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The Effect of Docosahexaenoic Acid (DHA) -Containing Phosphatidylcholine (PC) on
Liquid-Ordered and Liquid-Disordered Coexistence

by
Yongwen Gu

A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy
in
Applied Physics

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Abstract

Plasma membranes are essential to both the structure and function of mammalian cells. The first unifying paradigm of membrane structure, the Fluid Mosaic Model, is no longer considered adequate to describe the many non-homogeneous lipid structures that have been observed in both natural and model membranes over the past approximately thirty years. The field of membrane biophysics now appreciates that the complex mixture of different lipid species found in natural membranes produces a range of dynamic, laterally segregated, non-homogeneous structures which exist on time scales ranging from microseconds to minutes.

When sphingomyelin (SM), POPC and cholesterol are all present in a bilayer there is wide range of compositional ratios where the bilayer consists of a coexistence between two fluid phases designated liquid ordered (lo) and liquid disordered (ld). The lo phase is cholesterol-rich phase characterized by relatively high molecular order and slow rotational and translational motion, while the ld phase generally has low molecular order and relatively rapid rotational and translational motion. The driving force for the formation of these two phases is the ability of cholesterol to form favorable van der Waals contacts with the two saturated acyl chains on PSM and the one saturated acyl chain on POPC.

The ternary system is an important model system for examining the physical properties and functional implications of co-existing lo and ld phases. However, it does not include one of the most significant compositional variables found in many important mammalian

membranes. Membranes in the nervous system contain high concentrations of the highly polyunsaturated fatty acid docosahexaenoic acid (DHA), which contains 22 carbons and 6 double bonds. A wide range of experimental evidence shows that DHA-containing phospholipids are important for optimal performance of a number of membrane signaling systems and membrane protein functions. The goal of this study is to determine how addition of a DHA-containing phospholipid, PDPC alters the biologically important lo and ld co-existence region.

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Table of Contents

Abstract	i
Acknowledgements	iii
List of Tables	vii
List of Figures	viii
1. Introduction of Lipid membranes	1
1.1 Overview of Membrane Models.....	1
1.1.1 Historical Perspectives.....	1
1.1.2 Fluid Mosaic Models	2
1.1.3 Lipid Raft Hypothesis	3
1.2 Molecular Structures of Plasma Membranes.....	4
1.2.1 Phospholipid	5
1.2.2 Sphingomyelin	6
1.2.3 Cholesterol	7
1.2.2 Micelle, Liposome, and Bilayers	8
1.3 Polyunsaturated Fatty Acids.....	9
1.3.1 Trans Double Bonds and Cis Double Bonds in Fatty Acids.....	9
1.3.2 Polyunsaturated Fatty Acids	11
1.4 Phase Behavior	14

2. Lipid Composition and Fluorophores	19
2.1 Ternary Phase Diagrams Diagram	19
2.2 Compositions of Lipid Bilayer	22
2.3 Fluorescent Probes	23
2.3.1 NBD-DPPE	23
2.3.2 DPH.....	25
2.3.3 Rhod-DOPE	26
3. Material and Methods	28
3.1 Sample Preparation	28
3.2 Data Collection.....	32
4. Fluorescence Spectroscopy	35
4.1 Overview of Fluorescence.....	35
4.2 Jablonski Energy Diagram	37
4.3 Fluorescence Spectra.....	39
4.4 Stokes Shift	43
4.5 Förster Resonance Energy Transfer	43
4.6 Orientation Factor	48
5. Frequency-Domain Lifetime and Anisotropy Measurement	50
5.1 Time-resolved Fluorescence	50

5.2 Least-Squares Analysis of Frequency-Domain Intensity Decays	52
5.3 Time-resolved Anisotropy Decays	54
5.4 Rotational Diffusion Model	57
6. Instrumentation	61
6.1 ISS Chronos Spectrometer	61
6.2 Agilent 8453 UV-Visible spectrophotometer	67
6.3 ISS PC1 Photon Counting Spectrofluorometer	69
7. Results and Discussion	71
7.1 Lifetime Reports.....	71
7.2 Anisotropy Reports	84
8. Conclusion	96
References.....	98

List of Tables

Table 3.1 Table of Abbreviation	28
Table 3.2 List of Samples	31

List of Figures

Figure 1.1 From Cell Membrane to Phospholipid	4
Figure 1.2 Structure of POPC	5
Figure 1.3 Structure of Sphingomyelin.....	7
Figure 1.4 Structure of Cholesterol.....	8
Figure 1.5 Structure of Liposome and Micelle	9
Figure 1.6 Butyric Acid, a Short-Chain Fatty Acid	10
Figure 1.7 Acyl Chain with Cis and Trans Fatty Acids	11
Figure 1.8 Fatty Acid Numbering	12
Figure 1.9 Lipid Raft Lipid Organization Scheme	15
Figure 1.10 Phospholipid Bilayer with Cholesterol.....	16
Figure 1.11 Effects of Unsaturated Lipids on a Bilayer	17
Figure 1.12 Change of Phase Behavior with Temperature	18
Figure 2.1 Ternary Phase Diagram of POPC/SM/Chol Mixtures.....	20
Figure 2.2 Partitions of Fluorescence Probes in Lipid Bilayer	27
Figure 3.1 Flow Chart of Production of LUV	30
Figure 3.2 Absorption Spectrum for 535nm Band Pass and 405nm High Pass	33
Figure 4.1 Jablonski Energy Diagram of Fluorescence	38
Figure 4.2 Absorption of Light Passing through a Medium	40

Figure 4.3 Spectra of NBD-DPPE, DPH, and Rhod-DOPE	42
Figure 4.4 Stokes Shift.....	43
Figure 4.5 Overlap Spectrum of NBD-Rhod Pair and DPH-Rhod Pair	44
Figure 4.6 Resonance Energy Transfer Jablonski Diagram.....	45
Figure 4.7 FRET Efficiency as a Function of Distance	47
Figure 4.8 Dependence of the Orientation Factor κ^2 on the Direction of the Emission Dipole of the Donor and the Absorption Dipole of the Acceptor.....	49
Figure 5.1 Definition of the Phase Angle and Modulation of Emission.....	51
Figure 5.2 Frequency-Domain Measurements of Anisotropy Decay	55
Figure 5.3 Example of Anisotropy Decay	56
Figure 6.1 Schematic Drawing of ChronosFD, the Frequency-Domain Fluorometer from ISS.....	61
Figure 6.2 Magic Angle	62
Figure 6.3 Examples of Raw Lifetime Data	65
Figure 6.4 Example of Raw Anisotropy Data.....	66
Figure 6.5 A Representation of the Optical System Housed within the Agilent 8453 UV- Visible Spectrophotometer	68
Figure 6.6 A Schematic of the ISS PC1 Photon Counting Spectrofluorometer	70
Figure 7.1 Lifetime Change of NBD-DPPE and DPH with %I ₀ phase.....	71
Figure 7.2 Lifetime Change of NBD and DPH with Temperature	73

Figure 7.3 Lifetime Change of NBD-DPPE and DPH with Presence of Rhod-DOPE	75
Figure 7.4 FRET Efficiency Change Based on $R_0 = 49\text{\AA}$ and $R_0 = 36\text{\AA}$	76
Figure 7.5 FRET Efficiency of NBD-Rhod and DPH-Rhod	78
Figure 7.6 FRET Lifetime Change with PDPC	79
Figure 7.7 FRET Efficiency Change with PDPC	81
Figure 7.8 NBD-Rhod Efficiency Change with Temperature	82
Figure 7.9 DPH-Rhod Efficiency Change with Temperature	83
Figure 7.10 Order Parameter S, Orientational Freedom Parameter frand , and Rotational Correlation Time $\langle\phi\rangle$ Change with Lipid Composition	85
Figure 7.11 Order Parameter S and Disorder Parameter F_{rand}	86
Figure 7.12 Rotational Correlation Time $\langle\phi\rangle$ and Rotational Diffusion Coefficient $D_{\perp}$	87
Figure 7.13 Order Parameter Change with Temperature	89
Figure 7.14 Disorder Parameter Change with Temperature	90
Figure 7.15 Rotational Correlation Time Change with Temperature	91
Figure 7.16 Rotational Diffusion Coefficient Change with Temperature	93
Figure 7.17 Phase Segregation due to DHA	95

1. Introduction of Lipid Membranes

1.1 Overview of Membrane models

1.1.1 Historical Perspectives

The oldest artificial membrane systems reported was the so-called Langmuir films, which are monomolecular lipid films at the air water interface, from the 18th century [1]. The first documented evidence that cell lipids are arranged in a bilayer configuration was reported in 1925 by two Dutch physicians, Gorter and Grendel [1], when the presence of proteins as components of biological membranes was unknown. In 1935 Davson and Danielli proposed a model consisting of coats of globular proteins sandwiching the outer surfaces of the lipid bilayer using thermodynamic arguments [2]. In the 1950s Robertson extended this Davson-Danielli model and built the so-called unit membrane model, or the Davson-Danielli-Robertson (DDR) model [3]. The model support the concept of a “unit membrane” with a thickness of 6 to 8 nm and Robertson argued that this unit membrane was common to all biological membranes. In 1964, Alec Bangham observed artificial membranes formed by phospholipids in the form of liposomes by using electron microscopy (EM) [4].

In 1966, the DDR model was challenged by the observation from Green and Benson [5, 6]. These authors argued that the subunits containing lipid and proteins can be separated from the whole membrane and these subunits can be reconstituted to regain activity. Based on these observations a new model was proposed where the lipids work like solvent for embedded globular proteins [6, 7]. From later experiments using electron

microscopy (EM) to visualize the structures of frozen fractures of biological membranes, embedded proteins was clearly demonstrated in biological membranes, and the idea of integral membrane proteins was introduced for the first time.

1.1.2 Fluid Mosaic Model

In 1972, S. J. Singer and Garth Nicolson conceived a model, which is known as the fluid mosaic model, to describe the structure of plasma membranes [8]. Considering the controversy at the beginning of the 1970s, the fluid-mosaic model incorporated many relevant experimental facts for the first time. One key component of the fluid-mosaic model is the concept of fluidity in the lipid bilayer component of the membranes. Biological membranes can be considered as a pseudo-two-dimensional liquid in which both lipids and membrane-associated protein molecules move laterally or sideways throughout the membrane to allow for function. Thus the membrane is more like a fluid than a solid. The plasma membrane was postulated to be composed of different kinds of lipid-protein fluid macromolecules and the overall random appearance of the composite made the membrane looks like a mosaic.

Fluid mosaic model gives the unifying paradigm of membrane structure, however, in the past thirty years many non-homogeneous lipid structures have been observed in both natural and model membranes, thus the Singer-Nicholson model is no longer considered adequate. The field of membrane biophysics now appreciates that the complex mixture of different lipid species found in natural membranes produces a range of dynamic, laterally

segregated, non-homogeneous structures which exist on time scales ranging from microseconds to minutes.

1.1.3 Lipid Raft Hypothesis

A series of experimental evidence that lipids could laterally segregate in membranes under certain conditions of composition and temperature and form distinct lipid domains was reported at approximately the same time when the fluid-mosaic model was proposed [9,10]. Later several discussions proposed that the different membrane regions induced by lipid-protein interaction are the physical basis for membrane-mediated processes [10, 11]. In 1988, a particular functional aspect of specialized domains called lipid rafts was proposed by Simons and van Meer [12]. The idea was more formally developed in 1997 by Simons and Ikonen [13]. The raft hypothesis assumes that cholesterol and sphingolipids spontaneously associate with each other to form platforms for the segregation of proteins. The proposal of coexisting fluid phases met the requirement of high degree of mobility of a membrane and it provided a system for bilayer separation with different acyl chain orders and thickness.

Lipid rafts was originally characterized by their ability to be isolated from the remaining membrane treated at 4°C with the non-ionic detergent such as Triton X100 [14]. These detergent resistant membranes (DRMs) contain high proportion of cholesterol, sphingomyelin, a variety of phosphatidylcholines and glycosylphosphatidylinositol (GPI) anchored proteins. However, there has been controversy about the possibilities that detergent does not isolate a preexisting molecular domain and instead generates a completely different compositional arrangement [15]. In 2006 at the Keystone

Symposium of Lipid Rafts and Cell Function, lipid rafts were defined as “small (10-200nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein interactions” [16].

1.2 Molecular Structures of Plasma Membranes

Lipid-bilayer membranes are formed by self-assembling in aqueous media and are typically 5 to 8 nm thick, as shown in Fig. 1.1. Plasma membranes are essential to both the structure and function of mammalian cells; it encloses a cell and separates inside aqueous solution from outside environment. Membranes not only protect the cell but also regulate the molecular movement in and out of the cell. A typical membrane consists of as many as 1500 different lipid species and a hundred or more proteins [17]. Major lipid components of plasma membranes include phospholipid, sphingolipid and cholesterol.

Figure 1.1 From Cell Membrane to Phospholipid [18]

1.2.1 Phospholipid

Phospholipids are the major lipid components of the cell membrane and form a lipid bilayer. Most phospholipids contain a hydrophilic headgroup which contains glycerol, phosphate, or choline, and two hydrophobic fatty acid acyl chains. In 1847, phosphatidylcholine in the egg yolk was first identified as phospholipid by the French chemist and pharmacist Theodore Nicolas Gobley [19]. Typically the structure of the phospholipid molecule consists of a hydrophilic head group and hydrophobic tails. The hydrophilic head group contains glycerol and negatively charged phosphate group; the hydrophobic tails are two long fatty acid hydrocarbon chains. These properties allow the phospholipid bilayer structure to be formed in water. In a phospholipid bilayer the hydrophilic head groups face the water on both sides while the hydrophobic tails hide inside lining up against one another. In biological systems, the phospholipids with other molecules such as proteins, glycolipids, and sterols form a bilayer as cell membrane [20].

Figure 1.2 Structure of POPC [21]

Fig. 1.2 shows the structure of POPC (1-palmitoyl-2-oleoylphosphatidylcholine), or 16:0, 18:1 PC, a type of phospholipid to form the lipid bilayers. The different colors show the basic structural elements common to all phospholipids. The green part shows the three-

carbon glycerol backbone, which is the starting place for building the structure. To the right, the red phosphate group and blue choline group together form the hydrophilic phosphatidylcholine (PC) head group. The two hydrophobic acyl chains, shown in black, provide the “P” and “O” to the name POPC. Note that the palmitoyl chain has no double bonds, while the oleoyl chain has one double bond in the middle.

Phospholipid acyl chains can be saturated, i.e. all carbon-carbon bonds are saturated with hydrogen molecules and there is no double bond, or they can be unsaturated, i.e. with one or more double bonds in a chain. Phosphatidylcholines with saturated acyl chains or monounsaturated acyl chains have been heavily studied. However, the biological function of polyunsaturated acyl chains is not yet clear. Polyunsaturated fatty acids will be further discussed in Section 1.3.

1.2.2 Sphingomyelin

Sphingomyelin is a type of sphingolipid. It was discovered in the 1980s and was first isolated by Johann L.W. Thudicum [22]. Sphingomyelin is enriched in the membranous myelin sheath surrounding nerve cell axons and red blood cells [23]. Fig. 1.3 shows that sphingomyelin is composed by the phosphocholine headgroup, a sphingosine and a fatty acid acyl chains. Natural sources such as eggs or bovine brain often contain sphingomyelin with various fatty acid chain lengths. The lengths of the two hydrophobic chains are often very different, though the shape of sphingomyelin is almost cylindrical [24]. Sphingolipids typically have long, saturated acyl chains and thus sphingomyelin can form more compact structure with cholesterol than phospholipids to form a liquid-

ordered domain [25, 26]. Previous research has shown evidence that there may be a sphingomyelin pool in the inner leaflet of the membrane [27, 28].

Figure 1.3 Structure of Sphingomyelin [29]

1.2.3 Cholesterol

Cholesterol is the major sterol in animal tissues. It has a polar head group and a nonpolar hydrocarbon body. In its extended form it is as long as a 16-carbon fatty acid, as shown in Fig. 1.4. The hydroxyl group of cholesterol forms hydrogen bonds with polar head groups the phospholipids and the sphingolipids, while the steroid and the hydrocarbon chain are embedded in the membrane, alongside the nonpolar fatty acid chain of the other lipids.

Cholesterol modulates membrane fluidity and participates in membrane phases. Previous research has shown that cholesterol has a strong condensing effect, i.e. the area per molecule of a phospholipid-cholesterol mixture is much lower compared with ideal

mixing, and this is induced by cholesterol-lipid interaction. Because a membrane behaves as an incompressible fluid, a decrease of the area per molecule will result in a corresponding significant increase of the total thickness of the bilayer [31].

Figure 1.4 Structure of Cholesterol [30]

1.2.4 Micelle, Liposome, and Bilayers

Fig. 1.5 shows the structures of two three dimensional structures that lipids can spontaneously form in water: liposome and micelle. They both are composed of phospholipids, which have hydrophobic acyl chains and hydrophilic headgroups. Some lipids have only one acyl chain. Hence the cross sections of the headgroups are greater than that of the acyl chains; the individual units are wedge-shaped. In this case micelles instead of liposomes are formed. The hydrophilic headgroups protect the hydrophobic acyl chains from the water environment. Non-water structures such as dirt can be captured into the micelles. Thus micelles widely exist in detergent. Some other phospholipids have two acyl chains, which leads to that the cross sections of the

headgroups equal that of the acyl chains; the individual units are cylindrical. In this case lipid bilayers can be formed in liposomes.

Figure 1.5 Structures of Liposome and Micelle [32]

Small and large multilamellar and unilamellar vesicles have been widely used to study membranes. Unilamellar vesicles can be prepared from large multilamellar vesicles (LMV) which is a large “onion-like” structure consisting of multiple nested bilayers. Small unilamellar vesicles (SUV) are typically 15 to 30 nm in diameter and it can be prepared by sonication using a cuphorn, bath, or probe tip sonicator. Large unilamellar vesicles (LUV) range from 100 to 200 nm or larger [33], and the size of giant unilamellar vesicles (GUV) is on the order of a few tens of micrometers which is similar to the size of the plasma membrane of cells. LUV and GUV can be prepared by extrusion (as described in Chapter 4).

1.3 Polyunsaturated Fatty Acids

1.3.1 Trans Double Bonds and Cis Double Bonds in Fatty Acids

Fatty acids are long chain hydrocarbons possessing a carboxyl (COOH) group at one end, as shown in Fig. 1.6. If the fatty acids have no double bond, they are called saturated. If the fatty acids do have double bonds, they are called unsaturated.

Figure 1.6 Butyric Acid, a Short-Chain Fatty Acid [34]

There are two kinds of unsaturated fatty acids, one is called trans fatty acids, that is when the hydrogen atoms are on the opposite side of the double bonds of the carbon chain; when the hydrogen atoms are on the same side, it is called cis fatty acids. The difference between these two is shown in Fig. 1.7. Cis double bonds lead to a bent form of the acyl chain, while trans double bonds lead to straight form of the acyl chain.

In 2002 the Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids from the National Academy of Sciences (NAS) published their concerns regarding consumption of trans fatty acids [35]. Their reason were based on two important facts, one is that trans fatty acids are not essential and provide unknown effect to human health, and second is that trans fatty acids increase the risk of coronary heart disease while both saturated and unsaturated; because trans fatty acids increases low-density lipoprotein (LDL) cholesterol (also known as bad cholesterol) and lower high-density lipoprotein (HDL) cholesterol (also known as good cholesterol).

Figure 1.7 Acyl Chains with Cis and Trans Fatty Acids [36]

1.3.2 Polyunsaturated Fatty Acids

Polyunsaturated fatty acids (PUFA) contain more than one double bond in one acyl chain. PUFA includes many important compounds such as essential fatty acids (EFA). Essential fatty acids are indispensable for the health of mammalian animals. It can only be ingested from food and cannot be synthesized in human body. Only two EFAs are known for humans: alpha-linolenic acid (an omega-3 fatty acid) and linoleic acid (an omega-6 fatty acid) [37].

As shown in Fig. 1.8, the first carbon next to the carboxylate is marked as α , the next is β , and so forth. The last letter in the Greek alphabet ω is always used to label the last position in the carbon chain. For example, the term ω -6 means that the first double bond exists as the sixth carbon-carbon bond from the terminal CH_3 end (ω). The number of carbons and the number of double bonds are connected by a colon and go before or after the term indicating the position of the first double bond. For example, ω -3 22:6 or 22:6 ω -3 or 22:6 n-3 indicates a 22-carbon chain with 6 double bonds, and with the first double bond in the third position from the CH_3 end. If not noted, double bonds are *cis* and separated by a single methylene (CH_2) group.

Figure 1.8 Fatty Acid Numbering [38]

Docosahexaenoic acid (DHA) with 22-carbons and 6 double bonds is the most polyunsaturated fatty acid commonly found in biological systems [39, 40]. DHA is a primary structural component of the human brain cerebral cortex, sperm, testicles and retina [41]. It can be synthesized from alpha-linolenic acid or obtained directly from fish oil. The first double bond of DHA is the third bond from the terminal CH_3 end. Thus DHA is also known as 22:6(ω -3) fatty acids.

An early finding about health benefits of fish oil reported that due to the abundance of ω -3 long-chain fatty acids in deep-water fish, the incidence of heart disease among northern

Eskimos who consumes the fish is low despite of their high fat intake [42]. There are increasing amount of researches examining the benefit and/or preventative effects of ω -3 fatty acids, including cardiovascular disease (CVD), infant development, rheumatoid arthritis, asthma, cancer, and mental illness, among others [43]. It was argued that ω -3 fatty acids increases bleeding time, and erythrocyte deformability; decrease platelet aggregation, blood viscosity, and fibrinogen[40]. Based on the results of the studies, consumption of ω -3 fatty acids was encouraged.

It was assumed that membranes rich in DHA are thick and hardly permeable because DHA contains 22 carbons. However, in 1985 Dratz et al showed that membranes rich in DHA are thin and leaky [44], which is the opposite to what was expected. Studies on the structure of DHA have emerging using different methods including nuclear magnetic resonance (NMR), X-ray diffraction and molecular dynamics (MD) simulations [45- 47]. It is reported that the extreme flexibility of the PUFA chain in DHA is associated with the high “fluidity”, permeability, elasticity, fusion, enhanced flip-flop, and preference for non-lamellar phases in membranes [48]. A growing number of experimental observations supports that cholesterol has an aversion for PUFA. X-ray diffraction and solid-state ^2H NMR measurements demonstrate greatly reduced solubility of cholesterol in PUFA-containing membranes [49, 50]. Cholesterol interacts differently with PUFA and saturated fatty acids (SFA) and the difference was first indicated by ^2H NMR spectra [49]. The distinction of cholesterol orientation incorporated into dipolyunsaturated phospholipids compared to phospholipids with a saturated sn-1 chain is further demonstrated by neutron scattering measurements [51]. Within the highly disordered

environment of dipolyunsaturated membrane, cholesterol is more likely to be tipped over to lie flat between leaflets. Those results demonstrated that PUFA not only tends to force cholesterol out of the membrane, it also affects the orientation of the sterol within the membrane [48].

Despite of the progress made in recent years, the specific structure and biological function of DHA remains unclear. In this study, we report on how DHA affects the microdomain of lipid membrane with the co-existing liquid ordered and liquid disordered fluid phases using a fluorescent spectroscopy method.

1.4 Phase Behavior

In the two leaflets of the bilayer, the lipid distribution is not random. The sphingolipid and cholesterol enriched domains appear more tightly packed and stable than the surroundings. Such domains are the so-called liquid-ordered phase (lo). The rest less ordered surroundings in the membrane are in the so-called liquid-disordered phase (ld) [52- 54]. In a certain range of temperature depending on the kind of lipid present, the lo phase and the ld phase co-exist in the lipid bilayer. When the ld phase is the connected phase, the lipids that are in lo phase can float freely in those in ld phase, like an ice raft floating in the sea. Depending in the relative composition of the constituents it is also possible to have lo and ld coexistence where the lo phase is connected, which would like pools of water in a plain of ice. The current perception of a lipid raft has been refined by considering the dynamic entities and is commonly used to describe an arrangement containing various lipids and proteins. For the purpose of this study, we will regard a raft only as liquid-ordered domain in a lipid membrane.

Figure 1.9 Lipid Raft Lipid Organization Scheme [55]

Fig. 1.9 shows the structure of the lipid bilayer sheet. Although not all of the phospholipids within the raft are fully saturated, the hydrophobic chains of the lipids contained in the rafts are more saturated and tightly packed than the surrounding bilayer. Due to the enrichment of saturated phospholipids and cholesterol, the liquid-ordered phase in the middle shown in the middle of Fig. 1.9 is thicker than the surroundings.

Under normal biological conditions, that is when mixed with excess water at 37°C, which is beyond transition temperature of most lipid mixtures, phospholipids and sphingomyelin form stable bilayers with their hydrophobic acyl chains in the interior and hydrophilic head groups exposed to the water, as shown in Fig. 1.10. In this cartoon each blue head group corresponds to the phosphate and choline. This cartoon of a real bilayer also shows one aspect of how cholesterol disrupts the interactions between phospholipids in the fluid phase.

The unique physical characteristics of cholesterol exert complicated influence on lipid bilayer properties. It has been shown that cholesterol in a fluid phase bilayer decreases its permeability to water [56]. That is because cholesterol intercalates between lipid molecules, fills in free space and decreases the flexibility of surrounding lipid chains [57].

Figure 1.10 Phospholipid Bilayer with Cholesterol

The fluidity of the lipid molecules changes with temperature. At what is called “melting temperature”, lipid bilayer can transform from solid phase to liquid phase. Solid phase is commonly called the “gel” phase. The phase behavior of lipid bilayers is largely determined by the strength of the attractive Van der Waals interactions between adjacent lipid molecules. Generally, the lipids with longer acyl chains have higher melting temperature, due to more carbon molecules having more interaction energy. Also, at a given temperature, a short-tailed lipid will be more fluid than an otherwise identical long-tailed lipid [58]. Besides the length of the carbon chain, the unsaturation of the acyl chain can also affect the transition temperature. An extra double bond causes a kink on the acyl chain, which leads to extra space between bilayers and allows more flexibility of the adjacent chains, as shown in Fig. 1.11. Thus increasing double bonds can also lower the transit temperature.

Figure 1.11 Effects of Unsaturated Lipids on a Bilayer [59]

Fig. 1.12 demonstrates the phase change in membrane according to temperature change. In this diagram, the blue color represents liquid-disordered phase, the purple color represents the liquid-ordered phase, and the green color represents the solid, or gel, phase. Note that in liquid-ordered phase, the acyl chains of phospholipid are less wiggled and have more participation of cholesterol than that in liquid-disordered phase. In the solid-ordered phase, the cholesterol molecules have been “squeezed out” by the crystalline structure of the acyl chains.

From figure 1.12a through figure 1.12c the temperature goes down accordingly. Figure 1.12a shows that at 37°C (close to mammalian body temperature) the lipids are typically in ld phase with fast motions of lipid chains. Figure 1.12b shows that at lower temperature (23°C for example, which is room temperature), part of ld phase changes to lo phase as temperature goes down. This is because that lower temperature provides less

dynamic energy for lipid molecules to diffuse and exchange locations. Figure 1.12c show that at even lower temperature (10°C for example) liquid phase changes to solid (gel) phase. This is consistent with previously mentioned that at some certain temperature, lipid transforms from liquid phase to solid phase.

Figure 1.12 Change of Phase Behavior with Temperature [60]

Membrane lipids are free to diffuse laterally in the plane of the bilayer or to rotate about a molecular axis roughly normal to the bilayer plane, or “flip” between the two leaflets of the lipid bilayer. Individual phospholipids also possess many degrees of conformational freedom.

2. Lipid Composition and Fluorophores

2.1 Ternary Phase Diagrams

Though biological membranes have complicated components and structures, chemically simplified models for the mammalian outer leaflet have been studied to make substantial progress. Mixtures comprising a low melting temperature (low-T_m) lipid (e.g. phosphatidylcholine containing at least one unsaturated chain), a high-melting (high-T_m) lipid (di-saturated phosphatidylcholine or SM), and cholesterol exhibit key properties associated with lipid rafts [61]. The systems mimic the composition of specific biological membranes and can reproduce the coexistence of liquid-ordered (lo) and liquid-disordered (ld) domains and other complex phenomena. The compositional simplicity makes the minimal systems amenable to study composition and temperature-dependent behavior of lipid membranes [62].

POPC is an abundant low-T_m lipid in the outer leaflet of the plasma membrane [63]. Ternary mixtures containing POPC with a high-T_m lipid and Chol seem to exclusively exhibit nanoscale coexisting lo and ld phase heterogeneity [64]. The lo phase is cholesterol-rich phase characterized by relatively high molecular order and slow rotational and translational motion, while the ld phase generally has low molecular order and relatively rapid rotational and translational motion. The driving force for the formation of these two phases is the ability of cholesterol to form favorable van der Waals contacts with the two saturated acyl chains on SM and the one saturated acyl chain on POPC. In 2007, Zhao et al concluded that no macroscopic ld and lo phase coexistence

occurs in POPC/SM/Chol mixture by carefully observing the time dependence of domain formation while limiting the illumination intensity and duration with dilute probe concentrations [65]. This result implies that lipid raft must be smaller than the ~200nm resolution limit of light microscopy [62]. Researchers have published ternary phase diagrams for POPC/SM/Chol mixtures that include large regions of L_d and L_o coexisting phase using techniques with sensitivity to nanometer length scales [54, 66-69], although the locations of phase boundaries and the topology of the phase diagrams appear with large discrepancies. The discrepancies may be partially related the widespread use of fluorescence techniques in combination with excessive fluorophore concentration [62].

Figure 2.1 Ternary Phase Diagram of POPC/SM/Chol Mixtures

In 2013, Petruzielo et al reported phase behavior of POPC/SM/Chol from 15°C to 35°C using FRET with very low probe concentration and probe-free approaches including small-angle neutron scattering (SANS) and differential scanning calorimetry (DSC); they concluded that nanodomains exist in POPC/SM/Chol with radius smaller than ~7nm [62].

Fig. 2.1 is the ternary phase diagram at 25°C reported by Petruzielo et al in 2013. Each vertex corresponds to 100% composition of the labeled lipid. S in the diagram represent solid, or gel phase. The green dots in the blue tie line are the points corresponding to our sample composition. The percentage of each component for each dot is given in the green box. We assume that along the blue tie line across the coexisting range, the percentage change from ld phase to lo phase is even; thus the corresponding percentage of lo from point U to point S is 5%, 25%, 50%, 75%, and 90% respectively. This ternary system is an important model system for examining the physical properties and functional implications of co-existing lo/ ld phases. However, it does not include one of the most significant compositional variables found in many important mammalian membranes. A wide range of experimental evidence shows that DHA-containing phospholipids are important for optimal performance of a number of membrane signaling systems and membrane protein functions. One goal of this study is to determine how addition of DHA-containing phospholipid alters the phase diagram, particularly in the biologically important lo/ ld co-existence region.

2.2 Compositions of Lipid Bilayer

1-palmitoyl-2-oleoylphosphatidylcholine (POPC, or 16:0, 18:1 PC)

1-palmitoyl-2-docosahexaenoyl-sn-glycerco-3-phosphocholine (PDPC, or 16:0, 22:6 PC)

Sphingomyelin

Cholesterol

2.3 Fluorescent Probes

The applications of biophysical approaches, such as NMR spectroscopy, have resulted in a detailed understanding about the molecular arrangement within the different phases; however, NMR approaches do not provide much information of the different physical forms of the phases or domains [70, 71]. However, fluorescence microscopy by incorporating probes can directly visualize raft-like domains reporting on domain shape and size. The number of fluorescence probes has been steadily increased and it has been reported that the same fluorescent probe labeled with lipid analogues can have different partitioning phase preference in different binary and ternary systems containing mixtures of phospholipids and cholesterol [72]. For instance, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiIC₁₈) participates into ld phase in DOPC/SM/Chol mixture, but participates into lo phase in DLPC/SM/Chol mixture [73]. Lissamine rhodamine B-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (Rhod-DPPE) prefers ld phase in DMPC/DSPC mixture, but prefers in gel-phase in DLPC/DPPC mixture [74]. Fluorescent probe partitioning into particular phase domains is dependent upon local chemical environment of the lipid domains, instead of membrane phase state itself [75]. It is necessary to determine the partitioning phase preference of a particular probe in a given lipid system.

2.3.1 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DPPE)

The 7-nitrobenz-2-oxa-1,3-diazole-4-yl (NBD) group was first introduced by Ghosh and Whitehouse in 1968 [76] and has increasingly used as a fluorophore since then [77]. NBD group fluoresce weakly in water but strongly in organic solvents, membrane, and hydrophobic environments [78]. In membranology, NBD group has been synthesized with various phospholipids and cholesterol analogs to either the polar headgroup or to the nonpolar fatty acyl chain of the lipids [77]. These NBD probes have been used for studying membrane phase transitions, membrane fusion, lipid sorting in polarized cells, intracellular lipid transport and lipid metabolism in living cells, aminophospholipid translocase activity, lipid lateral diffusion, and lipid domains [78].

A few factors are critical for the validity of the lipid probe approach in the study of membranes, for example: the lipid probe must be similar to the native lipids, the probe location must be well defined within the membrane, and the probe must be randomly distributed within the host lipids. Probes where NBD group is attached with the polar headgroups or the acyl chains of phospholipids satisfy the first point, Fluorescence quenching studies in model membrane have shown that NBD group stays preferentially in the polar headgroup/hydrocarbon region in phosphatidylcholine. Most evidence so far has concluded that NBD-labeled lipids are miscible with the host lipids in the fluid phase at low concentrations. Serge Mazeres et al have demonstrated that the fluorescence properties of NBD-labeled phospholipids depend on the chemical structure and physical state of host lipids and on their own chemical structure, and NBD group are located in the region of lipid bilayer with higher polarity, which corresponds to the region of the lipid polar headgroups at the water-lipid interface [78].

In this project, we use NBD-DPPE, which is one of the rare fluorescent probes that have been consistently reported to preferentially partition into lo domain in several model membrane systems [72, 79- 81]. The chemical structure of NBD-DPPE is shown as following:

1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)
(ammonium salt)

2.3.2 1,6-diphenyl-1,3,5-hexatriene (DPH)

1,6-diphenyl-1,3,5-hexatriene (DPH) was first introduced by Shinitzky and Barneholz [82] and has become widely used in fluorescence anisotropy measurement. The orientation of DPH within lipid bilayers is loosely constrained. Generally it is assumed to be oriented parallel to the lipid acyl chain axis, but it can also reside in the center of the lipid bilayer parallel to the surface [83]. DPH is an ideal probe of the hydrophobic bilayer core for the study of phase separation in lipid bilayers because it partitions equally between it shows no partition preference between coexisting phases [84]. Its structure is shown as following.

1,6-Diphenyl-1,3,5-hexatriene (DPH)

2.3.3 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhod-DOPE)

Rhodamine (Rhod)- labeled lipids have been extensively used in the study of membrane lateral organization to address the properties of membranes displaying fluid/gel coexistence [85- 90]. Those probes have also been used in Förster resonance energy transfer (FRET) studies regarding the size of liquid-ordered cholesterol-enriched domains in binary phosphatidylcholine (PC)/Chol mixtures and in the raft system PC/sphingomyelin/Chol [87]. Rhod-probes have also been used in two-photon microscopy [91] and fluorescence lifetime imaging microscopy [86] due to their small Stokes shift, high photostability and emission in visible wavelengths [92].

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)

In our study, we use Rhod-DOPE (structure shown above) as acceptors to NBD-DPPE and DPH to form FRET pairs. Due to the two monounsaturated acyl chains which causes kinks and leads to inefficient lateral packing of the acyl chains, Rhod-DOPE partitions into *l_d* domain in the POPC/SM/Chol phase, compared to NBD-DPPE which partitions into *l_o* domain.

Figure 2.2 Partitions of Fluorescence Probes in Lipid Bilayer

Fig. 2.2 shows how the fluorescent probes attach to the lipid bilayer. NBD-PE and Rhod-PE attach to the head groups, while DPH sits in between the acyl chains or between the layers. Typically, a lipid bilayer is 5 nm thick, while DPH is about 1 nm long, thus the length of DPH is quite comparable with the length of acyl chains.

3. Materials and Methods

3.1 Sample Preparation

The phospholipids 16:0-18:1 PC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 16:0-22:6 PC, 1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (PDPC), and porcine brain sphingomyelin (SM) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Cholesterol was purchased from Calbiochem (La Jolla, CA). The fluorescently labeled phospholipids 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DPPE), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine -N-(lissamine rhodamine B sulfonyl) (Rhod-DOPE), and diphenylhexatriene (DPH) were purchased from Molecular Probes (Eugene, OR). Sphingomyelin and phospholipids were stored in chloroform when manufactured.

Table3.1 Table of Abbreviations

Abbreviation	Full Name
DHA	Docosahexaenoic Acid
POPC	1-palmitoyl-2-oleoylphosphatidylcholine
PDPC	1-palmitoyl-2-docosahexaenoyl-sn-glycerco-3-phosphocholine
SM	Sphingomyelin
Chol	Cholesterol
NBD-DPPE	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)
Rhod-DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)
DPH	1,6-Diphenyl-1,3,5-hexatriene
lo	liquid-ordered
ld	liquid-disordered

Cholesterol was in form of powder when purchased, which was very inconvenient to weigh the tiny amount needed in each sample. Therefore, during sample preparation, cholesterol was dissolved in chloroform (at the ratio of 4 mg cholesterol in 1 ml chloroform) for more accurate measurement. Cholesterol, sphingomyelin, and phospholipid were added to an 8 mL glass vial to give the required molar ratios of phospholipid: PSM: cholesterol. The compositions were mixed in a culture tube and vortexed. The chloroform was evaporated under a stream of nitrogen gas while the vial was rotated to produce a thin lipid film on the vial's surface. The thin lipid film was dissolved by adding cyclohexane to the vial [93] so the mixture can be frozen to solid phase for lyophilization. The mixture was frozen in dry ice or in a -40 °C freezer for at least half an hour. With the lids of the vial slightly loosened, the vial was placed in a pump vacuumed lyophilizer for at least three hours to completely remove the frozen cyclohexane. HEPES buffer (pH 7.22) was added to the vial, and the vial was vortexed thoroughly then put through four freeze and thaw cycles to produce multilamellar vesicles. Large unilamellar vesicles were formed by extruding [94] for at least seven times at 65°C through a pair of 0.1 micrometer membranes using a Lipex extruder (Vancouver, British Columbia, Canada) or Avanti Mini-Extruder (Alabaster, AL). The extruded vesicles are slowly cooled down in a water bath overnight from 65°C to room temperature. Then the vesicles are mixed with HEPES buffer (pH 7.22) in each cuvette for desired concentration and incubated in water bath at 65°C for at least 5 minutes.

All procedures involving polyunsaturated phospholipids PDPC were either carried out in an airtight glove box constantly flushed with argon or nitrogen, or under two steady

streams of nitrogen, in order to avoid chemical reaction between polyunsaturated acyl chains and oxygen, which is known as lipid peroxidation [95]. The multiple double bonds in polyunsaturated fatty acids provide reactive hydrogen and can produce lipid radical. Lipid radical is unstable and can easily react with oxygen to produce peroxy-lipid radical which can further react with other unsaturated lipid to produce lipid peroxide and more lipid radical. This circle reaction produce reactive aldehydes as final product and can damage cell membranes containing polyunsaturated fatty acids.

Figure 3.1 Flow Chart of Production of LUV

Fluorescent probes were added to the lipid mixture at different times depending on the structure of the probe. Fluorescent probes NBD-DPPE and Rhod-DOPE were added after the mixture was dissolved with cyclohexane before freeze thaw cycles. This is because these probes have a similar structure as phospholipid and they can participate into the

formation of vesicles. NBD-DPPE was at the molar ratio of 1000:1, lipid:NBD, for both cases with and without Rhod-DOPE. DPH was added into lipid mixture at the end in each cuvette. This is because DPH is embedded between lipid acyl chains or leaflets and it does not participate into the formation of vesicles. DPH was at the molar ratio of 1000:1, lipid:DPH, without Rhod-DOPE, and was at 400:1, lipid:DPH with Rhod-DOPE.

Table 3.2 List of Samples

Measurement	POPC	PDPC	SM	CHOL	% lo
1	57	0	27	16	
2	38	19	27	16	5
3	19	38	27	16	
4	46	0	34	20	
5	31	15	34	20	25
6	15	31	34	20	
7	0	46	34	20	
8	33	0	42	25	
9	22	11	42	25	50
10	11	22	42	25	
11	20	0	50	30	75
12	10	0	56	34	90

Note: all experiments used the following fluorescent probes

di-16:0-NBD-PE

di-16:0-NBD-PE + di-18:1-Rhod-
PE

DPH

DPH + di-18:1-Rhod-PE

Table 3.2 is the list of lipids composition used. Two FRET pairs were used: NBD-DPPE (donor) to Rhod-DOPE (acceptor), and DPH (donor) to Rhod-DOPE (acceptor), with Förster distance R_0 49Å and 36Å respectively [96]. For samples with

POPC:SM:CHOL=57:27:16, 46:34:20, and 33:42:25, one third and two thirds POPC were replaced by PDPC for studying the effect of DHA in vesicle membranes.

3.2 Data Collection

Fluorescence lifetime and anisotropy measurements were performed with a frequency domain Chronos Lifetime Spectrometer (ISS, Urbana, IL). Diode laser provides excitation at 477nm for NBD-DPPE, and 376nm for DPH. Lifetime and anisotropy decay data were acquired using decay acquisition software from ISS at 15°C, 23°C, 30°C, 40°C, and 50°C.

For lifetime and anisotropy measurements, 15 modulation frequencies were used, logarithmically spaced from 5 to 250 MHz. All lifetime measurements were made with the emission polarizer at the magic angle of 54.7° relative to the vertically polarized excitation beam. The reference cuvette for NBD was fluorescein in HEPES buffer (50mM, pH=8) with reference time 4.05ns, and for DPH the reference was POPOP in ethanol with reference time 1.35ns. The lifetime of the references were chosen to be comparable with the particular samples. For each anisotropy decay measurement, the instrumental polarization factors were measured and found to be between 1 and 1.05, and the appropriate correction factor was applied. Scattered excitation light was removed from the emission beam by 535 nm band pass or 405 nm high pass, for NBD and DPH respectively, as shown in Figure 3.2. At each frequency, data were accumulated until the standard deviations of the phase and modulation ratio were below 0.2 and 0.004, respectively, and these values were used as the standard deviation for the measured phase

and modulation ratio in all subsequent analysis. Both total intensity decay and differential polarization measurements were repeated at each temperature with each bilayer composition a minimum of three times.

Figure 3.2 Absorption Spectrum for 535nm Band Pass and 405nm High Pass

Figure 3.2 shows the absorption spectrum of 535 nm band pass filter and 405 nm high pass filter, for NBD and DPH respectively. The reason these filters are chosen is that we are interested in only the emission spectrum of donors and the effect of emission light of the acceptors should be eliminated. The emission peak of donor NBD-DPPE's is at 535 nm, while the emission peak of acceptor di-18:1-NBD-PE is at 583 nm, thus a band pass filter around 520-520 nm is chosen to select the emission light of NBD. The emission peak of donor DPH is at 452 nm, while the excitation and emission peak of the acceptor

Rhod-DOPE is at 560 nm and 583 nm respectively. Because of that only a small fraction of the emission from the donor would cause excitation of the acceptor, the emission intensity of the Rhod-DOPE is much smaller than DPH. Thus a 405 nm high pass is selected to gather the information of the emission light of DPH.

NBD-DPPE and Rhod-DOPE absorption spectra were measured in chloroform solvent, and DPH spectra was measured in tetrahydrofuran (THF) solvent, with Agilent 8453 UV-Visible spectrophotometer at 23°C using a 1-cm-path length quartz cuvette.

Emission spectra were recorded with ISS PC1 photon counting spectrofluorometer at 23°C with the excitation wavelength at the maximum value from the absorption spectra.

4. Fluorescence Spectroscopy

4.1 Overview of Fluorescence

Early studies of fluorescence were done by Newton [97] and Weber [98] in the 1950s and the initial utilizations of fluorescent probes were done by Chance and coworkers [99] and Tasaki et al [100]. Since the 1960s, based on the phenomenon of fluorescence many technological inventions were made, such as confocal microscopy, total internal reflection fluorescence (TIRF) microscopy, two-photon microscopy, fluorescence recovery after photobleaching (FRAP), fluorescence correlation spectroscopy (FCS), and Förster Resonance Energy Transfer (FRET). These fluorescence techniques revolutionized imaging and yielded access to dynamics on relevant timescales in cell biology that were previously inaccessible. Moreover, the introduction of fluorescent proteins accelerated the use of fluorescent techniques in living cells and organisms. Nowadays the fluorescent techniques enable us to explore systems down to the single molecule level with nanosecond time resolution.

Some proteins and various small molecules in cells are naturally fluorescent and are called intrinsic fluorophores, such as tryptophan. Extrinsic fluorophores are those added to a sample. Membranes typically do not display intrinsic fluorescence thus it is common to label membranes with probes. One of the most commonly used membrane probes is 1,6-diphenyl-1,3,5-hexatriene (DPH). DNA is weakly or non-fluorescent but it can spontaneously bind with a wide variety of dyes such as acridines and ethidium bromide. Therefore staining of the cells with dyes that bind to DNA is widely used to visualize and

identify chromosomes. Proteins can be labeled on free sulfhydryl groups using maleimide reagents such as Bodipy 499/508 maleimide.

Developments in fluorescence chemistry and technical discoveries have developed a vast selection of fluorophores which provide flexible and varied performances for research applications. In general, fluorophores can be divided into three general groups: organic dyes, biological fluorophores, and quantum dots. Synthetic organic dyes, such as fluorescein, were the first fluorescent probes used in biological research. The small size of organic dyes is advantageous over biological fluorophores because they be cross-linked to macromolecules without interfering with biological function. The first use of a biological fluorophore for research purposes was reported in 1994 when green fluorescent protein (GFP) was cloned from the jellyfish *Aequorea Victoria* and used as a genetic fluorescent probe [101]. It enabled visualizing proteins in their native environment and significantly improved our understanding of membrane proteins and their dynamics. Since then, derivatives of GFP and phycobiliproteins have been designed for use in biological expression systems. Other research developments include quantum dots, which are semiconductor nanocrystals. They were developed in the 1980s and have been increasingly used in fluorescence applications for biological research since the 1990s. When a quantum dot is excited, it emits photons at a wavelength proportional to the size of the particle; smaller quantum dots emit higher energy photons hence smaller wavelengths of light. Quantum dots have been reported to be more photostable than other fluorophores [102] and they can be coated for different biological applications such as

protein labeling. However, quantum dots are relatively expensive and there are reports of cell toxicity [103].

There are many other techniques that are used for membrane studies, including X-ray diffraction crystallography, nuclear magnetic resonance (NMR), electron spin resonance (ESR), and differential microcalorimetry [104, 105].

4.2 Jablonski Energy Diagram

Visible light is electromagnetic radiation with wavelength in the range of 400 to 700nm, which is between the infrared and ultraviolet. Visible light and all other types of electromagnetic waves are composed of photons. In 1950 Albert Einstein proposed that light of frequency ν could be regarded as a collection of discrete packets of energy, i.e. photon, each photon contains an amount of energy E given by

$$E = h\nu = \frac{hc}{\lambda} \quad (1)$$

where h is Planck's constant ($6.626 \times 10^{-34} \text{ J} \cdot \text{s}$), ν is the frequency of the photon, c is the speed of light, and λ is the wavelength of the photon.

Fluorescence is the decay of an excited state back to the ground state by emission of a photon. Fluorophores can exist in different electronic states. The ground electronic state is denoted as S_0 , and the excited electronic states are denoted as S_1 , S_2 , etc. At each electronic state, there are a number of vibrational energy levels, depicted as 0, 1, 2, etc, as shown in Figure 4.1. The larger energy difference between the S_0 and S_1 excited states is

generally too large for thermal population of S_1 , thus we use light instead of heat to induce fluorescence.

Figure 4.1 Jablonski Energy Diagram of Fluorescence

Following light absorption, a fluorophore is typically excited to a vibrational substate of the S_1 or S_2 from the ground state S_0 . Most of the molecules rapidly relax to the lowest vibrational level of S_1 via non-radioactive decay. This process is called internal conversion and usually takes place within $10^{-12}s$ or less. Since fluorescence lifetimes are typically near $10^{-8}s$, internal conversion is generally complete prior to photon emission. Therefore, emission of fluorophores is generally from the lowest vibrational level of S_1 . Fluorophores returning to different vibrational levels of S_0 state results in the emission spectrum. The emission wavelength is independent of the absorption wavelength because the electron always decays to the lowest vibrational energy level of the excited state before it emits the photon and moves back to the ground state. Interestingly, the emission spectrum is usually a mirror image of the absorption spectrum. Because according to the Franck-Condon principle, all electronic transitions are vertical and they occur without

change in the position of the nuclei. Thus if a particular transition probability between the 0th and 1st vibrational levels is largest in absorption, the reciprocal transition is also most probable in emission.

Molecules in the S_1 state can also decay to the first triplet state T_1 via an intersystem crossing. A transition from T_1 to the singlet state S_0 is forbidden, thus such a transition occurs over a larger time interval, from 0.1 ms to minutes even hours. Emission from T_1 to S_0 is called phosphorescence and it produces light with a longer wavelengths than fluorescence.

4.3 Fluorescence Spectra

When light passes through a substance, a fraction of the light is absorbed by the substance and it can be quantified by defining the term absorbance (A). Absorbance is given by Eq. 2

$$I = I_0 10^{-A} \quad (2)$$

Where I is the intensity of the light before it enters the substance and I_0 is the intensity of the light after it is transmitted through a substance. If we rearrange Eq. 2 it can be written as

$$A = \log\left(\frac{I_0}{I}\right) \quad (3)$$

Beer's states that the absorbance is proportional to the molar absorptivity of the medium ϵ (m^2/mol), the concentration c (mol/m^3), and the length of the medium the light passes through d (m), as shown in Eq. 4.

$$A = \epsilon cd = \alpha d \quad (4)$$

Here α (m^{-1}) is the absorption coefficient.

Figure 4.2 Absorption of Light Passing through a Medium

The absorbance is wavelength dependent and the absorption spectrum can be obtained using a spectrophotometer. When a photon is absorbed by a chromophore, an electron of the chromophore is lifted to a state higher in energy than the ground state. The excitation spectrum can be obtained by monitoring the fluorescent emission at a chosen wavelength such as the maximum intensity wavelength while exciting the fluorophore through a range of wavelengths. While the fluorophore is excited by various wavelengths only the chosen emission wavelength is allowed to pass to the detector and the intensity of the emission light is measured as a function of excitation wavelength. Generally the absorption spectrum and emission spectrum have the same shape and are superimposable.

The emission spectrum can be determined by scanning the fluorescence emission intensity over the entire series of emission wavelengths using a monochromator while exciting the fluorophore at the maximum absorption wavelength.

The absorption and emission spectra of fluorophores used in this study, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DPPE), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhod-DOPE), and diphenylhexatriene (DPH), are shown in Fig. 4.3.

Figure 4.3 Spectra of NBD-DPPE, DPH, and Rhod-DOPE

4.4 Stokes Shift

Fluorophores absorb a photon and enter an excited state before returning the ground state. The emitted photon has longer wavelength, i.e. lower energy, than the absorbed photon. The difference between the excitation and emission wavelength is called the Stokes shift, as shown in Figure 4.4. Energy loss between excitation and emission are observed universally for fluorescent molecules in solution and one common cause of the energy losses is the rapid decay to the lowest vibrational level of S_1 .

Figure 4.4 Stokes Shift

4.5 Förster Resonance Energy Transfer (FRET)

Whenever the emission spectrum of a fluorophore (donor), overlaps with the absorption spectrum of another molecule (acceptor), as shown in Figure 4.5, Förster Resonance Energy Transfer (FRET) may occur. FRET was first theoretically described by Perrin [107] and further explained by Förster [108]. FRET is widely used because the favorable

distances for energy transfer are typically the size of a protein or the thickness of a membrane. Fig. 4.5 shows the overlap spectra of NBD-DPPE with Rhod-DOPE pair and DPH with Rhod-DOPE pair.

Figure 4.5 Overlap Spectrum of NBD-Rhod Pair and DPH-Rhod Pair

FRET occurs when a donor fluorophore in an excited electronic state transfers its excitation energy to a nearby acceptor chromophore in a non-radiative long-range dipole-dipole interaction. The supporting theory is the concept of treating an excited fluorophore as an oscillating dipole that can exchange energy with a second dipole which has a similar resonance frequency. Thus, the donor and acceptor in FRET is analogous to coupled oscillators. FRET is a non-radiative process that does not require a collision and does not produce heat. The phenomenon can be observed by exciting a sample containing both donor and acceptor fluorophores with light at the maximum excitation wavelength of the donor and detect the emission light at the emission maximum wavelength of the acceptor.

Figure 4.6 Resonance Energy Transfer Jablonski Diagram

Fig. 4.6 is a Jablonski diagram illustrating the coupled transitions between the donor emission and acceptor excitation in FRET. Absorption and emission from the donor are represented by red and blue straight vertical arrows respectively. Vibrational relaxation is drawn by black dotted arrows. The coupled resonance energy transfer is drawn with

dashed arrows in blue and orange, for emission from donor and excitation from acceptor, respectively. The emission from the acceptor is represented by purple straight arrows.

In addition to the criteria that the emission spectrum of the donor has to overlap with the absorption spectrum of the acceptor, the two involved fluorophores also must be positioned within a certain range of each other in order for FRET to occur.

The measurement of efficiency must be related to the distance between the two chromophores in order to obtain useful structural information from FRET. This work has been done by Theodore Förster. He calculated that the rate of energy transfer from a donor to an acceptor $k_T(r)$ is given by

$$k_T(r) = \frac{1}{\tau_D} \left(\frac{R_0}{r} \right)^6 \quad (5)$$

where τ_D is the decay time of the donor in the absence of acceptor, and r is the donor-to-acceptor distance. The inverse sixth power is from the square of the dipole-dipole coupling. R_0 is the characteristic transfer distance and is called the Förster distance:

$$R_0 = 0.2108 \left(\kappa^2 \phi_D n^{-4} \int I(\lambda) \varepsilon(\lambda) \lambda^4 d\lambda \right)^{1/6} \quad (6)$$

where κ^2 is the orientation factor, ϕ_D is the donor quantum yield in the absence of acceptor which is defined as the ratio of emitted photons per absorbed photon, n is the refractive index, $I(\lambda)$ is the normalized donor emission spectrum, and $\varepsilon(\lambda)$ is the acceptor molar absorption spectrum (expressed in units of $M^{-1}cm^{-1}$). If the λ units are nm, then the calculated R_0 value has Å unit.

The efficiency of energy transfer is a measure of the fraction of photons absorbed by the donor that are transferred to the acceptor. This fraction is given by

$$E = \frac{k_T(r)}{\tau_D^{-1} + k_T(r)} \quad (7)$$

which is the ratio of the transfer rate to the total decay rate of the donor in the presence of acceptor. Combining with Eq. 5, one can rearrange Eq. 7 to yield

$$E = \frac{R_0^6}{R_0^6 + r^6} \quad (8)$$

Here we see that when the donor-acceptor distance equals to Förster distance, the FRET efficiency is 50%. Eq. 8 is plotted in Fig. 4.7 for a particular value of R_0 ($R_0 = 30\text{\AA}$). We can see that the transfer efficiency is strongly dependent on the donor-acceptor distance, and for distances near R_0 a measurement of efficiency can yield a quite accurate determination of the distance. For commonly used pairs of chromophores, R_0 varies from 20 to 60 $\text{\AA}$.

Figure 4.7 FRET Efficiency as a Function of Distance

The transfer efficiency can be calculated from the lifetimes of the donor with the absence (τ_D) and presence (τ_{DA}) of the acceptor [109]:

$$E = 1 - \frac{\tau_{DA}}{\tau_D} \quad (9)$$

Therefore, if the efficiency is calculated from the lifetime measurement and is the value of R_0 is known, one can calculate the distance between donor and acceptor based on the equation

4.6 Orientation Factor κ^2

The orientation factor κ^2 in FRET is a geometric factor that depends on the orientation of donor and acceptor. If both donor and acceptor are free to tumble rapidly on the time scale of fluorescence emission, κ^2 approaches a limiting value of $2/3$. It can be calculated from equation

$$\kappa^2 = (\cos \theta_{DA} - 3 \cos \theta_D \cos \theta_A)^2 \quad (10)$$

$$\kappa^2 = (\sin \theta_D \sin \theta_A \cos \varphi - 2 \cos \theta_D \cos \theta_A)^2 \quad (11)$$

where θ_{DA} is the angle between the emission transition dipole of the donor and the absorption transition dipole of the acceptor, θ_D and θ_A are the angles between these dipoles and the vector joining the donor and acceptor, and φ is the angle between the planes, as shown in Fig. 4.8.

Different relative orientation of donor and acceptor can lead to κ^2 values range from 0 to 4. If the transition dipoles are perpendicular to each other, $\kappa^2 = 0$; if the dipoles are oriented parallel to each other, $\kappa^2 = 1$; for head-to tail parallel transition dipoles $\kappa^2 = 4$.

From Eq. 8 we see that the sixth root is taken to calculate the Förster distance, a variation in κ^2 from 1 to 4 produces only a $(4^{1/6} - 1^{1/6})/1^{1/6} = 26\%$ change in the calculated distance. If the customarily assumed value of $\kappa^2 = 2/3$ is applied, the calculated distance can be in error by no more than $(4^{1/6} - 0.67^{1/6})/0.67^{1/6} = 35\%$.

Figure 4.8 Dependence of the Orientation Factor κ^2 on the Direction of the Emission Dipole of the Donor and the Absorption Dipole of the Acceptor.

5. Frequency-Domain Lifetime and Anisotropy Measurement

5.1 Time-resolved Fluorescence

Steady state fluorescence measurement does not provide the information of time-resolved decays of fluorescence intensity, thus time-resolved fluorescence measurement is an important tool in biochemical research [106, 110]. Time-resolved fluorescence decay may be measured in either time-domain or frequency –domain [111]. In time-domain measurements, the sample is excited by a pulsed light source, and usually the time – resolved emission light intensity is modeled as the sum of exponential decays. For a single exponential decay the lifetime is defined as the time when the value of intensity is $1/e$ of its initial value. For multi-exponential decay, the resulting time-dependent emission can be described by a sum of multi-exponential functions:

$$I(t) = \sum_i \alpha_i e^{-t/\tau_i} \quad (12)$$

where α_i are the pre-exponential factors and τ_i are the decay times.

The fraction of the intensity due to each multi-exponential component is given by

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \quad (13)$$

Note that although usually intensity decays are analyzed in terms of the multi-exponential model, the actual decay may not be exponential.

It is rather difficult to obtain accurate data for multiple exponential decay using time-resolved fluorescence measurement; because multiple decay exponential parameters are

highly correlated thus it is hard to identify each of them [112]. Frequency domain measurements offer the advantage of reducing measurement noise to a predefined value via continuous sampling. However, operating within the time domain, the signal from the reference is subtracted from the reference signal and the resulting curve has intensity close to the noise level of the measurement.

Figure 5.1 Definition of the Phase Angle and Modulation of Emission.

For frequency-domain fluorescence measurement, the intensity of the light source is modulated at a high frequency which is comparable to the reciprocal of the lifetime. This is necessary because according to Eq.14 and 15, if ω is very small the phase delay will become zero and the modulation ratio will approach 1. This is not practical for frequency domain applications because the measurements depend upon a finite phase delay and a modulation ratio smaller than 1. In Fig. 5.1, the blue curve is excitation intensity; the red dashed curve is emission intensity. The time lag between absorption and emission is

described as the phase shift (Φ_ω). The intensity decay of the emission light results in demodulation by a factor m_ω , where ω is the modulation frequency.

It may seem difficult to observe the phase delays and modulation ratio at high frequency, but the measurement is actually easy using cross-correlation detection. While the excitation light is modulated at frequency F , the detector is modulated at frequency $F+\delta F$ to avoid harmonics. The difference frequency δF is usually about 25 Hz.

Both time domain and frequency domain time-resolved measurements are designed to recover the parameters describing the time-dependent decay of the sample. For a single-exponential decay, lifetime τ can be calculated from phase shift ϕ_ω and modulation ratio m_ω by the following equations:

$$\tan\phi_\omega = \omega\tau \quad (14)$$

$$m_\omega = (1 + \omega^2\tau^2)^{-1/2} \quad (15)$$

5.2 Least-Squares Analysis of Frequency-Domain Intensity Decays

Usually frequency-domain data are analyzed by the method of nonlinear least squares. The phase and modulation values can be calculated using sine and cosine transforms of the intensity decay $I(t)$:

$$N_{\omega} = \frac{\int_0^{\infty} I(t) \sin \omega t \, dt}{\int_0^{\infty} I(t) \, dt} \quad (16)$$

$$D_{\omega} = \frac{\int_0^{\infty} I(t) \cos \omega t \, dt}{\int_0^{\infty} I(t) \, dt} \quad (17)$$

where ω is the circular modulation frequency (2π times the modulation frequency in Hz).

For a sum of exponentials the transforms are

$$N_{\omega} = \sum_i \frac{\alpha_i \omega \tau_i^2}{(1 + \omega^2 \tau_i^2)} / \sum_i \alpha_i \tau_i \quad (18)$$

$$D_{\omega} = \sum_i \frac{\alpha_i \tau_i}{(1 + \omega^2 \tau_i^2)} / \sum_i \alpha_i \tau_i \quad (19)$$

From the values of N_{ω} and D_{ω} , the value of phase shift ($\phi_{c\omega}$) and demodulation ($m_{c\omega}$) can be calculated by the following equations:

$$\tan \phi_{c\omega} = \frac{N_{\omega}}{D_{\omega}} \quad (20)$$

$$m_{c\omega} = (N_{\omega}^2 + D_{\omega}^2)^{1/2} \quad (21)$$

In the least-squares analysis, the parameters α_i and τ_i are varied to minimize the value of the goodness-of-fit parameter χ_R^2 :

$$\chi_R^2 = \frac{1}{v} \sum_{\omega} \left[\frac{\phi_{\omega} - \phi_{c\omega}}{\delta_{\phi}} \right]^2 + \frac{1}{v} \sum_{\omega} \left[\frac{m_{\omega} - m_{c\omega}}{\delta_m} \right]^2 \quad (22)$$

where v is the number of degrees of freedom, given by the number of data points subtracted by the number of parameters. ϕ_{ω} and m_{ω} are measured data of phase and modulation, δ_{ϕ} and δ_m are the standard deviation of the phase and modulation values.

In this study, a FORTRAN program is designed to find the best fit values of $\phi_{c\omega}$ and $m_{c\omega}$, so that the value of χ_R^2 is minimized.

5.3 Time-resolved Anisotropy Decays

The ground and excited state electronic distribution of any fluorophore define directions of the excitation and emission dipole moments within the molecule. The magnitudes of these directions determine the maximal probabilities of absorption or emission of a photon. When polarized exciting light is directed toward a fluorescent molecule, the light with electric vector aligned with the excitation dipole moment will be preferentially absorbed by the fluorophore. Since the process of absorption is much faster than molecular rotations, the oriented exciting light creates a population of preferentially oriented excited fluorophores; this is the so-called photo-selection. Because that the lifetime of fluorophores is much longer than the time required for absorption, fluorophores can often reorient before emission occurs. If the rotational correlation time of the excited molecule is less than or on the order of the excited state lifetime, the emission light will no longer be polarized parallel to the excitation light, even if the molecular excitation and emission dipoles are parallel within the fluorophore [83].

For time-dependent anisotropy decay measurement, the sample is excited by amplitude-modulated light, which is vertically polarized. The emission intensity is observed through a polarizer that is rotating between parallel and perpendicular directions for the values of parallel intensity $I_{\parallel}(t)$ and perpendicular intensity $I_{\perp}(t)$. The time-dependent anisotropy is defined as

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} \quad (23)$$

where the denominator is the total intensity $I(t) = I_{\parallel}(t) + 2I_{\perp}(t)$. If $I_{\parallel} = 3I_{\perp}$, anisotropy reaches maximum value 0.4.

From Eq. 23, we can see that for time domain measurement, the difference between two noisy signals of $I_{\parallel}$ and $I_{\perp}$ in the numerator yields a curve that has two times the noise of the regular signal. While in frequency-domain, we can sample until the noise in the difference falls below a preset value. Thus frequency domain measurement is superior to time domain for anisotropy measurement.

Figure 5.2 Frequency-Domain Measurements of Anisotropy Decay

Empirically all anisotropy decays can be described as a sum of exponentials:

$$r(t) = (r_0 - r_\infty) \left[\beta_1 e^{-t/\varphi_1} + \beta_2 e^{-t/\varphi_2} \right] + r_\infty \quad (24)$$

where φ_1 and φ_2 are the individual correlation times, r_0 is the fluorescence anisotropy at $t = 0$, and r_∞ is the non-decaying anisotropy, as shown in Figure 5.3.

Figure 5.3 Example of Anisotropy Decay

Similarly to lifetime measurement, for the anisotropy decay in the frequency domain, there are two characteristic quantities: the phase shift Δ_ω between the perpendicular and parallel components of the emission light, and the amplitude ratio Λ_ω of the parallel and perpendicular components of the modulated emission, where ω is the modulation frequency.

$$\Delta_\omega = \phi_\perp - \phi_\parallel \quad (25)$$

$$\Lambda_\omega = m_\parallel / m_\perp \quad (26)$$

A commonly used model-independent order parameter derived from anisotropy decays is the parameter S . The value of square root of r_∞ divided by r_0 is defined as order parameter S [119]

$$S = \sqrt{\frac{r_{\infty}}{r_0}} \quad (27)$$

The value of order parameter ranges from 0 to 1. When the value of r_{∞} is close to r_0 , the value of S is close to 1; when r_{∞} is much smaller than r_0 , then S is close to 0.

5.4 Rotational Diffusion Model

Although this empirical sum-of-exponentials model shown in Eq.24 provides information about fluorophore rotational correlation times, it does not provide the information regarding the range of fluorescent probe equilibrium angular orientations restricted by surrounding phospholipid acyl chains [113]. Therefore, the Brownian rotational diffusion (BRD) model [114] was used for anisotropy analysis. The BRD model is based on an approximate solution of the Smoluchowski equation [115, 116] and it yields the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ that can be used to construct an orientational distribution function $f(\theta)$. The equilibrium orientational distribution of a free-tumbling fluorescent probe reflects the equilibrium orientational order of the surrounding phospholipid acyl chains. In general the orientation of a cylindrically symmetric molecule in a lipid bilayer can be described by the angle θ between its symmetry axis and the normal at the local membrane. The orientational distribution function $f(\theta)$ is a series expansion of the Legendre polynomials $P_n(\cos \theta)$:

$$f(\theta) = \sum_n \frac{1}{2} (2n + 1) \langle P_n \rangle P_n(\cos \theta) \quad (28)$$

Where n is even and $\langle P_n \rangle$ is the n th rank orientational order parameter. $\langle P_n \rangle$ are defined as

$$\langle P_n \rangle = \int_0^\pi (\sin\theta) f(\theta) P_n(\cos\theta) d\theta \quad (29)$$

For macroscopically isotropic systems, only the first two order parameter $\langle P_2 \rangle$ and $\langle P_4 \rangle$ can be extracted from the experimental data. The BRD model relates the observed anisotropy decay with the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$, the diffusion coefficient of the symmetry axis of the molecule $D_\perp$, and r_0 , according to van der Meer et al.

$$r(t) = r_0 \left(\sum_{i=1}^3 g_i \exp(-t/\varphi_i) + g_4 \right) \quad (30)$$

where

$$g_1 = \frac{1}{5} + \frac{2}{7} \langle P_2 \rangle + \frac{18}{35} \langle P_4 \rangle - \langle P_2 \rangle^2$$

$$g_2 = \frac{2}{5} + \frac{2}{7} \langle P_2 \rangle - \frac{24}{35} \langle P_4 \rangle$$

$$g_3 = \frac{2}{5} - \frac{4}{7} \langle P_2 \rangle + \frac{6}{35} \langle P_4 \rangle$$

$$g_4 = \langle P_2 \rangle^2$$

$$\varphi_1 = g_1 / \left[6D_\perp \left(\frac{1}{5} + \frac{\langle P_2 \rangle}{7} - \frac{12}{35} \langle P_4 \rangle \right) \right]$$

$$\varphi_2 = g_2 / \left[12D_\perp \left(\frac{1}{5} + \frac{\langle P_2 \rangle}{14} + \frac{8}{35} \langle P_4 \rangle \right) \right]$$

$$\varphi_3 = g_3 / \left[12D_{\perp} \left(\frac{1}{5} - \frac{\langle P_2 \rangle}{7} - \frac{2}{35} \langle P_4 \rangle \right) \right]$$

The value of $\langle P_2 \rangle$, $\langle P_4 \rangle$, $D_{\perp}$, and r_0 can be obtained using least square analysis similar to intensity decay analysis introduced in Chapter 6.2. However, the resulting series of $\langle P_2 \rangle$ and $\langle P_4 \rangle$ can produce negative values of $f(\theta)$ using Eq.28. Considering that orientational probability function has to be positive and the total orientational probability has to be 1, the values of $\langle P_2 \rangle$ and $\langle P_4 \rangle$ must satisfy additional constraints so that

$$f(\theta) \geq 0 \quad (31a)$$

$$\int_0^{\pi} f(\theta) \sin \theta d\theta = 1 \quad (31b)$$

The resulting orientational distribution function $f(\theta)$ is symmetrical about $\theta = \pi/2$, and is based on maximizing the information entropy of $f(\theta)$ [117, 118],

$$f(\theta) = N^{-1} \exp[\lambda_2 P_2(\cos \theta) + \lambda_4 P_4(\cos \theta)] \quad (32)$$

Where λ_2 and λ_4 are constants determined by simultaneous solution of Eq. 29 for $\langle P_2 \rangle$ and $\langle P_4 \rangle$, and N is the normalization constant determined by Eq. 31b.

It is useful to calculate a single parameter that is corresponding to the equilibrium orientational freedom restricted by the phospholipid acyl chains. Thus a comparison of $f(\theta)$ and a random distribution was formulated.

$$f_{random} = 1 - \frac{1}{2} \int_0^{\pi} |f(\theta) - f(\theta)_{rand}| \sin \theta d\theta \quad (33)$$

Where $f(\theta)_{rand}$ is given by Eq. 32 with $\lambda_2 = \lambda_4 = 0$.

f_{random} results from a direct comparison of a random orientational distribution and the entire angular range from 0 to π , and it provides information regarding the orientational freedom of DPH^{vi}, thus f_{random} is a disorder parameter.

6. Instrumentation

6.1 ISS Chronos Spectrometer

Fluorescence lifetime and anisotropy measurements were performed with Chronos fluorescence lifetime spectrometer (ISS, Urbana, IL). This Chronos fluorometer contains the following components: light source, sample compartment, detectors, wavelength selection, polarizers, computer and software, as shown in Figure 6.1.

Figure 6.1 Schematic Drawing of ChronosFD, the Frequency-Domain Fluorometer from ISS [120].

A partially polarized laser diode is modulated at high frequency by an external function generator outputting a sinusoidal wave with a typical frequency range of 5-250 MHz. This time-varying laser signal is then sent through the excitation polarizer, which is

aligned with the laser so the maximum intensity is delivered to the sample cell. The alignment procedure is accomplished via an iterative process of tilting the laser diode assembly until the signal at the non-monochromator PMT is highest. Because lasers may vary in intensity with age and the output may have some variance in wavelength, a reference quantum counter and PMT must be used to correct for any changes. Photons originating from the laser diode are delivered to the reference arm with a 50/50 beam splitter. Predating the use of the photodiode, quantum counters were typically rhodamine-B or any fluorophore that has a constant fluorescent yield over a broad spectral range [121]. Fluorophore quantum counters have been replaced, however, due to the requirement of routine maintenance and possible variance in the quality of the fluorophore. The photodiode has an even broader spectral range while still offering negligible self-absorption [122]. Excitation photons are incident on the reference and subsequently the sample via stage rotation at each measurement point. The emission photons are collected at 90° relative to the path of the laser diode and sent through the emission polarizer. This polarizer is essential for distinguishing between fluorescent lifetimes and rotational information. If we place no polarizer in the detection pathway, we are measuring $I_{\parallel}(t) + I_{\perp}(t)$. However, this is not the total intensity $I_{\parallel}(t) + 2I_{\perp}(t)$. Therefore, we need to adjust the emission polarizer oriented under to the so-called “magic angle” for which $I_{\perp}$ would contribute twice as much as $I_{\parallel}$ in the passing light.

Figure 6.2 Magic Angle

By adjusting the emission polarizer to this angle the intensity seen at the non-monochromator PMT represents the total intensity. Therefore, the “magic angle” is conducive for measurements of fluorescent lifetimes. The non-reference PMT detects the modulated emission from the sample and the reference materials. The lifetime of the reference is known, thus by comparing the phase delay and modulation ratio of the sample and the reference, the lifetime of the sample can be calculated.

However, if interested in anisotropy, the emission polarizer must be dynamic throughout data collection rotating between the parallel and perpendicular positions for comparison of sample rotation (i.e. the relative position of the fluorophore). This dynamic anisotropy measurement is accomplished with a constant, vertical excitation polarization. Neutral density filters, optical elements with constant absorption across a broad spectrum, may be used to attenuate the emission or excitation signals.

Examples of raw data for lifetime and anisotropy measurements are shown in Figure 6.3 and 6.4.

In Fig. 6.3, lifetime measurement shows the change in phase delay (blue symbols, left axis) and modulation ratio (green symbols, right axis) with increasing modulation frequency due to the excited state lifetime of the fluorophore. In Fig. 6.3a, as modulation frequency increases, the phase delay increases from approximately 20 to 80, while modulation ratio decreases from approximately 1 to 0. Fig. 6.3b shows data from the same sample, the only difference is that Fig. 6.3a was taken under 15°C while Fig. 6.3b was taken under 50°C. Notice that the cross point was at a completely different place in Fig. 6.3b compared to Fig. 6.3a.

Similarly to lifetime, in Fig. 6.4 anisotropy decay measurement shows the change in phase delay and modulation ratio due to the difference between vertically and horizontally polarized excitation for vertically polarized emission. Note that in this case both the change in phase delay and modulation ratio covers a much smaller range than in lifetime measurement. And again, the only difference between Fig. 6.4a and 6.4b is temperature, we observe a clear change of the shape in the two phase-mod graphs.

Figure 6.3 Examples of Raw Lifetime Data

Figure 6.4 Examples of Raw Anisotropy Data

6.2 Agilent 8453 UV-Visible spectrophotometer

For absorbance measurements an Agilent 8453 UV-Visible spectrophotometer (UV-Vis) with a 1,024-element diode array was used. The UV wavelength range is supplied by a deuterium lamp equipped with a shine-through aperture [123]. The shine-through design is based upon the combination of visible and UV light by which the light from a halogen bulb is focused through a small aperture in the deuterium lamp [124]. This allows an axial configuration and improved UV-Vis intensity by placing the two sources in series. With the sources emitting simultaneously in series, the source light is then collimated by the source lens and passed through to the shutter and stray light filter assembly. The shutter is an electromechanical type and serves to block light from entering the sample while data is not being acquired so potential over-exposure effects may be mitigated. Another component installed adjacent to the shutter is the stray light correction filter. Stray light may come from many sources including: (1) scattering by the grating and mirror surfaces, (2) scattering from surfaces of filters and lenses, (3) distortions arising from thermal gradients present along the optical system, (4) Raleigh and Mie scattering by particles derived from air and dust within and without the instrument, (5) diffraction resulting from light passing through apertures within the optical system, and many other sources. The stray light correction filter acts to correct for stray light artifacts by introducing a filter that blocks 50% of light entering the detection array with a wavelength of 420 nm. The significance of this wavelength is derived experimentally as the amount of stray light will increase substantially at wavelengths below 400 nm. This issue could be addressed with a standard correction matrix; however, no single, standard

**Figure 6.5 A Representation of the Optical System Housed within the Agilent 8453
UV-Visible Spectrophotometer [125].**

UV or visible wavelength sources exist for comparison. Therefore, the Agilent 8453 first measures one spectrum with the filter present and then subtracts the intensity below 400 nm from the following spectra. This allows for acquisition of stray-light corrected spectra. Beyond the shutter and stray light correction filter assemblies lies the sample compartment. Here, cuvettes of the sample and reference may be placed with the addition of temperature control by a thermoelectric cooling element. The collimated light passing through the sample is then collected by the spectrograph lens and delivered to the optical grating through a slit. The slit is used to control the width of the incoming light so that each spectral component dispersed by the grating is addressing the appropriate photodiode. The grating acts to disperse the incoming light into its respective components (190 – 1100 nm for this instrument) with a concave geometry that both disperses light

and focuses the outgoing light to the detector thereby reducing the total number of optical components required.

6.3 ISS PC1 Photon Counting Spectrofluorometer

For fluorescence measurements an ISS PC1 photon counting spectrofluorometer was implemented. The light source is a xenon direct-current (DC) arc lamp. These lamps are the brightest available, manufactured light sources besides lasers and emit wavelengths from UV to the near-infrared (NIR). As the majority of the light emitted from xenon arc lamps is within the UV-Vis range, it makes them an excellent choice for steady-state fluorometry. Xenon arc lamps are filled with high purity xenon at pressures of 5 – 20 bar [126]. Large currents are passed between the anode and cathode and subsequently the pressure triples and intense light is generated. The xenon spectral lines are dominant between 750 – 1000 nm and the UV-Vis region emitted from the lamp is practically featureless. As the lamp emits a broad band of wavelengths, an excitation monochromator is necessary to select single excitation wavelengths. After the excitation monochromator, light is incident on a 50/50 beam splitter where light is both sent to the reference arm and towards the sample cell. With steady state measurements a quantum counter is used to correct for fluctuations in the lamp intensity. The ISS PC1 utilizes quantum counter solutions of high concentration. For the measurements executed in this thesis, a quantum counter solution of Rhodamine B is installed with a red filter. This filter allows for the fluorescence of the quantum counter solution to be detected versus the incident wavelength selected for excitation. This particular quantum counter works well up to the wavelength of 600 nm where other dyes such as HITC can be prepared in

Figure 6.6 A Schematic of the ISS PC1 Photon Counting Spectrofluorometer [128].

solution [127]. The cutoff of 600 nm is chosen because Rhodamine B emits above 610 nm. For all measurements present in this thesis, polarizers were not used as the steady-state emission spectra from each utilized fluorescent probe was desired. Excitation light is delivered to the sample, kept at constant temperature via circulating water chiller, and emission light from fluorescence of the sample is collected at 90° relative to the excitation optics axis. Incoming light is then dispersed with a holographic grating element and scanned across the excitation photomultiplier tube (PMT). Resulting spectra is plotted via ISS PC1 control software.

7. Results and Discussion

7.1 Lifetime Report

Figure 7.1 Lifetime Change of NBD-DPPE and DPH with %lo phase

Fig. 7.1 shows the comparison of lifetime of NBD-DPPE and DPH. The orange bars represent the lifetime of NBD-DPPE and the blue bars represent the lifetime of DPH at 23°C. The compositions of each lipid mixture along x-axis are: POPC/SM/Chol=57/27/16, 46/34/20, 33/42/25, 20/50/30, and 10/56/34, according to the ternary phase diagram.

Fig. 7.1 shows that lifetime of both NBD-DPPE and DPH increases with the increasing amount of cholesterol and sphingomyelin in the lipid. Overall NBD-DPPE has longer lifetime than DPH and lifetime of the range of NBD-DPPE lifetime change is bigger than DPH.

Mazereds et al had demonstrated that NBD groups are located in the region of the lipid headgroups at the water-lipid interface with higher polarity. Increasing temperature leads

to greater exposure of the NBD to water and thus increase the environmental dielectric constant. Therefore increasing dielectric constant due to rising temperature reduces NBD-DPPE lifetime [78].

The variation of the lifetime of DPH is most likely due to changes in water penetration into the bilayer induced by changes in the lipid composition. The increases of lifetime with cholesterol are similar to previous report showing that cholesterol reduces penetration of water into the membrane with laurdan fluorescence measurements [129].

Fig. 7.2 shows the effect of temperature in bilayers with a range of PDPC/POPC ratio. The column at the left hand side of Fig. 7.2 shows the lifetime of NBD-DPPE and the column at the right hand side demonstrate the lifetime of DPH. The blue diamond represent fluorophore lifetime in different lipid composition from the points in the ternary phase diagram, and there is no PDPC involve; the red blocks and green triangles are when 1/3 and 2/3 of POPC at each point are replaced by PDPC, the purple crosses represent the probe lifetime in the lipid composition when all of the POPC are replaced by PDPC; all the component ratio of the lipid are listed at the bottom of the figure. The percentage l_o phase in each sample is listed at the top of each graph.

In Fig. 7.2a, 7.2c, and 7.2e, the lifetime of NBD-DPPE universally goes down within the same lipid component when temperature increases. And all of the diagrams show that the curve of NBD-DPPE lifetime decreases when POPC is replaces by PDPC. Almost all of the results show that the more PDPC are added into the lipid, the more the curve of NBD-DPPE lifetime decreases. Since NBD-DPPE partitions in l_o domains, the fact that PDPC

Figure 7.2 Lifetime Change of NBD and DPH with Temperature

In Fig. 7.2, the left column shows the lifetime of NBD-DPPE in the composition of POPC/SM/Chol=57/27/16, 46/34/20, and 33/42/25; the right column shows the lifetime of DPH. The blue diamond represent sample with no PDPC; the red blocks and green triangles are when 1/3 and 2/3 of POPC are replaced by PDPC respectively, the purple crosses represent samples with all POPC replaced by PDPC.

reduces NBD-DPPE lifetime indicates that PDPC alters the headgroup packing in the lo phase.

In Fig. 7.2b, 7.2d, and 7.2f, lifetime of DPH decreases with rising temperature as well. And when PDPC is added into the mixture, lifetime of DPH also shows the decrease. The variation in lifetime of DPH with replacement of POPC with PDPC agrees with previous measurement that water penetration into the bilayer increases with increased acyl chain unsaturation .

When Rhod-DOPE is added into the lipid components, because of the overlap between NBD-DPPE emission and Rhod excitation, and between DPH emission and Rhod excitation, Förster Resonance Energy Transfer (FRET) occurs. Priyadarshini Pathak and Erwin London demonstrated that the Förster distance between NBD-DPPE and Rhod-DOPE is $49\text{\AA}$, between DPH and Rhod-DOPE is $36\text{\AA}$ [96]. In addition to the samples shown in Fig. 7.1 and 7.2, we prepared another set of samples with the same lipid composition but contains more than one fluorescent probe in each, half of the samples contain both NBD-DPPE and Rhod-DOPE, and the other half have both DPH and Rhod-DOPE, hence in each sample there is one FRET pair of either NBD-Rhod or DPH-Rhod. Due to FRET, the lifetime of both NBD-DPPE and DPH decrease with the presence of Rhod, and this is experimentally reported as shown in Fig. 7.3.

Figure 7.3 Lifetime Change of NBD-DPPE and DPH with Presence of Rhod-DOPE

Fig. 7.3 works as a baseline to show the difference in NBD-DPPE lifetime and DPH lifetime in different lipid composition before PDPC is added. The orange and blue bars represent the lifetime of NBD-DPPE without Rhod-DOPE, and lifetime of DPH without Rhod-DOPE respectively at 23°C, the purple bars in Fig. 7.3a and 7.3b shows the lifetime of NBD-DPPE and DPH with presence of Rhod-DOPE at 23°C.

Fig. 7.3 shows the lifetime change of NBD-DPPE and DPH with the presence of Rhod-DOPE. All the lipid mixture in Fig. 7.3 does not contain any PDPC and they work as a baseline to show the difference when more cholesterol and sphingomyelin is present in the lipid. The orange and blue bars represent the lifetime of NBD-DPPE and DPH without Rhod-DOPE, the purple bars in Fig. 7.3a and 7.3b shows the lifetime of NBD-DPPE and DPH with presence of Rhod-DOPE respectively. In both graphs we see that that the lifetime of both NBD-DPPE and DPH show obvious drop when Rhod-DOPE is present. Lifetime of NBD-DPPE shows more response to the presence of Rhod-DOPE comparing to the lifetime of DPH. In both graphs, at lowest lo phase percentage (5%)

lifetime of both NBD-DPPE and DPH drops most dramatically compared to the rest of the lipid composition, and when the lo phase domain is at 25%, 50%, and 75%, the lifetime of both NBD-DPPE and DPH barely show any response to the lipid position change. At highest lo domain percentage (90%), the change of lifetime of both NBD-DPPE and DPH is very small when Rhod-DOPE is added. For NBD-DPPE to Rhod-DOPE it looks like there are two significant changes: between 16% & 20% and between 30% & 34%.

Figure 7.4 FRET Efficiency Change Based on $R_0=49\text{\AA}$ and $R_0=36\text{\AA}$

Fig. 7.4 is plotting of Eq. 9 based on Förster distance $R_0=49\text{\AA}$ between NBD-DPPE and Rhod-DOPE pair, and $R_0=36\text{\AA}$ between DPH and Rhod-DOPE pair.

According to Eq. 9, the FRET efficiency can be calculated from the lifetime of donor (NBD-DPPE, and Rhod-DOPE) with and without the presence of acceptor (Rhod-DOPE). Förster distance R_0 is $49\text{\AA}$ for NBD-DPPE and Rhod-DOPE pair, and is $36\text{\AA}$ for DPH and Rhod-DOPE pair. The graph according to Eq. 9 based on $R_0=49\text{\AA}$ and $R_0=36\text{\AA}$ is shown in Fig. 7.4. Fig 7.4 shows that for NBD-DPPE and Rhod-DOPE pair with $R_0=49\text{\AA}$, FRET efficiency is sensitive to the distance between donar and acceptor when

they are approximately 40Å to 60Å apart; for DPH and Rhod-DOPE pair with $R_0 = 36\text{Å}$, FRET efficiency is sensitive to the donor-acceptor distance from approximately 30Å to 40Å.

The calculated result of FRET efficiency based on Fig. 7.3 is shown in Fig. 7.5. The small Förster distance between DPH and Rhod-DOPE pair leads to the result that the FRET efficiency shown in blue in Fig. 7.5 is much lower at all composition. At 5% l_o the transfer efficiency shown in orange and blue bar are close to each other, this is because the l_o domains are very small at 5% l_o , so that the difference in R_0 does not lead to a big difference in efficiency between the “long range” pair (NBD-DPPE and Rhod-DOPE) and the “short range” pair (DPH and Rhod-DOPE). At 25% there is clearly a large increase in domain size. At this composition the short range pair is not often close enough to interact and efficiency is only about one third as high as in 5% l_o . Interestingly, neither NBD-DPPE nor DPH shows a clear efficiency change when the percentage of l_o phase changes from 25% to 75%. This could be explained by the image when size of l_o domains are increasing but the number of l_o domains are decreasing thus the edge area where the donors and acceptors interact with each other remains roughly the same. At 90% l_o presumably l_o is the connected phase with ‘pools’ of l_d . The drop in efficiency for both pairs means that the pairs are not often within R_0 of each other in this system, suggesting that the pools of l_d are larger than the “islands” of l_o in the 5% l_o membrane.

Figure 7.5 FRET Efficiency of NBD-Rhod and DPH-Rhod

Fig. 7.5 shows energy transfer efficiency between two FRET pairs at 23°C calculated from data shown in Fig. 7.3. The orange bars represent efficiency between NBD-DPPE/Rhod-DOPE pair, and the blue bars represent efficiency between DPH/Rhod-DOPE pair.

Figure 7.6 FRET Lifetime Changes with PDPC

In Fig. 7.6, the orange bars at the left column show lifetime of NBD-DPPE with no presence of Rhod-DOPE changes along increasing ratio of PDPC/POPC. The blue bars at right hand side show lifetime of DPH with no Rhod-DOPE along increasing ratio of PDPC/POPC. The purple bars on the left and right columns show lifetime of NBD-DPPE and Rhod-DOPE respectively when Rhod-DOPE is present. All the data are at 23°C.

Fig. 7.6 shows the effect on fluorophore lifetime of replacing POPC with PDPC at different lipid composition. As shown in Chapter 1.3.2, PDPC has one chain with 22 carbons and 6 double bonds known as DHA. Fig. 7.6a, 7.6c, and 7.6e demonstrate that when more PDPC is replacing POPC, lifetime of NBD-DPPE shows a clear decreasing curve; but this trend of NBD-DPPE lifetime with the percentage change of PDPC/(PDPC+POPC) is not as clear when Rhod-DOPE is present. Fig. 7.6b, 7.6d, and 7.6f report that lifetime of DPH does not show an clear trend corresponding to the increasing amount of PDPC. When Rhod-DOPE is present in the lipid mixture, DPH lifetime shows a slight decrease.

Based on Eq. 9, the FRET efficiencies based on the data shown in Fig. 7.6 are also calculated and the results are shown in Fig. 7.7. Fig. 7.7 shows that adding PDPC reduces FRET efficiency between NBD-DPPE and Rhod-DOPE in all cases. This means that PDPC must be making lo domains larger; FRET pairs are not often within R_0 of each other. This long range pair suggests that the changes due to PDPC are bigger the more lo is present in the membrane. At 5% lo, 67% PDPC reduces FRET efficiency between NBD-DPPE and Rhod-DOPE by about 7%, in 25% it reduces the efficiency by roughly 22% and in 50% lo 67% PDPC reduces the efficiency by more than 50%.

The changes reported by the short range pair DPH and Rhod-DOPE show the same approximate trend. In 5% lo the increase in lo domain size with added PDPC is so large that the FRET efficiency is essentially zero. This efficiency in both 25% & 50% lo is essentially the same for all levels of PDPC. This is because before PDPC is added in these two membranes the starting lo domain size is so large, and the efficiency between

DPH and Rhod-DOPE is so small due to small Förster distance (36Å), thus for this pair that the addition of PDPC produces essentially no change.

Figure 7.7 FRET Efficiency Change with PDPC

In Fig. 7.7, orange bars represent FRET efficiency between NBD-DPPE and Rhod-DOPE pair at 23°C, blue bars represent FRET efficiency between DPH and Rhod-DOPE pair at 23°C. Along x-axis, the ratio of PDPC/POPC increases. The data shown in Fig. 7.6 are calculated based on data shown in Fig. 7.5.

Figure 7.8 NBD-Rhod Efficiency Change with Temperature

Fig. 7.8 shows FRET efficiency between NBD-DPPE and Rhod-DOPE changes along increasing temperature. From Fig 7.7a to Fig. 7.7d, the composition of lipids in each graph is POPC/SM/Chol=57/27/16, 46/34/20, 33/42/25, and 10/56/34, respectively. Along x-axis, temperature changes from 10°C to 50°C. Blue diamonds: PDPC/POPC=0, red blocks: PDPC/POPC=1/2, green triangles: PDPC/POPC=2/1, purple crosses: POPC/POPC=0.

Fig. 7.8 demonstrates how NBD-DPPE and Rhod-DOPE FRET efficiencies change with temperature. We see that PDPC decrease the energy transfer efficiency in almost all the cases.

Figure 7.9 DPH-Rhod Efficiency Change with Temperature

Fig. 7.9 shows FRET efficiency between DPH and Rhod-DOPE changes along increasing temperature. From Fig 7.7a to Fig. 7.7d, the composition of lipids in each graph is POPC/SM/Chol=57/27/16, 46/34/20, 33/42/25, and 10/56/34, respectively. Along x-axis, temperature changes from 10°C to 50°C. Blue diamonds: PDPC/POPC=0, red blocks: PDPC/POPC=1/2, green triangles: PDPC/POPC=2/1, purple crosses: POPC/PDPC=0.

Similar to Fig. 7.8, Fig. 7.9 shows the energy transfer efficiency between DPH and Rhod-DOPE pair. The transfer efficiency slightly increase when temperature rises. PDPC shows a dramatic effect at 5% lo, but shows almost no effect at 25% and 50% lo.

7.2 Anisotropy Reports

The principle and anisotropy measurement has been introduced in Chapter 5.3. From anisotropy measurement, there are four important parameters derived: order parameter S , orientational freedom parameter f_{rand} , rotational correlation time $\langle\phi\rangle$, and rotational diffusion coefficient $D_{\perp}$.

Fig. 7.10 shows an overview of how the order parameter, disorder parameter, and motion parameter change with lipid composition. We can see that when the percentage of lo phase increases, the order parameter increases from ~ 0.7 to ~ 0.9 , orientational freedom parameter decreases from ~ 0.3 to ~ 0.15 , and rotational correlation time decreases from ~ 0.3 ns to ~ 1 ns.

Fig. 7.11 and Fig. 7.12 demonstrate how those four parameters, S , F_{rand} , $\langle\phi\rangle$, and $D_{\perp}$, change with increasing PDPC in the lipids. Each graph compares the parameter at two temperatures: at 23 °C and at 50 °C. 50 °C is beyond the melting temperature of the lipids thus there is no more lo and ld coexistence at 50°C; everything is mixed. So differences between the compositions for each parameter at 50°C show differences in the average properties in a mixed membrane. However, at 23°C there is lo and ld phase each intact, so differences with PDPC correspond to differences in lo and ld properties with PDPC.

Figure 7.10 Order Parameter S , Orientational Freedom Parameter f_{rand} , and Rotational Correlation Time $\langle\phi\rangle$ Change with Lipid Composition

Fig 7.10a, Order Parameter S ; Fig 7.10b, Orientational Freedom Parameter f_{rand} , Fig 7.10c, Rotational Correlation Time $\langle\phi\rangle$. Along x-axis, the compositions of lipids are POPC/SM/Chol=57/27/16, 46/34/20, 33/42/25, and 10/56/34, from left to right. All data are taken at 23°C.

Fig. 7.11a, 7.11c, and 7.11e show that PDPC decrease order parameter and this agrees with previous report on highly unsaturated fatty acids [113].

Figure 7.11 Order Parameter S and Disorder Parameter F_{rand}

In Fig. 7.11, the left side column shows the change of order parameter S and the right side column shows the change of disorder parameter F_{rand} . The green bars represent S at 23 °C, and the purple bars represent F_{rand} at 23 °C. The orange bars represent S on the left and F_{rand} on the right at 50 °C.

Figure 7.12 Rotational Correlation Time and Rotational Diffusion Coefficient

In Fig. 7.12, the left side column shows the change of rotational correlation time $\langle\phi\rangle$ and the right side column shows the change of rotational diffusion coefficient $D_{\perp}$. The cyan bars represent $\langle\phi\rangle$ at 23°C, and the blue bars represent $D_{\perp}$ at 23°C. The orange bars represent $\langle\phi\rangle$ on the left and $D_{\perp}$ on the right at 50°C.

Fig. 7.11b, 7.11d, and 7.11f report the effect of PDPC on the orientational freedom parameter. As introduced in Chapter 5.4, frand is the overlap of the orientational distribution and a random orientational distribution. We see that at 23°C DPH gains more orientational freedom with increasing amount of PDPC, this agrees with previous studies on unsaturated acyl chain phosphatidylcholine vesicles [130, 131].

Fig. 7.12a, 7.12c, and 7.12e shows that PDPC decreases rotational correlation time. It tells us that DPH tumbles faster with more PDPC present in the lipids. Figure 7.12b, 7.12d, and 7.12f reports that PDPC increases rotational diffusion coefficient which means that DPH rotates faster along its longest symmetric axis when more PDPC is present in the lipids.

Fig. 7.13 shows the change in each parameter with temperature for each composition. It shows that order parameter decreases with rising temperature. With the percentage of lo phase increases from Fig. 7.13a through Fig. 7.13d, the curve of order parameter in each graph rises.

Fig. 7.13a corresponds to the lipid mixture with lowest cholesterol percentage from the ternary phase diagram, i.e. the percentage of POPC is highest; thus the amount of PDPC added into the lipid mixture is the highest proportionally. Therefore, in Fig. 7.13a we see an obvious drop of the curves corresponding to the lipid with PDPC. In Fig. 7.13b and 7.13c, the effect of PDPC on order parameter is less obvious. At low temperature, we can hardly distinguish the order parameter in the lipid with PDPC from those without. When temperature increases, it becomes easier to notice the difference and when it comes to

50°C, the decrement of order parameter due to the increasing amount of PDPC is quite obvious.

Figure 7.13 Order Parameter Change with Temperature

Fig. 7.13 shows the change of order parameter S with temperature increase. From Fig. 7.12a through 7.12d, the composition of lipids in each graph is POPC/SM/Chol=57/27/16, 46/34/20, 33/42/25, and 10/56/34, respectively. The x-axis shows the increase of temperature from 5°C to 50°C. Blue diamonds: PDPC/POPC=0, red blocks: PDPC/POPC=1/2, green triangles: PDPC/PDPC=2/1, purple crosses: POPC/PDPC=0.

Figure 7.14 Disorder Parameter Change with Temperature

Fig. 7.14 shows disorder parameter f_{rand} changes with temperature increase. From Fig. 7.13a through 7.13d, the composition of lipids in each graph is POPC/SM/Chol=57/27/16, 46/34/20, 33/42/25, and 10/56/34, respectively. The x-axis shows the increase of temperature from 5°C to 50°C. Blue diamonds: PDPC/POPC=0, red blocks: PDPC/POPC=1/2, green triangles: PDPC/PDPC=2/1, purple crosses: POPC/PDPC=0.

Figure 7.15 Rotational Correlation Time Change with Temperature

Fig. 7.15 shows rotational correlation time $\langle\phi\rangle$ changes with temperature increase. From Fig. 7.15a through 7.15d, the composition of lipids in each graph is POPC/SM/Chol=57/27/16, 46/34/20, 33/42/25, and 10/56/34, respectively. The x-axis shows the increase of temperature from 5°C to 50°C. Blue diamonds: PDPC/POPC=0, red blocks: PDPC/POPC=1/2, green triangles: PDPC/POPC=2/1, purple crosses: POPC/POPC=0.

Fig. 7.14 shows that rising temperature increase the value of f_{rand} . And as the percentage of lo phase increases from Fig. 7.14a to Fig. 7.14b, the curve of f_{rand} drops. Similar to the

results of order parameter, the effect of PDPC on f_{rand} is most obvious in Fig. 13a, where the most amount of POPC was contained and thus most amount of PDPC was involved proportionally. Although effect of PDPC is relatively hard to tell in Fig. 7.14b and 7.14c, we see that at lower temperature, PDPC brings down the value of f_{rand} , and this change become less noticeable at higher temp.

Fig. 7.15 shows that PDPC has most significant effect on rotational correlation time at lower temperature. Rotational correlation time decrease with rising temperature and PDPC reduces the value of rotational correlation time in general. Although at lower temperature with lower amount of PDPC the error bars are relatively high, at higher temperature the data become much more consistent and it becomes more obvious that as more PDPC is added into the mixture, the rotational correlation time becomes smaller.

Fig. 7.16 demonstrates that temperature increase of value of D_{perp} , which indicate the rotation rate of DPH along its longest axis. At higher temperature the effect of PDPC is most dramatic.

Figure 7.16 Rotational Diffusion Coefficient Change with Temperature

Fig. 7.16 shows rotational diffusion coefficient changes with temperature increase. From Fig. 7.16a through 7.16d, the composition of lipids in each graph is POPC/SM/Chol=57/27/16, 46/34/20, 33/42/25, and 10/56/34, respectively. The x-axis shows the increase of temperature from 5°C to 50°C. Blue diamonds: PDPC/POPC=0, red blocks: PDPC/POPC=1/2, green triangles: PDPC/POPC=2/1, purple crosses:

Overall, based on what we have seen from our lifetime and anisotropy data, we can build an image of how PDPC alters the lo and ld phase domain segregation, as visualized in Fig. 7.17.

The first row of pictures in Fig 7.17 demonstrate how the lo domains distributed in the lipids when lo rises from 5% to 90%. At each step of increasing lo percentage, each domain size increases however the number of lo domains decreases. From 5% lo to 25%, we observed a sudden drop in FRET efficiency. This is because at 5% lo, each lo domains are very small thus there are many donors and acceptors located within the Förster distance R_0 with each other; at 25% lo each lo domain expands and leads to the distance of many FRET pairs exceeding R_0 thus the efficiency drops. From 25% to 75% lo, the size of each lo domain increases but the number of the domains decreases, thus the edge area where the donors and acceptors are located within R_0 remains the same. While at 90% lo, presumably lo is the connected phase with “pools” of ld. The efficiencies drop of both FRET pairs suggests that the pools of ld are larger than the “islands” of lo in the 5% lo membranes.

When PDPC is added into the membrane, we observed FRET efficiency drop. This is because PDPC makes each lo domain larger without increasing the total lo percentage, thus the edge area where donors and acceptors are within R_0 decreases. Such gradual change is visualized in Fig. 7.17.

Figure 7.17 Phase Segregation due to DHA

8. Conclusion

Understanding the mechanisms of membrane function is one of the major challenges in the life sciences. Docosahexaenoic acid (DHA) is of special interest because it has been positively linked to the prevention of numerous human afflictions [133]. It has been proposed that one function of DHA may be to facilitate the formation of lateral domains [132]. Thus it is critical to understand the physical properties of the resulting domains enriched in highly polyunsaturated acyl chains.

Our data demonstrates that both increasing temperature and PDPC reduces the lifetime of NBD-DPPE that partitions in l_o phase. Mazereds et al. had demonstrated that NBD groups are located in the region of the lipid headgroups at the water-lipid interface with higher polarity. Increasing temperature leads to greater exposure of the NBD to water and thus increase the environmental dielectric constant. Therefore the increasing dielectric constant due to rising temperature reduces NBD-DPPE lifetime. Interestingly, PDPC showed a similar effect as temperature was increased. This indicates that PDPC alters headgroup packing in the l_o phase. Therefore we propose that a line tension around the boundary between l_o and l_d phase domains are changed by adding PDPC into the lipid mixture. Additionally, the decrease of DPH lifetime with a higher PDPC percentage demonstrates that DHA increases water permeability in the lipid bilayer; this result agrees with previous studies on water permeability of polyunsaturated lipid membranes using ^{17}O NMR done by Huster et al [134].

The change of FRET efficiency between the long range pair (NBD-DPPE with Rhod-DOPE) and short range pair (DPH with Rhod-DOPE) due to PDPC suggests that PDPC enlarges l_o domains. As shown in Fig. 7.4, FRET efficiency between NBD-DPPE and Rhod-DOPE is sensitive to the donor-acceptor distance between $40\text{\AA} \sim 60\text{\AA}$ with $R_0 = 49\text{\AA}$; while the efficiency of DPH and Rhod-DOPE pair is sensitive to the distance between $30\text{\AA} \sim 40\text{\AA}$ with $R_0 = 36\text{\AA}$. The decreased efficiency between the long range pair suggests an enlarged l_o domain size due to PDPC leads to FRET pairs less often within R_0 of each other; as more l_o is present in the membrane, the changes due to PDPC are larger. The short range pair suggests that at 25% to 90% l_o before PDPC is added, the domain sizes are too large compared to their small Förster distance. Thus, the DPH and Rhod-DOPE pair cannot report the enlarged domain size with additional PDPC in those lipid mixtures.

Although in Fig. 7.17, all l_o domains are notified in circular shapes, the actual l_o domain shape may be complicated. Jonathan Amazon et al has used Monte-Carlo simulation on the surface of giant unilamellar vesicles to show that competition between line tension and curvature, as specified by the Helfrich energy functional, can affect the morphology of modulated phase patterns [135]. Their simulation showed that the parameters of line tension and bending energies yield a range of different patterns of modulated phases, including striped, honeycomb, and macroscopic shapes [135].

Our analysis of DPH anisotropy data report that DHA increases acyl chain flexibility, reduces order, increases DPH random orientational distribution, and increases DPH rotation, which agrees with previous findings of the effect of DHA on membranes [136].

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