# Role of Ethanol in the Modulation of Miscibility Transition in Model Lipid Bilayers

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## Abstract

*The influence of ethanol on the miscibility transition of model bilayers containing cholesterol was investigated with fluorescence microscopy. A biphasic effect of the miscibility transition temperature and the presence of non-spherical vesicles were observed. Ethanol still has the disordering effect and reduces the transition temperature by ~2 °C at ~0.54M; nevertheless, this effect gets attenuated by 20 mol % cholesterol.*

## Introduction

As a simple model of cell membranes, ternary systems of DPPC, DOPC and cholesterol have been widely studied and miscibility transition temperatures of these mixtures with varying compositions are generally accessible (Veatch & Keller, 2003). Also, it is commonly believed that cholesterol tends to promote ordering in the acyl chains of lipids in a liquid phase, whereas ethanol is likely to induce disorder (Sun & Sun, 1985; Veatch & Keller, 2005). Therefore, it is interesting to study the competition between ethanol and cholesterol in the ordering of lipids and the effects of ethanol on the miscibility transition temperatures of model membranes composed of DPPC, DOPC and cholesterol. Besides, studies have shown that in animals chronic alcohol consumption can sometimes result in an increase in membrane content of cholesterol (Chin et al., 1978; Johnson et al., 1979; Smith & Gerhart, 1982; Wood & Schroeder, 1992). It is reasonable that cells can auto-tune their membrane compositions and produce more cholesterol in order to counteract the disordering effect of alcohol.

Furthermore, general anesthetic agents such as ethanol are used widely in that they can provide pain relief, immobility and unconsciousness. The lipid theory states that general anesthetics act through a common and nonspecific mechanism by dissolving in the membrane of nerve cells and causing structural changes in the lipid bilayer (Rudolph & Antkowiak, 2004). The structural perturbations can then lead to a cascade of lipid-protein and/or protein-protein interactions. So, understanding the molecular basis of the action of general anesthetics will be important for neuroscience as well as medicine.

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*Abbreviation used*: DPPC, dipalmitoylphosphatidylcholine (di (16:0) PC); DOPC, dioleoylphosphatidylcholine (di (18:1) PC); NMR, nuclear magnetic resonance.

Following the idea that general anesthetics need to get dissolved in the lipid membrane for action, we can easily understand the revised version of the famous Overton and Meyer rule proposed by Janoff et al (1981), which states that the more a general anesthesia partitions into a lipid bilayer, the higher its potency. Short chain alcohols with carbon number smaller than fourteen generally have high aqueous/lipid partition coefficient. Earlier studies have shown that short-chain alcohols lower the gel-liquid phase transition temperatures (Lee, 1976). Further NMR and nuclear Overhauser effect spectroscopy experiments have suggested that the location of short chain alcohols in the region of the headgroup nearest the tails causes the hydrocarbon chains to tilt and is responsible for the decreases in the gel-liquid transition temperatures (Barry & Gawrisch, 1994; Feller et al., 2002). Hence we hypothesize that ethanol will also promote lipid lateral mobility in our ternary model membrane and depress its miscibility transition temperature. We have carried out experiments with incorporating various amounts of ethanol into giant unilamellar vesicles (GUVs), and we might extend our studies to hexanol in the future.

## Materials and Methods

### Commercial Reagents

Cholesterol, DPPC and DOPC were all obtained from Avanti Polar Lipids (Birmingham, AL), and were used without further purification. Stock solutions of lipids and cholesterol were stored in chloroform at -20ºC until use. Texas Red dipalmitoyl-phosphatidylethanolamine (TR-DPPE) was obtained from Molecular Probes (Eugene, OR), and was used at 0.8 mol % as a dye for contrast between liquid phases. Ethanol ("200" proof) was purchased from Aaper Alcohol and Chemical Co. (Shelbyville, KT) and used as received.

### Sample Preparation

*Preparation of Giant Unilamellar Vesicles*

GUVs from ternary mixtures of DPPC, DOPC, and cholesterol (2:2:1 mole fraction) were produced using the electroformation method (Angelova et al., 1992) with modifications described in Veatch and Keller (2002) to increase yield. This particular composition was chosen because it resides within the liquid-liquid coexistence region in the phase diagram (Veatch & Keller, 2003). In our body, the cholesterol level can be as low as 6 mol % in mitochondria, and as high as 40 mol % in myelin membranes (Rosser et al., 1999). We chose 20 mol % of cholesterol so that our results can have potential physiological implications. In addition, Barry and Gawrisch's NMR experiments on phosphatidylcholines have suggested that high cholesterol level ($\geq$ 30 mol%) sharply attenuated the disordering effect ethanol; whereas lower concentration of cholesterol ($\leq$ 22 mol%) enhanced it (1995). 0.25 mg of lipids and cholesterol were dissolved in chloroform to ~8.75 mg / ml and spread evenly onto the conducting side of an ITO-coated glass slide. Lipid-coated slides were then placed under vacuum for at least 30 min to evaporate remaining solvent. A capacitor was made from a lipid-coated and uncoated slide coupled with a 0.3-mm Teflon spacer, filled either with water (>18 M$\Omega$ / cm) alone or with ethanol-water solutions, and sealed with

vacuum grease. Ethanol-water solutions were prepared using a glass volumetric flask and kept for 1 or 2 days. Vesicles were then grown for ~1h at 60 $\pm$ 5 ºC.  Simultaneously, an AC field of 10V was applied across the capacitor at 10 Hz. When finished, GUVs of diameters 10-50 μm were formed, and then stored warm for 1-2hs before observation.

There would be slight deviations in composition among vesicles. As a result, we observed a (narrow) range of temperatures during which the miscibility transition occurred. In this paper, we report an onset as well as a completion of the miscibility transition in temperature. The lipid composition corresponds to what was prepared in chloroform prior to GUV growth.

*Preparation of Coverslip Sample*

We diluted vesicle solution with whatever solution that had been used to fill the capacitor (pure water or ethanol-water mixture) for a clear view under microscope. The diluted solution was then placed between two coverslips and sealed with vacuum grease before viewing. We applied heat sink grease to attach the coverslip sandwich to a home-built temperature stage. The stage consists of a peltier thermoelectric heater/cooler and a thermistor temperature probe (0.2 ºC accuracy, Sensor Scientific, Fairfield, NJ) and connected to an Alpha-Omega temperature controller. A temperature range from 10 to 50 ºC was available for the sample.

*Viewing Samples with Fluorescence Microscope*

With a Nikon Hg lamp, a Nikon Microscope (Melville, NY), and a high sensitivity Photometrics FX CCD camera (Roper Scientific, Tucson, AZ), we were able to observe and capture frames of vesicles in coverslip samples through fluorescence microscopy. The depth of field was on the order of 10 μm, so either the top or the bottom half of a vesicle could be at focus at a time. Transitions were identified as the temperature at which micro-size domains (liquid-ordered phase) appeared as temperature was increased. Experimental errors in recorded transition temperatures came from autonomous fluctuations and delays in response of the temperature controller. We estimated the systematic error to be ~1 ºC. Error bars represent 95 % confidence level (twice the standard deviation) in both onset and completion transition temperatures.

**Methods**

*Approximate Ethanol Concentration Calculations[3]*

Early work done on tadpoles suggested that alkanols partition into the bilayer to cause disordering and anesthesia at a critical concentration, namely the local anesthetic blocking concentration ($ED_{50}$; Seeman, 1972). For ethanol, this anesthetic concentration is 0.070 moles / kg dry membrane. Knowing that membranes were formed from 0.25 mg of lipids and cholesterol, we calculated the number of moles of ethanol needed to be incorporated into them:

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[3] Since there was only 0.8 mol % of Texas Red added to the bilayer, we have assumed it is negligible in all calculations.

$$\frac{0.070\,moles\,ethanol}{1\,kg\,dry\,membrane}\times\frac{1\,kg}{10^{6}\,mg}\times 0.25\,mg\,membrane=1.75\times10^{-8}\,moles\,ethanol\,in\,membrane;\quad(1)$$

Out of the total amount of ethanol added to the membrane-suspended solution, only a certain proportion would partition into the membrane. The ratio of the molarity of ethanol in membrane to that in water, so-called the membrane-buffer partition coefficient for ethanol, is 0.2 according to Pringle (1981). In order to figure out the aqueous ethanol concentration easily, we need to know the volume of the membrane. Although only the molecular volume of DPPC in gel phase ($1145\pm1.1$ Å$^3$) is available (Raymond, 1998), it was good enough for approximation to assume the lipids and cholesterol have that molecular volume in liquid phase.

The next step would be to calculate the total number of moles of lipids and cholesterol out of the total mass, each of the molecular mass of DPPC, DOPC, and cholesterol, and their mole fractions. In order not to bore the reader, I will present this step in Appendix A and simply quote the result as $3.65\times10^{-7}$ moles here.

Subsequently,

$$Volume\,of\,membrane=3.65\times10^{-7}\,moles\times\frac{6.022\times10^{23}\,molecules}{1\,mole}$$
$$\times\frac{1145\,A^{3}}{1\,molecule}\times\frac{10^{-27}\,L}{1\,A^{3}}=2.52\times10^{-7}\,L;\qquad(2)$$

$$Molarity\,of\,ethanol\,in\,membrane=\frac{1.75\times10^{-8}\,moles}{2.52\times10^{-7}\,L}=0.070\,M;\qquad(3)$$

$$Molarity\,of\,ethanol\,in\,water=\frac{0.070\,M}{0.2}=0.35\,M\,;$$
$$=16\,mg\,ethanol\,/\,mL;$$
$$=2.0\%\,by\,volume;\qquad(4)$$

We considered this approximation to be good at least on the order of magnitude. Accordingly, we prepared ethanol-water solutions of concentrations ranging from 10 to 50 mg / mL.

**Results and Discussion**

*Ethanol Induced Shape Transformation of Vesicles*

Two kinds of GUVs were compared in this survey: (1) GUVs prepared in water (control samples) and (2) GUVs prepared in water-ethanol solutions (ethanol samples). Vesicles in control samples were generally spherical (Fig. 1); whereas membranes in ethanol samples were non-spherical (Fig. 2) and wobbling (Fig. 3).
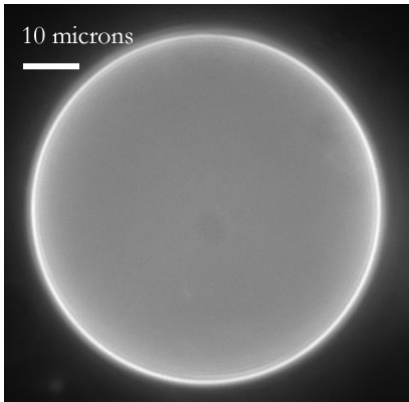
Fig. 1

Control Sample



Fig. 2

Ethanol Concentration:

50 mg / mL

Fig. 3

Ethanol Concentration:

10 mg / mL

Past works on pure lipid systems of both MLVs (multilamellar vesicles) and GUVs have shown that ethanol induces shape anomaly (Hønger et al., 1994; Angelova et al., 1999). Vierl et al. proposed that this wavy behavior is due to ethanol-headgroup association increasing the area-to-volume ratio of the vesicles (1993). For us, the swollen vesicles in the ethanol samples confirmed that ethanol did get incorporated into the membrane.

*The Role of Ethanol on Miscibility Phase Transition*

For studying the correlation between ethanol concentrations and depressions in miscibility transition ($T_m$) temperature, we prepared ethanol samples of 10, 25, and 50 mg / mL. At lower concentrations, ethanol seemed to reduce the $T_m$ as what we had hypothesized. At 10 mg / mL (0.21M), $T_m$ decreased by ~1.5 ℃, and 25 mg / mL (0.54M) of ethanol cut the $T_m$ by ~2 ℃. However, at 50 mg / mL, $T_m$ got lowered by only ~1.5 ℃. In other words, after some threshold point at ~20 mg / mL, a small increase in ethanol concentration allows the liquid-ordered phase to exist at a higher temperature. Fig. 4 is plotted to illustrate this interesting biphasic behavior.

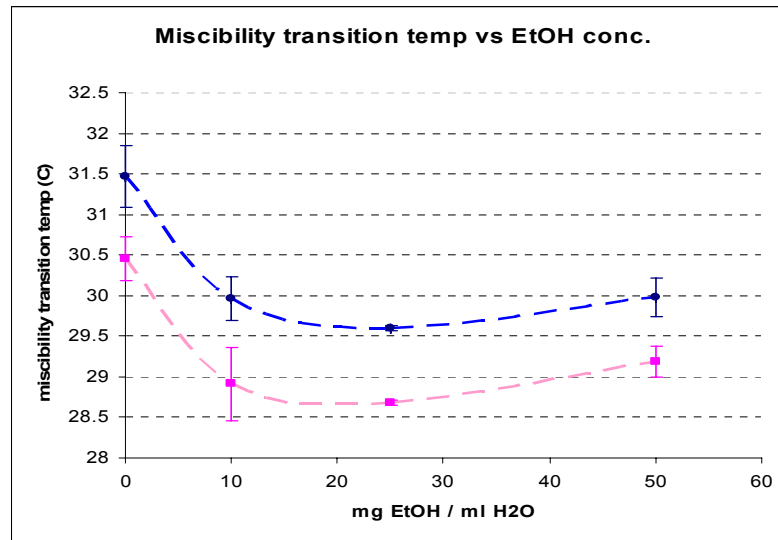**Miscibility transition temp vs EtOH conc.**

Fig. 4

This result indicates that after the biphasic point, ethanol promotes acyl chain order, rather than disrupt it. Early on, Elizabeth Rowe studied pure DPPC with spectrophotometry and saw a similar biphasic behavior with the gel-liquid transition temperature (1985); Rosser et al. also observed this when working on binary DPPC / 2% cholesterol systems using differential scanning calorimetry and fluorescence spectroscopy (1999). Both Rowe and Rosser have contributed this biphasic behavior to interdigitation (Fig. 5). Using X-ray diffraction and a light scattering method, McIntosh et al. and Nagel et al. separately verified Rowe's claim (1983, 1992).
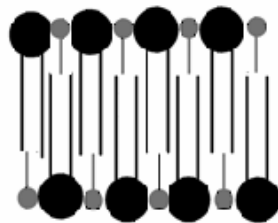


Fig. 5 Ethanol in grey and lipids in black.

Recall that at low concentration, ethanol disorders the hydrocarbon interior by tilting the lipid chain (Barry & Gawrisch, 1994; Feller et al., 2002). Nagel et al. proposed that at the threshold, the lipid tails cannot tilt any more, and they form interdigitation (1992). The interdigitated phase was characterized by acyl tails from opposing monolayers interpenetrating and interlocking each other in space, thus enhancing the order. Ethanol molecules promote interdigitation in that their small size and amphiphilic nature allow them to seal up the exposed hydrophobic end of the digits.

This biphasic behavior is very intriguing but might not be physiologically significant. Although phosphatidylcholines (PCs) can interdigitate under various conditions (Rowe & Komatsu, 1991 and reference therein), neural cell membranes might not since they are usually made up of not only PCs and cholesterol, but also phosphatidylethanolamines (PEs), gangliosides, sphingomyelin, etc. (Barry & Gawrisch, 1995[*]) Particularly, Rowe's spectrophotometry study has shown that the biphasic effect does not occur with PEs upon the addition of alcohol (Rowe, 1985). Moreover, according to Barry and Gawrisch,

the disordering effect of ethanol could be enhanced by the presence of 10 mol % brain gangliosides (1995). Therefore, in cell membranes, ethanol is more likely to induce disorder than order. Consequently, we propose that only concentrations below the biphasic point are biologically relevant.

*Ethanol vs Cholesterol*

20 mol % of cholesterol contributes to the formation of a liquid-ordered phase ($L_d$; Veatch & Keller, 2005), without the presence of ethanol. With the addition of ethanol, this $L_d$ phase exists, but at a temperature ~2 ºC lower at the biphasic point. Likewise, without cholesterol, ethanol can depress the gel-liquid transition temperature of a pure DPPC membrane at most by ~2.5 ºC (Rowe, 1985). We conclude that for a 1:1 DPPC:DOPC lipid bilayer, 20 mol % of cholesterol can slightly attenuate the disordering effect of ethanol. Similarly, Barry and Gawrisch studied pure DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) and claimed that high cholesterol levels of $\geq 30$ mol % rendered lipid membrane less susceptible to disordering by ethanol (1995[*]). These data supports the idea that cholesterol increases survivability for cells under exposure to ethanol.

Barry and Gawrisch also noted that lower concentrations of cholesterol < 22 mol % enhanced ethanol's effect (1995[*]). It would be interesting to lower the cholesterol level in our experiment and to see whether or not low cholesterol will boost disordering, however, there will be no recognizable liquid-ordered phase forming for cholesterol levels $\leq 10$ mol % (Veatch & Keller, 2003).

## Conclusion

Electroformation produces beautiful vesicles and fluorescence microscopy allows us to see micro-scale liquid-ordered phase fast and easy. Although it might not be of any physiological importance, in the near future we will increase the ethanol concentration even higher to test whether the miscibility transition temperature will level off or continue to rise. We can also extend our experiments with a long-chain alcohol, hexanol, to observe the effect of alcohol chain length on membrane ordering and miscibility transition temperature. We will compare the effects of hexane, n-hexanol and cyclohexane to test whether any effect on the lipid membrane is due to an anesthetic (hexanol), or to any 6-carbon molecule in the membrane. Moreover, since proteins play important roles in signal transduction among nerve cells, in the future our group would like to incorporate them into lipid membranes.

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## Appendix A.

Assume the total number of moles to be x.

Total mass = 0.25 mg.  (a)

Molecular mass of DPPC = 734.05 g / mol.  (b)

Molecular mass of DOPC = 786.15 g / mol.  (c)

Molecular mass of cholesterol = 386.66 g / mol.  (d)

Moles of DPPC : moles of DOPC : moles of cholesterol = 2 : 2 : 1  (e)

From (e), we knew that moles of DPPC = moles of DOPC = $0.40 \times x$;  (f)

$$\text{moles of cholesterol} = 0.20 \times x;$$  (g)

Therefore,

$$734.05\,g\,/\,mol \times (0.40 \times x\,mols) + 786.15\,g\,/\,mol \times (0.40 \times x\,mols)$$
$$+\,386.66\,g\,/\,mol \times (0.20 \times x\,mols) = 0.25\,mg \times \frac{1\,g}{10^3\,mg} = 2.5 \times 10^{-4}\,g \quad \text{(h)}$$

The answer to (h) is $3.65 \times 10^{-7}$ mols.