Detection and Characterization of Lipid Rafts by Fluorescence Spectroscopy

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Introduction

Since the proposal of the lipid raft hypothesis in 1992 [1], the importance of membrane heterogeneity in cellular processes has begun to be uncovered. However, although evidence is quickly being uncovered regarding the involvement of lipid rafts in cellular processes such as protein signaling, biosynthetic transport, and molecular sorting [2, 3, 4], significant questions remain about the nature of these cholesterol-enriched regions.

Indirect evidence of the existence of lipid rafts has been found, but the techniques used to gather this evidence, such as detergent extraction and protein cross-linking are highly intrusive, and the question remains as to whether these techniques help to visualize previously existing rafts or whether they instead cause the rafts to form [5]. Because of these ambiguities, researchers often turn to model systems, composed of just two or three components to study the molecular interactions involved in raft formation more systematically.

Although both the binary and ternary model systems include regions of phase coexistence, believed to be the region of interest in the study of lipid rafts, the difference in size between model and natural membranes has caused some concern. While the domains in ternary model membranes are quite large (micron-scale), lipid rafts in natural cell membranes are much smaller (nanometer-scale) [6, 7] What explains this significant difference in size? Many possible explanations to this question have been proposed such as the presence of proteins [8], the presence of a cytoskeleton [9], and the transient nature of rafts [10]. It is our belief that the answer is really a combination of these and other factors, and we have focused our attention on the difference in line tension between natural and model membranes to provide one part of the explanation for this size difference.

Line tension in model membranes arises due to the hydrophobic mismatch of the liquid ordered (l_o) and liquid disordered (l_{do}) phases. The l_o phase is characterized by fully extended acyl chains, while the acyl chains in the l_{do} phase are not extended. This results in a height difference between the two phases which causes a portion of the hydrophobic acyl chains of the l_o phase to be exposed to water as seen in Figure 1A. This interaction is highly unfavorable due to the hydrophobic effect, and as a result, any domains that are present in the membrane coalesce to alleviate the line tension. In natural membranes, it is believed that the variety of phospholipid types, with varying acyl chain lengths and extent of saturation, acts as a group of "lineactants", alleviating this line tension at the interface between the raft and nonraft phases (Figure 1B), resulting in smaller raft sizes than those seen in model membranes [11].

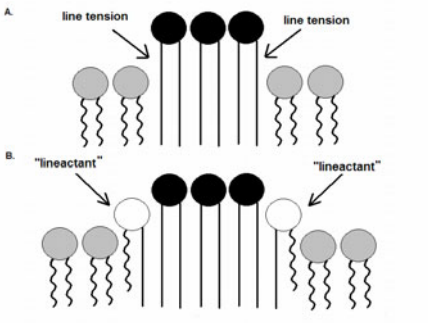

Figure 1. Line tension in model membranes (A) and alleviation of line tension in natural membranes (B).

This work investigates the applicability of two steady state fluorescence spectroscopy techniques to detect and measure membrane heterogeneities as a step towards a systematic study of line tension effects of model membranes. Initial work was performed on a binary 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)-cholesterol system so that the results could be compared to the well-established phase diagram. Current work in ternary systems utilizes the same techniques in more realistic systems to gather conclusions regarding the effect of line tension on domain/raft size.

Materials and Methods

Membrane Preparation

All model membranes were created using the rapid solvent exchange technique [12], in which heated aqueous buffer is added directly to the membrane components which have been dissolved in chloroform. The resulting solution is exposed to vacuum to allow the chloroform to evaporate, and MLVs are spontaneously formed.

The binary samples were made with cholesterol and DMPC, a fully saturated fourteen-carbon phospholipid (14:0). Ternary samples were made with either cholesterol, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) (18:1), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) (16:0) or cholesterol, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (16:0, 18:1), and DPPC. For both binary and ternary systems, spectral shift samples contained 1-acyl-2-[12-[(5-dimethylamino-1-naphthalenesufonyl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (DAN-PC), while Förster Resonance Energy Transfer (FRET) samples contained dehydroergosterol (DHE) and DAN-PC.

DAN-PC Spectral Shift

Many fluorophores exhibit an emission maximum shift due to the polarity of their local environment; DAN-PC is one of these polarity-dependent fluorophores. This property allows for determination of the local environment of the DAN-PC molecule, a tool that is especially useful in the study of amphiphilic membranes. Because of the cholesterol condensation effect, l_o membranes are less polar than l_{do} membranes [13, 14]. Therefore, it is possible to determine in which phase the DAN-PC resides, based on the emission maximum wavelength. Shifts to lower wavelengths (blue shifts) indicate a decrease in local polarity, while shifts to higher wavelengths (red shifts), indicate an increase in local polarity [15].

FRET

FRET is a commonly used fluorescent technique that is particularly useful in determining distances between an acceptor and donor molecule, due to its strong distance dependence [15].

As the donor molecule transfers energy to the acceptor molecule, its emission intensity decreases, and an efficiency of energy transfer (%E) can be defined in the following manner:

$$\%E = 1 - \frac{F_{DA}}{F_A} \qquad (1)$$

where F_D and F_{DA} are the intensity of the donor at its maximum wavelength in the absence and presence of acceptor, respectively [16].

In this work, the FRET profile is studied as a function of acceptor to lipid ratio (ALR) in order to fit the data to our model which relates efficiency of energy transfer as acceptor surface density is increased to an average distance of closest approach between the acceptor and donor molecules, a measure which provides an estimate of average domain size.

Binary DMPC-cholesterol system

In order to establish the efficacy of the two fluorescent techniques, a careful study was first performed on a binary DMPC-cholesterol system. This system has established phase diagrams [17, 18], and therefore allows for comparison of the obtained experimental results with known phase behavior.

Spectral Shift Data

The polarity-dependence of the DAN-PC molecule in membranes containing varying cholesterol compositions at 30°C is shown in Figure 2. The DAN-PC peak displays a fairly constant maximum at a wavelength of about 510 nm, but at a certain cholesterol composition, it undergoes a significant blue shift due to the cholesterol condensation effect. This cholesterol composition at which the membrane begins the blue shift varies depending on the temperature of the system and was found to be consistent with the cholesterol composition at which the membrane exits the two-phase region and becomes entirely l_o, as reported in two established phase diagrams [17, 18]. Table 1 shows the results of this experiment for temperatures of 30°C to 60°C, in comparison with these two phase diagrams.

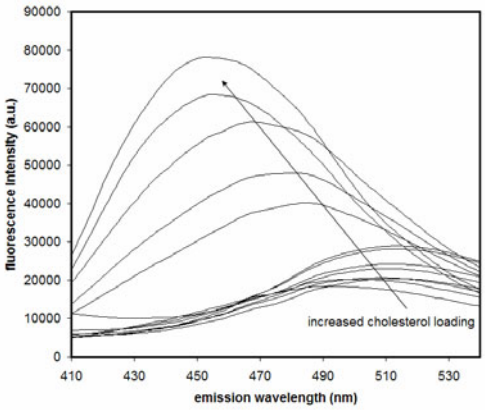

Figure 2. Emission scan of DAN-PC-labeled membranes containing increasing amounts of cholesterol at 30°C.

Table 1. Comparison of l_o phase boundary in DMPC-cholesterol membranes established using three different techniques.

Temperature	DAN-PC (x_{ch})	FRAP (x_{ch}) [17]	DSC (x_{ch}) [18]
30°C	.21	.28	.23
40°C	.30	.31	.25
50°C	.36	–	.29
60°C	.42	–	.38

It can be concluded that the DAN-PC molecule avoids the l_o phase as long as there is some l_{do} phase in the membrane; in other words, DAN-PC partitions preferentially into the l_{do} phase. Once the membrane becomes entirely l_o, DAN-PC is forced into the l_o phase, a phenomena that is marked by the observed blue shift. This technique allows for detection of the presence of an l_{do} phase.

FRET Data

Figure 3 shows the efficiency of energy transfer for membranes with varying amounts of cholesterol, at both 30°C and 60°C with lines as guides to the eye. In the 30°C case, a significant minimum in energy transfer is observed while the energy transfer remains constant in the 60°C case. At a temperature of 30°C, the membrane goes through a region of two-phase coexistence at cholesterol compositions between 8% and 28%, according to one phase diagram [17]. As the two phases form at this temperature, a minimum in energy transfer efficiency is observed due to the increased average separation between the two probes. From the DAN-PC spectral shift results, it is known that DAN-PC, the acceptor, resides in the l_{do} phase throughout the two-phase region. DHE, being very similar structurally to cholesterol, likely partitions similarly to cholesterol, favoring the l_o phase. This probe separation results in the observed energy transfer minimum as the two phases form. At 60°C, there is no two-

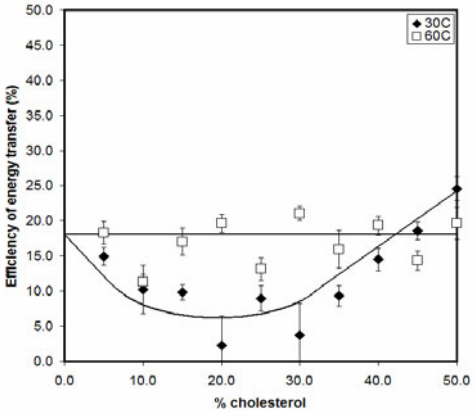

Figure 3. Efficiency of energy transfer for membranes containing varying amounts of cholesterol at 30°C and 60°C.

phase region, and therefore no probe separation. The fairly constant efficiency of energy transfer values are consistent with this lack of phase separation at 60°C.

Ternary systems

Once the behavior this particular FRET pair was established, it was possible to move to more representative ternary systems. In particular, the DOPC-DPPC-cholesterol and POPC-DPPC-cholesterol systems were chosen to investigate the effect of the third component on membrane heterogeneity. DOPC is a di-unsaturated phospholipid (18:1), while POPC contains one saturated and one unsaturated acyl chain (16:0, 18:1).

Preliminary Results

The spectral shift data for these two ternary systems show evidence of both l_{do} and l_o phases at 30°C. The same marked DAN-PC blue shift that was shown to mark the cholesterol composition at which the membrane becomes entirely l_o was found in both the DOPC-DPPC-cholesterol and POPC-DPPC-cholesterol systems. Initial phase diagrams for both systems have been published [19], which show two-phase coexistence in the DOPC-DPPC-cholesterol system but not in the POPC-DPPC-cholesterol system. The spectral shift data indicates that while there may not be membrane heterogeneity in the POPC-DPPC-cholesterol system, there likely is a direct phase transition from l_{do} to l_o at the compositions indicated by the DAN-PC spectral shift.

FRET results for these two systems show evidence of two-phase coexistence only in the DOPC-DPPC-cholesterol system. In Figure 4, the FRET profiles of DOPC-DPPC-cholesterol membranes containing 20% sterol are shown, as a function of ALR or acceptor surface density. At this constant cholesterol composition, the FRET profiles vary significantly as the DPPC composition is varied. A high efficiency of energy transfer is observed for membranes that contain 0% DPPC indicating small or no domains. As the DPPC composition is increased, the FRET profiles decrease indicating that larger domains are being formed at higher DPPC compositions. These results indicate the presence of a two-phase region, as was shown in the binary DMPC-cholesterol system. The FRET profiles vary throughout the two-phase region, depending on the size and number of domains present.

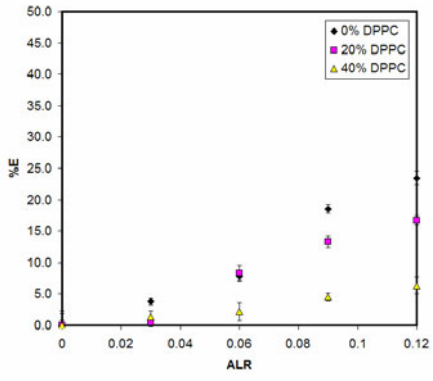

Figure 4. FRET profiles for membranes containing 20% cholesterol in a DOPC-DPPC-cholesterol system at 30°C.

In the POPC-DPPC-cholesterol system, no evidence of membrane heterogeneity is observed. Figure 5 shows the FRET profiles of POPC-DPPC-cholesterol membranes containing 20% sterol. In this case, the FRET profiles are fairly constant, indicating that there is no phase separation in this system.

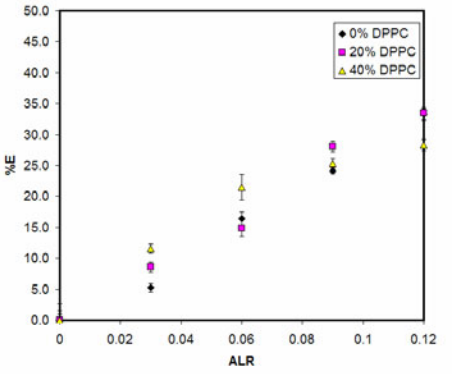

Figure 5. FRET profiles for membranes containing 20% cholesterol in a POPC-DPPC-cholesterol system at 30°C.

Discussion

The results shown in this paper demonstrate the applicability of the DAN-PC and DHE FRET pair to detect membrane heterogeneities in both binary and ternary systems. The obtained results are consistent with published phase diagrams. In addition, it has been shown that the two fluorescent techniques used in this work can be used in tandem to provide information about direct phase transitions, which are often not detectable using classical methods.

The dramatic influence of the third component in a ternary system has been demonstrated. In fact, changing only one acyl chain of the third component was shown to have significant effects on the phase behavior of the system. Future work will focus on the influence of this third component to further elucidate its effect on raft formation and size.

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