Photo-Sensitive Liposomes: Chemistry and Application in Drug Delivery

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Abstract: Photo-sensitive liposomes have been studied for a few decades and various photo-sensitive triggers have been developed so far. This review offers an overview of the different photo-triggering mechanisms for controlled pulsatile content release from liposomes, which have the potential of finding clinical applications as intelligent drug delivery systems.

Keywords: Liposomes, photo-sensitive, pulsatile, azobenzene, cholesterol.

INTRODUCTION

The single biggest challenge now facing drug delivery (for liposomes and indeed other carriers) is to initiate and produce release of the encapsulated drug only at the diseased site, at needed time, and at controllable rates. Liposomes have been considered as one of the most safety drug carrier system since their discovery in the early 1960s by Bangham and co-workers [1]. They still have stimulated ongoing interest in applications in drug delivery and controlled release. This is because liposomes with diameters approximately of 100 nm can be delivered to tumor tissues at high concentrations (passive targeting) and liposomes can encapsulate a wide range of drugs [2, 3]. Several systems employing pH, thermal, ultrasonic and photochemical triggering to achieve controllable release have been studied and reviewed in detail [4-8]. During which, photo-triggering offers an attractive alternative to the temperature or pH-driven modulation of drug release, as it provides a broad range of adjustable parameters (e.g., wavelength, duration, intensity) that can be optimized to suit a given application. The process results in the disruption of the lamellar integrity, liposome fusion, and eventually fast release of the encapsulated contents in the liposome. However, comparing to the pH-sensitive and thermal-sensitive liposomal systems, there are only limited photo triggering systems reported and the review paper focused on the literature appearing between January 1996–June 2001 [8]. Our laboratory have been working for over 5 years concerning the design and performance of the azobenzene-based drug delivery systems, which was found to be less spontaneous drug release, more controllable in both releasing rate and releasing amount in combination with light irradiation [9, 10].

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This review offers an overview of the different photo-triggering mechanisms for controlled content release from liposomes, not only including the reversible change in shape inducible in some molecules by the adsorption of light, but also the photo-chemical change in structure of some molecules.

PHOTO-SENSITIVE LIPOSOMES BASED ON PHOTO-INITIATED IRREVERSIBLE CHEMICAL REACTIONS.

Photo-Polymerization

Conjugated dienes and their derivatives contain double bonds separated by one single bond. These compounds can be easily polymerized under UV irradiation [11]. In 1992, Lamparski and O'Brien reported an interesting liposome composed of a 3:1 molar mixture of dioleoylphosphatidylethanolamine (DOPE) and 1,2-*bis*[10-(2`-hexadienoyloxy)decanoyl]-*sn*-glycero-3-phosphatidylcholine (*bis*-SorbPC, Scheme **1**) [12]. Upon UV light irradiation, *bis*-SorbPC was polymerized, and formed a cross-linked poly-SorbPC network. This process separated the liposome bilayer into polymeric and monomeric domains, which resulted in the disruption of the lamellar integrity, liposome fusion, and eventually fast release of the encapsulated contents in the liposomes (50% of the contents were released within a few minutes). O'Brien's studies also showed that the rate and extent of liposome fusion is dependent on the extent of photo-polymerization, the molar lipid ratio of *bis*-SorbPC, temperature, pH, and the presence of Mg^{2+} ions, and that the fusion was mediated *via* intermediates that associated with the lamellar to Q_{II} phase transition of the liposomal membrane [12, 13]. Further study showed that the fusion rates and extents of DOPE/mono-SorbPC liposomes are significantly smaller than that of DOPE/*bis*-SorbPC liposomes. This is partially because the photoactivated polymerization of *mono*-SorbPC produces only short linear oligomers of 3-10 repeat units, which can not necessarily produce a dramatic phase separation and the following fusion of the liposomes [12].

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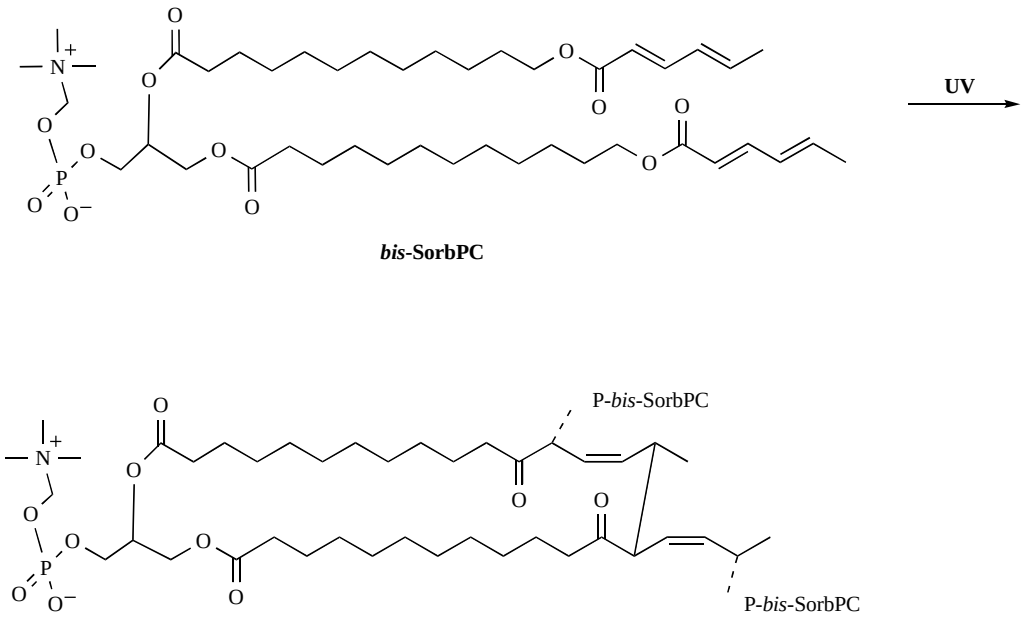


Scheme 1. Photo-polymerization of SorbPC.

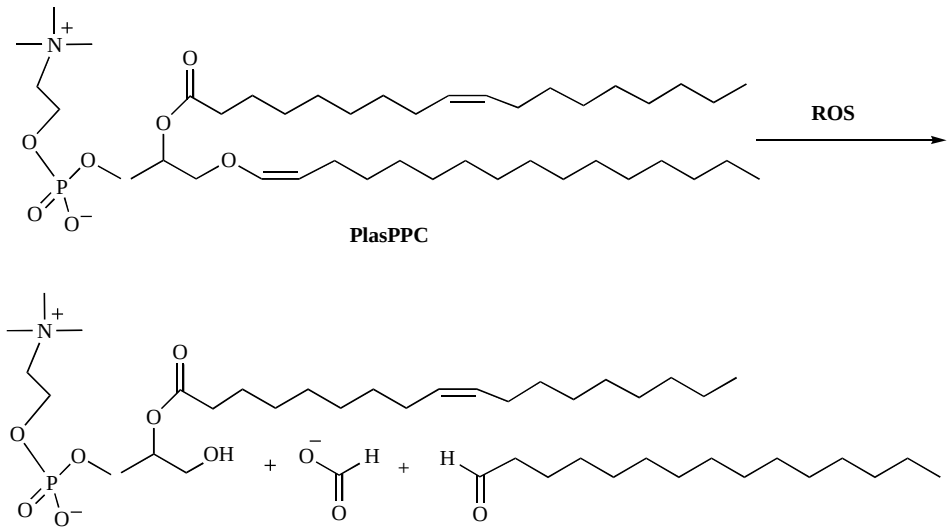
These initial studies opened the possibility of using photo-induced fusion of liposomes to trigger drug release. Taking advantage of the exceptional long circulation of polyethylene glycol-liposomes (PEG-liposomes, i.e., stealth liposomes), which have been found to accumulate preferentially in the interstitium of tumor sites because of the increased permeability of the vasculature at those sites, O'Brien *et al*. further optimized DOPE/*bis*-SorbPC photo-sensitive liposomes by incorporating PEG2000-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (PEG2000-DSPE) [14]. The liposomes were still very sensitive to UV irradiation; 2 minutes UV irradiation promoted a 90% loss of monomer and a nearly 60-fold increase in the rate of 1-amino-3,6,8-naphthalenetrisulfonic acid (ANTS) release from the liposomes [14-16]. Interestingly, the liposomal content release can even be triggered by visible light after incorporation of a green light absorbing dye [distearoyl indocarbocyanine, DiIC(18)3] into the bilayer of DOPC/*bis*-SorbPC/PEG-DOPE liposomes [14]. These observations demonstrate the possibility of using the available light source suitable for photodynamic therapy (PDT) to trigger content release from appropriately designed photo-liposomes.

Photo-Oxidation

Plasmalogens are a unique class of glycerophospholipids found in all mammalian cells. These compounds are characterized by the presence of a vinyl ether substituent at the *sn*-1 position of the glycerol backbone [17]. In 1988, Zoeller *et al*. found that animal cell mutants lacking plasmalogens are hypersensitive to UV irradiation; it seems that plasmalogens play a direct role in protecting animal cells from UV-initiated singlet oxygen [17]. The follow-up study showed that this protective process is through the cycloaddition of toxic singlet oxygen to the vinyl ether linkage of plasmalogen, followed by thermal decomposition of the resulting dioxetane intermediate. An additional mechanism is the formation of a 1'-allylic hydroperoxide derivative of the vinyl ether moiety by an "ene" reaction with singlet oxygen, or by radical-mediated oxidation, followed by metabolism or chemical decomposition of the hydroperoxide [18]. The photo-chemical reaction cleaves the plasmalogen lipids into single-chain surfactants, which possibly leads to the phase change of biomembranes.

Based on the photo-protective effects of plasmalogen in cells, Thompson's group in Purdue University developed a new type of photo-sensitive liposomes [8, 19, 20] (Scheme **2**). The liposomes consisted of three major functional components: (1) small unilamellar plasmalogen vesicles (SUVs) containing 1-alk-1'-enyl-2-palmitoyl-sn-glycero-3-phosphocholine (PlasPPC)/1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (8:1, mol/mol), (2) oxygen, and (3) a photo-sensitizer such as zinc phthalocyanine, tin octabutoxyphthalocyanine, or bacteriochlorophyll *a*, which was incorporated within the hydrophobic region of the SUV bilayers. The photo-sensitizers and oxygen were used to produce reactive oxygen species (ROS) by irradiation with 630-820 nm light. This wavelength regime was chosen for sensitization of the liposome because of its deep penetration property in many tissues [21]. Upon irradiation, the encapsulated contents, glucose or calcein, were rapidly released from the liposomes. For example, 100% encapsulated calcein was released in less than 20 min when bacteriochlorophyll *a* was incorporated into the liposomes as the photo-sensitizer.

Thompson's research results showed that the photo-induced release mechanism includes several steps: (1) lipid photo-degradation produces single chain surfactant species such as lysolipid and fatty aldehyde; (2) lateral diffusion of the degradation products within the liposome and inter-liposome leads to the accumulation of lysolipid/fatty aldehyde-rich domains and the formation of single chain surfactant stabilized membrane defects (pores and interlipidic particle formation); (3) the membrane defect formation facilitates the aggregation and fusion of photolyzed liposomes

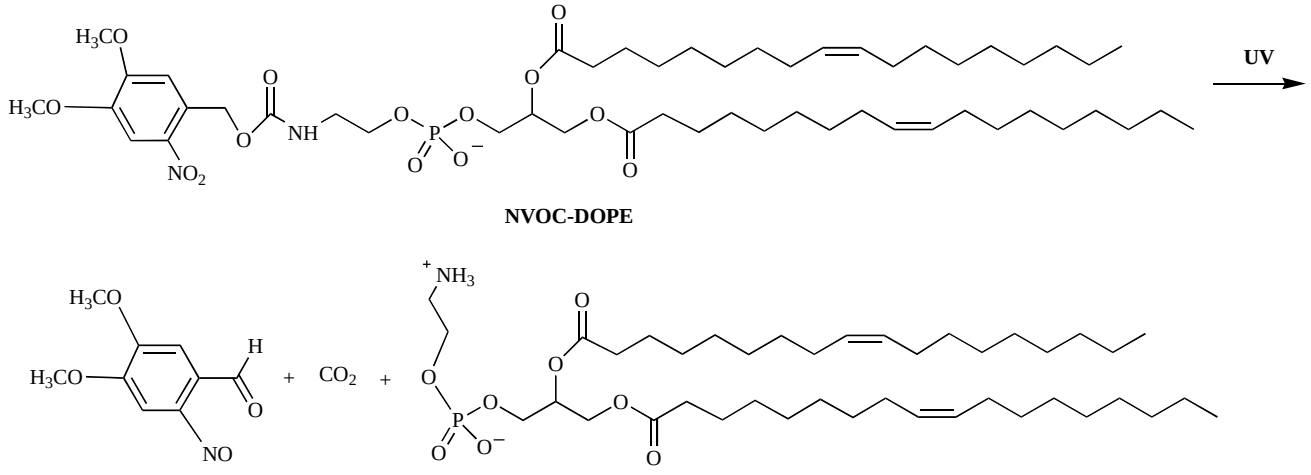


Scheme 2. Photo-oxidation of plasmalogen.

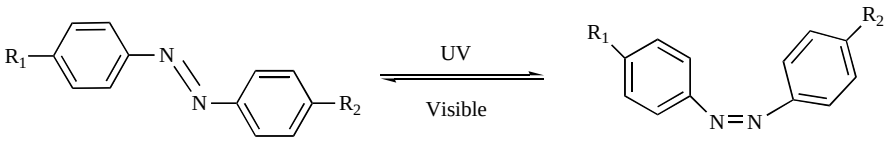
[20]. All of these steps account for the rapid content release from liposomes, and the content release rates depend on sensitizer localization, sensitizer concentration, temperature, total lipid concentration, light fluency, and the hydrophilic/hydrophobic balance of the encapsulated contents. To further expand this concept, Thompson developed a novel cascade-type triggering technique by utilizing the content released from the first liposome (photo-sensitive) to activate an enzyme, which is capable of destabilizing the second conventional liposome to allow rapid content release [22]. The photo-sensitive liposome was composed of synthetic diplasmalogen (1,2-dihexadec-1`-enyl-*sn*-glycero-3-phosphocholine, DPPlsCho), dihydrocholesterol (DHC), DPPE-PEG5000, and bacteriochlorophyll (BChl) with encapsulated Ca^{2+} as a signaling agent for phospholipase A_2 (PLA_2); the conventional liposomes were composed of 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine (DPPC) with encapsulated calcein. This cascade triggering approach has the potential to be used to activate a wide variety of Ca^{2+}-mediated or Ca^{2+}-dependent processes, and to assemble completely autonomous vesicular photo-sensitive drug delivery systems for the treatment of diseases by encapsulation of small Ca^{2+}-containing photo-sensitive liposomes within larger PLA_2- and drug-containing conventional liposomes.

Photo-Deprotection

Phosphatidylethanolamines (PE) are nonbilayer-forming lipids, which prefer inverse cubic and inverse hexagonal structures at physiological pH and have the function of activating bilayer fusion [23, 24]. However, N-acylated PEs can form kinetically stable liposomes [25]. In organic synthesis, nitroveratryloxycarbonyl can be used to acylate the amino group of PE for protective purposes, which can be cleaved by a photochemical approach. Taking advantage of this unique feature, Zhang and Smith synthesized a caged, photocleavable 6-nitroveratryloxycarbonylated 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (NVOC-DOPE, Scheme **3**) [26]. This compound itself, or with egg phosphatidylethanolamine (EPE), can form stable liposomes. Upon UV irradiation, NVOC-DOPE is rapidly converted to DOPE and 3,4-dimethoxy-2-nitrosobenzaldehyde byproduct. Increasing the concentration of DOPE in the liposomes induces fusion



Scheme 3. Photo-deprotection of NVOC-DOPE.



Scheme 4. Photoisomerization of azobenzene and possible photo-controlled release mechanism of liposomes.

and/or aggregation of the liposome, which finally leads to rapid release of encapsulated calcein. Zhang's results showed that a 20 min irradiation with a 150 W Xe lamp was needed to convert 90% of the NVOC-DOPE to DOPE, and that the calcein release rate can be controlled from 0.65% to 2.0% of the encapsulated calcein per minute in a linear fashion (depending on temperature, pH, and lipid concentration). The caged liposomes are likely to be useful in intelligent drug delivery, but the safety profile of the cleaved byproduct should be evaluated when this photo-deprotection strategy is considered for drug delivery.

Through continuous efforts in development, most of these photo-sensitive liposomes are characterized by rapid and transient release of encapsulated molecules within a short time-period immediately after photo irradiation [8]. These systems may significantly increase drug concentration in the disease site, and have the potential to increase the therapeutic efficacies of the encapsulated drugs. However, once the chemical reaction is initiated by photo irradiation, the encapsulated therapeutic agents will release rapidly from the liposomes, and there is no stopping mechanism because of the irreversible nature of photo-chemical reaction. This may cause drastic systemic or local toxicity of encapsulated toxic drugs in a clinical situation. Photo-sensitive liposomes with more controllable releasing mechanisms are desperately needed to optimize/control the drug release rate from liposomes to decrease/avoid the side effects caused by high local drug concentration. Liposome triggers based on photo-induced, non-cleavable, and reversible physical changes may resolve this problem.

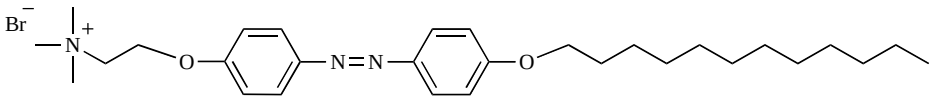
PHOTO-SENSITIVE LIPOSOMES BASED ON PHOTO-INDUCED REVERSIBLE PHYSICAL CHANGE

Photoisomerization refers to photo-initiated structural change between isomers of photoisomerizable molecules. It is a very important reversible process in biological systems. For example, photoisomerization of 11-*cis*-retinal to 11-*trans*-retinal triggers photo-transduction in the retinal photoreceptor cells and ultimately causes the sensation of vision [27]. There are many other photoisomerizable molecules that can be exploited to provide control and synchronization of biological processes. One particular example is azobenzene and many of its derivatives. Azobenzenes are characterized by reversible and reproducible transformations from the generally more stable *trans* isomer to the less stable *cis* isomer upon UV irradiation (about 320 nm), or from *cis* isomer to *trans* isomer through visible light irradiation (about 430 nm) or heating (Scheme **4**) [28]. This isomerization is accompanied with a large geometrical structural change: the distance between the para carbon atoms in the *trans* isomer of azobenzene is about 9.0 Å and the distance in the *cis* isomer is 5.5 Å, and the local contraction may be even greater. Furthermore, the *trans* isomer has a plannar and linear structure, while the two aromatic rings of the *cis* isomer are out of plane, and the long axes of the ring are oriented at an angle of about 56° to each other. These unique properties make azobenzenes an ideal option for liposomal photo-triggers in a non-disruptive reversible way (Scheme **4**).

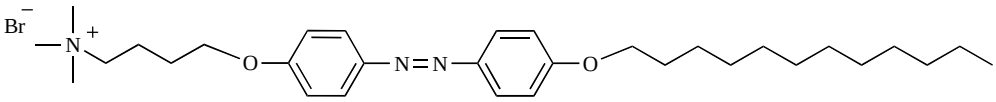
Single Chain Amphiphilic Azobenzene Derivatives

In 1980, Kano *et al.* reported firstly a novel photo-sensitive liposomes using synthetic single chain amphiphilic azobenzene derivatives, C_2-Azo-C_{12} and C_4-Azo-C_{12}, as photo-triggers (Scheme **5**) [29]. These azobenzene derivatives could form liposomes with dipalmitoyl-D,L-α-phosphatidylcholine (DPPC). However, the vesicle structure disappeared when the molar ratio of Azo/DPPC was higher than 1/5, implying C_2-Azo-C_{12} and C_4-Azo-C_{12} possibly destabilized the liposome's integrity. Upon UV irradiation (366 nm), more than 80% of *trans*-Azo could be converted to *cis*-isomer; and this isomerization was photo-reversible (*cis*-isomers could be quantitatively regenerated to *trans*-isomers upon irradiation with > 420 nm light). Bromothymole Blue (BTB) was then encapsulated into the photo-sensitive liposomes for release studies. Their results showed that the BTB release rate from the UV-irradiated liposomes (*cis*-isomers) was significantly higher than that of unirradiated liposomes (*trans*-isomers), and increasing the incorporation ratio of C_2-Azo-C_{12} and C_4-Azo-C_{12} led to an increased BTB release. Interestingly, C_4-Azo-C_{12} liposomes were more sensitive to UV-irradiated BTB release than C_2-Azo-C_{12} liposomes. This photo-induced release was interpreted to occur *via* large channels formed in the bilayers of the liposomes through the nonplanar and nonlinear structure of *cis*-Azo, and the content was released through these channels upon UV irradiation. However, it was not clear if visible light irradiation (*trans*-isomers) could switch off content release from liposomes.

To further understand the structural perturbation of liposomal bilayers induced by photoisomerization, Yamaguchi *et al.* studied the membrane fluidity changes by *trans*-*cis* photoisomerization of the azobenzene moiety at varying depths in the membrane of DPPC liposomes using Induced Circular Dichroism (ICD) [30]. Three synthetic single chain azobenzene amphiphiles were incorporated into DPPC liposomes with azobenzene moieties located at different sites in the lipid hydrocarbon tail. When the azobenzene moiety was located near the head group or at the center of the hydrocarbon tail, the amphiphiles were successfully incorporated into the bilayers of the DPPC liposomes. However, the amphiphiles with azobenzene moiety near the end of the hydrocarbon tail could not form stable liposomes with DPPC, suggesting these kinds of compounds destroy bilayer integrity. Azobenzene amphiphiles with the azobenzene moiety near the center of the hydrocarbon tail had the strongest photo-induced structural perturbation. However,



C_2-Azo-C_{12}



C_4-Azo-C_{12}

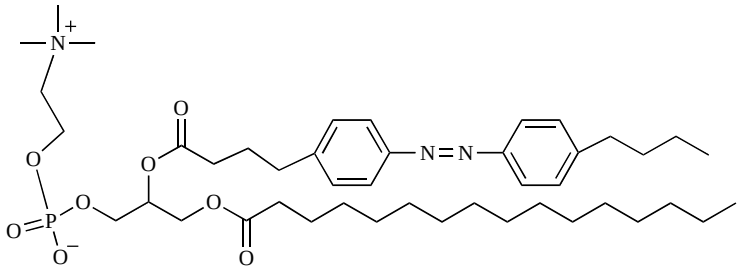
Scheme 5. Structure of C_2-Azo-C_{12} and C_4-Azo-C_{12}.

DSC results showed that these synthetic azobenzene amphiphiles could cause phase separation of DPPC liposomes; one domain is the mixture of synthetic amphiphile and DPPC, and the other domain is pure DPPC, which possibly increases the spontaneous release of liposomes.

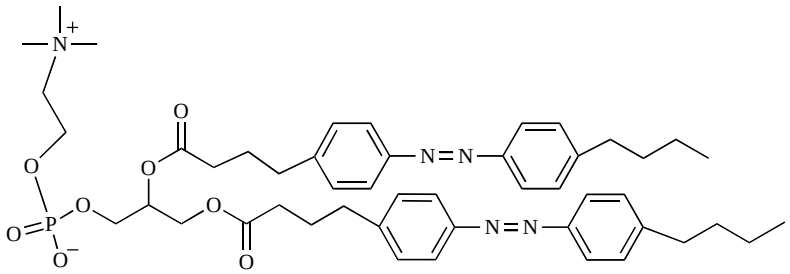
Phospholipid Mimic Azobenzene Derivatives

All the single chain amphiphilic azobenzene photo-triggers discussed above belong to cationic detergents, which inevitably contribute some negative effects to the integrity of liposomes, such as phase separation, fusion, and spontaneous content release of liposomes [31]. Morgan *et al.* proposed that phospholipid molecules containing the photo-isomerizable group may minimize perturbation to the bilayer compared to single chain amphiphilic azobenzene derivatives. They firstly synthesized a phospholipid molecule bearing an azobenzene moiety within one acyl chain (1-palmitoyl-2-(4-(4'-*n*-butylphenylazo)phenylbutyroyl)-phosphatidylcholine, P-Azo PC, Scheme **6**) [32]. This lipid behaved like a typical phospholipid, and could form typical vesicular structures by itself. After incorporating P-Azo PC into dipalmitoylphosphatidylcholine (DPPC) liposomes, Morgan found that photoisomerization of P-Azo PC in liposomes caused no loss of bilayer integrity, but enhanced water permeability and entrapped K^+ leakage. However, encapsulation of calcein (model drug) into this liposome showed no obviously enhanced release upon UV irradiation even though high concentration of P-Azo PC was incorporated and clear photoisomerization effects were observed.

To further increase the photo-sensitivity of Azo-lipid, Morgan *et al.* developed another photochromic phospholipid: 1,2-bis(4-(4'-*n*-butylphenylazo)phenylbutyroyl)-phosphatidylcholine (Bis-Azo PC, Scheme **6**), which resembles dipalmitoylphosphatidylcholine in size [32]. This lipid had a highly cooperative phase transition with DPPC, and did not show a very marked tendency of phase separation within the DPPC host. DPPC liposomes containing 6% (w/w) Bis-Azo PC were stable at room temperature (gel state), and contents did not significantly leak over weeks in the dark. Upon UV irradiation, the encapsulated calcein was immediately released from Bis-Azo PC/DPPC liposomes (on a time-scale of



P-Azo PC



Bis-Azo PC

Scheme 6. Structure of P-Azo PC and Bis-Azo PC.

milliseconds) below the phase transition temperature of DPPC (gel state), and total release of calcein was essentially instantaneous. It seemed that UV-irradiation led to locally nonlinear disordered bulky regions of *cis* Bis-Azo PC clusters to form a transient discontinuity in the lipid acyl chain packing, which quickly resulted in liposome fusion between 'photochromic' liposomes and those of pure lipid, followed by rapid clacein release. Morgan's fluorescence polarization assay and electron microscopy supported this hypothesis. On the other hand, UV-irradiation of P-AzoPC/DPPC liposomes caused no liposome fusion, which explains why P-Azo PC had little effect on bilayer permeability after photoisomerization. For Bis-Azo PC/DPPC liposomes, they ceased to trap calcein when the Bis-Azo PC content exceeded about 8% (mol:mol) of the total lipid, which implies that Bis-Azo PC may destabilize liposomes and promote the spontaneous content release. Further study confirmed that the Bis-Azo PC/DPPC liposomes showed increased spontaneous content release at temperatures nearby the phase transition of DPPC, while DPPC liposomes showed little solute release until the phase transition temperature was reached [33]. The photostationary state of azobenzene derivatives (such as Bis-Azo PC) exposed to room light contains some *cis*-isomers, which contributes the spontaneous release of liposomes to some extent. Morgan also found it difficult to control the rapid content release from Bis-AzoPC/DPPC liposomes by regulating the temperature, lipid host, and Bis-Azo PC concentration (> 2%) [33]. In order to expand the application of this photo-trigger, Morgan *et al*. also incorporated Bis-Azo PC into unsaturated PC liposomes (one and two unsaturated acyl chains). These liposomes could trap calcein efficiently, but showed no significant calcein release upon prolonged UV irradiation [34]. Bis-Azo PC was only sensitive to saturated host liposomes (DPPC, DSPC) below their phase transition temperature, which suggests that the rigidity of the bilayer must play a part role in the photo-induced content release. A rigid bilayer (DPPC) might not be able to accommodate the excess volume introduced by photoisomerization of Bis-Azo PC, while the greater free volume of the acyl chains in the unsaturated PC might be able to buffer the perturbation effect of photoisomerization of Bis-Azo PC in the bilayer.

Cholesterol is a well-documented lipid for the preparation of various liposomal drug delivery systems to modulate bilayer stability. Morgan *et al*. found that liposomes prepared from DPPC/Bis-Azo PC/cholesterol (79%/6%/15%, molar ratio) showed no significant spontaneous calcein release for at least 8 weeks at room temperature in the dark. However, liposomes containing higher concentrations of Bis-Azo PC (e.g. 10 mol%) in the absence cholesterol were stable overnight, but released their contents during long term storage, which implies that cholesterol can significantly decrease the spontaneous release from Bis-Azo PC/DPPC liposomes [34]. They also found that photo-induced calcein release rate was greatly enhanced with increased incorporation ratio of cholesterol, but the release rate decreased if more than 15% cholesterol (mol:mol relative to total lipid) was incorporated into saturated PC (DPPC, DSPC) liposomes. Interestingly, incorporation of cholesterol into P-Azo PC/DPPC liposomes could also cause rapid calcein release upon UV irradiation. These results suggest that inclusion of cholesterol can increase the photosensitivity of both Bis-Azo PC/DPPC liposomes and P-Azo PC/DPPC liposomes. However, the presence of up to 30% cholesterol (mol:mol relative to total lipid) did not induce significant content release from unsaturated PC liposomes, possibly because cholesterol could not increase the unsaturated bilayer rigidity enough to cause photo-induced content release. The increased photosensitivity of Bis-Azo PC/DPPC liposomes by cholesterol suggested a possible means to control the content release by changing the photo stationary state composition of Bis-Azo PC [28]. The photo stationary state of Bis-Azo PC is a *cis*-/*trans*-isomer mixture and the *cis*-isomer has a strong absorption peak at 420 nm, so irradiation with blue light (420 nm) could cause partial reversion to the *trans*-isomer. Morgan *et al*. proposed the *cis* to *trans* isomerization could also induce content release from DPPC/Bis-Azo PC/cholesterol liposomes because of the increased photosensitivity by cholesterol [35]. Their results showed that liposomes without cholesterol, or with about 5% cholesterol (molar ratio), were not sensitive to blue light irradiation, and no calcein was released even with prolonged blue light irradiation. However, very rapid calcein release happened at higher cholesterol levels (above 10% molar ratio). This phenomenon arised from the fact that cholesterol has the function of inducing phase separations in the bilayer [36]. The optimized features of Bis-Azo PC/DPPC liposomes by cholesterol suggest that this liposome has the potential for intelligent drug delivery. Further studies conducted by Morgan *et al*. found that their photo-sensitive liposomes could actively uptake the dye acridine orange and the anticancer drug doxorubicin, and the encapsulated drugs rapidly released from liposomes upon UV irradiation [37].

MULTI-PULSATILE PHOTO-SENSITIVE LIPOSOMES

The dependence of several body functions on circadian rhythm is well known; particular rhythms in the onset and extent of disease symptoms are also observed, such as in bronchial asthma, rheumatic disease, and diabetes [38]. Specifically delivering higher amounts of therapeutics in a burst manner at a specific time and site consistent with the pathological circadian rhythm of disease states can produce maximum therapeutic effects and minimum harmful effects [39]. To achieve this goal, numerous chronotherapeutics have been developed through various intelligent drug delivery systems. As an example, pulsatile drug delivery systems have attracted increasing interest in recent years [40, 41]. Pulsatile drug release is defined as the rapid and transient release of a certain amount of drug molecules within a short predetermined on-release period before an off-release period is initiated [40]. Pulsatile drug delivery systems can be categorized into single-pulsatile and multiple-pulsatile systems. All of the photo-sensitive liposomes discussed above display single-pulsatile release. Many disease treatments are long time processes, which may need sustained multiple-pulsatile drug delivery systems to achieve maximum therapeutic effects, such as multi-pulsatile photo-sensitive liposomes.

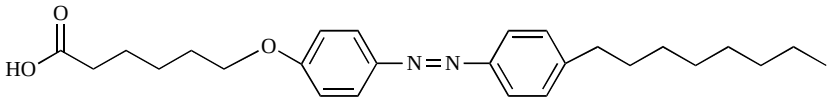
Single Chain Amphiphilic Azobenzene Derivatives

In 1991, Sato *et al*. firstly reported the experimental observation of the reversible control of ion permeability by

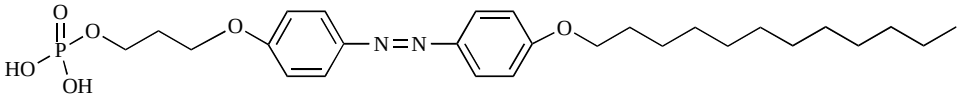
alternate irradiating photo-sensitive liposomes with UV and visible light [42]. The liposomes consisted of L-a-dimyristoyl phosphatidylcholine (DMPC), dicetyl phosphate (DCP), and 4-octyl-4'-(5-carboxypentamethyleneoxy)azoben-zene (8A5, Scheme **7**) (molar ratio, [DMPC]:[DCP]:[8A5] = 10:1:0-1). Photo-induced reversible *trans*-to-*cis* and *cis*-to-*trans* isomerization of 8A5 in liposomes could be repeated for several cycles, but the isomerization rate was considerably suppressed in the lipid membranes compared with that in chloroform. The permeability coefficient, P, of K^+ from liposomes was increased 5000 times after the onset of UV irradiation and the P constant could remain more than half an hour. When the liposome dispersion was irradiated with visible light, P decreased again to the original value. This on/off ion permeation from liposomes could be repeated for several cycles without destroying liposome integrity. After 100 min of irradiation, the K^+ trapped in the liposomes was almost completely released. The possible mechanism of enhanced ion permeation across the bilayer is the diffusion of ions through the transient pore formed in the bilayer by UV irradiation of 8A5 to *cis*-8A5, which has a bulkier structure than *trans*-8A5. It was estimated that the radius of the pore of this photo-sensitive liposome is 4 nm. Interestingly, photo-induced K^+ permeability thorough bilayer was significantly enhanced in DMPC/DCP/8A5 liposomes around the phase transition temperature (liquid crystalline state) [43], but almost 10% of the trapped K^+ was spontaneously released from liposomes after 20 h in the dark even in the gel state, implying that 8A5 contributed to the spontaneous release of liposomes. Similar to Sato's strategy, Lei *et al*. also synthesized another single chain amphiphilic azobenzene derivative, 4-dodecyl-4`-(3-phosphate-trimethyleneoxy) azobenzene(12A3, Scheme **7**), and then incorporated it into small unilamellar synthetic dihexadecyl phosphate (DHP) vesicles [44]. Their results showed that the photoisomerization of 12A3 and the photo-induced pulsatile K^+ release was similar to that of DMPC/DCP/8A5 liposomes, but the photo-controlled release rate was only slightly enhanced in the gel state of the liposomes and greatly increased in the liquid crystal state, which is different from Morgan's system. However, the spontaneous release of entrapped K^+ from this liposome was up to 1% and 3% after 1 hour at 25°C (gel phase) and 40°C (liquid-crystalline phase), respectively. In summary, the photo-induced pulsatile release rate and the spontaneous release rate from these multi-pulsatile liposomes were not particularly high, which is difficult to fulfill the chronotherapy duty of sustained release of encapsulated drugs consistent with the circadian rhythm of diseases.

Cholesterol in Liposomes

Cholesterol is composed of three six- and one five-membered rings which form a rigid planar structure. It is widely distributed in the biomembranes of living cells. Research results show that the interaction of cholesterol with the hydrocarbon chains of phospholipids leads to the formation of a molecular complex involving hydrogen bonding, which results in the well-known 'buffer' effects to the biomembrane rigidity. In the liquid crystalline phase, cholesterol increases the order and packing density within the biomembranes, while in the gel phase, the order is decreased. Due to this interaction, the presence of cholesterol can significantly suppress the phase transition of biomembranes over a wide temperature range in the liquid crystalline phase, and the phase transition is eliminated when 33 mol% or more of cholesterol is added into the biomembrane [45, 46]. A simulation model showed that seven acyl chains of phospholipids are required to surround each sterol nucleus and eliminate phase transition, corresponding to a phospholipid:cholesterol ratio of 2:1 [45]. Practically, cholesterol can be incorporated into biomembranes in concentrations up to 50 mol% to form a 1:1 phospholipid-cholesterol complex. Pure phospholipid bilayers, on the contrary, exhibit a liquid-disordered liquid crystalline phase above phase transition temperature and solid-ordered gel phase below. In the gel phase, biomembranes without cholesterol exhibit an unstable faceted polyhedron structure since curving gel phase bilayers into a sphere with high curvature is not possible; while in liquid crystalline phase, biomembranes with or without cholesterol show stable, smooth, and round structures. The major functions of cholesterol in biomembranes are to increase the stability, modulate the fluidity, and reduce the permeability of biomembranes [47, 48]. Due to these properties, cholesterol is widely used in the preparation of liposomal drug delivery systems. It can increase liposomal stability in the presence of plasma proteins and its circulating time in the blood (DaunoXome long-circulating liposomes) [49, 50]. For example, cholesteryl-3 β -carboxyamidoethylenedimethylamine (DC-Chol) was developed as non-viral gene delivery systems to resolve the problems other cationic liposomes faced in gene therapy, such as short lifetime of the complexes and inactivation by serum proteins [51].



8A5



12A3

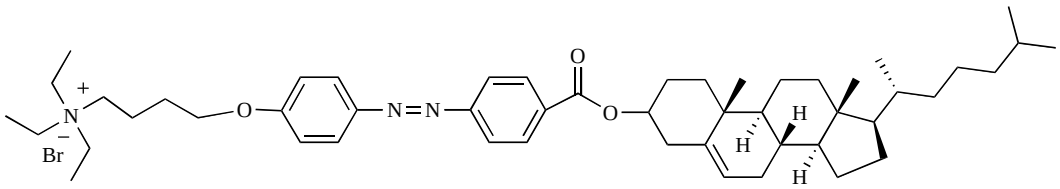
Scheme 7. Structure of 8A5 and 12A3.

Cholesterol Mimic Azobenzene Derivatives

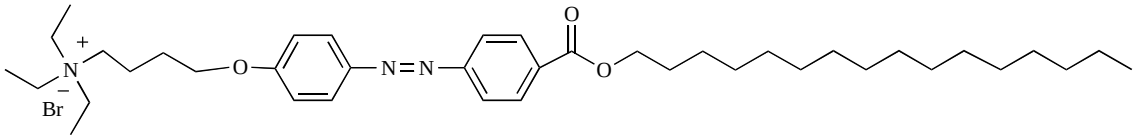
In 2000, Morgan *et al*. found an interesting phenomena after incorporating a high amount of cholesterol into DPPC/Bis-Azo PC liposomes (DPPC/cholesterol/Bis-Azo PC = 74%/20%/6%, molar ratio). Calcein release was significantly inhibited, and only happened during the period of UV irradiation. Furthermore, multi-pulsatile calcein release could be performed by intermittent UV irradiation for brief periods [35]. These results opened a possible way to decrease the photo-induced content release rate from liposomes by incorporating high concentrations of cholesterol. Wang's group proposed that cholesterol derivatives containing an azobenzene moiety may not only serve as photo-triggers for liposomes, but also retain cholesterol's intrinsic protective functions to the liposomal bilayers. They synthesized a new cholesterol mimic azobenzene derivative: 4-cholesteryloxy-carbonyl-4`-(4-aminium bromide-N, N, N, N-triethylbutoxy) azobenzene (AB lipid **1,** Scheme **8**) as a novel photo-trigger [9]. The photoisomerization of AB lipid **1** in egg PC liposomes could be reversibly repeated several times both in gel phase and liquid crystalline phase without destroying the bilayer integrity, and the rate of *cis*-to-*trans* isomerization of AB lipid **1** in liposomes was low in the dark. The calcein release rate was greatly increased after a few minutes of UV irradiation of AB lipid **1** to form the *cis* isomer (on state), and this increased content release was stable for a long time in the dark; the calcein release rate was greatly suppressed after a few minutes of visible light irradiation (off state). The photo-controlled on/off content release could be repeated several times without impacting the photosensitivity of the liposomes. Different from Bis-Azo PC, 8A5, and 12A3 [34, 44], the photo-controlled release from AB lipid **1**/egg PC liposomes was found both below (gel state) and above (liquid crystal state) the phase transition temperature of the liposomes. Thus, this drug delivery system can achieve multi-pulsatile drug release through UV (on) and visible light (off) irradiation. Interestingly, all of the UV light induced calcein release rates were similar except for a burst release caused by the first UV irradiation. Further study showed the diameter of the liposomes and the UV-Visible spectrum of AB lipid **1** did not change after periodic UV and visible light irradiation, implying that photoisomerization of AB lipid **1** did not induce phase separation and fusion of liposomes. The photo-induced calcein release mechanism was explained by diffusion of calcein through the pores formed by photoisomerization of AB lipid **1** to the *cis* isomer, which has a bulkier structure than the *trans* isomer, and the diffusion rate of calcein will increase/decrease when the pores formed/closed by the *cis*/*trans* isomer.

For clinical situations, it is important to have a regulated photo-induced content release rate and to have zero-order release. The total content release of the multi-pulsatile photosensitive liposomes discussed above was biphasic and characterized by an initial burst release stage (few hours), followed by a constant rate release stage (zero-order release stage). The release amount could be controlled either by regulating the *cis* isomer maintaining time with fixed ratio of AB lipid **1** or by regulating the ratio of the *cis* isomer state of AB lipid **1** with the fixed *cis* isomer maintaining time [9, 10].

Single chain amphiphilic or phospholipid mimic azobenzene photo-triggers appear to cause either an increase in the spontaneous content release or destabilize the integrity of liposomes (can't trap content). Wang's group found that the spontaneous calcein release from the AB lipid **1** lipsomes was less than that of the AB lipid **2** (Scheme **8**) in the absence of cholesterol moiety. Moreover, with the ratio of AB lipid **1** and **2** increased, the spontaneous release of AB lipid **1** liposomes had no change, while the spontaneous release of AB lipid **2** liposomes was dramatically increased. Since the photo-stationary state of azobenzene derivatives is the mixture of *cis*/*trans* isomers, it is reasonable to predict that the *cis*-azobenzene moiety of AB lipid **1** can increase the spontaneous content release, similar to other amphiphilic azobenzene derivatives reported. However, the cholesterol moiety in AB lipid **1** has a great inhibiting function against the spontaneous release, which may contribute to the sustained content release. These two contrary effects made AB lipid **1** have little effect on the spontaneous content release. Similar to other systems, the spontaneous release of AB lipid **1** liposomes could be greatly suppressed by addition cholesterol, without changing the photo-induced calcein release. Since the incorporation ratio of AB lipid **1** could be as high as 50%, this implies that the cholesterol moiety of AB lipid **1**



AB Lipid 1



AB Lipid 2

Scheme 8. Structure of AB Lipid 1 & 2.

has the same functions as cholesterol, by itself, in stabilizing liposomes. Wang's group found that the particle size of egg PC/AB lipid **1** liposomes were very stable and did not change dramatically after storage for six months at room temperature, while normal liposomes lacking AB lipid **1** were mostly fused and precipitated within one month at room temperature. This may be due to its rigid structure and intermolecular cationic repulsion. Furthermore, this drug delivery system has low cytotoxicity compared to DOTAP [*N*-(1-(2, 3-dioleoyloxy)propyl-*N*,*N*,*N*-trimethylammonium mesylate) [9, 10].

FUTURE DIRECTIONS OF PHOTO-SENSITIVE LIPOSOMES

Concerning to clinical practicability of the photo-sensitive liposomes, photodynamic therapy (PDT) is a relatively new therapeutic method, but it has become a good treatment option for various superficial skin cancers, such as actinic keratosis and basal cell carcinoma [52]. One of the liposomal formulations containing the photosensitizing drug Visudyne®, developed by Novartis Ophthalmicis, has been approved in Switzerland and USA for the treatment of the wet form of age-related macular degenerescence, which is characterized by the formation of choroidal neovasculature [53]. The mature technique of light source for PDT has stimulated various efforts to develop photo-induced release mechanisms for liposomal drug delivery systems. These kinds of intelligent liposomes are still in the early stages of development.

Although photo-sensitive liposomes can, in principle, trigger content release in a well-controlled and intelligent manner under appropriate conditions, most of them are prepared from conventional liposomes, especially for those with amphiphilic azobenzene photo-triggers. Their stability in plasma will be a major concern in clinical situations; developing photo-liposomes with long-circulation may improve this criteria. On the other hand, azobenzene derivatives might be toxic and/or carcinogenic (most of the amphiphilic azobenzene photo-triggers had no toxicity data available to evaluate their biocompatibility). The preliminary data shows that AB lipid **1**, a cholesterol derivative, has low toxicity and has the potential as a practical photo-trigger for liposomes, but more detailed studies are still needed to prove its safety profile. More importantly, most of the photo-sensitive liposomes are only sensitive to ultraviolet light (UV-A), which has a limited penetration in tissue. Topical application of these photo-sensitive liposomes might be possible, particularly for skin and eye diseases where near-UV radiation has sufficient penetration. But long-term irradiation with UV could be hazardous to the human body, which may limit their applications. Since near-infrared light (NIR) can penetrate deep into soft tissue, developing novel NIR triggers for photo-sensitive liposomes may be another promising strategy for developing these intelligent systems.

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