Polymerization of Vesicles Composed of *N*-(4-Vinylbenzoyl)phosphatidylethanolamine

Glenn E. Lawson,[†,||] J. J. Breen,[‡] Manuel Marquez,[§] Alok Singh,[||] and Bradley D. Smith*,[†]

Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556-5670, Department of Chemistry, Indiana University−Purdue University at Indianapolis, Indianapolis, Indiana 46202-3274, The Nanotechnology Lab, Kraft Foods R & D, 801 Waukegan Road, Glenview, Illinois 60025, and Naval Research Laboratory, Center for Bio/Molecular Science and Engineering, Code 6930, Overlook Avenue 4555, Washington, D.C. 20375

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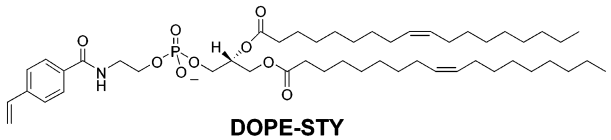
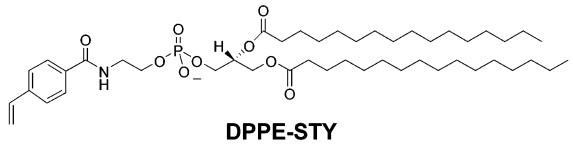
Introduction

Polymerized vesicles have a range of potentially useful applications in technological areas such as drug delivery, biosensing, biosynthesis, and microdevices.[1−5] A number of strategies have been employed to produce polymerized vesicles, but the most common approach is to utilize an amphiphile whose structure includes a polymerizable unit.[5] Commonly used unsaturated monomers include vinyl, dienoyl, acetylene, and diacetylene derivatives.[6] A concern with many of these compounds is their biocompatibility, high synthetic cost, and limited scalability. Our goal is to develop a series of inexpensive, polymerizable phospholipids that can be prepared on an industrial scale from readily available natural sources. We were attracted to the synthetic strategy of condensing polymerizable groups with phosphatidylethanolamines. This produces *N*-acyl-phosphatidylethanolamines (*N*-acyl-PEs) which are known to have a strong propensity to form vesicles.[7] Surprisingly, we can find no published report of an *N*-acyl-PE with a polymerizable headgroup. As a simple synthetic starting point, we chose to attach a styryl group.[8] Previous studies of styrene-modified amphiphiles have attached styryl groups to the ends of the nonpolar chains[9−11] and in two cases to the polar headgroup.[12,13] The polymerized vesicles were found to have enhanced structural stability and to resist the lysis action of detergents. On the other hand, it is known that polymerization can sometimes produce phase separation which may lead to vesicle destabilization and self-fusion.[14,15] Thus, the goals of this present study were to establish if vesicles composed of *N*-acyl-PEs can be polymerized and to ascertain if polymerization leads to altered vesicle properties. We present data demonstrating that vesicles composed of the phospholipids DOPE-STY or DPPE-STY can indeed be polymerized and that the polymerized vesicles have enhanced mechanical strength but do not appear to have altered membrane permeability.



DOPE-STY



DPPE-STY

Experimental Section

Synthesis of DOPE-STY and DPPE-STY. *DOPE-STY.* 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (100 mg, 0.081 mmol) and triethylamine (50 μL) were added dropwise to a mixture of 4-vinylbenzoic acid (24 mg, 0.174 mmol), 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride (34 mg, 0.180 mmol), and 1-hydroxy-7-azabenzotriazole (22 mg, 0.162 mmol) in dry chloroform (39 mL)/dimethylformamide (4 mL) over 30 min under an atmosphere of dry N_2 in the dark. The reaction was stirred at room temperature overnight and then evaporated to leave an oily residue. The residue was redissolved in $CHCl_3$, and the solution was washed with 4% Na_2CO_3, 10% aq. HCl, water, and then dried over Na_2SO_4. Column chromatography using silica gel and $CHCl_3/CH_3OH$ (5:1) afforded 100 mg (87%) of pure DOPE-STY ($R_f = 0.3$). ^{1}H NMR (500 MHz $CDCl_3$): $\delta =$ 7.91 (d, 2H, $J = 7.15$ Hz), 7.54 (d, 2H, $J = 7.69$ Hz), 6.75 (dd, 1H, $J = 11.0$ Hz), 5.91 (d, 1H, $J = 17.6$ Hz), 5.32 (d, 1H, $J = 7.00$), 5.22 (s, 4H), 5.21 (s, 1H), 4.33−3.79 (m, 8H), 2.29 (q, 4H, $J = 7.0$ Hz), 2.18 (s, 4H), 1.52 (m, 4H), 1.39 (m, 4H), 1.28 (s, 20H), 0.92 (t, 6H, $J = 6.5$ Hz). ^{13}C NMR (125 MHz $CDCl_3$): 17.0, 21.5, 22.5, 23.0, 30.0−31.0, 31.5, 34.0, 37.0, 40.0, 62.0−64.0, 70.0, 118.0, 126.0, 128.0, 130.0, 131.0, 136.0, 140.0, 172.0, 173.0. ESMS: 873.8 ($M + H^+$).

DPPE-STY. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (200 mg) was subjected to the above reaction and purification procedure to give 243 mg (98%) of pure DPPE-STY ($R_f =$ 0.35). ^{1}H NMR (500 MHz $CDCl_3$): $\delta = 7.91$ (d, 2H, $J = 7.2$ Hz), 7.54 (d, 2H, $J = 7.7$ Hz), 6.75 (d, 1H, $J = 11.0$ Hz), 5.92 (d, 1H, $J = 17.6$ Hz), 5.31 (d, 1H, $J = 7.0$), 5.22 (s, 1H), 4.33, 4.12, 3.97, 3.79 (m, 8H), 2.29 (q, 4H, $J = 6.4$ Hz), 2.18 (s, 4H), 1.52 (m, 4H), 1.39 (m, 4H), 1.28 (s, 20H), 0.92 (t, 6H, $J = 6.5$ Hz). ^{13}C NMR (125 MHz $CDCl_3$): 17.0, 21.0, 22.0, 34.0−36.0, 37.0, 41.0, 61.0, 62.0, 118.0, 128.0, 130.0, 132.0, 138.0, 141.0, 168.0, 172.0. FAB-MS: 844.5 ($M + Na^+$).

* Corresponding author. E-mail: smith.115@nd.edu.
† University of Notre Dame.
‡ Indiana University−Purdue University at Indianapolis.
§ Kraft Foods R & D.
|| Naval Research Laboratory.

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Vesicle Preparation and Polymerization. Vesicles composed of DOPE-STY or DPPE-STY were prepared at a lipid concentration of 5 mg/mL. An appropriate aqueous solution was added to a dried film of phospholipid, and the resulting dispersion was agitated using a vortex mixer. Pyrex glass helices were included to facilitate removal of the lipid film from the sides of the flask. After 5 freeze−thaw cycles, the vesicles were extruded 21 times through 100 nm nucleopore membrane filters at room temperature for DOPE-STY and 60 °C for DPPE-STY. Photopolymerization reactions were carried out in a thermostated quartz cell placed at a 10 cm distance from a 150 W xenon lamp.

Fluorescence Measurements. Fluorescence experiments were performed on a Perkin-Elmer luminescence spectrometer (LS 50B). Two probes were used to measure anisotropy: diphenylhexatriene (DPH) for the highly hydrophobic interior region and trimethylammonium diphenylhexatriene (TMA-DPH) for the interfacial region. Anisotropy measurements were obtained over the temperature range from 10 to 90 °C with a lipid to probe mole concentration ratio of 200:1. Calcein leakage was determined for unpolymerized and polymerized vesicles at 25 °C. Leakage was monitored for 90 min, after which 100 μL of a 20% Triton X-100 solution was injected to lyse the vesicles and give the fluorescence intensity for 100% calcein leakage.

Vesicle Imaging. Vesicle size and dispersity (based on a Gaussian distribution) was determined by dynamic light scattering (DLS) using a Coulter N4 Plus instrument with a detection angle of 90°.

The atomic force microscope (AFM) imaging experiments used atomically flat mica substrates that were chemically treated to have a positive surface charge. Approximately 60 μL of polymerized or unpolymerized vesicle suspension was deposited on a 2 mm by 2 mm piece of mica which had been treated with aminopropyltriethoxysilane (20 min in 1% ethanol solution). The vesicles were allowed to interact with the substrates for 1 h before transfer to the microscope followed by a gentle rinsing. AFM images were obtained at 25 °C in a closed liquid cell containing 10 mM NaCl using a Nanoscope III AFM operated in the contact mode (Digital Instruments, Santa Barbara, CA). The AFM was equipped with a 15 μm by 15 μm scanner (type E) and standard silicon nitride cantilevers (nominal force constant of 0.12 N/m and used as received). Conditions of minimal loading force, 1−5 nN, were employed while the scan rate was in the range of 4−7 Hz.

The transmission electron microscope (TEM) images were acquired using a Hitachi 8100 Zeiss EM-10 high-resolution transmission electron microscope at 60 kV. A drop of vesicle suspension was placed on a 200 mesh copper Formvar/carbon grid. A second drop of 1% uranyl acetate in water was contacted with the first for 10 s; then excess solution was removed by lightly touching the side of the grid with a tissue.

Results and Discussion

Phospholipid Monomer Synthesis and Vesicle Polymerization. The two *N*-acyl-PEs, DOPE-STY and DPPE-STY, were prepared in yields of 87% and 98%, respectively, by simply condensing the appropriate phosphatidylethanolamine with commercially available 4-vinylbenzoic acid in an organic solvent. Both DOPE-STY and DPPE-STY are stable upon storage at low temperature in the dark.

Vesicles were prepared by extrusion through filters with 100 nm pores. Vesicle polymerization was conveniently monitored by ^{1}H NMR analysis of samples that had been freeze-dried and then taken up in an appropriate organic solvent. Direct irradiation of DPPE-STY vesicles with a 150 W xenon lamp at 25 °C for 2 h produced very little polymerization. Therefore, the water-soluble initiator 2,2′-azobis(2-methyl-propionamidine) dihydrochloride (AAPH) was added to the DPPE-STY vesicles in the [phospholipid]/[initiator] mole ratio of 7:1. Irradiation of this vesicle/initiator sample for 90 min at 25 °C also produced very little (<10%) polymerization. Repeating the experiment at 65 °C resulted in complete consumption of the monomer over a 1 h period. The ^{1}H NMR spectra in Figure 1 show how the styryl vinylic signals disappear over time and are replaced by broadened polymeric signals in the spectral region of 7.3−8.2 ppm.

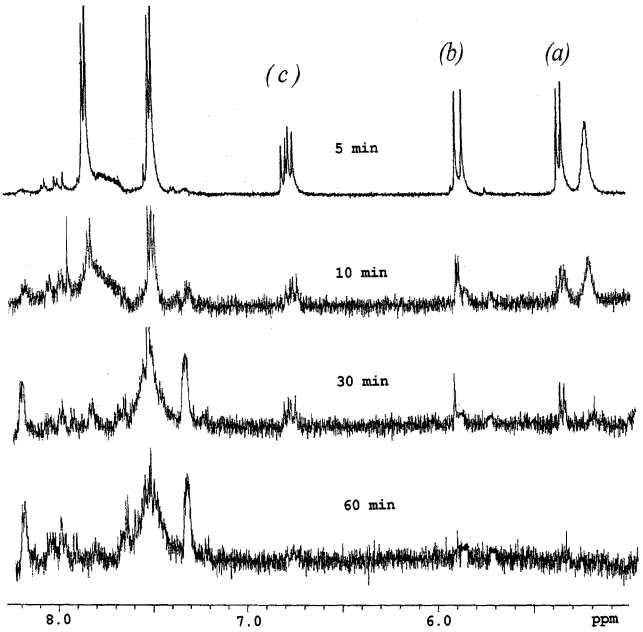

Figure 1. ^{1}H NMR spectra of DPPE-STY vesicles at 65 °C as a function of photolysis time. The proton signals denoted as (a), (b), and (c) are the vinylic protons of DPPE-STY.

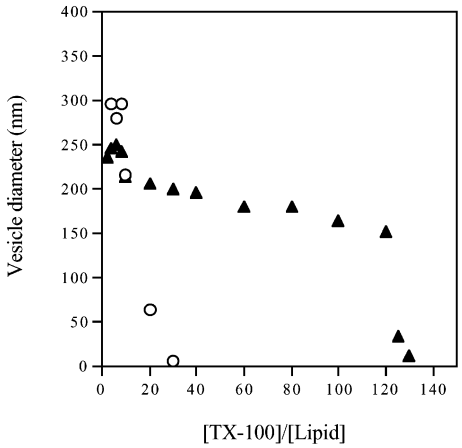

Figure 2. Diameter of DOPE-STY vesicles as a function of the Triton X-100 (TX-100) to lipid concentration ratio: unpolymerized vesicles (circles); polymerized vesicles (triangles).

The polymerization of DOPE-STY vesicles is significantly more facile. For example, photolysis at 25 °C for 90 min in the presence of AAPH results in the complete consumption of monomer. At 65 °C, the polymerization reaction is complete in less than 30 min. Qualitative evidence of polymer formation is the observation that the irradiated vesicles are insoluble in $CDCl_3$ whereas the unpolymerized vesicles are readily dissolved in $CDCl_3$. As reported by others,[13] the amphiphilic nature of the polymeric products prevents an assessment of the degree of polymerization by conventional size exclusion methods.

Characterization of Vesicle Size. Vesicle sizes were analyzed before and after irradiation using DLS. As seen in Figure 2, the average particle diameters for the unpolymerized and polymerized DOPE-STY vesicles are 250−300 nm. Treatment of the unpolymerized vesicle sample with increasing amounts of the nonionic detergent Triton X-100 leads to a sharp decrease in particle size

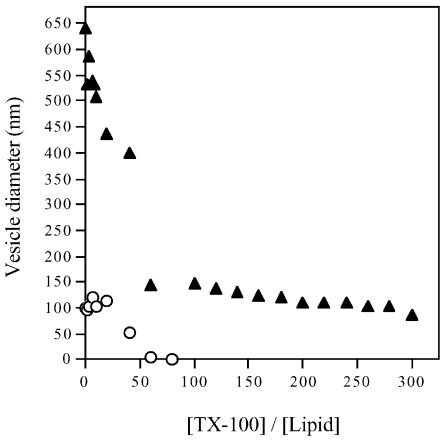

Figure 3. Diameter of DPPE-STY vesicles as a function of the Triton X-100 (TX-100) to lipid concentration ratio: unpolymerized vesicles (circles); polymerized vesicles (triangles).

until the vesicles are completely lysed at a [Triton X-100]/[lipid] ratio of 35. By comparison, the polymerized DOPE-STY vesicles show enhanced stability to detergent. The profile in Figure 2 shows a gradual decrease in particle diameter as the [Triton X-100]/[lipid] ratio is increased to 120, at which point sudden and complete lysis occurs. A similar trend is seen in the case of the DPPE-STY vesicles (Figure 3). About 50 molar equiv of Triton X-100 was needed to lyse the unpolymerized vesicles, which initially had an average diameter of 100 nm. Conversely, the vesicles of the polymerized DPPE-STY system initially have an average diameter of 650 nm, which is reduced to 140 nm upon addition of 50 equiv of Triton X-100 and then stays relatively constant up to a ratio [Triton X-100]/[lipid] of 300. It appears that the polymerized DPPE-STY vesicles are initially aggregated, and although the detergent can break up the aggregate it is unable to lyse the individual vesicles. The DLS data in Figure 3 illustrate the enhanced mechanical stability that is gained by phospholipid polymerization.

Since other explanations could potentially explain the large average size of the polymerized DPPE-STY vesicles (e.g., vesicle fusion), they were investigated further by acquiring AFM and TEM images. Depicted in Figure 4 are two topological AFM images of unpolymerized and polymerized DPPE-STY. In the case of unpolymerized vesicles, only a few vesicle-size objects (~150 nm in diameter, 50−100 nm in height) are seen in the 4 μm by 4 μm field. In contrast, for the case of polymerized vesicles, a larger number of vesicle-size spherical objects are observed clustered together and distributed throughout the 5 μm by 5 μm field. Higher resolution images of unpolymerized and polymerized DPPE-STY vesicles were gained from TEM analysis and are depicted as Figures 5 and 6. TEM images of the unpolymerized sample (Figure 5) show a uniform distribution of 100 nm diameter vesicles, whereas images of the polymerized sample (Figure 6) show the 100 nm vesicles aggregated into larger clumps. The data from both sets of microscopy experiments are in general agreement with the DLS data which indicates that DPPE-STY polymerization leads to vesicle aggregates. These vesicle aggregates appear to be held together by noncovalent forces since they are readily dispersed by detergent (see Figure 3).

Vesicle Leakage. Although the polymerized vesicles are mechanically stronger than the unpolymerized vesicles, there appears to be no difference in vesicle permeabilities as judged by calcein leakage experiments. The leakage experiments started with vesicles containing calcein,

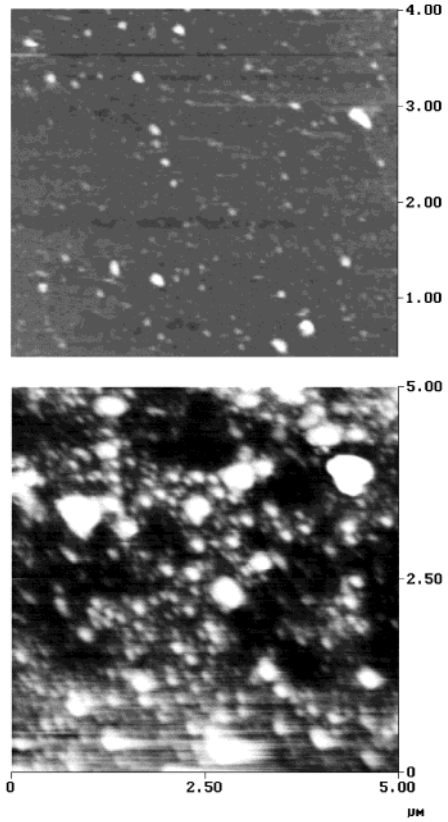

Figure 4. AFM image of DPPE-STY vesicles: unpolymerized (top); polymerized (bottom).

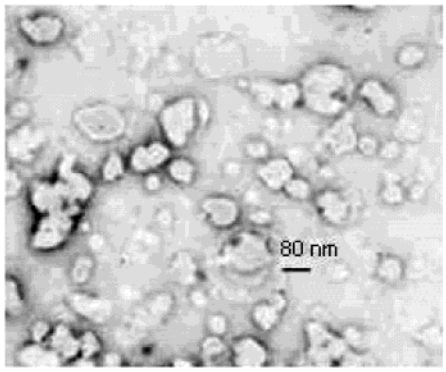

Figure 5. TEM image of unpolymerized DPPE-STY vesicles. Magnification, 40 000.

which is a self-quenching fluorescent dye. As the calcein leaks from the vesicles, its fluorescence emission intensity increases. In the case of DOPE-STY, approximately 35% of the calcein leaks from both unpolymerized and polymerized vesicles after 90 min at 25 °C. Similarly, in the case of DPPE-STY, approximately 18% of the entrapped calcein leaks from unpolymerized and polymerized vesicles after 90 min at 25 °C. The slower calcein leakage from the vesicles with saturated acyl chains is in agreement with literature expectations.[16] Our observation that polymerization does not alter membrane permeability is in line with the general trend that simple linear polymerization does not alter membrane permeability to a great extent compared to cross-linked polymerization.[6]

Membrane Fluidity. The radical-initiated polymerization of the DOPE-STY proceeds smoothly at 25 °C,

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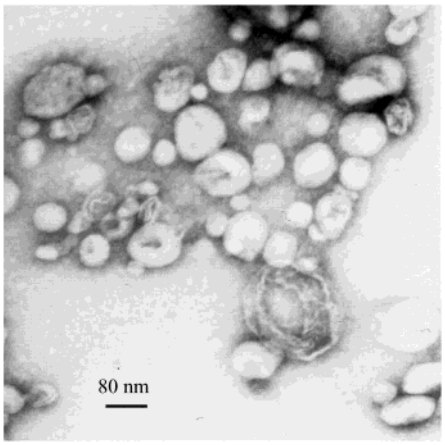

Figure 6. TEM image of polymerized DPPE-STY vesicles. Magnification, 60 000.

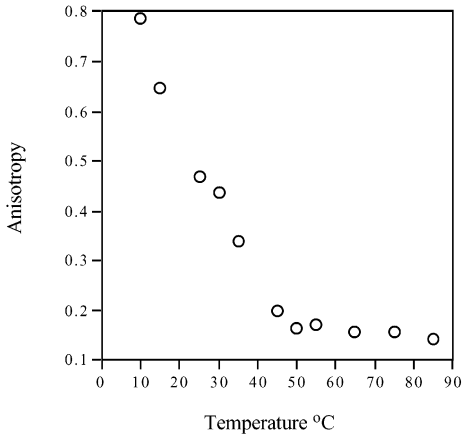

Figure 7. DPH anisotropy in unpolymerized DPPE-STY vesicles.

whereas the polymerization of DPPE-STY requires a higher temperature of 60 °C. This observation suggests that polymerization in the DPPE-STY system is inhibited by slower diffusion (lateral or transverse) through the vesicle membrane. To prove that membrane fluidity in DPPE-STY vesicles does indeed increase significantly upon heating, the anisotropy of two lipophilic fluorescent probes embedded in the vesicle membrane was measured as a function of temperature (Figures 7 and 8). The fluorescence anisotropy of the highly lipophilic probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), is generally thought to reflect the fluidity in the lipophilic interior of a bilayer membrane, whereas the more amphiphilic analogue, trimethylammonium diphenylhexatriene (TMA-DPH), is thought to reside closer to the interfacial region of the membrane. In both cases, the anisotropy is high at 10 °C,

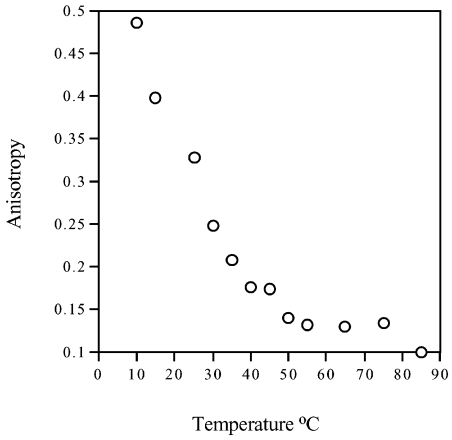

Figure 8. TMA-DPH anisotropy in unpolymerized DPPE-STY vesicles.

indicating only limited probe rotation in the excited lifetime. But the anisotropy drops sharply as the temperature is increased to 40 °C and then stays constant as the temperature is increased to 85 °C. These results suggest that the DPPE-STY membrane is in a fluid phase at temperatures above 40 °C and that the increase in polymerization for the DPPE-STY membrane at 60 °C is promoted in part by the increase in membrane fluidity.

Summary

Vesicles composed of an *N*-acyl-PE derivative with a single styrene headgroup do not readily undergo photopolymerization; the presence of a radical initiator is needed. The polymerization produces linear polymers within the vesicle membrane, which enhances the mechanical stability of the vesicles and inhibits detergent-induced lysis, but polymerization does not alter membrane permeability. This agrees with the literature expectation that linear polymerizations cause relatively modest changes in bilayer properties,[6] whereas cross-linking polymerizations can significantly decrease fluidity and permeability.[17−19]

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(19) It is known that radical polymerizations in bilayers tend to terminate by coupling of an initiator with the reactive polymer terminus (ref 6). It is possible that the high fraction of initiator used in these experiments produced low degrees of polymerization, which could be another reason for the absence of a membrane permeability change.