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Miranda J. Bradley
Portland State University

Marshall J. Colville
Portland State University

Miles J. Crumley
Portland State University

Drake C. Mitchell
Portland State University

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Differential Effects of Membrane Order on Membrane Permeability

Miranda J. Bradley, Marshall J. Colville, Miles J. Crumley, Drake C. Mitchell
Department of Physics, Portland State University

Portland State UNIVERSITY

ABSTRACT

Phospholipid membranes segregate into lateral domains of liquid ordered (l_o) and liquid disordered (l_d) phases when cholesterol and mixed species of lipids with saturated and unsaturated acyl chains are present. To examine membrane permeability and rate of vesicle rupture by Triton in pure l_o, pure l_d, and mixed l_o/l_d phases, LUVs were prepared based on the ternary phase diagram of POPC, sphingomyelin, and cholesterol. These LUVs were loaded with 2mM carboxyfluorescein (CF) and formed by extrusion at 65°C. Using a stopped-flow fluorometer, changes in CF fluorescence were measured when LUVs were exposed to sudden osmotic gradients, pH gradients, or 0.1% Triton. Acyl chain and phospholipid headgroup packing were assessed in all compositions with time-resolved measurements of DPH fluorescence lifetime and anisotropy decay. Water permeability was highest in the pure l_d phase, and a factor of more than 100 lower in the pure l_o phase. Proton permeability was lowest in the pure l_d phase, and approximately five-fold higher in the l_o phase. The rate of membrane rupture was higher in the pure l_d phase than in the pure l_o phase, with inconsistent results in the coexistence region. Water permeability was found to correlate with acyl chain packing, decreasing with increased membrane order. Proton permeability increased exponentially with increasing membrane order.

INTRODUCTION

Particular lipids were chosen for this study that are commonly found in biological membranes. This study focuses on changes in membrane permeability with lipid composition. Previous work has shown changes in permeability with the addition of cholesterol. These lipids have also been extensively studied as constituents of a ternary phase diagram[1] (Fig. 2, below) that predicts phase behavior based on percent composition. At 23°C, pure POPC membranes are in the liquid disordered (l_d) phase at room temperature. With the addition of cholesterol and sphingomyelin, the membranes exhibit liquid ordered (l_o) or solid (s_o) characteristics as shown in Figure 2.

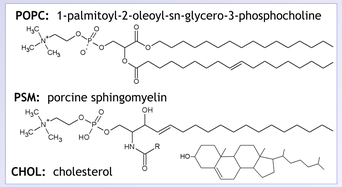

Figure 1: Membrane constituents used in this study

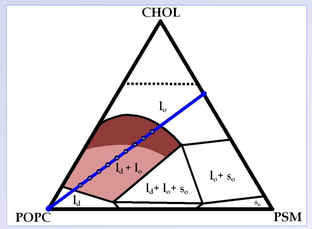

Figure 2: Ternary phase diagram at 23°C adapted from de Almeida[1]

Time resolved anisotropy decay measurements of diphenylhexatriene (DPH) embedded in membranes have also been shown to model membrane order (Mitchell[2]). DPH is incorporated into the bilayer where its lifetime and anisotropy measurements indicate packing properties of the bilayer acyl chains.

Questions:

Is there a correlation between membrane order as predicted by the ternary phase diagram and membrane permeability?

Is there a correlation between membrane order as predicted by time-resolved anisotropy measurements of DPH in the membrane and membrane permeability?

METHODS

Vesicle Preparation

Samples were prepared with POPC and PSM from Avanti Polar Lipids and CHOL from Sigma-Aldrich. Large Unilamellar Vesicles were prepared by mixing lipids suspended in chloroform, evaporating the chloroform under a stream of nitrogen, resuspending in cyclohexane, freezing, and lyophilizing overnight. Lyophilized samples were reconstituted with house buffer containing 2mM carboxyfluorescein, freeze/thawed 4X, and extruded with an Avanti mini-extruder at 65°C using 0.2μm membranes (0.1μm for lifetime/anisotropy measurements), then allowed to cool slowly to room temperature overnight.

Size-Exclusion Column Chromatography

Carboxyfluorescein (CF) outside of the vesicles was removed by running cooled samples over a column containing Sephadex G-25 Medium filtration gel. Clear separation of vesicles from external CF was observed, and verified by absorbance analysis of collected

Stopped-Flow Fluorimetry

Vesicle preparations were exposed to sudden changes in external environment by rapid mixing using the Applied Photophysics piStar-180 spectrometer with a mix time of approx. 3ms. CF was excited at 492nm, and emitted fluorescence was detected through a 550nm high-pass filter. For water permeability measurements, the vesicles were mixed with buffer containing added sucrose to bring the final osmotic potential to be twice that of the interior. The resulting gradient is assumed to cause the vesicles to shrink by 50%, the water efflux concentrates the CF and causes self-quenching.[3,4,5] The decrease in volume was fit as a single exponential decay. This equation for V(t) was fit into the osmotic permeability equation (1) and solved[6] for the water permeability coefficient P_W.

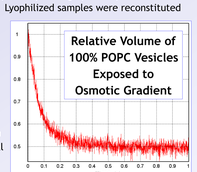

Figure 3: Normalized stopped-flow data

$$\frac{d}{dt}V(t) = (P_W \times MVW \times SAV) \times \left(\frac{C_{in}}{V_{(t)}} - C_{out}\right) \quad (1)$$

Proton permeability measurements were taken by mixing the same preparation of vesicles with buffer whose pH was lowered by titration of HCl, resulting in an external pH of 6.7 (and gradient of 0.52). Quenching of CF with lowered pH is monitored, internal pH is assumed to be 6.7 when the system comes to relative equilibrium (there was often a slow linear decay remaining after 5 minutes).[3,4,5,7,8,9] The change in fluorescence was fit with a single exponential decay equation, and the time constant from this equation was fit into the equation for proton flux (2) and solved for the proton permeability coefficient P_H.

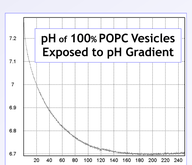

Figure 4: Normalized stopped-flow data

$$J_{H^+} = (P_{H^+})(SA)(\Delta C) = \left(\frac{\Delta pH}{t}\right)(BCV) \quad (2)$$

Finally, the same preparation of vesicles were mixed with buffer containing 0.1% TritonX-100, a concentration previously found sufficient to completely solubilize lipid membranes. CF fluorescence increased as it was released into solution, having been self-quenched at 2mM inside the vesicles. Vesicles were assumed to be entirely ruptured when the system came to equilibrium. The time constant of this exponential rise in fluorescence was reported.

Fluorescence Lifetime and Anisotropy Decay of DPH

Lifetime and Anisotropy Decay measurements were taken of vesicles incubated with DPH (400:1 molar ratio) using an ISS ChronosFD frequency domain fluorometer, in the interest of finding another measurement of order to compare against composition and permeability. The order parameter S (3) is a ratio of the non-decaying anisotropy (r_∞) with the anisotropy at time 0 (r_0); values determined by the empirical sum of exponentials model.[2]

$$S = \sqrt{\frac{r_\infty}{r_0}} \quad (3)$$

RESULTS

Water Permeability

The permeability coefficient of water through the membrane is plotted against both order as predicted by the ternary phase diagram and percent cholesterol in the membrane. There is a notable difference between the first two samples in the completely l_d phase, indicating that in this region there is a stronger correlation between percent cholesterol in the membrane than bulk membrane order.

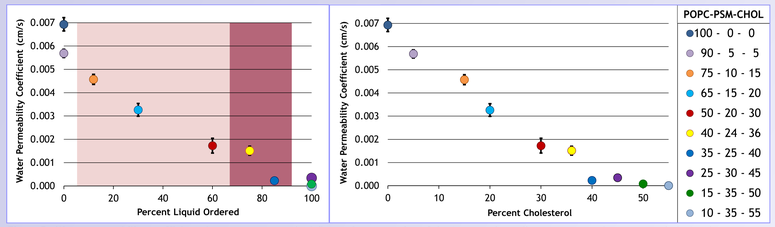

Figure 5: Water permeability coefficients by percent across the l_o/l_d coexistence region of the ternary phase diagram compared to percent cholesterol

Proton Permeability

Similar to the result seen with water permeability, there is a marked change in proton permeability while the membrane is in the completely l_d phase. Travelling through the coexistence region, there do appear to be plateaus in the differently interconnected regions. Once the membrane is in complete l_o phase, there is a marked increase in permeability with addition of cholesterol that indicates a stronger correlation to percent cholesterol.

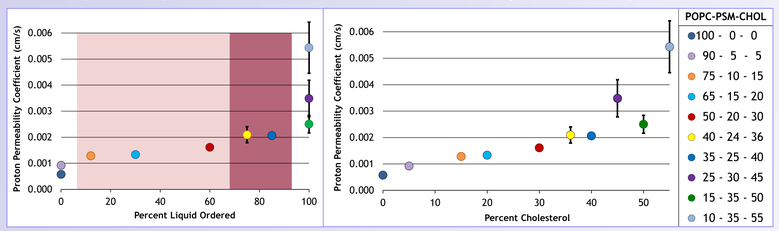

Figure 6: Proton permeability coefficients by percent across the l_o/l_d coexistence region of the ternary phase diagram compared to percent cholesterol

Vesicle Rupture by 0.1% TritonX-100

The results of vesicles ruptured by Triton were the least consistent from trial to trial, but are notable in that these are the only results that show consistent behavior in the 100% l_d and 100% l_o regions. In these regions, change in percent cholesterol does not significantly change the speed of vesicle rupture.

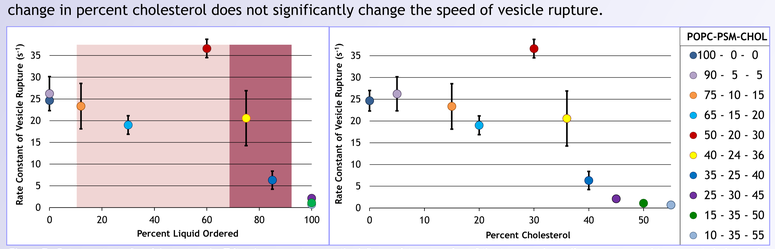

Figure 7: Rate constant of vesicle rupture by Triton by percent across the l_o/l_d coexistence region of the ternary phase diagram compared to percent cholesterol

Anisotropy Decay of DPH: Order Parameter S

The time resolved anisotropy decay order parameter S was found to correlate strongly with percent cholesterol in the membrane, increasing the fastest with the very small addition of cholesterol in the 100% l_d region. Once enough sphingomyelin and cholesterol had been added to produce a 100% l_o membrane, there was no change in order seen with the addition of cholesterol.

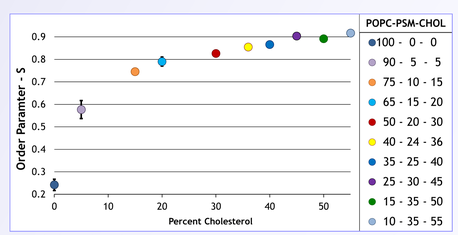

Figure 8: Ratio of non-decaying anisotropy with anisotropy at time zero compared to percent cholesterol in composition

DISCUSSION

There is a clear relationship between permeability and ensemble membrane order indicated by anisotropy decay measurements of DPH. Water permeability coefficients found in this study were comparable to those found in previous studies (Gensure[3], Lande[4]), decreasing when POPC in the membrane is replaced by PSM and/or CHOL. Proton permeability was found to be much lower than that of previous studies, although the trend of increasing permeability with decreased percentage of POPC was also seen by Gensure[3]. This difference could be explained by a difference in experimental procedure. In this study, each pH gradient mix was monitored for 250 seconds to ensure the system had come to equilibrium.

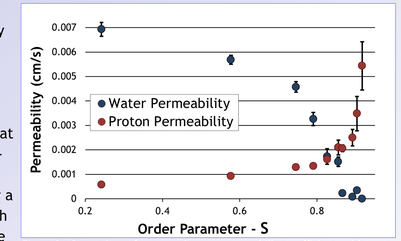

Figure 9: Comparison of water and proton permeability coefficients with ensemble membrane order as identified by anisotropy decay measurements of DPH.

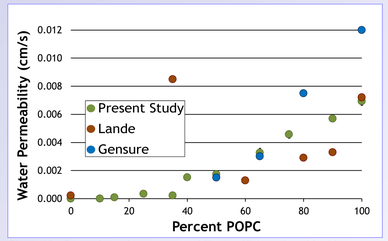

Figure 10: Comparison of water permeability coefficients found in this study to those found in previous studies, by percent POPC.

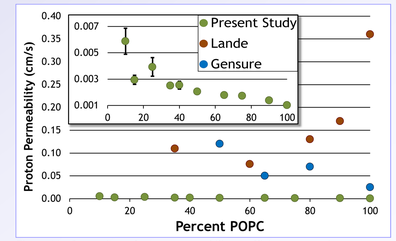

Figure 11: Comparison of proton permeability coefficients to previous studies, rescaled present study results in inset graph.

CONCLUSIONS

1. There is a strong correlation between membrane permeability and the DPH anisotropy-derived order parameter S (shown in Fig. 9); water permeability decreases with order and proton permeability increases with order.
2. Within the 100% l_d region of the ternary phase diagram, changes in percent cholesterol result in changes in water permeability (Fig. 6).
3. Within the 100% l_d and 100% l_o regions of the ternary phase diagram, changes in percent cholesterol result in changes in proton permeability (Fig. 7).
4. Within the 100% l_d and 100% l_o regions of the ternary phase diagram, changes in percent cholesterol do not affect the rate of vesicle rupture by Triton (Fig. 8).

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