n-Alcohol Length Governs Shift in \( L_o-L_d \) Mixing Temperatures in Synthetic and Cell-Derived Membranes

Caitlin E. Cornell,1 Nicola L. C. McCarthy,2 Kandice R. Levental,3 Ilya Levental,3 Nicholas J. Brooks,2 and Sarah L. Keller1,*

1University of Washington, Department of Chemistry, Seattle, Washington; 2Department of Chemistry, Imperial College London, London, United Kingdom; and 3Department of Integrative Biology and Pharmacology, McGovern Medical School at The University of Texas Medical Center, Houston, Texas

ABSTRACT A persistent challenge in membrane biophysics has been to quantitatively predict how membrane physical properties change upon addition of new amphiphiles (e.g., lipids, alcohols, peptides, or proteins) in order to assess whether the changes are large enough to plausibly result in biological ramifications. Because of their roles as general anesthetics, \( n \)-alcohols are perhaps the best-studied amphiphiles of this class. When \( n \)-alcohols are added to model and cell membranes, changes in membrane parameters tend to be modest. One striking exception is found in the large decrease in liquid-liquid miscibility transition temperatures (\( T_{\text{mix}} \)) observed when short-chain \( n \)-alcohols are incorporated into giant plasma membrane vesicles (GPMVs). Coexisting liquid-ordered and liquid-disordered phases are observed at temperatures below \( T_{\text{mix}} \) in GPMVs as well as in giant unilamellar vesicles (GUVs) composed of ternary mixtures of a lipid with a low melting temperature, a lipid with a high melting temperature, and cholesterol. Here, we find that when GUVs of canonical ternary mixtures are formed in aqueous solutions of short-chain \( n \)-alcohols (\( n \leq 10 \)), \( T_{\text{mix}} \) increases relative to GUVs in water. This shift is in the opposite direction from that reported for cell-derived GPMVs. The increase in \( T_{\text{mix}} \) is robust across GUVs of several types of lipids, ratios of lipids, types of short-chain \( n \)-alcohols, and concentrations of \( n \)-alcohols. However, as chain lengths of \( n \)-alcohols increase, nonmonotonic shifts in \( T_{\text{mix}} \) are observed. Alcohols with chain lengths of 10–14 carbons decrease \( T_{\text{mix}} \) in ternary GUVs of dioleoyl-PC/dipalmitoyl-PC/cholesterol, whereas 16 carbons increase \( T_{\text{mix}} \) again. Gray et al. observed a similar influence of the length of \( n \)-alcohols on the direction of the shift in \( T_{\text{mix}} \). These results are consistent with a scenario in which the relative partitioning of \( n \)-alcohols between liquid-ordered and liquid-disordered phases evolves as the chain length of the \( n \)-alcohol increases.

INTRODUCTION

Scientists have invested decades of research into understanding how \( n \)-alcohols affect model lipid membranes, largely with the goal of clarifying mechanisms by which ethanol consumption perturbs mammalian cell membranes. The results tell a compelling story: \( n \)-alcohols partition into membranes near lipid headgroups where they disorder carbon chains of neighboring lipids or probes (1–3). Concomitantly, \( n \)-alcohols alter physical properties of liquid-phase membranes: lipid lateral mobilities increase (4–6), ion channel cation permeabilities increase (7), membrane areas increase (8), thicknesses decrease (9), bending moduli decrease (8), area compressibilities decrease (8), interfacial tensions decrease (8), gel-liquid transition temperatures decrease (10), \( L_o-H_{II} \) transition temperatures shift (11), and lateral pressure profiles shift (6,12). However, the magnitudes of most of these effects are modest. For example, relatively high concentrations of ethanol (120 mM) decrease membrane gel-liquid melting temperatures by only 0.3°C (10).

One striking exception to the rule that \( n \)-alcohols tend to have a minimal effect on physical properties of membranes was recently reported by Veatch and colleagues (13). Using cell-derived giant plasma membrane vesicles (GPMVs), they found that short-chain \( n \)-alcohols dramatically decreased miscibility transition temperatures (\( T_{\text{mix}} \)). The shift in \( T_{\text{mix}} \) (~4°C for 120 mM ethanol) is more than an order of magnitude larger than ethanol’s effect on membrane melting temperatures (10,13). 120 mM ethanol is the concentration reported by Pringle et al. (14) as the anesthetic concentration (AC50) at which 50% of tadpoles lose their righting reflex. The ethanol concentration at
which proteins begin to denature is at least an order of magnitude higher (15). Strikingly, the result that short-chain n-alcohols decrease $T_{\text{mix}}$ of GPMVs by $\sim 4^\circ \text{C}$ holds equally well for ethanol, propanol, octanol, and decanol at the AC50 concentration (13).

Cell-derived GPMVs have several advantages as experimental systems. They are large enough ($\sim 10 \ \mu \text{m}$) to image by conventional microscopy; they contain significant amounts of functioning, native proteins; they retain extraordinary complexity in their lipid and protein compositions (similar to cell plasma membranes); and the spatial distribution of the lipids and proteins in their membranes can be probed by fluorophores (16–21). At high temperatures, GPMV membranes appear uniform by epifluorescence microscopy. Below $T_{\text{mix}}$, GPMV lipids and proteins demix into coexisting liquid-ordered ($L_o$) and liquid-disordered ($L_d$) phases (18–21).

The result that n-alcohols dramatically shift miscibility transition temperatures in GPMVs leads to the clear question of whether n-alcohols also shift $T_{\text{mix}}$ in simpler membranes of giant unilamellar vesicles (GUVs) composed of ternary lipid mixtures. We are motivated to ask this question because the phenomenon of membranes demixing into $L_o$ and $L_d$ phases has been largely understood in the context of GUVs composed of ternary mixtures of a lipid with a high melting temperature, a lipid with a low melting temperature, and cholesterol. The relative amounts of each lipid type can be quantitatively tuned in GUVs (22), making them an ideal system for mapping phase diagrams. General features within phase diagrams of ternary membranes are well understood. For example, researchers know how to tune lipid ratios to achieve membranes that are likely to exhibit gel phases, coexisting $L_o$ and $L_d$ phases, or critical phenomena, and they have used this information to provide a broader context to interpret results from specific cell-derived membranes (21,23,24). However, an enduring challenge has been to quantitatively predict the effect of substituting or adding new membrane components (19,25), including n-alcohols.

Here, we find that the addition of short-chain n-alcohols to ternary GUVs significantly shifts miscibility transition temperatures, and that the magnitude of the shift is large, as in cell-derived GPMVs. However, to our surprise, we find that the direction of the shift is opposite in the two systems. We describe experiments that explore this phenomenon, and we offer a plausible speculation to explain why short-chain n-alcohols decrease miscibility transition temperatures in GPMVs and increase them in GUVs.

**MATERIALS AND METHODS**

**Materials**

Phosphocholine (PC) lipids including DOPC (dioleoyl-PC, or di-18:1-PC), DPPC (dipalmitoyl-PC or di-16:0-PC), POPC (palmitoyl-oleoyl-PC or 16:0-18:1-PC), di16:1-Δ6cis-PC, di18:1-Δ6cis-PC, di14:1-Δ9cis-PC, lysol(18:0)-PC, and palmitoyl sphingomyelin (PSM or 18:1-16:0 SM) were from Avanti Polar Lipids (Alabaster, AL). Texas Red dihexadecanoyl-phosphoethanolamine was from Life Technologies (Grand Island, NY), and cholesterol was from Sigma Aldrich (St. Louis, MO). Lipid structures appear in Fig. S1. Stock solutions of laurdan (Invitrogen, Carlsbad, CA) and C-laurdan (a gift from B. R. Cho, Seoul, Korea) were prepared in ethanol and dimethyl sulfoxide (DMSO). Alcohols (ethanol, propanol, butanol, pentanol, hexanol, octanol, decanol, tetradecanol, hexacontanol, propofol, and 2,6-di-tert-butylphenol), DMSO, and all additional reagents were from Sigma Aldrich unless specified. All alcohols were purchased at their highest available purity. All materials were used as from the manufacturer without further purification.

**Production of GUVs for $T_{\text{mix}}$ measurements**

GUVs with diameters in the order of $10^3–10^5 \mu \text{m}$ were electroformed (26) in either pure (18 MΩ-cm) water or in alcohol solutions (0.0025–480 mM alcohol in 18 MΩ-cm water). At ethanol concentrations above 1.2 M, PC-membranes are partially solubilized; above 7 M, mixed micelles form (27). Alcohols with low water solubilities (tetradecanol, hexadecanol, and 2,6-di-tert-butylphenol) were first dissolved in DMSO before being dissolved in water. For these alcohols, the maximum final concentration of DMSO in aqueous solution was 210 mM, too small to measurably affect miscibility transition temperatures. Control experiments were conducted to verify that shifts in $T_{\text{mix}}$ values due to producing GUVs of 35:35:30 DOPC/DPPC/cholesterol in 18 MΩ-cm water ($T_{\text{mix}} = 30.4 \pm 0.29^\circ \text{C}$) versus in DMSO solutions $\sim 7$-fold more concentrated (1.4 M) than those used in n-alcohol experiments ($T_{\text{mix}} = 31.1 \pm 0.04^\circ \text{C}$) were smaller than shifts in $T_{\text{mix}}$ due to the type of n-alcohol used.

GUVs used in measurements of $T_{\text{mix}}$ were electroformed as follows. An aliquot of 0.25 mg of lipids in chloroform was spread evenly on an indium-tin-oxide-coated glass slide (Delta Technologies, Loveland, CO). The lipid mixture contained 0.8 mol% Texas Red dihexadecanoyl-phosphoethanolamine, a dye-labeled lipid that selectively partitions to the $L_d$ phase (28). The slide was placed under vacuum for $> 30 \text{ min}$ to evaporate the chloroform. A capacitor was created by separating two indium-tin-oxide-coated slides with two rectangular Teflon bars (0.3mm thick). The gap between the bars was filled with water or alcohol solution, and all edges were sealed with vacuum grease. An AC voltage of 10 Hz and 1.5 V was applied to the capacitor for 1 h at 60° C. Vesicles were then extracted from the capacitor and diluted 5–10-fold in water or alcohol solution at 60° C to make a stock solution.

**Measurement of $T_{\text{mix}}$**

Electroformation produces populations of vesicles with distributions of miscibility transition temperatures, reflecting slight differences in lipid ratios from vesicle to vesicle. Uncertainties in ratios of PC-lipids in electroformed vesicles have been estimated at $< 2 \text{ mol\%}$ (29). Techniques for minimizing uncertainties in $T_{\text{mix}}$ (30) were followed.

To image vesicles, several drops of vesicle stock solution were deposited between glass cover slips, and the edges were sealed with vacuum grease. This assembly was thermally coupled to a home-built temperature stage for an epifluorescence microscope (Y-FL; Nikon, Melville, NY) via a layer of thermal grease (Omega Engineering, Stamford, CT). An Alpha-Omega (Lincoln, RI) controller adjusted temperature via a thermoelectric heater/cooling using feedback from a thermistor (0.02 °C accuracy; Sensor Scientific, Fairfield, NJ). Images were captured through an air objective using a CoolSnapFX camera (Photometrics, Tucson, AZ) and processed using ImageJ (public domain http://rsbweb.nih.gov/ij). The percent of vesicles that exhibited coexisting $L_o$ and $L_d$ phases was recorded over temperature steps between 10 and 50° C. Typically, ~100 vesicles were imaged at each step, over three fields of view. A nonlinear least squares fit to a
sigmoidal curve of % phase separated = $100 \times (1 - (1/(1 + e^{-(T-T_{\text{mix}})/B})))$ yields $T_{\text{max}}$, at which 50% of vesicles are phase separated, and $B$, which relates to the width of the transition (13). The shift in $T_{\text{max}}$ $\Delta T_{\text{mix}}$ is defined as $T_{\text{mix}}$ (with alcohol) – $T_{\text{mix}}$ (without alcohol). Uncertainties for each measurement correspond to 95% confidence intervals of the fit, as shown in Fig. 1. For the data in Fig. 1, the dimensionless term $\Delta T_{\text{mix}}$ $T_{\text{mix}} = 0.00626 \pm 0.00115$ (for temperatures expressed in Kelvin). Uncertainties for a set of measurements due to day-to-day variation is typically < 0.5 °C. For example, four experiments on different days using dilute concentrations of (0.05–1 mM) butanol yielded $\Delta T_{\text{mix}}$ of 0.43 ± 0.14 °C.

**AC50 values**

To compare results with Gray et al. (13), we used AC50 values from Pringle et al. (14): 120 ± 10 mM for ethanol, 54 ± 6 mM for propanol, 12 ± 1 mM for butanol, 0.7 ± 0.1 mM for hexanol, 0.06 ± 0.9 mM for octanol, and 0.013 ± 0.2 mM for decanol. An exponential regression of these values gives 2.96 mM for pentanol. More recent AC50 values by Alifimoff et al. (31) are similar: 190 ± 16 mM for ethanol, 73 ± 2.4 mM for propanol, 10.8 ± 0.77 mM for butanol, 2.9 ± 0.11 mM for pentanol, 0.57 ± 0.37 mM for hexanol, 0.059 ± 0.0031 mM for octanol, and 0.0126 ± 0.00048 mM for decanol. At the AC50, n-alcohols are several mol% of membranes (14).

**Cell culture and GPMV isolation**

Rat basophilic leukemia (RBL) cells were maintained at 37°C in humidified 5% CO2 in growth medium containing 60% modified Eagle’s medium, 30% RPMI medium, 10% fetal calf serum, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. GPMVs were isolated and imaged as previously described (32). Briefly, cells were washed in GPMV-buffer (10 mM HEPES, 150 mM NaCl, 2 mM CaCl2, pH 7.4) and incubated for 1 h at 37°C in GPMV-buffer supplemented with 25 mM parafomaldehyde and 2 mM dithiothreitol. Differences between results in GUVs and GPMVs are not due to parafomaldehyde and dithiothreitol; miscibility temperatures of GUVs in 18 MΩ-cm water ($T_{\text{mix}} = 31.8 \pm 0.15°C$, for GUVs of 35:35:30 DOPC/DPPC/cholesterol) are indistinguishable from temperatures of GUVs in 25 mM parafomaldehyde and 2 mM dithiothreitol ($T_{\text{mix}} = 31.9 \pm 0.09°C$), where uncertainties are determined as in Fig. 1 for single experiments. We verified that our conditions sufficiently replicated the conditions of Gray et al. (13): we found that a short-chain n-alcohol (butanol) decreases $T_{\text{mix}}$ in GPMVs, whereas a long-chain n-alcohol (hexadecanol) increases $T_{\text{mix}}$ (Fig. S6).

**Laurdan and C-laurdan microscopy**

GUVs used for laurdan microscopy were electroformed by a method described previously (32) that is functionally equivalent to the method used to produce GUVs for measurements of $T_{\text{mix}}$. Specifically, 1 μL of a chloroform/methanol (2:1 v/v) solution containing 5 mg/mL of lipids was spread on two platinum electrodes within a Teflon chamber. The lipid mixture contained 0.5 mol% laurdan. The chamber was placed under vacuum for 1 h to dry the solvent and form a lipid film. Next, 350 μL of an aqueous solution (0.2 M sucrose with or without alcohol) was added to the chamber. GUVs were grown by applying an AC voltage of 10 Hz and 2.5 V across the electrodes for 1 h at 52°C. GUVs were then collected and diluted in 1 mL of 0.2 M glucose with or without alcohol.

GUVs and GPMVs were imaged at 20 and 5°C, respectively, by confocal microscopy on a Nikon A1R with spectral imaging at 60× and an excitation of 405 nm. The emission was collected in two bands: 433–463 nm and 473–503 nm. MATLAB (The MathWorks, Natick, MA) was used to calculate two-dimensional general polarization (2D GP) maps, where GP for each pixel was calculated from a ratio of the two fluorescence channels as previously described (33). Briefly, each image was background subtracted and thresholded to retain only pixels with intensities 3–5 standard deviations (SDs) greater than the background in both channels (32). The difference in generalized polarization (ΔGP) between $L_o$ and $L_d$ phases was determined for each vesicle, where GP for each phase was derived from average pixel intensities ($I$) from large, representative areas via $GP = [\sum_{\psi=0}^{463} I_{\psi} - \sum_{\psi=463}^{503} I_{\psi}]/[\sum_{\psi=463}^{503} I_{\psi} + \sum_{\psi=433}^{433} I_{\psi}]$.

**Area fractions**

Micrographs of vesicle equatorial sections yielded vesicle diameters that were ~80–250 μm. Videos of these equatorial sections were collected such that the top, spherical cap of the vesicle lay inside the < 5 μm depth of field of the microscope objective; the remainder of the vesicle appeared as a bright ring. Centers of vesicles whose bright ring remained in the field of view were identified by custom MATLAB code available by public license in the “Track_Vesicle” program (34,35). Drift of free-floating vesicles in the $x$-$y$ plane was corrected by stacking video frames on vesicle centers. Areas out of focus were excluded, yielding squares with edges ~15–60 μm. Pixel intensities were thresholded so the $L_o$ phase was white and the $L_d$ phase was black. Images within the 2D squares were projected onto 3D spherical surfaces using MATLAB code by Sarah Veatch (36). The area fraction of the $L_d$ phase was the 3D-projected area of all white pixels divided by the projected area of all pixels in the image.

**High-pressure microscopy**

GUVs were electroformed as in the production of GUVs for $T_{\text{mix}}$ measurements, except that a 0.5 mm PDMS O-ring was used instead of Teflon bars. After electroformation, GUVs were transferred into a custom-built, high-pressure cell mounted on a Nikon Eclipse TE2000-E inverted microscope equipped with a Zyla SCMO5-based camera (Andor Technology, DSCU CF080A).
RESULTS AND DISCUSSION

n-Alcohols increase $T_{\text{mix}}$ in model membranes

When ternary GUVs are formed in aqueous solutions of short-chain $n$-alcohols, the temperatures at which the vesicles demix into coexisting $L_o$ and $L_d$ phases increase relative to GUVs in water. For example, in Fig. 1, $T_{\text{mix}}$ increases by 1.9°C for vesicles of 35/35/30 DOPC/DPPC/cholesterol in 100 mM butanol. This shift is in the opposite direction to that observed in cell-derived GPMVs (13). The difference between the GUV and GPMV results is not due to proteins denaturing, which occurs at butanol concentrations that are roughly an order of magnitude higher (15).

The increase in $T_{\text{mix}}$ that we observe in model GUVs is robust across a range of short-chain $n$-alcohols and is proportional to the concentration of alcohol in solution (Fig. 2A). When the concentration of each $n$-alcohol is scaled by its AC50 value (14), the data within the shaded area of Fig. 2A collapse (Fig. 2B). The same result holds when AC50 values from (31) are used (Fig. S2). A similar scaling occurs in cell-derived GPMVs (13). Because the AC50 value of an $n$-alcohol is proportional to its partition coefficient from water into PC bilayers (39), the observation of scaling implies that the magnitude of $\Delta T_{\text{mix}}$ is colligative: the value of $\Delta T_{\text{mix}}$ depends only on the mole fraction short chain $n$-alcohol in the membrane.

Increase in $T_{\text{mix}}$ is robust across GUV lipid ratios and lipid types

The result that short-chain $n$-alcohols increase $T_{\text{mix}}$ by several degrees in model GUV membranes is robust. Fig. 3 shows that this result holds for membranes composed of different ratios of DOPC, DPPC, and cholesterol. The Gibbs triangle in Fig. 3 plots $T_{\text{mix}}$ values for control GUVs (without butanol) with five different lipid ratios. When GUVs of these same ratios are produced in butanol solutions, $T_{\text{mix}}$ increases independent of whether the ratios are varied along a vertical (Fig. 3A) or horizontal (Fig. 3B) path in the triangle. A corollary is that short-chain $n$-alcohols increase $T_{\text{mix}}$ independent of whether the majority of the GUV area is the $L_o$ phase or by the $L_d$ phase. Similarly, $T_{\text{mix}}$ increases independent of whether the GUV membrane is close to or far from a miscibility critical point.

The result that short-chain $n$-alcohols increase $T_{\text{mix}}$ is also robust for membranes composed of lipids with different shapes. Lysolipids are cone-shaped lipids with a single acyl tail, which means that lysolipid head groups have larger cross-sectional areas than lysolipid tails. One consequence of this shape is that lysolipids are thought to shield membrane cholesterol, whereas alkanols displace it (40). In Fig. 4, we replace half of the unsaturated DOPC lipids with lyso(18:0)-PC lipids, and we find that butanol still
FIGURE 4 Miscibility transition temperatures of GUVs composed of 17.5/17.5/35/30 lyso(18:0)-PC/DOPC/DPPC/cholesterol (green, top) and composed of 35/30 DOPC/DPPC/cholesterol (gray, bottom) increase with the concentration of butanol in solution (increasing darkness of the points). Each point represents a single experiment for which uncertainties are smaller than symbols. $\Delta T_{\text{mix}}$ is with respect to vesicles with no butanol. To see this figure in color, go online.

Butanol increases $L_o$-$L_d$ contrast in GUVs, but not in GPMVs

Why does the addition of short-chain $n$-alcohols increase $T_{\text{mix}}$ in model GUV membranes (Figs. 1, 2, 3, 4, and 5) and decrease $T_{\text{mix}}$ in cell-derived GPMVs (13)? The simplest explanation is that short-chain $n$-alcohols in GUVs partition much more strongly into one of the membrane phases than the other (44–46). Given that short-chain alcohols lie directly below lipid headgroups in membranes (1), we expect these alcohols to strongly partition to the $L_d$ phase of GUVs instead of the $L_o$ phase. GPMVs are more complex; it is difficult to predict how short-chain alcohols would partition between the $L_d$ and $L_o$ phases of a GPMV. If an alcohol were to instead partition roughly equally between the two membrane phases as an “inert diluent,” $T_{\text{mix}}$ would decrease over all lipid ratios (46,47). At the very least, we can state that our results in Figs. 3 and 5 are not consistent with the phase boundary merely translating within the plane of the Gibbs phase triangle, such that $T_{\text{mix}}$ would increase at some lipid ratios and decrease at others. To illustrate these concepts, Fig. 6 A shows $T_{\text{mix}}$ increasing over all lipid ratios, and Fig. 6 B shows the phase boundary translating.

Leung and Thewalt (48) found that a particular probe (napthopyrene) partitions strongly to $L_d$ phases in GUVs, increases the difference in lipid chain order between the two phases, and increases $T_{\text{mix}}$. They found that an alternate probe (laurdan) partitions weakly between the two membrane phases and does not have these effects (48). If short-chain $n$-alcohols partition strongly to $L_d$ phases in GUVs and weakly between the two membrane phases in GPMVs, then we would expect short-chain $n$-alcohols to increase the difference in lipid chain order between $L_o$ and $L_d$ phases in GUVs and to not increase it substantially in GPMVs. To test this idea, we used the GP of laurdan as a qualitative measure of the difference between $L_o$ and $L_d$ phases in GUVs and in GPMVs. Laurdan’s emission spectrum reflects its exposure to aqueous solvent, which in
Alcohol Length Governs $L_o$-$L_d$ $T_{mix}$ Shift

To review, short-chain $n$-alcohols increase membrane $T_{mix}$ in GUVs. One of these alcohols, butanol, increases the difference in laurdan GP between the $L_o$ and $L_d$ phases of GUVs, presumably as a result of strong preferential partitioning of the butanol to the $L_d$ phase. As the length of $n$-alcohols increases, we expect a crossover in behavior. Specifically, we expect $n$-alcohols of medium lengths to partition more equally between the $L_d$ and $L_o$ phases, resulting in a decrease in $T_{mix}$ and no significant increase in $\Delta$GP. Once the number of carbons in $n$-alcohols exceeds a second threshold, we expect the alcohols to again strongly partition to only one of the membrane phases, this time to the $L_o$ phase. This expectation is reasonable given that a recent calculation predicts that short, saturated alkyl chains partition preferentially to an $L_d$ phase, that medium chains...
partition equally, and that long chains partition preferentially to an \( L_o \) phase \( (64) \).

Fig. 8 supports the notion that a crossover does indeed occur. Fig. 8 shows that \( T_{\text{mix}} \) increases when GUVs of 35/35/30 DOPC/DPPC/cholesterol are produced in solutions containing \( n \)-alcohols with short alkyl chains (\( \leq 8 \) carbons). In contrast, for medium chains (10–14 carbons), \( T_{\text{mix}} \) decreases. For long chains (\( \geq 16 \) carbons), \( T_{\text{mix}} \) increases again. The data in Fig. S5 C are consistent with this view: as a medium chain \( n \)-alcohol (tetradeanol) is introduced to a GUV solution, there is no significant increase in the difference in laurdan GP between the \( L_o \) and \( L_d \) phases. Recent theory suggests that the greatest decrease in \( T_{\text{mix}} \) should occur when the \( n \)-alcohol slightly prefers the \( L_d \) phase (M. Schick and D.W. Allender, personal communication).

The designation of \( n \)-alcohols as “short,” “medium,” and “long” invokes relative terms that depend upon characteristics of the membrane in which the alcohol is embedded. Upon switching from GUVs to GPMVs, \( n \)-alcohols with 2–10 carbons behave as alcohols in our “medium” category: they decrease \( T_{\text{mix}} \) in GPMVs \( (11) \). A length of 16 carbons qualifies as “long” in both types of membranes: in GPMVs, hexadecanol increases \( T_{\text{mix}} \) \( (45) \). A switch from “medium” to “long” behavior in GPMVs, where \( \Delta T_{\text{mix}} \approx 0 \), occurs at an alkanol length of 14 carbons \( (13) \) (Fig. S4). Addition of tetradeanol to GPMVs results in no significant increase in the difference in laurdan GP values (Fig. S5 D). Gray et al. \( (15) \) discuss this switch in behavior as an analogy of the “cutoff effect,” the decrease in efficacy of alcohols as general anesthetics when \( n \)-alcohols exceed a cutoff length \( (39,65) \).

Three natural length scales arise in Fig. 8: the length of the alcohol’s alkyl chain, the length of the lipid’s acyl chain, and the length from the lipid’s glycerol backbone to its double bond. These lengths are shown in Fig. S1. In Fig. 9, we decrease the length of \( n \)-alcohols that qualify as “short” by replacing DOPC with two types of analogous lipids. The first type of replacement uses a lipid with the same length of carbon chain as DOPC and a double bond at a new position (the \( \Delta 6 \) position rather than the \( \Delta 9 \) position). With this substitution, the crossover in behavior occurs at six carbons (i.e., only \( n \)-alcohols with two, four, or six carbons result in a significant increase \( T_{\text{mix}} \) whereas \( n \)-alcohols with 8 or 10 carbons do not). The second type of replacement uses lipids with shorter acyl chains, retaining the double bond at the \( \Delta 9 \) position. These new substitutions push the crossover length of the \( n \)-alcohol down to four carbons (for 16:1\( \Delta 9 \)PC lipids) and down to two carbons (for 14:1\( \Delta 9 \)PC lipids).

These results imply that the length of the lipids and the position of each lipid’s double bond determine how an \( n \)-alcohol partitions into \( L_d \) versus \( L_o \) phases. One of the primary differences between GUV and GPMV membranes is in the length and unsaturation of the lipids. Given the high occurrence of polyunsaturated lipids in GPMVs \( (32) \), and that \( n \)-alcohol partition coefficients are highly sensitive to lipid polyunsaturation \( (66) \), it is plausible that different \( n \)-alcohols qualify as “short,” “medium,” and “long” in GPMV membranes than in GUV membranes. It is also plausible that the significant protein content of GPMVs contributes to differences in partitioning of \( n \)-alcohols between \( L_d \) and \( L_o \) phases of GUVs versus GPMVs. Generalizing from \( n \)-alcohols to other types of amphiphiles, a long list of molecules are known to partition differently into \( L_d \) versus \( L_o \) phases in...
GUVs than in GPMVs (18). To highlight the magnitude of this differential partitioning, at least one probe’s preference for the Ld versus the Lo phase is reversed in the two types of membranes (19). In summary, differences in how n-alcohols partition between the two phases in GUVs and GPMVs may explain why short-chain alcohols increase Tmix in GUVs and decrease Tmix in GPMVs.

Alcohol antiintoxicants increase Tmix in GUVs

If we consider shifts in Tmix as a consequence of how impurities partition into Lo and Ld phases of membranes, we gain a method of predicting whether small molecules will increase or decrease Tmix. Here, we focus on dihydromyricetin (DHM) and Ro15-4513. Both are expected to partition to membranes: Ro15-4513 is roughly twice as hydrophobic as butanol. DHM and Ro15-4513 are termed “antiintoxicants” because they reverse the effects of ethanol in cultured neurons as well as whole organisms, at least at concentrations of 3 μM and 100 nM, respectively (67,68). These two compounds are also antiintoxicants in terms of reversing the effect of ethanol on Tmix in cell-derived GPMVs (45). Namely, ethanol decreases Tmix in GPMVs, whereas DHM and Ro15-4513 increase it (45). The increase in Tmix implies that DHM and Ro15-4513 partition strongly to only one of the membrane phases in GPMVs. Given that DHM and Ro15-4513 both feature polar groups and bulky ring structures, they are expected to strongly partition near lipid headgroups in the Ld phase of GUVs and to thereby increase Tmix in GUVs. Fig. 10 shows that Tmix of GUVs incubated in 3 μM DHM is indeed higher (by ~0.5°C) than for GUVs in water. Likewise, Fig. 10 shows that Tmix for GUVs incubated in 100 nM Ro15-4513 is ~1.3°C higher than for GUVs in water.

Minor structural changes in alcohols result in large shifts in membrane Tmix

In Fig. 10, a cursory description of the bulky, amphiphilic structures of DHM and Ro15-4513 led to a prediction that the compounds would partition strongly into the Ld phase and increase Tmix. However, partition coefficients are not always straightforward to predict, as for propofol and its membrane-soluble structural analog 2,6-di-tert-butylphenol (Fig. 11). This pair is particularly interesting because propofol is a general anesthetic, whereas 2,6-di-tert-butylphenol is not (69). Gray et al. (13) previously found that propofol decreases Tmix in cell-derived GPMVs, whereas 2,6-di-tert-butylphenol does not shift Tmix. In other words, propofol behaves as a short-chain alcohol does in GPMVs.

In Fig. 11, we find that propofol also behaves as a short-chain alcohol in GUVs: ternary vesicles in propofol have higher values of Tmix than vesicles in water. No significant shift in Tmix occurs for the nonanesthetic analog at identical concentrations. We are unaware of any current models that would predict the partitioning of propofol and 2,6-di-tert-butylphenol into Ld versus Lo phases. Future models to predict partitioning might incorporate area-to-volume ratios of molecules, as in (70).

Hydrostatic pressure increases Tmix in GUVs

Because Tmix of a GUV membrane is an equilibrium property, it can be tuned by adjusting thermodynamic parameters. For example, Tmix increases as model vesicles are subjected to increasing hydrostatic pressure (71), indicating that a demixed membrane fills less volume than a uniformly mixed membrane. This result, that Tmix increases with pressure, holds whether the membrane originates from a model GUV or from a cell-derived GPMV (45).

Although GUVs and GPMVs are alike in that Tmix increases with pressure, we have seen in Figs. 1, 2, 3, 4, and 5 that the two systems are dissimilar in their response to short-chain n-alcohols. Fig. 12 provides an equivalent illustration of this point. Increasing concentrations of butanol decrease the miscibility transition pressure, Pmix, in model GUVs at constant temperature. These GUV data

![FIGURE 10](image-url) 35/35/30 DOPC/DPPC/cholesterol GUVs produced in 3 μM DHM or 100 nM Ro15-4513 (white bars) have higher miscibility transition temperatures than control GUVs in water (ΔTmix). Similarly, GUVs produced in 120 mM ethanol (EtOH, black bars) have higher miscibility temperatures under all conditions in the figure. Each bar represents a single experiment for which uncertainties are calculated as in Fig. 1.

![FIGURE 11](image-url) Propofol, a general anesthetic, increases Tmix in 35/35/30 DOPC/DPPC/cholesterol GUVs. In contrast, 2,6-di-tert-butylphenol, which is structurally similar but anesthetically inactive, does not increase Tmix. Each point represents a single experiment. In all cases, symbols are larger than uncertainties determined as in Fig. 1.
are consistent with the hypothesis that butanol strongly partitions into only one membrane phase in GUV membranes, such that membranes spontaneously demix over a wider range of conditions. In contrast, in GPMVs, increasing concentrations of butanol increase $P_{mix}$ in cell-derived GPMVs (45). Specifically, 12 mM butanol increases $P_{mix}$ by $240 \pm 30$ bar (45). These GPMV data are consistent with the hypothesis that butanol partitions roughly equally into both membrane phases in GPMV membranes (45), such that membranes spontaneously demix over a narrower range of conditions.

**Speculations**

In Figs. 1, 2, 3, 4, 5, 6, 7, 8, and 9, we observe shifts in $T_{mix}$ and $\Delta G_P$ that are consistent with 1) short-chain $n$-alcohols partitioning strongly to $L_d$ phases in ternary GUVs, 2) medium-chain alcohols partitioning roughly equally between $L_d$ and $L_o$ phases, and 3) long-chain alcohols partitioning strongly to $L_o$ phases. In this model, the magnitude of the shift in $T_{mix}$ depends on the concentration of $n$-alcohol in a membrane. The experimental question of whether $n$-alcohol concentrations are roughly equivalent in model and cell membranes is not well resolved (39,72). Our focus on differential partitioning of $n$-alcohols between $L_o$ and $L_d$ phases does not exclude other possible modes of action that may differ between GUV and GPMV systems, e.g., $n$-alcohols behaving as lineactants, interacting with proteins, distributing at different distances from the membrane midplane, or competing with cholesterol for phospholipid association.

We speculate that the tendency of an $n$-alcohol to partition differentially between $L_o$ and $L_d$ phases depends on characteristic length scales of membrane lipids, set by the location of double bonds and by the length of lipid chains. If this speculation is valid, then GPMVs with different lipid compositions [perhaps due to cell cycle, state, or growth conditions (61,73)] would have different crossover lengths of $n$-alcohols (see Fig. 9). Similarly, we speculate that any compound that partitions strongly to only the $L_o$ or $L_d$ phase will prove to raise $T_{mix}$ in membranes. Predicting how subtle structural differences between compounds (e.g., between propofol and 2,6-di-tert-butylphenol) are manifested in their partitioning between $L_o$ and $L_d$ phases within an all-atom simulation would be expensive given current capabilities. However, the tendency of a compound to strongly preferentially partition to one membrane phase over another may prove to map onto other membrane physical parameters, such as changes in lateral pressure profiles (66) that may be easier to calculate or simulate.

Models that invoke differential partitioning of molecules between $L_o$ and $L_d$ phases predict a variety of results. Dilute concentrations of an impurity cause critical temperatures (and hence $T_{mix}$) to increase in models that consider differential solubilities (46; M. Schick and D.W. Allender, personal communication) or differential partitioning within an Ising model (44). Experiments in which dilute impurities were added to two-component bulk mixtures found that critical temperatures increased when the impurity had a low solubility in one of the components and decreased when the impurity was likely to be soluble in both components (74). In another model, Schick (75) used the well-known result (76) that for a one-component membrane undergoing a phase transition, an impurity that preferentially partitions into the membrane phase with higher entropy decreases the transition temperature. He showed that a term that relates partitioning of the impurity to the change in transition temperature would also appear in equations describing multi-component membranes. This term, combined with a predicted differential partitioning of alkyl chains (64), could explain why short-chain alcohols decrease $T_{mix}$ in GPMVs, but not why they increase $T_{mix}$ in GUVs.

**CONCLUSIONS**

Here we show that $n$-alcohols with $\leq 8$ carbons increase $T_{mix}$ for membranes of ternary GUV membranes over a range of $n$-alcohol concentrations. This increase is robust for membranes of several lipid types and ratios. As chain lengths of alcohols increase, their effect on membranes is nonmonotonic: $n$-alcohols with 10–14 carbons decrease $T_{mix}$ in the GUVs in this study; for $n = 16$, $T_{mix}$ increases. Previous experiments using cell-derived GPMVs also found that as chain lengths of alcohols increase, their effect on membranes evolves: $n$-alcohols with $\leq 10$ carbons decrease $T_{mix}$ in GPMVs; for $n = 16$, $T_{mix}$ increases (13,45). A full summary of how the GUV data compare with previous GPMV data appears in Table S1.

Results from GUVs and GPMVs are equally consistent with a scenario in which the partitioning of $n$-alcohols into $L_d$ versus $L_o$ phases changes as the length of the alcohol increases (45). In this scenario, alcohols that are shorter than
a characteristic length scale set by the membrane would strongly preferentially partition to the $L_a$ phase, increasing $T_{\text{mix}}$. One piece of evidence in support of this scenario is that for GUVs in butanol solutions, laurdan $\Delta G_P$ values increase with the butanol concentration, as shown in Fig. 7. In contrast, $n$-alcohols of medium length (e.g. tetradecanol, $n = 14$) decrease $T_{\text{mix}}$ with no significant effect on laurdan $\Delta G_P$ (Fig. S5).

**SUPPORTING MATERIAL**

Six figures and one table are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(17)30757-9.

**AUTHOR CONTRIBUTIONS**

C.E.C., K.R.L., I.L., N.J.B., and S.L.K. designed experiments. C.E.C., K.R.L., I.L., and N.L.C.M. performed experiments. C.E.C. and S.L.K. wrote the manuscript.

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