Abstract
Optical control of phospholipids is an attractive option for rapid, reversible, and tunable manipulation of membrane structure and dynamics. Azo-PC, a lipid with an azobenzene group within one acyl chain, undergoes a light-induced trans-to-cis isomerization and thus arises as a powerful tool for manipulating lipid order and dynamics. Here we report on vesicle-scale micropipette measurements and atomistic simulations to probe the elastic stretching modulus, water permeability, toughness, thickness, and membrane area upon isomerization. We investigated both dynamics and the steady state properties. In pure azo-PC membranes, we found that the molecular area in trans was 16% smaller than in cis, the membrane’s stretching modulus $k_A$ was $2.5 \pm 0.3$ times greater, and the water permeability $P_W$ was $3.5 \pm 0.5$ times smaller. We also studied mixtures of azo-PC with a miscible, unsaturated lipid DOPC. Atomistic molecular dynamics simulations show how the membrane thickness, chain-order, and correlations across membrane leaflets explain the experimental data. Together these data show how rotating a single bond changes the molecular-scale and membrane-scale properties. These results will be useful for photopharmacology and for developing new materials whose permeability, elasticity and toughness may be switched on demand.

Introduction
Thanks to their fluidity and flexibility, lipid bilayer membranes are the site of crucial biochemical processes that are essential for life. In live cells, lipid bilayer membranes carry out functions such as transducing chemical or mechanical signals, phagocytosis, endocytosis, cell junctions in tissues, and active/passive transport. These cellular functions also inspire the design of synthetic materials that could find application in stimulated release or uptake of nutraceuticals, stimulated switching from fluid to solid, or even directed motility. The search for ways to switch membrane properties rapidly and reversibly has led to development of stimuli-responsive lipids. Light is among the most sought-after stimuli used in photoswitchable chemistry owing to its unique capabilities for ultra-fast temporal control, precise spatial control, and dose level control.¹ Photoswitchable molecules contain light-responsive moieties such as azobenzene that alter a bond orientation by photoisomerization, which can result in dramatic changes in overall membrane properties.² In live cells, photoswitchable lipids based on azobenzene create opportunities for photopharmacology by which, for example, lipid-based signaling pathways can be turned off or on by switching one bond in the lipid’s tail between the cis and trans states.³⁴ In bio-inspired synthetic membranes, photoswitchable lipids have the potential to turn on or off permeability to particular solutes,⁵ rupture,¹² trigger a change of membrane shape,¹³¹⁴ or shift phase coexistence.¹⁵
Experimental measurements of shape, elasticity and dynamics require ‘giant’ vesicles that are approximately the sizes of cells, i.e., at least 5 µm in diameter, to allow optical imaging and mechanical measurements in situ. Vesicles that were partially functionalized with azobenzene (azo) were introduced in the 1980s but were below the size for imaging and manipulation.16,17 The first giant unilamellar vesicles (GUVs) had azobenzene incorporated into the polar headgroups of some of the lipids.13,15 More recently, Pernpeintner, et al. developed a two-tailed, azo-modified phospholipid, 1-stearyl-2-[(E)-4-(4-((4-butylphenyl)diazanyl)phenyl)butanoyl]-sn-glycero-3-phosphocholine, referred to as azo-PC. Azo-PC forms GUVs with up to 100 mol% azo-modified lipid,14 which opened the door to measuring morphology and mechanics for the full range of compositions. Azo-PC, shown in Fig. 1A, consists of a phosphatidylcholine headgroup, one saturated acyl tail (stearoyl, 18:0) and one azo-modified tail. After exposure to blue radiation (wavelength = 465 nm), the lipids remain in the straight trans configuration. With exposure to UV (365 nm), a fraction of the azo-PC switch to the metastable cis configuration with a kinked tail. In cis, the mean diameter of sub-micron vesicles increased by a few % and in GUVs the bending modulus decreased roughly 5-fold.14,18 Later work from those authors and collaborators reported on mixtures of azo-PC with a non-photoactive DPhPC lipid containing bulky methylated tails. They found a blue shift in the trans absorption peak as the molar fraction of azo-PC was increased, indicating association of azo-PC lipids as H-aggregates and providing a way to measure molar fraction in situ.18 Pure azo-PC membranes were also found to become transiently permeable to a fluorescent dye during photoisomerization.11 These exciting results inspire further questions about the response to light, namely, how much do the per-molecule area and thickness change, how does the inter-molecular packing change, and at the continuum scale how do elasticity, toughness and permeability change? How fast are these changes and what sets the rates?

Here we report on the time-dependent change of the material properties of membranes composed of the azo-PC photoisomerizing lipid. Atomistic simulations provide a crucial link between our continuum-scale experiments and the molecular-scale changes. We study pure azo-PC membranes as well as mixtures with the monounsaturated lipid 18:1 DOPC, which resembles cis azo-PC. Photoisomerizing azo-PC significantly changes lipid packing and membrane properties without phase separation. We investigated dynamics of the response and the steady state properties, all as functions of molar fraction of azo-PC ($X_{azo-PC}$). As in prior studies of LUVs,19 the GUV surface area expanded reversibly for each cycle of UV and blue excitations. We found that the area change followed single-exponential dynamics with a $1/e$ time ($\tau$) that ranged from 4 to 20 s depending on intensity and composition. Based on our measurements of area and $\tau$, we found that the steady-state fraction of the cis isomer ranged from 45%-75% depending on the excitation intensity. In steady state measurements with pure azo-PC, we found that the total membrane area in trans was up to 12% smaller than in cis, the stretching modulus $k_A$ was 2.5 ± 0.3 times greater, and the water permeability $P_W$ was 3.5 ± 0.5 times smaller. In mixtures of azo-PC and DOPC, the ratio of $k_A$ values for trans vs. cis increased rapidly with $X_{azo-PC}$, showing that the isomerization-driven stiffening is a cooperative effect. We found no evidence of enhanced glucose or sucrose permeability, except for indirect evidence of limited glucose exchange with pure azo-PC. This finding agrees with a recent study of azo-PC using sugars20 but disagrees with prior work using fluorescent solute,11 which suggests that photoactivation of the solute might play a role in the latter case.

In our simulations, we measured values of area change per lipid and stretching modulus that were comparable to the experiments. We also found that the trans state had greater membrane thickness, larger chain order parameter ($S_{CD}$), and stronger nearest-neighbor peaks in the radial distribution function $g(r)$. These data shed light on the increased $k_A$ and lower $P_W$ of trans. To our surprise, though, the
experimental values of $\tau$, $k_A$, and $P_W$ were non-monotonic functions of $X_{azo-PC}$. Simulations explained these trends. The potential of mean force for trans-to-cis has a barrier that depends on the cis or trans state of the neighboring lipids, so that the isomerization rate should vary with composition; we compare this finding to the experiments. Finally, the simulations showed a striking tendency of the azo-PC lipids to align opposite to DOPC lipids in the other leaflet, which reduces packing frustration when $X_{azo-PC} = 0.5$. The simulation results nicely explain the variation of $k_A$ and $P_W$ with $X_{azo-PC}$.

Together, our experiments and simulations show how changes to a single bond alter the molecular shape and in-plane order, and thus alter structure, elasticity, and permeability for the entire membrane. We have elucidated the time- and energy scales for these changes and we discuss how azo-PC may be mixed with a passive lipid to tune the response. The results pave the way toward understanding stimulated response in photopharmacological settings and in developing new materials whose permeability, elasticity and toughness may be switched on demand.

RESULTS AND DISCUSSION

Membrane area expansion and contraction

Figure 1 shows the most pronounced effect of optical excitation: a rapid expansion of the membrane area on exposure to ultraviolet light (UV), which excites trans-to-cis. On exposure to blue light (exciting cis to trans), the area returns to the original value. For accurate measurements of membrane area and interior volume, we held the vesicles in place using micropipette aspiration (see Fig. S1.) We applied a tension of 1 mN/m, which gave the vesicles a well-defined shape of a sphere plus a cylindrical ‘tongue’ and a spherical cap, extending into the pipette. With this known geometry, we could measure the membrane area and interior volume. We continuously shone blue light (intensity of 9 mW/cm²) for imaging. Immediately after exposing GUVs to UV intensity of 8 mW/cm² (with blue light still on), the ‘tongue’ inside the pipette extended (Fig. 1B) and the membrane area increased by an amount $\Delta A$ (Fig. 1C). After the UV was turned off, the lipids reverted to the trans configuration and the area reduced to its original value, $A_{init}$. Under these same UV conditions, the absorbance spectrum of sub-µm vesicles formed
by extrusion shifted to a new absorbance spectrum, indicating trans-to-cis isomerization of azobenzene (Fig S4).18,21 We further found that as $X_{azo-PC}$ was increased, the trans absorbance peak shifted toward the blue, which shows that the azo-PC is successfully incorporated into the membrane. As described previously, this blue shift also shows that the trans azo-PC fraction associated with one another and formed H-aggregates, whereas cis did not.18

Figure 1D shows the steady-state membrane area change with UV exposure, $\Delta A/A_{ini}$, which we call $\alpha_{\infty}$. As expected, pure DOPC membranes showed no response to UV. With increasing $X_{azo-PC}$, the value of $\alpha_{\infty}$ increased to a maximum value of approximately 0.07 (UV intensity 8mW/cm²). Four cycles of UV on/off produced a highly repeatable and reversible response. Repeating these measurements with a greater applied tension, 3 mN/m, had no discernible effect on $\alpha_{\infty}$ (Fig. S3). The value of $\alpha_{\infty}$ did, however, depend on the excitation intensity: either increasing the UV intensity or decreasing the blue intensity led to a greater expansion. For example, when GUVs were irradiated by UV light (8 mW/cm²) combined with green light excitation (instead of blue light), the measured $\alpha_{\infty}$ increased by 0.01-0.03 compared to the blue + UV condition. When the UV intensity was increased from 8 to 15 mW/cm², the maximum value of $\alpha_{\infty}$ increased to 0.12 (Fig. 1D). Our observation that the magnitude of the response depends on the light intensities indicates that the azo-PC lipids were in a dynamical steady state, switching back and forth between the two isomers. The response time $\tau$ and the steady state population were set by a competition between the trans-to-cis and cis-to-trans rates, as will be described in the Conclusions section.

We found that the area expansion followed single-exponential dynamics, as shown in Fig. 1C. We fit the surface area to exponential functions of time, $e^{-t/\tau}$, and found the $1/e$ time ($\tau$). (See other examples in Fig. S2) Figure 2 shows the best-fit values with blue+UV excitation (trans-to-cis, $\tau_{BU}$) and with blue-only (cis-to-trans, $\tau_B$). The values were in the range of 4-20 s and varied with both light intensity and $X_{azo-PC}$. With green-light only (no blue), the cis-to-trans relaxation time slowed to several hundred seconds because of the weak absorbance of green light by cis-azo-PC. For the UV-driven trans-to-cis process, the fastest response occurred with $X_{azo-PC} = 0.3$ ($\tau_{BU} = 3.9$ s). Increasing the UV intensity from 8 to 15 mW/cm² had no measurable effect on $\tau_B$ but did enhance the UV-driven trans-to-cis rate by an average factor of 1.5. We observed no significant change in timescale when the GUVs were under higher membrane tension, 3 mN/m (Fig. S3B). These measured timescales are similar to a very recent study of azo-PC GUVs measurements that reported 1/e times of approximately 0.5 s; the response may have been faster owing to a more intense excitation.20 Our timescales are also comparable to azobenzene-functionalized surfactants.22 In the Conclusions section, we show that the $\tau_B$, $\tau_{BU}$ data are related to the rates of isomerization and the steady-state fraction of cis and trans molecules.

All vesicles discussed here had the same solution osmolarity on the inside and outside or had a slightly higher external osmolarity to

![Figure 2](image_url)
make the vesicles floppy. (See Methods in SI). The vesicle interiors were 170 mOsm/kg sucrose and the exterior were a slightly hypertonic (180 mOsm/kg) glucose solution. Despite the presence of impermeable sugar, we found a general trend in which low UV intensity and low $X_{\text{azo-PC}}$ slightly reduced volume by as much as 2%. With high intensity or high $X_{\text{azo-PC}}$, however, the volume slightly expanded with UV, by as much as 2%. When we plotted the fraction volume change as a function of $\alpha_\infty$, we found that the data collapsed to a consistent trend: when $\alpha_\infty \leq 0.06$ (low UV or low $X_{\text{azo-PC}}$) led to modest volume reduction, whereas larger $\alpha_\infty$ led to modest volume expansion (Fig. S5). We noticed that in all GUVs with $X_{\text{azo-PC}} \leq 0.8$, the vesicle volume returned to its initial condition upon turning off the UV (Fig. S5B, C). For $X_{\text{azo-PC}} = 1.0$, to our surprise, the vesicle volume in 8 out of 20 GUVs did not fully return to its initial state, but instead remained in a slightly expanded state. We attribute this change to a transient permeation of glucose from the exterior into the GUV; we will return to this point in the Conclusions section.

Free-floating GUVs also expanded in response to UV. Images of an example azo-PC/DOPC system with $X_{\text{azo-PC}} = 0.5$ are shown in Fig. S6. We measured an increase of the perimeter and interior area of an image slice (Fig. S6) but were unable to extract true membrane area or volume because the full three-dimensional shape was not known. In this example, we also observed intriguing formation of a bud and a tether as the membrane expanded. We saw this in vesicles that were initially floppy but not in ones that were initially spherical (tense), similar to earlier reports with a single-tailed azobenzene-labeled lipid called F-azo.

Our atomistic molecular dynamic simulations of an azo-PC membrane ($X_{\text{azo-PC}} = 1$), show that in equilibrium the area per lipid of the cis isomer was 13% higher than for the trans lipids, which is close to our high-intensity UV data of Fig. 1D. Snapshots of typical lipid configurations show qualitatively how the highly bent tail of the cis form takes up greater area (Fig. 1A). The areas for azo-PC were $64.5 \pm 0.2 \text{ Å}^2$ and $73.5 \pm 0.3 \text{ Å}^2$ and for trans and cis, respectively (Fig. 3A). Our simulated thicknesses, defined as the distance between P atoms in opposite leaflets, were $38.5 \pm 0.1 \text{ Å}$ and $34.5 \pm 0.1 \text{ Å}$ for trans and cis (Figs. 3A, S11). Hence the bilayer for all-cis was thinner than for trans by $4.0 \pm 0.1 \text{ Å}$ or (10.0 \pm 0.4%). Two recent X-ray measurements reported a slightly large shift of $5.3 \text{ Å}^2$25 or a similar shift of $4.2 \text{ Å}$ in de-ionized water (though the thickness shift is enhanced with added salt).26 The simulation results agree with the x-ray data for cis but appear slightly to underestimate the thickness of trans.

For later reference, we note that the area for DOPC found using the same simulation package and force field is in between the two azo-PC values: $69.0 \pm 0.3 \text{ Å}^2$.27 Experimental values for DOPC are similar, ranging from $67.4 \text{ Å}^2$ to $72.4 \pm 0.5 \text{ Å}^2$.28,29 Again for reference, the thickness of DOPC bilayers using the same simulation approach was $36.2 \pm 0.2 \text{ Å}$27 (similar to experimental values, which range between 35.3 Å and 37.1 Å.29-31) Hence, the molecular area and thickness of DOPC are intermediate between cis and trans. These data are shown in Fig. 3A.

### Lipid tilt order, packing, and diffusion

The chain order parameter, $S_{\text{CD}}$, provides a measure of the alignment of the hydrocarbon chains in the membrane with respect to the bilayer normal: $S_{\text{CD}} = \frac{3}{2} \langle \cos^2(\theta) \rangle - \frac{1}{2}$. The order parameter ranges from -0.5 to 1. A value of 1 indicates perfect parallel order and smaller values indicate weaker alignment that is characteristic of more flexible or disordered regions.32,33 In a membrane with $X_{\text{azo-PC}} = 1$, we found that the alkyl chains in a pure-trans membrane were significantly more ordered ($S_{\text{CD}} = 0.4-0.5$) than in a pure-cis membrane ($S_{\text{CD}} = 0.2-0.3$) for all methylene groups in the chain (Fig. S12B). The $S_{\text{CD}}$ values lie within the range that is typical of the liquid-ordered and liquid-disordered phases in lipid membranes.34
Simulation measurements of the in-plane radial distribution function \(g(r)\) show a more structured packing for trans than for cis. In all cases, \(g(r)\) showed two main peaks and a less pronounced third peak corresponding to successive lipid shells. The first shell extends roughly from 4.0 to 7.0 Å and the second one from 7.5 to 10 Å. When \(X_{azo-PC} = 1\), we found an enhanced and slightly inward-shifted peak for trans relative to cis. When \(X_{azo-PC} = 0.5\), we found a significantly higher peak at first- and second-nearest neighbor distances for trans than for cis and DOPC, which indicates a preferential ordering of trans molecules near one another (Fig. S13A, B). This ordering of trans is consistent with the experimentally found blue shift of the azo-PC absorbance that, as described above and in the literature,\(^{18}\) indicated H-aggregate formation.

Motivated by these differences in lipid packing, we used the simulations to investigate whether the environment around a lipid molecule affected the energy change of isomerization. We compared two environments: (1) the target azo-PC immersed in a bilayer where all the neighbors are trans and (2) the opposite: all the surrounding lipids are cis. The C-N=N-C dihedral angle \(\phi\) was selected as the reaction coordinate and was varied from 180° (trans) to 0° (cis)- See Methods for details. Figure 3B shows the potential of mean force (PMF). Five independent replicas are reported in each condition. We observed that the free-energy barrier for trans-to-cis isomerization for an azo-PC surrounded by trans neighbors was approximately 16 kcal/mol (approx. 27 \(k_B T\)). The barrier dropped significantly to 6 kcal/mol (approx. 10 \(k_B T\)) for an azo-PC surrounded by the cis neighbors. The difference between these two PMF curves tells us the free energy changes arising from inter-molecular forces, entropy, and lipid packing effects. We emphasize that these results should be understood only in relative terms: the intramolecular energy differences between cis and trans are not fully accounted for. Prior \textit{ab initio} calculations show that the potential-energy barrier for the cis to trans conversion is over 25 kcal/mol (42 \(k_B T\),\(^{35,36}\) which explains why we found isomerization timescales for cis-to-trans in the range of seconds rather than ns. While we cannot estimate rates of isomerization from our potentials of mean force (PMF), we can infer from the relative free-energy barriers that the rate of trans-to-cis isomerization should be slower with trans neighbors. This greater energy barrier likely arises from the greater membrane thickness and chain order, which means that the lipid packing must change to accommodate the new cis configuration. By contrast, we found negligible change of the free-energy barrier for the cis-to-trans conversion. Therefore, the rate of cis-to-trans isomerization is expected to be independent of the identity of neighbors. We return to these rates in the Conclusions section, below.

With simulations, we measured the mean square displacement of DOPC and azo-PC phosphate groups in a mixed membrane \((X_{azo-PC} = 0.5)\). We found that the diffusion constant for azo-PC was 1.7 times faster in cis than in trans. \((D = 1.25 \text{ and } 0.75 \mu m^2/s, \text{ respectively (Fig. S13C, D)})\). Previous experiments with supported lipid bilayers with \(X_{azo-PC} = 1\) using fluorescence recovery after photobleaching (FRAP) yielded a similar ratio, though the measured values of \(D\) were about 35% lower.\(^{25}\) Interestingly, the DOPC lipids
in our mixed membranes diffused more than 2× faster than azo-PC: $D_{\text{DOPC}} = 2.75 \, \mu m^2/s$ and $2.5 \, \mu m^2/s$ when mixed with cis and trans. The faster diffusion of DOPC vs. cis-azo-PC vs. trans-azo-PC is correlated with a lower chain order parameter $S_{\text{CD}}$ and weaker inter-molecular associations.

Stretching elasticity, cohesion, and water permeability of mixed membranes

We measured the elastic stretching modulus, $k_A$, using both experiments and simulations. In experiments, we used micropipette aspiration to sweep the applied tension and measure the change of membrane area. (See SI for methods.) At the start of the measurements, the thermal undulations were smoothed out by an applied initial tension, $\sigma_0$, in the range of 0.5-2 mN/m. As tension was increased, the measured area strain increased linearly and $k_A$ was obtained from the slope of these curves (See Fig. S7).

Figure 4A shows the results for azo-PC/DOPC vesicles that were continuously exposed to either blue light (filled symbols, trans) or UV + blue (open symbols; cis). The red points and lines show mean and standard error. The inset shows the same data in an expanded scale. (B) The ratio of $k_A$ in the trans (blue-excited) to $k_A$ in the cis (UV+blue). The sharply increasing trend points to collective interactions among the trans lipids.
We found a surprising local peak in $k_A$ at the symmetric point, $X_{azo-PC} = 0.5$. In other words, the behavior was non-monotonic, with $X_{azo-PC} = 0.1$ and 0.8 having unexpectedly low $k_A$. We attribute this behavior to disruption of membrane packing due to mismatch of the chain lengths of cis azo-PC and DOPC. As was pointed out in prior dissipative particle dynamics simulations, adding a small amount of a lipid with a different chain length diminishes $k_A$ because the mismatched chain lengths of adjacent lipids frustrate close packing. However, a 50 mol% composition is a special case: the mis-matched lipids can arrange themselves on opposite leaflets and maintain a uniform thickness, resulting in less packing disruption and a higher value of $k_A$. This results in a trend of $k_A(X_{azo-PC})$ with a peak at 0.5, as in Fig. 4A. It happened that DOPC and cis-azo-PC had very similar $k_A$, so that a symmetric mixture had the same value as $X_{azo-PC} = 0$ or 1, while asymmetric mixtures always had smaller $k_A$ owing to the chain-length mismatch. The blue-excited (trans) membrane also shows a non-monotonic response for the same reason: frustration from chain-length mismatch is minimized at $X_{azo-PC} = 0.5$ by arranging the longer trans azo-PC opposite the shorter DOPC. As will be shown in the next subsection, snapshots from our atomistic simulation nicely show this anti-correlated composition in the two leaflets.

By increasing the applied tension until the vesicles rupture, we measured the total amount of elastic energy stored in the membrane before lysis (known as the cohesive energy density). Surprisingly, this value was dramatically decreased by a factor of $2.5 \pm 0.1$ for trans-azo-PC membrane compared to cis-azo-PC membrane (Fig. S8). In other words, the pure trans-azoPC membrane was stiffer (larger $k_A$) but it was also more brittle and stored substantially less stretching energy than did the cis membrane.

We measured experimentally the rate at which water permeated the membrane in response to a change of osmotic stress. The volume of water flowing per unit time per unit membrane area is known as the permeation coefficient, $P_w$. We measured $P_w$ experimentally by monitoring the change in interior volume versus time after the vesicle was moved into a hypertonic solution, i.e., with osmotic stress pulling water out. (See SI for Methods and Fig. S9.) The volume decayed steadily over a time of 120 s. We found that the vesicle volumes returned to their original values after GUVs were moved back to their starting solution, which indicates the expected reversibility. Figure 5 shows how the water permeability of the membrane varied with $X_{azo-PC}$. The pure-DOPC system had a relatively large $P_w = 40 \pm 2 \mu m/s$ and (as expected) did not respond to UV light. This value agrees well with the literature. For pure azo-PC ($X_{azo-PC} = 1$), the cis permeability was quite similar to that of DOPC. By contrast, the trans-azo-PC membrane was $3.5 \pm 0.5$ times less permeable to water than cis. We associate this significantly lower $P_w$ to the higher degree of chain order, tighter in-plane packing, and greater thickness of trans found in our simulations. The lower $P_w$ correlates with the higher $k_A$.

As a function of $X_{azo-PC}$, $P_w$ varied only weakly in the cis form, suggesting that the cis- isomer mixed well with DOPC and they were equally permeable. For the trans case, however, we found a significant variation. A pure-trans membrane had low $P_w$ but adding a small amount of DOPC significantly raised $P_w$, presumably because of the disrupted lipid packing. On the other hand, starting with pure DOPC and adding
a small amount of trans-azo-PC sharply reduced \( P_w \), perhaps because the trans molecules enhanced the ordering of the lipids. This enhanced DOPC ordering is shown by stronger peaks in \( g(r) \) extracted from our simulations, Fig. S13A. The strikingly low \( P_w \) at \( X_{azo-PC} = 0.5 \) arose because the longer trans lipids arranged in the leaflet opposite DOPC as discussed just above, for \( k_A \). By minimizing packing defects, the membrane achieved a low \( P_w \).

Anti-correlation across the membrane, correlations in the plane for trans

Our results show several measures by which cis-azo-PC closely resembles DOPC (in \( k_A, P_w \), and \( S_{CD} \)) and differs from trans-azo-PC. This observation led us to wonder under what conditions these lipids might demix. One possibility, as pointed out by Illya, et al., \(^{37} \) is that lipids with different areas per molecule tend to phase separate in the plane. To investigate this possibility, we performed MD simulations of azo-PC/DOPC mixtures with \( X_{azo-PC} = 0.5 \). We arranged the system initially with a nanodomain or cluster of azo-PC lipids. We then tested the stability of this cluster with the photolipids in cis or trans state by running 6.0 \( \mu s \) of classical MD simulation at 300 K. (During 200 ns, the mean-square displacement, MSD, of a freely diffusing lipid is approximately 3 lipid diameters, sufficient for the nanodomain to break up in the course of 6.0 \( \mu s \) unless it is energetically favorable). Figure 6A,B (top) shows one leaflet of the steady-state configuration. With cis, we found that the initial nanodomains were not stable, and instead diffused away. Hence, cis-azo-PC and DOPC mix (Fig. 6B).

By contrast, our same study with the trans isomer showed persistent clustering in each leaflet but no large-scale phase separation. The clustering is obvious in Fig. 6A and in the strong peaks in \( g(r) \) (Fig. S13A). Atomic simulations in steady state beautifully show how the lipids arrange themselves on the two adjacent leaflets of the membrane. The lateral (edge-on) views of Fig. 6A show a striking tendency for trans-azo-PC to become enriched in mesoscopic domains in one leaflet, adjacent to DOPC-enriched domains in the other leaflet. The compositions of the two leaflets are anti-correlated: each trans-azo-PC tends to reside opposite a DOPC. For a membrane with equal number densities of the two lipids, this configuration maintains a consistent thickness even when the molecules are not randomly placed within each leaflet. This conclusion agrees with prior experiments that showed a tendency of short lipids to lie opposite long lipids (longer by 4 C-C bonds) in the other leaflet, even in the absence of phase separation.\(^ {38} \)

For our system, this anti-correlation of the two leaflets (or “crosstalk”\(^ {38} \)) explains why \( k_A \) shows a local peak and \( P_w \) shows a local minimum with a 50/50 composition.

In experiments, we probed for domain formation in azo-PC/DOPC vesicles. We added labeled lipids of Rh-DPPE (saturated) and Rh-DOPE with a concentration of 0.1 mol%. Under blue excitation (trans state), there were no observed domains under any circumstances in temperatures as low as 10 °C. This finding ruled out phase separation, which is consistent with the simulation results of Fig. 6 because the correlated regions were too small to resolve optically.
CONCLUSIONS

In summary, with trans-to-cis isomerization of the photolipids, we observed reversible area changes, $\alpha_{\infty} = \frac{\Delta A}{A_{\text{init}}}$, as large as 12% at the highest UV intensity (15 mW/cm$^2$). Atomistic simulations showed a similar area expansion (13%) and also revealed that the membrane thickness decreased by 10%. The time-response of area was found in experiments to be single-exponential with decay times in the range of 4-20 s, depending on UV intensity and $X_{\text{azo-PC}}$ and independent of membrane tension. Free-floating membranes with low tension (< mN/m range) also expanded with UV excitation and sometimes generated tubules.

These results are explained by UV-excited cis-azo-PC molecules pushing away from one another as they adopt the cis form, thereby placing the membrane in compressive stress. The resulting compressive

Figure 6. Classical atomistic simulation images of azo-PC:DOPC mixture with $X_{\text{azo-PC}} = 0.5$. Initially, azo-PC and DOPC lipids were arranged in separate domains and then the system was equilibrated for 4 μs and the results shown here. A) All the azo-PC photolipids in the trans state (blue). DOPC molecules are shown in yellow. Above: top view of one leaflet of a membrane with 512 lipids. Below: lateral (edge-on) views showing both leaflets, with azo-PC tending to lie opposite DOPC (anti-correlation across the leaflets). B) All of the azo-PC photolipids were in the cis state (red). Above: top view of one leaflet of a membrane with 128 lipids. Below: edge-on view showing both leaflets. (Note that the size scales differ in A) and B.)
stress then relaxes as the area expands to the new steady-state value. We normally associate expansion with an applied tensile stress that increases the area per molecule above the energetically preferred value. This case differs, however, because the preferred area per molecule increases during photomerization to cis. This point also explains why the area change can easily exceed the typical membrane lysis strain of about 4%. When we attempt to stretch a trans membrane by more than about 4%, it ruptures but isomerization changes the equilibrium area without inducing tension. The transient compression following UV excitation also explains why membranes form buds or tubules (Fig S6). In general, thin sheets are highly susceptible to bending and wrinkling when compressed along their surface. By contrast, stretching a membrane should cause the tension to suppress shape fluctuations and tubule formation.

A simple first-order isomerization reaction rate model explains many of our results and lets us estimate the per-molecule area change and the steady-state population of cis. Defining the trans-to-cis rate as \( q_{UV} \) and a cis-to-trans rate as \( q_{Blue} \), one can predict an exponential approach to steady state. Under blue-only excitation, \( q_{UV} = 0 \) and the 1/e time is predicted to be \( q_{Blue} \). Consistent with this prediction, the measured 1/e time (called \( \tau_0 \)) did not depend on the UV intensity. Under UV+blue excitation, the 1/e time is predicted to be \( (q_{UV} + q_{Blue}) \). In this model, the steady-state fraction of cis molecules is set by a competition between the rates \( q_{UV} \) and \( q_{Blue} \) and is equal to \( X_{AZO-PC} \) \( q_{UV}/(q_{UV} + q_{Blue}) = X_{AZO-PC} (1 - \tau_0/\tau_B) \). The area change is then equal to this steady-state cis fraction multiplied by the area change per lipid, \( \Delta a_0 \).

To test this scaling, we plotted the measured \( \alpha_\infty \) vs. this predicted function. For the data measured with the lower UV intensity of 8 mW/cm², we found a strong linear correlation, which further confirms that the competing isomerization rates determine the steady state (Fig. S10). From the best-fit slope, we found \( \Delta a_0 = 0.16 \). (This value agrees very well with the extracted slope of \( \alpha_\infty \) vs. \( X_{AZO-PC} \) near \( X_{AZO-PC} = 0 \), Fig. 1D.) Comparing the measured \( \alpha_\infty \) to \( \Delta a_0 \), and assuming that the blue-excited membrane is all trans, we can then find the fraction of azo-PC molecules that are in the cis form in steady state. For the pure azo-PC membranes, we found that UV intensities of 8 and 15 mW/cm² lead to 45% and 75% conversion to cis, respectively.

With the higher UV intensity of 15 mW/cm², we found that the cis population predicted from the timescales has a strongly nonlinear trend with the measured area expansion. In particular, the data corresponding to the shortest measured \( \tau_BU \) are shifted farthest from the linear trend in the direction indicating that the measured \( \tau_BU \) is longer than expected (Fig. S10). From this comparison, we conclude that the measured timescale was slower than the isomerization time \( (q_{UV} + q_{Blue}) \). The most likely explanation is hydrodynamic friction as the water and lipid flowed to accommodate the vesicle’s size change. We can roughly estimate what the limiting timescale might be from the characteristic viscous damping times for membrane undulation. Earlier theoretical work predicted that the viscous damping time is proportional to \( \eta R/\sigma \), where \( R \) is the vesicle radius, \( \sigma \) is the membrane tension and \( \eta \) is the viscosity of the surrounding solution. A typical value of viscous damping time for 10 µm vesicles is a few seconds for floppy vesicles, or less for tense vesicles. We propose, therefore, that \( \tau_BU \) measured from area expansion cannot be substantially shorter than this viscous damping timescale. This predicted \( R \) scaling of the viscous time suggests that small vesicles (e.g., 0.1 µm vesicles, so-called LUVs) could have much faster timescales. This scaling offers an exciting prospect for millisecond-scale fast triggered response.

From the potential of mean force (Fig. 3B), we concluded that the energy barrier for converting from trans to cis is much smaller in a neighborhood surrounded by cis molecules, presumably because of their looser packing and less ordered tails. Therefore, the rate of formation of cis should be higher. We repeat the point that cis is very like DOPC in terms of area per lipid, \( S_{CD} \), \( k_A \) and \( P_W \). Therefore, we anticipate that
the presence of DOPC should, like cis-azo-PC, enhance the rate of trans-to-cis isomerization. Hence, a large amount of DOPC (small $X_{azo-PC}$) should correspond to having cis-like neighbors, a relatively lower free-energy barrier to cis, a higher cis-formation rate, a larger population of cis relative to trans, and a larger area expansion. Conversely, with high $X_{azo-PC}$ we should find a slower trans-to-cis rate and relatively lower cis population. This argument also explains qualitatively why $\tau_{BU}$ increases with $X_{azo-PC}$ beyond the minimum near 0.4. This argument also explains qualitatively why $\alpha_{\infty}$ expands roughly linearly with $X_{azo-PC}$ at small $X_{azo-PC}$ but then reaches a plateau, as reported in Fig. 1 and in a separate recent study.20

The stretching modulus, toughness, and water permeability all change strikingly with photoswitching in pure-azo-PC membranes ($X_{azo-PC} = 1$). All-trans membranes are $2.5 \pm 0.3$ times stiffer and $3.5 \pm 0.5$ times less permeable than cis membranes. The trans state also has higher tilt order $S_{CD}$, a 1.7-times slower diffusion constant, an increased inter-molecular associations (inferred from nearest-neighbor peak in $g(r)$ and the blue-shift in the trans absorption$^{18}$). These changes arise from the loss of chain orientational order with trans-to-cis isomerization as the lipid tail curves away from the normal direction and hence frustrates lipid packing. In most phosphocholine (PC) lipid membranes, the $k_A$ values vary weakly (by < 10%) with either tail length or chain unsaturation$^{19,44}$ so our measured 2.5× change between cis and trans is remarkably large. We propose that the stiffness is enhanced by the alignment of the aromatic moieties in the azobenzene. For comparison, we refer to the well-known result that adding cholesterol to unsaturated-lipid bilayers can significantly raise $k_A$ (especially when mixed with lipids having one saturated tail$^{46}$) and also lower $P_w$.44,46 The effect is partly attributed to cholesterol’s increasing the orientational order in the membrane with its rigid aromatic rings.45,47 We propose that the aromatic ring in the trans-azo-PC has a similar effect on ordering the nearby trans-azo-PC lipids. The tilt and non-planar orientation of the aromatic rings in the cis conformation may then prevent this ordering effect and thus reduce $k_A$ and increase $P_w$ to values that resemble DOPC.

With isomerization into cis, the reduction of the (stretching) $k_A$ and the thickness should lead to a lower bending modulus. We can estimate the magnitude of the change using simple a continuum model, assuming constant inter-leaflet coupling, which predicts that $\kappa \propto k_A h^2$, where $\kappa$ is the bending modulus and $h$ is the membrane thickness.$^{19}$ Using our experimental and simulation measurements for $X_{azo-PC} = 1$, we find that the ratio of $k_A h^2$ for trans to cis is $3.1 \pm 0.4$. The larger contribution to this ratio comes from $k_A$ rather than $h^2$. Our expected change of $\kappa$ is comparable to, though less than, prior measurements of a 5× increase for the trans relative to cis.$^{14,18}$ A more recent study, however, reported a factor-of-14 increase of bending modulus for trans relative to cis, using the method of electrodeformation. This large enhancement was attributed to enhanced coupling between the two leaflets in the trans state.$^{20}$

We did not observe any contrast-loss associated with sucrose or glucose permeation, nor did we see a significant loss of volume that would result from the tension in aspiration if sucrose could permeate outward. We did, however, find a modest (~1%) irreversible volume expansion with UV irradiation with pure azo-PC, which might indicate a small amount of glucose permeating the membrane. Overall, our results agree with a recent study of azo-PC that found no exchange of sugar across the membrane.$^{20}$ By contrast, another recent study reported higher permeability of a fluorophore when azo-PC was in cis compared to trans.$^{11}$ (The UV intensity at the sample was not reported but the timescales for the response appear comparable to ours, suggesting that the light intensity was similar.) Comparing our findings with glucose to the prior work, we conclude that photochemistry of a solute may affect permeability.$^{48}$ Further studies of permeability of non-fluorescent solutes will be useful.

Our results show how rotation of a single bond in the lipid tail changes the properties. With combined simulations and experiments, we found how the molecular-scale changes propagate to the continuum
scale. Our results show how future work could tune the response by mixing azo-PC with passive lipids. For example, by mixing azo-PC with a shorter unsaturated lipid (e.g., 16:1) that better matches cis in thickness but differs more strongly from trans, it may be possible to accentuate the effect of isomerization and possibly drive separation into two fluid phases. Conversely, mixing azo-PC with a saturated lipid that more closely resembles trans may drive separation into coexisting phases in the cis state. Overall, our results point the way to photoactive lipids that can trigger specific property changes for photopharmacology or triggered-delivery applications.

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REFERENCES


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Mechanical pipette measurements

Atomistic simulations

Bond rotation
membrane expansion,
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