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Photoisomerisable cholesterol derivatives as photo-trigger of liposomes: Effect of lipid polarity, temperature, incorporation ratio, and cholesterol

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Abstract

Three cholesterol derivatives containing an azobenzene moiety with different polarities were designed and synthesized (AB lipids **1** to **3**). The effects of structure, temperature and incorporation ratio on liposomes were studied, with the results showing that the polarity in 4-substituent and in some cases, 4′-substituent may be important for their incorporation feasibility and photoisomerizability in liposomes. Liposomes incorporated with AB lipid **3** could release multi-pulsatilely upon UV and visible light irradiation both in gel state and liquid crystal state of liposomes. An increase in the incorporation ratio of AB lipid **3** enhanced the amount of drug released greatly. Unlike other azobenzene photo-triggers reported, AB lipid **3** did not increase the spontaneous release of liposomes. Furthermore, cholesterol suppressed the spontaneous release of liposomes.

Keywords: Liposome; Cholesterol; Azobenzene; Drug delivery system

1. Introduction

Photochromism has recently received considerable interest in biochemistry. The role of photoisomerization in vision is well known [1]. There are however many other areas where photochromism can be exploited to provide control and synchronization of biological systems. Particular examples include the modulation of ions or drug permeability through model biomembranes (for example, liposomes) using azobenzene derivatives to undergo photoisomerization upon ultra violet (UV) light and visible light irradiation. This photo-control process has advantages of providing a broad range of adjustable parameters (e.g., wavelength, duration, intensity) that can be optimized in a non-disruptive manner, while other photo-control processes through photo-polymerization [2–4] or photo-oxidation [5–7] of membrane lipids can disrupt the integrity of the lipid bilayer to enhance ions or drugs release from liposomes.

Several different photo-triggering approaches employing azobenzene derivatives as the photo-control switch have been developed. The Yonezawa [8] and Hurst [9] groups incorporated single chain azobenzene derivatives into liposomes and could release drug multi-pulsatilely, but it has been reported that single chain amphiphilic azobenzene derivatives promote phase separation and fusion of liposomes [10,11], thus promoting the spontaneous release of liposomes. Morgan et al. believe that phospholipid molecules containing the isomerisable group can minimize perturbation to bilayer membranes of single chain amphiphilic azobenzene derivatives. They incorporated bis-azo phosphatidylcholine (Bis-Azo PC) into liposomes as a photo-trigger [12–15]. This system led to a rapid release upon UV light irradiation (a few minutes to cause total release), and was not applicable to the clinical situations that needed sustained and multi-pulsatile release. Furthermore, liposomes ceased to trap model drug calcein when the Bis-Azo PC content exceeded about 8% (mol:mol) of the total lipid, which implies that Bis-Azo PC destabilized liposomes and promoted the spontaneous release of drugs from liposomes.

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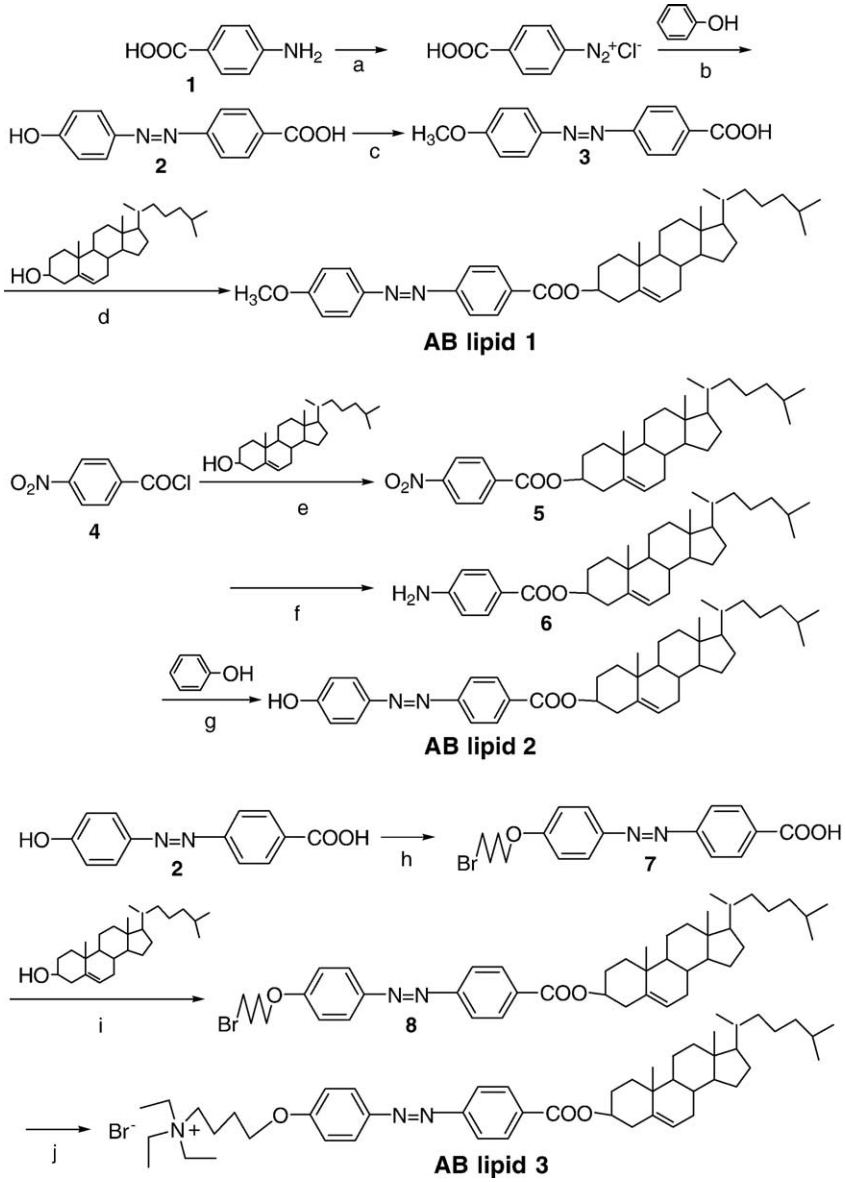
In order to minimize perturbation and spontaneous release to bilayer membranes used, it is desirable to find new molecules containing an azobenzene group. Cholesterol is an important component in biomembranes and has functions to modulate the fluidity of biomembranes, increasing the mechanical strength and decreasing the permeability of the biomembranes [16]. It is worth studying whether cholesterol derivatives containing an azobenzene moiety could not only serve as a photo-trigger, but also perform such functions as cholesterol. Research results also showed that cholesterol and its derivatives cholesteryl methyl ether and 3-β-[*N*-(*N*′*N*′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) with different polarities can be easily incorporated into liposomes [17,18]. In this paper, three cholesterol derivatives containing an azobenzene moiety (AB lipid **1** to **3**) with different polarities were designed and synthesized, and their incorporation feasibility into liposomes, their effects on photo-controlled release and the effect of cholesterol were investigated.

2. Materials and methods

Egg phosphatidylcholine (PC), cholesterol and Triton X-100 were purchased from Sigma. Calcein was purchased from ACROS, and Sephadex G-50 was purchased from Amersham Biosciences. The buffer used was a phosphate type at pH 7.4. Other reagents were the best grade commercially available, which were distilled, recrystallized, or used with further purification, as appropriate. All reactions were conducted under dry N_2 conditions unless otherwise stated.

2.1. Lipid synthesis

The synthetic routes of the cholesterol derivatives containing azobenzene moiety were given in Scheme 1. After diazo coupling of *p*-aminobenzoic acid with phenol gave 2, which reacted with methyl iodide resulting in **3**. Esterification of **3** with cholesterol gave AB lipid **1**. Esterification of **4** with



Scheme 1. Synthesis of AB lipid 1, 2, 3: (a) HCl, $NaNO_2$, 0 °C; (b) NaOH (75%), 0 °C; (c) CH_3I, KOH, KI, reflux (78%); (d) DCC, DMAP, r.t. (50%); (e) Pyridine, r.t. (80%); (f) $SnCl_2$, reflux (90%), 0 °C; (g) HCl, $NaNO_2$ then NaOH, phenol, 0 °C (50%); (h) 1,4-dibromobutane, K_2CO_3, 18-crown-6, reflux (90%); (i) DCC, DMAP, r.t. (55%); (j) Et_3N, reflux (50%).

cholesterol gave 5, which reduced by $SnCl_2$ gave **6**. Diazo coupling of **6** with phenol gave AB lipid **2**. Compound **2** reacting with 1,4-dibromobutane gave **7**, and esterification of **7** with cholesterol followed by reacting with Et_3N gave AB lipid **3**.

2.2. Liposome preparation

The preparation of the small unilamellar liposomes (SUV) was carried out using the standard sonication method under nitrogen. Briefly, stock lipids in $CHCl_3$ were transferred from their stock solutions into flasks and dried by evaporation under nitrogen stream. The samples were then stored under vacuum for 4 h at 4 °C. The thin lipid film formed on the wall of flask was hydrated with a 0.15-M phosphate-buffered saline solution (PBS, pH 7.4) and sonicated under nitrogen for 10 min (30 s on and 30 s off for each cycle) with a VC 130 Probe sonicator at maximum power output (SONICS and MATERIALS, INC.). Temperature was controlled with an ice water bath and no lipid degradation was found as examined by thin-layer chromatography. The total lipid concentration was 0.1 mM. Subsequent centrifugation with 100,000×*g* was carried out to remove untrapped lipids and titanium. For release and leakage measurements, the thin lipid film was hydrated with 100 mM calcein at pH 7.4 before sonication and untrapped calcein was separated by gel filtration on Sephadex G-50 column using the above buffers as eluents.

2.3. Differential scanning calorimetry (DSC)

The thermal behavior of lipid samples was studied with Perkin-Elmer DSC-7. Data were processed with Origin software. The samples were examined at a scanning rate of 10 °C/min by applying two heating and one cooling cycle between −25 °C and 60 °C. The peak from the second heating cycle was noted. A typical sample size was 20 μmol taken from the dried lipid film in an aluminum pan for measurements.

2.4. Photoisomerization of AB lipids in liposomes

A diluted AB lipids/egg PC mixed liposome dispersion was irradiated with a 400 W high-pressure Hg lamp (LCE-9, Zhengzhou, China). A bandpass filter (λ_{max}=365 nm) was used for UV light; and a cut filter of λ>420 nm (Fujifilm, Japan) was used for visible light (irradiation 10 min per 4 h). After a certain period, photoisomerization of AB lipids upon UV light was traced by the Ultraviolet-Visible (UV-Vis) spectra of the liposome dispersion using a UV-Vis spectrophotometer (UNICAM UV 500, UK).

2.5. Liposome release measurements

The liposome release behavior was observed by monitoring the release of calcein from liposomes. The liposome dispersion was added to PBS buffer. After UV or visible light irradiation for 10 to 15 min every 4 h, the calcein release from the liposomes was measured by fluorescence spectroscopy (Perkin-Elmer LS 550, excitation at 494 nm, emission at 514 nm). The 100% release of calcein was estimated by the fluorescence intensity after complete destruction of liposomes by the addition of Triton X-100 (final concentration: 0.3%). The amount of calcein released (%) from liposomes was calculated by the equation: calcein released (%)=$100\times(I_t-I_0)/(I_{max}-I_0)$, where I_0 is the fluorescence intensity of the liposome dispersion containing calcein at the initial time, I_{max} is the maximum fluorescence intensity after Triton X-100 addition, and I_t is the measured fluorescence intensity with respect to time.

3. Results and discussion

3.1. Liposome preparation

The gel filtration curve of the liposome dispersion had a single peak, indicating that most liposomes were in the form of SUV. The absorption peak of liposomes and the centrifugal precipitate indicated that the AB lipid **2** and AB lipid **3** were incorporated into liposomes and were located between the lipid bilayer, not in the interior aqueous phase of liposomes, while AB lipid **1** was not incorporated into liposomes. This may be due to their different polarities. AB lipid **2** and AB lipid **3** are amphiphilic compounds, just like cholesterol and DC-Chol, which can be used as components of liposomes. Cholesteryl methyl ether is a non-polar compound that can be inserted into the hydrocarbon chains of PC. AB lipid **1** has a methoxy group and cholesterol group that are much like cholesteryl methyl ether, but it also has an ester bond linked with the azobenzene group and the cholesterol group, so it is less amphiphilic and failed to incorporate into the liposomes.

3.2. Differential scanning calorimetry

So far, considerable amounts of data indicate that cholesterol interacts with the membrane phospholipids [16]. It can modulate the fluidity, increase the mechanical strength, and decrease the permeability of the biomembrane. These properties induced by cholesterol in the biomembrane have been widely studied by DSC. The DSC of cholesterol/PC mixtures showed a decrease in the heat of phase transition peak by increasing of cholesterol content until the phase transition peak vanished at 50 mol% [19]. The DSC results showed that the phase transition peak of the AB lipid 2/PC mixture also vanished when AB lipid **2** was at 50 mol% of the total lipids, so AB lipid **2** may perform the same functions as cholesterol in liposomes (Fig. 1A). But the phase transition peak of AB lipid 3/PC mixtures changed so little with the mole percent of AB lipid **3** increased, which indicated that no phase separation in liposomes occurred (Fig. 1B) [20]. It has been reported that the permeability of lipid membranes increases accompanied with the phase transition [21]. So temperature below and above the phase transition temperature of liposomes should have greater effects on the arrangement of lipid molecules of AB lipid 3/PC liposomes even at high ratios of incorporation, while this would not exist in AB lipid 2/PC liposomes because AB lipid 2/PC liposomes did not have a phase transition peak at about 33% of incorporation rate.

3.3. Photoisomerization of AB lipids in liposomes

It has been known that azobenzene derivatives in $CHCl_3$ solution undergo *trans*-to-*cis* isomerization by UV light irradiation and *cis*-to-*trans* isomerization by visible light irradiation. The liposome dispersion incorporated with AB lipid **3** was irradiated with light at 360 nm and the UV-Vis spectra are shown in Fig. 2A. *Trans*-AB lipid **3** in liposomes converted to *cis*-AB lipid **3** as evident from the decrease of the strong peak at λ=360 nm and the concurrent increase of the weak peak at λ=450 nm. It is estimated from the spectral change that 65% of *trans*-AB lipid **3** in liposomes was converted into the *cis*-AB lipid **3** to reach the photostationary state after 10 min of UV light irradiation; while in $CHCl_3$ solution, it only took 6 min of UV light irradiation to reach the state that 90% of *trans*-AB lipid **3** was converted into the *cis*-AB lipid **3** (Fig. 2B). The rate and ratio of *trans*-to-*cis* isomerization by UV light irradiation was slower and lower in liposomes than in $CHCl_3$

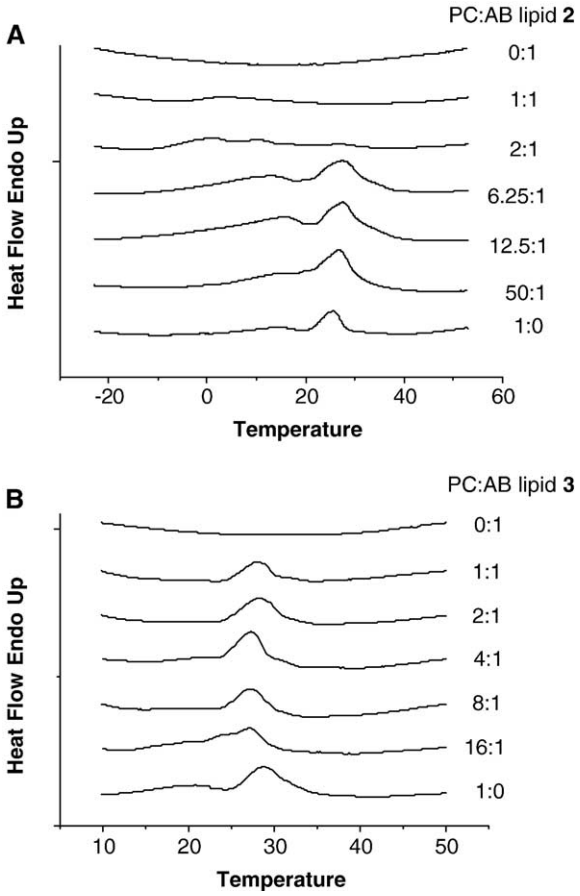


Fig. 1. DSC of AB lipid 2/PC samples (A) and AB lipid 3/PC samples (B).

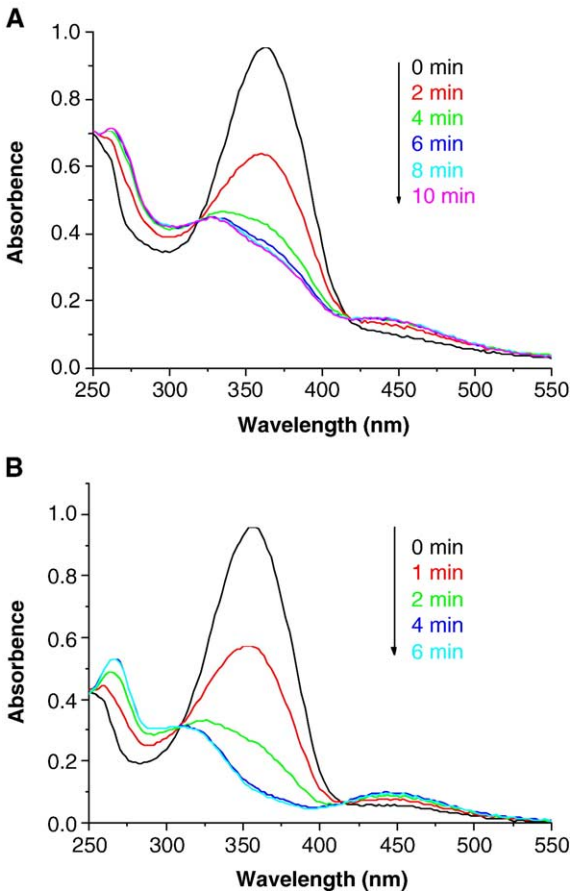


Fig. 2. UV-Visible spectral change following UV light irradiation of AB lipid **3** in liposomes (A, egg PC:AB lipid **3**=4:1, molar ratio) and in $CHCl_3$ (B) at 360 nm for different times.

solution, implying that AB lipid **3** was incorporated into liposomes. It is likely that *trans*-to-*cis* isomerization, along with enlargement of bulk structure of the AB lipid **3** molecule, is considerably suppressed in the lipid membrane [22]. In the dark, the rate of *cis*-to-*trans* isomerization of AB lipid **3** in liposomes was very slow. Upon irradiation with light wavelengths longer than 420 nm, the *cis*-to-*trans* isomerization happened very quickly and it reach to the photostationary state after 10 min of visible light irradiation. This *trans*–*cis* isomerization could be repeated several times.

In the case of liposomes incorporated with AB lipid 2, the spectral change of *trans*-AB lipid **2** was hardly observed upon irradiation of 360 nm even after 30 min (Fig. 3), which indicated that AB lipid **2** in liposomes is very difficult to be isomerized from *trans* to *cis* form. A possible explanation is the existence of azo-hydrazone tautomerization equilibrium [23]. Ionization of phenolic hydroxyl is another possibility that deactivates the excited state and makes AB lipid **2** not realize *trans*-to-*cis* isomerization. Ongoing study should further refine a complete view of this phenomenon. Anyway, this kind of compound was not suitable as photo-triggers of liposomes.

3.4. Effect of temperature on the photo-controlled release of liposomes

Hurst's results showed that the photo-controlled release rate was only slightly enhanced in gel state of liposomes and greatly increased in liquid crystal state of liposomes by photo-conversion of the single chain azobenzene derivative to its *cis* form [9]. Morgan's results showed that liposomes of Bis-Azo-PC/egg PC in liquid crystal state did not release calcein upon UV light irradiation, even at the highest concentration of Bis-Azo-PC/egg PC tested (20%, mol:mol), and that only synthetic saturated phospholipids in the gel state resulted in a photo-controlled release [12]. The photo-controlled release of AB

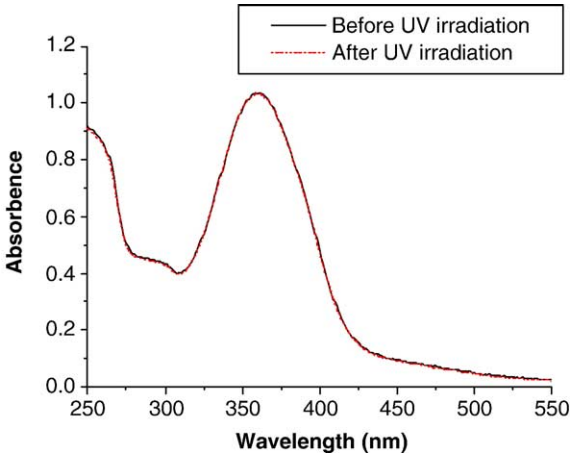


Fig. 3. UV-Vis spectra of egg PC/AB lipid **2** liposomes before and after UV light irradiation at 360 nm for 30 min (egg PC:AB lipid **2**=4:1, molar ratio).

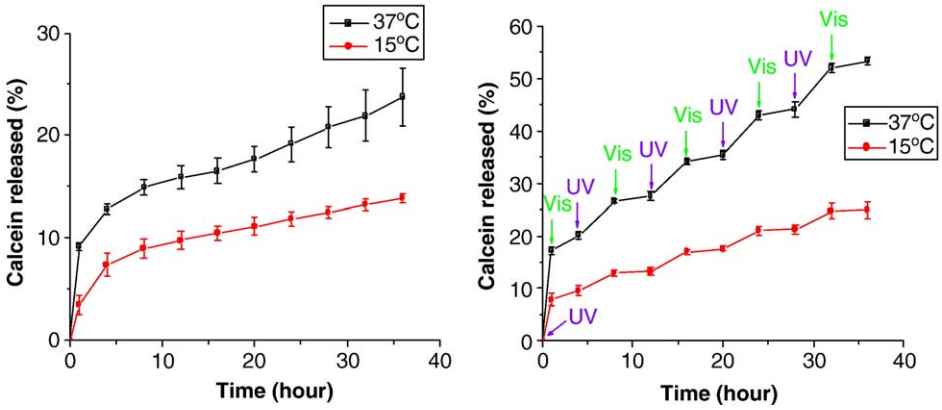


Fig. 4. The spontaneous release rate (left) and the photo-controlled release rate (right) of calcein from the mixed systems of egg PC and AB lipid **3** (egg PC:AB lipid **3**=1:1 in molar ratio) at 15 °C and 37 °C. Periodical UV and visible light irradiation (UV, 10 min; Visible, 15 min) was carried out. Each point represents the mean±S.D. (n=3).

lipid 3/PC mixed liposomes (AB lipid 3/PC=1:1, mol:mol) was investigated both in the gel state (15 °C) and the liquid crystal state (37 °C) of liposomes. The liposome dispersion was periodically (every 4 h) irradiated with 10 min of UV light and 15 min of visible light. As shown in Fig. 4, the calcein release rate was greatly increased by UV light irradiation. Upon irradiation with visible light, the calcein release rate was greatly suppressed. This calcein release rate controlled by UV and visible light could be repeated several times without destroying the liposome integrity both in the gel state and in the liquid crystal state.

An important factor for controlling such an on–off release system was to provide a perfect off state. Although there is always a very small amount of calcein spontaneously released from liposomes, the amount was dependent on the lipid used in liposomes. We could suppress the rate of calcein release by visible light irradiation. According to our previous results, the photo-controlled release across the bilayer membrane is a diffusion mechanism [24]. From Fig. 4, both the spontaneous release rate and the photo-controlled release rate of liposomes at 37 °C was faster than those at 15 °C. One of the reasons was that the diffusion coefficient of drug at higher temperatures is higher than that at lower temperatures. Moreover, the DSC results showed that the phase transition temperature of AB lipid 3/PC mixtures was about 25 °C and it did not vanish as the mole percentage of AB lipid **3** increased to 50% of the total lipids (Fig. 1). It is well known that the diameter of the bilayer of liposomes in liquid crystal state is shorter than that in gel state. Therefore, another reason for the difference was the drug diffusion route at 37 °C is shorter than that at 15 °C [21].

3.5. Effect of AB lipid 3 on the photo-controlled release and spontaneous release of liposomes

Morgan et al. found that it took only a few minutes for total release of the drug from the liposomes and the liposomes cease to trap calcein when the Bis-Azo PC content exceeds about 8% (mol:mol) of the total lipid content of Bis-Azo PC and dipalmitoyl-L-α-phosphatidylcholine (DPPC) [12]. This means that the rate of photo-controlled release in their system is difficult to regulate. With our systems, however, multi-pulsatile drug release can be achieved through UV and visible light irradiation at the ratio range of 1–12 (PC:AB lipid 3, mol:mol; Fig. 4 and Fig. 5 right). The photo-controlled release rate increased with increasing molar ratio of AB lipid **3** to PC, proving that the photo-controlled release rate can be regulated by altering the incorporation ratio of AB lipid **3**. Moreover, with the ratio of AB lipid **3** increased, the spontaneous release did not be enhanced (Fig. 5 left), which means that AB lipid **3**

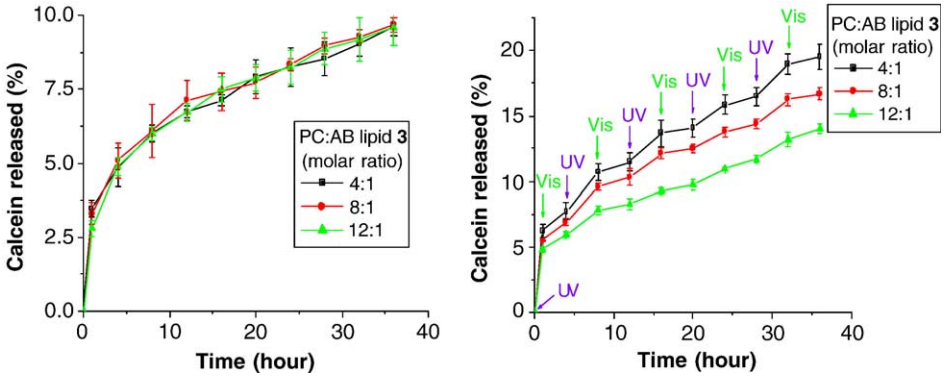


Fig. 5. Effect of the addition of different ratio of AB lipid **3** on the spontaneous release (left) and the photo-controlled release (right) of calcein from liposomes at 15 °C. Periodical UV and visible light irradiation (UV, 10 min; Visible, 15 min) was carried out. Each point represents the mean±S.D. (n=3).

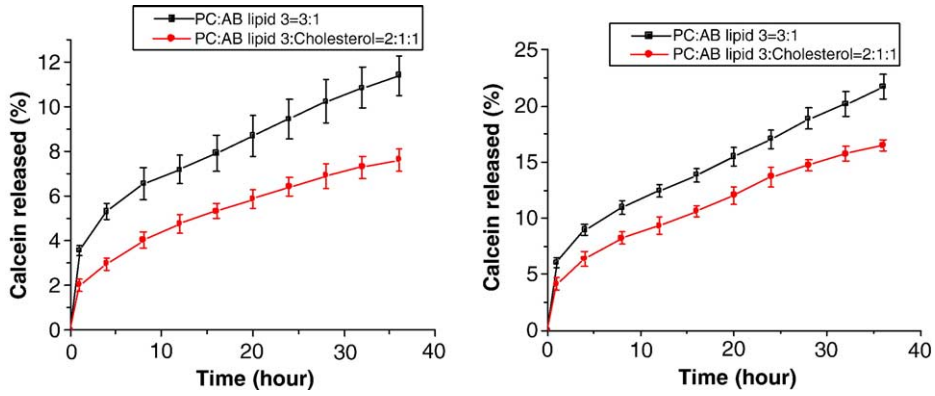


Fig. 6. Effect of the addition of cholesterol on the spontaneous release of calcein from liposomes at 15 °C (left) and 37 °C (right). Each point represents the mean ± S.D. (n = 3).

had little effect on the spontaneous release of liposomes. It is well known that the thermodynamically stable form of azobenzene derivatives is the *trans*-isomer, but this will not exist in a pure form except in freshly synthesized samples which never expose to visible light. The photostationary state of azobenzene derivatives exposed to a room light will be a mixture of *cis/trans* isomers. It is reasonable to consider that the *cis*-isomer of azobenzene moiety in AB lipid **3** can increase the spontaneous release of calcein from liposomes, just like other single chain amphiphilic molecules and phospholipid molecules containing an azobenzene group reported. But the cholesterol moiety in AB lipid **3** has a great inhibiting function against the spontaneous release of liposomes. These two contrary effects led to AB lipid **3** having little effect on the spontaneous release of liposomes.

3.6. Effect of cholesterol on the spontaneous release and photo-controlled release of liposomes

To suppress the spontaneous release of calcein from liposomes, the effect of the incorporation of cholesterol was examined because cholesterol can greatly stabilize bilayer membranes [16]. Fig. 6 illustrates that when cholesterol was added to the egg PC/AB lipid **3** system, the spontaneous release of liposomes was greatly suppressed both in the gel state (15 °C) and the liquid crystal state (37 °C), which can be attributed to the enhancement of a hydrophobic interaction between the hydrophobic moieties of PC and cholesterol.

Morgan et al. found that both UV light and visible light increased drug release from liposomes after the addition of cholesterol [13]. Our results showed that the calcein release was greatly suppressed upon irradiation with visible light after the addition of cholesterol, but the multi-pulsatile release through UV and visible light irradiation could still be achieved both in the gel state (15 °C) and the liquid crystal state (37 °C), especially in a higher temperature (Fig. 7). The photo-controlled release rate decreased with the decreasing the molar ratio of AB lipid **3** to cholesterol, the function of cholesterol against biomembrane permeability may have contributed to this result (Fig. 8). Furthermore, the burst release was also greatly suppressed by the addition of cholesterol, therefore decreasing the toxic effect caused by burst release.

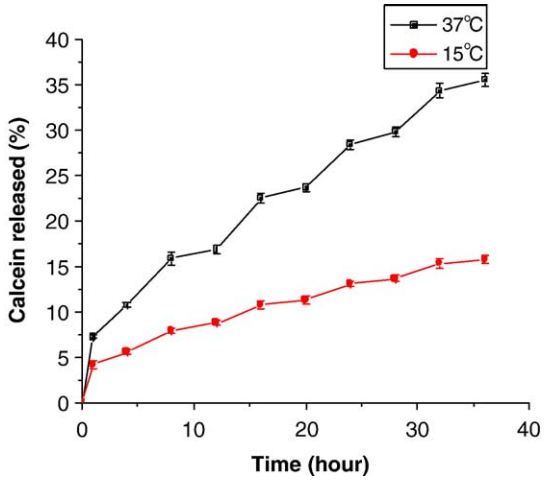


Fig. 7. The photo-controlled release rate of calcein from the mixed systems of egg PC and AB lipid **3** in the presence of cholesterol (egg PC:cholesterol:AB lipid **3** = 2:1:1 in molar ratio) at 15 °C and 37 °C. Periodical UV and visible light irradiation (UV, 10 min; Visible, 10 min) was carried out every 4 h. Each point represents the mean ± S.D. (n = 3).

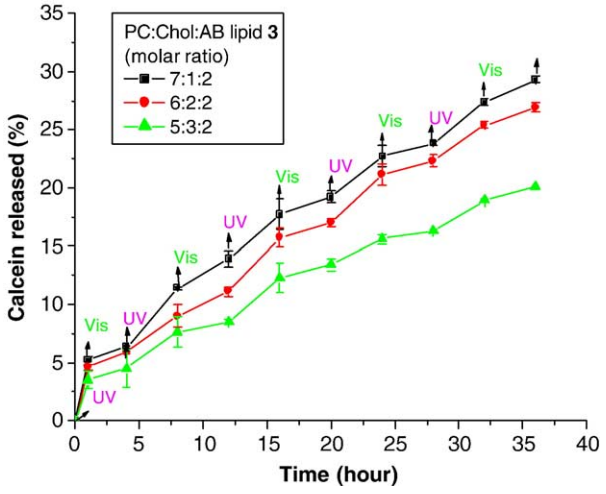


Fig. 8. The effect of the addition of different ratio of cholesterol on the photo-controlled release of calcein from liposomes as a function of periodical UV and visible light irradiation at 15 °C (UV, 10 min; Visible, 10 min). Each point represents the mean ± S.D. (n = 3).

4. Conclusions

In conclusion, we studied the effects of three cholesterol derivatives containing an azobenzene moiety with different polarities on the release function of liposomes. The polarity in 4-substituent was important for the incorporation feasibility and photoisomerizability in liposomes. The phase state of liposomes and the incorporation amount of AB lipid **3** had great effects on the photo-controlled release of liposomes. Unlike other azobenzene photo-triggers reported, AB lipid **3** did not increase the spontaneous release of liposomes. Furthermore, cholesterol greatly suppressed the spontaneous release of liposomes, while multi-pulsatile release could be achieved when AB lipid **3** was used as a photo-trigger.

Acknowledgments

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