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Liposomes from Novel Photolabile Phospholipids: Light-Induced Unloading of Small Molecules As Monitored by PFG NMR

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Light-triggered unloading of liposomes offers an attractive alternative to the temperature or pH-driven modulation of membrane permeability. The known approaches to photoresponsive vesicles are based on disrupting the integrity of the lipid bilayer through (i) photoinduced polymerization of, e.g., sorbate-based lipids causing phase changes in the bilayer and enhanced leakage at the boundaries of polymerized domains,[1] (ii) dramatic conformational changes due to photoisomerization in the incorporated azobenzene or alkene fragment,[2] (iii) photoinduced release of a masked hydrophilic group in the hydrophobic region of the bilayer membrane[3] or, finally, (iv) phototransformation of lipid headgroups, e.g via oxygenation of a double bond.[4] While severing hydrophilic headgroups appears to be a simple and straightforward way to disrupt the lipid bilayer, this approach did not receive as much attention.

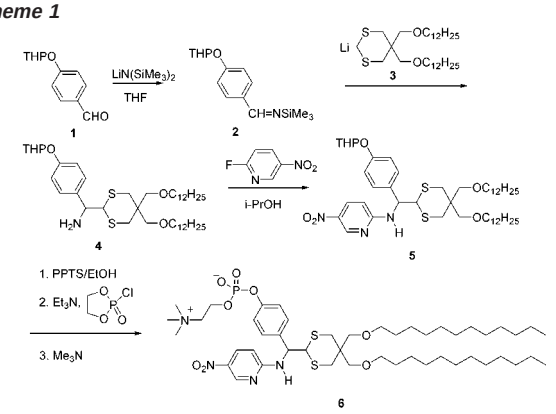
Recently we have developed a novel strategy for the assembly of photolabile molecular systems based on carbonyl additions of substituted di- and trithianes. This methodology allows us to link various molecular blocks with photolabile latches that can be unfastened on demand via photoinduced electron transfer.[5] In this communication we report on the synthesis of novel phosphatidylcholine-like lipids, in which the hydrophilic phosphocholine headgroups are connected to the hydrophobic tails via a photolabile, dithiane-based tether. The photocleavable unit can also be outfitted with the dual-purpose nitropyridinamino group serving as an internal ET-sensitizer and as a model element of molecular recognition.[5d] Here we show that such photolabile lipids can be used in formulations with egg palmitoyl-oleoyl phosphatidylcholine (POPC) and cholesterol to form vesicles capable of unloading their content upon irradiation.

Synthetic approaches to the photolabile phospholipids are shown in Schemes 1 and 2. 5,5-Bis(hydroxymethyl)-1,3-dithiane[5e] is alkylated to attach two hydrophobic hydrocarbon tails, lithiated and added to a solution of in situ generated *N*-silylimine **2**. Amine **4** is reacted with 2-fluoro-5-nitropyridine, the phenol is deprotected, and phosphocholine is introduced via a standard procedure[6] involving 1-chloro-phosphadioxolane to furnish photolabile lipid **6**, Scheme 1.

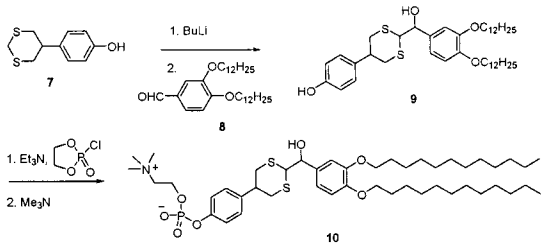
Alternatively, 5-(4-hydroxyphenyl)-1,3-dithiane[7] (**7**) can be utilized to carry the hydrophilic headgroup (Scheme 2). In this example the hydrophobic tails are attached to the carbonyl component, e.g. 3,4-dihydroxybenzaldehyde (**8**), and thus hydroxyalkyl dithiane **10** is produced. This lipid requires external sensitization for cleavage.

The liposomes were then prepared from a three-component mixture of POPC(egg)−cholesterol−photolipid in the ratio 5:3:2. First, a thoroughly dried lipid film was hydrated with a 0.15 M phosphate buffered saline solution (PBS, pH 7.0) containing the probe molecule. The suspension was subjected to 4 freeze−thaw cycles to disrupt large multilamellar vesicles (LMV) and extruded 21 times at 55 °C through a polycarbonate filter with 100 nm pores to form large unilamellar vesicles (LUV). Subsequent gel filtration on Sephadex was carried out to remove untrapped probes.

Scheme 1



Scheme 2



Conventional assays of liposome leakage are based on fluorescence recovery derived from reduced efficiency of collisional quenching in the bulk solution as opposed to nearly total quenching inside the vesicle due to the higher internal concentration of fluorophore quencher.[1b,c,g] While these are very simple assays for studying liposome leakage induced by changes in pH, temperature, and other nonradiative chemical processes, we detected considerable photobleaching of several fluorophores during the photochemical experiments, rendering fluorescent assays less suitable for quantitative monitoring of photoinitiated release. Also, the light absorption by the probe molecule may interfere with the desired photochemistry, especially when accurate quantum yield measurements are required. We suggest that these shortcomings in photochemical experiments can be overcome by utilizing the Pulse Field Gradients (PFG) NMR technique[8] for determining the self-diffusion coefficients of small probe molecules, which do not have any interfering UV absorption. When the probe is confined inside the vesicle its apparent diffusion coefficient is equal to that of the carrier liposome. It changes by orders of magnitude, depending on the hydrodynamic size ratio, when the probe is released into the bulk solution. We further suggest using probes that carry fluorine-containing groups,

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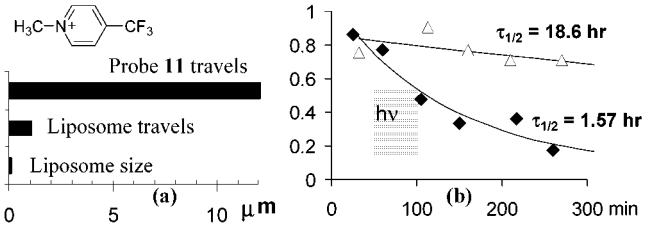

Figure 1. (a) Relative size and distances traveled in 0.15 s and (b) release of **11** from POPC-cholesterol-**6** in the dark (△) and irradiated (◆).

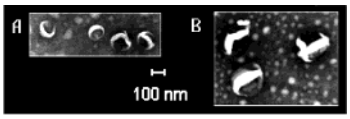

Figure 2. TEM images of photolabile liposomes before (A) and after irradiation (B)—same scale.

e.g. trifluoromethyl, for easy detection with ^{19}F PFG NMR.[9] In such a case, the monitoring is reduced to observing one signal with no interference from other peaks and no need for water suppression.

We tested several probe molecules for ^{19}F and ^{1}H PFG NMR monitoring of membrane permeability. Small inorganic anions showed unacceptably high rates of dark leakage, even when control experiments were run with liposomes made of well-documented stable POPC-cholesterol formulations: at room temperature the lifetimes of leakage for salts KF ($D_S = 1.33 \times 10^{-5}$ cm^2 s^{-1}), KPF_6 ($D_S = 1.14 \times 10^{-5}$ cm^2 s^{-1}), and KOTf ($D_S = 9.08 \times 10^{-6}$ cm^2 s^{-1}) were less than 0.5 h, making them impractical for monitoring. Larger cations were escaping much slower, for example, the lifetime of dark leakage for *N*-methyl-4-trifluoromethylpyridinium (**11**) exceeded 18 h. While pyridine **11** was our probe of choice for this study, we note that sodium 2-(trimethylsilyl)ethanesulfonate ($Me_3SiCH_2CH_2SO_3Na$, $D_S = 3.9 \times 10^{-6}$ cm^2 s^{-1}), commonly used as an ^{1}H NMR reference for aqueous solutions, also is an adequate PFG probe molecule, with an easily identifiable rightmost peak in the proton spectrum.

The diffusion coefficient of **11** in PBS solution at 20 °C was found to be 5.36×10^{-6} cm^2 s^{-1}, whereas the apparent diffusion coefficient of this probe entrapped inside the liposome is 4.3×10^{-8} cm^2 s^{-1}. This value is in remarkably good agreement with the 100 nm average size of the vesicles, provided self-diffusion of liposomes is subject to the Stokes–Einstein equation,[10] $D_S = kT/6\pi\eta R$. The 2 orders of magnitude difference in diffusion coefficients is sufficient to accurately monitor the probe release (for details refer to the Supporting Information).

The results of photorelease of **11** from liposomes prepared with photolipid **6**, which has λ_{max} of 350 nm, are shown in Figure 1. While the dark leakage lifetime was on the order of 20 h, irradiation of the sample at $\lambda > 300$ nm (medium-pressure mercury UV lamp, Pyrex filter) for 75 min decreased the lifetime to 1.5 h (Figure 1b). NMR showed that 55 to 60% of the photolabile lipid was cleaved, which corresponds to 11–12% of the total bilayer material.

The TEM images of the negatively stained vesicles suggested that some fusion occurred during irradiation (Figure 2). Judging by the size, two to three 100 nm liposomes fuse into larger unilamellar vesicles. The resulting vesicles have almost "normal" spherical shape, although the staining pattern was slightly different (conceivably due to accumulation of the fragmented lipid, which increased positive staining by stain exclusion). We hypothesize that irradiation uniformly affected the permeability of photolabile liposomes, although we cannot rule out that the increase in liposome fusability is also related to photorelease of the entrapped material.

In conclusion, we have developed a dithiane-based modular approach for assembly of photolabile lipids capable of forming liposomes in formulations with POPC and cholesterol. The lipids can be equipped with hydrogen bond-based elements of molecular recognition offering a possibility to rationally modify the surface of vesicles. Obviously, before this chemistry could be used to solve "real" problems of drug delivery, the efficiency of the photofragmentation and subsequent release will have to be improved.

We also have developed a simple assay to monitor the release of small organic molecules based on PFG NMR. The potential advantage of ^{19}F monitoring is that many biomedically relevant compounds of interest can be labeled via, for example, trifluoroacetylation and their unloading from liposomes or other delivery vehicles can then be followed easily.

Work is in progress in our laboratories to improve the dark stability of photosensitive liposomes and the efficiency of photorelease, and to model recognition events based on vesicle surface modification.

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Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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