MeOH/EtOH (10 mL), and the mixture was stirred under a balloon of hydrogen for 48 h. The solvent was evaporated and the residue was chromatographed with 5% EtOAc in hexane. Eluting first was the major 2*S*,4*R* isomer **18a** (155 mg, 25%) followed by a mixture of minor 2*S*,4*S* isomer **18b** and starting material **23** (395 mg, 62%). The mixture of minor isomer **18b** and **23** (375 mg, 0.9 mmol) in MeOH (6 mL) was resubjected to $Rh(P(C_6H_5)_3)_3Cl$ (140 mg, 0.15 mmol) in EtOH (4 mL) and stirred under a hydrogen atmosphere for 48 h. Removal of the solvent and chromatography yielded 315 mg of the diastereomers **18**.

(2*S*,3*R*,4*R*)-2-Amino-4-phenyl-3-pentanol (24a). (2*S*,3*R*,4*R*)-4-Phenyl-2-[*N*-(9-phenylfluoren-9-yl)amino]-3-pentanol (**23a**, 640 mg, 1.5 mmol) was dissolved in methanol (20 mL) and to this solution was added three drops of concentrated HCl followed by palladium on carbon (300 mg of 10%). The suspension was shaken in a Parr apparatus under hydrogen at 55 psi for 26 h. The mixture was filtered through Celite, which was washed with MeOH (20 mL) and EtOAc (20 mL), and the combined organic phase was concentrated to a solid which was partitioned between H_2O (15 mL) and toluene (10 mL). The aqueous layer was washed with toluene (2 × 10 mL) and then basified with saturated K_2CO_3 (20 mL), diluted with brine (10 mL), and extracted with 3/1 $CHCl_3$/isopropyl alcohol (3 × 15 mL). After drying and evaporating, the combined organic phase left **24a**: yield, 260 mg, 95%; mp 74-76 °C; ^1H NMR (CD_3OD) δ 1.21, (d, 3 H, J = 7.1), 1.27 (d, 3 H, J = 6.7), 2.77 (m, 1 H), 3.5 (m, 1 H), 3.84 (dd, 1 H, J = 9.5, 2.2), 7.2 (m, 5 H).

Preparation of Cyclic Carbamates. To a solution of (2*S*,3*S*,4*R*)- and (2*S*,3*S*,4*S*)-2-amino-4-phenyl-3-pentanol (**24b**, 70 mg, 0.47 mmol) in 3/1 THF/DMF (4 mL) at 0 °C was added carbonyldiimidazole (175 mg, 1.08 mmol), and the reaction vessel was flushed with nitrogen, and stoppered, and the contents were stirred for 14 h at 0 °C. Water (4 mL) was added to the suspension and the mixture was extracted with EtOAc (3 × 5 mL). The combined organic phase was washed with brine (10 mL), dried, and concentrated to an oil which was chromatographed (radial chromatography) with a gradient of 25-50% EtOAc in hexane as eluant. Concentration of the collected fraction yielded 40 mg (50%) of **25b** as a 2/1 mixture of diastereomers: TLC (3/1 EtOAc/hexane) R_f 0.39; ^1H NMR δ 0.96 (d, 3 H), 1.2 (d, 3 H), 1.42 (d, 3 H), 1.45 (d, 3 H), 2.92 (m, 1 H), 3.05 (m, 1 H), 3.58 (m, 1 H), 3.63 (m, 1 H), 4.17 (dd, 1 H, J = 8.5, 6), 4.28 (dd, 1 H, J = 5.9, 6), 7.3 (m, 5 H). Carbamate **25a** was prepared in a similar manner and purified on a plate of silica gel (1000-μm thickness): mp 154-155 °C; R_f 0.35; $[\alpha]^{20}_D$ 24° (*c* 0.75, $CHCl_3$); ^1H NMR δ 1.25 (d, 3 H, J = 7.2), 1.25 (d, 3 H, J = 6), 3.04 (m, 1 H), 3.9 (dq, 1 H, J = 6.4, 6.4), 4.6 (dd, 1 H, J = 10.6, 6.4), 7.3 (m, 5 H). Anal. Calcd for $C_{12}H_{15}NO_2$: C, 70.2; H, 7.4; N, 6.8. Found: C, 70.4; H, 7.5; N, 6.5.

***tert*-Butyldimethylsilyl Enol Ether 26.** To a solution of amino ketone **9** (200 mg, 0.54 mmol) in 6 mL of THF was added a 1 M solution of potassium hexamethyldisilazide in THF (0.76 mL) at -78 °C; the solution was stirred for 1 h at -78 °C and then *tert*-butyldimethylsilyl chloride (0.8 mmol) was added. The solution was stirred for 1 h at -78 °C, the solvent was evaporated, and the residue was chromatographed on a chromatotron with 5% EtOAc and 0.25% Et_3N in hexanes as eluant. **26**: ^1H NMR δ 0.0 (s, 3 H), 0.17 (s, 3 H), 1.15 (s, 9 H), 1.24 (t, 3 H, J = 6.2), 1.24 (d, 3 H, J = 6.7), 1.64 (m, 2 H), 2.2 (m, 2 H), 2.77 (q, 1 H, J = 6.7), 5.27 (t, 1 H, J = 7.1), 7.45-8.1 (m, 13 H).

Acknowledgment. We thank Adrian Pohlit, Undergraduate Research Participant, for his assistance in the preparation of intermediates and Edgar R. Civitello for help in the NMR work.

H^+-Induced Release of Contents of Phosphatidylcholine Vesicles Bearing Surface-Bound Polyelectrolyte Chains

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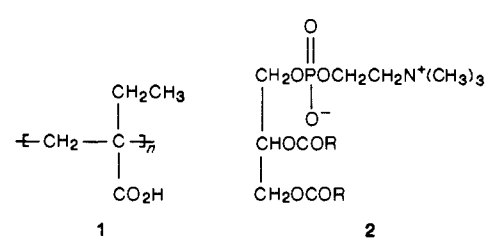
Abstract: A semisynthetic vesicular membrane was constructed by immobilization of a synthetic polyelectrolyte [poly(2-ethylacrylic acid)] on the surface of a phosphatidylcholine bilayer. Immobilization was accomplished via Michael addition of polymer-bound thiol functions to a reactive maleimide handle incorporated into the lipid membrane in the form of dimyristoyl-*N*-[[4-(maleimidomethyl)cyclohexyl]carbonyl]phosphatidylethanolamine. Semisynthetic membranes prepared in this way are sensitive to hydrogen ion concentration and are subject to large variations in permeability with small changes in pH. Rapid and quantitative release of vesicle contents can be achieved by mild acidification within the physiological pH range.

Although conformational transitions in membrane-bound macromolecules provide the most general and powerful mechanisms for chemical and physical signaling processes in biology, the molecular details of such processes are not well understood. For example, it is only very recently that even the primary structure of the synaptic vesicle protein synaptophysin has been reported,[1] despite the critical role that such membrane proteins must play in the exocytic release of neurotransmitters from their vesicular storage sites.[2] The complexity of the natural membrane precludes, for the present, a precise description of the role of protein conformation. We describe in the present paper the construction of a much simpler model system: a "semisynthetic" vesicular membrane in which a well-defined conformational transition in a surface-bound polyelectrolyte chain causes rapid and quantitative release of vesicle contents. The semisynthetic membrane thus shares with its natural counterparts important elements of structure and function, but retains a remarkable simplicity.

The design of the membrane builds on our previous work on the interactions of the hydrophobic polyelectrolyte poly(2-ethylacrylic acid) (PEAA, **1**) with aqueous dispersions of natural or synthetic phosphatidylcholines (**2**). PEAA in aqueous solutions



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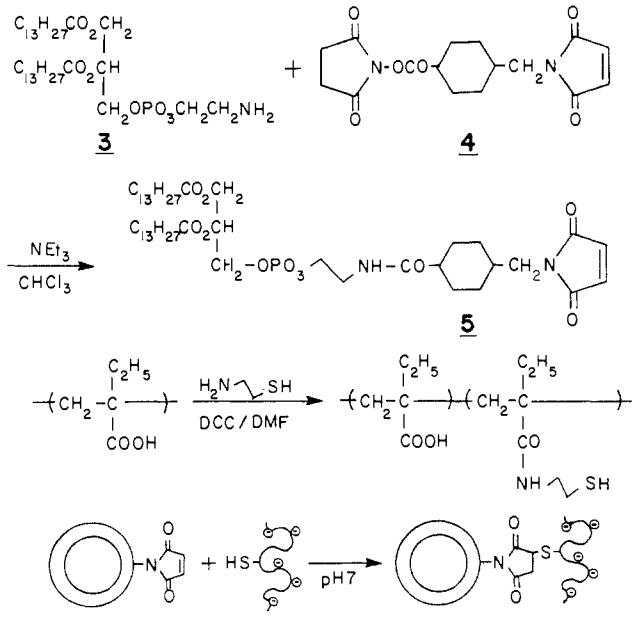
Scheme I



undergoes a well-defined conformational transition from an expanded, hydrophilic coil at high pH to a compact, globular structure upon acidification.[3] The globular from binds strongly to vesicular phosphatidylcholine membranes, and the resulting membrane reorganization leads to large and useful changes in membrane permeability. We have exploited this phenomenon to prepare suspensions of phosphatidylcholine vesicles that show permeabilities subject to regulation by pH, temperature, glucose concentration, or light.[4]

But the utility of such membranes—and their analogy to natural secretory vesicles—is limited by the reversibility of the polymer–bilayer interaction. In the present paper, we describe an improved membrane design, in which an essentially irreversible binding of PEAA to phosphatidylcholine membranes is accomplished by the method illustrated in Scheme I.[5] An amphiphilic "handle" is constructed by the condensation of dimyristoylphosphatidylethanolamine (**3**) with succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (**4**) to provide dimyristoyl-*N*-[[4-(maleimidomethyl)cyclohexyl]carbonyl]phosphatidylethanolamine (**5**). Lipid vesicles prepared from a 9:1 mixture of egg yolk phosphatidylcholine and **5** are then conjugated with a modified PEAA bearing a small number of thiol functions. The semisynthetic membranes prepared in this way are exquisitely sensitive to H^+, as a result of the pH-dependent conformational transition of the surface-bound polyelectrolyte chains. The preparation and behavior of such membranes are described herein.[6]

Experimental Section

Materials. Dimyristoylphosphatidylethanolamine (DMPE) and egg yolk phosphatidylcholine (EYPC) were purchased from Avanti Polar Lipids, Inc., and were used as received. Calcein and dithiothreitol (DTT) were used as received from Sigma Chemical Co. Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)], 2-aminoethanethiol, 4-pyrrolidinopyridine, 1,3-dicyclohexylcarbodiimide (DCC), fluorescamine, and succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (SMCC) were obtained from Aldrich Chemical Co. and used without further purification. Azobisisobutyronitrile (AIBN, Aldrich) was recrystallized from methanol. *N,N*-Dimethylformamide (DMF, Fisher Scientific Co.) was distilled under reduced pressure. Triton X-100 was purchased from Fisher Scientific Co. and used without purification. 2-Ethylacrylic acid was prepared by alkaline hydrolysis of methyl 2-ethylacrylate[7] and was fractionally distilled (bp 52 °C/1 mmHg). 1-Pyreneacrylic acid was prepared from 1-pyrenecarboxyaldehyde and malonic acid[8] and was recrystallized from methanol/acetone. Sephadex G-25 prepacked columns (PD-10) and Sepharose CL-6B were purchased from Pharmacia, Inc.

Table I. Introduction of Thiol Groups into PEAA[a]

PEAA, mg	4-PP[b] (mg)	2-AET[c], mg	DCC,[d] mg	SH content,[e] mol %
225.0 (2.25 mmol)	10.0 (0.07 mmol)	48.0 (0.62 mmol)	51.0 (0.25 mmol)	3.4
226.6 (2.27 mmol)	10.9 (0.08 mmol)	57.4 (0.74 mmol)	100.4 (0.49 mmol)	1.6
226.2 (2.26 mmol)	20.0 (0.14 mmol)	98.9 (1.28 mmol)	101.4 (0.50 mmol)	0.6

[a] Reactions were run for 24 h at room temperature in 22 mL of DMF. [b] 4-Pyrrolidinopyridine. [c] 2-Aminoethanethiol. [d] Dicyclohexylcarbodiimide. [e] Thiol content of product PEAA-SH.

Thiolated Poly(2-ethylacrylic acid) (PEAA-SH). Copolymerization of 2-ethylacrylic acid (2.8 g, 28 mmol) and 1-pyreneacrylic acid (22 mg, 0.08 mmol) was carried out in bulk in a vacuum-sealed ampule at 60 °C for 72 h with 0.5 mol % AIBN as initiator. The reaction mixture was dissolved in 20 mL of methanol and the copolymer precipitated by addition of the solution to 2 L of ether. Filtration and vacuum drying afforded 1.6 g (57%) of the copolymer as a white solid of M_n = 18 000 and M_W = 40 000 by aqueous gel permeation chromatography. The pyreneacrylic acid content of the copolymer was determined to be 0.1 mol % by ultraviolet (UV) absorption spectroscopy.

The copolymer was modified by the introduction of thiol groups as shown in Scheme I. 2-Aminoethanethiol (48 mg, 0.62 mmol), copolymer (225 mg, 2.25 mmol repeating units), and 4-pyrrolidinopyridine (10 mg, 0.07 mmol) were dissolved in 20 mL of DMF. Dicyclohexylcarbodiimide (DCC, 51 mg, 0.25 mmol) dissolved in 2 mL of DMF was added to the reaction mixture. After 24 h at room temperature, the precipitated dicyclohexylurea was removed by filtration, and the filtrate was concentrated to a volume of 5 mL. Dropwise addition of the concentrated solution into 1 L of stirred methanol afforded 70 mg of thiol-modified copolymer (PEAA-SH). Analysis of the copolymer by the use of Ellman's reagent indicated incorporation of 3.4 mol % thiol functionality. Three preparations of PEAA-SH were used in the course of this work; thiol content varied from 0.6 to 3.4 mol % within this set (Table I).

Dimyristoyl-*N*-[[4-(maleimidomethyl)cyclohexyl]carbonyl]phosphatidylethanolamine (Mal-PE). Mal-PE was prepared by a modification of the method of Martin and Papahadjopoulos[5] (Scheme I). DMPE (63.5 mg, 0.1 mmol) was dissolved in 5 mL of chloroform. Triethylamine (10 mg, 0.1 mmol) and SMCC (45 mg, 1.35 mmol) were added, and the reaction mixture was allowed to stand at room temperature for 24 h. After extraction of the reaction mixture with two 50-mL portions of 1% aqueous NaCl, the chloroform phase was dried with $CaCl_2$ and chromatographed on a 2 × 15 cm column of silica gel. Reaction products were eluted with 120 mL of $CHCl_3$, 120 mL of 20:1 $CHCl_3/CH_3OH$, and finally 160 mL of 15:1 $CHCl_3/CH_3OH$. Maleimide-containing fractions were identified by UV-absorption maxima at 301 nm and concentrated under reduced pressure. The yield of Mal-PE was 42 mg (52%); λ_{max} = 301 nm, $\epsilon = 6.4 \times 10^2$, TLC (silica gel, $CHCl_3/CH_3OH$ 15:1) produced a single spot, R_f 0.46. The ^1H NMR spectrum of Mal-PE in $CDCl_3$ showed a singlet at 6.7 ppm which was assigned to the two vinyl protons of the maleimide group.

Surface Conjugation of PEAA-SH. Egg yolk phosphatidylcholine (EYPC, 14 mg, 18 μmol) was dissolved in a mixture of 2 mL of chloroform and 1.8 mg (2 μmol) of Mal-PE. The chloroform was removed under reduced pressure to leave a thin lipid film. The lipid film was hydrated at room temperature by vortex agitation with 2 mL of 100 mM phosphate buffer, pH 7.4. The suspension was then sonicated in an ice bath with a Branson Sonifier 185 at a setting of 20 W for 30 min.

PEAA-SH (15 mg) was reduced with 2 mL of 50 mM DTT in 100 mM phosphate buffer, pH 7.4, for 15 min at room temperature. DTT was removed on a 1.5 × 5 cm Sephadex G-25 column, and the combined polymer fractions, which eluted between 2.5 and 5.5 mL and which displayed the characteristic fluorescence of the bound pyrene chromo-

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phore, were incubated with the vesicle suspension (prepared as described above) for 24 h at room temperature at pH 7.4. Separation of vesicles from unreacted PEAA-SH was accomplished by passage of the reaction mixture through a 2.6 × 20 cm Sepharose CL-6B column at 5 °C (eluent: 100 mM phoshpate buffer, pH 7.4). The column was preequilibrated with sonicated EYPC vesicles prior to use. Polymer and phospholipid concentrations in each 2.25-mL fraction were estimated by determination of fluorescence emission intensity (λ_{ex} = 345 nm, λ_{em} = 379 nm) and optical density (250 nm), respectively.

Thiol Analysis.[9] PEAA-SH was reduced with DTT and then separated from the reducing agent as described above, and fluorescence spectra of the polymer fractions were recorded on a Perkin-Elmer MPF-66 spectrophotometer. Polymer concentration was determined by comparison of the pyrene fluorescence intensity at 379 nm with a calibration curve prepared by using a series of polymer solutions of increasing concentration. PEAA-SH fractions (0.1 mL) were mixed with equal volumes of phosphate-buffered 10 mM DTNB solution, pH 8, and then diluted with 2 mL of buffer. As a reference, 0.1 mL of DTNB solution was added to 2.1 mL of buffer. The UV spectra of the sample and reference solutions were recorded on a Beckman DU-7 spectrophotometer. Thiol concentration was calculated from the absorbance at 412 nm, on the basis of a molar extinction coefficient of 1.36×10^4 M^{-1} cm^{-1}, for the DTNB-derived thiolate anion.

Amine Analysis.[10] Fluorescamine was dissolved in reagent-grade acetone (EM Science) at a concentration of 0.2 mg/mL. A 0.2-mL aliquot of this solution was added to 2 mL of reduced PEAA-SH at pH 7.4. Fluorescence spectra were recorded on a Perkin-Elmer MPF-66 spectrophotometer with λ_{ex} = 390 nm, an excitation slit of 5 nm, and an emission slit of 3 nm. Primary amine concentration was determined by comparison with a calibration curve of fluorescence emission intensity at 495 nm vs concentration of 2-aminoethanethiol.

Gel Column Chromatography of PEAA-SH. Polymer solutions were applied to a Sepharose CL-6B column (2.6 × 20 cm) and eluted with 100 mM phosphate buffer (pH 7.4) at 5 °C. Effluent fractions of 2.25 mL (45 drops) were collected and fluorescence intensity at 379 nm (excitation at 345 nm) was measured.

Dye Entrapment and Release. Vesicles prepared from a 9:1 mixture of EYPC and Mal-PE were hydrated as described above in a 50 mM Tris·HCl buffer, pH 7.0, that contained 200 mM calcein. PEAA-SH was then conjugated as described, and free calcein and PEAA-SH were removed on a Sepharose CL-6B column. Vesicle fractions 23 and 24 (elution volume 49.5–54 mL) were collected, and the time dependence of the fluorescence intensity was monitored at 530 nm at 25 °C under steady-state excitation at 495 nm. The initial pH of 7.0 was maintained for a period of 10–20 min, after which the pH was adjusted to 6.5 by addition of ca. 5 μL of 1 N HCl. The total fluorescence of each sample was subsequently determined after addition of 100 μL of 10% aqueous Triton X-100. Similar results were obtained in vesicle suspensions buffered with phosphate, rather than Tris·HCl.

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, Toyo Soda Mfg. Co.) and a differential refractometer detector. Calibration was done with nine PEO samples of narrow molecular weight distribution with average molecular weights in the range from monomer up to 10^6 (Toyo Soda). 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.[11] A similar solvent system with a pH of 11 gave the same results.

Results and Discussion

Preparation of Reactive Phosphatidylcholine Vesicles. The strategy adopted in this work for the binding of polyelectrolyte chains to phosphatidylcholine surfaces relies on the Michael addition of polymer-bound thiol groups to a small number of *N*-alkylmaleimide functions introduced to the membrane surface in the form of the modified phospholipid **5**. Compound **5** was readily prepared in a single step from commercially available dimyristoylphosphatidylethanolamine (**3**) and succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (**4**) (Scheme 1). Hydration of a 9:1 mixture of EYPC and **5**, followed by sonication of the hydrated mixture for 30 min at 20 W, afforded a vesicle suspension that eluted from Sepharose CL-6B in a manner identical with that of pure EYPC vesicles prepared in similar fashion.

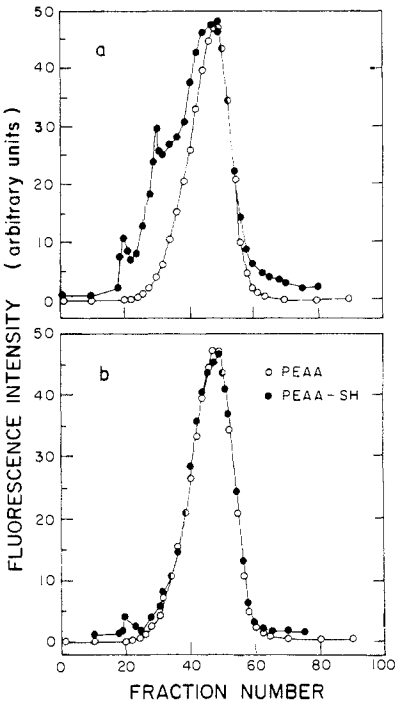

Figure 1. (a) Sepharose CL-6B chromatography of PEAA (○) and PEAA-SH (1.6 mol % SH) prior to reduction with DTT (●). (b) Sepharose CL-6B chromatography of PEAA (○) and PEAA-SH (1.6 mol % SH) subsequent to reduction with DTT (●).

Preparation of PEAA-SH. The introduction of thiol functionality into the polyelectrolyte was accomplished in a straightforward fashion by a carbodiimide-promoted coupling of PEAA with 2-aminoethanethiol. Table I summarizes the results of three such preparations of PEAA-SH; although some variation in the thiol content of the products is apparent, this method consistently resulted in modification of PEAA to the extent of 0.6–3.4 mol % of the polymer-bound carboxylic acid functionality. Given the number-average degree of polymerization (X_n) of the PEAA used in these experiments (X_n = 200 by aqueous gel permeation chromatography), the four samples of PEAA-SH listed in Table I carry an average of one to seven thiol groups per chain. Analysis of the thiol-modified polymers for pendant amine functionality (which would have been introduced by carbodiimide-promoted thioester coupling) placed the amine content below the limit of detection of the method (5×10^{-3} mol %, or less than 1% of the thiol content).

The introduction of thiol functionality into PEAA caused no irreversible changes in the molecular weight of the polyelectrolyte. Figure 1 shows the results of gel column chromatography of PEAA and a sample of PEAA-SH bearing 1.6 mol % thiol groups. The chromatogram of the modified polymer shows a distinct tail on the high molecular weight edge of the main elution peak (Figure 1a). That this is a result of the formation of reversible intermolecular disulfide linkages is illustrated in Figure 1b; treatment of PEAA-SH with the reducing agent DTT regenerates the original elution profile of PEAA. We therefore adopted a surface-conjugation protocol in which PEAA-SH was reduced with DTT immediately prior to the coupling reaction. Excess DTT was then effectively removed from PEAA-SH by gel filtration (as shown by an absence of reducing activity in the appropriate fractions in polymer-free controls), and surface conjugation was allowed to proceed.

Surface Conjugation of PEAA-SH. Attachment of PEAA-SH to the surface of EYPC vesicles was accomplished by incubation of the polymer with vesicles composed of a 9:1 mixture of EYPC and **5**. Reaction for 24 h at room temperature was followed by separation of bound and free polyelectrolyte by gel column chromatography. We observed no visible aggregation of vesicles

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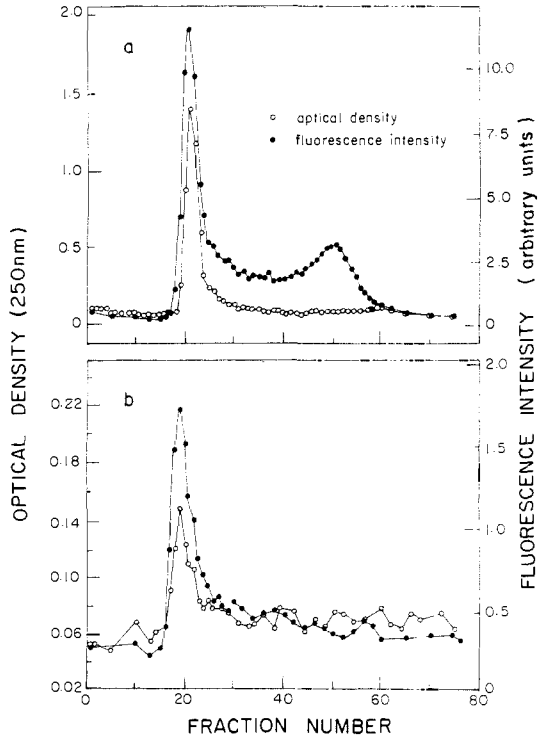

Figure 2. (a) Sepharose CL-6B chromatography of reaction mixture from surface conjugation of PEAA-SH (1.6 mol % SH) with 9:1 mixture of EYPC and **5**. Elution profiles were determined by measurements of optical density (○) and fluorescence intensity (●) to monitor elution of vesicles and PEAA-SH, respectively. (b) Rechromatography of fractions 20–23 from Figure 2a. Elution profiles determined by measurements of optical density (○) and fluorescence intensity (●).

Table II. Summary of Results of Conjugation of PEAA-SH and Phosphatidylcholine Vesicles

sample no.	EYPC, μmol	Mal-PE, μmol	PEAA-SH SH content, mol %	PEAA-SH SH, μmol[a]	PEAA-SH linked, μg/mol of lipid
1	12	1.3	3.5	0.67	64
2	10	1.3	3.3	0.29	47
3	18	2.0	1.6	0.75	77
4	19	1.0	0.6	0.65	40
5	10	1.2	0	0	5

[a] Amount of polymer-bound thiol functionality added to surface-conjugation reaction mixture.

treated with PEAA-SH, and the chromatographic elution profiles of polymer-treated vesicular dispersions were indistinguishable from those of polymer-free controls. Figure 2a shows typical elution profiles as monitored by optical density (which reports vesicle elution) and fluorescence emission intensity (which reports PEAA-SH by virtue of the polymer-bound pyrene chromosphore). The figure shows results for a sample of PEAA-SH bearing 1.6 mol % thiol functionality. Two elution peaks are apparent in the fluorescence profile; an estimated 40% of the polymer coelutes with vesicles in fractions 20–25 and the remaining polymer appears much later, in fractions 45–60. The latter fractions coincide with the elution of free PEAA-SH and so must contain polymer that was not successfully surface conjugated. Rechromatography of fractions 20–23 verifies the irreversible nature of the surface conjugation, in that no free PEAA-SH is detected on the second run (Figure 2b).

The amount of PEAA-SH bound to the membrane surface can be estimated from the intensity of fluorescence emitted from the polymer-bound pyrene chromophore. Table II summarizes the results of four coupling reactions, as well as a control experiment in which thiol-free PEAA was incubated with the lipid mixture. In each coupling experiment, approximately 50 μg of PEAA-SH was immobilized per mg of lipid. That this represents *covalent*

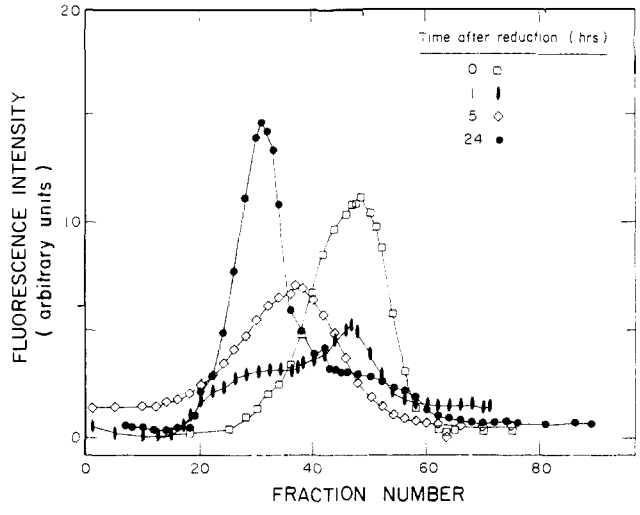

Figure 3. Sepharose CL-6B chromatography of PEAA-SH (1.6 mol % SH) subsequent to reduction by DTT. Time after reduction: 0 h (□), 1 h (❙), 5 h (◇), 24 h (●).

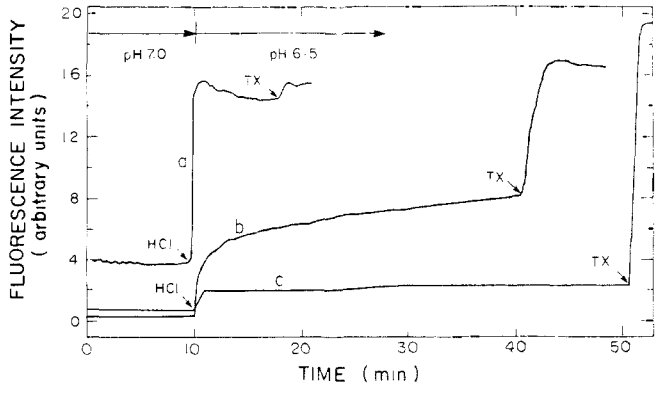

Figure 4. H^+-induced release of calcein from phospholipid vesicles constructed from (a) a 9:1 mixture of EYPC and **5** bearing surface-conjugated PEAA-SH (3.4 mol % SH), (b) a 9:1 mixture of EYPC and **5** treated with thiolfree PEAA, and (c) EYPC treated with thiolfree PEAA. In each case, the polymer-lipid mixture was subjected to Sepharose CL-6B chromatography prior to the dye release experiment. Calcein-loaded vesicles were incubated at pH 7.0 for 10 min, after which time HCl was added (arrow) to reduce the pH to 6.5. The total fluorescence of each sample was determined subsequently by addition of Triton X-100 (TX arrow).

attachment is clear from the results of the control experiment, in which the level of binding was reduced by 1 order of magnitude (Table II, sample 5). Covalent attachment is also consistent with the observed stability of the conjugate upon a second passage through the Sepharose column, as discussed above. Finally, the possibility that oxidative cross-linking results in fortuitous coelution of vesicles and PEAA-SH is ruled out by observation of the time evolution of the molecular weight distribution of the polymer (Figure 3). The figure shows results from a sample of PEAA-SH bearing 1.6 mol % thiol functionality. The molecular weight of PEAA-SH does indeed increase on standing subsequent to reduction by DTT and removal of the reducing agent, but the elution volume of the polymer remains substantially greater than that of the vesicle preparation, even after 24 h.

H^+-Induced Release of Vesicle Contents. Figure 4 shows the time dependence of the intensity of fluorescence emitted by the dye calcein initially entrapped at a concentration of 200 mM in PEAA-conjugated vesicles prepared from a 9:1 mixture of EYPC and **5**. The figure shows results for a sample of PEAA-SH bearing 3.4 mol % thiol functionality. The calcein fluorescence is self-quenched in the vesicle interior,[12] so that release of contents is

(12) Allen, T. J. In *Liposome Technology*; Gregoriadis, G., Ed.; CRC: Boca Raton, FL, 1984; Vol. 3, p 177.

revealed by an increase in emission as the quenching condition is relieved.

When calcein-loaded vesicle fraction 23 or 24 (Figure 2a)[13] is incubated at room temperature, pH 7.0, the fluorescence intensity remains constant over a period of 10 min. Acidification of the suspension to pH 6.5 then causes a rapid increase in emission intensity (Figure 4, curve a). Subsequent addition of the detergent Triton X-100 causes no further increase in fluorescence and thus confirms that the H^+-induced release of the dye from vesicles bearing surface-bound polyelectrolyte chains is both rapid and quantitative. The effectiveness of the polyelectrolyte is remarkable, given the modest concentration of polymer on the membrane surface (ca. 5% of the membrane by weight).

Figure 4 also shows the results of two control experiments. First, coincubation of EYPC and thiol-free PEAA, followed by passage of the mixture through the Sepharose column, afforded a vesicle suspension that was nearly pH-insensitive. Acidification of this suspension resulted in a fluorescence increase only 10% of that observed on subsequent addition of detergent (Figure 4, curve c). Chromatographic separation thus reduces the concentration of the polyelectrolyte to a level that is insufficient to cause membrane reorganization. In a second control experiment, thiol-free PEAA was incubated with the 9:1 mixture of EYPC and **5** and the mixture was chromatographed. In that case, the release of calcein on acidification was approximately 30% within 2 min and ca. 40% after 30 min (Figure 4, curve b). The presence of 10 mol % of **5** thus changes the behavior of the lipid membrane, either by increasing the adsorption of PEAA or by sensitizing the bilayer to reorganization by small amounts of adsorbed polymer. In any case, the results of the experiments summarized in Figure 4 demonstrate the effectiveness of a covalent surface conjugation procedure in the construction of pH-sensitive, semisynthetic phosphatidylcholine membranes.

(13) Similar experiments carried out on earlier vesicle fractions consistently resulted in partial, rather than quantitative, release of entrapped calcein upon acidification. We believe this to be a result of variations in vesicle structure (e.g., in the number of concentric bilayers), which would correlate with vesicle size and elution volume. We have not pursued this point. Selection of vesicle fraction 23 or 24 assured reproducible, rapid, and quantitative release of contents.

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Nitrogen Protonation of *N*-Nitrosodimethylamine†

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Abstract: Evidence for the presence of the $Me_2N(H)NO^+$ ion at kinetically significant concentrations in aqueous solutions of *N*-nitrosodimethylamine (NDMA) at pH $\leq$ 2 has been found. To obtain this evidence, the methyl group syn to the oxygen of NDMA was selectively deuteriated and the rate of $Z \rightleftarrows E$ equilibration in the resulting NDMA-d_3 was measured by nuclear magnetic resonance spectrometry as a function of pD. The reaction was first order in $[D^+]$, with the plot of observed first-order rate constants versus $[D^+]$ having a slope of $k/[D^+] = 10^{-2}$ M^{-1} s^{-1} at 3 °C and an ionic strength of 0.2 M. The observed rate data were used to estimate K_a for N-protonated NDMA as 10^{12}–10^{13} M by assuming that the rates of rotation about the N–N bond in $Me_2N(H)NO^+$ and of its deprotonation are similar to those for the isoelectronic N-protonated carboxamide function. The trisubstituted nitrogen of the nitrosamine thus appears to be several orders of magnitude less basic than that of the carboxamides.

N-Nitrosoammonium ions such as **2** (Scheme I) have been implicated in several important transformations, including diazotization and deamination of primary amines,[1,2] nitrosation[1,2] and transnitrosation[2,3] of secondary amines, and photolysis[4] as well as protolysis,[5,6] rearrangement,[7] and mass spectral fragmentation[8] of carcinogenic *N*-nitroso compounds, but little is known about the lifetimes of these ions in solution. Indeed, many of the above reactions are so rapid as to suggest that steady-state concentrations of **2** must be immeasurably small, with a wealth of published data firmly establishing the oxygen atom, rather than nitrogen, as the preferred site of stable electrophilic attachment to the potentially ambident nitrosamino function.[6,9-12] Thus, the O-protonated tautomer **3a** is the only conjugate acid of *N*-nitrosodimethylamine (**1a**) that is macroscopically observable by nuclear magnetic resonance (NMR) spectrometry in acidic solutions of **1a**.[6,9,12]

We now present evidence that reversible N-protonation of the simplest nitrosamine, **1a**, does occur to a detectable extent in dilute

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