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Using Fluorescence Lifetimes to Characterize Lipid Behavior in Nanodiscs

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Using Fluorescence Lifetimes to Characterize Lipid Behavior in Nanodiscs

By

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ABSTRACT

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Cellular uptake of molecules, including drugs, can be affected by the fluidity of the membrane. Nanoparticles have been hypothesized to alter membrane fluidity resulting in inflammation and its related clinical effects. Variations in phospholipids can alter membrane structure and its interaction with drugs or nanoparticles. To study membrane lipid differences and dynamics, we are using nanodiscs and liposomes as model systems. Nanodiscs are a lipid bilayer surrounded by a membrane scaffold protein, which is a derivative of Apolipoprotein A1, a protein involved in the removal of cholesterol from the body. There are important unresolved questions about how the belt protein affects the fluidity of the lipid bilayer. The goal of this project is to learn more about the behavior of lipid-protein interactions and how that affects membrane fluidity. Using nanodiscs made of either DMPC, DOPC, DOPS and cardiolipin with 5% NBD labeled lipid, we can take lifetimes of the nanodiscs at distinct wavelength intervals, which in concert can yield information about the relaxation rate of the lipid bilayers. Fluorescence lifetime is the time it takes between the fluorophores being excited by light and returning to the ground state by releasing photons. Liposomes of similar lipid compositions will be used as a control model system. This study will examine the effects of length and saturation of hydrocarbon tails, temperature, and the overall charge of the lipid to study the relaxation rates of the membranes.

Using Fluorescence Lifetimes to Understand Lipid Behavior in Nanodiscs

Introduction

Cytoplasmic membranes surround cells to keep the contents enclosed and protected from the environment. Cytoplasmic membranes are composed mainly of lipids and these lipids have polar headgroups attached to nonpolar hydrocarbon tails. Because of the amphipathic character of lipids, due to the polar head and hydrophobic tail groups, the lipids in the membrane form a bilayer, with the headgroups on the outside surfaces. The tail groups aggregate together because the nonpolar tails do not repel each other, whereas the polar headgroups interact with water.

Nonpolar molecules and gases can easily diffuse through the membrane because the head groups of the lipids do not repel the nonpolar molecules and gases. Channels within the membrane, however, can allow transport of these polar molecules into the cell.

The amphipathic characteristics of the lipid membrane are especially important in drug delivery. When drugs cross the lipid bilayer, the drug can interact with specific proteins within the cell to inhibit or initiate downstream signaling cascades. Signaling cascades control the functions of the different parts of the cell, and may lead to production of more or different kinds of protein. Signaling cascades can even lead to cell death, a process known as apoptosis.

The fluidity of cellular membranes alters drug delivery and the effectiveness of the drug in the cell. If the cellular membrane is less fluid, drugs may not diffuse through the membrane efficiently. Therefore, studying the fluidity of membranes can aid in therapeutic drug design by enhancing the drug's ability to cross the lipid bilayer (Stubbs, 1984). Cellular membranes are dynamic systems, as the different lipids and other parts of the lipid bilayer move around the cellular membrane's surface, making the bilayer fluid. It is thought that an increase in the fluidity of the membrane may increase the transport of drugs across the plasma membrane. Studying lipid dynamics and lead to an understanding of membrane fluidity.

Studying lipid dynamics can be done using nanodiscs, a newer system than the previously used liposomes. Nanodiscs are a lipid bilayer surrounded by a membrane scaffold protein referred to as a belt protein, as shown in Figure 1. The membrane scaffold protein used in our model studies is a derivative of Apolipoprotein A1, which is a protein that transports cholesterol to the liver to be excreted (Glickman, 1977). Nanodiscs have recently been determined to be an effective model for studying lipid bilayers and solubilizing integral membrane proteins (Nath, 2006). This study uses nanodiscs to show that they are an appropriate system to study membrane fluidity and compares different lipid structures in nanodiscs and liposomes.

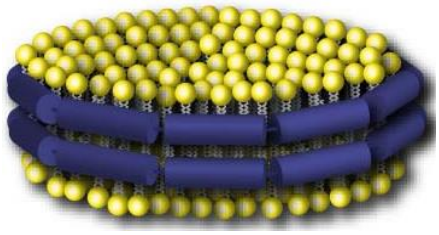

Figure 1: Proposed structure of Nanodisc
Source: www.uni-duesseldorf.de/MathNat/ipb//index.php

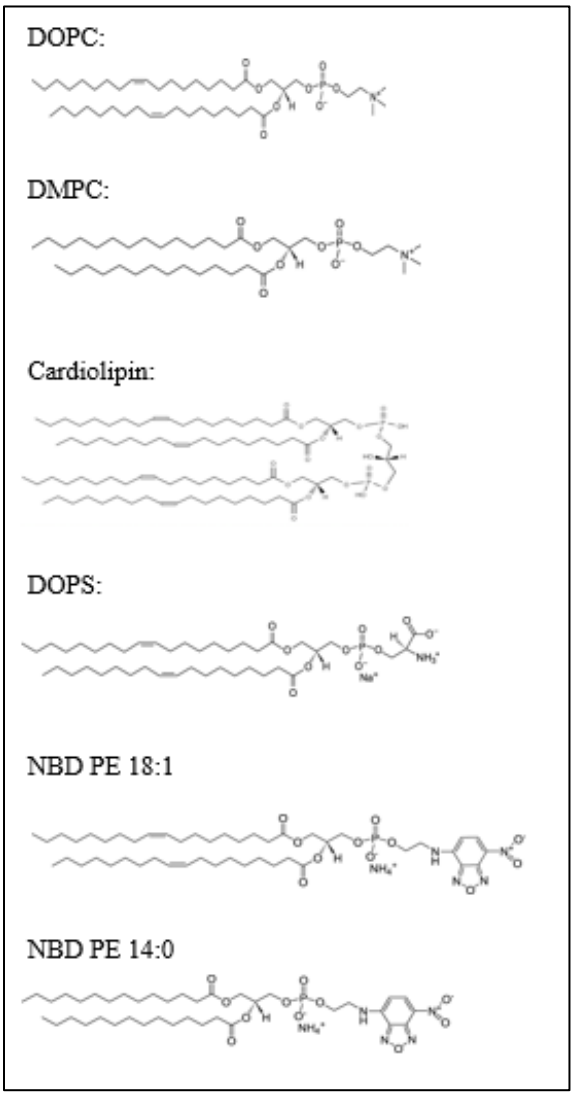

Figure 2: Structures of the different lipids and fluorophores used. (Avanti Polar Lipids, Alabaster, AL)

This study will employ various lipid structures to analyze lipid membrane dynamics to compare liposomes to nanodiscs. One lipid being studied is 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) which is a neutral phospholipid with an 18:1 carbon chain. To determine how tail length, and degree of unsaturation may affect the dynamics of lipids, DOPC was compared to 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) as DMPC is a neutral phospholipid with a 14:0 carbon chain. Variations in the number of tails and negative charges of phosphate head groups may influence the dynamic properties of lipids. The negative charges in the phosphate head groups may lead to electrostatic repulsion between lipids, while the

increase in the number of tails may lead to tighter packing. To determine how an increase in hydrocarbon tails and negative charges may influence dynamics, 1',3'-bis[1,2-dioleoyl-*sn*-glycero-3-phospho]-*sn*-glycerol (Cardiolipin) was compared to DOPC and DMPC, as cardiolipin is a phospholipid containing a double negative charge with four 18:1 carbon chain tails. To compare the effects of negatively charged headgroups in nanodiscs, this study also used 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) as the lipid contains 18:1 carbon chain tails and has an overall -1 charge.

This study utilizes fluorophores to study the lipid dynamics. The fluorophores used were 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD PE 14:0) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD PE 18:1) as they have very similar structures to the lipids that are already being used. In the nanodiscs and liposomes, the fluorophore tail length would match the lipid of interest's tail length. For example, if the composition of a nanodiscs is made of DOPS, NBD PE 18:1 would be used as the fluorophore as this would keep the tail lengths consistent throughout macromolecular complexes. This study used these different lipid compositions with a 5% NBD labeled lipid to study the effects that different overall lipid charge, hydrocarbon tail length and saturation, and temperature have on the fluidity of cellular membranes (Kinosita, 1977). To ensure that the belt protein was not influencing lipid dynamics, liposomes lacking protein were used as a control containing the same lipid and fluorophore compositions as the nanodiscs. This study is attempting to reflect that nanodiscs would be an ideal model membrane to study lipid-protein dynamics like those *in vivo* cellular membranes.

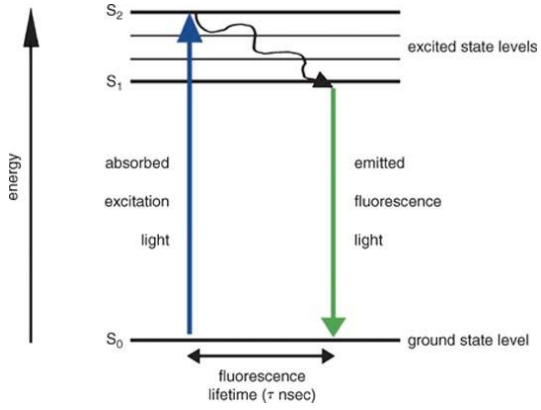

Figure 3: Jablonski diagram showing the excitation and emission of photons that characterizes fluorescence. Wikipedia.

Fluorescence lifetimes are being used to collect the quantitative data. To observe fluorescence, light energy at a discrete wavelength excites a molecule into a higher energy state that returns to the ground state by releasing a photon. A fluorescence lifetime is the average time it takes for the excited to fluorophore to return to the ground state. Though not every substance is able to fluoresce, molecules with conjugated double bonds can fluoresce. Collecting photons that are being emitted at discrete wavelengths

provides information about the different processes and changes within the lipid bilayer as it is excited and relaxed in different chemical environments. Fluorescence lifetimes are important in lipid dynamics because they can help infer the behavior of the lipids in our model systems because the fluorophores in the different lipid environments would have different lifetimes. These different lifetimes reflect relaxation rates, which can then be used to determine whether nanodiscs are reasonable model membrane systems for these lipids.

Experimental Procedures

Materials:

The following lipids were purchased from Avanti Polar Lipids (Alabaster, AL): 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1',3'-bis[1,2-dioleoyl-*sn*-glycero-3-phospho]-*sn*-glycerol (Cardiolipin), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD PE 18:1), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD PE 14:0), and a mini-extruder were purchased from Avanti Polar Lipids. The highest grade of reagents were used in all experiments.

MSP Buffer is prepared using the composition outlined by the Sligar lab (Sligar, 2008).

Preparation of Nanodiscs and Liposomes:

Nanodiscs were synthesized using the standard protocol developed by the Sligar Lab and is outlined below. The lipids and fluorophore are pipetted into a glass test tube which is determined by the molar ratio, with the values used in table 1. The molar ratio for phospholipid to fluorophore varies between the different lipids being used, due to differing molar masses, but is consistent with fluorophores being 5% of the total lipid composition.

Lipid	Lipid Concentration	Lipid Volume (µL)	Fluorophore Concentration	Fluorophore Volume (µL)
DOPC	25 mg/mL	225.16	1 mg/mL	61.50
DMPC	25 mg/mL	217.36	1 mg/mL	73.44
Cardiolipin	25 mg/mL	160.52	1 mg/mL	22.95
DOPS	25 mg/mL	62.79	1 mg/mL	18.85

Table 1: Table of different lipid compositions and the corresponding amount of fluorophore (NBD) that was used.

Preparing liposomes is a very similar process, omitting the detergent and the belt protein. After drying the lipids, they would then immediately go into the overnight freeze in the -20°C freezer, and then be rehydrated using MSP buffer, as outlined in Table 2.

Lipid	Volume of 1x MSP Buffer (µL)
DOPC	250
DMPC	300
Cardiolipin	300
DOPS	200

Table 2: Table of different volumes of 1x MSP buffer needed to bring up the film of dried lipids.

Data Collection:

The FLASC 1000 (QNW) is a fluorimeter and TimeHarp timing board (PicoQuant) were used to collect the data, which comes as single-photon counts. The FLASC 1000 was used along with a 470 nm laser and the pulse repetition rate was set between 2.5 and 10 MHz to excite the liposome or nanodisc samples. An example of the resulting data looks like the graph shown in Figure 3, which is a histogram of the lifetimes, which has a maximum peak of 40,000 counts.

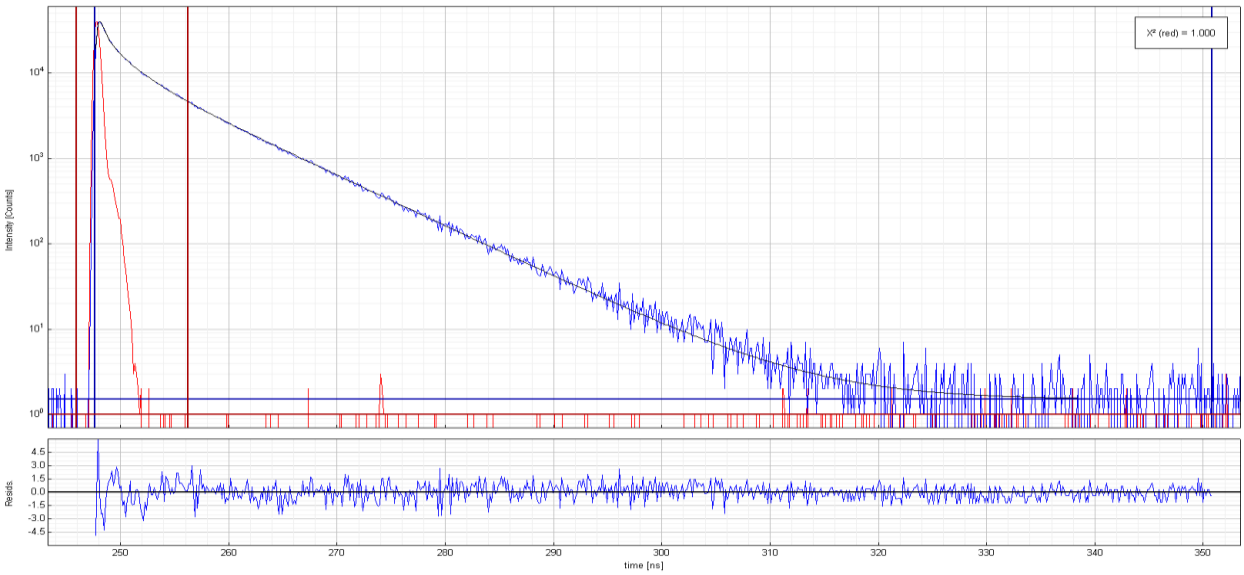

Figure 3: Example of Data Collected.

The lifetimes were obtained by exciting the fluorophores in the nanodiscs or liposomes using vertically-polarized light and the emission was detected in a magic angle (54.7° from the vertical) polarized filter. The intensity of light is depicted by the equation: $I_T = I_{||} + 2I_{\perp}$. The total intensity of light is equal to the intensity of light parallel and twice the intensity of the perpendicular. The magic angle is the angle in which this equation gives the maximum total

intensity because it would best account for the rotation of the molecule in a cone-like shape (Lakowicz).

Data Analysis:

The data was analyzed using Fluofit (PicoQuant, Berlin, Germany). The data analysis fits the data curve with exponential equations, which can be used to describe the intensity decay. The intensity decay is shown as:

$$I(t) = \sum a_n e^{-\frac{t}{\tau_n}}$$

Where α is the amplitude of the exponential, t is the time, and τ is the lifetimes (Kinosita, 1982). FluoFit fits different parts of the decay curve, starting from when the histogram first reaches 30,000 counts to the point in which the histogram returns to the baseline, with exponentials. The average of the exponential terms is the average lifetime of the fluorophore in that environment at that discrete wavelength of emission. For example, Figure 3 is fit to three different exponential terms, which represents the three different portions of the curve that corresponds to the slow, fast, and medium lifetimes.

Results and Discussion

Through the analyses, there were many differences that were reflected in the experiments. As hypothesized, there were significant differences in the lifetimes of the fluorophore between nanodiscs and liposomes, charged and uncharged environments, environments with shorter lipid tail length, and different amounts of lipid tails, which are outlined below.

As temperature increases, lifetimes become shorter due to more kinetic energy present in the system at higher temperatures, which allows for more movement in the nanodiscs and liposomes. When there is more movement in these large macromolecular complexes, there is more opportunities for relaxation by releasing a fluorophore.

As wavelength increases, there is less energy in the system, therefore at higher wavelengths of emission, lifetimes are longer because in less energetic environments there would be less kinetic energy present and therefore less movement. Because of this, it would take longer to release a photon and return to the ground state.

The lifetimes of the fluorophores in nanodiscs were shorter than in liposomes. Figure 4 is a representative graph of the different lifetimes at different temperatures are plotted against

wavelengths of emission. This could be due to the proposed lateral pressure provided by the belt protein in the nanodiscs, but this will be investigated more using time-resolved emission spectroscopy, explained further in my future directions.

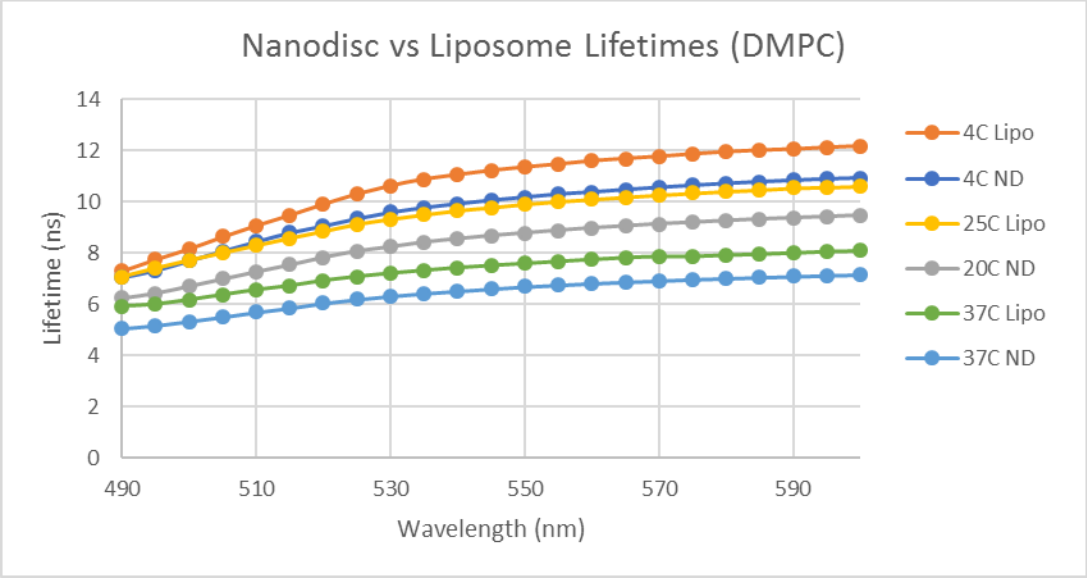

Figure 4: Chart of Nanodisc vs Liposome lifetimes at three different temperatures over 490-600 nm emission wavelengths.

The difference in behavior between lipids of different tail lengths and saturation is shown when comparing DMPC and DOPC lifetimes. These lipids have similar head group structures, but they have different tail lengths. Carbon chains with unsaturated bonds have kinks in the tails, which would prevent the lipids from packing closely together. DMPC has a longer lifetime across different temperatures than DOPC because of the differences in packing, as shown in Figure 5. DOPC's *kink* in the tail would allow for more space between the lipids and because of differences in how tightly the lipids pack in the nanodiscs and liposomes, fluorophores in nanodiscs with less tightly packed lipid tails have shorter lifetimes.

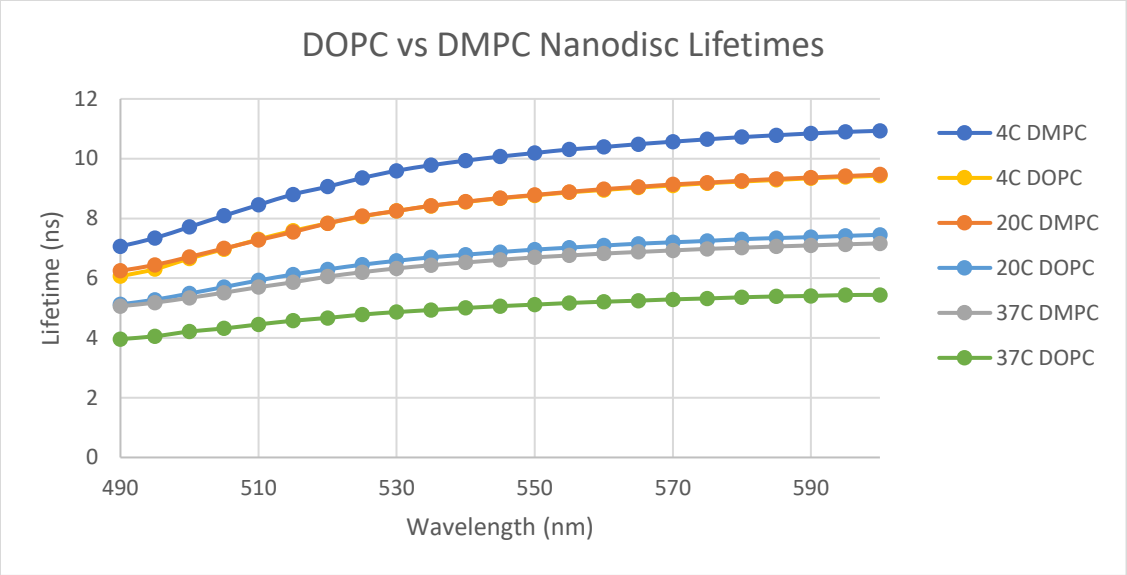

Figure 5: Chart of the different lifetimes vs wavelength of DMPC and DOPC nanodiscs at different temperatures.

Charged molecular environments can affect the lifetime of the fluorophore. In order to study this, compared DOPC vs DOPS nanodiscs and liposomes. DOPC and DOPS have 18:1 carbon chains for tails, but DOPS's headgroup has two negative charges, whereas DOPC is an overall neutrally charged molecule. As shown in Figure 6, the fluorophore in a charged environment has a shorter lifetime. This is due to the charges repelling each other and would allow for more movement between the lipids, which would cause the nanodiscs and liposomes to relax at a faster pace than in a neutral environment.

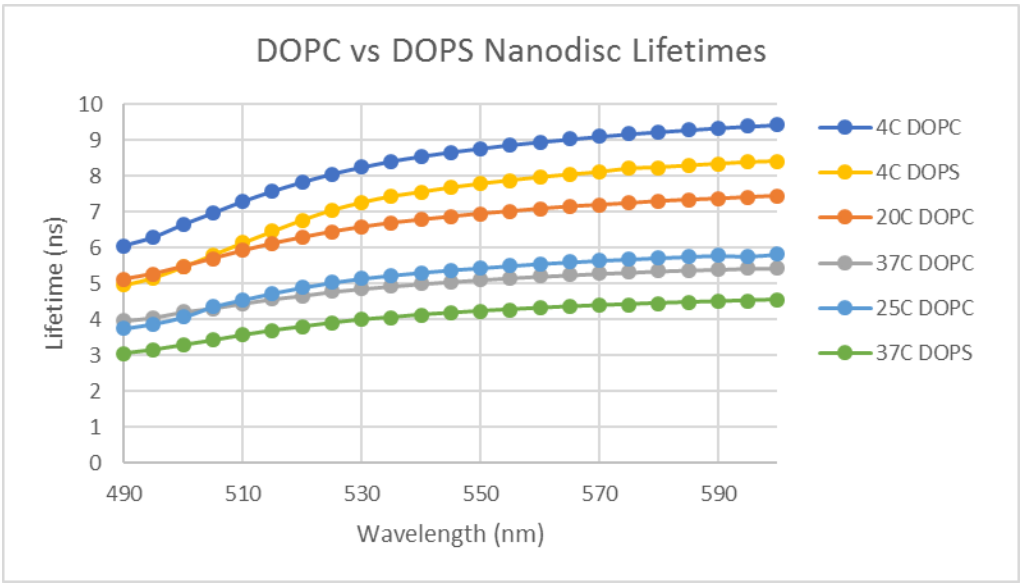

Figure 6: Wavelength vs Lifetime of DOPS and DOPC nanodiscs across different temperatures.

The last comparison made was between charged environments and numbers of tails. Cardiolipin has an overall -2 charge and four hydrocarbon tails, whereas DOPS has a single negative charge and only two hydrocarbon tails. The fluorophores in the more charged environment with more hydrocarbon tails had higher lifetimes. This could be due to tighter packing with the cardiolipin lipids that they would not be able to relax as quickly as in the smaller lipids of DOPS.

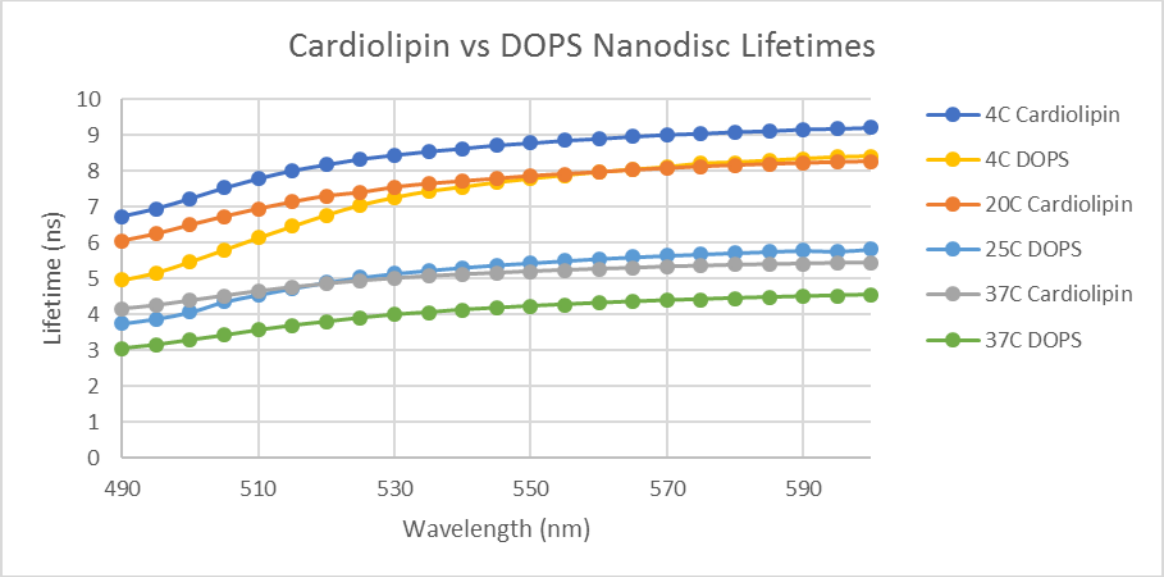

Figure 7: Wavelength vs lifetime of Cardiolipin and DOPS nanodiscs.

Conclusion, Implications, and Future Directions

From these studies, many of the differences of lipid environment and the effects it has on the relaxation rate of fluorophores in those environments were explored. The different lipid environments reflected that nanodiscs are appropriate models for cellular membranes as they are more fluid. Cells membranes, in vivo, are fluid as lipids move around and form rafts which can move around the cellular surface. Because the nanodiscs were interacting with protein, they were more cell-like in character, and they were able to relax quickly, which can be correlated to its fluidity.

Next steps include performing time-resolved emissions spectroscopy (TRES) measurements to better understand what processes are happening in the lipid environments. TRES studies compare the lifetimes to the amount of counts at each wavelength. TRES creates a 3D model of the intensities vs time vs wavelength of the emitted photons (Easter, 1976). By

studying TRES data, there can be better inferences in the different processes in the different lipid systems. Another direction to explore would be to understand the differences between fluorophores on the tail groups instead of the head groups of the lipids. The study conducted used head group fluorophores which reflect head group relaxation, but tail group behavior was not well-investigated. Using fluorophores on the tails would help to better understand what the tail groups are doing in these systems.

Other studies can be done in the future to put proteins within the nanodisc to be able to crystallize integral proteins to investigate their structure in their native conformations in membranes, which can be used to study the structure and function of these proteins. If these proteins and process are studied more thoroughly, drug delivery can be made more effective.

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