown pyrene to be a useful photophysical probe of polyelectrolyte conformation in aqueous solutions. The transition of the PEAA chain 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 peak III) and 373 nm (peak I). These changes in the vibrational fine structure of the fluorescence emission are consistent with a decrease in the polarity of the pyrene microenvironment in PEAA solutions at low pH.

In Figure 1 are plotted the intensities of peak I and III and the III/I intensity ratio as functions of pH, for aqueous pyrene solutions containing EAA/MAAd copolymers bearing different amounts of adenine. Results are also shown for the PEAA control. In each sample, the emission intensity rises sharply as the pH is depressed, and the inflection points in the intensity curves are shifted to higher pH as the adenine content of the copolymers is increased. The transition midpoints lie at pH 6.3, 6.6, and 6.8 for samples bearing 5.6, 9, and 11.7 mol % of pendant adenine, respectively, compared to a transition midpoint for PEAA of pH 6.0. Figure 1c shows the III/I intensity ratio as a function of pH. A rise in III/I upon acidification signals

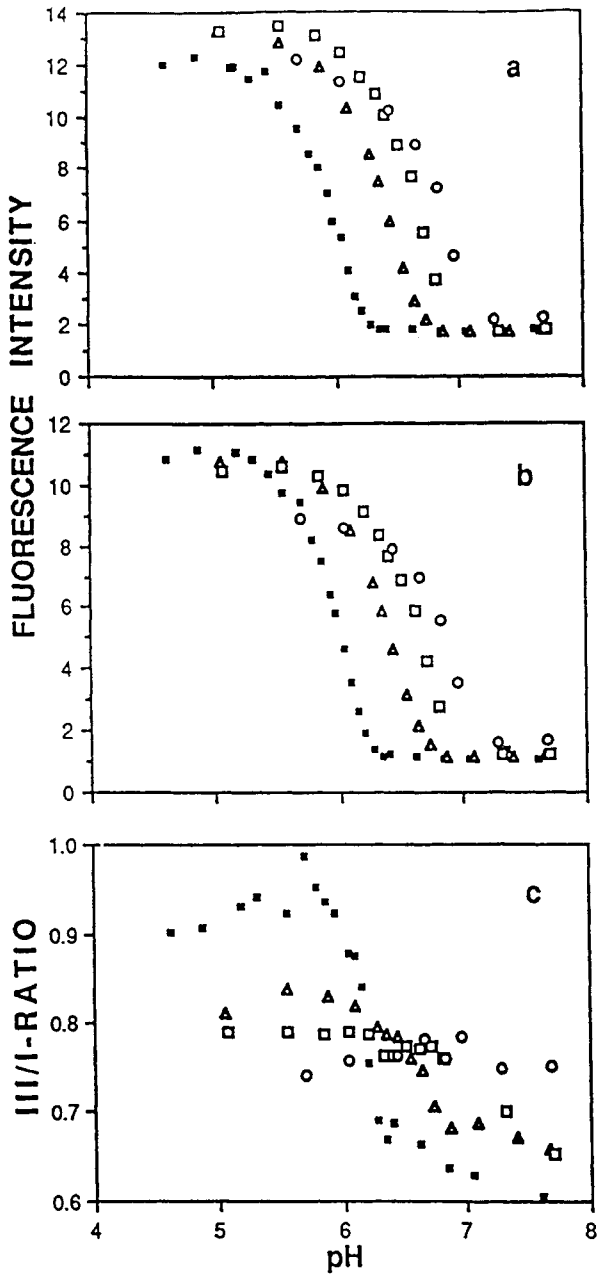
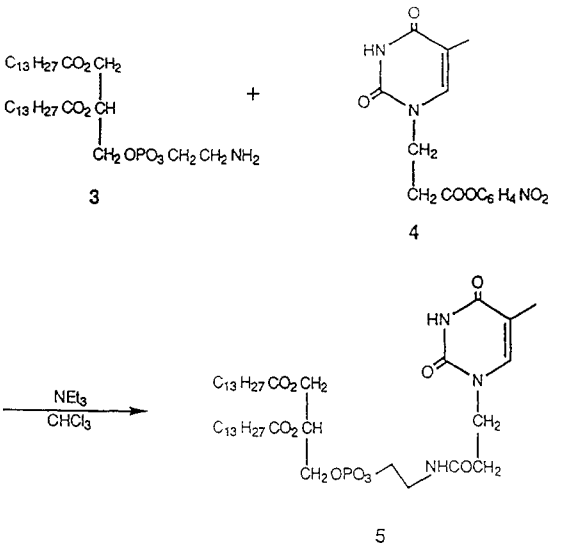


Figure 1. Fluorescence intensities at 373 nm (peak I; a) and at 384 nm (peak III; b) and the ratio of intensities emitted at 384 and 373 nm (III/I-Ratio; c) from pyrene dissolved in Tris·HCl buffered solutions of copolymers bearing adenine at concentrations of 5.6 (△), 9 (□), and 11.7 mol % (○) and of the PEAA control (■).

a decrease in the polarity of the environment sampled by the pyrene probe. The apparent change in polarity is large in the case of PEAA but grows progressively smaller as the content of adenine in the copolymer is increased. These results suggest that, as the content of adenine is increased, the expanded form of the polymer becomes less hydrophilic, while the collapsed form excludes water less effectively from the globular coil.

Preparation of Thymine-Labeled Vesicles and H+-Induced Release of Vesicle Contents. The thymine-bearing lipid **5** (DMPE-Thy) was prepared from commercially available dimyristoylphosphatidylethanolamine (**3**) and 1-(*p*-nitrophenyl-2-carboxyethyl)thymine (**4**) (Scheme 2). Hydration of a 9:1 mixture of EYPC and DMPE-Thy, followed by sonication of the hydrated mixture, afforded a vesicle suspension that eluted from Sepharose CL-4B in a manner identical with that of pure EYPC vesicles prepared in a similar fashion.

Scheme 2



The membrane selectivity of the adenine-bearing copolymers was assessed in a 1:1 mixture of thymine-labeled and unlabeled liposomes. Copolymers were added to the lipid suspension at pH 7.6, with the molar concentration of adenine units adjusted to approximately that of the surface-bound thymine sites. Calcein was entrapped in *either* the thymine-labeled vesicle population *or* the unlabeled population, *but not in both*. The test of membrane selectivity then involves acidification and the question of whether release occurs from labeled vesicles under conditions where unlabeled vesicles retain their contents.

Figure 2 shows the results of these experiments. In the case of the copolymer containing 5.6 mol % adenine (Figure 2a), acidification of the suspension causes a rapid increase in the emission intensity at about pH 6.6. However, the copolymer does not discriminate between labeled and unlabeled vesicles, as indicated by essentially identical release profiles regardless of which population carried the dye. A similar experiment with the copolymer bearing 9 mol % adenine reveals a remarkable difference in the sensitivities of the labeled and unlabeled membranes (Figure 2b). In this case, if the dye is entrapped in the unlabeled liposomes, the release of calcein is nearly insensitive to acidification until pH 6.9, at which point the intensity of fluorescence progressively increases, reaching approximately 30% release at pH 5.27. However, if the dye is entrapped in the thymine-labeled liposomes, the fluorescence intensity increases sharply on acidification and reaches approximately 80% release at pH 5.3. Figure 2c shows that an increase in the concentration of adenine in the copolymer does not enhance discrimination between labeled and unlabeled liposomes; the release curves are displaced *less* for the 11 mol % copolymer than for the derivative carrying 9 mol % adenine. The reduced selectivity of the more heavily labeled copolymer probably reflects the reduced hydrophilicity of the chain (cf. Figure 1), which in turn increases nonspecific binding to the membrane surface.

The role of nonspecific interactions was also examined with unmodified PEAA. As shown in Figure 3, the adenine-free homopolymer effects release from both vesicular populations according to the same pH profile and in this respect behaves in a manner similar to that of the copolymer bearing 5.6 mol % adenine (Figure 2a). These observations indicate that the selective release of contents from thymine-labeled liposomes shown in parts b and c of Figure 2 is not simply a consequence of increased

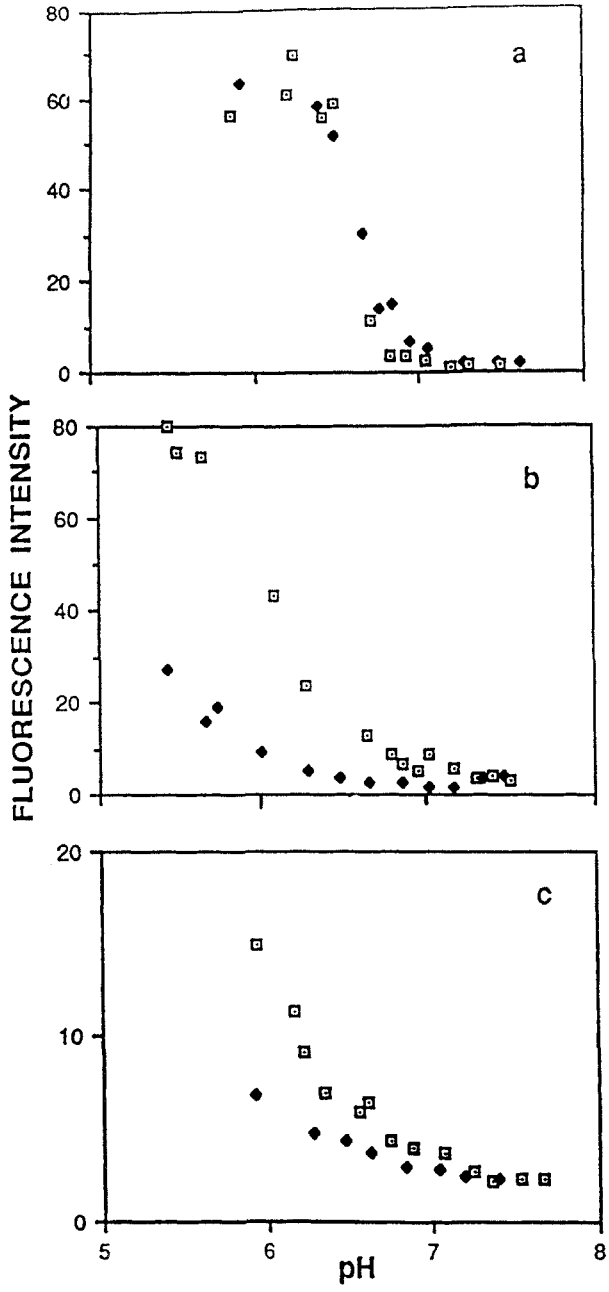


Figure 2. H^+-induced release of calcein from phospholipid vesicles constructed from a 1:1 mixture of (◆) thymine-labeled EYPC and calcein-loaded EYPC and (□) calcein-loaded, thymine-labeled EYPC and EYPC. Vesicles were suspended in Tris·HCl buffered solutions containing copolymers bearing adenine at concentrations of 5.6 (a), 9 (b), and 11.7 mol % (c). Suspensions were incubated at 10 °C for 24 h before measurement. Fluorescence intensity has been normalized to correspond to the percentage of dye released.

fragility of the thymine-labeled membranes. We propose instead that interactions between polymer-bound adenine and membrane-bound thymine groups account for the observed selectivity. It is not possible to determine on the basis of these experiments whether recognition occurs via hydrogen-bonding or base-stacking interactions. Furthermore, it remains to be determined whether discrimination among alternative bases can be accomplished in such simple systems. For example, does adenine-labeled polymer bind selectively to thymine-bearing surfaces in preference to those bearing cytosine or guanine?

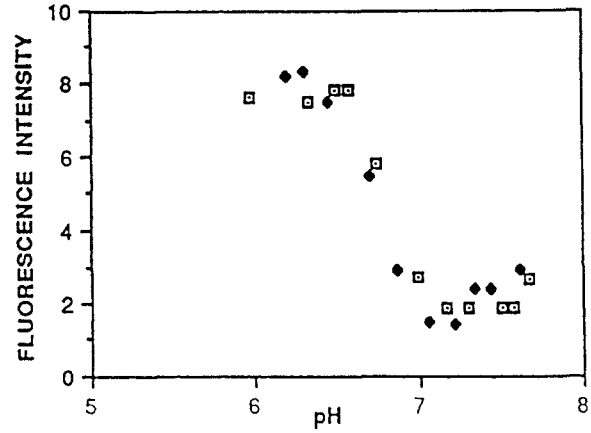


Figure 3. H^+-induced release of calcein from phospholipid vesicles constructed from a 1:1 mixture of (◆) thymine-labeled EYPC and calcein-loaded EYPC and (□) calcein-loaded, thymine-labeled EYPC and EYPC. Vesicles were suspended in Tris·HCl buffered solutions containing PEAA. Suspensions were incubated at 10 °C for 24 h before measurement.

Acknowledgment. This work was supported by the NSF Materials Research Laboratory at the University of Massachusetts. Financial support by the Direccion General de Investigacion Cientifica y Tecnica for M.B.M. is gratefully acknowledged.

References and Notes

(1) Sugai, S.; Nitta, K.; Ohno, N.; Nakano, H. *Colloid Polym. Sci.* **1983**, *261*, 159.
(2) Joyce, D. E.; Kuruscev, T. *Polymer* **1981**, *22*, 415.
(3) Fichtner, F.; Schonert, H. *Colloid Polym. Sci.* **1977**, *255*, 230.
(4) Tirrell, D. A.; Takigawa, D. Y.; Seki, K. *Ann. N.Y. Acad. Sci.* **1985**, *446*, 237.
(5) Seki, K.; Tirrell, D. A. *Macromolecules* **1984**, *17*, 1692.
(6) Devlin, B. P.; Tirrell, D. A. *Macromolecules* **1986**, *19*, 2465.
(7) Ferritto, M. S.; Tirrell, D. A. *Macromolecules* **1988**, *21*, 3117.
(8) Ahlers, M.; Ringsdorf, H.; Rosemeyer, H.; Seela, F. *Colloid Polym. Sci.* **1990**, *268*, 132. Ringsdorf and co-workers have examined surface recognition reactions involving nucleic acid bases bound to Langmuir monolayers.
(9) Ferritto, M. S.; Tirrell, D. A. *Macromol. Synth.* **1992**, *11*, 59.
(10) Schaeffer, H. J.; Gurwara, S.; Vince, R.; Bittner, S. *J. Med. Chem.* **1971**, *14*, 367. In this paper, the condensation of 1-(benzyloxy)-2-(chloromethoxy)ethane and 6-chloropurine gave 9-[[(benzyloxy)ethoxy]methyl]-6-chloropurine as an oil. In our hands, this compound crystallized from ethanol: mp 56–58 °C; ^{1}H NMR ($CDCl_3$, 80 MHz) δ 3.61 (m, 2H, CH_2O), 3.74 (m, 2H, CH_2O), 4.51 (s, 2H, CH_2Ph), 5.76 (s, 2H, NCH_2O), 7.30 (m, 5H, Ph), 8.28 (s, 1H, purine CH), 8.77 (s, 1H, purine CH). Anal. Calcd for $C_{15}H_{15}ClN_4O_2$: C, 56.52; H, 4.74; N, 17.57. Found: C, 56.50; H, 4.78; N, 17.25. Debenzylation was carried out in absolute ethanol using palladium hydroxide on carbon as catalyst. The mixture was hydrogenated (45 psi) at room temperature for 48 h; yield 78% (lit. yield 54%).
(11) Overberger, C. G.; Inaki, Y. *J. Polym. Sci. Polym. Chem. Ed.* **1979**, *17*, 1739.
(12) Charles, J.; Stewart, M. *Anal. Biochem.* **1980**, *104*, 10.
(13) Kalyanasundaram, K.; Thomas, J. K. *J. Am. Chem. Soc.* **1977**, *99*, 2039.
(14) Borden, K. A.; Eum, K. M.; Langley, K. H.; Tirrell, D. A. *Macromolecules* **1987**, *20*, 454.
(15) Schroeder, U. K. O.; Tirrell, D. A. *Macromolecules* **1989**, *22*, 765.
(16) Chen, T. S.; Thomas, J. K. *J. Polym. Sci., Polym. Chem. Ed.* **1979**, *17*, 1103.
(17) Chu, D. Y.; Thomas, J. K. *Macromolecules* **1984**, *17*, 2142.
(18) Thomas, J. K. *The Chemistry of Excitation of Interfaces*; American Chemical Society: Washington, DC, 1984; p 283.