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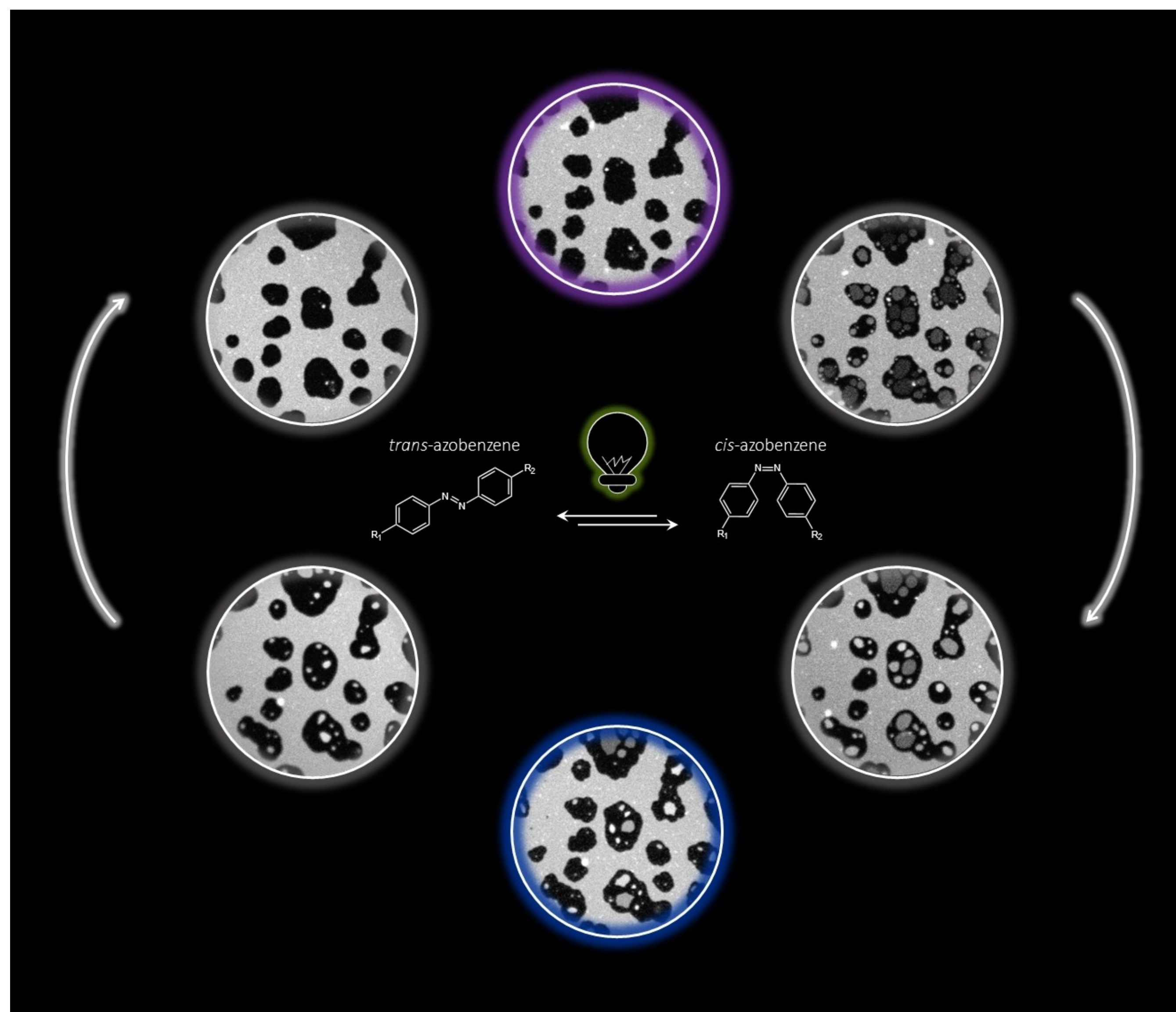
Photo-Lipids: Light-Sensitive Nano-Switches to Control Membrane Properties

Larissa Socrier*[a] and Claudia Steinem[b]



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Biological membranes are described as a complex mixture of lipids and proteins organized according to thermodynamic principles. This chemical and spatial complexity can lead to specialized functional membrane domains enriched with specific lipids and proteins. The interaction between lipids and proteins restricts their lateral diffusion and range of motion, thus altering their function. One approach to investigating these membrane properties is to use chemically accessible probes. In particular, photo-lipids, which contain a light-sensitive azobenzene moiety that changes its configuration from *trans*- to *cis*- upon light irradiation, have recently gained popularity for modifying membrane properties. These azobenzene-derived lipids serve as nanotools for manipulating lipid membranes *in vitro* and *in vivo*. Here, we will discuss the use of these compounds in artificial and biological membranes as well as their application in drug delivery. We will focus mainly on changes in the membrane's physical properties as well as lipid membrane domains in phase-separated liquid-ordered/liquid-disordered bilayers driven by light, and how these changes in membrane physical properties alter transmembrane protein function.

1. Introduction

50 years ago, biological membranes were described as a fluid mosaic according to thermodynamic principles, and the available data at that time provided information about the bilayer structure, the lateral mobility of the components within the membrane plane, and the ability of proteins to transiently or permanently bind to the membrane.[1] Over the intervening years, it became, however, more and more clear, that the chemical and spatial complexity of biological membranes[2] is key to understanding their dynamics and functions on various length scales. The lipid and protein composition of different membrane types has been analysed as well as the asymmetry of the two leaflets of the membrane. Furthermore, information has been gathered that shows the importance of specialized membrane domains, such as ordered domains, often referred to as lipid rafts,[3–5] and protein complexes highlighting the heterogeneous structure and dynamics of biological membranes. These membrane domains, observed in artificial and cell membranes using fluorescent probes,[6,7] are essential for the function of cellular membranes being involved in signalling and trafficking thus adding a new layer of complexity and hierarchy to the system.

To investigate the heterogeneity and different functional units of cellular membranes, photochromic molecules, in particular azobenzene-derived probes, serving as nanotools have been introduced in the field of membrane biophysics in the mid-80's.[8–12] Since then, the azobenzene group has been functionalized with a wide variety of chemical moieties for various biological applications.[13,14] The use of azobenzene derivatives gained great popularity in the membrane community over the last decade.[15] The ability of azobenzene to change its configuration from *trans* to *cis* upon light irradiation enables dynamic, quasi-non-invasive photocontrol to influence the properties of membrane components by light. The electronic absorption spectrum of the *trans*-azobenzene shows a strong band in the UV region between 320–350 nm which is associated with a π-π^* transition. A second band, much less pronounced and corresponding to an n-π^* transition, is visible in the blue region with a maximum of around 450 nm. In *cis*-configuration, the intensity of the UV band significantly diminishes while that in the blue region slightly increases (Figure 1A). The change of configuration of the azobenzene group, *i.e.*, its isomerization, can also be triggered by thermal, mechanical, or electrostatic stimulation and can be repeated for thousands of cycles without fatigue. Four mechanisms have been proposed to describe the *trans-cis* isomerization process (Figure 1B).[16] i) The rotational mechanism involves breakage of the N=N double bond allowing a phenyl moiety to rotate until the *cis*-configuration is reached. ii) In the case of the inversion mechanism, no bond breakage is observed but an extension of one N=N–C angle to 180° is observed. iii) For the isomerization to occur by a concerted inversion, also no bond breakage takes place, but both N=N–C bond angles increase to 180° to form a linear transition state. iv) Inversion-assisted rotation combines N=N bond breakage and large changes in the C–N=N–C dihedral angle and N=N–C angles. Dependent on the substitutions on the phenyl rings, the solvent, and the excitation mode, the isomerization mechanism as well as the yield may vary. In the case of light irradiation, it is presumed that *trans*- to *cis*-isomerization occurs via a rotational or inversion pathway while the *cis*- to *trans*- occurs via an inversion mechanism.[17]

Due to the possibility to switch the azobenzene moiety simply by light, azobenzene-derived molecules have been applied to regulate oligonucleotide structures and function[18] and to control peptides and proteins.[19] More recently, however, an emphasis was put on azobenzene-derived lipids serving as nanotools to manipulate lipids and lipid membranes *in vitro* and *in vivo*.[15] In this review, we will discuss the use of these compounds in artificial and biological membranes as well as their application in drug delivery. We will focus mainly on studies dealing with changing the physical properties of lipid

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membranes such as their mechanics and organization as well as the influence on membrane-anchored proteins.

2. Azobenzene-derived photo-lipids

2.1. Control of membrane physico-chemical properties by photo-lipids

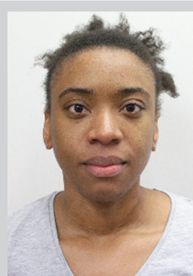
Several processes such as fusion and fission, vesicle trafficking, and budding take place in the plane of cellular membranes. These confined processes, which are essential for living cells, require a deformation of the bilayer.[20,21] Owing to their impact on lipid geometry, photo-lipids appear to be well-suited candidates to manipulate the membrane's physico-chemical properties. Backus *et al.*[22] investigated the impact of dialkyl-5-phosphate azobenzene (DT Azo-5P, Figure S2A) on 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) monolayers. Inserting 17 mol% of DT Azo-5P in DPPC monolayers enabled them to manipulate the packing and molecular ordering of the lipid. Lately, Urban *et al.*[23] worked with an azobenzene compound resembling the structure of a phospholipid. They prepared supported lipid bilayers containing 1-stearoyl-2-[(E)-4-(4-((4-butylphenyl)diazenyl)phenyl)butanoyl]-*sn*-glycero-3-phosphocholine (18:0-azoPC), a photo-lipid derived from phosphatidylcholine, in which the azobenzene was attached to the sn_2 chain (Figure S1C). By means of fluorescence microscopy and small-angle X-ray scattering, they found that the *trans*- to *cis*-isomerization of the 18:0-azoPC led to an increase in the lipid bilayer's fluidity with a simultaneous reduction of its thickness. With a similar approach, Ober *et al.*[24] observed a decrease in lipid vesicle thickness of about 8 Å, if 18:0-azoPC is in *cis*-configuration.

Using giant unilamellar vesicles (GUVs) composed of pure 18:0-azoPC (Figure 2A), Pernpeintner *et al.*[25] were able to control the membrane mechanics and vesicle's morphology on a time scale of only milliseconds to seconds. *Trans*- to *cis*-isomerization induced various vesicle shape transformations, such as budding, pearling and tube formation (Figure 2B–C). These structures could be reversed upon *cis*- to *trans*-isomerization, demonstrating the efficiency of photo-lipids to manipulate the mechanical properties of these lipid bilayers in a non-invasive manner. Based on these observations, Pernpeintner *et al.*[25] even exploited these photo-lipids to induce a large structural change *i.e.*, membrane tubule fission using strong irradiation (Figure 2D).

Although this study provided valuable information about the impact of isomerization on the membrane's mechanical properties, the results were obtained with pure 18:0-azoPC vesicles. It would be more interesting to see what the impact of the azobenzene lipid is in more complex lipid mixtures resembling the situation found in natural membranes. Following this idea, Aleksanyan *et al.*[26] recently investigated the impact of the isomerization of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) monolayers and GUVs containing different 18:0-azoPC concentrations ranging from 5–50 mol%. They showed that the isomerization-induced reversible membrane deformation, as well as membrane thinning, and area increase were a function of the photo-lipid concentration.

Morstein *et al.* evidenced that photo-lipids can also alter the cell membrane morphology. They treated human embryonic kidney (HEK) and neuronal cell lines with an azobenzene lysophosphatidic acid (AzoLPA, Figure S1G). In HEK cells, the *cis*-AzoLPA isomer greatly modified the activity of LPA receptors while in neuronal cells, it triggered a retractation of neurite branches 30 minutes after UV irradiation.[27]

Although photo-lipids are frequently used tools and were applied in various studies, the exact mechanisms by which these compounds modify the physico-chemical properties of membranes still need to be further investigated. Simulations have shown that the azobenzene group adopts a planar cylindrical shape in *trans*-configuration while in *cis*-configuration the shape is more conical.[28,29] In accordance with those simulations, surface tension and X ray scattering experiments revealed that the *trans*- to *cis*- isomerization increases the mean molecular area of lipids[30] while reducing their length.[23] These



Larissa Socrier received her B.Sc. and M.Sc. in biochemistry from the University of the French West-Indies and the University Claude Bernard Lyon 1 (France), respectively. She then obtained a Ph.D. in the fields of biotechnology and biophysics at the University of Technology of Compiegne (France) where she studied the mechanisms of action of nitrone and flaxseed antioxidants to inhibit membrane lipid oxidation. Larissa Socrier currently works as a post-doctoral fellow at the Max Planck Institute for Dynamics and Self-Organization in Göttingen (Germany). She investigates membrane lateral organization as well as the interaction between bacterial proteins and glycolipid receptors to understand the mechanisms leading to the internalization of toxins into cells.

Claudia Steinem got trained in biology and chemistry and obtained a Ph.D. in biochemistry at the University of Münster (Germany). She then worked as a post-doctoral researcher at the Scripps research institute in La Jolla, (CA, USA). After her habilitation at the University of Münster, she got appointed associate professor at the University of Regensburg (Germany). In 2006 she moved to the University of Göttingen (Germany) as a full professor of Biomolecular Chemistry. As a principal investigator at the Institute of Organic and Biomolecular Chemistry, she investigates membrane-confined processes in a bottom-up approach. Using artificial membrane systems, preferentially on solid and porous substrates, she investigates lipid-protein interactions, membrane fusion, and peptide- and protein-mediated transport across membranes.

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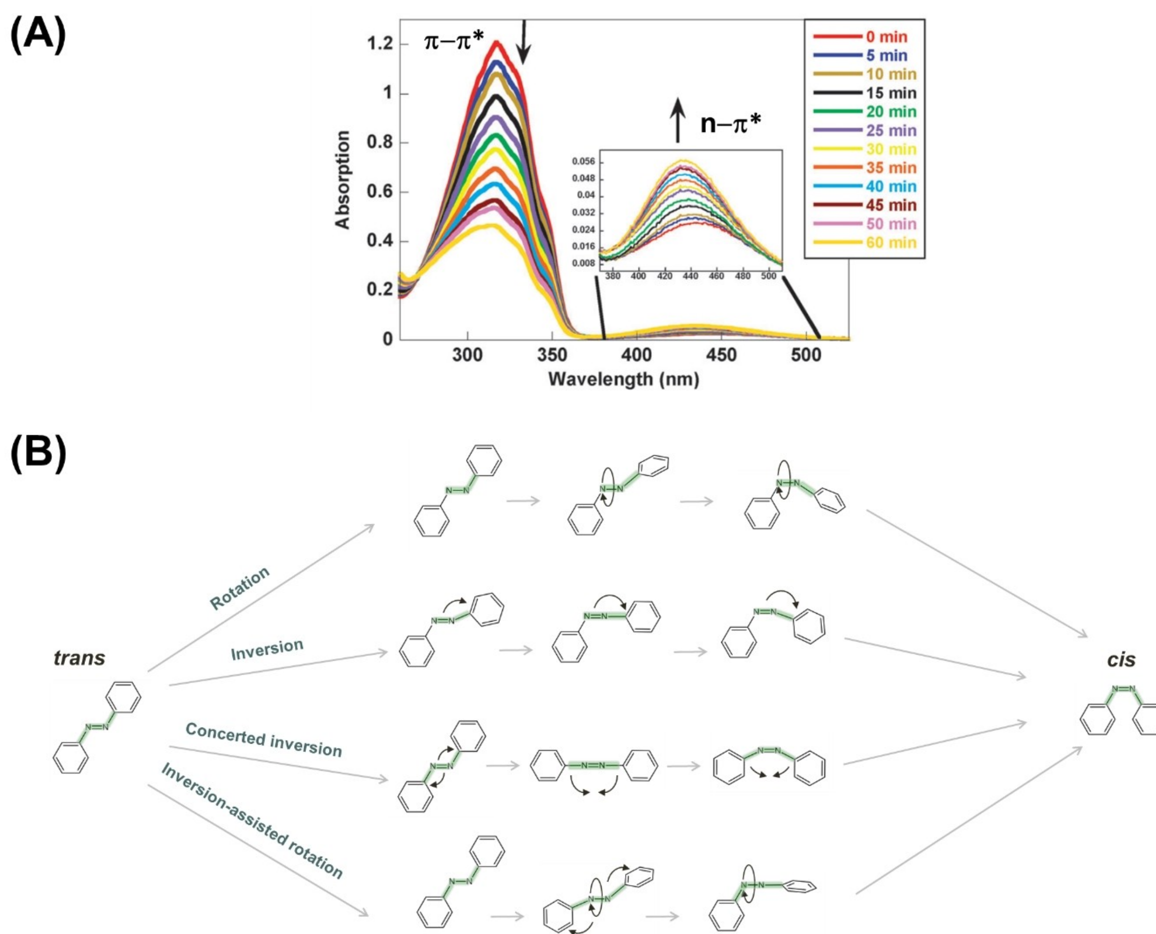

Figure 1. Photochromism of azobenzene. (A) Evolution of the electronic absorption spectra of *trans*-azobenzene upon conversion to *cis*-azobenzene. The intensity of the UV band greatly diminishes while that of the blue slightly increases. (B) Proposed mechanisms of the *trans-cis* isomerization of azobenzene. Adapted with permission from Ref. [16]. Copyright: 2023, Royal Society of Chemistry.

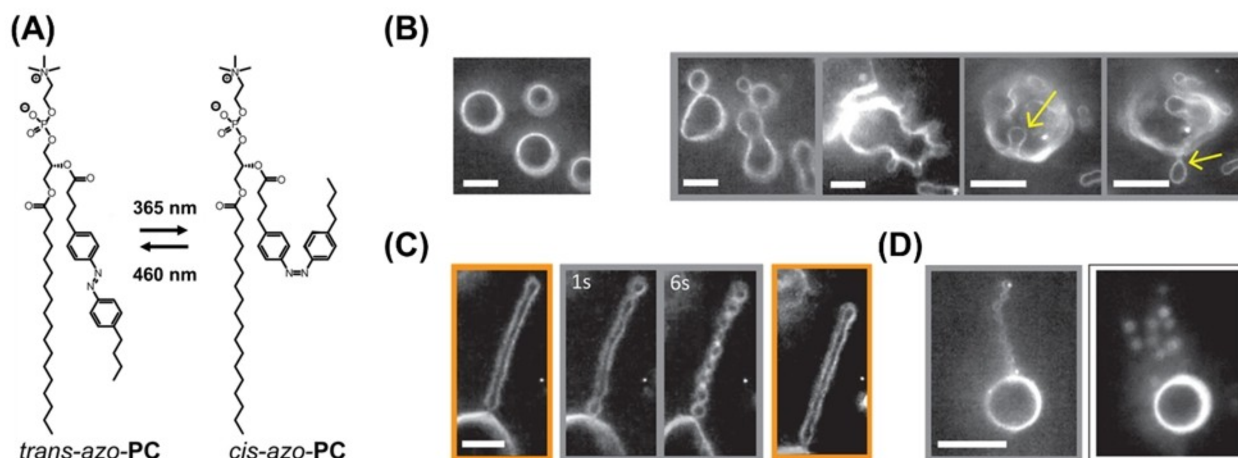

Figure 2. Light-induced membrane deformation. (A) Chemical structure and isomerization of 18 : 0-azoPC. (B) Membrane buds and (C) pearling structures induced after *trans*- to *cis*-isomerization. (D) Fission of a pearling structure after light irradiation. Scale bars: 10 μm. Adapted with permission from Ref. [25]. Copyright: 2023, American Chemical Society.

modifications can alter lipid packing which can lead to membrane deformations[25,26] but can also increase the membrane permeability[31,32] as used for drug delivery, which is further discussed below.

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2.2. Control of membrane domains by photo-lipids

It is well accepted that the biological membrane is very heterogeneous with a large number of lipid components. To mimic this situation, model membranes with an increasing number of lipid components are investigated including phase-separated membranes to access membrane domains. In particular, liquid-liquid phase separated membranes have significantly contributed to our understanding on the composition, size and dynamics of the so-called liquid ordered (l_o) domains.[33–35] To address the question of domain reorganization and to be able to manipulate such domains, photo-lipids have been added to the lipid mixtures.

Caged lipids, which bear a cleavable group that can be removed by light, are mainly used to investigate signalling pathways,[36] but these compounds have been seldomly applied to modify domains in artificial lipid bilayers. Carter-Ramirez and colleagues reported the insertion of caged ceramides (Figure S3) in phase-separated DOPC/SM/cholesterol (8:7:4, n/n) supported lipid bilayers.[37] By means of atomic force microscopy imaging, they evidenced that the light-induced removal of the photo-labile group modified the morphology of l_o domains, which exhibited small pockets that presented a similar height to that of the l_d phase. Although a spatio-temporal control of domain morphology was achieved, the reaction required long irradiation times (10–50 minutes) and was not reversible. In that sense, azobenzene photo-lipids, due to the reversibility of isomerization, which can occur several times without fatigue,[14,16] appear more suited to investigate domain organization especially that several studies reported the application of these probes in this field.

For instance, Yasuhara *et al.*[38] added 5 mol% of an azobenzene-modified cholesterol in DOPC/DPPC/cholesterol (2:2:1, *n/n*) phase-separated vesicles. The azobenzene group was attached to the OH-group of cholesterol (AzCh, Figure S1B). UV irradiation modified the shape of these azobenzene-modified cholesterol GUVs and led to a complete disappearance of the l_o domains. Likewise, Hamada *et al.*[39] introduced 40 mol% of the photo responsive amphiphile KAON12 in GUVs of similar composition but with a different lipid ratio, *i.e.* DOPC/DPPC/cholesterol (2:1:2, *n/n*). The azobenzene moiety was attached to the headgroup of the amphiphile (Figure S2B). In this case, UV irradiation increased the area of the l_o domains. This apparent discrepancy between the results may be attributed to the position of the azobenzene moity in the headgroup region. Modifying lipid head groups limits the control on the membrane properties in the vicinity of the polar region and is known to alter lipid l_o/l_d partitioning.[40,41]

More recently, Urban *et al.*[42] produced phase-separated vesicles using several lipid mixtures composed of DPhPC, cholesterol and 18:0-azoPC, in which the azobenzene is attached on the fatty acid (Figure S1C). In this ternary lipid mixture, the size of l_o domains increased if the concentration of 18:0-azoPC was increased from 20 to 60 mol% while keeping the cholesterol concentration constant. These l_o domains could be repeatedly controlled with light: upon UV irradiation, which isomerizes the 18:0-azoPC in the *cis*-configuration, a demixing of the l_o phase was observed. Upon blue light irradiation which restored the photo-lipids in the *trans*-configuration, l_o domains became again visible. Kol and colleagues reported similar observations using photoswitchable ceramides (ca-Cer, Figure S1A) and a different experimental approach.[43] They prepared DOPC/cholesterol/SM_{18}/ca-Cer supported lipid bilayers (10:6.7:5:5, *n/n*) which were imaged by atomic force microscopy following light irradiation. *Cis*-isomerization led to a decrease of the l_o phase area that was characterized by the appearance of small holes in the l_o domains, termed "l_d lakes". Isomerization of ca-Cer back to *trans*-configuration increased membrane order, visible by a shrinkage of the l_d lakes. The changes occurred a few seconds after light irradiation and could be repeated over multiple cycles.

In general, the structure of photo-lipids with the azobenzene group in the fatty acid was found to significantly influence the membrane reorganization. The Trauner group reported on the synthesis of photo-ceramides (Figure S1A) bearing the azobenzene group at different positions on the fatty acid.[44] After inserting these compounds in phase-separated supported lipid bilayers, they recorded fluorescence and atomic force micrographs revealing that domain rearrangement is dose-dependent and more pronounced if the azobenzene is deeply embedded in the membrane. In a similar study, they synthesized photo-switchable sphingolipids with different polar head groups while the azobenzene was kept in a constant position of the fatty acid (Figure S1F). Regardless of the size and nature of the polar headgroup, all compounds presented the ability to remodel DOPC/cholesterol/SM_{18}/photo-sphingolipid (10:6.7:5:5, n/n) phase-separated supported lipid bilayers and the light-induced *cis*-isomerization of the azobenzene group increased the line tension and height mismatch at the l_o/l_d phase boundary. The polarity of the sphingoid backbone had a great impact on the architecture of l_o domains. Compounds bearing a free sphingoid were found to induce a demixing of the l_o phase upon *cis*-isomerization. Conversely, when the OH group of the sphingoid was blocked, the demixing of l_o domains was less pronounced. In that case, the morphology of the l_d phase was more impacted as l_o lakes were formed upon *cis*-isomerization. It was suggested these discrepancies could be the result of a different repartition of the compounds between the l_o and l_d domains. In particular, the absence of possible H-bonding at the sphingosine backbone may have caused a redistribution of the compounds in the l_d phase, which limited their ability to remodel l_o domains.[45]

In most studies, membrane domains were observed with one fluorescent dye which does not allow to distinguish between the outer and inner leaflet of a bilayer. By means of fluorescence microscopy, the Pohl group investigated domain formation in both leaflets using two different lipid dyes.[46] Two lipid fluorophores which preferentially partition in the l_d phase were inserted in the top and bottom leaflets of phase-separated free-standing lipid bilayers containing 17 mol% of photo-switchable diacylglycerol (Pho-DAG) (Figure 3A). The domains in both leaflets registered (Figure 3B) and, as observed in other studies,[42,44,45] *trans-* to *cis*-isomerization led to a fluidification of the membrane while the opposite triggered the formation of ordered domains (Figure 3C). They also observed a size-depend-

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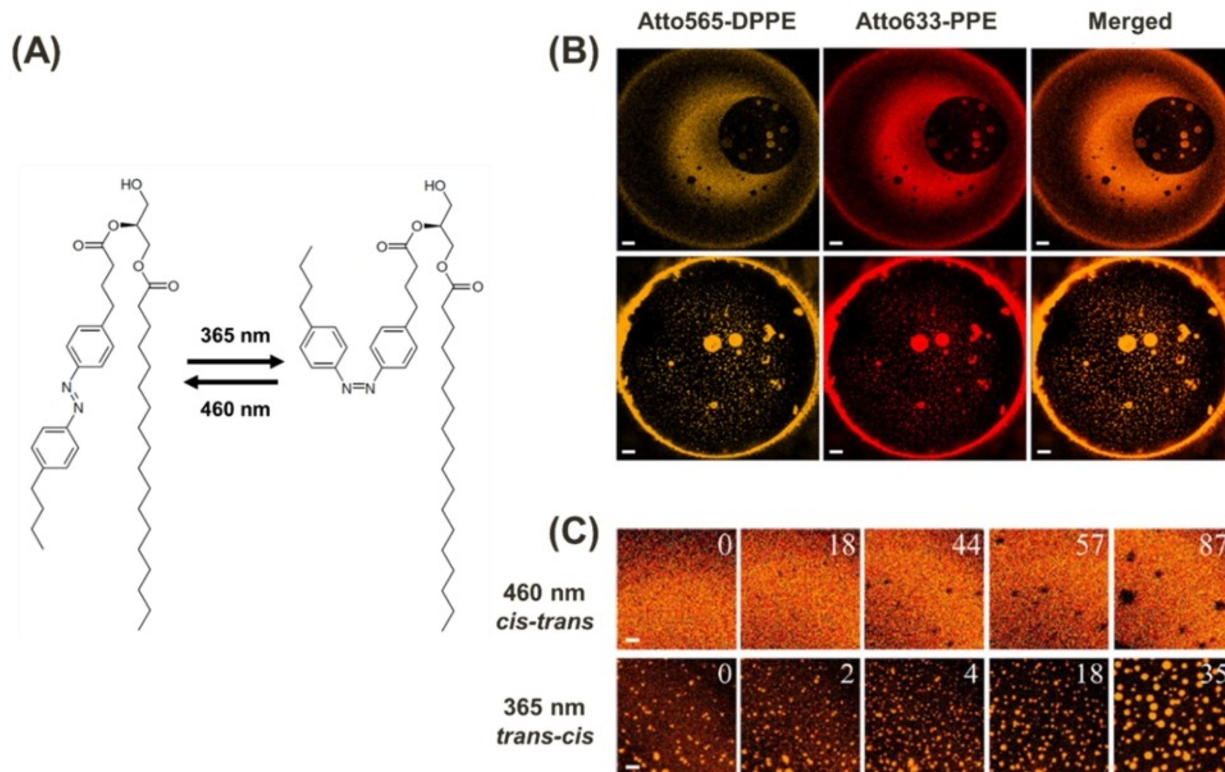

Figure 3. Light-induced domain reorganization in phase-separated bilayers. (A) Chemical structure and isomerization of Pho-DAG. (B) Domain registration observed in phase-separated GUVs. The external leaflet is labelled with Atto565-DPPE and the internal one with Atto633-PPE. Scale bar: 20 μm. (C) Light-induced formation of ordered domains (top) and disordered domains (bottom). The time after irradiation (in seconds) is indicated in the upper right corner of the images. Scale bar: 5 μm. Adapted from Ref. [46] with permitted reuse. Copyright 2023: American Physical Society.

ent domain diffusion[46] and later demonstrated that isomerization inhibits domain fusion as a result of a reduction of line tension at the l_o/l_d phase boundary.[47]

In general, the observed disordering of the *cis*-isomerized azobenzene lipid 18:0-azoPC not only changes the membrane structurally and mechanically but also facilitates other membrane-confined processes such as fusion, as a result of splayed lipids, whose tails transiently contact the headgroup region of the bilayer.[48,49]

Glycosphingolipids are a minor but pivotal component of ordered domains. They constitute the entry site for various pathogens including bacterial toxins, which bind with very high specificity to individual glycosphingolipids on the surface of cells.[50,51] These bacterial proteins such as cholera toxin, which recognizes the ganglioside G_{M1}, have been widely used as l_o phase markers in *in vitro* studies using l_o/l_d coexisting membranes.[52–56] To our knowledge, azobenzene derivatives of glycosphingolipids serving as receptors for bacterial proteins have as yet not been investigated. These molecules are, however of great interest as they allow to alter domain reorganization reversibly by light, which may also impact the lipid-protein interactions.[57,58]

An influence of protein binding as a result of lipid membrane domain formation was shown by Carter-Ramirez *et al.*[59] They used caged ceramide molecules in fluid POPC-supported lipid bilayers (1:9, *n*/*n*) that was randomly distributed in the bilayer. Upon UV light irradiation, uncaging occurred and small ordered ceramide domains were formed that phase separated from the fluid membrane. Photo-uncaging of the caged C16-ceramide (Figure S3) in POPC membranes doped with the ganglioside G_{M1} (9:1, *n*/*n*) resulted in ordered ceramide enriched domains to which the B-subunits of cholera toxin bound. However, this process is very slow (30–60 minutes) and not reversible unlike in the case of azobenzene lipids, as uncaging of these lipids is an irreversible process, and thus reversible domain dispersion cannot be investigated.

Recently, the globotriaosylceramide (Gb_3) which constitutes a natural binding site for Shiga toxin,[60] was functionalized with the azobenzene group at different positions on the fatty acid (Figure S1D).[61] Concentrations of 5–20 mol% of the photo-Gb_3 glycosphingolipids in a membrane reversibly triggered the demixing and mixing of phase-separated supported lipid bilayers in a dose-dependent manner. These concentrations are generally used also for other azobenzene lipids.[42,44,45] The impact of the azobenzene isomerization on domain reorganization was largest for Gb_3 molecules with the azobenzene group at the end of the fatty acid (Figure 4A), which is in agreement with a previous result using photo switchable ceramides.[44] This observation can be rationalized by the fact that the structure of the fatty acid of the glycosphingolipid Gb_3 influences the miscibility with l_o and l_d phase lipids.[62] It is well conceivable that the *trans*- and *cis*-configuration of the Gb_3 species partition

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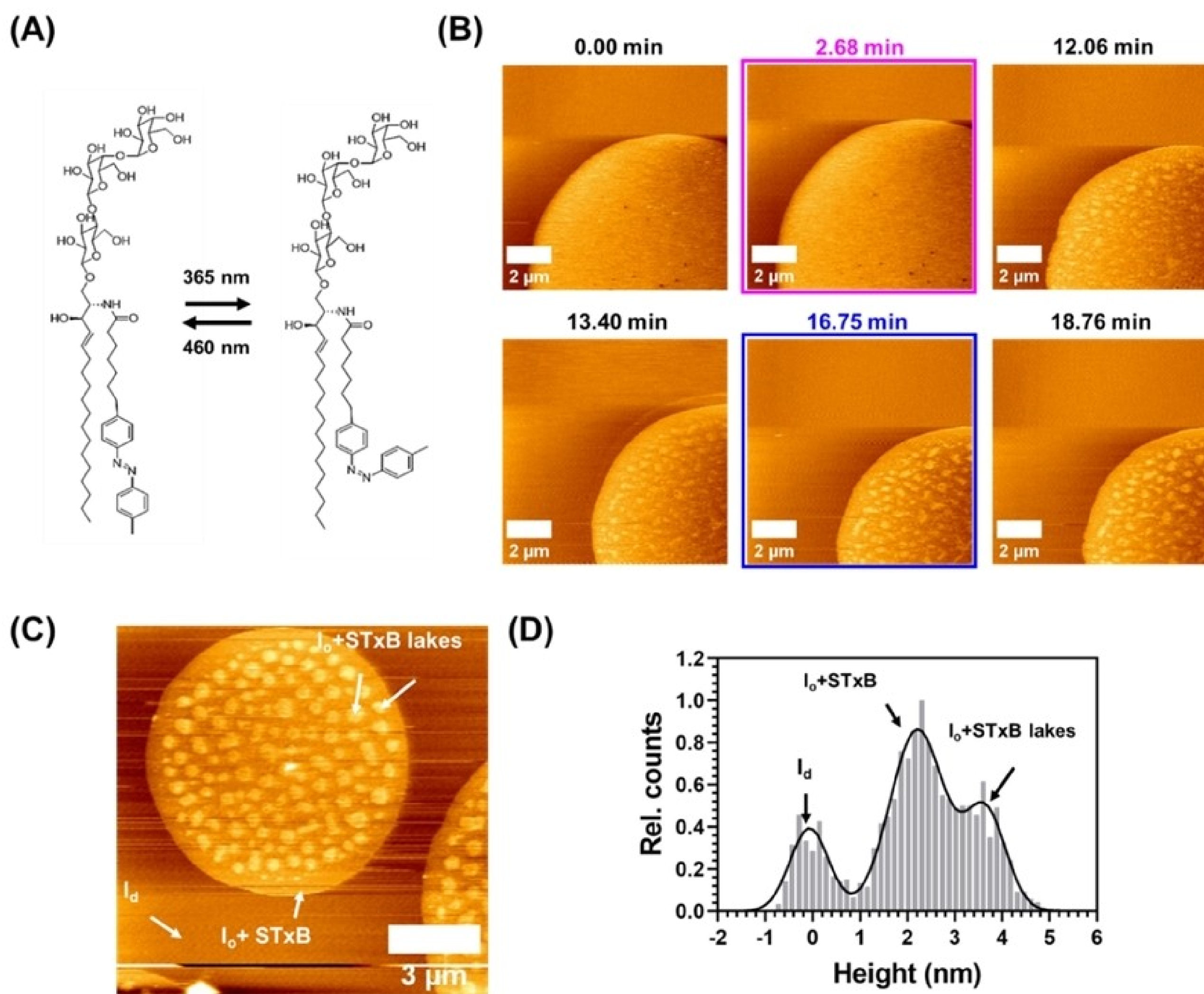

Figure 4. Light-induced redistribution of Shiga toxin B in a liquid-liquid phase-separated supported lipid bilayer. (A) Chemical structure and isomerization of a photo-Gb_3. (B) Time-lapse of domain rearrangement after UV and blue light irradiation. (C) AFM micrograph of a domain with protein clusters and (D) histogram analysis. Adapted from Ref. [61] with permitted reuse. Copyright 2023: Wiley-VCH GmbH.

differently in the l_o and l_d phase. In addition, the headgroup of the sphingolipid, experiencing a different H-bonding as a function of the *cis*- to *trans*-isomerization can also influence the membrane remodelling.[45]

The Gb_3 azobenzene derivative with the largest domain influence was then used to investigate the influence of *trans*- to *cis*-isomerization on the binding of the B-subunits of Shiga toxin (STxB). Prior to light-irradiation, STxB bound solely on the ordered domains of liquid-liquid phase-separated bilayers. *Trans*- to *cis*-isomerization caused a slow redistribution of the protein with the formation of protein clusters. The clusters, which became discernible 10 minutes after UV irradiation, were named "protein lakes" (Figure 4B). Interestingly, in contrast to the results obtained for protein-free ordered domains, where *cis*- to *trans*-isomerization dispersed the l_o domains, 'protein lakes' were not dispersed (Figure 4B–C). We discussed this finding in terms of a limited diffusion of the protein-bound Gb_3 lipids.

2.3. Impact of photo-lipids on membrane proteins

Biological membranes are the interfaces that participate in the regulation of the cells' exchange with the environment given their ability to sense and transduce external signals. Signals are primarily transduced by membrane proteins being a major element of eukaryotic plasma membranes. More than 60% of therapeutic drug targets interact with these membrane proteins.[63,64] External stimuli such as tensile, compressive and shear stresses can induce a modification of the shape and mechanical properties of the membrane, which can be detected by membrane proteins.[65,66] In this context, the interaction of a number of photo-switchable compounds with proteins have been investigated. The interested reader is referred to two comprehensive reviews covering this topic.[13,17] In this review we limit ourselves to the impact of photo-lipids on membrane structure and organization, and thus we will only discuss

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membrane proteins that react to changes in the physico-chemical properties of the bilayer in this section.

A simple transmembrane peptide is gramicidin A. Pfefferman *et al.*[67] showed a relationship between gramicidin A channel activity and the physico-chemical properties of the membrane. Single channel, micro-aspiration and small-angle X-ray scattering measurements were performed on DOPC or DPhPC planar bilayers and lipid vesicles doped with 10 mol% of the photo-lipid OptoDArG. OptoDArG is composed of a glycerol backbone bearing two esterified azobenzene-containing acyl chains (Figure 5A). Their results revealed that *trans*- to *cis*-isomerization led to a decrease of spontaneous curvature, hydrophobic thickness, and bending rigidity. Concomitant with these changes of physical properties of the bilayer, an increase in bilayer conductance as well as an increase in the lifetime of channel openings were observed (Figure 5B–C).

Channels that are directly influenced by the physico-chemical properties of a membrane are mechanosensitive channels. The mechanosensitive channel of large conductance (MscL), which protects bacteria against strong osmotic variations, is a prototype of mechanosensitive protein channels.[68] Although it was suggested that the protein senses variations of membrane thickness,[69] the exact mechanism by which MscL is activated is not fully understood.[70] In an early study, it was found that the isomerization of di-(5-{[4-(4-butylphenyl)azo]-phenoxy}pentyl)phosphate (4-Azo-5P), in which both acyl chains are functionalized with an azobenzene group (Figure S2C), can increase the opening probability of MscL in a reversible manner following long irradiation times (30–140 seconds).[71] Later on, Booth and co-workers[72] reconstituted MscL in DOPC/DOPG (1:1, *w/v*) lipid vesicles doped with up to 10% (w:v) of 4-Azo-5P. They found that the light induced 4-Azo-5P switch that manipulates the lipid bilayer properties influenced the folding and assembly of the pentameric bacterial mechanosensitive channel MscL. Just recently, both the MscL conformation and its channel activity were studied in DOPC nanodiscs doped with the well-studied 18:0-azoPC (1:1, n/n) with an approach combining infrared spectroscopy (IR) and patch-clamp experiments.[73] Whereas IR spectroscopy revealed differences in the amide I range indicating reversible conformational changes in MscL as a direct consequence of light switching, the patch-clamp results demonstrated that MscL channels can be activated by blue-light illumination of *cis*-18:0-azoPC leading to spontaneous openings of the channel for a few milliseconds.

18:0-azoPC turned out to be an invaluable tool to investigate also other mechanosensitive proteins. By means of NMR spectroscopy, the Glaubitz group studied the impact of 18:0-azoPC on diacylglycerol (DAG) kinase,[74] a membrane protein found in *E. coli* that was suggested to participate in the response to tensile stress induced by osmotic variations via its implication in glycerol metabolism.[75] Isomerization of POPE/POPG (4:1) vesicles containing 15 mol% of 18:0-azoPC increased the membrane fluidity both in the absence and

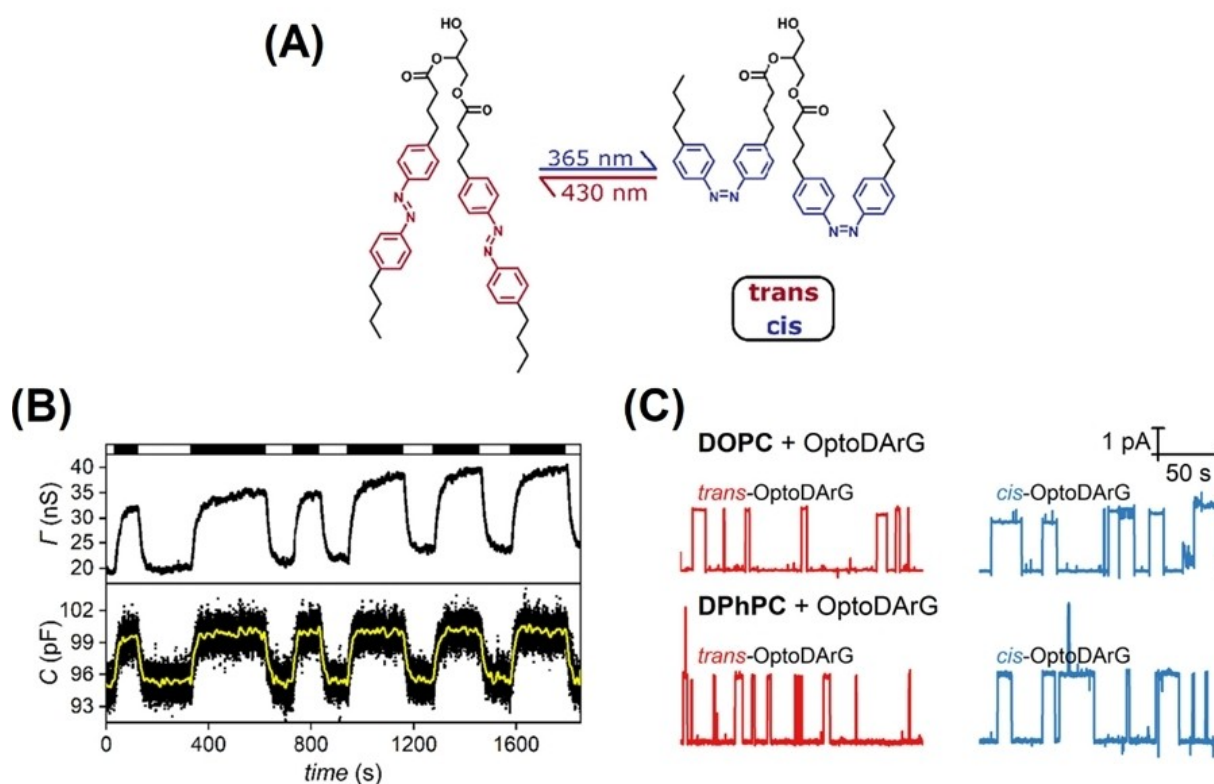

Figure 5. Impact of OptoDArG on the activity of gramicidin A. (A) Chemical structure and light-induced isomerization of OptoDArG. (B) Modification of membrane conductance (top) and capacitance (bottom) and (C) channel opening events of gramicidin A in DOPC and DPhPC, respectively doped with OptoDArG in either *trans*- or *cis*-configuration. Adapted from Ref. [67] with permitted reuse. Copyright 2023: Elsevier B.V.

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presence of DAG kinase. While a modification of the protein conformation was observed by NMR spectroscopy upon isomerization, the impact on its enzymatic activity was not investigated.

All these *in vitro* studies were performed on peptides and proteins of bacterial origin. For eukaryotic mammalian cells, photo-lipids were primarily used to study signalling pathways leading to apoptosis,[76] cytokine production,[77] lipid signalling,[78,79] and biosynthesis[43,80] or nociception.[81,82] Rather recently, the impact of photo-lipids on transient receptor potential canonical channels (TRCP) was also reported.[83–85] Although it was widely suggested that TRCP channels are activated by DAG,[86,87] the mechanism of activation of these channels is not fully understood, given the fact that a direct interaction with DAG is yet to be reported.[88] Lichtenegger *et al.*[83] demonstrated that the TRCP3 activity could be regulated using OptoDArG in human embryonic kidney 293 cells (HEK293). The *trans-* to *cis*-isomerization of this photo-lipid increased the membrane current density. Based on a homology model of human TRPC3 based on the cryo electron microscopy study of the transient receptor potential cation channel V1 (TRPV1) and the direct mutation of the amino acid residue 652 (G652A) they concluded that this amino acid is pivotal for a lipid-induced activation of the channel. In a similar manner, photo-lipids termed PhoDAGs were found to modulate the activity of TCRP3 and other TRPC isoforms in mouse vomeronasal sensory neurons (VSNs) and HEK293 cells. PhoDAGs has a similar structure as OptoDArG but only one fatty acid chain is substituted with the azobenzene (Figure S1H). Its isomerization from *trans* to *cis* caused channel opening events and altered cellular calcium ion levels.[84] Nonetheless, OptoDArG appears to be more efficient than PhoDAGs to control the TCRP channel activity, probably because of a better stability in the membrane.[85]

3. Drug release based on photo-sensitive vesicles

The *trans*-to *cis*-isomerization of the azobenzene group has been shown to induce regions of low order in the membrane, which impacts the permeability of the membrane. Based on this knowledge, single-chain derivatives, such as trans-4-octyl-4′-(5-carboxyl-pentamethylene-oxy)-azobenzene (8A5, Figure S2D), were introduced early on to design photo-sensitive vesicles. Upon isomerization, a potassium ion leakage was observed.[89,90] These single-chain azobenzene derivatives act, however more as a surfactant.[91,92] To include azobenzene moieties in lipid membranes with a more natural chemical structure, phospholipids with an azobenzene-group in the fatty acid were favoured to produce photo-responsive vesicles. The use of PC vesicles containing up to 50 mol% of photo-isomerisable cholesterol derivatives (AB lipids 1–3, Figure S1B),[32] 10-70 mol% of 4-Azo-5P (Figure S2C)[31] and cell-sized GUVs containing 100% 18:0-azoPC[30] in combination with fluorescence microscopy and patch-clamp experiments revealed that the *trans-* to *cis*-isomerization triggered a significant dye leakage out of the vesicle lumen and a modification of the ion permeability of the membrane in a few seconds. Together with surface tension experiments using a Langmuir-trough showing that the lipid mean molecular area can be reversibly modified upon azobenzene isomerization, it was concluded that elevated membrane fluctuations and lipid mobility result in an increased likelihood for pore formation as a consequence of the change in membrane compressibility.[30]

The ability of photo-sensitive lipids to alter membrane properties, in particular their permeability, opens up a path for applications in pharmacology and drug delivery. In fact, various strategies involving light to release bioactive substances from lipid vesicles have been developed. For more details on this topic, the reader may refer to the following reviews.[93–95] Recently, Chander *et al.*[96] showed that the incorporation of 10 mol% of 18:0-azoPC in small unilamellar vesicles (SUVs), which they called photoactivatable long-circulating lipid nanoparticles (paLNP), leads to vesicles with similar structural integrity, drug loading capacity, and size distribution as the well-established parent long-circulating lipid nanoparticles (LNP) comprising DSPC-cholesterol vesicles (Figure 6A/B). Pulsed light irradiation switched 18:0-azoPC from *trans-* to *cis*-configuration, resulting in a progressive release up to 65–70% of the encapsulated doxorubicin drug within a time window of 24 hours. Treatment of hepatocyte derived carcinoma cells (HuH7) demonstrated that the photoactivation of the paLNP successfully released doxorubicin (Figure 6C) and fluorescence imaging revealed that the cells internalized the drug (Figure 6D). As poly ethylene glycol (PEG) is known to enhance the stability of lipid vesicles and prolong their circulation time,[97] Xiong *et al.*[29] recently inserted PEG lipids in their formulation to produce functionalized vesicles containing 12 mol% of 18:0-azoPC, which were termed "azosomes". With this approach, they were able to limit the spontaneous release of calcein while achieving a controlled and sequential release of the cargo. Azosomes were then used to transport a dopamine agonist to neuronal cells. Cells could be repeatedly activated upon pulsating UV irradiation. Similarly, photo-phosphatidylserine (phoPS, Figure S1E) vesicles were recently introduced for potential immunotherapy applications.[98] In this study, Yang *et al.*[98] exploited the ability of phosphatidylserine to bind Tim-3, an inhibitory receptor, present on the surface of natural killer (NK) cells, which are known to participate in the autoimmune response. Binding of phosphatidylserine containing vesicles to Tim-3 reduced the NK92 cell-mediated killing activity as well as the production of the pro-inflammatory cytokines IFN-γ and TNF-α. Interestingly, when cells were treated with pure phoPS liposomes irradiated at 365 nm, these effects were more pronounced. In contrast, blue-light irradiation restored the NK cell activity. These results show that phoPS repeatedly switches between the *cis-* and *trans*-configuration, resulting in an active/inactive Tim-3 ligand, thus modulating the function of NK cells.

In conclusion, given their ability to control lipid membrane properties, azobenzene derived photo-lipids appear to be a promising nanotool in the field of pharmacology in which lipid vesicles are used to transport bioactive substances.[97] However,

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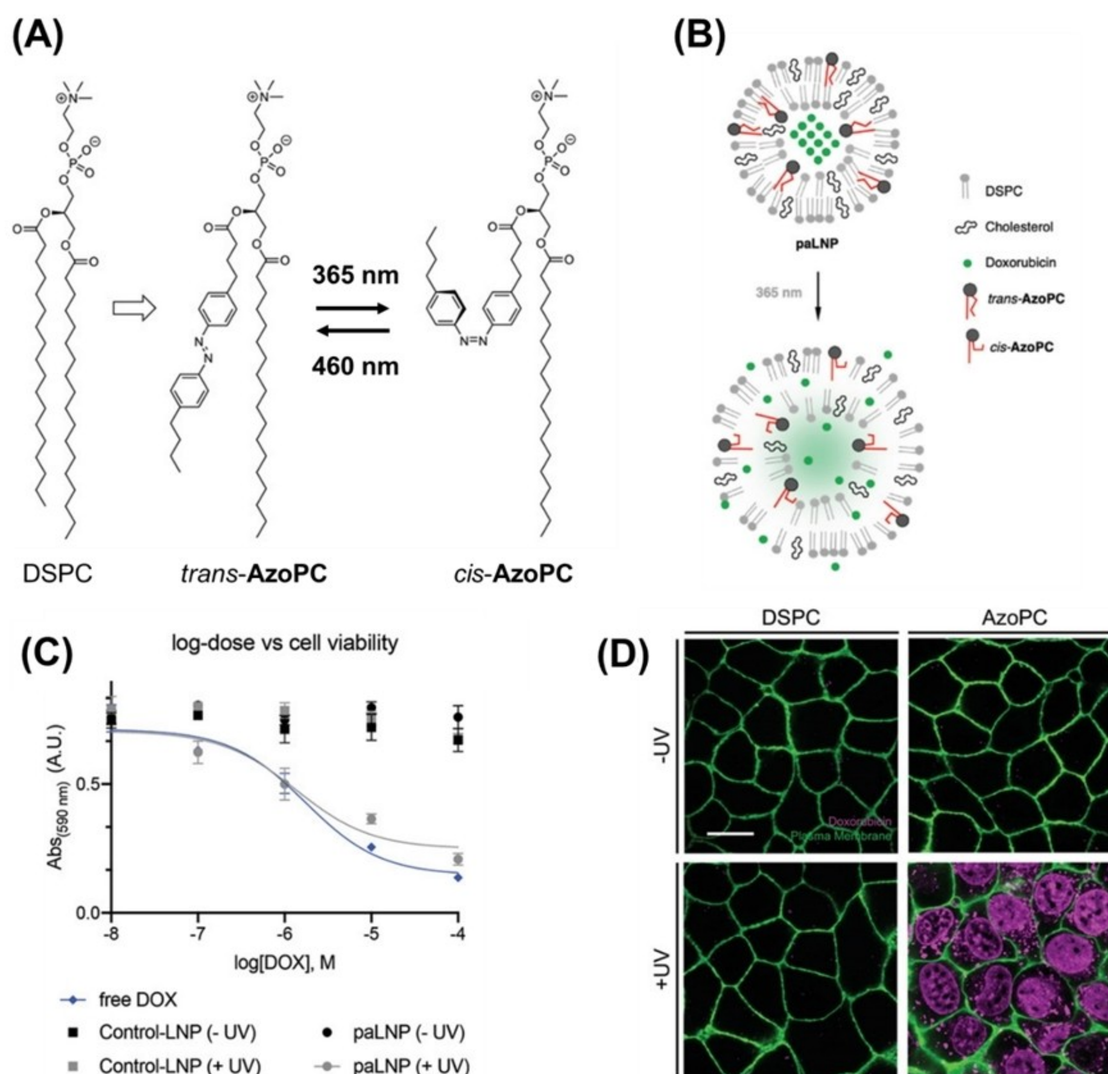

Figure 6. Delivery of doxorubicin into cells using paLNPs. (A) Isomerization of 18:0-azoPC. (B) Photoactivation of the paLNP. (C) Viability of HuH7 cells incubated with control-LNP and paLNP without (−UV) and with (+UV) irradiation. The cells were irradiated for 5 min at 365 nm and then subjected to pulsed irradiation at 365 nm for 24 hours. (D) Fluorescence micrographs of HuH7 cells treated with control-LNP (DSPC) and paLNP (AzoPC) without (−UV) and with (+UV) UV irradiation. Adapted from Ref. [96] with permitted reuse. Copyright 2023: Wiley-VCH GmbH.

one issue that needs to be considered is that UV irradiation is required to trigger the *trans*- to *cis*-isomerization. For biological applications, longer wavelengths are highly desirable to avoid irradiation-induced cytotoxic effects and to penetrate deeper into a cell tissue. Thus, various strategies have been proposed to trigger the azobenzene isomerization using longer wavelengths. A promising approach is to attach voluminous moieties such as chlorine on the planar azobenzene.[99,100] The induced steric hindrance and electron withdrawing ability of chlorine results results in the separation of the n-π^* band of the *trans* and *cis* isomers, allowing a selective excitation with light of specific wavelengths. Further details about the influence of these substituents can be found in these references.[14,16,101]

Recently, Pritzl *et al.*[102] exploited this approach. They introduced a red-shifted version of 18:0-azoPC (red-azoPC, Figure S1C). By replacing the azobenzene by tetra-ortho-chloro-azobenzene, the UV irradiation of the azobenzene moiety was shifted to the longer wavelength regime with a *cis*- to *trans*-configuration at 660 nm. The chlorinated azobenzene group behaved very similarly and the chlorine groups did not influence the ability of the compounds to alter the physico-chemical properties of artificial lipid membranes. Experiments performed *in vivo* with zebrafish demonstrated that lipid vesicles containing 10 mol% of red-azoPC showed excellent systemic circulation resulting in pronounced tissue extravasation 24 hours post-injection, which is typical for long-circulating vesicles. Upon light radiation, doxorubicin was effectively released into the cells.[96]

4. Summary and Outlook

Over the past decade, azobenzene-derived lipids have been devised as nanotools to manipulate structural and mechanical

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properties of membranes in *in vitro* artificial lipid systems as well as cellular membranes. Even if a control of the model membranes' morphology, fluidity, permeability and domain structure was achieved, it is still challenging to fully comprehend the impact of these modifications on domain rearrangement and protein organisation at the membrane surface. While isomerization often altered the activity of proteins, its impact on their distribution within coexisting l_o/l_d membranes is still only partially understood, even though the formation of protein domains was recently achieved.

To envision working with living cell membranes, it is essential to circumvent the limitations of the cytotoxic UV irradiation required to initiate photo-lipid isomerization. In that sense, the substitution of the azobenzene to shift its absorbance bands towards longer wavelengths appears a straight forward synthetic strategy. Promising red-shifted photo-lipids were recently introduced, opening a path for *in vivo* studies and biological applications.

Supporting Information

The authors have cited an additional reference within the supporting information.[103]

Author Contributions

L. S. wrote the manuscript with the support of C. S.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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REVIEW

An optical control: The structure and organization of eukaryotic plasma membranes has been widely discussed since the introduction of the fluid mosaic model. Light-sensitive lipids were recently introduced in artificial lipid systems and cells to study lipid-lipid and lipid-protein interactions. Here, we review the application of these probes to investigate membrane properties and lateral organization.

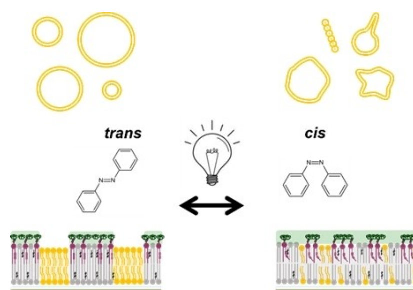

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Photo-Lipids: Light-Sensitive Nano-Switches to Control Membrane Properties

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