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Non-Phospholipid Fluid Liposomes with Switchable Photo-Controlled Release

*Zhong-Kai Cui, Thida Phoeung, Pierre-Antoine Rousseau, Gaulthier Rydzek, Qian Zhang, C. Geraldine Bazuin, and Michel Lafleur**

Department of Chemistry, Center for Self-Assembled Chemical Structures (CSACS),

Université de Montréal, C.P. 6128, Succ. Centre Ville, Montréal, Québec, Canada H3C 3J7.

E-mail: michel.lafleur@umontreal.ca

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Abstract

We created novel non-phospholipid photosensitive liposomes from a mixture of a monoacylated azobenzene amphiphile ($AzoC_{10}N^+$), and cholesterol sulfate (Schol). This system belongs to the family of sterol-enriched non-phospholipid liposomes that were shown to form stable large unilamellar vesicles (LUVs) with enhanced impermeability. Fluid bilayers were successfully prepared from $AzoC_{10}N^+$/Schol (25/75, mol/mol) mixtures and LUVs could be derived at room temperature using standard extrusion methods. The isomerization process of the bilayer-inserted $AzoC_{10}N^+$ was characterized. Leakage from these liposomes could be induced by the photoconversion of the $AzoC_{10}N^+$ from its *trans* to its *cis* form. This photo-controlled release is obtained from fluid liposomes, contrasting with phospholipid-based azo-containing liposomes, which are generally required to be in the gel phase in order to be photosensitive. It is proposed that the very high conformational order of the monoalkylated amphiphile and the tight packing of the hydrophobic core of the $AzoC_{10}N^+$/Schol liposomes make them responsive to the presence of the bulky *cis* azo isomer. Interestingly, the liposome impermeability could be fully restored by the photoisomerization of the *cis* form back to the *trans* form, providing a sharp on-and-off control of payload release. In addition, these non-phospholipid liposomes display a very limited passive release. Therefore, it is shown that $AzoC_{10}N^+$/Schol LUVs can be used as nanocontainers, whose content can be released by light in a controlled and switchable manner.

1. Introduction

Light is a versatile stimulus for controlling the release of entrapped materials from nanocontainers. A broad range of parameters, such as frequency, quantity, and duration, can be straightforwardly optimized for a given application. In addition, this approach confers local control of the nanocontainer permeability, because of the use of light sources such as lasers. Various strategies (e.g. photo-polymerisation[1-2] and photo-oxidation[3-4]) have been exploited to obtain photo-controlled release from nanovectors. Among these, the isomerization of photosensitive groups, such as spiropyran,[5-7] spirooxazine,[8] and azobenzene,[9-11] constitutes an interesting approach to induce photo-controlled release from vesicles, as it has the advantage of being reversible. The azobenzene group is a functional group that can undergo reversible *trans–cis* isomerization under irradiation of UV or visible light.[9-11] Thermodynamically, the *trans* isomer is favored because of the minimized steric hindrance. Because of the greater stability of the *trans* form, there is thermal relaxation of the system from the *cis* to the *trans* isomer; this relaxation process is generally much slower than the photoconversion from one isomer to the other.[12] It has been shown that when an azobenzene derivative is inserted in phospholipid liposomes, the *trans-cis* isomerization of the azobenzene group can induce defects in bilayers that lead to the release of the entrapped payload, including K^+,[10] calcein,[13-16] and doxorubicin.[17] This controlled release is typically obtained with gel-phase liposomes where tight chain packing cannot accommodate the defects associated with the bulky *cis* conformers. It was shown that fluid bilayers prepared with phospholipid would remain impermeable despite the photoformation of bent *cis* isomers due to the flexibility of a fluid hydrophobic core.[14, 18]

In this paper, we demonstrate the possibility of making fluid-phase photosensitive non-phospholipid liposomes formed by a monoacylated amphiphile, decyl-azobenzyl-

triethylammonium ($AzoC_{10}N^+$, **Figure 1**), and a sterol, cholesterol sulfate (Schol). Mixtures of a single-chain amphiphile (between 25 and 50 mol%) and a very high quantity of sterol (75 to 50 mol%) form stable liquid-ordered (lo) phases under certain conditions (for a review, see ref 19) and large unilamellar vesicles (LUVs) can be prepared from these mixtures by a simple extrusion process. This distinct composition confers to these non-phospholipid LUVs distinct advantages for the formation of photosensitive liposomes. First, these nanovectors show enhanced impermeability compared to conventional phospholipid-based liposomes. For example, LUVs prepared from palmitic acid (PA) and cholesterol, in a molar ratio of 30/70, release only 30% of the entrapped calcein after 18 months.[20] Second, the high sterol content leads to very high chain order, and very tight chain packing, while maintaining the fluid character of the bilayers.[21-22] It is hypothesized that the high degree of order should make the liposome bilayers responsive to the *trans-cis* isomerization of the azobenzene group. According to this hypothesis, these liposomes would be photoresponsive in the fluid but highly ordered phase, a feature that presents advantages with respect to their use as drug nanovectors, including the possibility to extrude liposomes at room temperature,[23] and an efficient drug loading.[23-24] Third, single-chain amphiphile/sterol mixtures can display an advantageous chemical stability compared to phospholipids, which are degraded by indigenous phospholipases and whose ester links are susceptible to hydrolysis under extreme pH conditions.[25] Thus, our aim is to obtain the combined properties of very stable yet fluid liposomes, due to the high sterol content, and photo-triggered delivery of encapsulated materials, due to the azobenzene-containing single-chain amphiphile.

$AzoC_{10}N^+$ was designed in such a way that its length matches that of Schol, a prerequisite to ensure the stability of fluid bilayers.[26] The azobenzene moiety is located at the end of the acyl chain and, as a consequence, should be located mainly at the center of the self-

assembled bilayers. The presence of the positively charged ammonium group is required in order to provide proper lipid mixing with the sterol and to ensure suitable interfacial hydration.[27-28] The design of the $AzoC_{10}N^+$/Schol system is based on comparable mixtures of cetylpyridinium chloride (CPC)/sterol[28] and stearylammonium/sterol,[27] which were shown to form fluid bilayers. On the basis of the behavior of the well characterized CPC/Schol systems, we explored the behavior of the mixture composed of 25 mol% $AzoC_{10}N^+$ and 75 mol% Schol, since this ratio is predicted to be a good candidate for forming fluid lamellar phases with very tight chain packing. First, we determined whether the $AzoC_{10}N^+$/Schol system forms fluid bilayers and whether LUVs could be generated. Subsequently, we characterized the kinetics of the *trans-cis* isomerization process of the azobenzene derivative inserted in $AzoC_{10}N^+$/Schol bilayers as well as solubilized in $CHCl_3$ as a control. Finally, we carried out release experiments in order to assess the efficiency of the photo-control of the payload release that can be obtained from the reversible photo-isomerization of the azobenzene group. We specifically investigated the possibility of exploiting the photoreversible isomerization for switching the payload release on and off. As shown below, this mixture provides a very tight switchable control of the release of the entrapped material to an extent that has never been reported before.

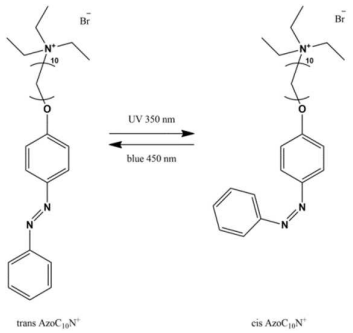

Figure 1. Chemical structure and photo-isomerization of $AzoC_{10}N^+$.

2. Experimental

Materials

The monoacylated azobenzene derivative, $AzoC_{10}N^+$, was synthesised according to a procedure published elsewhere.[29] Schol, PA (99%), Triton X-100, ethylenediaminetetraacetic acid (EDTA, 99%), and tris(hydroxymethyl)aminomethane (TRIS, 99%) were purchased from Sigma-Aldrich (St Louis, MO). Sulforhodamine B (SRB) was purchased from Invitrogen (Eugene, OR). 2-[*N*-morpholino]ethanesulfonic acid (MES, > 99%) and NaCl (> 99%) were obtained from EMD Chemicals (Gibbstown, NJ) and Anachemia (Montréal, QC, Canada), respectively.

Methods

$AzoC_{10}N^+$/Schol and PA/Schol mixtures were prepared by mixing weighed quantities of each compound solubilized in a benzene/methanol (75/25 v/v) mixture, followed by freeze-drying for at least 16 h.

For UV-Vis experiments, a stock liposome dispersion was prepared by hydrating the lipid mixtures in a MES/TRIS buffer (140 mM NaCl, 50 mM MES, 50 mM TRIS and 0.5 mM EDTA, pH 7.4 for $AzoC_{10}N^+$/Schol, and pH 7.0 for PA/Schol). The dispersions were then extruded 15 times through two stacked polycarbonate filters with a 100-nm pore size, using a handheld Liposofast extruder (Avestin Corp., Ottawa, ON, Canada) at room temperature. The size of the resulting LUVs was determined at 25 °C, using a Malvern Zetasizer. The lipid concentration was adjusted to ~20 μM for these measurements.

The isomerization of the azobenzene group was initiated using the xenon lamp (75 W) of a fluorimeter (QuantaMaster, Photon Technology International Inc.) with slit widths of 350 and 450 nm providing a power density of 500 $\mu W/cm^2$ at the sample. The samples were constantly stirred and equilibrated at 25 °C throughout the irradiation. UV-Vis measurements were carried out using a CaryWin spectrometer. Typically, the $AzoC_{10}N^+$ concentration in the samples was adjusted to ~0.05 mM. The conversion from *trans* to *cis* isomers was induced using light at 350 nm, a wavelength at which the molar absorptivity coefficient of the *trans* isomer is much larger than that of the *cis* isomer. Photo-isomerization from *cis* to *trans* was

effected using light at 450 nm. The proportion of $AzoC_{10}N^+$ in the *trans* form at time *t* ($AzoC_{10}N^+{}_{trans}$) was determined using **Equation 1**:

$$AzoC_{10}N^+{}_{trans} = \left(\frac{A(t)-A_c}{A_t-A_c}\right) \times 100 \quad (1)$$

where *A(t)* is the absorbance at 350 nm at time *t* and A_t and A_c are the absorbances of the pure *trans* and *cis* forms, respectively, at 350 nm. It was assumed that there were only *trans* isomers when the absorbance at 350 nm was constant for samples kept in the dark and only *cis* isomers when illumination at 350 nm reached a constant value. Measurements were carried out in at least triplicate. The percentages and uncertainties reported are the average values and the standard deviations, respectively.

The isomerization kinetics were fitted according to an exponential process. The *trans* to *cis* and *cis* to *trans* isomerizations are described by **Equations 2a** and **2b**, respectively.

$$AzoC_{10}N^+{}_{trans} = AzoC_{10}N^{+0}{}_{trans} \cdot e^{-t/\tau} \quad (2a)$$

$$AzoC_{10}N^+{}_{trans} = AzoC_{10}N^{+0}{}_{trans} + \left[\left(1 - e^{-t/\tau}\right)\left(AzoC_{10}N^{+\infty}{}_{trans} - AzoC_{10}N^{+0}{}_{trans}\right)\right] \quad (2b)$$

where $AzoC_{10}N^{+0}{}_{trans}$ and $AzoC_{10}N^{+\infty}{}_{trans}$ are the percentages of the $AzoC_{10}N^+$ *trans* isomer at $t = 0$ and ∞, respectively, and τ is the characteristic time of the exponential isomerization process.

For the release experiments, SRB, a fluorescent probe, was added to the buffer used for lipid hydration: 10 mg of lipid mixtures were hydrated in 300 µL of a MES/TRIS buffer containing 50 mM SRB (70 mM NaCl, 50 mM MES, 50 mM TRIS, 0.5 mM EDTA), referred to as the internal buffer. At this concentration, SRB fluorescence is essentially self-quenched.[30] After the extrusion of the LUVs, free SRB was separated from the probe-loaded LUVs by gel exclusion chromatography using a column (diameter, 1.5 cm; length, 25 cm) filled with Sephadex G-50 medium (Pharmacia Biotech, Uppsala, Sweden) equilibrated with an iso-osmotic MES/TRIS buffer; this external buffer was composed of 140 mM NaCl, 50 mM MES, 50 mM TRIS and 0.5 mM EDTA. The pH of both the internal and external buffers

was set to 7.4 for the $AzoC_{10}N^+$/Schol system and to 7.0 for the control PA/Schol LUVs. This exclusion chromatography step also ensured that $AzoC_{10}N^+$ was completely inserted in the collected LUVs.

Fluorescence measurements were carried out using a Photon Technology International QuantaMaster fluorimeter. LUV suspensions were diluted with the external buffer to obtain a lipid concentration of ~70 μM. The excitation and fluorescence wavelengths of SRB were 567 and 583 nm, respectively. The bandwidths of the excitation and emission monochromators were set to 1.5 and 2 nm, respectively. The percentage of SRB release was calculated according to **Equation 3**:

$$\%\ of\ release = 100 - \left(\frac{((I_T - I_F)/I_T)}{((I_{T,0} - I_{b,0})/I_{T,0})}\right) \times 100 \qquad (3)$$

where $I_{b,0}$ and $I_{T,0}$ are the fluorescence intensities measured from the collected SRB-loaded LUVs immediately after the gel exclusion chromatography step, before and after the addition of Triton X-100 (final concentration ~0.1 vol%), respectively. Triton X-100 led to the complete release of the probe and the resulting fluorescence intensity was used to normalize the release curves. I_F and I_T are the SRB fluorescence intensities measured at a given time t, before and after addition of Triton X-100, respectively. The experiments were carried out in independent triplicates. The reported release percentages and their uncertainties correspond to the average values and the standard deviations, respectively.

3. Results and Discussion

The first step was to establish that the $AzoC_{10}N^+$/Schol (25/75, mol/mol) mixture forms fluid bilayers. To this end, we examined if LUVs can be produced. We also determined if the putative LUVs can encapsulate SRB, a hydrophilic fluorophore, when the lipid mixture was hydrated with a buffer containing SRB and the resulting suspension was extruded. Dynamic light scattering measurements of this preparation indicated that LUVs were formed. Their size distribution was unimodal with an average diameter of 140 nm. In addition, SRB fluorescence measurements showed that the fluorophore was encapsulated in the LUVs with a self-quenching efficiency of about 90%, a value consistent with trapped SRB at a

concentration of 50 mM. From these results, it is concluded that the $AzoC_{10}N^+$/Schol (25/75, mol/mol) mixture behaves similarly to other mixtures of a long acyl chain ammonium and a sterol,[27-28] and that it also forms fluid bilayers that can be extruded to provide LUVs.

The variations of the UV-Vis spectrum associated with the isomerization of $AzoC_{10}N^+$ in chloroform solution and of the bilayer-inserted $AzoC_{10}N^+$ are presented in **Figure 2**. Prior to irradiation, the spectrum of $AzoC_{10}N^+$ in organic solution was dominated by a peak at 348 nm accompanied by a weak component at 440 nm (Figure 2A). The most intense band corresponds to the $\pi \rightarrow \pi^*$ transition of the azobenzene *trans* form, whereas the weak component is assigned to its $n \rightarrow \pi^*$ transition.[29, 31-34] When the sample was irradiated at 350 nm, the intensity of the band at 348 nm decreased, whereas the intensity of two weak bands located at 315 and 440 nm increased. These two bands correspond to the $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions, respectively, of the *cis* isomer.[9] A similar pattern was observed for the bilayer-inserted $AzoC_{10}N^+$ (Figure 2B). The intense band for the $\pi \rightarrow \pi^*$ transition of the *trans* form was slightly blue-shifted to 330 nm. This observation has been related to the less polar environment of the azobenzene group when inserted in the hydrophobic core of the bilayers compared to when it is solubilized in chloroform.[35] It has also been attributed to weak H-aggregation[13] or dimer-like formation[36] of the azobenzene chromophores. Therefore, this shift is a strong indication that $AzoC_{10}N^+$ was inserted in the self-assembled structures formed in the presence of Schol.

Upon illumination at 350 nm, the intensity of the band associated with the azobenzene *trans* isomer decreased, whereas that of the *cis* isomer bands increased. The spectra of free and bilayer-inserted $AzoC_{10}N^+$ before any irradiation (corresponding to the *trans* form) and at the end of the 350-nm irradiation period ($t = \infty$, corresponding to the *cis* form) (**Figure** 2) were fitted using two Gaussian functions that were representative of the two isomer bands.

The spectra were fitted between 300 and 377 nm and between 276 and 377 nm for the $CHCl_3$-solubilized and the bilayer-inserted $AzoC_{10}N^+$, respectively. Prior to illumination, the spectra could be essentially reproduced by the high-wavelength component associated with the *trans* form, the low-wavelength component contributing less than 10 % of the total area. Analogously, at $t = \infty$, the spectra could be simulated by the low-wavelength band representing the *cis* form, the band associated with the *trans* form representing less than 5 % of the total area. These results suggest that, in both conditions, a single isomer is predominant. The presence of clearly defined isosbestic points (λ = 410 nm for the solubilized $AzoC_{10}N^+$, λ = 300 and 397 nm for the bilayer-inserted form) demonstrates the transition between the *trans* and *cis* forms, without the accumulation of significant quantities of any intermediate form.

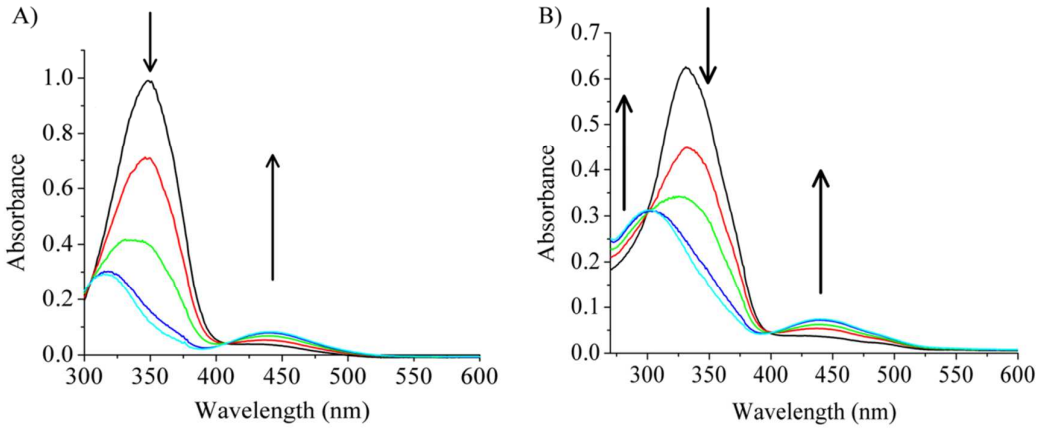

Figure 2. UV-Vis spectra of $AzoC_{10}N^+$ recorded during the *trans-cis* photo-isomerization process. Black lines are the spectra obtained at $t = 0$ (before irradiation). Turquoise lines are the spectra obtained at $t = \infty$ (at the end of the irradiation process, i.e. when no further evolution of the spectrum was observed). The arrows show the intensity change directions upon irradiation at 350 nm. A) $AzoC_{10}N^+$ solubilized in $CHCl_3$, B) $AzoC_{10}N^+$ inserted in bilayers formed with Schol.

The kinetics of the *trans* to *cis* conversion, photo-induced by irradiating at 350 nm, is presented in **Figure 3**. It is clearly observed that the *trans* to *cis* conversion is more rapid for the single-chain azobenzene derivative solubilized in $CHCl_3$ compared to when it is inserted in Schol bilayers. The variation of the absorbance as a function of illumination time can be reproduced well using single-exponential functions. The extracted characteristic times, presented in Table 1, indicate that the photo-conversion of $AzoC_{10}N^+$ is about 3 times slower in the bilayer environment than in organic solution. This phenomenon can be partly associated with the fact that 350 nm corresponds more closely to the maximum of the absorption band of the *trans* form for the compound solubilized in $CHCl_3$ compared to that inserted in bilayers. The tight chain packing existing in the bilayers[28] can also inhibit the isomerization process, as has been reported for similar azobenzene derivatives inserted in bilayers.[10, 16, 37] The free volume accessible to $AzoC_{10}N^+$ when inserted in bilayers is expected to be considerably more limited than that of the form solubilized in organic solution. It was previously reported that the mobility of the chromophore is crucial for efficient isomerization.[9] It is therefore a positive step that AzoC10N$^+$ inserted in the highly ordered bilayers prepared with Schol could undergo a *trans-cis* isomerization at an acceptable rate.

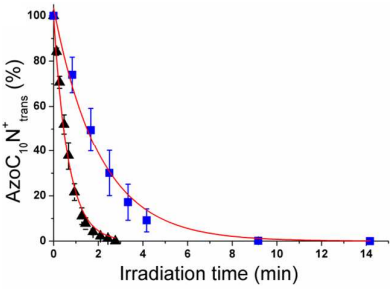

Figure 3. Kinetics of the *trans-cis* photo-isomerization, induced by irradiation at 350 nm, of $AzoC_{10}N^+$ solubilized in $CHCl_3$ (▲) and bilayer-inserted $AzoC_{10}N^+$ (■). The red lines

represent the fitted exponential functions.

Table 1. Characteristic times, τ, for the *trans-cis* photo-isomerization, *cis-trans* photo-isomerization, and *cis-trans* thermal relaxation of $CHCl_3$-solubilized and bilayer-inserted $AzoC_{10}N^+$.

	$AzoC_{10}N^+$ in $CHCl_3$	$AzoC_{10}N^+$/Schol
Trans-cis photo-isomerization (min)	0.65 ± 0.02	2.1 ± 0.1
Cis-trans photo-isomerization (min)	2.3 ± 0.1	3.0 ± 0.1
Cis-trans thermal relaxation (days)	3.3 ± 0.1	1.3 ± 0.1

The kinetics of the *cis-trans* isomerization induced by irradiation at 450 nm was also examined (**Figure 4A**), and was found to be a little slower than the kinetics observed for *trans-cis* photo-isomerization (**Table 1**). A stationary state was reached after ~10 min of illumination. Furthermore, the *cis-trans* isomerization was slightly slower for bilayer-inserted $AzoC_{10}N^+$ compared to the free form (3.0 vs. 2.3 min), which can be attributed to the structured environment of the apolar core of the bilayers and/or to different absorption efficiencies of the azobenzene moiety in different environments.

At the stationary phase reached after the 10-min illumination period, about 70 % of the initial *trans* $AzoC_{10}N^+$ content was observed for $AzoC_{10}N^+$ solubilized in $CHCl_3$, whereas it was about 90 % for the bilayer-inserted $AzoC_{10}N^+$. This indicates that the photo-isomerization

process was not completely reversible, a phenomenon that has also been observed for other azobenzene systems.[31, 38] This might be due to the proximity of the absorption bands associated with each isomer that may result in a steady-state photo-exchange between the two isomers. On the basis of this rationale, the greater recovery of the *trans* isomer for bilayer-inserted $AzoC_{10}N^+$ compared to the $CHCl_3$-solubilized $AzoC_{10}N^+$ is consistent with its increased energy separation between the $\pi \rightarrow \pi^*$ band maximum and the light wavelength used to induce the *cis-trans* isomerization (450 nm).

Figure 4B shows the thermal relaxation of the system, leading to the recovery of the *trans* isomers when the samples with the photo-induced *cis* form were kept in the dark. The kinetics, on the order of days (Table 1), are clearly considerably slower than those observed for photo-isomerization at 450 nm. These results demonstrate that the *cis* $AzoC_{10}N^+$ resulting from 350-nm irradiation remained stable for several hours, a feature that was subsequently exploited in the photo-controlled release of the payload as discussed below.

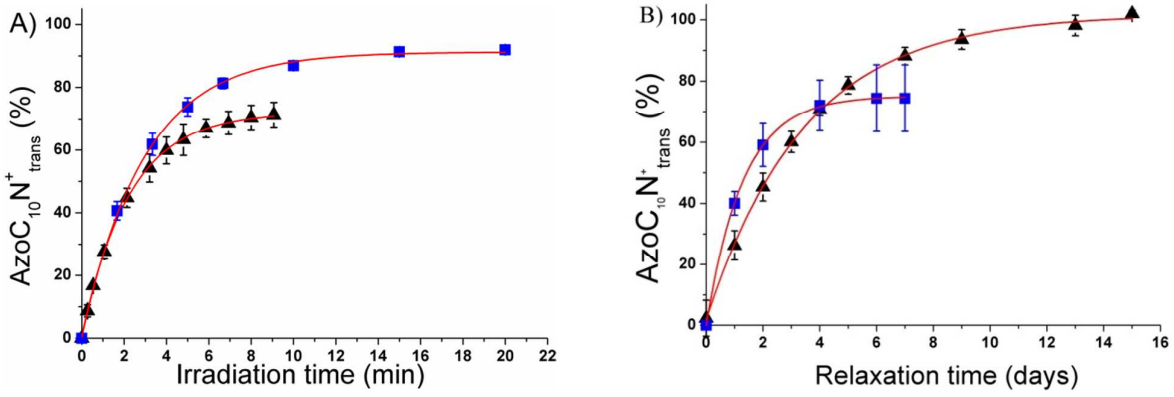

Figure 4. Kinetics of the *cis-trans* isomerization of $AzoC_{10}N^+$ solubilized in $CHCl_3$ (▲) and bilayer-inserted $AzoC_{10}N^+$ (■): A) photo-induced by illumination at 450 nm and B) induced by thermal relaxation. The red lines represent the fitted exponential functions.

Figure 5 illustrates that it is possible to cycle the isomerization process for $AzoC_{10}N^+$. In the case of $AzoC_{10}N^+$ solubilized in $CHCl_3$, the sample was irradiated for 3 min at 350 nm to induce the *trans-cis* isomerization, the proportion of remaining *trans* form was measured, the reverse process was achieved by a 10-min irradiation at 450 nm, and the proportion of *trans* form was measured again. This cycle was repeated three times. A similar cycling protocol was used for bilayer-inserted $AzoC_{10}N^+$ except that the illumination at 350 nm was for 5 min. The results show that it is possible to photocycle between the *trans* and *cis* conformers of $AzoC_{10}N^+$ for both systems. A similar reversibility was reported for a monoacylated azobenzene derivative inserted in dihexadecyl phosphate micelles.[39] It was possible to photo-convert the *trans* to the *cis* isomer almost completely, whereas the levels of photo-conversion from *cis* to *trans* form, which is constant after the first cycle, is consistent with the photo-kinetics displayed in Figure 2, supporting the hypothesis of steady-state photo-conversion between the two isomers. It should also be noted that our results indicate that illumination during several consecutive short periods led to an extent of conversion that is very similar to that resulting from continuous illumination (for example, in Figure 3, the 3-min illumination leading to the *trans-cis* photo-isomerization was divided into 15-s periods, separated by the recording of the UV-Vis spectrum, whereas it was continuous for each period in the experiments in Figure 5). This observation is associated with the slow *cis-trans* thermal relaxation described above, as well as the photo-stability of the azobenzene derivative under these illumination conditions.

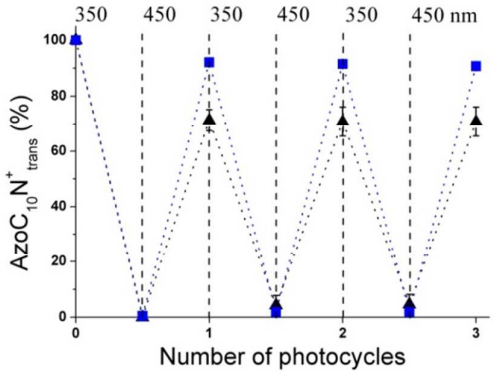

Figure 5. Photo-cycling of the *cis-trans* isomerization of $CHCl_3$-solubilized $AzoC_{10}N^+$ (▲) and bilayer-inserted $AzoC_{10}N^+$ (■). One cycle included illumination at 350 nm to generate the *cis* isomer (3 min for $CHCl_3$-solubilized and 5 min for bilayer-inserted $AzoC_{10}N^+$), followed by 10-min irradiation at 450 nm to generate the *trans* form.

With the isomerization behavior of the bilayer-inserted azobenzene characterized, we proceeded to examine the possibility of triggering the release of entrapped payload from the $AzoC_{10}N^+$/Schol LUVs by photo-conversion of the azobenzene group. **Figure 6A** displays the extent of SRB release from the LUVs upon the irradiation at 350 nm, with $t = 0$ corresponding to the beginning of illumination. The samples were illuminated for 30-s periods and the level of material release was measured between these periods. No release of the probe was observed during the first 60 s. After this time lag, a progressive release was measured and ~75 % of the SRB had leaked out from the LUVs after 5 min. It was inferred, on the basis of the isomerization kinetics, that a threshold amount of azobenzene groups should be in the *cis* form in order to initiate leakage. In our system, ~40% of the azobenzene groups are in the *cis* form after a 60-s illumination period. The conclusion that the release observed for $AzoC_{10}N^+$/Schol LUVs is due to the presence of the inserted azobenzene derivative and is

effectively photo-triggered was validated using PA/Schol LUVs as a negative control; in this control, the photosensitive Azo amphiphile was replaced by a linear saturated fatty acid. As expected, 350-nm irradiation of PA/Schol did not lead to any significant payload release (Figure 6A).

Time lags for photo-induced leakage were also observed for other azobenzene derivatives inserted in phospholipid liposomes.[10, 14, 40] To rationalize these lags between the isomerization and the release, it was proposed that the bulky *cis* isomers need to rearrange in the plane of the bilayers in order to create leaky defects.[14, 40] Laser-induced photo-isomerization and release studies of azobenzene-containing liposomes[14, 40] showed that, despite very rapid *trans*-*cis* isomerization due to a high laser light intensity, the release of the entrapped payload displayed a time lag. It was inferred that the release was not associated with the isomerization process per se, but with the lateral reorganization of the bulky *cis* azobenzene conformers in the liposome walls to create a defect.

It was also established previously that the *trans*-*cis* isomerization in phospholipid liposomes more generally induced leakage of the entrapped payload in gel-phase bilayers. It was concluded that the fluid bilayers prepared from phospholipids, whose chain packing is relatively loose, can accommodate the bent *cis* isomers in such a way that bilayer permeability is preserved and no release is observed despite the photoisomerization of the Azo group.[14, 40] Likewise, it was reported recently that gel-phase domains or a large fraction of liquid-ordered domains were required for *cis* conformers to induce the bursting of Giant Unilamellar Vesicles (GUVs).[11] In the present case, the single-chain amphiphile/sterol bilayers formed display very high orientational order in the apolar core, which is a consequence of their high sterol content,[22, 26] but, nevertheless, the bilayers remain fluid, as exemplified by the fast rotational motion (relative to the ^{2}H-NMR timescale) of the monoacylated amphiphile and of

the sterol.[22, 27-28, 41] The tight chain packing is probably essential for the enhancement of liposome permeability by the *cis* conformers reported here. Thus, the LUVs presented in the current work represent a class of liposomes that combine fluidity and photosensitivity.

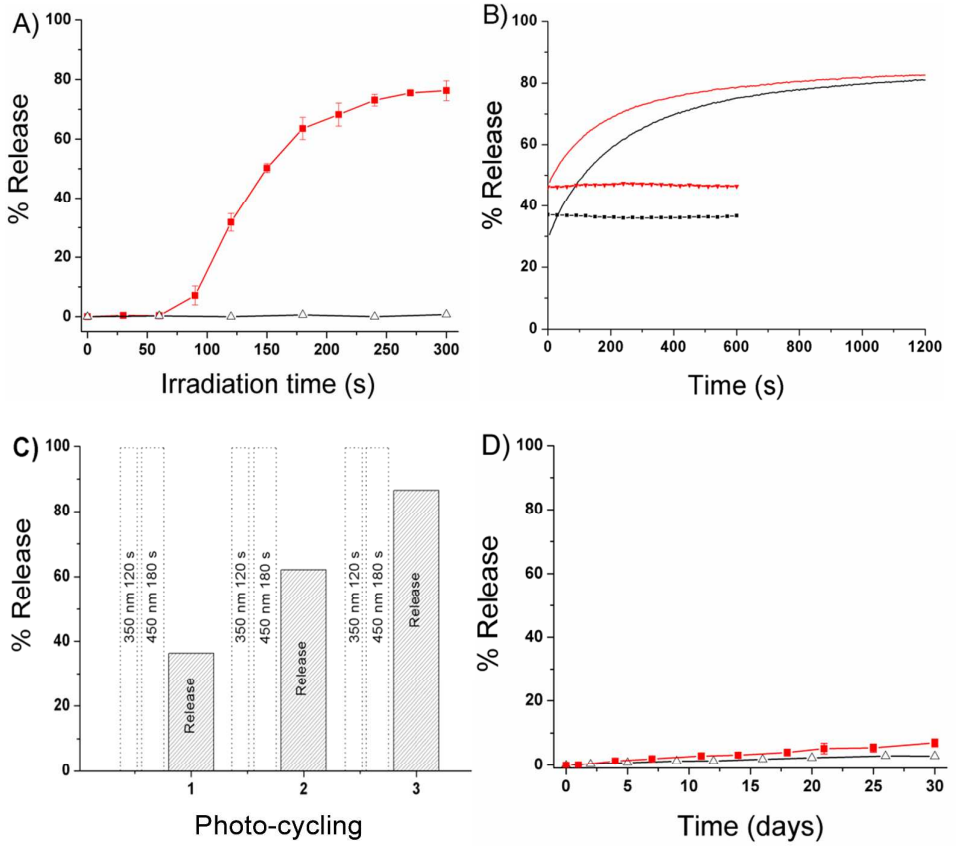

Figure 6. A) Photo-induced SRB release from $AzoC_{10}N^+$/Schol (■) and PA/Schol (Δ) LUVs by 350-nm irradiation. B) SRB release from $AzoC_{10}N^+$/Schol LUVs induced by 350-nm irradiation for 120 s (–) and for 150 s (–); SRB release induced by 350-nm irradiation for 120 s (■–) and 150 s (▼–), followed by 450-nm irradiation for 180 s (the time was set to 0 at the end of this two-step irradiation process). C) SRB release from $AzoC_{10}N^+$/Schol LUVs after 350–450 nm photo-cycling for 120–180 s. D) Passive SRB release from $AzoC_{10}N^+$/Schol (■) and PA/Schol (Δ) LUVs.

In a second series of experiments, we examined the impact of the presence of *cis* azobenzene conformers on the bilayer permeability. First, we continuously illuminated the $AzoC_{10}N^+$/Schol LUVs at 350 nm for 120 or 150 s, and then measured the SRB release as a function of time (Figure 6B). The initial release levels, measured immediately after the illumination period, corresponded to those obtained when the samples were illuminated in a quasi-continuous manner (Figure 6A). Despite the fact that the samples were no longer illuminated at 350 nm, the SRB release continued in a fashion similar to that obtained with continuous 350-nm illumination. These results indicate that the leaks introduced by the *trans-cis* photo-conversion remained, even after illumination was stopped. This finding is consistent with the fact that thermal relaxation to the *trans* form is much slower than the time scale of the release measurements (days versus seconds). Therefore, once "static" leaky structures associated with the *cis* isomer were formed, the release of the material could be observed over the 20-min period. A similar observation was made for the permeability of K^+ from phospholipid liposomes containing an azobenzene derivative, where, after turning off the light causing *trans-cis* isomerization, the K^+ release from the liposomes continued at the same rate as before.[10]

We showed above that it was possible to restore the *trans* form rapidly by illumination at 450 nm. With this in mind, we examined the possibility of stopping payload release by restoring the *trans* isomers. The experiment consisted of continuously illuminating the $AzoC_{10}N^+$/Schol LUVs at 350 nm for 120 or for 150 s (to form the *cis* conformer), followed by illumination at 450 nm for 180 s (to reform the *trans* conformer), and then measuring the SRB release as a function of time (Figure 6B). The initial release levels were typical of those obtained for similar illumination periods at 350 nm. However, with this illumination protocol, further release was completely arrested, which can only be a result of the reformation of tight

vesicle walls with *trans* azobenzene conformers. Therefore, it can be concluded that the photo-control of the azobenzene conformation in the LUVs is an efficient approach for switching on as well as for turning off the delivery of the encapsulated material.

In addition, we successfully achieved precisely controlled release of the encapsulated SRB by exploiting photo-cycling. During a cycle, the $AzoC_{10}N^+$/Schol LUVs were irradiated first at 350 nm for 120 s and then at 450 nm for 180 s, after which the probe release was measured for a 300-s period. Figure 6C shows that this allows the release of a specific amount of the material (determined by the duration of the illumination period at 350 nm, after which it is stopped by photo-induced restoration of the *trans* azobenzene form due to illumination at 450 nm). Moreover, the delivery of the material can be performed in multidose format by applying several cycles, as illustrated in Figure 6C, which shows 3 distinct amounts of SRB release from the LUVs.

It should be pointed out that the $AzoC_{10}N^+$/Schol LUVs are very efficient nanocontainers, since only ~7% of the encapsulated SRB had leaked out passively after 30 days (Figure 6D). This impressive impermeability is due to the high content of cholesterol sulfate in the bilayer: this sterol has a strong ability to order neighboring acyl chains[22, 28] and, consequently, to reduce the passive permeability of bilayers. A similar limited permeability was reported for analogous non-phospholipid liposomes prepared from a single-chain amphiphile and a sterol.[20, 41] The restricted passive release compares advantageously with that of other photo-sensitive formulations. For example, photo-sensitive liposomes of dimyristoyl phosphatidylcholine/dicetyl phosphate/4-octyl-4'-(5-carboxypentamethy1eneoxy)azobenzene (DMPC/DCP/8A5) showed 10% leakage over 20 h,[10] whereas those prepared with egg phosphatidylcholine and cholesterol onto which an azobenzene moiety was grafted displayed passive release between 10 and 30% over 40 hours.[16, 42] Because of their very limited passive

permeability, the on-and-off release provided by $AzoC_{10}N^+$/Schol LUVs is sharper than those previously reported with phospholipid-based Azo-containing LUVs for which residual leakage could be observed after the illumination restoring the trans Azo conformers.[10, 16]

4. Conclusion

We demonstrated that it is possible to prepare fluid-phase liposomes from $AzoC_{10}N^+$ and Schol that combine very low passive permeability and photo-control of the entrapped payload. The high proportion of sterol in the bilayers, which is similar to that reported for other non-phospholipid liposomes prepared from monoacylated amphiphiles and sterols,[19-22, 27-28, 43] leads to very limited permeability. The presence of the azobenzene derivative makes these LUVs light sensitive and allows precise control of the release of the encapsulated material. It thus appears that the *trans* form of the azobenzene is compatible with the molecular packing of the bilayer, giving impermeable membranes. On the other hand, the *cis* form introduces defects in these fluid bilayers because of the tightly packed alkyl chains, thereby allowing the photo-induced leakage of the encapsulated material. Because the azobenzene conformer can be photo-selected by illumination with the proper energy, it is possible to begin as well as to arrest the release of the entrapped solutes by photo-inducing the *cis* and the *trans* azobenzene conformers, respectively. Therefore, it is feasible to deliver, at will, a defined quantity of solute from these liposomes. Furthermore, multidose release can be controlled in a straightforward manner by photo-cycling between the *cis* (to initiate the release) and the *trans* (to stop the release) azobenzene isomers. Passive release was found to be minimal. These distinct characteristics make $AzoC_{10}N^+$/Schol LUVs a very versatile type of photo-sensitive nanocontainer.

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