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SHORT COMMUNICATION

A photo-responsive cholesterol capable of inducing a morphological transformation of the liquid-ordered microdomain in lipid bilayers

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Abstract An azobenzene-modified cholesterol was designed and synthesized for photo-induced domain transformation in lipid bilayer membranes. Upon UV-light irradiation, the cholesterol derivative changes the conformation through photoisomerization of the azobenzene moiety from *trans*- to *cis*-form. The photoisomerization effectively occurred both in liquid-ordered (L_o) and liquid-disordered (L_d) phases. Phase-contrast and fluorescence microscopic observation revealed that photoisomerization of the azobenzene-modified cholesterol induced the shape transformation of giant unilamellar vesicle (GUV) and the reorganization of L_o domain structure. Such a photo-induced transformation of lipid domain gave two different pathways dependent on the lipid composition of GUV; disappearance of the L_o domain or appearance of a small L_d domain with in the L_o domain.

Keywords Liquid-ordered domain · Lipid raft · Azobenzene · Photoisomerization · Molecular packing

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Introduction

Microdomain formation is one of the unique characteristics of lipid bilayers. The lateral phase separation between liquid-ordered (L_o) and liquid-disordered (L_d) phases is considered an especially interesting behavior in relation to the lipid raft hypothesis [1–3]. In this hypothesis, the L_o domain or lipid raft in the cytoplasmic membrane is thought to play a crucial role in a wide range of cellular processes. For instance, recent reports have pointed out that lipid rafts participate in signal transduction, protein sorting and membrane trafficking. Additionally, they play a role in viral infection, Alzheimer's diseases and prion disorders [4–7]. However, fundamental characteristics, such as the mechanism of lipid raft formation, remain the subject of current investigations. For example, the reconstitution of L_o domains as model lipid rafts within L_d membranes has been also extensively studied from biophysical and physicochemical standpoints [8–9].

In general, cholesterol and saturated phospholipids including sphingomyelin are tightly packed with maintaining the fluidity to form the L_o phase within lipid raft domains [10]. It has been shown that the structural affinity of cholesterol to the surrounding lipids plays an important role in the formation of L_o domains. In particular, increasing the head group bulkiness or decreasing the steroid ring planarity diminishes domain formation [11–14]. These results imply that formation of the L_o domain can be controlled by changing the structure of cholesterol.

In this work, we describe photo-sensitive microdomain formation as schematically shown in Fig. 1. In this system, light irradiation to a lipid bilayer membrane containing L_o and L_d domains perturbs in molecular packing of the lipids in the L_o domain. This involves disorganization of the L_o domain. For this purpose, a novel photo-responsive

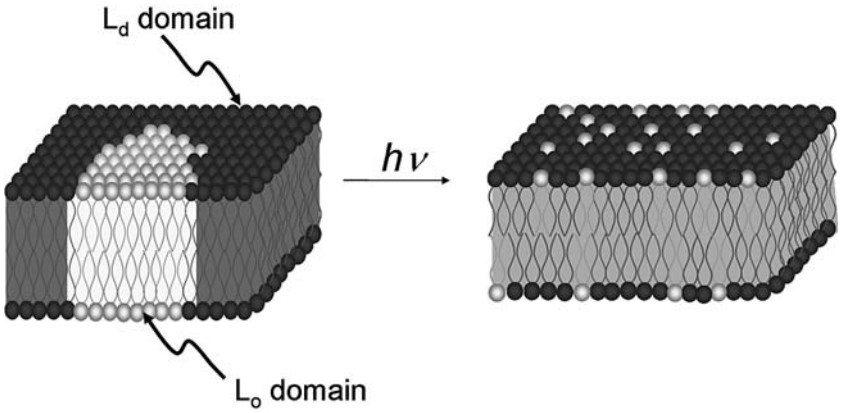
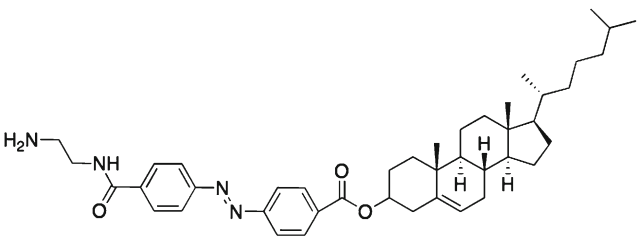


Fig. 1 Schematic representation of photo-induced disorganization of liquid-ordered domain formed in a lipid bilayer membrane

cholesterol derivative (AzCh) was designed and synthesized. We employed azobenzene as a photo-responsive moiety, since its photoisomerization behavior was well characterized not only in homogeneous solutions but also in lipid membranes [15–18]. Previously, various azobenzene-conjugated lipids have been designed to adopt photo-responsive function to biochemical and biophysical applications of liposomal membrane systems, such as controlled release of protons or DNA and switching of organic or enzymatic reactions [19–23]. However, such methodology has not been applied to the study of the photo-responsive transformation of lipid domain. We report the effect of lipid phase state on photoisomerization behavior of AzCh by means of electronic absorption spectroscopy. Furthermore, we show that the L_o domain formed in giant vesicles embedding AzCh is photosensitive using phase-contrast and fluorescent microscopy. The results provide insights into the nature of lipid packing within the L_o domain. Such knowledge will potentially be useful in understanding the structure of lipid rafts.



AzCh

Experimental

Materials

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids, Inc. (AL, USA). Cholesterol and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl), ammonium salt (Rho-DOPE) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Molecular Probes (OR, USA), respectively. All of the reagents used in this study were analytical grade and used without further purification. We designed a photo-responsive amphipathic cholesterol derivative, 4-(2-aminoethylcarbamoly)-4′-(cholesteryloxycarbonyl)azobenzene (AzCh), composed of a hydrophilic head group, a photo-responsive azobenzene unit and a hydrophobic cholesterol moiety. AzCh was synthesized by condensation of 4.4′-dichloroformylazobenzene with cholesterol and *N*-*t*-butoxycarbonylethylenediamine (*N*-Boc-ethylenediamine), followed by deprotection of the Boc group (48% yield, details in supplementary data).

Preparation of liposomal membranes

Large unilamellar vesicles (LUVs) were prepared by hydration of lipid thin films as follows. Appropriate amounts of phospholipids, cholesterol and AzCh were dissolved in chloroform–THF (95:5 *v*/*v*). The solvent was evaporated under nitrogen gas flow and the residual trace solvent was completely removed *in vacuo* for 3 h to give a thin film on the wall of a glass vial. Hydration of the lipid thin film was performed above the phase transition temperature with an appropriate amount of HEPES buffer (100 mM, pH 7.0), followed by five freeze-and-thaw cycles at −196 and 50 °C. The liposome dispersion thus obtained was extruded 11 times through stacked 100-nm polycarbonate membrane filters and a LiposoFast miniextruder from Avestin above the phase transition temperature.

Giant unilamellar vesicles (GUVs) were prepared by the gentle hydration of a lipid film. Appropriate amounts of phospholipids, cholesterol, AzCh, and Rho-DOPE were dissolved in chloroform–THF (95:5 *v*/*v*). The solvent was evaporated under nitrogen gas flow and the residual trace solvent was completely removed in vacuo for 3 h to give a thin film on a wall of round glass vessel. The film was hydrated with aqueous sucrose solution (12 mM) at 55 °C

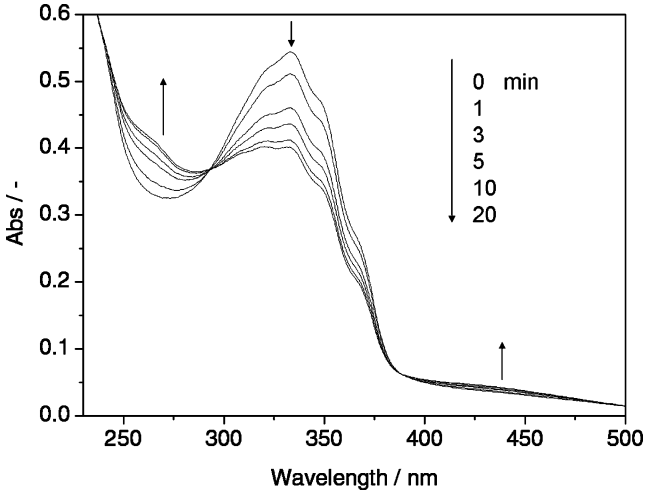


Fig. 2 Changes in electronic absorption spectra for photoisomerization of AzCh (25 μM) embedded in the DMPC liposome (500 μM) in HEPES buffer (100 mM) at pH 7.0 and 30 °C. UV-light irradiation time, 0, 1, 3, 5, 10, and 20 min

overnight. The giant liposome thus obtained was incubated for 1 h at 25 °C prior to microscopic observation. The total concentration of the lipids was set to 500 μM.

Photoisomerization of AzCh

Photoisomerization of AzCh embedded in liposomal membrane was performed by irradiation with a 500-W Xe lamp (SX-UID500X; Ushio Inc.). A UV band-pass filter (D33S; Toshiba Ltd.) was used for UV-light irradiation. During the photo-irradiation, the temperature of a sample cuvette was kept constant using a circulating water bath incubator. The photoisomerization behavior of AzCh was evaluated by electronic absorption spectra recorded with a Shimadzu UV-2400 spectrophotometer.

Membrane solubilization experiment

Aqueous dispersion of LUV containing 500 μM phospholipid was used for the experiment. Membrane solubilization was carried out by adding 10% (v/v) aqueous Triton X-100 (10 μl) to the GUV dispersion (990 μl). After incubation for 4 h at 25 °C, light-scattering intensity was measured at 400 nm with a Hitachi F-4500 spectrofluorometer.

Microscopic observation

Microscopic observation was carried out using an Olympus IX71 epifluorescence microscope. The images were recorded on an Olympus DP70 color CCD camera. Photoisomerization of AzCh embedded in GUV was performed on the stage by irradiation with a 100-W extra-high-pressure mercury lamp and an appropriate dichroic mirror unit installed in the microscope.

Results and discussion

Effect of the phase state on the photoisomerization of AzCh

It is well-known that azobenzene changes conformation in response to UV/visible light irradiation. We evaluated the photoisomerization behavior of AzCh embedded in lipid bilayer membranes by means of electronic absorption spectroscopy. As shown in Fig. 2, the electronic absorption spectra of AzCh embedded in LUV formed with DMPC at pH 7.0 and 30 °C exhibited photo-sensitivity. Since AzCh is sufficiently hydrophobic, this molecule is preferentially incorporated in the lipid membrane phase as well as cholesterol. An absorption characteristic to the *trans*-AzCh appeared at 330 nm before photo-irradiation (time=0 min). Upon UV-light irradiation, absorption maxima at 265 and 435 nm, which are assigned to the *cis*-isomer, increased with a concomitant decrease of the absorption at 330 nm. Isobestic points are observed at 291 and 382 nm. The absorption spectra were unchanged by UV-light irradiation for >20 min, showing that the photoisomerization of AzCh reached photostationary state on this time scale. To examine the effect of lipid phase state, the absorption decrease at 330 nm was measured in several lipid matrices and summarized in Table 1. It was found that the phase state of the lipids surrounding AzCh affects the photo-sensitivity.

Table 1 Photoisomerization behavior of AzCh embedded in various lipid membranes

Entry	Phospholipid	Cholesterol derivative	Phase state[a]	ΔAbs_{330}[b]
1	DPPC	AzCh	S	−0.05
2	DPPC	AzCh + cholesterol	S + L_o	−0.10
3	DMPC	AzCh	L_d	−0.14
4	DMPC	AzCh + cholesterol	L_d + L_o	−0.14

[phospholipid] = 500 μM, [AzCh] = 25 μM, [cholesterol] = 50 μM in HEPES buffer (100 mM) at pH 7.0 and 30 °C

[a] *S* gel phase, L_o liquid-ordered phase, L_d liquid-disordered phase. Phase state was evaluated from the phase diagrams for the corresponding phospholipid–cholesterol systems reported in Refs. [24] and [25]

[b] Absorbance change at 330 nm upon UV-light irradiation for 20 min

The phase states of each system are reported based on phase diagrams in the literature [24–25]. We assumed that effect of AzCh in the *trans*-form is comparable to native cholesterol.

In the DPPC bilayer, the absorbance of AzCh at 330 nm is not highly photo-sensitive (entry 1 in Table 1). This result suggests that the azobenzene moiety of AzCh is mainly present in the gel (S) phase. Tight molecular packing of the lipids in the S phase does not allow the photo-induced conformational change of AzCh to occur. In contrast, AzCh is highly photo-sensitive within DMPC bilayers in the L_d phase (entry 3 in Table 1). This is likely due to the fact that DMPC membrane in the L_d phase has greater fluidity than the S state of DPPC bilayers. In DPPC bilayers containing cholesterol and AzCh (100:10:5), the absorption at 330 nm was moderately sensitive to UV-light irradiation (entry 2 in Table 1). The S phase and the liquid-ordered (L_o) phase coexist in this membrane. AzCh molecules in the L_o domain are able to change their conformation much more easily than those in the S domain. Inclusion of cholesterol in DMPC bilayers does not significantly impact the ΔAbs_{330} value (entry 4 in Table 1). Accordingly, AzCh can change the conformation in a photo-responsive manner in L_o and L_d phases. In addition, it has been reported that lipid molecules are tightly packed in the L_o phase, although the membrane fluidity is greater than that of the S phase [10]. Thus, we expect that photo-induced conformational changes of the AzCh molecules in the L_o phase would make a significant effect to induce the structural change of the L_o domain in lipid bilayers.

Photo-induced shape transformation of giant vesicle

Giant unilamellar vesicles (GUVs) are a powerful tool for the direct visualization of membrane-related events [26]. In order to determine the effect of AzCh photoisomerization on the morphology of lipid bilayers, we observed GUVs containing AzCh by optical microscopy. For this purpose, we employed a ternary lipid matrix system composed of DOPC, DPPC and cholesterol in a molar ratio of 2:2:1, respectively. This gave GUVs with phase-separated L_o and L_d domains [27]. Phase-contrast microscopic images of GUVs containing AzCh (5 mol% of the total lipids) revealed their sensitivity to UV-light irradiation as shown in Fig. 3. Before photo-irradiation, the vesicular surface exhibits a wavy structure indicating the coexistence of multiple domains with different curvatures. It has been reported that the microscopic curvature of mixed lipid membranes is strongly related to the structural property of lipid domains, or difference in molecular packing of the component lipids [28–30]. In particular, L_d domains prefer relatively low curvature as compared with L_o domains due to the differences in elasticity. Thus, the observed wavy structure reflects the separation of L_o and L_d phases. Upon

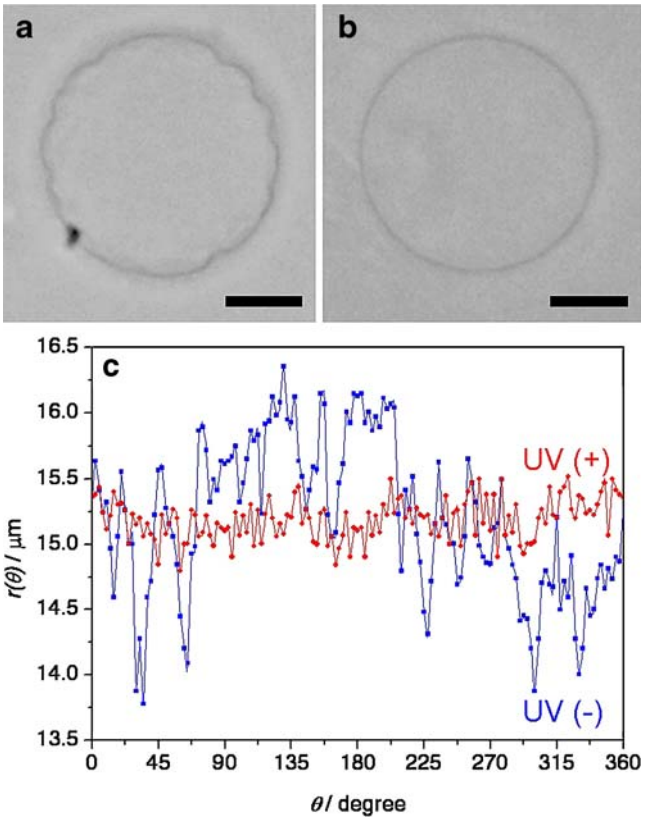


Fig. 3 Phase-contrast microscopic images of GUV formed with DPPC–DOPC–cholesterol–AzCh (38:38:19:5 in molar ratio) before (**a**) and after (**b**) UV-light irradiation and analysis of the vesicular waviness (**c**). *Bar*=10 μm

UV-light irradiation, the GUV surface became smooth, which strongly suggests that a structural reorganization of the membrane took place.

To evaluate the membrane waviness, the radius of the vesicle, $r(\theta)$, was plotted against the center angle, θ (Fig. 3c). While the average radius of the vesicle was nearly constant before and after photo-irradiation, the fluctuation of $r(\theta)$ significantly decreased in the latter case. This clearly shows that photoisomerization of AzCh causes the standard deviations of $r(\theta)$ to decrease from 0.58 to 0.16 μm. Recently, Hamada et al. have reported that a photo-responsive double-chain lipid with an azobenzene moiety on the head group induces a shape transformation in GUVs. This occurs due to the increase of cross-sectional area of the lipid molecule upon *trans*- to *cis*-isomerization [31]. The transformation of vesicular shape observed in this study implies that AzCh has potential to control micro-domain structures through its photoisomerization.

Effect of photoisomerization of AzCh on lipid microdomain structures

Phase-separated microdomains on a GUV can be clearly visualized with fluorescence microscopy using an appro-

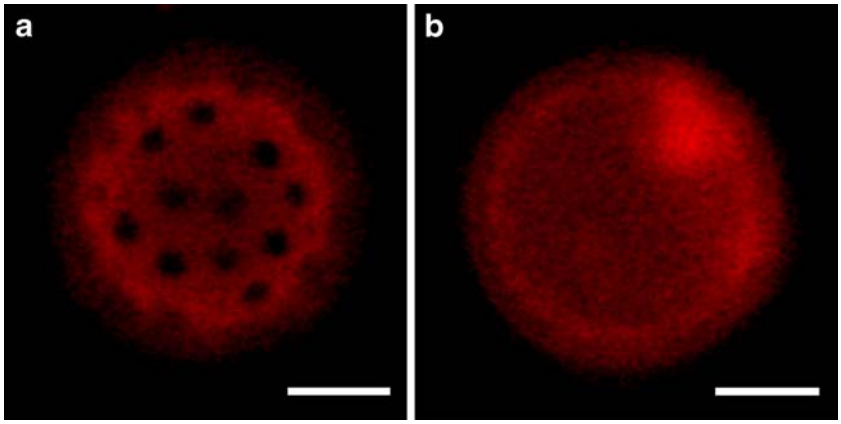


Fig. 4 Fluorescence microscopic images of GUV formed with DPPC–DOPC–cholesterol–AzCh (38:38:19:5 in molar ratio) before (**a**) and after (**b**) UV-light irradiation. L_d phase in the GUV was stained with Rho-DOPE (0.01 mol% of the total lipids). *Bar*=10 μm

priate fluorescent probe [27, 32]. For the visualization of domain structures in the present work, GUVs were stained with Rho-DOPE (0.01 mol% of the total lipids), a well-known fluorescent probe, which is known to selectively stain the L_d phase [33]. Figure 4a and b show the fluorescent microscopic images of the giant vesicle consisting of the same lipid composition before and after UV-light irradiation, respectively. Upon UV-light irradiation, microdomains on the GUV became homogeneous. Before UV-light irradiation, dark spots were observed on the surface of the GUV, suggesting that the GUV has phase-separated domains. Based on the property of Rho-DOPE, it is regarded that the dark spots and red regions on the vesicle are corresponding to L_o and L_d phases, respectively. The preparation procedure used in this study gave good reproducibility in formation of the L_o circular domains in the L_d phase matrix. After UV-light irradiation, the color of the GUV image became homogenous, indicating that the L_o domain structure on the GUV vanished or shrank to a size smaller than the limit of the microscopic resolution. Therefore, the photo-responsive disorganization of the L_o phase is regarded as a consequence of the photoisomerization of AzCh. For quantitative analysis of the domain disorganization, we evaluated the amount of lipids resistant to solubilization by a surfactant. Since lipid molecules are tightly packed in the L_o phase as compared with L_d phase, the former phase is resistant to solubilization by nonionic surfactants such as Triton X-100 (TX-100). Thus, the TX-100 solubilization has been employed as a common technique for evaluation of the L_o domain formation [34]. We found that UV-light irradiation causes a 20% decrease in the amount of detergent-resistant lipids at equilibrium based on light-scattering measurement (see supporting information Table S1). Accordingly, we conclude that the L_o domain in the GUV was destabilized by photoisomerization of *trans*-AzCh to the corresponding *cis*-form.

Next, we examined photo-induced domain transformation of GUV formed with phospholipids at higher cholesterol content. For this purpose, the GUVs consisting of DOPC, DPPC and cholesterol (molar ratio 1:1:1) with AzCh (5 mol% of the total lipids) were prepared. With this lipid system, a somewhat different behavior was observed as shown in Fig. 5. Before UV-light irradiation, phase segregated structure was also observed in a similar manner to the GUV with low cholesterol content. However, total area of the L_o domain relative to that of L_d is greater in this GUV system (Fig. 5a). Similarly, Crane and Tamm reported

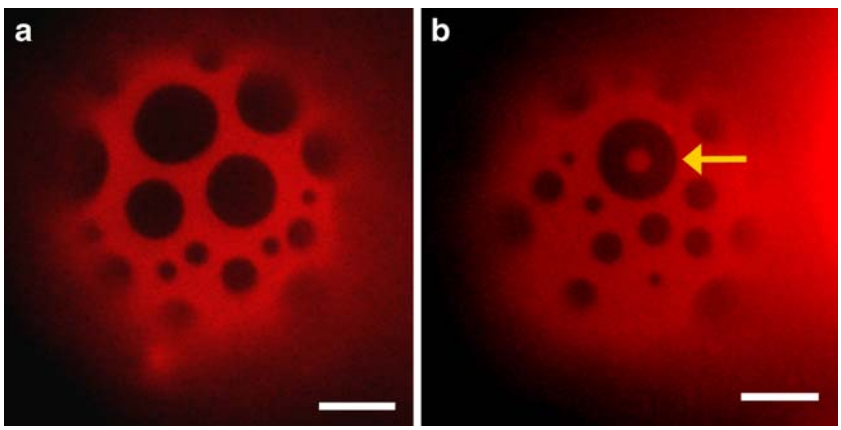


Fig. 5 UV-light induced transformation of domain structure. Fluorescence microscopic images of GUV formed with DPPC–DOPC–cholesterol–AzCh (32:32:32:5 in molar ratio) before (**a**) and after (**b**) UV-light irradiation. L_d phase in the GUV was stained with Rho-DOPE (0.01 mol% of the total lipids). *Arrow* indicates a newly appeared domain upon the irradiation. *Bar*=10 μm

that an increase of the cholesterol content enhances L_o phase formation in the porcine brain phosphatidylcholine–porcine brain sphingomyelin–cholesterol system [35]. Upon UV-light irradiation, the present GUV system underwent a unique domain transformation behavior (Fig. 5b). The microscopic image suggested that the photoisomerization of AzCh induced two different pathways dependent on the size of L_o domain. One is a decrease in phase separation as observed in the GUV with lower cholesterol content. Another is the appearance of a small red domain (L_d phase) within a large dark domain (L_o phase) as shown by an arrow in Fig. 5b. The appearance of the small red domain within a dark domain was observed for the other GUV samples with good reproducibility (additional microscopic image in supporting information). Although a detailed mechanism remains unclear, we speculate that the photoisomerization of AzCh perturbs the lipid packing of the L_o domains, resulting the formation of a small L_d domain within a relatively large L_o domain. The unique microdomain structure observed here may be potentially useful in the design of highly sophisticated supramolecular architectures, such as artificial protein organization system [36]. Hence we expect that this technique can establish a membrane platform with hierarchical lateral phase separation that can be regarded as the artificial lipid raft system.

Conclusion

In the present work, we developed an amphipathic cholesterol derivative (AzCh) with a photo-responsive azobenzene moiety capable of inducing a transformation of the L_o domain structure in lipid bilayers. The photoisomerization of AzCh was strongly affected by the phase state of the lipid matrix. AzCh effectively undergoes photoisomerization when embedded in the L_d and L_o phases, but is less responsive in the S phase. Phase-contrast and fluorescence images revealed that photoisomerization of AzCh drastically perturbed the L_o domain structure in GUVs consisting of DPPC, DOPC and cholesterol. Interestingly, the domain transformation behavior was dependent on the initial size of L_o domain. That is, upon UV-light irradiation, a tiny L_d domain appeared within a relatively large L_o domain, while the smaller L_o domain diminished. The present photo-responsive cholesterol has the potential to act as a trigger to control structure and function of lipid-raft-related membrane system.

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