J. Membrane Biol. 166, 37–49 (1998)

The Journal of
Membrane
Biology

Membrane Fusion and Rupture in Liposomes: Effect of Biodegradable pH-Sensitive Surfactants

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Received: 18 February 1998/Revised: 14 July 1998

Abstract. Biodegradable pH-sensitive surfactants (BPS) are a unique family of easily metabolized compounds that demonstrate pH-dependent surface activity. These agents, in combination with other delivery systems, have demonstrated effects in enhancing transnucleic acid activity. The increased activity has been hypothesized to occur from a release of endosomal contents. Simply, the BPS delivery system containing nucleic acids enters the cell through an endocytotoic process. It encounters an acidic pH and becomes surface active leading to defects in the endosomal membrane. In the current study, an in vitro model membrane was used to better understand the liposome defect mechanisms that BPS elicit. Using this system, it is shown that BPS can induce both liposome fusion and rupture depending upon the pH and mole ratio of BPS to membrane lipids. Futhermore, liposome fusion induced by BPS was dependent on the total numbers of liposome particles while rupture was independent of interacting liposome particles. The generated data indicate that BPS agents act differently from other typical surface active agents and fuosgenic compounds. Instead of facilitating membrane fusion through the hexagonal II phase, BPS appeared to contribute and participate in the membrane fusion at different stages.

Key words: Drug delivery — Liposomes — Membrane fusion — Membrane rupture — Surfactants

Introduction

The therapeutic approach of using nucleic acids as drugs is attractive due to their ability to combat diseases lacking current treatment options. However, there are several barriers that must be surmounted. An impediment to the therapeutic use of nucleic acids is intracellular transport. It has been demonstrated that most nucleic acids (e.g., oligodeoxynucleotide (ODN) and plasmid DNA (pDNA)) and their carriers (e.g., liposomes and cationic macromolecules) enter cells via endocytosis (Zabner et al., 1995). These molecules initially accumulate in endosomes, with a majority of the nucleic acids later degraded by enzymes within lysosomes (McGraw & Maxfield, 1991). Since nucleic acids must escape from the endocytotic pathway to have an effect, the endosomal membrane presents a barrier to nucleic acid therapy.

A method to increase the amount of nucleic acids reaching the cytoplasm is to enhance the efficacy of nucleic acid escape from endosomes. Systems that have been shown to disrupt cellular membranes include virosomes (Hug & Sleight, 1994), pH-sensitive liposomes (Chu et al., 1990), viral peptides (Yu et al., 1994), and synthetic alpha-helical forming peptides (Fattal et al., 1994). All these delivery methods increase the biological effect of the associated nucleic acids but have their own related limitations.

Dodecyl 2-(1′-imidazolyl) propionate (DIP), a member of the biodegradable pH-sensitive surfactants (BPS), has been previously demonstrated to increase cytoplasm delivery of ODN (Hughes et al., 1996) and pDNA (Liang & Hughes, 1998). BPS are lipophilic amines with pKa values between 5 and 7 which become protonated at acidic intra-endosomal pH (Firestone, Pisano & Bonney, 1979; de Duve et al., 1974). At neutral pH in the cytoplasm or intracellular fluid, BPS are un-ionized with little surface activity. Upon protonation, the surface activity of the BPS is increased due to the expansion in the hydrophilicity of the head group that activates the compound leading to membrane destabilization. BPS are synthesized with an easily metabolized bond (e.g., ester) connecting the head and aliphatic tail groups. The incorporation of BPS into particulate delivery systems (e.g., cationic liposomes) forces the agent to

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enter cells through an endocytotic process along with the nucleic acids of interest. However, it remains unclear how BPS destabilize the endosomal membrane. To further utilize and modify BPS in intracellular macromolecule delivery, it is essential to understand the mechanisms responsible for membrane destabilization.

In addition to DIP, two other pH-sensitive surfactants were synthesized to investigate their mechanisms. Dodecyl imidazole (DI) is a non-ester containing pH-sensitive surfactant that was originally synthesized by Firestone et al. (1979) as an anticancer agent. DI was selected as a reference compound to address the importance of the linker. The second agent, methyl 1-imidazolyl laureate (MIL), is biodegradable and similar to DIP but has the ester bond in the opposite direction. The rearrangement of the ester will enhance the biodegradability of the compound and also alter the attached imidazole's pKa value. In this report, all three agents were loosely grouped in the BPS family.

Instead of serving as a nucleic acid delivery system, liposomes were used as the experimental membrane model to study the possible membrane destabilization mechanisms. To maintain simplicity in these model studies, only neutral lipids (e.g., lecithin) were used in the liposome system. While the liposomes may not fully represent events occurring in biological situations they still served as excellent models in addressing potential mechanisms of lipid membrane disruption. An aqueous space fusion assay (Smolarsky et al., 1977) and a lipid mixing resonance energy transfer assay (Struck, Hoekstra & Pagano, 1981) were used to observe membrane destabilization through fusion. A liposome leakage assay of a marker compound, calcein, was used to observe membrane destabilization through rupture. Using these systems, it was demonstrated that BPS could lead to both membrane fusion and rupture in a pH-dependent manner via a similar pathway.

Materials and Methods

Materials

Calcein and N,N-dimethylformamide (DMF) were purchased from Aldrich (Milwaukee, WI). N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (Rh-PE), N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (NBD-PE), L-α-lecithin, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), dioleoylphosphatidyl ethanolamine (DOPE), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Dodecanol, 2-bromopropionyl bromide, imidazole, 12-bromo-1-dodecanol, lauric acid, and N,N′-dicyclohexylcarbodimide were purchased from Fluka (Ronkonkoma, NY). N,N′-p-xylylenebis-(pyridinium bromide) (DPX) and 1-aminonaphthalene-3,6,8-trisulfonic acid (ANTS) were purchased from Molecular Probes (Eugene, OR).

All buffers were adjusted with NaCl to an equal ionic strength. The pH of the buffers and their chemical compositions were as follows: pH 4.2 (150 nM sodium acetate and 350 mM glacial acetic acid), pH 5.0 (300 mM KH_2PO_4 and 50 mM Na_2HPO_4), pH 6.0 (150 mM KH_2OP_4 and 100 mM Na_2HPO_4), pH 6.5 (120 mM KH_2PO_4 and 100 mM Na_2HPO_4), pH 7.0 (80 mM KH_2PO_4 and 120 mM Na_2HPO_4), and pH 8.0 (200 mM KH_2PO_4 and 188 mM NaOH).

Methods

Preparation of biodegradable pH-sensitive surfactants (BPS)

Dodecyl 2-(1′-imidazolyl) propionate (DIP) was synthesized as previously reported (Hughes et al., 1996) by mixing dodecanol (0.05 mol) and 2-bromopropionyl bromide (0.025 mol) in 50 ml of $CHCl_3$ at room temperature for 24 hr to yield crude dodecyl 2-bromopropionate. After purification, dodecyl 2-bromopropionate (0.015 mol) was mixed with imidazole (0.03 mol) in 50 ml of $CHCl_3$ and refluxed overnight yielding DIP. Dodecyl imidazole (DI) was synthesized by reacting imidazole (0.05 mol) and 12-bromo-1-dodecanol (0.025 mol) in 50 ml of N,N-dimethylformamide (DMF) at 75°C for 24 hr. A mixture of 1-imidazole methanol (0.05 mol) (supplied by Dr. Kenneth Sloan, Department of Medicinal Chemistry, University of Florida), lauric acid (0.025 mol) and N,N′-dicyclohexylcarbodiimide (0.025 mol) in 50 ml of DMF was stirred overnight at 75°C yielding methyl 1-imidazolyl laureate (MIL). Crude DIP, MIL, and DI were purified through silica gel flash chromatography and their structures (*see* Fig. 1) confirmed through ^{1}H-NMR, mass spectroscopy, and elemental analysis.

Liposome Preparation

Liposomes (L-α-lecithin: DMPC: cholesterol; mole ratio 6:1:8) were prepared and used in most experiments to evaluate fusion and rupture events. The lipid rehydration method was used to produce vesicles (Hughes et al., 1994). The liposomes were passed through a high pressure extruder (Lipex Biomembrane, Vancouver, BC) with 600 nm polycarbonate membranes three times. The size distribution (volume weight Gaussian distribution) was determined to be 578 ± 113 nm (standard deviation) with a NICOMP 380 ZLS Zeta Potential/Particle Sizer (Santa Barbara, CA). The concentration of total phospholipid was determined by a spectrophotometric technique as previously described (Stewart, 1980).

Time Course Fusion Assay

Increasing mole ratios (R = 0, R = 0.2, and R = 0.4) of BPS to 75 mmol/ml of lipids (L-α-lecithin: DMPC: cholesterol; mole ratio 6:1:8) were incorporated into liposomes to monitor fusion mechanism over time in a pH 5.0 buffer solution. Vesicle-vesicle fusion was characterized by measuring fluorescence resonnance energy transfer between two lipid head groups (Struck et al., 1981). N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (NBD-PE) and N-(lissamine rhodamine β sulfonyl)-phosphatidylethanolamine (Rh-PE) were incorporated into two separate populations of vesicles at 1% (mol) each. As the two liposome groups interacted, the fluorescence energy emitted from NBD-PE labeled liposomes was transferred to the Rh-PE labeled lipsomes resulting in a decreased fluorescence signal. The NBD-PE liposomes were initially added into various pH buffers with the Rh-PE liposomes and fluorescence intensity measured over 30-min incubation period at 25°C. The liposomal suspensions were excited at a wavelength of 470 nm and observed at 530 nm with a Perkin-Elmer Luminescence Spectrophotometer LS-50B. The percentage of fusion was defined by the following relationship: % Fusion = $100 \cdot (1\text{-}F/F_0)$

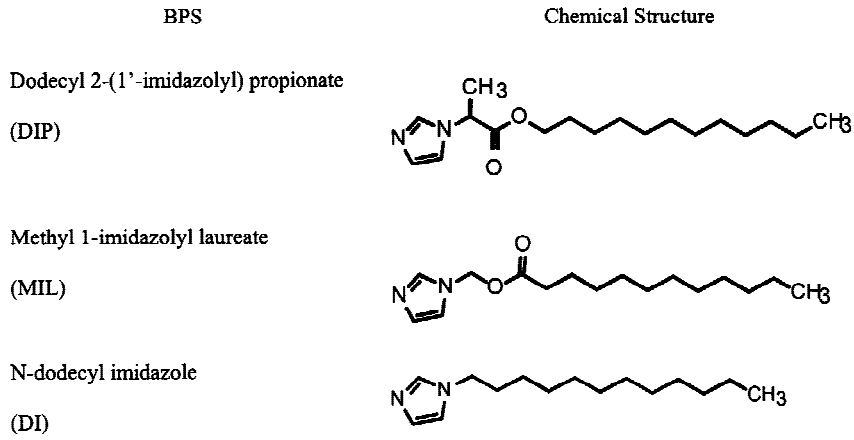

Fig. 1. Names and chemical structures of three biodegradable pH-sensitive surfactants (BPS).

where F and F_0 are the fluorescence intensities in the presence and absence of the Rh-PE group, respectively (Struck et al., 1981).

To corroborate membrane fusion, an aqueous content mixing method was used (Smolarsky et al., 1977). In this assay, 25 mM of 1-aminonaphthalene-3,6,8-trisulfonic acid (ANTS) and 90 mM of N,N′-p-xylylenebis-(pyridinium bromide) (DPX) were encapsulated into two separate lipsomes (L-α-lecithin: DMPC: cholesterol; mole ratio 6:1:8). Unencapsulated ANTS and DPX were later removed through centrifugation (10,000 rpm, 5 min) five times and washed with a pH 7.4 phosphate buffer solution. These two lipsome populations (75 mmol/ml) were mixed and the fluorescence intensity recorded over time at an excitation wavelength of 353 nm and an emission wavelength of 525 nm. Mixing of the aqueous contents of ANTS and DPX containing liposomes resulted in a decrease in fluorescence due to the quenching of ANTS by DPX. The percentage of fusion was calibrated similarly with the above equation where F is the fluorescence intensity in the presence of DPX group and F_0 is the fluorescence intensity in the absence of DPX group in different pH buffers.

BPS-induced Membrane Fusion

Both aqueous and lipid mixing techniques were further applied to characterize the liposome fusion induced by different mole ratios of BPS after 30 mins. Increasing mole ratios (R = 0, R = 0.2, and R = 0.4) of BPS were incorporated into 75 mmol/ml of liposomes (L-α-lecithin: DMPC: cholesterol; mole ratio 6:1:8) to monitor fusion with changing pH (5.0–8.0). The fluorescence intensities were then quantified after 30 min in both assays.

BPS-induced Membrane Rupture

Liposomes (L-α-lecithin: DMPC: cholesterol; mole ratio 6:1:8, 75 mmol/ml) containing 100 mM calcein (> self-quenching concentration) were prepared using increasing mole ratios of BPS (R = 0, R = 0.1, R = 0.2, and R = 0.4). The liposomes were used to observe whether membrane rupture occurred. The BPS-liposome preparations were incubated with phosphate buffers (pH 5.0–8.0) for 30 min and calcein release quantified. The released calcein was excited at 496 nm and observed at 517 nm at 25°C. The percentage of released calcein was calculated by the equation $I(\%) = (I_a\text{-}I_b)/(I_x\text{-}I_b) \cdot 100$ (Liu & Regen, 1993), where I_x is 100% fluorescence intensity value after adding excess Triton X-100 in different pH buffers. I_a and I_b are the florescence intensities after incubation with and without BPS, respectively.

Comparison of BPS-induced Membrane Defects when either Added Externally or via Direct Incorporation into Liposomes

To investigate the effects of partitioning of BPS between membrane and aqueous medium, studies were conducted which varied the amount of BPS in a solution of calcein containing liposomes (L-α-lecithin: DMPC: cholesterol; mole ratio 6:1:8, 10 mmol/ml). Increasing amounts of BPS were either added from external environmental or incorporated into liposomes in various buffer solutions. The liposome suspensions were incubated for 30 min at 25°C and the percentage of calcein release was calculated by the equation $I(\%) = (I_a\text{-}I_b)/(I_x\text{-}I_b) \cdot 100$ as previously described (Liu & Regen, 1993).

Effects of Liposome Concentration on Membrane Fusion and Rupture

Increasing concentrations of liposomes with the R = 0.2 BPS were used to determine their dependency on membrane rupture (3 mmol/ml, 15 mmol/ml, and 75 mmol/ml) and fusion (37.5 mmol/ml, 75 mmol/ml, and 150 mmol/ml) in a pH 5.0 buffer solution. Similar lipid mixing assay protocols were used as described above.

Effect of Cholesterol and of DOPE on Membrane Fusion

Liposomes (135 mmol/ml) containing L-α-lecithin, with or without cholesterol (weight ratio 6:4; mole ratio 4:5) at two mole ratios (R = 0.2 and R = 0.4) of DIP were used to determine the impact of the addition of cholesterol on fusion. A similar study was conducted with cholesterol replaced by DOPE (weight ratio 6:4; mole ratio 3:2). The percentage of fusion using the lipid mixing assay were determined in different pH buffers after 30 min.

To further characterize the difference of fusion events caused by cholesterol and DOPE, liposomes (135 mmol/ml) with an equal mole ratio of cholesterol or DOPE to L-α-lecithin were incorporated with DIP at mole ratio 0.4 (R = 0.4). The percentages of fusion using the lipid mixing assay were determined with different pH buffer after 30 min.

Statistical Analysis

Statistical differences between the treatments were determined using analysis of variance where appropriate (StatView 4.53, Abacus Con-

cepts, Berkeley, CA) with $P < 0.05$ considered statistically significant and Fisher's (PLSD) post hoc *t*-test was applied.

Abbreviations

ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid; BPS, biodegradable pH-sensitive surfactants; DI, dodecyl imidazole DIP, dodecyl 2-(1′-imidazolyl) propionate; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DOPE, dioleoylphosphatidyl ethanolamine; DPX, N, N′-p-xylylenebis-(pyridinium bromide); MIL, methyl 1-imidazolyl laureate; NBD-PE, N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine; ODN(s), oligodeoxynucleotide(s); pDNA, plasmid DNA; R, mole ratio of the biodegradable pH-sensitive surfactants to the other lipids; Rh-PE, N-(lissamine rhodamine β sulfonyl)-phosphatidylethanolamine

Results

Time Course Fusion Assay

To identify possible events in the membrane destabilization sequence, fusion assays were applied with each BPS-liposome system using a pH 5.0 buffer and observed over time. The percentage of membrane fusion that was determined by the lipid mixing assay demonstrated a rapid initial event followed by a plateau (Fig. 2*A*). For the aqueous mixing assay, however, the percentages of fusion increased slightly in the early period and continued over the entire test time course (Fig. 2*B*). Liposome fusion was significantly different ($P < 0.001$) among all the mole ratios in each BPS over time. No significant difference in fusion among the three agents was observed.

BPS-Induced Membrane Fusion

To investigate the fusogenic properties elicited by inclusion of BPS on membranes, increasing amounts of the three BPS agents were incorporated into liposomes followed by incubation at different pHs (Fig. 3). When fusion was determined with the lipid mixing assay, throughout all pHs tested, the percentage of fusion with the control liposomes ($R = 0$) increased slightly as the pH decreased. After incorporating BPS into the liposomes, the percentage of fusion was statistically higher at all observed pHs. As the mole ratio of BPS in the liposomes was raised, fusion was significantly increased ($P < 0.001$) in pHs lower than 6.0 (Fig. 3*A*). All three BPS agents exhibited a similar fusion profile and there was no significant difference between the fusion events among the three agents.

For the fluorescence aqueous mixing method, the percentages of fusion by control liposomes ($R = 0$) demonstrated no significant difference at all tested pHs. After the addition of BPS, the percentage of fusion increased at all pHs. As the mole ratio of PBS was raised, fusion increased significantly ($P < 0.001$) below pH 6.0 (Fig. 3*B*). Comparable to the lipid mixing assay, the three BPS agents exhibited similar fusion profiles with no significant difference among the three BPS evaluated.

BPS-induced Membrane Rupture

We determined the ability of liposome incorporated unionized BPS to facilitate the release of entrapped solutes at acidic pHs. Liposomes containing calcein were prepared with increasing amounts of BPS and incubated at decreasing pHs. Minimal calcein release was observed at the lower BPS/liposome mole ratio group ($R = 0.1$), but at the $R = 0.4$ group, calcein release increased significantly ($P < 0.001$) at all pHs (Fig. 4). At pH 5.0 and 6.0, significant differences ($P < 0.05$) in the amount of released calcein were observed among all mole ratios. Comparing these three BPS, the percentages of release were significantly different ($P < 0.01$) at the $R = 0.4$ group in all observed pHs. However, no significant difference was observed at the other two ratio groups ($R = 0.1$ and $R = 0.2$) among the three BPS liposomes.

Comparison of BPS-induced Membrane Defects when either Added Externally or via Direct Incorporation into Liposomes

When DIP was added from the external environment into the liposomes, significant differences ($P < 0.01$) of the calcein release were seen when the mole ratio of DIP to total lipids reached two or above among all pH groups (Fig. 5*A*). A similar trend was seen when DIP was replaced by MIL (Fig. 5*A*). However, when DI was evaluated, no significant differences of the calcein release were seen at any mole ratios with respect to the pH of the testing buffer (Fig. 5*A*). On the other hand, when BPS was incorporated into liposomes, similar profiles were observed for all three agents as was observed in the BPS-induced membrane rupture experiment set (Fig. 5*B*).

Effects of Liposome Concentration on Membrane Fusion and Rupture

To quantify the liposome concentration dependency on membrane fusion and rupture, we measured the fusion and rupture behavior of liposomes at three concentrations. Measurements were made with the $R = 0.2$ BPS containing liposomes in a pH 5.0 buffer solution. Due to the sensitivity of the assays, different liposome concentrations were used in the studies presented. Significant differences of fusion ($P < 0.05$) were observed among the three liposome concentrations with the lipid mixing assay (Fig. 6*A*). The vesicle-vesicle fusion was concen-

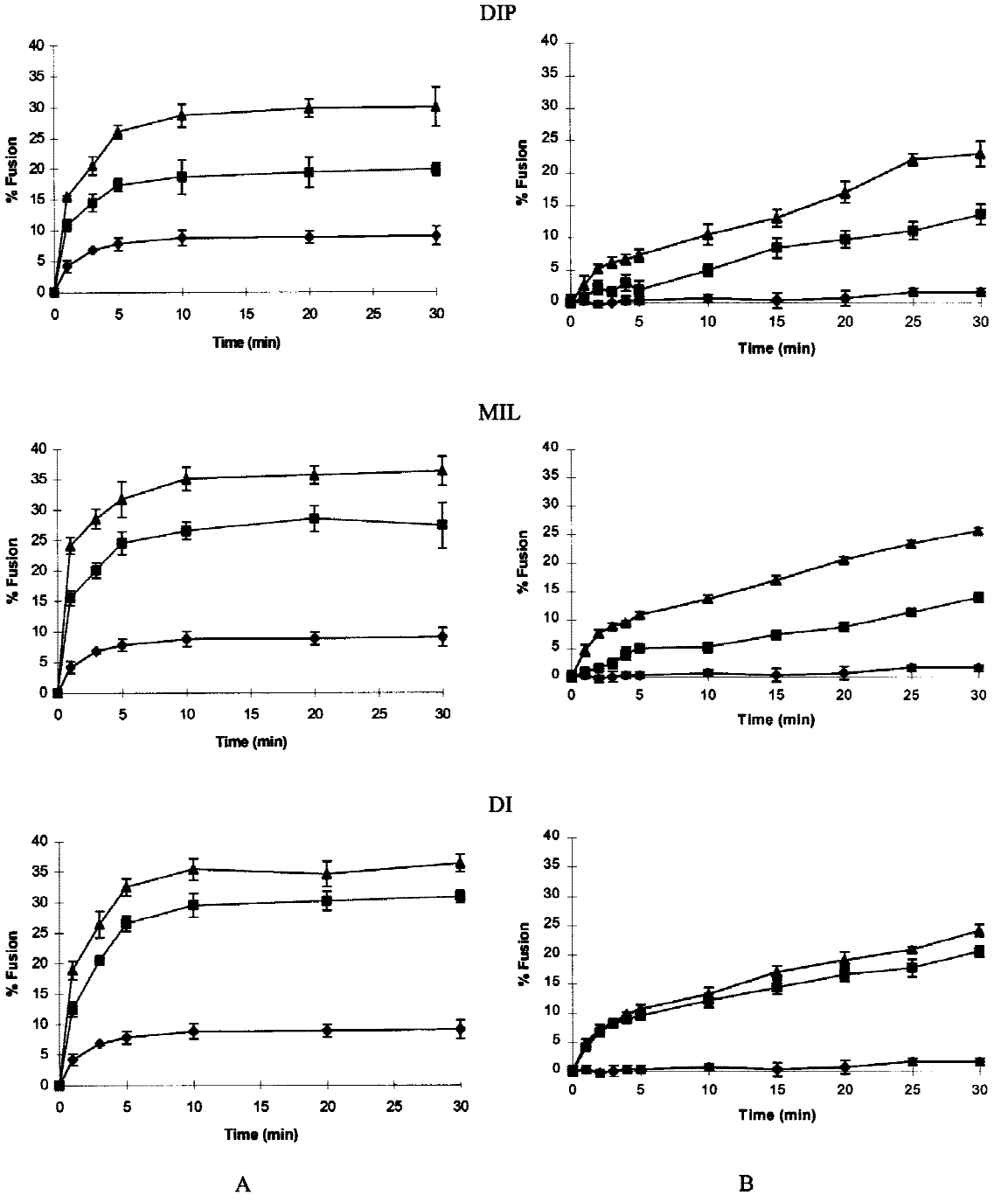

Fig. 2. Time course of liposome fusion at three mole ratios (R = 0 (♦), R = 0.2 (■), and R = 0.4 (▲)) of BPS in a pH 5.0 buffer solution. *(A)* The NBD-PE labeled liposomes were incubated with Rh-PE labeled liposomes for 30 min ($n = 3$). *(B)* Liposomes containing 25 mM of ANTS were incubated with liposomes containing 90 mM of DPX for 30 min ($n = 3$). Data are expressed as mean ± SD.

tration dependent on each individual BPS. However, no significant difference in released calcein was observed among the three liposome concentrations on the rupture behavior (Fig. 6*B*) indicating the concentration independence of BPS-liposome lysis profiles.

Effect of Cholesterol and of DOPE on Membrane Fusion

For liposomes without any other additive, the percentage of fusion increased significantly ($P < 0.05$) as the pH

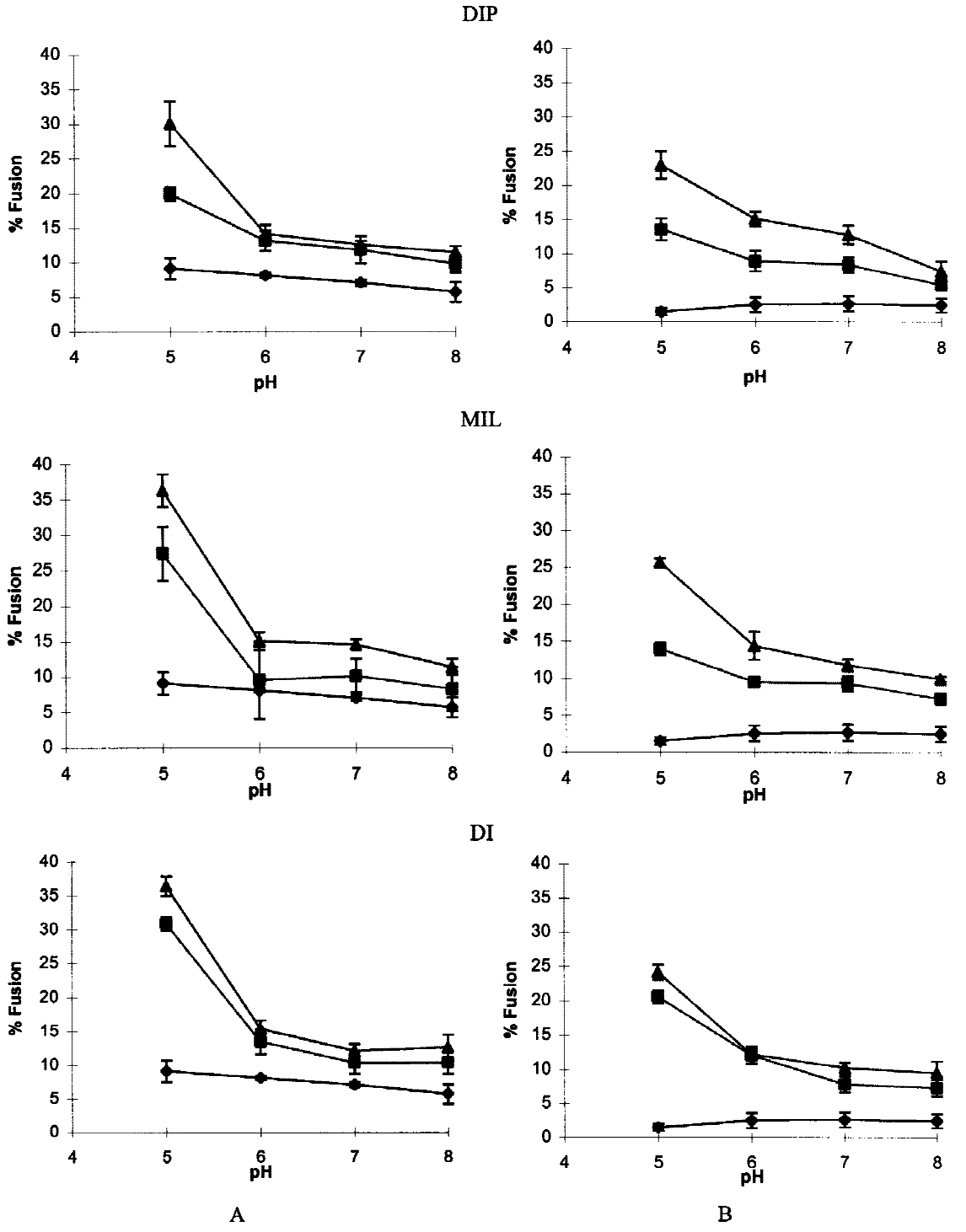

Fig. 3. Fusogenic properties of liposomes at three mole ratios (R = 0 (♦), R = 0.2 (■), and R = 0.4 (▲)) of BPS at different pHs. *(A)* The NBD-PE labeled liposomes were incubated with Rh-PE labeled liposomes for 30 min and the fluorescence intensity measured at 530 nm ($n = 3$). *(B)* Liposomes containing 25 mM of ANTS were incubated with liposomes containing 90 mM of DPX for 30 min and the fluorescence intensity measured at 535 nm ($n = 3$). Data are expressed as mean ± SD.

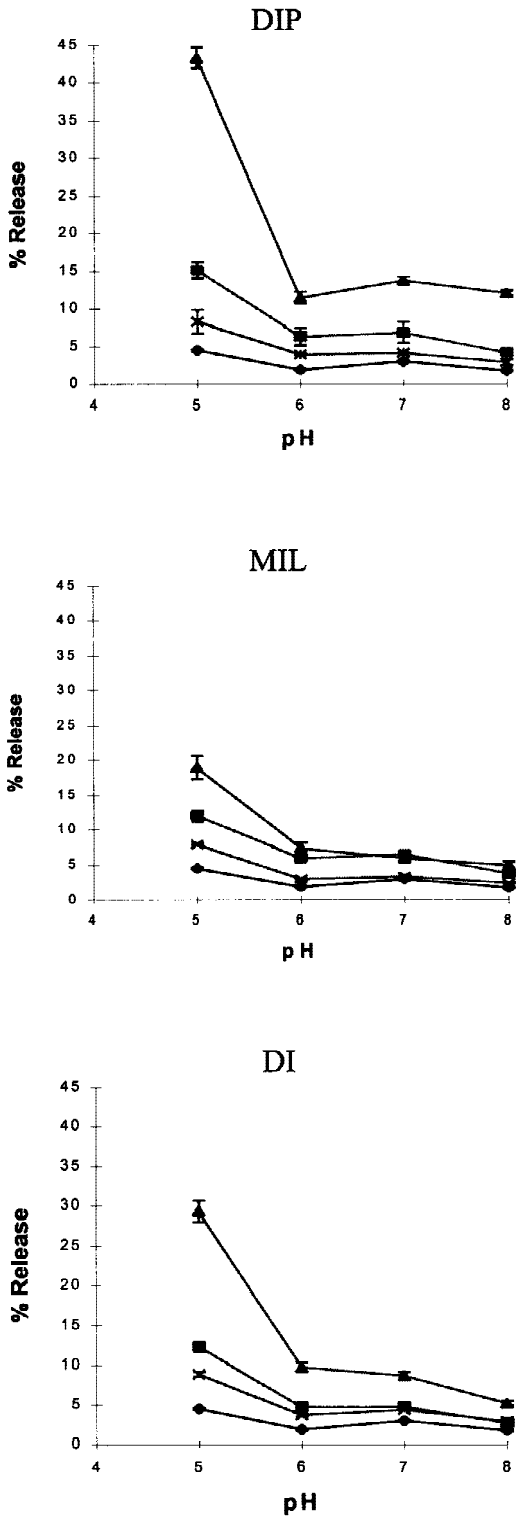

Fig. 4. Membrane rupture profile with the incorporation of BPS into liposomes. The membrane lysis effects with BPS/liposome ratios (R = 0 (♦), R = 0.1 (✕), R = 0.2 (■), and R = 0.4 (▲)) at different pHs were determined by calcein release (mean ± SD) after 30 min (n = 3). There were significant differences among all mole ratio groups (R = 0.1, R = 0.2, and R = 0.4) at all observed pHs.

decreased to 5.0 in both ratio groups (R = 0.2 and R = 0.4). Greater fusion was observed at the higher ratio groups (R = 0.4) than in lower ones (R = 0.2) (Fig. 7). The incorporation of 40% (weight) cholesterol into the liposome system resulted in enhanced fusion with both groups. However, when cholesterol was replaced by DOPE, fusion decreased significantly ($P < 0.001$). Significant differences ($P < 0.001$) were observed among the three separate formulations in both ratio groups when the pH was dropped to 5.0.

With the same mole ratio to a neutral lipid, DOPE and cholesterol contributed distinctively in the DIP-liposome fusion behaviors over the observed pH (Fig. 8). The incorporation of cholesterol into the liposomes resulted in additional fusion ($P < 0.01$) than the liposomes with L-α-lecithin only as pH dropped to 7.0 or lower. When cholesterol was replaced by DOPE, the fusion decreased significantly ($P < 0.001$) as pH decreased to 5.0. The fusion affected by cholesterol was significantly higher than that affected by DOPE ($P < 0.001$) at pH 5.0. When the pH was raised to 6.0 or above, the fusion affected by DOPE was significantly higher than that affected by cholesterol ($P < 0.001$).

Discussion

Nucleic acid (e.g., plasmid DNA and oligonucleotide) therapy is a promising approach for the treatment of a variety of disorders (Sokol & Gewirtz, 1996). Two delivery methods, viral and non-viral, are being studied for cellular delivery of these nucleic acid agents. The non-viral systems (e.g., cationic liposomes) are attractive due to the ease of production, the ability to transfect a variety of cell types, and the lower chance of immune reactions (Lee & Huang; 1997). While non-viral systems are currently somewhat inefficient, they will undoubtedly improve with the determination of rate-limiting mechanisms that govern transgene expression (Hughes et al., 1996).

DIP, the prototypical member of the BPS family, has been shown to increase the effectiveness of nucleic acid delivery (Liang & Hughes, 1998; Hughes et al., 1996). However, it is unclear how BPS destabilize the endosomal membrane and elicit the transfer of ODNs and pDNA or if this is its main mechanism of action.

Both membrane fusion and rupture elicited by BPS were pH and mole ratio dependent. As the pH became acidic or BPS intraliposomal amounts increased (e.g., increasing the amount of ionized BPS), the fusion and rupture events increased. The extent of membrane rupture and fusion was also elevated when the non-charged BPS was equal to a mole ratio of 0.4. This effect is possibly due to the alternations in lipid packing (New, 1990). We were unable to form BPS-liposomes as the mole ratio of BPS to total lipids equaled 0.5 (R = 0.5) or above.

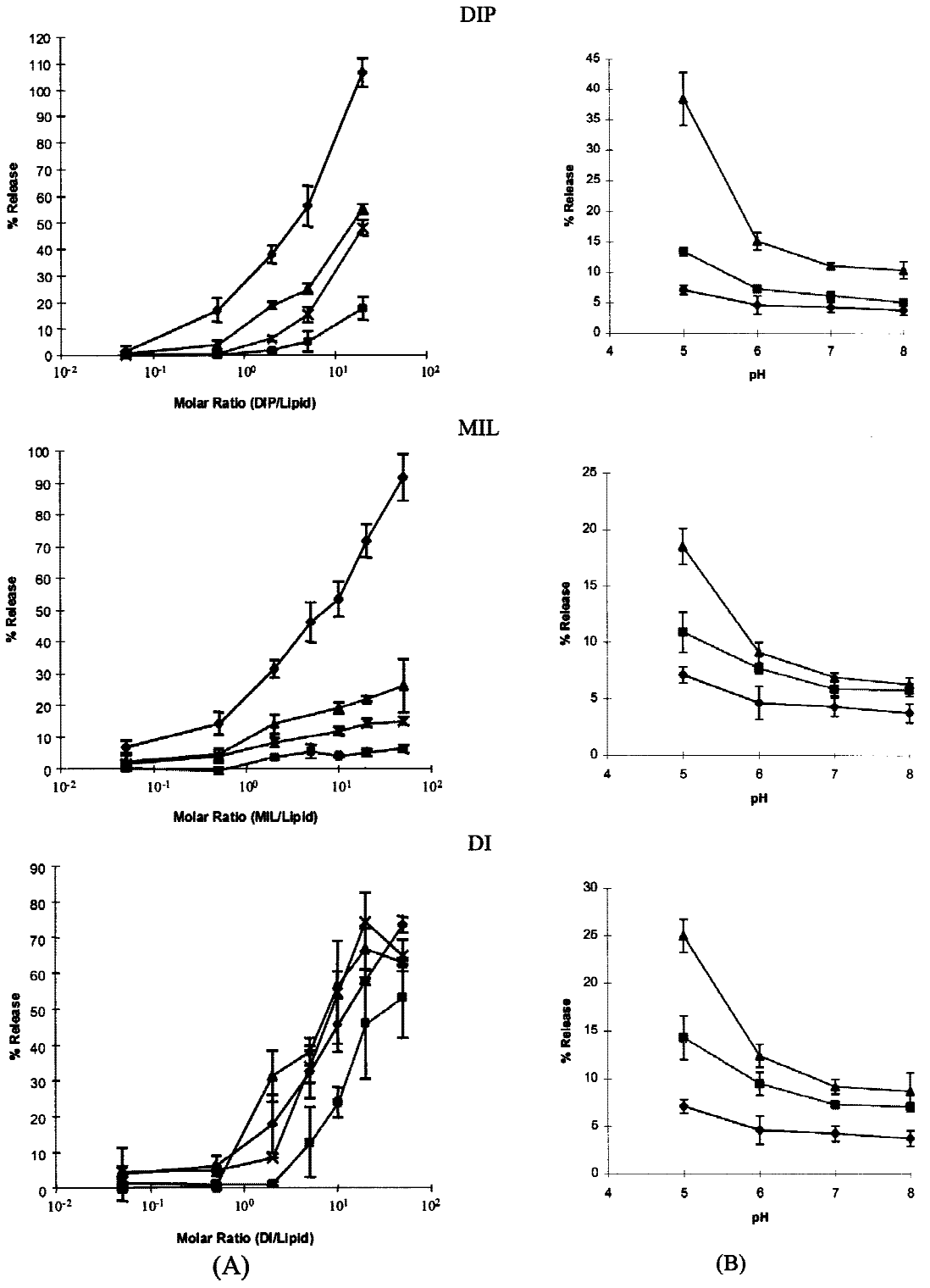

Fig. 5. Comparison of BPS-induced membrane rupture. *(A)* The effect of BPS on calcein release from liposomes when added from the external environment ($n = 3$). The percentages of release were calculated after a 30-min incubation in four buffer solutions (pH 4.2 (♦), 6.0 (▲), 6.5 (✕), and 8.0 (■). *(B)* The membrane lysis profile of BPS when incorporated into liposomes at different mole ratios ($R = 0$ (♦), 0.2 (■), and 0.4 (▲)). BPS-induced calcein release was obtained after 30 min ($n = 3$). Data are expressed as mean ± SD.

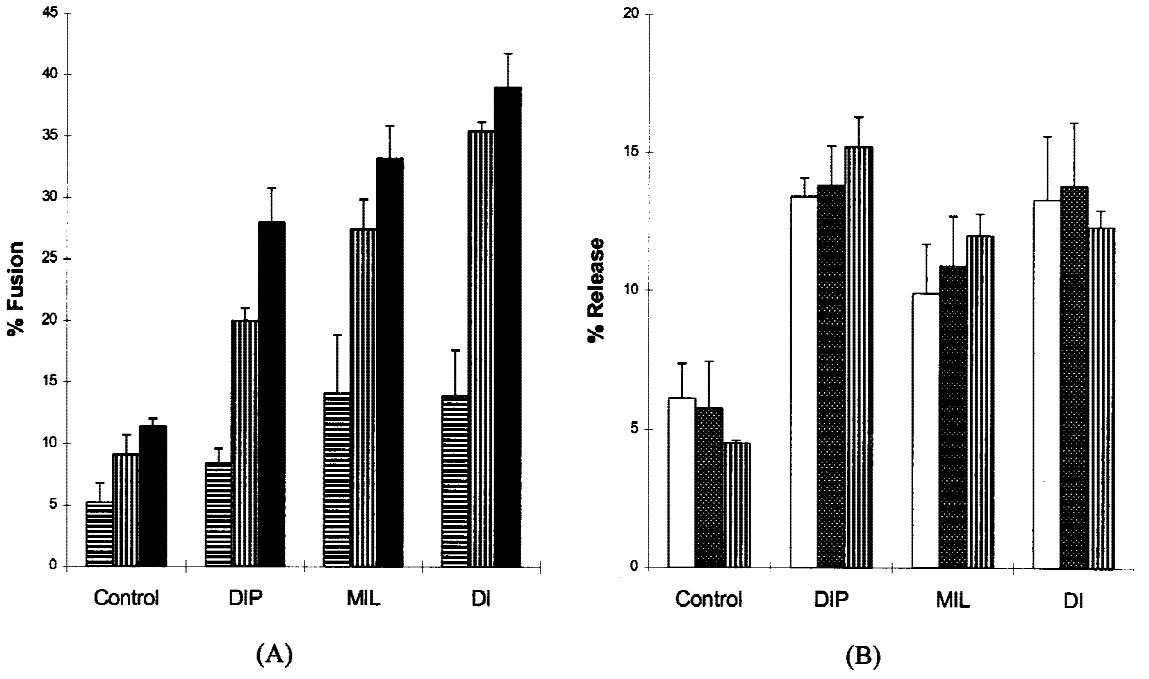

Fig. 6. Effect of liposome concentration on membrane fusion and lysis events. The control group indicates the liposomal membrane without BPS. *(A)* A lipid mixing assay was used to quantify fusion at various liposome concentrations (37.5 mmol/ml (horizontal line bar), 75 mmol/ml (vertical line bar), and 150 mmol/ml (solid bar)) of a R = 0.2 BPS-liposome in a pH 5.0 buffer solution. Significant differences of the liposome fusion were observed among all three concentrations. *(B)* A calcein release measurement was used to determine the liposome lysis behavior at increasing lipsome concentrations (3 mmol/ml (open bar), 25 mmol/ml (dotted bar), and 75 mmol/ml (vertical line bar)) of R = 0.2 BPS-liposome in a pH 5.0 buffer solution. No significant difference of liposome rupture was seen among three different concentrations of any BPS. Data are expressed as mean ± SD (n = 4).

When comparing membrane fusion by these two assays, it was found fusion determined by the lipid mixing assay was consistently (i.e., 5–10%) higher than those by the aqueous content mixing assay. This discrepancy was attributed to the sensitivity of lipid aggregation for the lipid-mixing assay. When membranes aggregate, the fluorescence energy from one liposome group can also transfer to the other group (Duzgunes et al., 1985; Yeagle, 1993*a*). The difference in the two assays could be also due to the hemifusion stage in liposome-liposome interaction. At this stage, lipids from the external leaflets would mix together which proceeds liposomal content mixing. Both events may then lead to a perceivable higher percentage of fusion. However, with the membrane aggregation or at the hemifusion stage, no aqueous mixing events would be observed.

The time course study using both fusion assays supports the above findings. Liposome fusion reached a plateau in the early period with the lipid-mixing assay. With the aqueous mixing assay, however, fusion gradually increased during the entire time period. This indicates the liposome fusion process caused by the BPS is at least composed of three components, an initial liposome aggregation event followed by lipid mixing and aqueous mixing.

An interesting finding was observed between release of liposome entrapped calcein when the BPS agent was added externally as compared to direct incorporation into the liposome matrix. All three BPS agents demonstrated similar release profiles when the un-ionized BPS agent was incorporated into the liposome illustrating that they most likely have similar mobility within the bilayer (Fig. 5*B*). When the three agents were added from the external phase (Fig. 5*A*) to performed liposomes there was a drastic difference between the release profiles of the BPS agents with ester linkages (e.g., DIP and MIL) as compared to the alkyl chain analogue (DI) which demonstrated little pH dependence. It is unlikely that this lack of pH dependency is due solely to changes in the pKa of BPS. The different profiles may reflect the ease of the BPS to partition into the bilayer. BPS agents with a more polar head group (e.g., ester containing) would be expected to have added resistance in membrane partitioning. Regardless of the mechanism this is an interesting finding which will be investigated in future studies.

Membrane fusion is not only dependent on the pH and liposome formulation, but it is also directly proportional to the probability of the liposomes to associate with each other (Ellens, Bentz & Szoka, 1984). It is therefore an intermembrane interaction and liposome

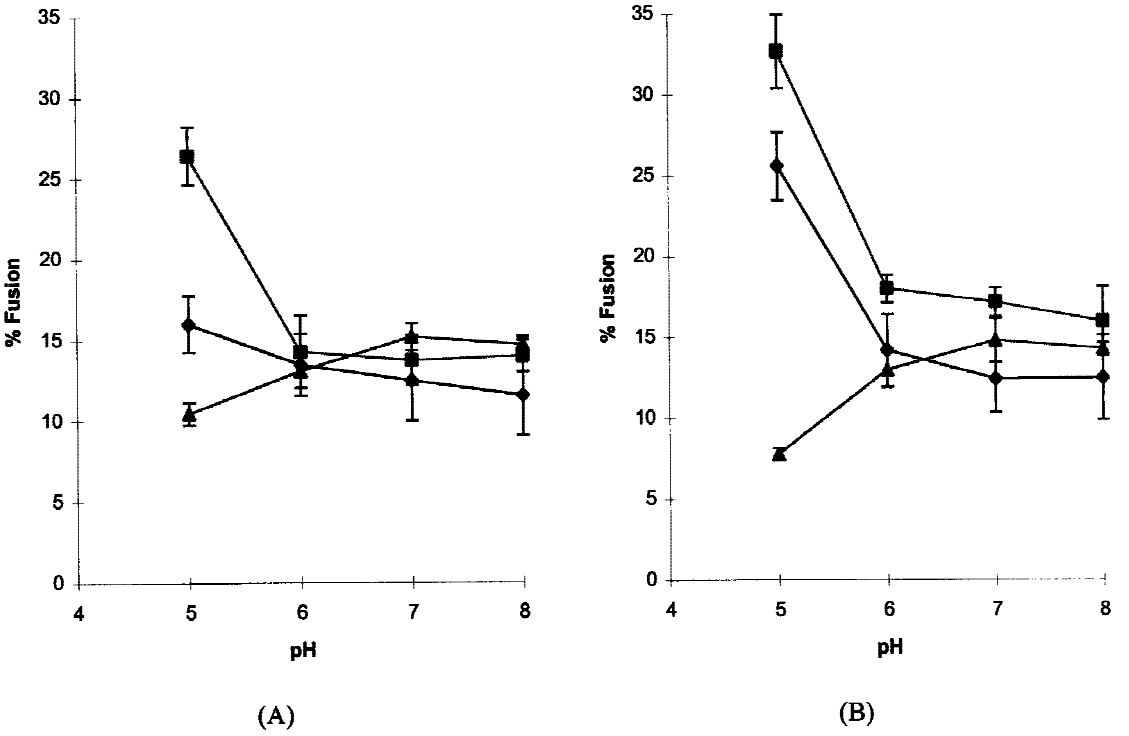

Fig. 7. Effect of cholesterol and of DOPE on membrane fusion. Liposomes without any co-lipid (♦), liposomes containing 40% (weight) cholesterol (■) and liposomes with 40% (weight) DOPE (▲) at two mole ratio groups R = 0.2 *(A)* and R = 0.4 *(B)* were compared in different pH environments. The NBD-Rh fusion assay was applied and each sample was excited at 470 nm and measured at 530 nm (n = 4). Significant differences were observed among three formulations in both mole ratio groups after the pH dropped to 5.0. Data are expressed as mean ± SD.

concentration dependent (Fig. 6*A*). If the calcein release is the result of leakage during the observed period caused by fusion, it should be concentration dependent (Ellens, 1984). On the other hand, if membrane rupture, an intramembrane effect, is responsible for the majority of the calcein release, it should be independent from the liposome concentration. The extent of calcein release caused by BPS depended only on the pH and liposome formulation (Fig. 6*B*). Similar behaviors were also seen with the R = 0.4 BPS-liposome groups (*data not shown*). This indicated that both rupture and fusion are responsible for the BPS-induced membrane defect.

To understand BPS-induced membrane destabilization, it is necessary to comprehend the interactions between the various lipid molecules. A simple means to explain the lipid molecule interaction (e.g., hexagonal II (H_{II}) phase, lipid bilayer phase and micellar phase) is to describe the packing of lipids into a bilayer by considering the shape of the molecule. This can be represented more quantitatively by $P_r = \frac{A_h}{A_c}$ where P_r is the packing ratio, A_h is the effective cross-sectional area of the head group and A_c is the effective cross-sectional area of the hydrocarbon chain region (Yeagle, 1993*b*). When $P_r < 1$ (e.g., phosphatidylethanolamine), the inverse cone shape would lead to an inverse packing (H_{II} phase). When $P_r \approx 1$ (e.g., phosphatidylcholine), the cross-sectional area of the head group is similar to the cross-sectional area of the hydrocarbon chains. The resulting cylindrical shape would lead to a lipid bilayer. When $P_r > 1$ (e.g., Triton X-100), cone shapes would lead to micelle formation.

To support the above conclusion that BPS membrane fusion and rupture are parts of the same membrane destabilization continuum, additional studies were conducted with alternative liposome formulations. In these studies, the impact of cholesterol and DOPE on liposome fusion was assayed. Cholesterol is known to increase the rigidity of the liposome structure (New, 1990) while DOPE can destablize the liposome structure with its tendency to form the H_{II} phase (Yeagle, 1994; Yeagle, 1993*b*; Siegel, 1986). When cholesterol was present in a lecithin liposome, the addition of BPS resulted in increased fusion at the acidic pH. Cholesterol has also been reported to promote fusion of lipid bilayers in other studies (Nussbaum, Rott & Loyter 1992; Vogel et al., 1992). However, when cholesterol was replaced by DOPE, the liposome fusion events were significantly decreased.

It is thought that this alteration in membrane fusion is due to the chemical structure configurations among the liposome formulations. Cholesterol can fill the gaps between the hydrocarbon chains of adjacent lipid mol-

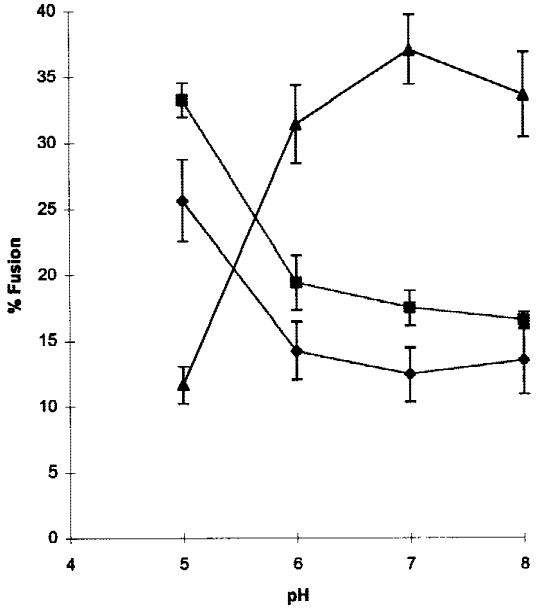

Fig. 8. Effect of equal mole ratio of cholesterol and of DOPE to L-α-lecithin on membrane fusion. Liposomes without any adjuvant (♦), liposomes containing cholesterol (■) and DOPE (▲) at a mole ratio group R = 0.4 DIP were compared in different pH environments. The NBD-Rh fusion assay was applied and each sample was excited at 470 nm and measured at 530 nm ($n = 4$). Significant differences were observed among three formulations at observed pHs. Data are expressed as mean ± SD.

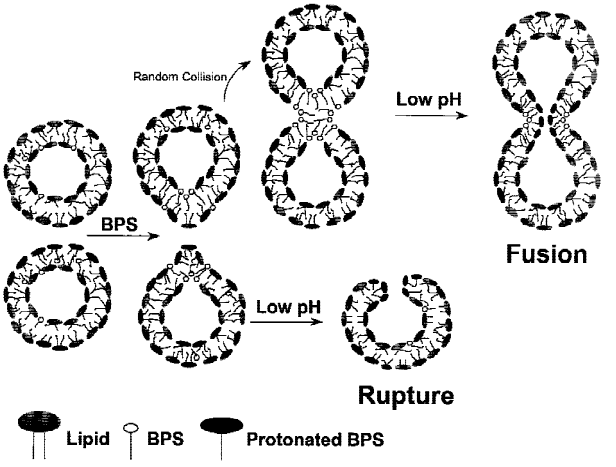

Fig. 9. Proposed mechanism of liposome destabilization elicited by BPS. At an alkaline pH, the BPS will be neutral and reside within the lipid bilayer. When additional BPS are incorporated into the liposomes, a humpbacked mixed bilayer will be built from the swollen mixed bilayer. With less dehydration required to form the stalk, the humpbacked mixed bilayer membrane may interact with each other more easily. As a consequence of the decreased pH, a portion of the BPS will be protonated and facilitate the formation of a stalk pore at which the fusion process will be finished. Without interaction with other mixed bilayers, the humpbacked mixed bilayer will be transformed into a mixed bilayer sheet leading to rupture.

ecules, which will result in similar ratios of head group to tail group and solidify the liposome. The ensuing rigidity of the cholesterol-liposome with reduced ability to accommodate other lipophilic compounds within the bilayer can then facilitate membrane fusion after the conversion of the protonated BPS. When DOPE was present in the liposome formula, the packing ratio of BPS ($P_r <$ 1) was similar to the H_{II} phase of DOPE ($P_r < 1$) which made the liposome more fusogenic than liposomes without DOPE at an alkaline pH (Figs. 7 and 8). This effect was more evident in the presence of extra 10% (mol) of DOPE in the liposomes (Fig. 8). As the pH decreased, the protonated BPS ($P_r > 1$) became complementary to DOPE ($P_r < 1$). Instead of causing fusion, the liposomes would then tend to remain as an intact bilayer structure as the pH decreased. When the amount of PBS was 20% (mol) more than that of DOPE at which the BPS outweighed DOPE, the fusion was relatively stable in the pH range of 6–8 and increased at pH 5.0 (*data not shown*). However, as expected, the increased fusion was less than the liposomes without DOPE.

Based upon the generated data and existing literature regarding membrane fusion and rupture mechanism, we proposed a BPS-induced membrane defect mechanism (Fig. 9). It is suggested that at an alkaline pH, un-ionized BPS having a relatively small head group as compared to the ionized species will reside within the lipid bilayer or inner leaflet. With the losse lipid packing from the incorporation of un-ionized BPS, the swollen mixed bilayers tend to be less stable, leading to easier fusion and leakage (Figs. 3 and 4), corresponding to the increasing amounts of BPS packed within the bilayer. As the amount of BPS in the membrane increases, the swollen mixed bilayer may be transformed into a humpbacked mixed bilayer at which the BPS will reside within regions of high negative curvature. The imidazole head group which is more hydrophobic than lecithin's head group would require less dehydration to form the stalk, the first step in the membrane fusion process, thus favoring this transition and resulting in the lipid mixing effects (Figs. 2*A* and 3*A*). When a portion of the BPS is protonated ($P_r > 1$) as a consequence of the acidic pH present in the system, a stalk pore will be created to complete the fusion process resulting the content mixing effects (Figs. 2*B* and 3*B*). Without any further interaction between other humpbacked mixed bilayers (Fig. 6), the protonated BPS may transform the humpbacked mixed bilayer to a mixed bilayer sheet (Lasch, 1995) in which only a rupture event will be observed (Fig. 4). The above hypothetical membrane fusion description is only one of the reported mechanisms that have been proposed for membrane fusion (Yeagle, 1997). Other models of fusion such as inverted micelle intermediates may also show BPS dependency but were not addressed in the current study.

BPS may induce membrane defects leading to fusion and rupture by alternative routes in comparison of other fusogenic compounds. The difference in events is partially attributed to BPS's unique chemical features. When BPS are un-ionized (pH 7.4), it can be incorporated into the liposome hydrophobic regions but once ionization occurs, it will behave as a surfactant. Traditionally, commonly studied surfactants (e.g., sodium dodecyl sulfate) have always interacted with the first membrane encountered; in most cases the outer lipid leaflet of a bilayer. When surfactants are added to the external aqueous environment of biological membranes, they will interact with the membranes leading to mixed micelles. A recent report (Melikyan et al., 1997) indicated the inner membrane leaflet controlled membrane fusion when a hemagglutinin-peptide was the triggering agent. If the results from this study can be applied to our model, agents which alter the inner leaflet may then increase membrane fusion. Due to the uncharged lipophilic nature of BPS at pH 7.4, it was hypothesized that BPS would distribute to the inner leaflet similar to other compounds with relative small head groups (Michaelson, Horwitz & Klein, 1973; Huang et al., 1974) and demonstrate a portion of its activity at this location. In the current discussion the influence of the transmembrane pH gradient formed by incubating the liposomes at acidic pH was not addressed. Ongoing studies will study this phenomenon which may result in redistribution of BPS within the lipid bilayer.

In the preceding discussion we have considered the three BPS agents (DIP, MIL, and DI) as one category. At the onset of the experiments a greater difference was expected between the individual BPS members in regards to fusion and rupture. In previous studies, by an interfacial tensiometer the critical micelle concentration (CMC) of the BPS members in an ionized stat were determined to be 1.0 mM, 0.7 mM, and 1.0 mM for DIP, MIL, and DI, respectively (*data not shown*). It was speculated that there would be a correlation between these values and membrane destabilization. Furthermore, with the various chemistries used in the attachment of the imidazole head group to the aliphatic hydrocarbon tail, the pKa values for the three head groups would be altered. The difference in pKa was expected to affect the pH dependency of BPS membrane destabilization this effect was not observed when incorporated into the liposome bilayer and an unexpected result was obtained when the BPS agents was added to the external environment where DI lost its pH dependency. These findings indicate that the various BPS agents will likely have different partioning into lipid bilayers but may demonstrate similar intrabilayer movement. The only statistical difference between the various BPS agents was observed in the release of calcein. In these experiment DIP was noted to have the greatest effect on membrane leakage. It is unclear why DIP increases leakage over the other two agents but this may be related to the branched methyl group near the head group whose steric effect may facilitate membrane destabilization.

In conclusion, to enhance membrane fusion, negative curvature ($P_r < 1$) compounds can facilitate the formation of the stalk and positive curvature ($P_r > 1$) compounds are preferred for the formation of the stalk pore to complete the membrane fusion process (Chernomordik, Kozlov & Zimmerberg, 1995). The unique chemical structure of BPS allows coexistence of both un-ionized ($P_r < 1$) and ionized BPS ($P_r > 1$) species during the fusion process. Instead of causing membrane fusion with the H_{II} phase similar to DOPE, BPS seemed to be able to participate in membrane fusion at different stages (stalk and stalk pore). The above results implied examples of how BPS agents can induce both membrane fusion and rupture in pH and concentration dependent manner. It should be noted, however, that there was little structure activity relationship between the BPS agents and their membrane destabilization effects in the study. To clarify the structure activity relationship and more fully understand the unique membrane destabilization effects of the BPS agents, more work regarding diverse head groups, linkers, and aliphatic tails is required.

The authors with to express their gratitude to National Institutes of Health (P01-AG10485-07 and R29 HL55770-03) for the funding and Dr. M.J. Cho, Dr. Gayle Brazeau, and Ms. Marla N. Rosenblatt for their assistance in preparing the manuscript. Support from the Centers of Gene Therapy and Neurobiology of Aging at the University of Florida is also gratefully acknowledged.

References

Chernomordik, L., Kozlov, M.M., Zimmerberg, J. 1995. Lipids in biological membrane fusion. *J. Membrane Biol.* **146:**1–14

Chu, C.J., Dijkstra, J., Lai, M.Z., Hong, K., Szoka, F.C. 1990. Efficiency of cytoplasmic delivery by pH-sensitive liposomes to cells in culture. *Pharm. Res.* **7:**824–834

De Duve, C., De Barsy, T., Poole, B., Trouet, A., Tulkens, P., Van Hoof, F. 1974. *Biochem. Pharmacol.* **23:**2495–2531

Duzgunes, N., Straubinger, R.M., Baldwin, P.A., Friend, D.S., Papahadjopoulos, D. 1985. Proton-induced fusion of oleic acid-phosphatidylethanolamine liposomes. *Biochemistry* **24:**3019–3098

Ellens, H., Bentz, J., Szoka, F.C. 1984. pH-induced destabilization of phosphatidylethanolamine-containing liposomes: role of bilayer contact. *Biochemistry* **23:**1532–1538

Fattal, E., Nir, S., Parente, R.A., Szoka, F.C. 1994. Pore-forming peptides induce rapid phospholipid flip-flop in membranes. *Biochemistry* **33:**6721–6731

Firestone, R.A., Pisano, J.M., Bonney, R.J. 1979. Lysosomotropic Agents. 1. Synthesis and Cytotoxic Action of Lysosomotropic Detergents. *J. Med. Chem.* **22:**1130–1133

Huang, C.H., Sipe, J.P., Chow, S.T., Martin, R.B. 1974. Differential interaction of cholesterol with phosphatidylcholine on the inner and outer surfaces of lipid bilayer vesicles. *Proc. Natl. Acad. Sci. USA* **71:**359–362

Hug, P., Sleight, R.G. 1994. Fusogenic virosomes prepared by parti-

tioning of vesicular stomatitis virus G protein into preformed vesicles. *J. Biol. Chem.* **269:**4050–4056

Hughes, J.A., Aronsohn, A.I., Avrutskaya, A.V., Juliano, R.L. 1996. Evaluation of adjuvants that enhance the effectiveness of antisense oligodeoxynucleotides. *Pharm. Res.* **13:**404–410

Hughes, J.A., Bennett, C.F., Cook, P.D., Guinosso, C.J., Mirabelli, C.K., Juliano, R.L. 1994. Lipid membrane permeability of 2′-modified derivatives of phosphorothioate oligonucleotides. *J. Pharm. Sci.* **83:**597–600

Lasch, J. 1995. Interaction of detergents with lipid vesicles. *Biochim. Biophys. Acta* **1241:**269–292

Lee, R.J., Huang, L. 1997. Lipidic vector systems for gene transfer. *Crit. Rev. Ther. Drug. Carrier. Systm.* **14:**173–206

Liang, E., Hughes, J.A. 1998. Characterization of a pH-sensitive Surfactant, Dodecyl-2-(1′-imidazolyl) propionate (DIP), and Preliminary Studies in Liposome Mediated Gene Transfer. *Biochim. Biophys. Acta* **1369:**39–50

Liu, Y., Regen, S.L. 1993. Control over vesicle rupture and leakage by membrane packing and by the aggregation state of an attacking surfactant. *J. Am. Chem. Soc.* **115:**708–713

McGraw, T.E., Maxfield, F.R. 1991. Internalization and sorting of macromolecules: endocytosis. *In:* Targeted Drug Delivery. R.L. Juliano, editor. pp. 11–42. Springer-Verlag, New York

Melikyan, G.B., Brener, S.A., Ok, D.C., Cohen, F.S. 1997. Inner but not outer membrane leaflets control the transition from glycosylphosphatidylinositol-anchored influenza hemagglutinin-induced hemifusion to full fusion. *J. Cell Biol.* **136:**995–1005

Michaelson, D.M., Horwitz, A.F., Klein, M.P. 1973. Head group modulation of membrane fluidity in sonicated phospholipid dispersions. *Biochemistry* **12:**2637–2645

New, R.R.C. 1990. Introduction. *In:* Liposomes: A Practical Approach R.R.C. New, editor. pp. 1–32. Oxford University Press, New York

Nussbaum, O., Rott, R., Loyter, A. 1992. Fusion of influenza virus particles with liposomes: requirement for cholesterol and virus receptors to allow fusion with and lysis of neutral but not of negatively charged liposomes. *J. Gen. Virol.* **72:**2831–2837

Siegel, D.P. 1986. Inverted micellar intermediates and the transitions between lamellar, cubic, and inverted hexagonal lipid phases. II. Implications for membrane-membrane interactions and membrane fusion. *Biophys. J.* **49:**1171–1183

Smolarsky, M., Teitelbaum, D., Sela, M., Gitler, C. 1977. A simple fluorescent method to determine complement-mediated liposome immune lysis. *J. Immunol. Methods* **15:**255–265

Sokol, D.L., Gewirtz, A.M. 1996. Gene therapy: basic concepts and recent advances. *Crit. Rev. Eukaryot. Gene Expr.* **6:**29–57

Stewart, J.C.M. 1980. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. *Anal. Biochem.* **104:**10–14

Struck, D.K., Hoekstra, D., Pagano, R.E. 1981. Use of resonance energy transfer to monitor membrane fusion. *Biochemistry* **20:**4093–4099

Vogel, S.S., Chernomordik, L.V., Zimmberberg, J. 1992. Calcium-triggered fusion of exocytotic granules requires proteins in only one membrane. *J. Biol. Chem.* **267:**25640–25643

Yeagle, P.L. 1993*a.* Membrane fusion. *In:* The Membranes of Cells. P.L. Yeagle, editor. pp. 263–280. Academic Press, San Diego

Yeagle, P.L. 1993*b.* Lipid properties in membranes. *In:* The Membranes of Cells. P.L. Yeagle, editor. pp. 69–139. Academic Press, San Diego

Yeagle, P.L. 1994. Lipids and lipid-intermediate structures in the fusion of biological membranes. *Curr. Top. Membr.* **40:**197–214

Yeagle, P.L. 1997. Membrane Fusion Intermediates. *Curr. Top. Membr.* **44:**375–401

Yu, Y.G., King, D.S., Shin, Y.K. 1994. Insertion of a coiled-coil peptide from influenza virus hemagglutin into membranes. *Science* **266:**274–276

Zabner, J., Fasbender, A.J., Moninger, T., Foellinger, K.A., Welsh, M.J. 1995. Cellular and molecular barriers to gene transfer by a cationic lipid. *J. Biol. Chem.* **270:**18977–19007