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Design and Characterization of Synthetic Bilayer Vesicles with a Polymerized Inner Bilayer Leaflet

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Four new phospholipid derivatives containing a β-nitrostyrene unit linked to the phosphate headgroup have been synthesized and converted into unilamellar vesicles. The vesicles were characterized by freeze-fracture transmission electron microscopy (FFEM), cryo-scanning electron microscopy (cryo-SEM), light scattering, and differential scanning calorimetry (DSC). At pH 11.5 and at $T < T_m$, the β-nitrostyrene unit can be cleaved specifically *exo*-vesicularly. The cleavage process was analyzed in terms of a model providing independent rates of hydrolysis and *flip-flop*. Furthermore, the β-nitrostyrene units can be polymerized yielding a polystyrene derivative. Neither the *exo*-vesicular cleavage nor the polymerization influence the morphology of the vesicles (FFEM, cryo-SEM, and light scattering). *Exo*-vesicular cleavage followed by polymerization of the remaining *endo*-vesicular β-nitrostyrene units results in the first examples of vesicles containing a polymer-immobilized inner bilayer leaflet and a "monomeric" outer bilayer leaflet. Such vesicles are of interest for studies of the mechanism of fusion of bilayers formed from synthetic amphiphiles.

Introduction

Many of the molecular details of the rearrangement of the phospholipid bilayer membrane during the ubiquitious process of membrane fusion are still unknown. The approach of charged bilayers from equilibrium distances, the establishment of a contact zone between the different bilayers, and the subsequent rearrangement of lipid molecules are known to involve high Gibbs energy barriers.[1] Consequently, the lifetimes of intermediate structures will be relatively short and identification of transient bilayer conformations and/or structures that are formed during the fusion event is difficult. These structures have been the subject of theoretical considerations,[2−5] and particularly, Siegel, Talmon, and co-workers[6] collected experimental evidence for transient intermediates using sophisticated cryo-electron microscopic techniques.

Membrane mimetic systems have been fruitful models to gain more insight into many membrane characteristics,[7] including the fusion process.[8] Since bilayers repel each other and fusion implies an approximation to contact distance, fusogenic agents (*e.g.* divalent metal ions for phosphate-based amphiphiles,[9] dianions for positively charged amphiphiles,[10] water-soluble polymers in various cases,[11] and proteins *in vivo*) are normally required to reduce inter-bilayer repulsion and bring the bilayers together. In certain cases, this aggregation phenomenon has been resolved kinetically; it preceeds the actual rate-determining fusion process.[12] For Ca^{2+}-induced fusion of phosphate amphiphiles, formation of "trans" inter-bilayer complexes has been proposed.[10a,b] For triggering the fusion step, bilayers must depart (albeit locally) from the equilibrium bilayer structure. Reports of lipidic particles (contact sites of fusing bilayers) have been frequent.[13] However, due to their short lifetime, the molecular structure of these contact sites remains elusive. Siegel and Chernomordik and co-workers[2,3,6] contend that the outer leaflets make contact and merge, leading to a short-lived hemifusion intermediate with a continuous outer bilayer leaflet and two separate inner bilayer leaflets. At the fusion contact site, a nonlamellar structure is formed. Whether inverted micellar structures,[2] "stalks",[3] local interdigitated structures,[4] or lipids with extended alkyl chains[5] participate awaits further study. Nevertheless, the order of events during bilayer fusion—aggregation, outer leaflet fusion (hemifusion), inner leaflet fusion and pore formation (fusion)—has been confirmed by fluorescence and capacitance measurements, and the interference of stalklike intermediates has been substantiated from the effect of varying lipid composition on bilayer fusion.[14]

Scheme 1. Hydrolytic Cleavage of 4-Hydroxy-β-nitrostyrene

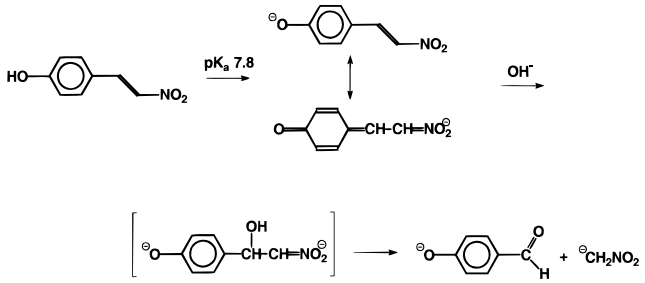

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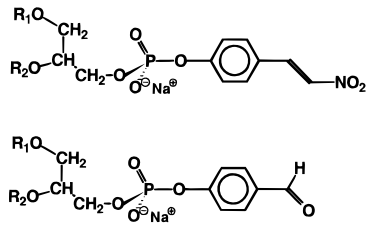
Chart 1. 1,2-Bis(*n*-alkyloxy)prop-3-yl 4-(*β*-Nitrovinyl)phenyl Phosphates and 1,2-Bis(*n*-alkyloxy)prop-3-yl-4-(Formyl)phenyl Phosphates



$R_1 = R_2 = n\text{-}C_{12}H_{25}$ (DDPBNS, 3)
$n\text{-}C_{14}H_{29}$ (DTPBNS, 4)
$n\text{-}C_{16}H_{33}$ (DHPBNS, 5)
$n\text{-}C_{18}H_{37}$ (DOPBNS, 6)

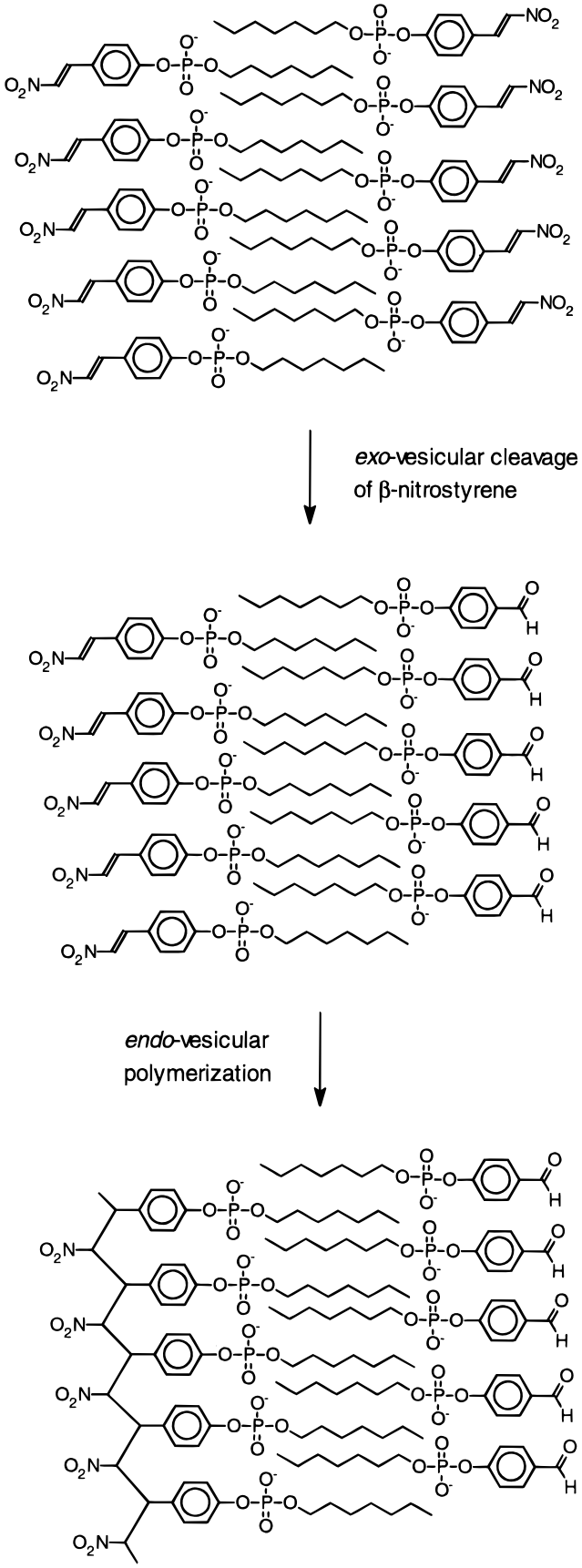
$R_1 = R_2 = n\text{-}C_{16}H_{33}$ (DHPPFP, 8)

However, due to the rapid "collapse" of all intermediate structures, experimental characterization is very difficult.

Our aim is to develop new bilayer mimics that stabilize fusion intermediates which possess a fused, continuous outer bilayer leaflet and two separate, not-yet-fused inner bilayer leaflets. In other words, our studies have been aimed at examining the fusogenic behavior of bilayers with a fusogenic outer leaflet and a nonfusogenic inner leaflet. This poses two challenging problems: firstly, it is uncertain what exactly determines fusogenicity, and secondly, this approach demands vesicles with a high degree of surface differentiation. However, it is well established in the literature that polymerization of amphiphiles in the bilayer improves vesicle stability and strongly reduces lipid mobility within the bilayer.[15] It is reasonable to assume that polymerization may in this way inhibit vesicle fusion.[16] Thus, we set out to develop vesicles containing a polymerized inner bilayer leaflet and a monomeric outer bilayer leaflet.

In this paper we report vesicles formed from a novel class of synthetic phospholipids containing a bifunctional *β*-nitrostyrene (BNS) unit. The *β*-nitrostyrene unit is covalently attached to the phosphate headgroup and will reside at the vesicle surface, partitioned between the inner bilayer leaflet (*endo*-surface) and the outer bilayer leaflet (*exo*-surface). Surface differentiation of the vesicles was obtained by two simple reactions of the *β*-nitrostyrene units. On the one hand, *β*-nitrostyrenes are polymerizable,[17] and on the other hand they can undergo rapid cleavage in alkaline aqueous solution.[18] The cleavage reaction involves rate-determining nucleophilic attack by hydroxide ion at the α-position of the styrene, leading to an intermediate that rapidly splits into a benzaldehyde and the anion of nitromethane (Scheme 1). In order to probe the minimal membrane stability required to differentiate between the *exo*- and *endo*-bilayer leaflet, we tested amphiphiles with *n*-dodecyl, *n*-tetradecyl, *n*-hexadecyl, and *n*-octadecyl chains (Chart 1). Since the cleavage reaction occurs in alkaline solution, we used nonhydrolyzable 1,2-bis(*n*-alkoxy)propanols rather than 1,2-bis(*n*-acyloxy)propanols. The product that results from hydrolytic cleavage of the *β*-nitrostyrene group was synthesized independently. In the bis(*n*-hexadecyl) and bis(*n*-octadecyl) systems we carried out selective *exo*-vesicular cleavage of the *β*-nitrostyrene unit, followed by rapid UV-initiated polymerization of the remaining *endo*-vesicular units (Scheme 2). Thus, vesicles were obtained which contain a polymerized and immobilized inner bilayer leaflet and a monomeric (dynamic) outer bilayer leaflet.

Scheme 2. *Exo*-vesicular Cleavage followed by *Endo*-vesicular Polymerization of *β*-Nitrostyrene in Bilayers[a]



[a] The drawn alkyl chains denote 1,2-bis(*n*-alkyloxy)propyl units.

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(16) Preliminary lipid mixing assays indicate that for vesicles of DHPBNS (**5**) this is indeed the case.

(17) There is literature precedent for dimerization as well as for polymerization of *β*-nitrostyrene. See: (a) Miller, D. B. Ph.D. Thesis, Ohio State University, 1957 (*Dissertation Abstr.* **1958**, *18*, 1981). (b) Topchiev, A. V.; Alaniya, V. P. *J. Polym. Sci. A* **1963**, *1*, 599.

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To the best of our knowledge, these vesicles represent the first examples of surface-specifically polymerized bilayers; only vesicles in which the counterions were polymerized in a surface-specific manner have been decribed previously.[19] We contend that vesicles containing a polymerized inner bilayer leaflet are of great interest in studies of bilayer−bilayer interactions and of the fusion of bilayers in particular.

Experimental Section

Synthesis. 1,2-Bis(*n*-alkoxy)propanols were prepared by alkylation of 1-benzyloxy-2,3-propanediol with *n*-alkyl triflates, followed by hydrogenation to remove the benzyl group. These reactions are rapid and more efficient than other procedures previously reported.[20] All bis(*n*-alkoxy)propanols have been described.[20] The phosphorylation reactions were carried out under nitrogen atmosphere. NMR spectra were recorded in deuterated chloroform solution using 200 or 300 MHz Varian machines, unless indicated otherwise. Elemental analyses were carried out in the analytical department of our laboratory. The analyses of **3**, **6**, and **8** were hampered by the hygroscopic nature of these compounds.

***n*-Octadecyl Triflate.** *n*-Octadecanol (7.03 g, 26.0 mmol, 1.0 equiv) in dry CH_2Cl_2 (50 mL) was dropped into a suspension of Na_2CO_3 (2.50 g, 23.6 mmol, 0.7 equiv) and trifluoromethanesulfonic acid anhydride (4.8 mL, 28.4 mmol, 1.1 equiv) in dry CH_2Cl_2 (50 mL). The mixture was stirred for 2 h. The solids were removed by column filtration (2.5 cm, 30 g silica). The column was rinsed with 100 mL of CH_2Cl_2. The solvent was evaporated and a colorless solid (9.00 g, 22.4 mmol, 86%) was obtained. ^{1}H-NMR: $\delta = 4.55$ (t, 2H, $TfOCH_2$), 1.82 (m, 2H, CH_2), 1.27 (m, 22H, chain), 0.89 (t, 3H, CH_3) ppm.

1-(Benzyloxy)-2,3-bis(*n*-octadecyloxy)propane. 1-(Benzyloxy)propanol (2.00 g, 11.0 mmol, 1.0 equiv) in benzene (15 mL) was slowly added to a suspension of NaH (0.93 g, 22.5 mmol, 2.1 equiv, rinsed with 2 × 5 mL of benzene) in benzene (50 mL). The mixture was refluxed for 1.5 h. After the mixture was cooled to room temperature, a solution of *n*-octadecyl triflate (9.00 g, 22.4 mmol, 2.1 equiv) in benzene (15 mL) was added to the reaction mixture. After 2 h a small amount of NaH was added again. The gel-like solution was refluxed for 8 h. Subsequently the solution was diluted with ether (200 mL), washed (3 × 100 mL saturated $NaCl_{(aq)}$), and dried on Na_2SO_4. The solvent was removed, and the crude product was crystallized from acetone, yielding a white solid (5.27 g, 7.68 mmol, 70%). ^{1}H-NMR: $\delta = 7.33$ (s, 5H, Ph), 4.55 (s, 2H, OCH_2Ph), 3.56 (m, 9H, 4 × CH_2O + CHO), 1.59 (m, 4H, 2 × OCH_2CH_2), 1.25 (s, 60 H, chains), 0.88 (t, 6H, 2 × CH_3) ppm. ^{13}C-NMR: δ = 128.29 (CH), 127.58 (CH), 127.48 (CH), 77.91 (CHO), 73.34 (CH_2O), 71.66 (CH_2O), 70.72 (CH_2O), 70.62 (CH_2O), 70.28 (CH_2O), 31.94 (CH_2), 30.12 (CH_2), 29.71 (CH_2), 29.66 (CH_2), 29.52 (CH_2), 29.37 (CH_2), 26.12 (CH_2), 22.70 (CH_2), 14.14 (CH_3) ppm.

1,2-Bis(*n*-octadecyloxy)propanol. A solution of 1-(benzyloxy)-2,3-bis(*n*-octadecyloxy)propane (5.27 g, 7.68 mmol) in hexane (75 mL) with palladium on carbon (0.3 g, 5% Pd) was stirred under H_2 for 16 h. Some product precipitated; it was solubilized by the addition of 50 mL of $CHCl_3$. The catalyst was removed by column filtration (1.5 cm, 5 g of Celite). The column was rinsed with $CHCl_3$ (100 mL). The solvent mixture was removed, and a solid was obtained (4.55 g, 99%). Mp = 65−66 °C. ^{1}H-NMR: $\delta = 3.52$ (m, 9H, 4 × OCH_2 + OCH), 2.23 (br s, 1H, OH), 1.57 (m, 4H, 2 × CH_2CH_2O), 1.27 (s, 60 H, chains), 0.89 (t, 6H, 2 × CH_3) ppm. ^{13}C-NMR: $\delta = 78.23$ (CHO), 71.85 (CH_2O), 70.92 (CH_2O), 70.40 (CH_2O), 63.12 (CH_2OH), 31.93 (CH_2), 30.08 (CH_2), 29.71 (CH_2), 29.63 (CH_2), 29.48 (CH_2), 29.37 (CH_2), 26.10 (CH_2), 22.70 (CH_2), 14.12 (CH_3) ppm.

4-Hydroxy-β-nitrostyrene (1). The product was prepared in 40% yield from 4-hydroxybenzaldehyde and nitromethane according to a literature procedure.[21] ^{1}H-NMR (DMSO-d_6): δ = 8.04 (s, 2H), 7.70 (d, $J_{ortho} = 8.5$ Hz, 2H), 6.82 (d, $J_{ortho} = 8.5$ Hz, 2H) ppm. ^{1}H-NMR (benzene-d_6): $\delta = 7.79$ (d, $J_{trans} = 13$ Hz, 1H), 7.33 (d, $J_{trans} = 13$ Hz, 1H), 7.15 (br s, 2H), 7.02 (br s, 2H) ppm. ^{13}C-NMR (DMSO-d_6): $\delta = 161.52$ (C_q), 139.86 (CH), 134.81 (CH), 132.28 (CH), 121.08 (C_q), 116.15 (CH) ppm.

4-(β-Nitrovinyl)phenyl Phosphorodichloridate (2). A solution of pyridine (0.27 mL, 3.5 mmol, 1.0 equiv) in benzene (3 mL) was slowly added to a mixture of 4-hydroxy-β-nitrostyrene (580 mg, 3.5 mmol, 1.0 equiv) and $POCl_3$ (5.0 mL, 55 mmol, 15.7 equiv) in benzene (10 mL). The mixture was stirred for 1 h. Subsequently ether (20 mL) was added. The solids were removed by filtration. The solvents were removed, and ether (20 mL) was added again. The remaining solids were removed, and the solvent was evaporated. The surplus of $POCl_3$ was removed in vacuum (<1 mmHg). A yellow solid (820 mg, 2.9 mmol, 83%) was obtained. Mp = 85−88 °C. It colors upon standing and should be used within a few hours. ^{1}H-NMR: $\delta = 8.00$ (d, $J_{trans} = 13.7$ Hz, 1H), 7.62 (d, $J_{ortho} = 9$Hz, 2H), 7.57 (d, $J_{trans} = 13.7$ Hz, 1H), 7.40 (dd, $J_{ortho} = 9$ Hz, $J_{PH} = 2$ Hz, 2H) ppm. ^{13}C-NMR: $\delta = 151.90$ (C_q), 137.95 (CH), 137.00 (CH), 131.08 (CH,d, $J_{PC} = 2.4$ Hz), 129.12 (C_q, d, $J_{PC} = 2.4$ Hz), 121.69 (CH, d, $J_{PC} = 6.4$ Hz) ppm. ^{31}P-NMR: $\delta = 3.45$ ppm.

***1,2*-Bis(*n*-dodecyloxy)prop-3-yl-4-(β-Nitrovinyl)phenyl Phosphoric Acid (DDPBNS, 3).** A solution of pyridine (0.15 mL, 1.97 mmol) and 1,2-bis(*n*-dodecyloxy)propanol (720 mg, 1.68 mmol) in THF (3 mL) was slowly added to **2** (500 mg, 1.77 mmol) in THF (3 mL). The mixture was stirred for 1.5 h. The solvent was partly evaporated, and the residue was diluted with ether (10 mL). The reaction was quenched with 0.1 M HCl (5 mL). After stirring for 20 min the aqueous layer was removed and washed with ether (10 mL). The ether solution was dried on Na_2SO_4, and the solvent was removed. A yellow solid (778 mg, 1.19 mmol, 71%) was obtained, with a melting point around room temperature. ^{1}H-NMR: $\delta = 7.93$ (d, $J_{trans} = 13.5$ Hz, 1H), 7.55 (d, $J_{ortho} = 10$ Hz, 2H), 7.55 (d, J_{trans}, 1H), 7.29 (d, J_o, 2H), 4.20 (m, 2H), 3.47 (m, 7H, 3 × OCH_2 + OCH), 1.52 (m, 4H, 2 × O−CH_2−CH_2), 1.25 (s, 36 H, chain), 0.88 (t, 6H, 2 × CH_3) ppm. ^{31}P-NMR: $\delta = -6.00$ ppm. Anal. Calc for $C_{35}H_{62}NPO_8$: C, 64.08; H, 9.53; N, 2.14. Found: C, 64.82; H 10.09; N, 1.61.

1,2-Bis(*n*-tetradecyloxy)prop-3-yl-4-(β-Nitrovinyl)-phenyl Phosphoric Acid (DTPBNS, 4). The product was prepared from **2** (410 mg, 1.45 mmol) and 1,2-bis(*n*-tetradecyloxy)-propanol (480 mg, 1.0 mmol) as described for **3**, using CH_2Cl_2 as a solvent. A yellow solid (700 mg, 0.98 mmol, 98%) was obtained. Mp = 43−45 °C. ^{1}H-NMR: $\delta = 7.95$ (d, $J_{trans} = 14$ Hz, 1H), 7.55 (d, J_{trans}, 1H), 7.55 (d, $J_{ortho} = 9$ Hz, 2H), 7.30 (d, J_{ortho}, 2H), 4.25 (m, 2H), 3.55 (m, 7H), 1.55 (m, 4H), 1.30 (s, 44 H, chain), 0.90 (t, 6H, 2 × CH_3) ppm. ^{31}P-NMR: $\delta = -5.04$ ppm. Anal. Calc for $C_{39}H_{70}NPO_8$: C, 65.82; H 9.85; N, 1.97; P, 4.36. Found: C, 65.64; H, 9.74; N, 1.91; P, 4.15.

1,2-Bis(*n*-hexadecyloxy)prop-3-yl-4-(β-Nitrovinyl)-phenyl Phosphoric Acid (DHPBNS, 5). The product was prepared from **2** (410 mg, 1.45 mmol) and 1,2-bis(*n*-hexadecyloxy)-propanol (540 mg, 1.0 mmol) as described for **4**. The crude product was crystallized from methanol (0.90 g in 40 mL). Pure product (420 mg, 0.55 mmol, 55%) with mp = 49−52 °C was isolated. ^{1}H-NMR: $\delta = 7.95$ (d, $J_{trans} = 13.5$ Hz, 1H), 7.51 (d, $J_{ortho} = 8.5$ Hz, 2H), 7.51 (d, J_{trans}, 1H), 7.27 (d, J_{ortho}, 2H), 4.20 (m, 2H, CH_2OP), 3.48 (m, 7H, 3 × CH_2O + CHO), 1.52 (m, 4H, 2 × O−CH_2−CH_2), 1.25 (s, 52 H, chain), 0.90 (t, 6H, 2 × CH_3) ppm. ^{13}C-NMR: δ = 153.41 (C_q), 137.67 (CH), 136.81 (CH), 130.67 (CH,d, $J_{PC} = 8.5$ Hz), 126.77 (C_q), 121.06 (CH,d, $J_{PC} = 4.9$ Hz), 71.83 (CH_2O), 70.79 (CH_2O), 69.17 (CH_2O), 67.71 (CH_2OP, d, $J_{PC} = 6.1$ Hz), 31.82 (CH_2), 29.96 (CH_2), 29.84 (CH_2), 29.80 (CH_2), 29.73 (CH_2), 29.62 (CH_2), 29.57 (CH_2), 29.47 (CH_2), 29.39 (CH_2), 29.26 (CH_2), 25.96 (CH_2), 25.88 (CH_2), 22.60 (CH_2), 14.02 (CH_3) ppm (CHO signal hidden under solvent signal). ^{31}P-NMR: $\delta = -5.90$ ppm. Anal. Calc for $C_{43}H_{78}NPO_8$: C, 67.28; H, 10.17; N, 1.83; P, 4.04. Found: C, 67.88; H, 10.52; N, 1.59; P, 3.82.

1,2-Bis(*n*-octadecyloxy)prop-3-yl-4-(β-Nitrovinyl)-phenyl Phosphoric Acid (DOPBNS, 6). The product was prepared from **2** (841 mg, 3.00 mmol) and 1,2-bis(*n*-octadecyloxy)-propanol (1.40 g, 2.34 mmol) as described for **4**. The reaction mixture was stirred with aqueous hydrochloric acid for 3 h to

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obtain complete hydrolysis of the phosphoromonochloridate. The crude product was crystallized from methanol (80 mL) and from a mixture of acetone (25 mL) and acetonitrile (5 mL). Pure product (1.17 mg, 1.42 mmol, 61%) with mp = 55−57 °C was isolated. ^{1}H-NMR: δ = 7.96 (d, J_{trans} = 13.5 Hz, 1H), 7.53 (d, J_{ortho} = 8.5 Hz, 2H), 7.53 (d, J_{trans}, 1H), 7.30 (d, J_{ortho}, 2H), 4.17 (m, 2H, CH_2OP), 3.49 (m, 7H, 3 nm × CH_2O + CHO), 1.54 (m, 4H, 2 × O−CH_2−CH_2), 1.26 (s, 60H, chain), 0.89 (t, 6H, 2 × CH_3) ppm. ^{13}C-NMR: δ = 153.43 (C_q), 137.65 (CH), 136.87 (CH), 130.61 (CH), 126.84 (C_q), 121.10 (CH, d, J_{PC} = 6.1Hz), 77.00 (CHO), 71.84 (CH_2O), 70.83 (CH_2O), 69.24 (CH_2O), 67.77 (CH_2OP, d, J_{PC} = 6.1Hz), 31.84 (CH_2), 29.81 (CH_2), 29.80 (CH_2), 29.64 (CH_2), 29.57 (CH_2), 29.49 (CH_2), 29.41 (CH_2), 29.28 (CH_2), 25.98 (CH_2), 25.91 (CH_2), 22.60 (CH_2), 14.02 (CH_3) ppm. ^{31}P-NMR: δ = −5.35 ppm. Anal. Calc for $C_{47}H_{86}NPO_8$: C, 68.49; H, 10.52; N, 1.70; P, 3.76. Found: C, 67.52; H, 10.59; N, 1.44 P, 3.43.

4-Formylphenyl Phosphorodichloridate (7). The product was prepared from *4*-hydroxybenzaldehyde (366 mg, 3.0 mmol) and $POCl_3$ (4.0 mL, 42 mmol) as described for the synthesis of **2** from **1** and $POCl_3$. A greenish oil (410 mg, 1.7 mmol, 57%) was obtained. ^{1}H-NMR: δ = 10.03 (s, 1H), 7.98 (d, J_{ortho} = 9 Hz, 2H), 7.49 (d, J_{ortho} = 9 Hz, 2H) ppm. ^{13}C-NMR: δ = 190.31 (CH), 153.60 (C_q, d, J_{PC} = 11 Hz), 134.86 (C_q, d, J_{PC} = 3.2 Hz), 131.92 (CH), 127.35 (C_q), 121.32 (CH, d, J_{PC} = 6.4 Hz) ppm. ^{31}P-NMR: δ = 3.10 ppm.

1,2-Bis(*n*-hexadecyloxy)prop-3-yl 4-Formylphenyl Phosphoric Acid (DHPPFP, 8). The product was prepared from **7** (353 mg, 1.48 mmol) and 1,2-bis(*n*-hexadecyloxy)propanol (540 mg, 1.0 mmol) as described for the synthesis of **4**. The crude product was crystallized from methanol (0.70 g in 20 mL). Pure product (450 mg, 0.62 mmol, 62%) with mp = 41−42 °C was isolated. ^{1}H-NMR: δ = 9.94 (s, 1H), 7.85 (d, J_{ortho} = 8 Hz, 2H), 7.35 (d, J_{ortho} = 8 Hz, 2H), 6.85 (br s, 1H, POH), 4.20 (m, 2H, CH_2OP), 3.48 (m, 7H, 3 × CH_2O + CHO), 1.51 (m, 4H, 2 × O−CH_2−CH_2), 1.24 (s, 52H, chain), 0.87 (t, 6H, 2 × CH_3) ppm. ^{13}C-NMR: δ = 190.61 (CH), 155.29 (C_q, d, J_{PC} = 6.1 Hz), 133.29 (C_q), 131.55 (CH), 120.69 (CH, d, J_{PC} = 6.1 Hz), 77.11 (CHO), 71.88 (CH_2O), 70.87 (CH_2O), 69.33 (CH_2O), 67.79 (CH_2OP, d, J_{PC} = 7.1 Hz), 31.93 (CH_2), 29.90 (CH_2), 29.71 (CH_2), 29.66 (CH_2), 29.56 (CH_2), 29.50 (CH_2), 29.37 (CH_2), 26.05 (CH_2), 25.98 (CH_2), 22.70 (CH_2), 14.12 (CH_3) ppm. ^{31}P-NMR: δ = −5.99 ppm. Anal. Calc for $C_{42}H_{77}PO_7$: C, 69.58; H, 10.70; P, 4.27. Found: C, 68.97; H, 10.49; P, 4.13.

Sodium Salts of 3−6 and 8. All sodium phosphate salts were prepared by slow and careful addition of 1.0 equiv of a 0.149 mmol/g NaOEt solution in ethanol to a 0.1 M solution of the phosphoric acid in anhydrous ethanol or methanol. The products were isolated by evaporation and stored as stock solutions in $CHCl_3$ (usually 10 mM) in a cold and dark place. Characteristic shifts were observed in the ^{1}H- and ^{31}P-NMR spectra of the sodium salts relative to those of the acids. For the sodium salt of **5**: ^{1}H-NMR (MeOH-d_4): δ = 7.85 (d, J_{trans}, 1H), 7.40 (d, J_{trans}, 1H), 7.33 (s, 4H), 3.95 (m, 2H), 3.40 (m, 7H), 1.40 (m, 4H), 1.25 (s, 52H), 0.90 (t, 6H) ppm. ^{31}P-NMR (MeOH-d_4): δ = −4.43 ppm.

Vesicle Preparation. Vesicle solutions were prepared from 10 mM stock solutions of the sodium salts of **3**−**6** in chloroform. Aliquots of these stock solutions were rotary-evaporated in pyrex tubes to yield thin lipid films, which were dried *in vacuo* for 1 h. Subsequently, 0.5−2.0 mM vesicle solutions were prepared by dispersion of the lipid films in double-distilled water or 5.0 mM HEPES/NaAc buffer (pH 7.4) at temperatures ca. 10 °C above T_m by means of a Branson B15 sonication immersion tip (2 min) or by the ethanol injection method (2.0 μmol of lipid/100 μL of ethanol/2.0 mL of water).

Freeze−Fracture Transmission and Cryo-Scanning Electron Microscopy. Samples were prepared and examined as described previously.[22]

Quasi-Elastic Light Scattering. Vesicle solutions (1−2 mM) were analyzed in a Nicomp Submicron Particle Sizer Model 370. Diameters reported are mean diameters derived from volume-weighted Gaussian analysis at 25 °C.

(22) (a) Sein, A.; Engberts, J. B. F. N.; Linden, E. van der; Pas, J. C. van de. *Langmuir* **1993**, *9*, 1714. (b) Jeffree, C. E; Read, N. in *Electron Microscopy of Plant Cells*; Hall, J. L., Hawes, C., Eds.; Academic Press: London, 1991, pp 313−413.

Differential Scanning Calorimetry. DSC enthalpograms were recorded on a MC-2 differential scanning microcalorimeter (MicroCal Ltd., experiments carried out at the University of Leicester (U.K.), [lipid] = 2.0 mM) or a Perkin-Elmer DSC-7 ([lipid] = 2−5 mass %). Scans were reproduced five times.

Kinetics. Cleavage of the β-nitrostyrene unit was quantified by monitoring the decrease of its intense ($\epsilon \approx 10\,000$ M^{-1} cm^{-1}) characteristic absorption at 334 nm using a Perkin-Elmer λ5 spectrophotometer equipped with a thermostated cell compartment. Data were analyzed in terms of pseudo-first-order rate constants or in terms of a double exponential decay. Usually, 100 μL aliquots of a 1.0 mM vesicle solution prepared at neutral pH were added rapidly to 1.9 mL of double-distilled water with pH adjusted to 11.5 by the addition of a NaOH solution. The pH was measured with an Orion SA 720 pH electrode.

Polymerization. Polymerization of β-nitrostyrene in the bilayer was achieved by irradiation of 1.0 mL samples of 0.5−2.0 mM solutions in quartz cuvettes or Pyrex NMR tubes for 5 min with a Hanau SN81 medium-pressure mercury lamp. Samples were placed at a distance of 1 cm from the lamp. The extent of polymerization was determined by the disappearance of the characteristic UV−vis absorption and the ^{1}H-NMR signal of the vinyl protons of the monomer. A sample of fully polymerized material was freeze-dried. It dissolves readily in chloroform and THF, and its ^{1}H- and ^{31}P-NMR spectra are characteristic for a high molecular weight polymer. The sample was analyzed by gel permeation chromatography ($CHCl_3$), electrospray mass spectrometry (CH_2Cl_2), and vapor pressure osmometry (THF).

Results and Discussion

Synthesis. The procedures described in this paper present a straightforward and efficient approach toward a new class of phosphate amphiphiles, carrying a β-nitrostyrene unit. 4-Hydroxy-β-nitrostyrene was prepared following a literature procedure.[20] Phosphorylation was carried out using an excess of $POCl_3$; the phosphorodichloridate thus obtained was not stable and was reacted as soon as possible with the 1,2-bis(*n*-alkyloxy)propanols to yield the desired phosphodiesters. Most likely, the phosphorylation reaction is applicable to the selective phosphorylation of any phenol and any primary alcohol.

Vesicle Characterization. Vesicle solutions were prepared from 10 mM stock solutions of sodium salts **3**−**6** in chloroform. Vesicle solutions (0.5−2.0 mM) were prepared by dispersion of the corresponding lipid films in water or HEPES/NaAc buffer by means of a sonication immersion tip or by the ethanol injection method. In all cases FFEM showed the formation of unilamellar vesicles with diameters of 100−300 nm (Figure 1a). Light scattering revealed average diameters of 150−200 nm for vesicles of DDPBNS (**3**) and DTPBNS (**4**), 250 nm for DHPBNS (**5**), and 275 nm for DOPBNS (**6**). These average diameters were also observed using cryo-SEM (Figure 1b). Differential scanning microcalorimetry revealed a main phase transition temperature (T_m) of 53.8 °C (ΔH = 37.8 kJ/mol) for DOPBNS (**6**), 40.7 °C (ΔH = 42.3 kJ/mol) for DHPBNS (**5**), and 21.6 °C (ΔH = 74.3 kJ/mol, this includes secondary transitions around 40 °C) for DTPBNS (**4**) and no T_m above 5 °C for DDPBNS (**3**). Temperature-dependent ^{31}P-NMR spectra of a DHPBNS (**5**) vesicle solution in D_2O showed a constant line width from 70 °C down to 40 °C, followed by a steady increase from 40 to 10 °C, indicating a phase transition of the bilayer around 40 °C; thus, NMR and DSC provide consistent results. Vesicle solutions of **3** and **4** are colloidally stable for several days, whereas solutions of **5** and **6** tend to flocculate after about 1 day at room temperature. In any case, to avoid spontaneous polymerization, samples should be protected from sunlight and prolonged heating (T > 50 °C).

Cleavage of the β-Nitrostyrene (BNS) Moiety. Hydrolytic cleavage experiments were initiated by dilution of an aliquot of a solution of vesicles prepared at neutral

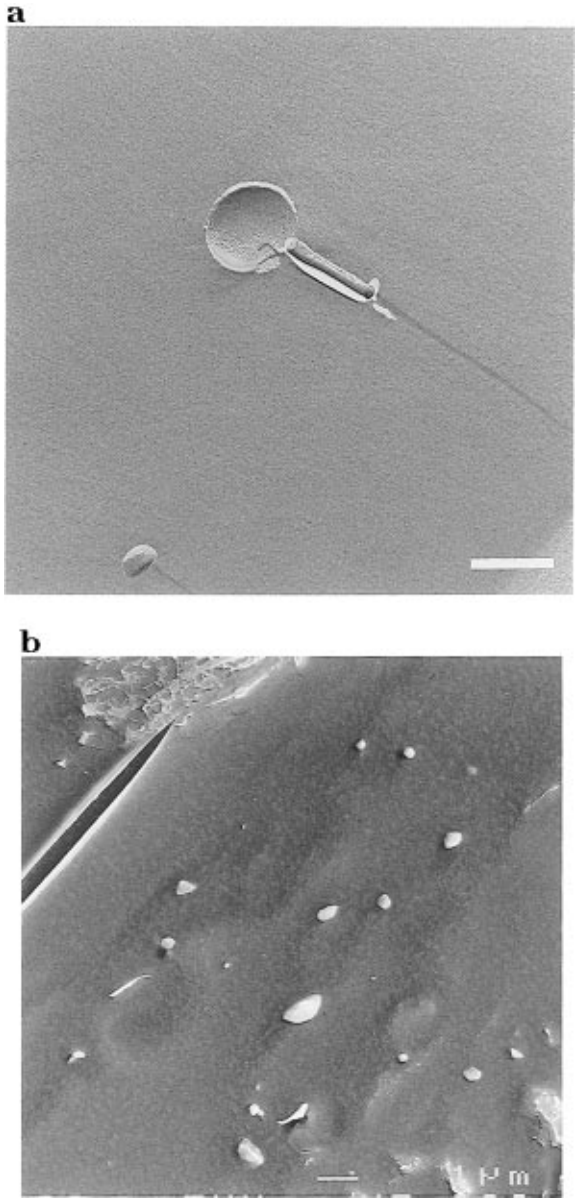

Figure 1. (a) FFEM specimen of DHPBNS (**5**) vesicles (1.0 mM). Bar represents 250 nm. (b) Cryo-SEM specimen of DDPBNS (**3**) vesicles (1.0 mM). Bar represents 1000 nm.

pH into an alkaline solution (pH 11.5). In this way, vesicles with neutral internal pH and high external pH were obtained. Surface differentiation now critically depends on the permeability of the bilayer to hydroxide ion (which we presume to be low, since the bilayer is negatively charged; we have obtained experimental evidence for this assumption[23]), as well as the rate of exchange of lipid molecules between the two bilayer leaflets (*flip-flop*). Surface differentiation is favored by an increase of the length of the alkyl chains and by low temperatures ($T < T_m$),[24] because *flip-flop* is retarded by these factors. However, the rate of *flip-flop* also depends on the nature of the hydrophilic headgroup.[24]

In a first approximation, we have analyzed the cleavage process in terms of a model in which we assume that the BNS units are partitioned between the *exo*-vesicular and *endo*-vesicular surfaces of the vesicles. The ratio of $[BNS]_{exo,0}$ to $[BNS]_{endo,0}$ depends on the vesicle diameter and the thickness of the bilayer and may be estimated from the ratio of *endo*-surface to *exo*-surface.[25] We analyzed our data assuming that the *exo*-vesicular part of the BNS groups is easily accessible to nucleophilic attack by hydroxide ion and will undergo a relatively rapid cleavage reaction. On the other hand, the *endo*-BNS groups are much less easily accessible (since prior to cleavage they will have to translocate over the membrane) and will undergo a much slower cleavage. In kinetic equations:

$$d[BNS]_{exo}/dt = -k_{fast}[OH^-][BNS]_{exo} \quad \text{and}$$
$$d[BNS]_{endo}/dt = -k_{slow}[OH^-][BNS]_{endo}$$

Since by UV−vis spectroscopy there is no way to discriminate between $[BNS]_{exo}$ and $[BNS]_{endo}$, we monitor the decrease of $[BNS]_{exo+endo}$ in time:

$$[BNS]_{exo+endo,t} = [BNS]_{exo,0}\exp(-k_{fast}[OH^-]t) + [BNS]_{endo,0}\exp(-k_{slow}[OH^-]\,t)$$

which may be simplified to a double pseudo-first-order rate equation

$$[BNS]_{exo+endo,t} = [BNS]_{exo,0}\exp(-k'_{fast}t) + [BNS]_{endo,0}\exp(-k'_{slow}t)$$

In a system in which the *endo*-BNS groups are as easily accessible to hydroxide as the *exo*-BNS groups ($k_{fast} \approx k_{slow}$), no surface differentiation is possible and the kinetics simplify to a pseudo-first-order rate equation:

$$[BNS]_{tot,t} = [BNS]_{tot,0}\exp(-k''t)$$

The results of several experimental runs are presented in Table 1. As expected, the cleavage of free 4-hydroxy-β-nitrostyrene (deprotonated above pH 7.8) is slower than that of the monomeric amphiphilic BNS derivatives (run 1 *vs* runs 2 and 7). Also, the cleavage in monomeric BNS amphiphile solutions (aqueous solutions containing 25 mol % of ethanol) is more rapid than the cleavage of amphiphiles organized in vesicles (runs 2 and 7 *vs* runs 3, 4 and 8, 10). Obviously, the negatively charged membrane retards the nucleophilic attack by hydroxide ion. Disrupture of the bilayer structure by CTAB leads to a 15−25-fold increase of the rate of cleavage (runs 3, 4 and 8, 10 *vs* runs 5 and 11). In the presence of CTAB, the reaction is even accelerated 10-fold relative to the cleavage in monomeric solution (runs 2 and 7 *vs* runs 5 and 11), which suggests a catalytic effect of CTAB micelles.

Runs 1−5 and 7 and 11 all follow smooth first-order kinetics, indicating equal accessibility of all BNS groups present to hydroxide nucleophilic attack. Apparently, in vesicles of DDPBNS (**3**), $k'_{slow} = k'_{fast}$ and surface differentiation is impossible.

However, cleavage of the BNS unit in vesicles formed from DTPBNS (**4**), DHPBNS (**5**), and DOPBNS (**6**) can be

(23) Neutral red was cosonicated in water with DDPBNS (**3**) and with DHPBNS (**5**). At 25 °C, in the case of DHPBNS, the decrease of the absorbance of the protonated indicator ($\lambda_{max} = 515$ nm), representing the fraction of material included in the vesicles, amounted to less than 5% over the time scale of the cleavage experiments. When CTAB was added, the absorbance at 515 nm immediately disappeared. We conclude that in vesicles of DHPBNS the pH gradient is maintained during the cleavage experiments and that hydroxide ion permeation is negligible. For DDPBNS, all protonated indicator was lost over the time scale of the cleavage experiments: therefore, at $T > T_m$, hydroxide leakage is considerable.

(24) Moss, R. A. *Pure Appl. Chem.* **1994**, *66*, 851 and references therein.

(25) For spherical vesicles with a bilayer thickness of 5 nm, the *exo*-vesicular surface area as a fraction of the total surface area is 0.57 for a diameter of 80 nm, 0.55 for a diameter of 100 nm, and 0.53 for a diameter of 200 nm.

Table 1. Cleavage of (β-Nitrovinyl)phenyl Functionalized Amphiphiles

run	experiment[a]	k'_{fast}	k'_{slow}	k'_2	k_1	k'_2/k_1	BNS_{endo}/BNS_{tot}
1	BNS (**1**)[b]	2.56					
2	DDPBNS (**3**)[c], 25% EtOH	12.8					
3	tip	10.2				1.0[d]	
4	injection	9.89					
5	+ CTAB[e]	142					
6	DTPBNS (**4**),[c] tip	5.11	0.584	4.32	0.680	6.4	<0.20
7	DHPBNS (**5**),[c] 25% EtOH	13.3					
8	tip	5.49	0.316	5.13	0.343	15	0.43
9	tip[f]	21.4					
10	injection	7.33	0.769	6.32	0.893	7.1	<0.30
11	+ CTAB[e]	133					
12	DOPBNS (**6**),[c] tip	6.24	0.095	6.15	0.100	62	0.47

[a] Vesicle solutions were prepared by sonication with an immersion tip (tip) or by the ethanol injection method (injection) in HEPES/NaAc buffer of pH 7.4. The external pH was 11.5 and the temperature was 25 °C. Rate constants are reported in 10^{-4} s^{-1} and are averages of at least four reproducible runs. [b] $[BNS]_0 = 3.1 \times 10^{-5}$ M. [c] $[BNS\text{-lipid}]_0 = 5.0 \times 10^{-5}$ M. [d] Hypothetical value. [e] $[CTAB] = 1.0 \times 10^{-3}$ M. [f] Temperature 46 °C.

treated much more accurately in terms of a double exponential decay, reflecting fast cleavage of the *exo*-vesicular BNS and slow cleavage of the *endo*-vesicular BNS (runs 6, 8, and 12). In these cases, we found the analysis in terms of a fast and a slow process satisfying but perhaps oversimplified. Therefore we extended our model to allow for *flip-flop*. The kinetic scheme thus became as follows:

$$BNS_{endo} \rightleftarrows BNS_{exo} \rightarrow \text{product}$$

with *flip-flop* characterized by k_1 and k_{-1} and product formation by $k'_2 = k_2[OH^-]$. Now:

$$d[BNS]_{endo}/dt = -k_1[BNS]_{endo} + k_{-1}[BNS]_{exo}$$

$$d[BNS]_{exo}/dt = k_1[BNS]_{endo} - k_{-1}[BNS]_{exo} - k'_2[BNS]_{exo}$$

Leading to an equation of the form

$$[BNS]_{endo+exo,t} = C_a \exp(-k_a t) + C_b \exp(-k_b t)$$

The solution for $[BNS]_{endo+exo,t}$ may be found using Laplace transformation.[26] C_a, C_b, k_a and k_b are combinations of $[BNS]_{exo,0}$, $[BNS]_{endo,0}$, k_1, k_{-1} and k'_2. Thus, assuming $k_1 \approx k_{-1}$, the values of k_1 and k'_2 may be calculated from the experimentally determined values of k_a and k_b, which were previously reported as k'_{slow} and k'_{fast}. The results of this analysis are reported in Table 1.

It is obvious that the second model is superior to the first one, since whereas k'_2 values are quite similar for all BNS derivatives (we attribute the differences to small variations in external pH and perhaps vesicle composition), there is a clear trend in the k_1 values. As can be seen from Table 1 (k'_2/k_1 values), *endo*-vesicular cleavage is most significant in the system with the shortest alkyl chain (DTPBNS, run 6), in which the most rapid *flip-flop* is anticipated. At 25 °C, surface differentiation in this system is difficult to carry out experimentally, since k_1 is relatively large. On the other hand, in vesicles of DHPBNS and DOPBNS, k_1 is small relative to k'_2 (runs 8 and 12); surface differentiation is easily achieved and can be maintained over periods of several hours (the half-life of *flip-flop* in vesicles of DHPBNS is more than 6 h). These rates of *flip-flop* are similar to those reported for comparable model systems.[27] The percentage of BNS remaining in the DHPBNS and DOPBNS vesicle systems after more than 4 half-lifes of the *exo*-vesicular cleavage reaction corresponds to unreacted *endo*-BNS. The relative amounts of *exo*-BNS and *endo*-BNS match expectations from theory.[25]

At temperatures above T_m, $k'_2 \approx k_1$ and the cleavage reaction follows first-order kinetics (runs 3, 4, and 9). We propose this is due to much faster *flip-flop* above T_m. There is literature precedent indicating that the persistence of surface differentiation above T_m strongly depends on the nature of the headgroup of the lipid.[24,28] Also, above T_m considerable hydroxide leakage is expected.[23] In either case, for the present system this explains why DDPBNS (**3**) is not appropriate for our goals: its T_m is too low to allow specific *exo*-vesicular cleavage at room temperature.

In vesicles of DHPBNS prepared by the ethanol injection method (run 10), a much higher k_1 was found. We suppose that the small amount of ethanol in the system induces faster *flip-flop* and concomitantly more hydroxide leakage.[29] Therefore, it is unadvisable to use the ethanol injection method for preparation of surface differentiated vesicles.

FFEM and light scattering showed that vesicles of DHPPFP (**8**), the product of the hydrolysis of DHPBNS (**6**), have similar size as compared to vesicles of **6**. In the DSC enthalpogram of DHPPFP (**8**), a T_m of 40.4 °C was observed. The enthalpy of transition is 44.6 kJ/mol. For **6**, these values are 40.7 °C and 42.3 kJ/mol, respectively (*vide supra*). We therefore contend that the cleavage reaction influences neither the vesicle size nor bilayer dynamics and melting behavior.

Polymerization of the (β-Nitrovinyl)phenyl Moiety. Rapid polymerization of the (β-nitrovinyl)phenyl moiety was achieved by intense UV irradiation. No initiator was required, and no monomer could be detected (^{1}H-NMR, UV−vis) after irradiation for 5 min. It is known that polymerization reactions greatly benefit from the close proximity of the monomers in the bilayer.[30] In fact, it became apparent that polymerization also takes place under the influence of sunlight and heating (*vide supra*). Light scattering, FFEM, and cryo-SEM showed that the average size and morphology of the vesicles were unaffected by the polymerization (Figure 2a and b). However, the stability of the vesicles increases: no flocculation was observed in samples of poly-DHPBNS (poly-**5**) and poly-

(26) We refer to the Supporting Information for more detail.

(27) Moss, R. A.; Bhattacharya, S. *J. Am. Chem. Soc.*. **1995**, *117*, 8689.

(28) Moss, R. A.; Swarup, S. *J. Am. Chem. Soc.* **1986**, *108*, 5341.

(29) Even small amounts of ethanol are known to affect bilayer phase transitions and dynamics. See: (a) Blandamer, M. J.; Briggs, B.; Cullis, P. M.; Engberts, J. B. F. N. *Chem. Soc. Rev.* **1995**, 251. (b) Blandamer, M. J.; Briggs, B.; Butt, M. D.; Waters, M.; Cullis, P. M.; Engberts, J. B. F. N.; Hoekstra, D.; Mohanty, R. K. *Langmuir* **1994**, *10*, 3488.

(30) Reed, W.; Guterman, L.; Tundo, P.; Fendler, J. H. *J. Am. Chem. Soc.* **1984**, *106*, 1897.

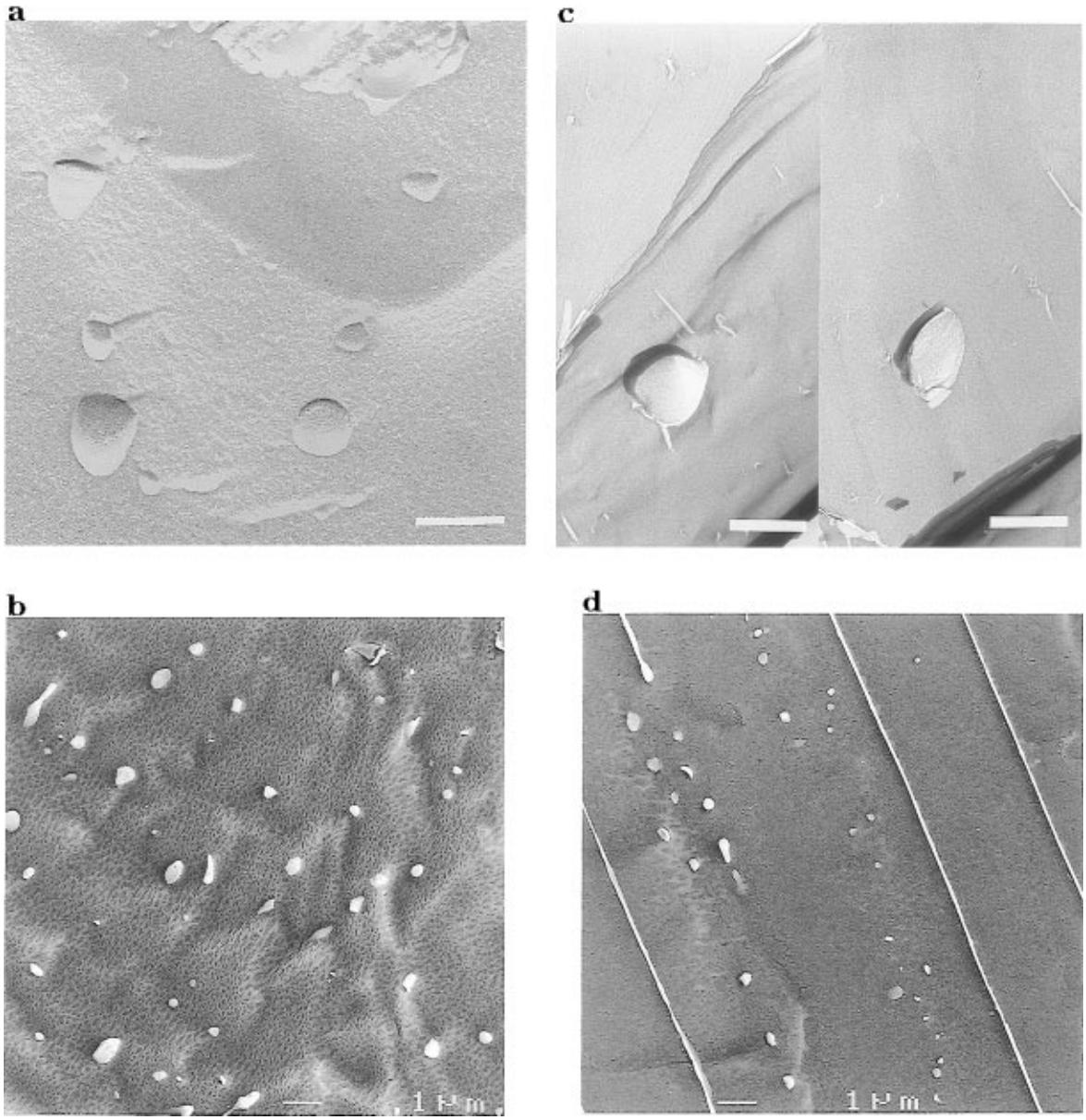

Figure 2. (a) FFEM specimen of DHPBNS (**5**) vesicles (1.0 mM) irradiated with UV for 5 min. Bar represents 250 nm. (b) Cryo-SEM specimen of DHPBNS (**5**) vesicles (1.0 mM) irradiated with UV for 5 min. Bar represents 1000 nm. (c) FFEM specimen of DHPBNS (**5**) vesicles (1.0 mM) exposed to external pH of 11.5 for 1.5 h, followed by UV irradiation for 5 min. Bar represents 250 nm. (d) Cryo-SEM specimen of DHPBNS (**5**) vesicles (1.0 mM) exposed to external pH of 11.5 for 1.5 h followed by UV irradiation for 5 min. Bar represents 1000 nm.

DOPBNS (poly-**6**) after several days. Strong line broadening in ^{1}H- and ^{31}P-NMR suggests a high molecular weight for the polymer. Also a characteristic signal was observed at 3.8 ppm in the ^{1}H-NMR spectrum. Gel permeation chromatography of poly-**5** was not successful. Vapor pressure osmometry yielded an average molecular weight for poly-**3** of 2469, corresponding to an average polymerization degree of 3.8. No cyclobutane-like dimers[17a] could be detected in the electrospray mass spectrum of poly-**3**. DSC of poly-**5** indicated that T_m after polymerization shifts to a slightly higher temperature and the peak in the enthalpogram becomes broader. It seems likely that, upon polymerization of the headgroups, the phase transition of the hydrocarbon interior of the bilayer occurs at a similar temperature but in a less cooperative manner as compared to the unpolymerized vesicles. There is literature precedent for these observations.[31]

Hydrolysis followed by Polymerization. Vesicles of DHPBNS (**5**) and DOPBNS (**6**) were exposed to pH 11.5 during *ca.* 1 h, after which *exo*-vesicular cleavage is complete (compare Table 1, runs 6, 8, and 12). Subsequently, the vesicle solution was neutralized and irradiated with UV for 5 min. In this way, vesicles containing a polymerized inner bilayer leaflet and a monomeric outer bilayer leaflet were obtained. We assume that the degree of polymerization is similar to that determined for the *endo*- and *exo*-polymerized vesicles. Samples were examined by light scattering, FFEM, and cryo-SEM, and it was apparent that neither the hydrolytic cleavage nor the polymerization had affected the average size and morphology of the vesicles (Figure 2c and d). No flocculation was observed in either of the samples within several days.

Conclusions

We have explored a novel class of bifunctional vesicle-forming amphiphiles containing β-nitrostyrene units linked to the phosphate headgroup. Under the appropri-

(31) Hirano, K.; Fukuda, H.; Regen, S. L. *Langmuir* **1991**, *7*, 1045.

ate experimental conditions, these units are amenable to *exo*-vesicular hydrolysis and can undergo rapid polymerization in the bilayer. Light scattering, FFEM, and cryo-SEM show that vesicle size and morphology are not affected by these manipulations. It is possible to generate vesicles containing a polymerized inner bilayer leaflet and a monomeric outer bilayer leaflet. We have started a detailed study of the fusogenic behavior of such bilayers. It might now be feasible that, due to the polymerization (*i.e.* immobilization) of the inner bilayer leaflet, the fusion process will be inhibited after the stage(s) in which a local contact zone between the outer leaflets is established. Further studies are aimed at characterizing the structures that are formed by (electron) microscopy and fluorescence and NMR spectroscopy.

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Supporting Information Available: Schematic analysis of the kinetic data (2 pages). Ordering information is given on any current masthead page.

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