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Published in:
Langmuir

DOI:
10.1021/la960322+

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1996

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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

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Received April 4, 1996. In Final Form: August 19, 1996

Four new phospholipid derivatives containing a \(\beta\)-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 \(\beta\)-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 \(\beta\)-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 \(\beta\)-nitrostyrene units results in the formation 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 ubiquitous 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. 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 and particularly, Siegel, Talmon, and co-workers collected experimental evidence for transient intermediates using sophisticated cryo-electron microscopic techniques.

Membrane mimetic systems have been fruitful models to gain more insight into membrane characteristics, including the fusion process. Since bilayers repel each other and fusion implies an approximation to contact distance, fusogenic agents (e.g., divalent metal ions for phosphate-based amphiphiles, dianions for positively charged amphiphiles, water-soluble polymers in various cases, 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 precedes the actual rate-determining fusion process. For \(Ca^{2+}\)-induced fusion of phospholipid vesicles, formation of "trans" inter-bilayer complexes has been proposed. 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. However, due to their short lifetime, the molecular structure of these contact sites remains elusive. Siegel and Chernomordik and co-workers 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, "stalks," local interdigitated structures, or lipids with extended alkyl chains 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.

Scheme 1. Hydrolytic Cleavage of 4-Hydroxy-\(\beta\)-nitrostyrene

\[
\begin{align*}
\text{HO} & \quad \text{NO}_2 \\
\text{OH} & \quad \text{CH}_2 & \quad \text{NO}_2 \\
\end{align*}
\]

Abstract published in Advance ACS Abstracts, November 1, 1996.


(2) Siegel, D. P. Biophys. J. 1984, 45, 399.


(6) Siegel, D. P.; Green, W. J.; Talmon, Y. Biophys. J. 1994, 66, 402.


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. It is reasonable to assume that polymerization may in this way inhibit vesicle fusion. 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 \( \beta \)-nitrostyrene (BNS) unit. The \( \beta \)-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 \( \beta \)-nitrostyrene units. On the one hand, \( \beta \)-nitrostyrenes are polymerizable, and on the other hand they can undergo rapid cleavage in alkaline aqueous solution. The cleavage reaction involves rate-determining nucleophilic attack by hydroxide ion at the \( \alpha \)-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(\( n \)-acycloxy)propanols. The product that results from hydrolytic cleavage of the \( \beta \)-nitrostyrene group was synthesized independently. In the bis(\( n \)-hexadecyl) and bis(\( n \)-octadecyl) systems we carried out selective exo-vesicular cleavage of the \( \beta \)-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.

![Chart 1](image1)

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

![Scheme 2](image2)

**Scheme 2.** Exo-vesicular Cleavage followed by Endo-vesicular Polymerization of \( \beta \)-Nitrostyrene in Bilayers

\( ^a \) 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.

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 described previously.\textsuperscript{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-benzoxyl-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.\textsuperscript{20} All bis(n-alkoxy)propanols have been described.\textsuperscript{21}

#### Analyses

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\textsubscript{2}Cl\textsubscript{2} (50 mL) was dropped into a suspension of NaH (0.93 g, 22.5 mmol, 0.7 equiv) and trifluoromethane sulfonic acid anhydride (4.8 mL, 28.4 mmol, 1.1 equiv) in dry CH\textsubscript{2}Cl\textsubscript{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 washed with 100 mL of CH\textsubscript{2}Cl\textsubscript{2}. The solvent was evaporated. A colorless liquid (9.00 g, 22.4 mmol, 86%) was obtained.\textsuperscript{4,5} \textsuperscript{13}C-NMR: δ 22.70 (CH\textsubscript{2}), 14.12 (CH\textsubscript{3}) ppm.

#### 1-Methyl-3,6-bis(hexadecyloxy)propane

1-Methyl-3,6-bis(hexadecyloxy)propane (20.0 g, 110 mmol, 1.0 equiv) in benzene (15 mL) was slowly added to a suspension of NaN\textsubscript{3} (0.93 g, 22.5 mmol, 1.0 equiv, 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 POCl\textsubscript{3} (1.97 mmol) and trifluoromethanesulfonic acid anhydride (2.17 mmol) was added. The mixture was allowed to stir for 5 h. The solvent was partly evaporated, and the residue was diluted with ether (3 mL). The mixture was stirred with aqueous hydrochloric acid for 3 h. The mixture was transferred to a separatory funnel and washed with 3 × 50 mL of saturated Na\textsubscript{2}CO\textsubscript{3} and then with 3 × 50 mL of brine. The organic layer was dried over Na\textsubscript{2}SO\textsubscript{4}, and the solvent was removed.

#### 2,3-Bis(hexadecyloxy)propene

A solution of pyridine (0.15 mL, 1.97 mmol) and 1,2-bis(hexadecyloxy)propene (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\textsubscript{2}SO\textsubscript{4}, and the solvent was removed. A yellow solid (778 mg, 1.19 mmol, 71%) was obtained, with a melting point around room temperature. \textsuperscript{4,5} \textsuperscript{13}C-NMR: δ 7.93 (d, J\textsubscript{ortho} = 13.5 Hz, 1H), 7.55 (d, J\textsubscript{ortho} = 10 Hz, 2H), 7.55 (d, J\textsubscript{ortho} = 13 Hz, 1H), 7.55 (d, J\textsubscript{ortho} = 13 Hz, 1H), 7.55 (m, 7H, 3 × OCH\textsubscript{3} + OCH\textsubscript{2}), 1.52 (m, 4H, 2 × O−C−CH\textsubscript{2}), 1.25 (s, 36 H, chain), 0.88 (t, 2H, 2 × CH\textsubscript{2}) ppm. \textsuperscript{31}P-NMR: δ 6.00 ppm. Anal. Calc for C\textsubscript{43}H\textsubscript{78}NPO\textsubscript{8}: C, 68.08; H, 9.53; N, 2.14. Found: C, 68.82; H, 10.09; N, 1.61.

### 2,3-Bis(tetradecyloxy)prop-3-yl-4-\textendash{(\textit{i}-Nitrovinyl)phenyl} Phosphonic Acid (DDBPNs, 3)

The product was prepared from 2(410 mg, 1.45 mmol) and 1,2-bis(tetradecyloxy)propene (480 mg, 1.0 mmol) as described for 3, using CH\textsubscript{2}Cl\textsubscript{2} as a solvent. A yellow solid (700 mg, 0.98 mmol, 98%) was obtained. Mp = 43–45°C. \textsuperscript{13}C-NMR: δ = 7.95 (d, J\textsubscript{ortho} = 14 Hz, 1H), 7.55 (d, J\textsubscript{ortho} = 11 Hz, 1H), 7.55 (d, J\textsubscript{ortho} = 14 Hz, 1H), 7.55 (d, J\textsubscript{ortho} = 14 Hz, 1H), 7.55 (m, 7H, 3 × OCH\textsubscript{3} + OCH\textsubscript{2}), 1.52 (m, 4H, 2 × O−C−CH\textsubscript{2}), 1.25 (s, 36 H, chain), 0.88 (t, 2H, 2 × CH\textsubscript{2}) ppm. \textsuperscript{31}P-NMR: δ = −5.04 ppm. Anal. Calc for C\textsubscript{46}H\textsubscript{86}NPO\textsubscript{8}: C, 65.64; H, 9.85; chain), 0.88 (t, 2H, 2 × CH\textsubscript{2}) ppm. \textsuperscript{31}P-NMR: δ = −5.04 ppm. Anal. Calc for C\textsubscript{46}H\textsubscript{86}NPO\textsubscript{8}: C, 65.64; H, 9.85; N, 1.97; P, 4.15.

### 1,2-Bis(hexadecyloxy)prop-3-yl-4-\textendash{(\textit{i}-Nitrovinyl)phenyl} Phosphonic Acid (DDBPNs, 5)

The product was prepared from 2(410 mg, 1.45 mmol) and 1,2-bis(hexadecyloxy)propene (540 mg, 1.0 mmol). 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.

### 4-Hydroxy-\textit{β}-Nitrostyrene (1)

The product was prepared in 40% yield from 4-hydroxybenzaldehyde and nitromethane


obtain complete hydrolysis of the phosphoramidochloridate. 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. 4-HN-NMR: δ = 7.96 (d, J = 13.5 Hz, 1H), 7.53 (d, J = 8.5 Hz, 2H), 7.53 (d, J = 13.5 Hz, 2H), 7.30 (d, J = 8.5 Hz, 2H), 1.47 (m, H, CH2O), 3.48 (m, H, 2 × CH2O + CH2O), 1.54 (m, 4H, 2 × O–CH2–CH2–), 1.26 (s, 60H, chain), 0.89 (t, 6H, 2 × CH3) ppm. 13C-NMR: δ = 153.43 (Cα), 137.65 (CH3), 136.87 (CH), 130.61 (CH), 126.84 (Cβ), 121.10 (CH, d, J = 6.1Hz), 77.00 (CHO), 71.84 (CHO), 70.83 (CHO), 69.24 (CHO), 67.77 (CH2OP, d, Jpc = 6.1Hz), 31.84 (CH2), 29.81 (CH2), 29.80 (CH2), 29.64 (CH2), 29.57 (CH2), 29.49 (CH2), 29.41 (CH2), 29.28 (CH2), 29.98 (CH2), 25.91 (CH2), 22.60 (CH2), 14.02 (CH3) ppm. 31P-NMR: δ = –5.38 ppm. Anal. Calc. for C42H77PO7: C, 69.58; H, 10.70; P, 4.27. Found: C, 68.97; H, 10.52; P, 3.76. 

### 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. Phosphorylation was carried out using an excess of POCl3; the phosphochloridate thus obtained was not stable and was reacted as soon as possible with the 1,2-bis(n-alkyloxy)propanols to yield the desired phosphodiester. 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 of 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 FFFM 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 DHBPNS (5), and 275 nm for DOPBNS (6). These average diameters were also observed using cryo-SEM (Figure 1b). DSC enthalpograms were recorded on a MC-2 differential scanning calorimeter (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 (ε = 10 000 M–1 cm–1) characteristic absorption at 334 nm using a PC-16 Perkin-Elmer 15 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 of S-(β-Nitrostyrene in the bilayer was achieved by irradiation of 1.0 mM 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 4-HN-NMR signal of the vinyl protons of the monomer. A sample of fully polymerized material was freeze-dried and redissolved in THF, and its 1H- and 31P-NMR spectra are characteristic for a high molecular weight polymer. The sample was analyzed by gel permeation chromatography (CHCl3), electro spray mass spectrometry (CH2Cl2), and vapor pressure osmometry (THF).

**Freeze–Fracture Transmission and Cryo-Scanning Electron Microscopy.** Samples were prepared and examined as described previously. For Differential Scanning Calorimetry. DSC enthalpograms were recorded on a MC-2 differential scanning calorimeter (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. The crude product was crystallized from methanol (0.7 g in 20 mL). Pure product (450 mg, 0.62 mmol, 62%) with mp = 41–42 °C was isolated. 4-HN-NMR: δ = 9.94 (s, 1H), 7.85 (d, J = 8.5 Hz, 2H), 6.85 (br s, 1H, POH), 4.20 (m, 2H, CH2OP), 3.48 (m, 7H, 3 × CH2O + CH2O), 1.51 (m, 4H, 2 × O–CH2–CH2–), 1.24 (s, 52H, chain), 0.87 (t, 6H, 2 × CH3) ppm. 13C-NMR: δ = 190.61 (CHO), 155.29 (Cα, d, Jpc = 6.1Hz), 133.29 (Cβ), 131.55 (Cγ), 120.69 (d, Jpc = 6.1 Hz), 77.11 (CHO), 71.88 (CHO), 70.87 (CHO), 69.33 (CHO), 67.79 (CH2OP, d, Jpc = 6.1 Hz), 31.90 (CH2), 29.11 (CH2), 29.71 (CH2), 29.65 (CH2), 29.56 (CH2), 29.54 (CH2), 29.37 (CH2CH2), 25.98 (CH2), 22.70 (CH2), 14.12 (CH3) ppm. 31P-NMR: δ = –5.09 ppm. Anal. Calc. for C42H77PO7: 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 4-Formylphenyl phosphorodichloridate thus obtained was not stable and was reacted as soon as possible with the 1,2-bis(n-alkyloxy)propanol to yield the desired phosphodiester. Most likely, the phosphorylation reaction is applicable to the selective phosphorylation of any phenol and any primary alcohol.

**Vesicle Preparation.** Vesicle solutions were prepared from 10 mM stock solutions of the sodium salts of 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 FFFM 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 DHBPNS (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 of 54.3°C (ΔH = 37.8 kJ/mol) for DOPBNS (6), 40.7°C (ΔH = 42.3 kJ/mol) for DTPBNS (5), and 21.6°C (ΔH = 74.3 kJ/mol), this includes secondary transitions around 40°C for DTPBNS (4) and no T2 above 5°C for DDPBNS (3). Temperature-dependent 31P-NMR spectra of a DHPBNS (5) vesicle solution in D2O 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).**
Surface differentiation is favored by an increase of the molecules between the two bilayer leaflets (T > Tm), as well as the rate of exchange of lipid charged; we have obtained experimental evidence for this assumption \(^{(23)}\). 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-
\(\beta\)-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 amphiphiles solutions (aqueous solutions containing 25 mol \% of ethanol) is more rapid than in the cleavage of amphiphiles organized in vesicles (runs 2 and 7 vs runs 3 and 8, 10). Obviously, the negatively charged membrane retards the nucleophilic attack by hydroxide ion. Disruption 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

\[(25)\] 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.

\[(23)\] 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 (\(\lambda_{abs} = 515\) 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 > Tm, hydroxide leakage is considerable.

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:

$$\text{BNS}_{\text{endo}} \rightarrow \text{BNS}_{\text{exo}}$$

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

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

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

Leading to an equation of the form

$$[BNS]_{\text{endo} \rightarrow \text{exo}} = C_a \exp(-k_1 t) + C_b \exp(-k_{-1} t)$$

The solution for $[BNS]_{\text{endo} \rightarrow \text{exo}}$ may be found using the Laplace transformation.\(^{(26)}\) $C_a$, $C_b$, $k_1$ and $k_2$ are combinations of $[\text{BNS}]_{\text{endo} \rightarrow \text{exo}}$, $k_{-1}$, $k_1$ and $k'$. Thus, assuming $k_1 \approx k_{-1}$, the values of $k_1$ and $k_2$ may be calculated from the experimentally determined values of $k_0$ and $k_2$, which were previously reported as $k_{\text{slow}}$ and $k_{\text{fast}}$. 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 $k_1$ 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 $k_2$ values. As can be seen from Table 1 ($k_2/k_{-1}$ values), endo-vesicular cleavage is not 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 $k_2$ 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); thus, 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-lives 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 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 $k_2$ 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.

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

<table>
<thead>
<tr>
<th>run</th>
<th>experiment</th>
<th>$k'_{\text{fast}}$</th>
<th>$k'_{\text{slow}}$</th>
<th>$k_2$</th>
<th>$k_1$</th>
<th>$k'_{2}/k_1$</th>
<th>BNS$<em>{\text{endo}}$/BNS$</em>{\text{tot}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BNS (1)</td>
<td>2.56</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>DDPBNS (3)</td>
<td>12.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>tip</td>
<td>10.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>injection</td>
<td>9.89</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>+ CTAB</td>
<td>142</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>DTPBNS (4)</td>
<td>5.11</td>
<td>0.584</td>
<td>4.32</td>
<td>0.680</td>
<td>6.4</td>
<td>&lt;0.20</td>
</tr>
<tr>
<td>7</td>
<td>DHPBNS (5)</td>
<td>13.3</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>8</td>
<td>tip</td>
<td>5.49</td>
<td>0.316</td>
<td>5.13</td>
<td>0.343</td>
<td>15</td>
<td>0.43</td>
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<tr>
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<td>0.893</td>
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<tr>
<td>11</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>DOPBNS (6)</td>
<td>6.24</td>
<td>0.095</td>
<td>6.15</td>
<td>0.100</td>
<td>62</td>
<td>0.47</td>
</tr>
</tbody>
</table>

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\(^{(26)}\) We refer to the Supporting Information for more detail.


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 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.

**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. 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 $\beta$-nitrostyrene units linked to the phosphate headgroup. Under the appropriate conditions, the vesicles undergo hydrolytic cleavage of the phosphate ester to form a polymerized bilayer leaflet. The degree of polymerization can be controlled by adjusting the pH and the irradiation time. The resulting vesicles maintain their morphology and size, indicating that the polymerization process does not significantly affect the vesicle structure.

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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.

Acknowledgment. We express our gratitude to K. Siegel (ERASMUS exchange student from the University of Goettingen, Germany) for the synthesis of DTPBNS \(^4\), to Dr. A. Sein and to Mr. I. Stokroos (Laboratory of Cell Biology and Electron Microscopy) for their help in the preparation of several EM samples, to Prof. A. D. R. Brisson (Institute of Electron Microscopy) for hospitality in his laboratory, and to Prof. M. J. Blandamer and Dr. B. Briggs (University of Leicester, U.K.) who performed the DSC measurements. We thank Prof. D. Hoekstra (Department of Physiological Chemistry) for stimulating discussions and hospitality in his laboratory. Dr. K. Surkov (University of St. Petersburg, Russia) is acknowledged for his help in the analysis of our kinetic data. We acknowledge with gratitude the contributions of Dr. L. A. M. Rupert in the initial stages of the project.

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