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Visible-Light-Stimulated Destabilization of PEG-Liposomes

Anja Mueller, Bruce Bondurant, and David F. O'Brien*

Department of Chemistry, C. S. Marvel Laboratories, University of Arizona, Tucson, Arizona 85721

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ABSTRACT: In recent years several groups have described methods to prepare and utilize poly(ethylene glycol) conjugated phosphatidylethanolamine (PEG-PE) to sterically stabilize liposomes in order to avoid uptake of the liposomes by the reticuloendothelial system and increase their period of circulation. Sterically stabilized liposomes (PEG-liposomes) preferentially accumulate in the interstitium of tumor sites. If PEG-liposomes are to be effective for delivery of therapeutic agents, their drug permeability must be low enough that little passive release occurs during the time the liposomes are circulating in the bloodstream. However, once PEG-liposomes reach tumor sites, slow passive release of the encapsulated drug may not be optimal. Therefore, it is important to find methods to trigger the release of agents from suitably designed PEG-liposomes. The photoinduced destabilization of liposomes offers an attractive method to couple the temporal and spatial control of light to drug delivery. Bondurant and O'Brien [*J. Am. Chem. Soc.* **1998**, *120*, 13541–13542] showed that UV-induced cross-linking of lipids could destabilize certain PEG-liposomes and increase the bilayer permeability by greater than 10^2 . Here we show that photochemical-induced destabilization of PEG-liposomes can be sensitized to visible light by the incorporation of a cyanine dye into the bilayer wall of PEG-liposomes. These observations demonstrate that light of wavelengths suitable for photodynamic therapy can also be used to increase the permeability of appropriately designed liposomes.

Introduction

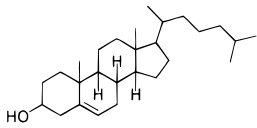
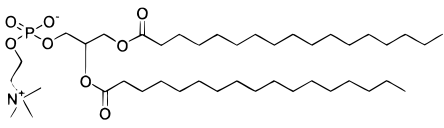
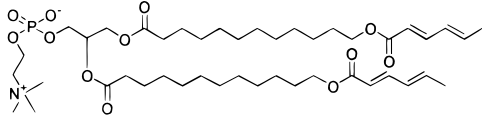
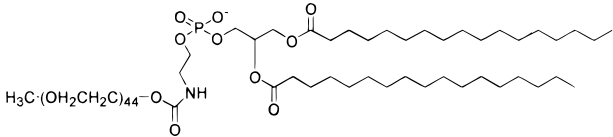
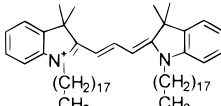
A considerable research effort has been devoted to the development of liposomes for the delivery and buffering of drugs in the body. In recent years several groups have described methods to prepare and utilize poly(ethylene glycol) conjugated phosphatidylethanolamine (PEG-PE) to sterically stabilize liposomes in order to avoid uptake of the liposomes by the reticuloendothelial system and increase their circulation time.^{1,2} Sterically stabilized liposomes (PEG-liposomes) have also been found to accumulate preferentially in the interstitium of tumor sites,^{3–7} because of the increased permeability of the vasculature at those sites.

If PEG-liposomes are to be effective for drug delivery at tumor sites, there should be little drug leakage during the time the liposomes are circulating in the bloodstream. However, once the PEG-liposomes accumulate at tumor sites, the passive release of the encapsulated therapeutic agent may be too slow for optimal delivery to the tumor. Therefore, it is important to find methods to trigger the release of agents from suitably designed PEG-liposomes. Among the various published methods to destabilize liposomes, the photoinduced destabilization of liposomes offers an attractive method to couple the temporal and spatial control of light to drug delivery. Liposomes can be made photosensitive by the use of lipids that either isomerize, fragment, or polymerize upon photoexcitation.^{8,9} The desirability of a stimulus of visible light rather than ultraviolet light is a consequence of the eventual need to deliver the light to liposomes in the body, where it is necessary to utilize longer wavelength light to minimize photoinduced damage to cellular nuclei and to increase the depth of light penetration into tissues. Although a variety of photochemical methods to increase liposome permeability have been described, few have been effectively used with PEG-liposomes,^{10,11} and only one report to date has described a photosensitive liposome that is sensitive to visible or near-infrared light. In that case bacteriochlorophyll was used to sensitize the photocleavage of

dipalmitoylcholine in liposomes.¹¹ Irradiation of the bacteriochlorophyll with 800 nm wavelength light in the presence of oxygen produced singlet oxygen, which is postulated to photooxidize the dipalmitoylcholine forming lysolipid(s), thereby increasing the liposome permeability.

An alternative to photofragmentation of lipids is photopolymerization, especially because it is multiplicative and may therefore require lower light exposures. Moreover, bis-substituted photosensitive lipids, e.g., 1,2-bis[10-(2',4'-hexadienoyloxy)decanoyl]-*sn*-glycero-3-phosphocholine (bis-SorbPC), react to form cross-linked polymer networks that significantly alter bilayer properties.¹² Both the Ringsdorf and O'Brien groups found that the cross-linking polymerization of bis-substituted lipids in the presence of unreactive lipids causes the formation of lipid domains.¹³ Indeed, Armitage et al. demonstrated that cross-linking polymerization of bis-substituted lipids could be used to concentrate charged lipids that are repelled by electrostatic interactions.¹⁴ Bondurant and O'Brien showed that UV-induced cross-linking of lipids could effectively destabilize appropriately designed PEG-liposomes.^{10,15} The cross-linking polymerization of bis-SorbPC with UV light in liposomes, that also are composed of cholesterol, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and PEG₂₀₀₀-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (PEG-DOPE), caused a $>10^2$ -fold increase in the permeability of an encapsulated fluorescent dye. Now we are able to report that this photochemical-induced destabilization of PEG-liposomes can be sensitized to visible light by the incorporation of a cyanine dye into the bilayer wall of PEG-liposomes. Previously, Clapp et al. showed that the addition of ballasted cyanine dyes to liposomes composed of polymerizable lipids, e.g., bis-SorbPC, rendered the liposomes sensitive to the wavelength of light absorbed by the dye.¹⁶ A particularly effective sensitizer was the green light absorbing 1,1'-dioctadecyl-

Scheme 1. Liposome Components and Their Main Phase Transition, Where Applicable

			T_m (°C)
Cholesterol		1	
Distearoyl phosphatidylcholine (DSPC)		2	55.5
bis-SorbPC		3	28.8
Distearoyl poly(ethyleneglycol) phosphatidyl ethanolamine (PEG ₂₀₀₀ -DSPE)		4	
Distearoyl indocarbocyanine (DiI C(18)3)		5	

3,3,3',3'-tetramethylindocarbocyanine, DiI(18)3. The rate of photosensitized polymerization was relatively independent of temperature. Here we report the visible-light-induced destabilization and leakage of bis-SorbPC containing PEG-liposomes (PEG-SorbLUV).

Results and Discussion

Polymerization. The PEG-SorbLUV used in this study were composed of 15 mol % bis-SorbPC, 30–39 mol % 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 6–15 mol % PEG₂₀₀₀-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (PEG-DSPE), and 40 mol % cholesterol (Scheme 1). Polymerization of the monomeric bis-SorbPC yields cross-linked lipid networks, because of the presence of a reactive group in each alkyl chain. Cholesterol and DSPC were selected to yield relatively rigid bilayer membranes with low dark permeability. PEG₂₀₀₀-DSPE anchors the PEG chains in the bilayer and provides steric stabilization of the liposome. The stearoyl chains in the PEG lipid improve mixing with the DSPC in the liposome due to packing and similar mobility of the alkyl chains. This liposome composition was shown to have low dark leakage and a high photosensitivity in UV polymerizations.¹⁵

A ballasted indocarbocyanine, DiI(18)3, was chosen as the photosensitizer (Scheme 1). It is an especially efficient initiator for the polymerization of bis-SorbPC-containing liposomes, because it strongly absorbs the light output of Xe(Hg) lamps of the type used here.¹⁶ Photoactivation of DiI(18)3 in the presence of oxygen produces oxygen radicals which can initiate the polymerization of bis-SorbPC, especially if the dye and monomer are in the same bilayer membrane. Therefore, the stearoyl chains are needed to anchor the dye in the bilayer.

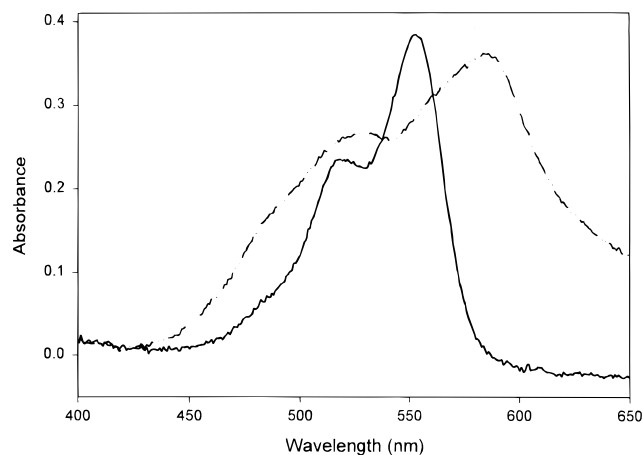


Figure 1. Absorption spectra of DiI(18)3 in either the bilayer of a PEG-liposome (solid line) or a solution of methoxy-PEG (dashed line).

Normally, PEG-SorbLUV were incubated with 5 mol % of the DiI(18)3 at the temperature used for polymerization. To ensure that the dye was predominantly in the bilayer rather than associated with the PEG groups, the absorbance of the dye was determined. Cyanine dyes are solvatochromic; i.e., their absorbance maximum is sensitive to the solvent polarity. Figure 1 shows the absorbance of DiI(18)3 in methoxy-PEG, λ_{\max} 600 nm, which is distinct from that observed for the dye incubated with PEG-liposomes, λ_{\max} 550 nm. In the latter case no evidence for dye absorbance at 600 nm was observed; therefore, little if any of the dye was associated with the PEG-groups. Moreover, it was shown that the percent loss of monomer was dependent on the extent of photolysis of the dye, i.e., dye bleaching, but

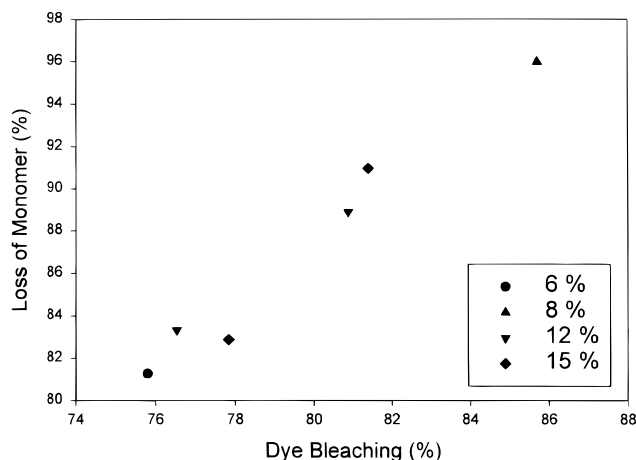


Figure 2. Loss of monomer bis-SorbPC vs the photobleaching of the sensitizing dye DiI(18)3 for samples with different mole fractions of PEG-PE in the liposomes composed of 15 mol % bis-SorbPC, 30–39 mol % DOPC, 6–15 mol % PEG₂₀₀₀-DOPE, and 40 mol % cholesterol.

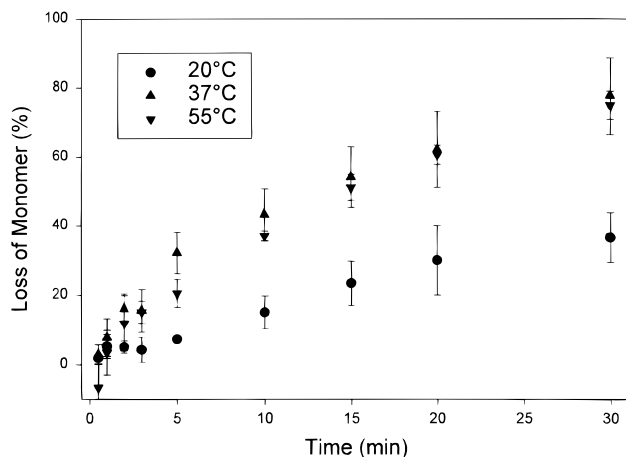


Figure 3. Rate of loss of monomeric bisSorbPC in PEG-SorbLUV at 20 °C (●), 37 °C (▲), and 55 °C (▼).

not on the mole fraction of PEG-lipid in the PEG-SorbLUV (Figure 2).

The progress of the photosensitized reaction of the DiI-PEG-SorbLUV could be followed by absorption spectroscopy. The DiI-PEG-SorbLUV suspensions were irradiated with green light, and aliquots were removed at selected times and then immediately quenched in methanol, and the UV/vis spectrum was measured. The loss of monomer was followed by the disappearance of the bis-SorbPC absorption at 260 nm. Note that a photoproduct of DiI(18)3 also absorbs at 260 nm and must be accounted for in the calculation of monomer loss.¹⁶ Surprisingly, the photoreaction was temperature-dependent (Figure 3). At temperatures above the main phase transition temperature, T_m , of the bis-SorbPC, the rate of monomer loss was approximately twice that at temperatures below the T_m .

Leakage with UV Polymerization. When PEG-SorbLUV were polymerized by exposure to UV light, a very high conversion of monomer was needed to generate leakage of the contents. Leakage was measured with the ANTS/DPX assay introduced by Ellens and Bentz.¹⁷ In this assay the fluorescent dye ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid) and its collisional quencher DPX (*p*-xylenebis(pyridinium bromide)) were incorporated into the liposomes by hydrating the lipid

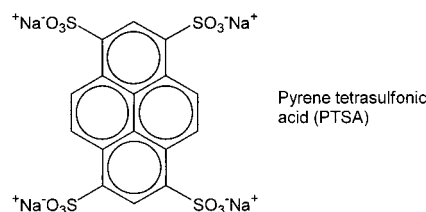
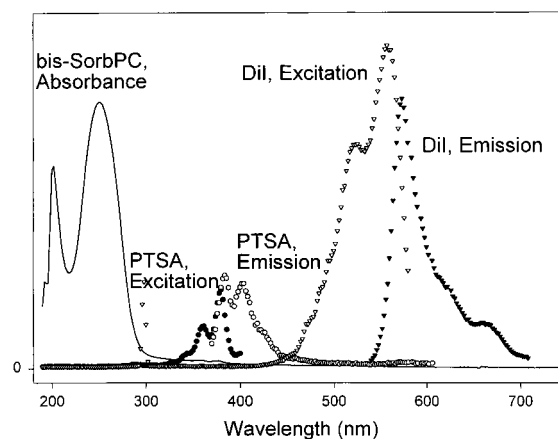


Figure 4. Structure of PTSA and its fluorescence excitation/emission spectra compared with the excitation/emission spectra of DiI C(18)3 and the absorption spectrum of monomeric bisSorbPC.

mixture with an ANTS/DPX buffer. Excess dye-containing buffer outside of the liposomes was exchanged for non-dye buffer by gel chromatography. When the dye escapes the liposomes, the dye quenching is diminished by dilution. The ANTS permeability of PEG-SorbLUV at 25 °C was substantially increased at a high UV photoreaction of bis-SorbPC.^{10,15} Control PEG-liposomes that did not contain bis-SorbPC were not photosensitive.

Leakage Assay. Although the ANTS/DPX assay was useful for the studies of undyed (unsensitized) LUV, it was less suitable for the dye-sensitized LUV because the fluorescence emission of ANTS at 520 nm overlaps the absorbance spectrum of DiI(18)3. Therefore, other assays for leakage were examined. Pyrenetetrasulfonic acid (PTSA) was selected to replace ANTS in a new leakage assay related to the one developed by Ellens and Bentz.¹⁷ The PTSA fluorescence emission has very little overlap with DiI(18)3 absorption spectrum (Figure 4). The fluorescence of PTSA in the presence of DPX (1:6 molar ratio) was less than 30% of the value in the absence of DPX. Because the extinction coefficient of PTSA is high ($\epsilon = 51\,000$), a 3 mM concentration of PTSA was sufficient for the dye encapsulation and permeability studies.

Dark Leakage. The dark leakage PTSA from unphotolyzed DiI-PEG-SorbLUV was measured with 30 s scans over the period of several hours. The PTSA dark leakage was temperature-dependent. It was insignificant at 33 °C or cooler but was substantial at 37 °C, suggesting the possibility of a phase transition between 33 and 37 °C (Figure 5). Consequently, the photoinduced leakage was measured at 25 °C, which is well below the proposed phase transition temperature.

An unexpected finding, which will be described in more detail in future papers, was the observation that photoactivation of PTSA [rather than DiI(18)3] can cause some loss of monomeric bis-SorbPC. Irradiation of the PEG-SorbLUV (1–12 min) with the 374 nm

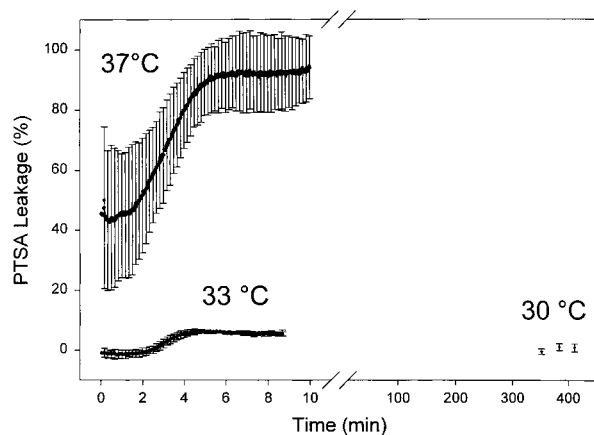


Figure 5. Rate of leakage of PTSA from unphotolyzed PEG-SorbLUV at different temperatures (error bars indicate the average of three experiments each).

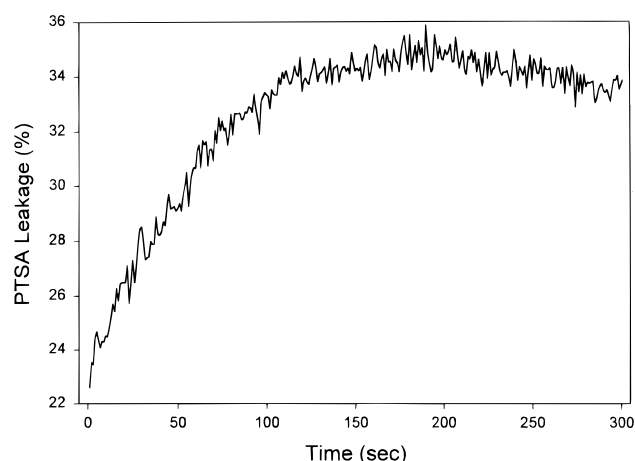


Figure 6. Leakage of PTSA vs time after 15 min irradiation of DiI-PEG-SorbLUV with green light.

excitation light of the fluorimeter (slit width of 1 mm) caused a 2–12% loss of monomer. These data suggest that some of the measured dark leakage at temperatures ≤ 33 °C was probably due to PTSA-sensitized polymerization of the PEG-SorbLUV.

Visible-Light-Induced Leakage. Exposure of DiI-PEG-SorbLUV to green light results in substantial release of the encapsulated PTSA. In a typical experiment (Figure 6) the sample is irradiated for a period of time (15 min), and the increase in emission due to diminished PTSA quenching continues for a few minutes and then levels off. The amount of PTSA leakage from photolyzed DiI-PEG-SorbLUV increased with the initial loss of monomer and then leveled off after about 10% loss of monomer (Figure 7). The observed initial rate appeared to decrease with increased irradiation time, but that is because more of the dye has already leaked by the time the measurement is started, so that the measured rate is calculated with points closer to the breaking point in the leakage curve. The average initial leakage rate was ca. 20 PTSA molecules (LUV) $^{-1}$ s $^{-1}$. We estimate that about two-thirds of the observed leakage is due to the green light photolysis of DiI-PEG-SorbLUV, and the balance is a consequence of increase in the PTSA emission during analysis as well as leakage induced by the 374 nm excitation of PTSA during the analysis.

The thermal behavior of DiI-PEG-SorbLUV indicates that monomeric bis-SorbPC forms separate domains

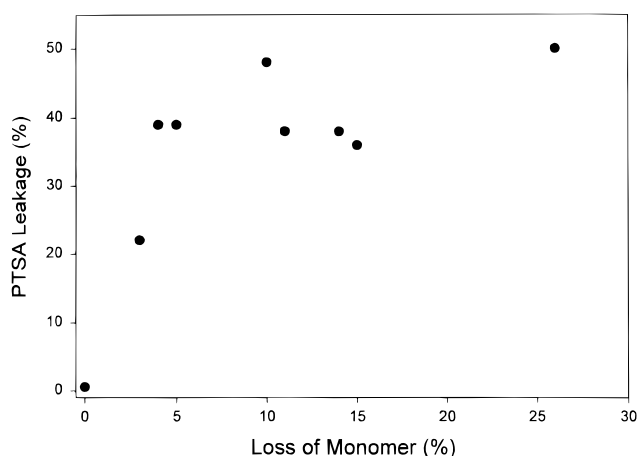


Figure 7. Photoinduced leakage of PTSA from DiI-PEG-SorbLUV liposomes vs the loss of monomeric bis-SorbPC.

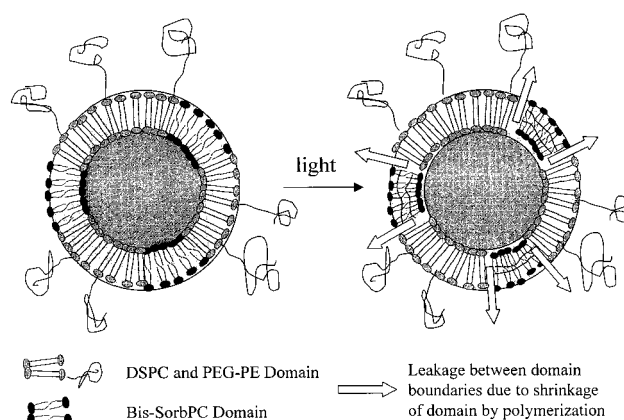


Figure 8. Schematic cross section of PEG-SorbLUV that represents the domains of bis-SorbPC and stearyl lipids. The proposed photopolymerization-induced reduction in the surface area of bis-SorbPC domains is shown on the right.

from the distearoyl lipids. Calorimetry of the PEG-SorbLUV shows a phase transition near 30 °C (bis-SorbPC) and a second near 55 °C (DSPC). The presence of these two transitions indicates that the bilayers are composed of domains that are enriched in bis-SorbPC and distearoyl lipids, respectively. A schematic representation of a cross section of a PEG-SorbLUV is shown in Figure 8. When both lipid domains are in a solidlike phase, i.e., at temperatures below the T_m of bis-SorbPC, the observed permeability of the PEG-SorbLUV is low. When the PEG-SorbLUV are warmed to a temperature at or above the phase transition of bis-SorbPC, the melting of the lipid tails results in an increase in liposome permeability due to disorder at the domain boundaries. The observed increase in permeability of ca. 10^2 is consistent with previous observations of increased permeability at lipid phase transitions.¹⁸ The photopolymerization-induced increase in permeability of DiI-PEG-SorbLUV at temperatures below the phase transition temperature is most likely due to disorder at the domain boundaries. Chain polymerization of vinyl monomers occurs with a reduction in volume. In a similar manner the polymerization of monolayers of reactive amphiphiles proceeds with a reduction in occupied surface area. When bilayer vesicles (liposomes) composed of a single reactive amphiphile are polymerized, any reduction of the surface area is not readily detected because it modestly alters the liposome diameter, which is only measured as an average value of an ensemble

of liposomes. However, when lipid domains within a liposome, such as PEG-SorbLUV, are polymerized, the reduction in surface area of the domains of reactive amphiphile, i.e., bis-SorbPC, should cause discontinuities at the interface with the solidlike stearyl lipid domains, leading to increased permeability as observed. After a period of time the initial stress may be relieved by reorganization of the unreactive lipids, and the liposome permeability decreases.

Conclusions

Steric stabilization of liposomes increases their circulation time in a manner that increases their potential for drug delivery. However, once PEG-liposomes accumulate at tumor sites, it is desirable to enhance the release of encapsulated contents. We originally demonstrated that UV-activated polymerization of bis-SorbPC-containing liposomes could serve as a successful trigger to increase the permeability of water-soluble encapsulated compounds by $>10^2$ -fold.¹⁰ Here we show that the polymerization of similar PEG-liposomes can be sensitized to visible light by use of a sensitizing dye, e.g., DiIC(18)3, and that this polymerization process can photodestabilize the DiI-PEG-SorbLUV with release of encapsulated contents. The change in bilayer properties resulting from the cross-linking polymerization of domains of the liposome effectively increases the bilayer permeability. These observations demonstrate that light of wavelengths suitable for photodynamic therapy, i.e., green to infrared light, can also be used to increase the permeability of appropriately designed liposomes.

Experimental Section

Materials and Methods. The bis-SorbPC was synthesized as described previously.¹⁹ The other lipids were purchased from Avanti Polar Lipids. 8-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS), 1,4,6,8-pyrenetetrasulfonic acid (PTSA), *p*-xylenebis-(pyridinium bromide) (DPX), and DiIC(18)3 were obtained from Molecular Probes. The osmolarity of the buffers were determined with an Osmette S osmometer. UV/vis absorption spectra were measured with a Varian DMS 200 double-beam UV/vis spectrophotometer.

Liposome Preparation. PEG₂₀₀₀-DSPE, cholesterol, DSPC, and bis-SorbPC were each dried under an argon stream followed by drying under vacuum for at least 2 h and then weighed. The lipids in the molar ratio of 15/40/15/30 were hydrated by either phosphate buffer saline (PBS) or buffer containing either ANTS or PTSA as the water-soluble fluorescent marker and DPX as their collisional quencher. Photosensitive PEG-LUV (100 ± 10 nm diameter large unilamellar liposomes) were prepared by freeze/thawing and conventional extrusion procedures.²⁰ The ANTS/DPX or PTSA/DPX containing PEG-LUV was eluted through a Sephadex G75 column with an isoosmotic pH 7.0 phosphate buffered saline (PBS) to remove unencapsulated dye and quencher. A suspension of PEG-LUV was treated with a methanol solution of DiIC(18)3 at the experimental temperature. The volume of the added methanol was less than 1% of the volume of the LUV buffer suspension. Liposome size distributions were measured using dynamic light scattering (Brookhaven BI-8000AT correlator with a 5 mW He-Ne polarized laser source). LUV were examined at a total lipid concentration of 100 μM at angles of 60°, 90°, and 120°. Two fitting methods, nonnegative least squares and CONTIN, were used to extract the set of exponential functions that made up the autocorrelation functions.

Photosensitized Polymerization. The PEG-LUV suspensions were thermostated and then irradiated with light from a 200 W Xe(Hg) arc lamp filtered through a CS3-72 cutoff filter (>470 nm). The incident light intensity at the sample distance of 3.5 cm was 1.3 W over an area of 2 cm in diameter. Aliquots were removed from the sample cuvette at various times,

immediately quenched in methanol, and their UV/vis spectra were measured. Because the visible light photolysis did not normally cause complete loss of monomeric bis-SorbPC, the point of complete conversion of monomer was measured after an additional 8 min irradiation with UV light (254 nm, Hg pen lamp, with a CS9-54 cutoff filter).

Fluorescence Measurements. Fluorescence spectra were obtained with a SPEX FluoroLog2 fluorometer with a slit width of 1 mm and a band-pass of 3.6 nm. PTSA fluorescence excitation spectra were obtained at an emission wavelength of 410 nm, scanning from 190 to 410 nm. PTSA fluorescence emission spectra were obtained at an excitation wavelength of 355 nm, scanning from 370 to 550 nm. DiI fluorescence excitation spectra were obtained at an emission wavelength of 590 nm, scanning from 370 to 580 nm. DiIC(18)3 fluorescence emission spectra were obtained at an excitation wavelength of 525 nm, scanning from 540 to 670 nm. The change in PTSA fluorescence emission over time was determined with an excitation wavelength of 374 nm, at an emission of 403 nm and a slit width of 1 mm. The extent of unencapsulated ANTS/DPX or PTSA/DPX was determined from the sample fluorescence prior to photolysis. Immediately after sample photolysis with UV or visible light the loss of monomeric bis-SorbPC was determined by UV absorbance, and the sample fluorescence was measured continuously over several minutes. Finally, the sample was lysed with an aliquot of Triton X-100 solution (5% v/v) to determine the 100% leakage after correcting for the bleaching of ANTS or PTSA during photolysis and the dilution factor due to the Triton X-100. The percent ANTS or PTSA bleaching was determined by comparing the fluorescence measurement after Triton X-100 treatment of a photolyzed sample to a similar measurement performed on a sample of nonphotolyzed liposomes that was of the same total lipid concentration. Dark leakage samples were treated accordingly with the exception that they were not photolyzed, and the fluorescence was measured in 30 s scans over the period of several hours.

The percent leakage at time t is given by

$$\% \text{ leakage} = (I_t - bI_0)/(1.16I_{100} - bI_0)$$

where I_t is the fluorescence intensity at time t , I_0 is the fluorescence intensity prior to photolysis, I_{100} is the fluorescence intensity after addition of Triton X-100, and b is the bleaching factor. The initial rate of leakage was calculated from the initial linear region using a least-squares fit. The fluorescence of a solution of ANTS/DPX or PTSA/DPX is linear over the concentration range used in this experiment. Therefore, it was possible to determine both the amount of ANTS or PTSA originally encapsulated and the amount that has leaked at any time directly from the equation.

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