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Photoinitiated destabilization of sterically stabilized liposomes

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Abstract

A considerable effort has been devoted to the development of liposomes for the transport and buffering of drugs in the body. Several research groups have reported the increased localization of sterically stabilized liposomes (PEG-liposomes) at tumor sites. If PEG-liposomes are to be effective carriers of therapeutic agents, their drug permeability must be sufficiently low that little passive release occurs during the circulation time of the PEG-liposomes. However, once PEG-liposomes reach tumor sites, it may be desirable to accelerate the release of the encapsulated drug. The use of light to stimulate the release of encapsulated compounds from liposomes is attractive, because it is possible to control the spatial and temporal delivery of the radiation. PEG-liposomes composed in part of the photosensitive lipid, bis-SorbPC, can be prepared in a manner that effectively encapsulates water soluble compounds, yet releases them upon exposure to ultraviolet light in the presence of oxygen. The observed increase in liposome permeability is about 200-fold at high photoconversion of the monomeric bis-SorbPC. The increase in permeability is dependent on the extent of photolysis, but independent of both the charge on the PEG-lipid and the mole fraction of PEG-lipid included in the liposome. Therefore the photoinitiated destabilization of these PEG-liposomes is not a consequence of micellization of the PEG-lipid, but probably due to the formation of defects in the bilayer during crosslinking of the bis-SorbPC. The photoinduced increase in liposome permeability is great enough to make it possible to release therapeutic agents from PEG-liposomes at specific sites in a manner of tens of minutes to hours.

Keywords: PEG-liposome; Phospholipid; Photoinduced leakage; Photopolymerization; Reactive lipid

1. Introduction

The encapsulation characteristics and biocompatibility of liposomes recommend them as carriers for therapeutic agents. Consequently, a considerable effort has been devoted to the development of liposomes for the transport and buffering of drugs in the body. The delivery of liposomes to tumor sites depends in part on long circulation times. This can be accomplished by reducing the uptake of liposomes by the reticuloendothelial system (RES), which has been achieved by steric stabilization of liposomes [1,2]. An especially useful stabilization method is the incorporation of a poly(ethylene glycol) conjugated phosphatidylethanolamine (PEG-PE) into the liposomes. The successful delivery of liposomes to tumor sites requires the liposomes to enter the interstitium. Several research groups have reported the increased localization of sterically stabilized liposomes (PEG-liposomes) at tumor sites [3–6]. The increased permeability of the vasculature at these sites allows PEG-liposomes to escape the capillaries to

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reach the tumor interstitial space. However, once the PEG-liposomes are at the tumor site the PEG groups can interfere with the rapid release of the encapsulated reagents. Consequently it continues to be important to find methods for the triggered release of reagents from PEG-liposomes.

The use of light to stimulate the release of encapsulated compounds from liposomes is attractive because it is possible to control the spatial and temporal delivery of the radiation. Liposomes may be made photosensitive by the use of uniquely designed lipids that can alter the liposome properties via photoisomerization, photocleavage, or photopolymerization [7]. A particularly useful characteristic of the latter is the multiplicative nature of the polymerization process [8]. Ultraviolet light directly initiates the polymerization of phospholipids having the hexa-2,3-dienoyl (sorbyl) functionality at the chain ends. The photopolymerization reaction produces polymers with a low kinetic chain length of about 10 [9]. However, if lipids are substituted with polymerizable groups in both acyl chains, high molecular weight, crosslinked polymers are formed [10]. Although UV initiated polymerization is not suitable for biological applications due to the high absorbance of UV light by many biomolecules, it does provide a convenient method to test lipid compositions which could, in a clinical setting, be polymerized by more biocompatible techniques such as photosensitization with longer wavelength light [11,12], or by exposure to therapeutic doses of ionizing radiation.

Whatever the means of destabilization, it is important to be able to substantially increase the solute permeability of PEG-liposomes at the desired location. Obviously the permeability must be low during the several minutes to hours the PEG-liposomes circulate in the bloodstream. Therefore if less than 1% of the encapsulated agent leaked during a circulation period of 10 h, it would then take more than a month for the rest of the drug to escape the liposome at the tumor site. In cases where it is desirable to release the drug in hours rather than weeks, the permeability must be increased by two orders of magnitude. In a preliminary report we showed that goal can be achieved [13]. Here we provide a more complete description of a successful design of photosensitive PEG-liposomes.

2. Experimental

2.1. Materials

The fluorescent probe, 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), was purchased from Molecular Probes and used without further purification. The collisional quencher, bis-pyridinium-1,4-xylene dibromide (DPX) was prepared from dibromo-1,4-xylene and pyridine. Bis-SorbPC was prepared by a modification of the published procedure [8]. 3-Oleoloamidyl-2-(monomethoxy PEG_{2000}-succinyl)-1-oleoyl-3-aminopropane-1,2-diol (PEG-S-DOAPD) was prepared by a similar procedure as described earlier [15]. 1,2-Dioleoyl-*sn*-phosphatidylcholine (DOPC) and PEG_{2000}-1,2-dioleoyl-*sn*-phosphatidylethanolamine (PEG-DOPE) were obtained from Avanti Polar Lipids. Cholesterol was purchased from Sigma Chemicals.

2.2. Liposome preparation

Lipids were measured volumetrically into a 10 ml round bottom flask from stock solutions of known concentrations. After the addition of each lipid, the solution was dried under an argon stream followed by high vacuum (4 h). The flask was then weighed to obtain the weight (approx. 0.2 mg). The lipids were then suspended in a sufficient amount of dye containing pH 7 phosphate buffer to make a 10 mM total lipid solution. The buffer used for the hydration of the lipids contained ANTS (25 mM), DPX (90 mM) and sodium phosphate (10 mM). The osmolarity of this buffer solution was found to be 277 mosmol. The lipid suspension was subjected to ten freeze thaw cycles and then extruded three times through two stacked 200 nm Nuclepore membranes followed by ten times through two stacked 100 nm Nuclepore membranes. The resulting ANTS/DPX containing liposomes were eluted through a Sephadex G-75 column with pH 7 buffer solution containing sodium phosphate (10 mM), sodium chloride (139 mM), and having an osmolarity of 277 mosmol. The concentration of the resulting liposome solutions were determined by UV absorbance at 260 nm (sorbyl $\lambda_{max} = 258_{MeOH}$, $\varepsilon = 47\,100$) of a 30 μl aliquot in 0.97 ml of HPLC grade methanol. The total lipid concentrations of resulting liposome solutions were

between 1 and 3 mM. In some cases the phosphate buffer saline (PBS) was alternately outgassed under vacuum, then flushed with argon gas to remove oxygen.

2.3. QELS measurements

PEG-liposomes for light scattering experiments were composed of PEG-S-DOAPD and DOPC in molar fractions of 0, 0.20, 0.30, 0.50 and 1.00 PEG-S-DOAPD. Liposome samples were diluted to between 500 μM and 1 mM total lipid concentration in order to give a scattering intensity of 20–150 kHz at a 90° angle. Light scattering measurements were made at 25°C and at angles of 60°, 90°, and 120° using a Brookhaven Instruments BI-200SM goniometer and BI8000 correlator (Brookhaven Instruments) equipped with a 15 mW He/Ne laser. At least three measurements were taken at each angle for each sample. Sample duration time for all but the 100% PEG-S-DOAPD sample was 3×10^8 μs. This time was doubled for the 100% PEG-S-DOAPD sample to compensate for the low scattering intensity even at 1 mM. The data analysis was done using the non-negative least squares method for each angle, followed by averaging of all results for all angles. The average for those samples having a bimodal distribution was calculated separately for each maximum.

2.4. ^1H NMR measurements

Proton NMR measurements were obtained at 25°C using a Varian Unity 300 MHz instrument with a 4-nucleus probe. A 45° pulse, acquisition time of 3.744 s and delay of 0 s was used to create 1024 transients of each sample. The line width at half-height of the oleoyl-methylenes (1.3 ppm) was measured for each sample.

2.5. Fluorescence measurements

Fluorescence time based scans were done on 3 ml, 0.15 mM dilutions of the liposome suspensions in pH 7.0 PBS, with 360 nm excitation and 520 nm emission on a Spex Fluorolog 2 fluorimeter. The slit width for both excitation and emission monochrometers was 4 mm. Complete leakage was determined by addition of 0.3 ml of 5% (v/v) aqueous Triton X-100 to a 3 ml sample. Photopolymerization was carried to between 20 and 99% by exposures of 1 s up to 8 min to light from a low pressure Hg pen lamp at 0.02 to 0.04 W/cm^2. The percent conversion was determined by the change in UV absorbance at 258 nm. A Corning CS-9-54 filter (>230 nm) was used to prevent photolysis of the polymerization product. Monomer conversion was calculated as

$$\%\ \text{conversion} = (A_0^{254}-A_t^{254})(A'^{254}_0)/(A'^{254}_0-A'^{254}_{20})(A_0^{254})$$

where A_0 is the initial absorbance of the sample, A'_0 is the initial absorbance of the standard sample, A_t is the absorbance of the sample after time (t) of irradiation, and A'_{20} is the absorbance of the standard sample after 20 min of irradiation.

2.6. Determination of encapsulated ANTS

The total amount of ANTS encapsulated in the liposome was determined shortly (within 1–2 h) after chromatographic separation of the unencapsulated dye. A 3 ml sample of liposomes having a total lipid concentration of 150 μM was prepared, and the fluorescence at 520 nm with excitation of 360 nm was measured for 45 s. Triton X-100 (0.3 ml at 5% aq. v/v) was added, and the fluorescence measurement was continued for an additional 45 s. The emission intensity of the sample after addition of Triton X-100 was multiplied by 1.1 to adjust for the dilution by the detergent solution and the difference between this and the initial measurement was compared to the emission intensity of standard solutions of ANTS/DPX (5:18 ratio).

2.7. Determination of liposome leakage

In order to determine the percent leakage of liposome encapsulated ANTS/DPX, the fluorescence of each sample was measured over 30 s immediately before photolysis. Immediately after photolysis, the percent conversion was determined from the sample absorbance with a diode array spectrophotometer, and the fluorescence was measured continuously over several minutes. After the leakage measurement, a 90 s time scan was performed during which a 5% solution of Triton X-100 was added at 45 s. The

fluorescence due to 100% leakage was determined from this measurement after factoring out the bleaching of ANTS during photolysis and the dilution factor due to the Triton X-100 solution.

The fluorescent marker, ANTS, has a minor absorption maximum at 258 nm. For this reason, irradiation of the liposome solution at 230–300 nm causes some bleaching of the ANTS. The percent ANTS bleaching was determined by comparing the fluorescence measurement after the addition of Triton X-100 for a photolyzed sample to a similar measurement performed on a sample of non-photolyzed liposomes after dividing each by the UV absorbance at 254 nm to factor out any differences in sample preparation,

$$b = I_{\text{photolyzed}}/I_{\text{non-photolyzed}} \times A_{\text{non-photolyzed}}/A_{\text{photolyzed}}$$

A bleaching factor of between 0.8 and 1.0, depending on length of photolysis, was obtained, which adjusts the baseline (I_0) to what it would be if the amount of ANTS present before photolysis were equal to that present after photolysis.

The percent leakage at any time is given by the following expression:

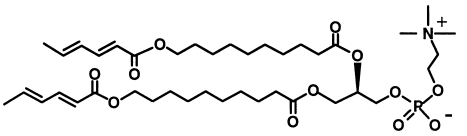
$$\%\ \text{leakage} = 100 \times (I_t - bI_0)/(1.1I_{100} - bI_0)$$

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. Because the initial change in concentration inside the liposomes is relatively small, the initial leakage is pseudo-zero order, and the plot is a straight line. The rate of leakage was calculated from the linear region of the plot using a least squares fit.

3. Results and discussion

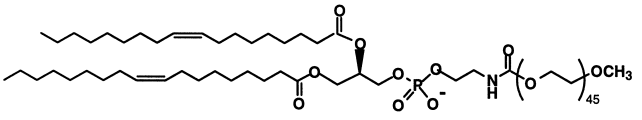
3.1. Liposome composition and encapsulation

The photosensitive PEG-liposomes were composed of four components, the photopolymerizable bis-SorbPC, DOPC, cholesterol, and a pegylated lipid, either PEG-DOPE or PEG-S-DOAPD. The bis-SorbPC was used to confer photosensitivity to the liposomes. Cholesterol was included in the liposomes to reduce dark leakage of the encapsulated fluorescent probe, ANTS and its collisional quencher, DPX [14].

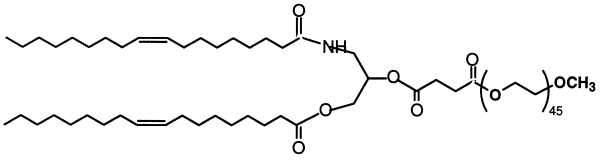


bis-SorbPC

In order to ascertain the effect of lipid charge on the photoinitiated destabilization of the PEG-LUV a neutral PEG lipid, PEG-S-DOAPD, was prepared and the photosensitivity of PEG-liposome partly composed of PEG-DOPE or PEG-S-DOAPD were compared. Efremova et al. have previously shown that bilayers composed in part of neutral PEG-lipids lack the longer range electrostatic repulsion exhibited by PEG-lipids prepared from PE [15]. Both neutral and anionic PEG-lipids impart similar steric repulsion to the bilayers.



PEG-DOPE



PEG-S-DOAPD

The maximum amount of encapsulation of a fluorescent marker or drug molecule into liposomes, without the use of a pH gradient or other means of increasing the concentration inside the liposomes relative to the concentration outside the liposomes, is the product of the total internal volume of a liposome solution and the concentration of the molecule to be encapsulated. The internal volume of a liposome suspension was calculated for various sizes of unilamellar liposomes (LUV) of POPC by Chapman et al. [16]. Liposomes in the leakage experiments presented here ranged between 90 and 120 nm in diameter. Because the volume is proportional to r^3 the encapsulated volume in a population of liposomes should be dominated by the larger liposomes. The

highest values for dye encapsulation in these leakage experiments were about 33% of that corresponding to calculated maximum internal volume for 100 nm POPC LUV.

Possible reasons for the lower encapsulation include: (1) leakage during or after chromatography, (2) liposome volume occupied by PEG chains [17], (3) the assumed area per lipid molecule was incorrect, or (4) the formation of a sub-population of non-encapsulating or non-spherical structures such as disc shaped liposomes, toroidal liposomes, rod shaped micelles, or spherical micelles. Although a detailed analysis of these factors appears elsewhere [18], it was found that the greatest decrease in encapsulation efficiency occurs at high PEG-lipid concentrations, and/or high bis-SorbPC concentrations. In contrast to conventional LUV, the internal volume of PEG-LUV is reduced by the volume occupied by the hydrated polymer. The thickness of a hydrated PEG layer for a PEG_{2000}-grafted, supported bilayer was determined to be 5.2 nm for a mushroom conformation in the weak overlap regime (4–5% PEG-lipid) and 6.2 nm for a brush regime (>10% PEG-lipid) [15]. The calculated volume per liposome for 90–120 nm LUV indicates the PEG chains could reduce the encapsulation volume by as much as 20%. In addition we found that the dark leakage of fluorescent markers from the LUV was too high for effective encapsulation, if the LUV were composed of 30% or more bis-SorbPC with no cholesterol. When 20–40 mole% cholesterol was included in the liposome composition, the dark leakage was reduced by two orders of magnitude. The effect of bis-SorbPC on encapsulation efficiency is probably a result of the polar ester function in the hydrophobic chains.

3.2. Liposome photolysis and photoinduced leakage

Fig. 1 shows the UV spectra of LUV suspensions with a total lipid concentration of 150 μM at several photolysis time points for LUV composed of PEG-DOPE, bis-SorbPC, cholesterol, and DOPC. Photolysis of the LUV suspension results in a decrease in the absorbance at approx. 260 nm with a lesser increase in the absorbance at 204 nm, which corresponds to the isolated double bond formed during the photopolymerization. Initially the photochemical reaction appears to yield a single photoproduct,

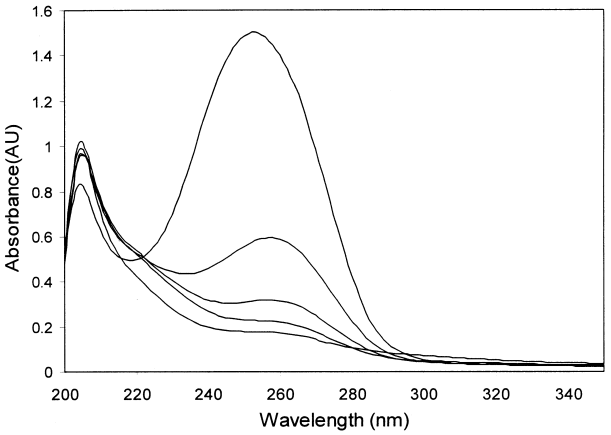


Fig. 1. Absorption spectra of a 0.15 mM PEG-liposome suspension composed of PEG-DOPE, bis-SorbPC, cholesterol, and DOPC (molar ratio 15:30:40:15). The absorption maximum decreases with increasing photolysis time (0, 15, 30, 60, and 600 s) at 37°C and 0.04 W/cm^2 intensity.

based on the presence of an isosbestic region; however, at longer exposure times the isosbestic region is lost (Fig. 1). Lamparski and O'Brien found that the major product from the polymerization of pure bis-SorbPC liposomes in the absence of oxygen is due to 1,4-addition [9]. The conditions of polymerization in these experiments were somewhat different (mixed liposomes and presence of atmospheric oxygen), therefore the previous observations only provide a reasonable prediction that has not been rigorously demonstrated in this case.

Earlier reports from this group demonstrated that polymerization of phospholipids having polymerizable groups at the ends of both acyl chains causes the formation of separate domains of polymerized lipid and of unreactive lipids in liposomes [8,19,20]. Phase separation, prior to polymerization, between the fluid dioleoyl lipids and polymerizable lipids was not observed in these experiments. The initial strategy adopted here to induce leakage in sterically stabilized liposomes was to use the photopolymerization to create domains of polymerized lipid, while concentrating the PEG-lipid into the non-polymerized domains. At high mole fractions, PEG-lipids form micelles rather than bilayers. In order to promote lipid mixing and rapid diffusion of lipids into their perspective domains upon polymerization, fluid lipids (DOPC, PEG-S-DOAPD, and PEG-DOPE) were used. These lipids have two oleoyl chains, which

give them a low T_m, and are in the fluid, liquid crystalline phase at room temperature.

The photolysis of PEG-liposomes composed of PEG-DOPE, bis-SorbPC, cholesterol, and DOPC (molar ratio 15:30:40:15) not only causes a loss of monomeric bis-SorbPC, but also causes an increase in the liposome permeability of the encapsulated ANTS/DPX. The increase in fluorescence of the ANTS upon photolysis of the PEG-liposomes was used to measure the rate of ANTS release from the liposomes. A set of three independent experiments, i.e. separate PEG-liposome preparations, are shown in Fig. 2. The open symbols indicate the loss of monomeric bis-SorbPC with irradiation time at 37°C, and the filled symbols show the log rate of ANTS leakage at the same times. There is a 10-fold increase in permeability after 80% loss of monomer, plus an additional 20-fold increase when the photolysis exceeds 95% conversion. Therefore at high photoconversion the PEG-liposome permeability was increased about 200-fold. The highest observed initial rates of leakage were about 0.1% per second, which would release the contents in 16 min. Of course the initial rate of leakage is attenuated as the entrapped ANTS concentration is diminished. Consequently at high photoconversion of bis-SorbPC most of the ANTS is released in 50–60 min.

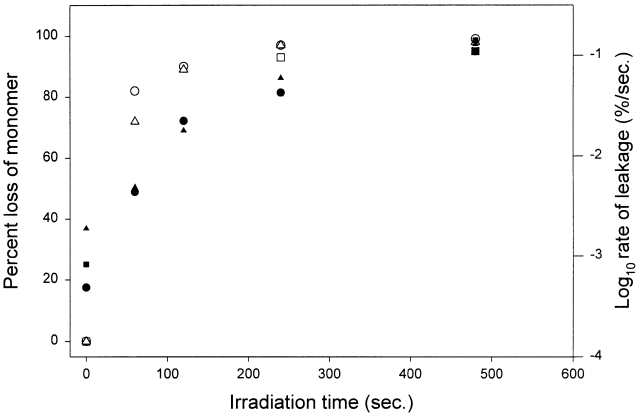


Fig. 2. Effect of the photolysis of PEG-liposomes composed of PEG-DOPE, bis-SorbPC, cholesterol, and DOPC (molar ratio 15:30:40:15). Both the percent loss of monomeric bis-SorbPC (open symbols, left axis) and the log of the initial rate of ANTS release expressed in percent per second from the liposomes (filled symbols, right axis) are shown for three experiments as a function of the sample irradiation time at 0.02 W/cm^2 intensity and 37°C.

3.3. Phase behavior of PEG-liposomes

While PEG-lipids mixed, in low concentrations, with conventional lipids form a protective, hydrated polymer brush at the liposome surface, the PEG-lipid molecule is an amphiphile with detergent-like properties, because of the large cross-section of the head group compared to that of the lipid tail. At concentrations greater than 60 mole%, Lasic et al. have observed that PEG-lipids convert liquid crystalline bilayers into micelles [21]. Hristova and Needham calculated that the maximum concentration of PEG_{2000}-lipid that can be incorporated into a liquid crystalline phase bilayer is 18 mole% [22]. Kenworthy et al. performed extensive phase characterization experiments on mixtures of the anionic, saturated PEG lipid, PEG_{2000}-DSPE with DSPC both above and below the main phase transition temperature (55°C) [23]. In these experiments, it was found that, at concentrations of PEG_{2000}-DSPE greater than approx. 20 mole%, there is a broad range at which lamellar and micellar phases coexist. Above 60 mole% PEG-DSPE, only the micellar phase was observed. It is necessary for our studies of neutral PEG-lipids to determine the concentration at which non-lamellar phases form for a DOPC/PEG-S-DOAPD system.

The methylene line widths of the lipid compositions examined (Fig. 3) show a dramatic decrease in the line width between 20 and 30 mole% PEG-S-DOAPD, indicating that there is a large increase in the mobility of the oleoyl-methylene protons. NMR line width is proportional to the inverse of the relaxation time ($\Delta\nu. = 1/2\pi T_2$). The relaxation time (T_2) decreases dramatically as the mobility of the observed nuclei decreases (as in a large solid-like particle). For this reason the line width of the of the NMR spectrum of the hydrophobic tails ($\delta = 0.8$ and 1.3 ppm) decreases as the lipid phase goes from lamellar to micellar. These data parallel QELS data that show an increase in the population of small particles at the same compositions. Because a spectrum with a narrow line width has a higher signal to noise ratio, the change in line width should be much more sensitive to small structures in the presence of large ones. Accordingly, the NMR experiment compliments the QELS experiment.

The significant increase in the population of small lipid aggregates observed between 20 and 30 mole%

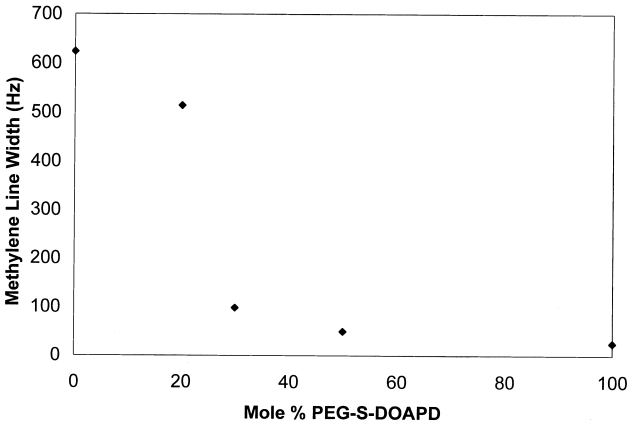


Fig. 3. Effect of the mole fraction of PEG-S-DOAPD in a hydrated mixture of this neutral PEG-lipid with DOPC on the ^{1}H NMR acyl chain methylene line width.

PEG-S-DOAPD indicates the onset of a lamellar to micellar phase transition. These data agree well with the previous studies of PEG_{2000}-DSPE/DSPC mixtures that found a broad transition from the lamellar to micellar phase beginning at 20 mole% PEG_{2000}-DSPE [23]. Complete polymerization of the bis-SorbPC in LUV composed of PEG-lipid/bis-SorbPC/DOPC (15:30:55) would make the mole% PEG-lipid (relative to monomeric lipid) somewhat greater than 20%. Inclusion of more than 20 mole% cholesterol was necessary to control the LUV dark leakage, and facilitated encapsulation of the ANTS/DPX solution. The expected cholesterol induced condensation of the surface area per lipid has the effect of increasing the mole% of the PEG-lipid in completely polymerized LUV to 29%, an ideal value for formation of mixed micelles and membrane destabilization. In light of the above considerations, the formulation of LUV used in the initial experiments consisted of 15 mole% PEG-S-DOAPD, 25–30 mole% bis-SorbPC, and 40 mole% cholesterol with the remainder being made up of DOPC.

3.4. Effect of surface charge on photodestabilization

The increase in free energy that results from bringing together two tethered polymer chains in the brush conformation was predicted by Alexander to be proportional to $TN(a/D)^{5/3}$ where T is the absolute temperature, N is the degree of polymerization, a is the length of each monomer unit, and D is the distance between the grafting sites [24]. A more recent theory developed by Milner et al. placed this dependence at $TN(a/D)^{4/3}$ [25]. In addition, the energy required to bring two point charges together is equal to the integral from the initial distance to the final distance of the electrostatic force equation: e z_1z_2/r^2, or e$(1/r_2-1/r_1)$, if $z_1 = z_2 = \pm 1$. The work required to move two charged PEG-lipids together would be a sum of these two functions. This work must come from the free energy of the polymerization, which is fairly limited and decreases with increasing temperature.

In order to determine if PEG-lipid charge influences the efficiency of the photoinitiated destabilization of the PEG-LUV, liposomes composed in part of either PEG-DOPE or PEG-DOAPD were studied. Figs. 2 and 4 summarize some of the results of leakage experiments with liposomes that differ only in the charge of the PEG-lipid. With the exception of a 5 times higher rate of dark leakage in the neutral LUV (which could be a result of higher encapsulation), there is little difference in photosensitivity or of these LUV. Therefore the negative charge of the PEG-DOPE does not inhibit the photoinduced leakage.

3.5. Effect of PEG-lipid concentration

If the hypothesis that photoinitiated leakage re-

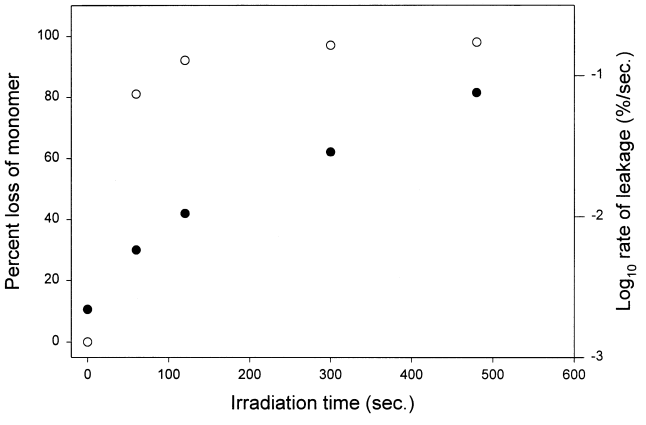


Fig. 4. Effect of the photolysis of PEG-liposomes composed of PEG-S-DOAPD, bis-SorbPC, cholesterol, and DOPC (molar ratio 15:30:40:15). Both the percent loss of monomeric bis-SorbPC (open symbols, left axis) and the log of the initial rate of ANTS release expressed in percent per second from the liposomes (filled symbols, right axis) are shown as a function of the sample irradiation time at 0.02 W/cm^2 intensity and 37°C.

sults from an increased concentration of PEG-lipid in non-polymerized domains is true, then reduction of the PEG-lipid concentration should reduce the photosensitivity of the LUV, and elimination of the PEG-lipid entirely should eliminate the photosensitivity. For this reason, leakage experiments were carried out in which the concentration of PEG-lipid was reduced from 15% to 5% and 0%. Contrary to expectation, it was found that the concentration of PEG-lipid has little effect on the rate of photoinduced leakage at high conversion. Fig. 5 summarizes the results of leakage experiments on liposomes composed of PEG-S-DOAPD/bis-SorbPC/cholesterol/DOPC (5:30:40:25 and 15:30:40:15). The rate of leakage at high conversion is not significantly different in these experiments. At intermediate conversion the leakage rate for the 5% PEG-lipid composition is somewhat lower, but this could also be the result of differences in polymerization extent and conditions. These data provide no clear evidence of any significant difference in the photosensitivity of the two liposome formulations. Moreover, when leakage experiments were carried out with LUV composed of bis-SorbPC/cholesterol/DOPC (25:40:35), i.e. no PEG-lipid in the LUV, the LUV were found to exhibit a similar photosensitivity as the PEG-liposomes. The photosensitivity of non-pegylated LUV demonstrates that the concentration of PEG-lipid into non-polymerized domains leading to the formation of mixed micelles is not the major mechanism for photoinduced leakage in PEG-liposomes containing dioleoyl lipids.

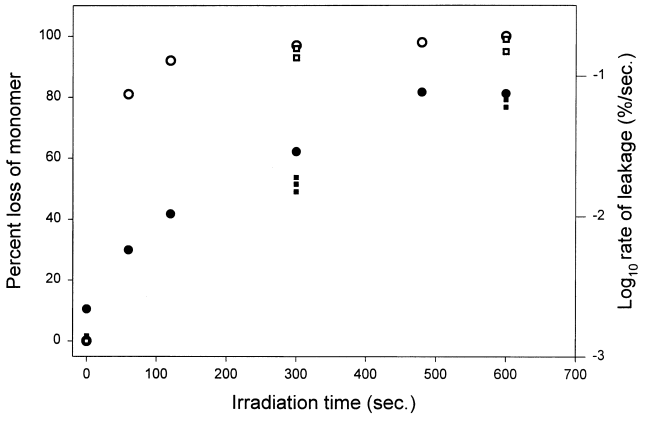


Fig. 5. Effect of the PEG-lipid mole fraction on the photosensitivity of liposomes composed of PEG-S-DOAPD, bis-SorbPC, cholesterol, and DOPC in a molar ratio of 15:30:40:15 (circles) or a molar ratio of 5:30:40:25 (squares). Both the percent loss of monomeric bis-SorbPC (open symbols, left axis) and the log of the initial rate of ANTS release expressed in percent per second from the liposomes (filled symbols, right axis) are shown vs. irradiation time at 37°C.

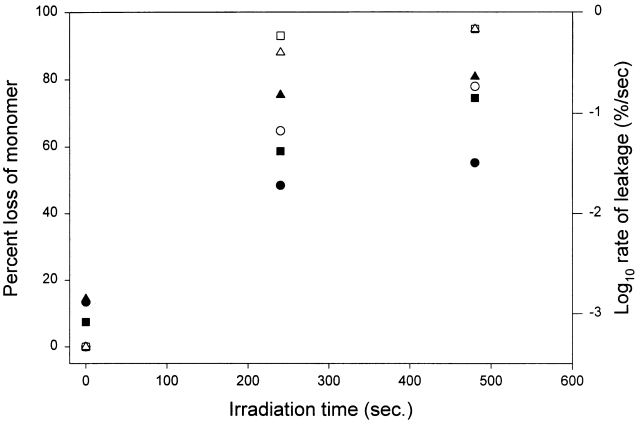


Fig. 6. Effect of oxygen on the photosensitivity of PEG-liposomes composed of PEG-DOPE, bis-SorbPC, cholesterol, and DOPC (molar ratio 15:30:40:15). The liposome suspension was either purged with argon (circles), not purged (squares), or bubbled with oxygen gas (triangles). The percent loss of monomeric bis-SorbPC (open symbols, left axis) and the log of the initial rate of ANTS release expressed in percent per second from the liposomes (filled symbols, right axis) are shown vs. irradiation time at 37°C.

An alternative explanation for the increased permeability of the liposomes could be the disordering of polymerized domains by crosslinking of polymer chains. The crosslinking of adjacent chains can change the distance between the lipids involved in the crosslinked polymer. Any resulting defects in lipid packing would not be filled in because of the relative lack of mobility of the polymerized lipid. These defects are necessarily small because the rate of leakage is only increased by two orders of magnitude, whereas large pores would result in immediate and complete leakage. The disordering that occurs between domains of lipids at the main phase transition temperature is sufficient to increase bilayer permeability by 10^2 [26]. In the case of photosensitive PEG-LUV, the suggested defects in the membrane must persist over several minutes, because the rate of leakage does not decrease significantly.

3.6. Effect of oxygen

Molecular oxygen is often considered to be an inhibitor of radical polymerizations. Although poly-

merization of the sorbyl group by direct irradiation is considered to be a successive photoaddition process rather than a radical chain reaction, there is some possibility that oxygen could take part in side reactions that could decrease the efficiency of the polymerization. It was of interest, therefore, to perform polymerization and leakage assays in the absence of oxygen to determine if higher photosensitivity can be achieved. An experiment was designed in which liposomes from the same stock solution were diluted to 100 μM in PBS buffer that was either purged with argon, prepared in and exposed to air as in other experiments as a control, or bubbled with pure oxygen through a Pasteur pipette for 1 min immediately prior to use (Fig. 6). Contrary to expectation, it was found that the exclusion of oxygen from the system resulted in a decrease both in the rate of polymerization and in photosensitivity. On the other hand, saturation with oxygen lead to significantly higher photoinduced leakage compared to the control, though the rate of monomer loss was not significantly higher than that of the control. These results indicate that oxygen plays a role in the destabilization of these liposomes. One possible explanation for this effect is that oxygen is involved either as a comonomer, or as a crosslinking agent in such a way that it increases the crosslinking density of the polymerized domains, thereby making more and larger defects through which leakage occurs. Evidence for inclusion of oxygen during the polymerization of the sorbyl group in the solid state has been observed by Matsumoto et al. [27]. Octadecyl sorbate crystallized from ethanol and photopolymerized in the presence of oxygen produced an alternating 3,4-polysorb-co-peroxide. This structure was determined by ^{13}C and ^{1}H NMR because of a downfield shift in the methine carbon and proton signals.

4. Conclusions

PEG-liposomes composed in part of the photosensitive lipid, bis-SorbPC, can be prepared in a manner that effectively encapsulates water soluble compounds, yet releases them upon exposure to ultraviolet light in the presence of oxygen. The surface compositions of these photosensitive liposomes are similar to those that have been shown to have prolonged circulation times in the body. The observed increase in liposome permeability is about 200-fold at high photoconversion of monomeric bis-SorbPC. The increase in permeability is dependent on the extent of photolysis, but independent of both the charge on the PEG-lipid and the mole fraction of PEG-lipid included in the liposome. Therefore the photoinitiated destabilization of the liposomes is not a consequence of micellization of the PEG-lipid, but probably due to the formation of defects in the bilayer during crosslinking of the bis-SorbPC. The photoinduced increase in liposome permeability is great enough to make it possible to release entrapped water soluble compounds from PEG-liposomes at specific sites in a manner of tens of minutes to hours, rather than over the course of weeks. In order to perform such experiments in vivo it will be necessary to utilize PEG-liposomes that are sensitive to longer wavelength light [12], or to ionizing radiation [28]. Both of these possibilities will be described in more detail in the near future.

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