Synthesis and Characterization of NVOC-DOPE, a Caged Photoactivatable Derivative of Dioleoylphosphatidylethanolamine

Zhi-Yi Zhang and Bradley D. Smith*

Department of Chemistry and Biochemistry, 251 Nieuwland Science Hall, University of Notre Dame, Notre Dame, Indiana 46556-5670. Received June 23, 1999; Revised Manuscript Received September 9, 1999

A caged, photocleavable derivative of dioleoylphosphatidylethanolamine (DOPE) called NVOC-DOPE was prepared by reaction of DOPE with 6-nitroveratryloxycarbonyl chloride. In contrast to egg phosphatidylethanolamine (EPE), NVOC-DOPE or its 1:1 mixture with EPE forms liposomes at both pH 7.4 and 5.0. Photolysis ($\lambda > 300$ nm) of aqueous liposomal dispersions of NVOC-DOPE at pH 9.0, 7.4, or 5.0 results in complete conversion to DOPE and subsequent release of entrapped calcein dye. The temporal and spatial control associated with the photorelease technique suggests that NVOC-DOPE can be used to study a range of important dynamic membrane processes such as membrane fusion and the action of membrane-associated enzymes.

INTRODUCTION

The presence of nonbilayer-forming lipids, such as phosphatidylethanolamine (PE), is known to activate important biological membrane processes such as bilayer fusion (Kinnunen, 1996b; Siegel, 1999) and the action of membrane-associated enzymes (Hunter et al., 1999; Kinnunen, 1996a; Stubbs and Slater, 1996; Williams, 1998). These dynamic supramolecular events are spatially localized and short-lived, which makes them hard to observe and characterize by physical methods (Chanturiya et al., 1997; Siegel and Epand, 1997; Siegel et al., 1994; Walter and Siegel, 1993). Thus, there is a need to develop triggered, biophysical characterization methods that have a short time frame. A classical photochemical approach is to synthesize an inert "caged" precursor of the active compound and use flash photolysis to rapidly release the compound in a spatially controlled manner (Adams and Tsien, 1993; Corrie and Trentham, 1993). Here we describe a caged version of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) called NVOC-DOPE and show that it is able to form stable liposomes. Upon irradiation, NVOC-DOPE is rapidly converted to DOPE (Scheme 1), which induces bilayer instability and liposome leakage.

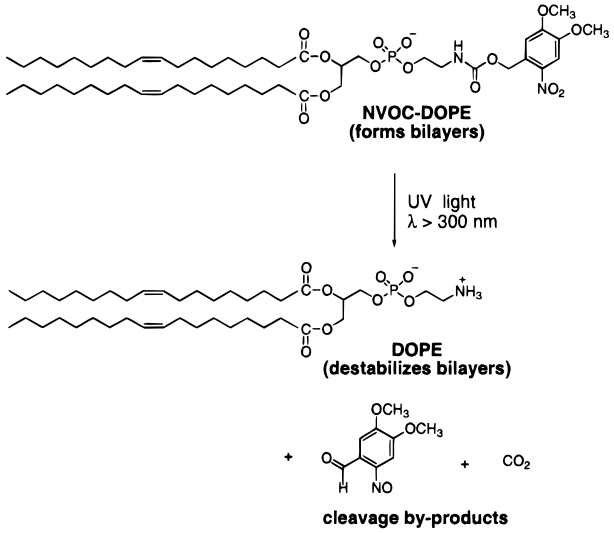
EXPERIMENTAL PROCEDURES

Reagents. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and egg phosphatidylethanolamine (EPE) were purchased from Avanti. Calcein, dithiothreitol (DTT), and 6-nitroveratryloxycarbonyl chloride (NVOC−Chloride, or 4,5-dimethoxy-2-nitrobenzyl chloroformate) were purchased from Aldrich.

Synthesis of NVOC-DOPE. DOPE (100 mg, 134 μmol) and 6-nitroveratryloxycarbonyl chloride (1.5 molar equivalents) were mixed in dry methylene chloride in the presence of triethylamine (4.0 molar equivalents). The mixture was stirred at 40 °C for 12 h under nitrogen in the dark. The reaction mixture was cooled to room temperature and washed successively with cold solutions of 4% Na_2CO_3, H_2O, 5% HCl, and H_2O. The organic layer was dried with Mg_2SO_4, and then evaporated. The residue was purified by column chromatography (silica gel) using $CHCl_3$ and subsequently $CH_3OH-CHCl_3$ (1:4, v/v) as eluant to give NVOC-DOPE as a yellow wax: yield 75%. ^{1}H NMR (300 MHz, CD_3OD) 7.74 (s, 1H), 7.20 (s, 1H), 5.46 (s, 2H), 5.34 (t, $J = 5.0$ Hz, 4H), 5.19 (m, 1H), 4.40 (dd, $J = 11.7$, 3.3 Hz, 1H), 4.15 (q, $J = 6.2$ Hz, 1H), 4.00−3.91 (m, 10H), 3.39 (t, $J = 5.4$ Hz, 2H), 2.33−2.26 (m, 4H), 2.02 (d, $J = 5.4$, 8H), 1.58 (m, 4H), 1.30 (m, 40H), 0.90 (t, $J = 6.6$ Hz, 6H) ppm; IR ($CHCl_3$ solution deposited on a NaCl plate) 1738, 1715, 1651, 1609, 1518 cm^{-1}; HRMS (FAB) observed *m/z* 1026.5579, calcd for $C_{51}H_{85}O_{14}N_2PNa_2$ [M + 2Na − 2H] 1026.5534; TLC R_f = 0.37 (silica gel/$CH_3OH-CHCl_3$, 1:4, v/v).

Liposome Preparation. A chloroform solution containing an appropriate amount of lipid was evaporated using a rotary evaporator (<30 °C), and the lipid film dried under vacuum for at least 1 h. The liposomes were dispersed in a solution of 50 mM calcein in either pH 5.0 buffer (100 mM NaCl/50 mM sodium acetate), pH 7.4 buffer [100 mM NaCl/5 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid], or pH 9.0 buffer (100

Scheme 1



* To whom correspondence should be addressed. Phone: (219) 631 8632. Fax: (219) 631 6652. E-mail: smith.115@nd.edu.

10.1021/bc990087h CCC: $18.00
Published on Web 10/26/1999

mM NaCl/50 mM sodium carbonate) with the aid of vortex mixer. Pyrex glass beads were added before vortexing to facilitate removal of the lipid film from the sides of the flask (final lipid concentration: 10 mM). The resulting opaque dispersion was frozen in an ethanol/dry ice bath and then allowed to thaw in a water bath at 35−40 °C. This freeze−thaw cycle was repeated 10 times. The resulting mixture was extruded, at room temperature, 29 times through a 19 mm polycarbonate filter (Nucleopore) with 100 nm diameter pores using a hand-held Basic LiposoFast device purchased from Avestin. Untrapped calcein was separated by dialysis against isoosmotic buffer.

Photoinduced Calcein Release. A solution of NVOC-DOPE (300 μM, 300 μL) was placed in a 1 mL glass cuvette situated 20 cm from the output lens of a 150 W Xe lamp (filter cut off λ < 300 nm). The temperature was controlled by a thermostated cell holder. After irradiation, a 30 μL aliquot was diluted to 3.0 mL with isoosmotic buffer. The fluorescence intensity was measured at 25 °C. Excitation was set at 490 nm and fluorescence emission was measured at 520 nm. The fluorescence corresponding to 100% leakage was determined after addition of 75 μL of Triton X-100 (20% w/v). The fluorescence intensity for 100% leakage of a control dark sample was measured after liposome lysis with Triton. The percent leakage of calcein was calculated as $\{[I_t - (I_{100}/I_{100}^{dark})I_0]/[1.025\ I_{100} - (I_{100}/I_{100}^{dark})I_0]\} \times 100\%$ (Bondurant and O'Brien, 1998).

Extent of NVOC-DOPE Cleavage in Calcein-Filled Liposomes. The calcein loaded liposomes (300 μM, 300 μL) were lysed with excess ethanol. After removal of solvents and drying under vacuum, 3 mL of $CHCl_3$ was added to take up the lipids. The solution was passed through a 0.8 μm filter to remove the calcein particles and the absence of calcein was confirmed by UV. The extent of photoconversion was calculated from the decrease in absorbance at 350 nm and confirmed by TLC (silica gel; $CHCl_3$:CH_3OH:H_2O, 80:20:1; R_f NVOC-DOPE = 0.56, R_f DOPE = 0.46).

RESULTS AND DISCUSSION

PE does not form a bilayer membrane at physiological pH, but instead adopts inverse cubic and inverse hexagonal structures (Cullis and De Kruijff, 1976; Litzinger and Huang, 1992). Conversely, N-acylated PEs are negatively charged phospholipids and can form kinetically stable liposomes (Domingo et al., 1995; Shangguan et al., 1998). Triggered liposomal systems have been reported with chemically cleavable N-acyl PEs (Kirpotin et al., 1996; Pak et al., 1998), but to our knowledge there is no published example of a photocleavable N-acyl PE; however, polar lipids with caged headgroups (Tuchinsky and Zehavi, 1998) and hydrocarbon tails (Kusumi et al., 1989) are known. NVOC-DOPE is prepared in one step by reaction of DOPE with 6-nitroveratryloxycarbonyl chloride. After purification; NVOC-DOPE is stable to low-temperature storage in the dark. In contrast to pure DOPE, large unilamellar vesicles (LUVs) composed solely of NVOC-DOPE or a 1:1 mixture of EPE/NVOC-DOPE can be prepared at pH 7.4 or 5.0. Liposomes are not readily formed at neutral pH using a 7:3 mixture of EPE/NVOC-DOPE.

The photocleavage of NVOC-DOPE occurs readily in organic solvent, but an isosbestic point is not observed in the UV spectrum (Figure 1). Most likely, the released DOPE condenses with the 3,4-dimethoxy-2-nitrosobenzaldehyde byproduct, which is a common concern with this photocleavage method (Corrie and Trentham, 1993). The side reaction is not observed in aqueous solution, where reasonably concentrated aqueous dispersions of NVOC-DOPE LUVs (300 μM) at pH 9.0, 7.4, or 5.0 are completely converted to DOPE after irradiation with a 500 W Hg lamp (λ < 300 nm) for 4 min. TLC analysis shows that DOPE is liberated in a stoichiometric amount and that the presence of dithiothreitol, which acts as scavenger of 3,4-dimethoxy-2-nitrosobenzaldehyde (Corrie and Trentham, 1993), has no effect on the photocleavage reaction. A dynamic light scattering analysis of the NVOC-DOPE LUVs at pH 5 before photolysis indicated a mean size of 116 ± 47 nm. After photolysis, the dispersion was slightly turbid, and dynamic light scattering indicated a mean size of 495 ± 191 nm. Thus, concomitant with the photoconversion to DOPE, the vesicles undergo fusion and/or aggregation to form larger particles.

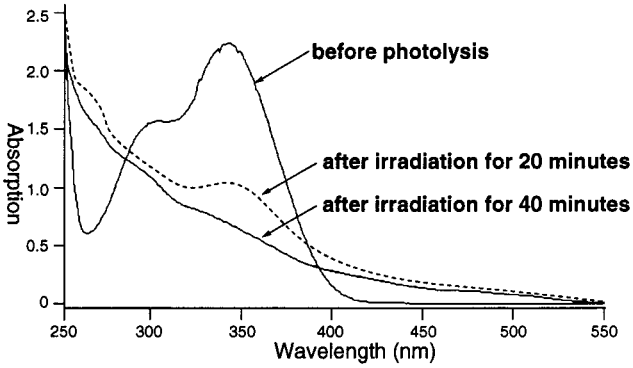


Figure 1. Absorption spectrum of NVOC-DOPE (300 μM) in chloroform.

Calcein leakage experiments show that increasing the fraction of DOPE in the liposomes by photolysis of NVOC-DOPE leads to liposome disruption. The fluorescence assay is hindered by two technical difficulties, the strongly absorbing calcein inhibits the photolysis of NVOC-DOPE, and the calcein emission is decreased due to photobleaching. For example, irradiation of calcein-containing LUVs made from 1:1 EPE/NVOC-DOPE with a 500 W Hg lamp results in rapid and complete leakage of all the calcein, but calcein photobleaching is extensive (~50%). Clearer mechanistic insight was gained using a less intense 150 W Xe lamp that required a longer irradiation time to complete the photocleavage, but produced a much smaller amount of calcein photobleaching and a slower leakage rate. As shown in Figure 2, a 20 min irradiation was needed to convert 90% of the NVOC-DOPE to DOPE, and the amount of escaped calcein initially increased in a slow, linear fashion. Initial calcein leakage rates were measured as a function of temperature, pH, and concentration. The results (Table 1) indicate that photoinduced leakage from 1:1 EPE/NVOC-DOPE LUVs increases with temperature (compare entries 1, 3, and 5), acidity (compare entries 3 and 7), and liposome concentration (compare entries 3 and 8). These trends are in agreement with previous studies of leakage from PE-containing liposomes (Litzinger and Huang, 1992) and show that leakage is promoted by experimental conditions that increase the amount of liposome-liposome contact.

In conclusion, caged derivatives of DOPE, such as NVOC-DOPE reported here, are likely to be useful as photochemical triggers of a range of dynamic membrane processes, especially membrane fusion and the action of

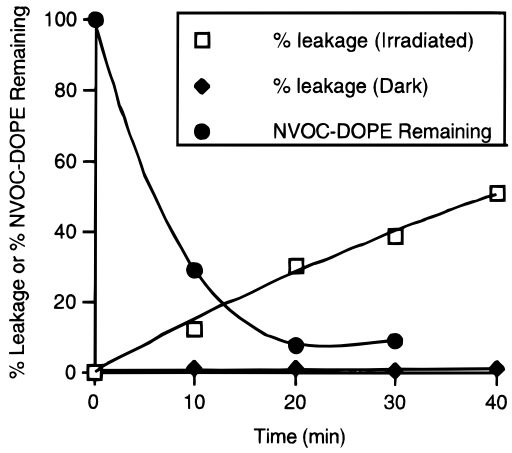


Figure 2. Photocleavage of NVOC-DOPE and calcein release from 1:1 EPE/NVOC-DOPE liposomes (300 μM) at pH 5 and 37 °C, upon irradiation with 150 W lamp.

Table 1. Photoinduced Calcein Leakage from 1:1 EPE/ NVOC-DOPE Liposomes[a]

entry	lipid concentrated (μM)	pH	temp (°C)	light (λ > 300 nm)	leakage rate[a] (% per min)
1	300	5.0	25	UV	0.65
2	300	5.0	25	dark	0
3	300	5.0	37	UV	1.5
4	300	5.0	37	dark	0.05
5	300	5.0	50	UV	2
6	300	5.0	50	dark	0.15
7	300	6.9	37	UV	0.9
8	100	5.0	37	UV	0.8

[a] Initial leakage rate is the extent of leakage after irradiating the sample with a 150 W lamp for 20 min which converted ~90% of the NVOC-DOPE to DOPE (see Figure 2 and Experimental Procedures).

membrane-associated enzymes. They can be employed in studies that require rapid, spatially controlled activation or where mechanical mixing is impractical. A technical drawback with NVOC-DOPE is that the UV light required to induce cleavage may interfere with some of the optical assays used to monitor the subsequent membrane process. The recent introduction of photolabile protecting groups that can be cleaved by two photon IR radiation, however, suggests that this potential problem can be overcome (Furata et al., 1999). Finally, we note that the development of photoactive liposomes for drug or reagent delivery is an active research area (Bondurant and O'Brien, 1998; Wymer et al., 1998) and that "caged" liposomes may be useful as general, triggered-release capsules for solutes (e.g., inorganic ions, peptides, and enzymes, etc.) that are difficult to modify by chemical methods (Morgan et al., 1995a,b).

ACKNOWLEDGMENT

This work was supported by the National Institutes of Health (GM 59078). We thank Professor G. Hartland for access to his DLS instrument.

Supporting Information Available: ^{1}H NMR spectrum of NVOC-DOPE. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

Adams, S. R., and Tsien, R. Y. (1993) *Annu. Rev. Physiol. 55*, 755−784.
Bondurant, B., and O'Brien, D. F. (1998) *J. Am. Chem. Soc. 51*, 13541−13542.
Chanturiya, A., Chernomordik, L. V., and Zimmerberg, J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 14423−14428.
Corrie, J. E. T., and Trentham, D. R. (1993) In *Bioorganic Photochemistry* (H. Morrison, Ed.) pp 243−305, Wiley, New York.
Cullis, P. R., and De Kruijff, B. (1976) *Biochim. Biophys. Acta 436*, 523−540.
Domingo, J. C., Mora, M., and De Madriaga, M. A. (1995) *Chem. Phys. Lipids 75*, 15−25.
Furata, T., Wang, S. S.-H., Dantzker, J. L., Dore, T. M., Bybee, W. J., Callaway, E. M., Denk, W., and Tsien, R. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 1193−1200.
Hunter, G. W., Negash, S., and Squier, T. C. (1999) *Biochemistry 38*, 1356−1364.
Kinnunen, P. K. J. (1996a) *Chem. Phys. Lipids 81*, 151−166.
Kinnunen, P. K. J. (1996b) In *Handbook of Nonmedical Applications of Liposomes* (D. D. Lasic and Y. Barenholz, Eds.) Vol. 1, Chap. 6, CRC, Boca Raton.
Kirpotin, D., Hong, K., Mullah, N., Papahadjopoulos, D., and Zalipsky, S. (1996) *FEBS Lett. 388*, 115−118.
Kusumi, A., Nakahama, S., and Yamaguchi, K. (1989) *Chem. Lett.* 433−436.
Litzinger, D. C., and Huang, L. (1992) *Biochim. Biophys. Acta 1113*, 201−227.
Morgan, C. G., Bisby, R. H., Johnson, S. A., and Mitchell, A. C. (1995a) *FEBS Lett. 375*, 113−116.
Morgan, C. G., Yianni, Y. P., Sandhu, S., S., and Mitchell, A. C. (1995b) *Photochem. Photobiol. 62*, 24−29.
O'Brien, D. F., and Tirrell, D. A. (1993) In *Bioorganic Photochemistry* (H. Morrison, Ed.) pp 243−305, Wiley, New York.
Pak, C. C., Ali, S., Janoff, A. S., and Meers, P. (1998) *Biochim. Biophys. Acta 1372*, 13−27.
Shangguan, T., Pak, C. C., Ali, S., Janoff, A. S., and Meers, P. (1998) *Biochim. Biophys. Acta 1368*, 171−183.
Siegel, D. P. (1999) *Biophys. J. 76*, 291−313.
Siegel, D. P., and Epand, R. M. (1997) *Biophys. J. 73*, 3089−3111.
Siegel, D. P., Green, W. J., and Talman, Y. (1994) *Biophys. J. 66*, 402−414.
Stubbs, C. D., and Slater, S. J. (1996) *Chem. Phys. Lipids 81*, 185−195.
Tuchinsky, A., and Zehavi, U. (1998) *Chem. Phys. Lipids 92*, 91−97.
Walter, A., and Siegel, D. P. (1993) *Biochemistry 32*, 3271−3281.
Williams, E. E. (1998) *Am. Zool. 38*, 280−290.
Wymer, N. J., Gerasimov, O. V., and Thompson, D. H. (1998) *Bioconjugate Chem. 9*, 305−308.

BC990087H