

TEMPERATURE DEPENDENT SOLVATION
IN PHOSPHOLIPID MEMBRANES

by

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DEDICATION

I dedicate this dissertation to my loving family:

Dad- for teaching me how to respect and love, for keeping your expectations high, and for helping me find my passions.

Mom- for being my role model of a “Proverbs 31” woman, for teaching me unconditional love and compassionate trust, and for your constant willingness to talk, advise, and hug.

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ABSTRACT

Experiments described in this dissertation were designed to systematically investigate solute partitioning in phospholipid bilayers as a function of phospholipid identity, solute identity, membrane phase, and membrane composition. Experiments use time-resolved fluorescence, steady-state fluorescence, dynamic light scattering, and differential scanning calorimetry to experimentally quantify solute partitioning in three specific regions of a model membrane, as well as track how solutes migrate into and out of lipid bilayers as a function of temperature.

Phosphatidylcholine vesicles were comprised of DLPC (12:0 PC), DMPC (14:0 PC), and DPPC (16:0 PC). In all three lipid systems, coumarin 152 (C152) showed partitioning behavior that was qualitatively similar but quantitatively different. Partitioning into a gel phase membrane was slightly exothermic and slightly entropically unfavorable. Partitioning of C152 near the lipid membrane melting temperature was entropically driven and endothermic. Well above the melting temperature, exsolvation of C152 from the membrane back into the aqueous buffer was enthalpically driven but entropically unfavorable. Regardless of solution temperature, relatively little (<20%) C152 partitioned into the hydrophobic core of the membrane. The magnitudes of the thermodynamic forces driving C152 partitioning systematically increased with alkyl chain length (DLPC < DMPC < DPPC).

C152 and C461 differ solely in the 4-position where C152 has a trifluoro methyl group in place of C461's $-\text{CH}_3$ group. Fluorescence amplitudes were used to calculate absolute partition coefficients and average number of solutes per DPPC vesicle. C152 shows a ~10-fold greater affinity than C461 for lipid bilayers, despite both solutes having similar log P values.

Differential scanning calorimetry traces of vesicles composed of binary mixtures of lipids show moderate miscibility between DLPC and DMPC and low miscibility between DMPC and DMPE. Time-resolved fluorescence decays indicate C152 partitioning into mixed PC membranes is nearly ideal; that is, even if mixed PC vesicles *do* form single lipid domains, C152 partitioning behavior is largely unaffected. Time-resolved fluorescence decays show C152 partitioning behavior into PC/PE membranes is distinctly non-ideal, but the cause of this non-ideal behavior requires further studies.

CHAPTER ONE

INTRODUCTION

1.1 Motivation

Biological membranes are nature's selective barriers to intra and extracellular environments as they control the exchange of molecules between cells and their surroundings.¹ Membranes can both mediate transmembrane solute movement and act as physical barriers to specific solute diffusion. In short, membranes are the structures that enable life itself. Membranes, however, are also permeable to some solutes and membrane solubility controls the activity of certain pharmaceuticals and the bioaccumulation of toxins. In this context, membrane solubility refers to a solute's tendency to partition into a biological membrane from bulk solution. While this description seems simple enough, membrane solubility reflects a subtle balance of noncovalent forces between the solute and its bulk solution limits versus the solute in the heterogeneous solvation environment created within biological membranes. Consequently, quantitative and predictive descriptions of membrane solubility are difficult to formulate.

Lipophilicity, the affinity of a solute for a lipophilic environment such as those found in biological membranes, is a general term used to describe complex interactions occurring in a given system. Lipophilicity has historically been determined using the "shake-flask" method, where the ratio of concentrations of solute between water and an

immiscible “lipid-like” environment (e.g. octanol) is found and expressed as a log P value.^{2,3} Log P refers to the logarithm of the partitioning coefficient:

$$\log P = \log \left(\frac{[\text{solute}]_{\text{octanol}}}{[\text{solute}]_{\text{water}}} \right) \quad (1.1)$$

Newer approaches to determine the log P value include reverse phase chromatography^{4,5} and computational simulations.⁶ Log P values are commonly used to determine parameters for drug design and are even considered in government measures to preserve human health and environmental safety. For example, bioconcentration factors (BCFs) are parameters routinely investigated for new pharmaceuticals, pesticides, and even chemicals such as polychlorinated biphenyls (PCBs) that have not been produced for generations.⁷

The log P scale is one example of a high throughput screening (HTS) method used to empirically predict a solute’s tendency to partition into cell membranes. Although a multitude of HTS methods are used to quickly and cost efficiently describe the equilibrium position of a solute within a lipid membrane, lacking from these analyses is a molecular description of membrane solvation and the sensitive balance of the driving thermodynamic forces. Solute partitioning has been shown to change with temperature, lipid acyl chain length, degree of unsaturation in the acyl chain, and presence of domains or lateral heterogeneity. Simple HTS-derived descriptions of solute solubility in lipid membranes are unable to account for these lipid-specific differences.

In the sections below, solute partitioning is discussed in context of drug design in the pharmaceutical industry as well as the relevance of partitioning from an ecological standpoint. The following sections summarize the ways membrane partitioning has been

studied over the years, including many specific examples. Although both the experimental techniques and studied systems are drastically different throughout the examples listed below, lacking from each analysis is a thorough discussion of the molecular interactions that form the basis of solute partitioning. The remainder of this thesis describes research that systematically investigated fundamental driving forces behind solute partitioning in lipid membranes and answer three specific questions:

- 1) *How much* of a solute partitions into the membrane?
- 2) *Where* do solutes partition in lipid membranes?
- 3) *How* do specific factors (system temperature, membrane composition, solute structure) alter solute partitioning in membranes (and *why*)?

To provide context and motivation for these studies, the consequences of membrane partitioning are considered in two specific cases: nonspecific membrane soluble pharmaceuticals and bioaccumulation of organic toxins in living systems.

1.2 Pharmaceuticals

The pharmaceutical industry relies on collections of empirical constants to identify promising drug candidates.⁸⁻¹⁰ Membrane-drug interactions affect pharmacokinetical properties of compounds, including the ADMET (adsorption, diffusion, metabolism, elimination, and toxicology).¹⁰ The most notable of the methods used to determine drug likeness is termed the “Lipinski Rule of Five.” The rule of five states that a compound is likely to be membrane permeable and adsorbed by the body if:

- 1) the compound has a molecular weight less than 500,
- 2) the compound's log P (octanol-water) is <5 ,
- 3) the number of hydrogen donating atoms is <5 , and
- 4) the number of hydrogen bond acceptors is <10 .^{6,11}

The consideration of a drug candidate starts experimentally with a log P analysis, as 90% of effective, marketable drugs have a log P value ranging from 0 to 5 (Figure 1.1).¹² Solutes with a log P <0 partition weakly into membranes, and thus are generally clinically unsuitable drug candidates (unless the molecule is otherwise transported in/across the membrane via active transport). Drug candidates with moderate lipophilicity ($0 < \log P < 3$) are both sufficiently hydrophilic for efficient oral delivery and retain enough lipophilic behavior to passively diffuse through cell membranes. Molecules with large log P values (>5) are virtually insoluble in water, and thus not frequently considered for oral delivery. When the log P value of a compound exceeds ~ 5 , it will accumulate in membranes, eliminating itself only after a large amount of time (weeks to years). Thus drug candidates with log P > 5 are usually unsuitable for clinical use.¹²

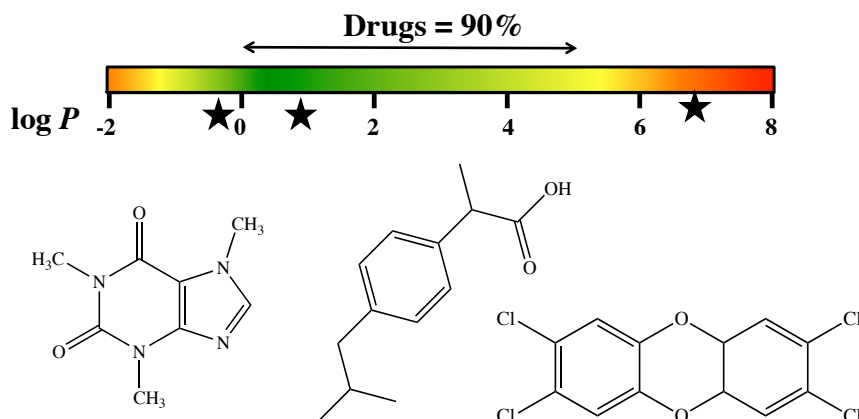


Figure 1.1. Log P values for ideal drug candidates. Pictured from left to right are the molecular structures of caffeine (log P = -0.04),¹³ ibuprofen (log P = 1.27),¹³ and a polychlorinated biphenyl (2,3,7,8 Tetrachlorodibenzo-*p*-dioxin, log P = 6.80).¹⁴

Models characterizing partitioning behavior tend to rely on collections of constants (such as the log P value)¹⁹, empirical correlations (such as Lipinski's Rule of 5),^{15,16} or qualitative structure-permeability relationships (QSPRs).^{9,15,17} Currently lacking from the description of drug effectiveness are description of specific solute-lipid interactions within the membrane bilayers themselves. These noncovalent interactions are often responsible for the pharmaceutical's biological activity and metabolism.¹⁸

Transdermal drug delivery is becoming a popular technique to administer drugs otherwise ineffective due to their low oral bioavailability.¹⁹ Diffusion through the skin is difficult: solutes must pass from skin surfaces to the stratum corneum to the epidermis, through the dermis, and into the blood. To increase the permeability of transdermal drugs, penetration enhancers (azone^{20,21} and short chain amphiphilic molecules^{22,23}) may be added to the drug delivery system (commonly a patch). Although the mechanism behind penetration enhancers is still somewhat vague, these molecules are thought to temporarily diminish the impermeability of the skin by creating microdomain to form in

the lipid bilayer, thus increasing membrane fluidity and increasing the number of sites in which a drug may partition.

The pharmaceutical industry also manipulates membranes (and model membranes) for targeted drug delivery and controlled release.²⁴⁻²⁸ Lipid-based drug carrier systems can target interstitial tumor tissues, however these systems are generally created to be “slow-release” as controlling a quick release (seconds) of a large amount of drug directly into a tumor is difficult. A plethora of drug delivery (triggered release) ideas are being studied, including environment-sensitive lipids that respond to a change in pH,^{29,30} thermal changes,³¹⁻³³ endogenous enzymes,³⁴ or electric fields.³⁵ For example, Needham *et al.* have successfully fine-tuned the melting temperature of a vesicle drug delivery system (and thus the amount of an anti-cancer drug released) by adding small amounts of a different amphiphilic molecule (such as DMPC) to pure DPPC vesicles.²⁵

Although the applications of membranes in drug delivery systems are promising, the pharmaceutical industry is remains result oriented, thus not investing the money or time in critically assessing the sensitive interactions that occur in membranes as a solute partitions into the hydrophobic core.

1.3 Biological and Ecological Partitioning

A second example of solute accumulation in biological membranes focuses on toxin bioaccumulation. More than 1.25 billion pounds of polychlorinated biphenyls (PCBs) were produced in the United States from 1929 to 1977 and used in hydraulic and lubricating fluids, pump oils, pesticides, plasticizers, adhesives, and flame-retardants.³⁶

Dr. Paul Muller's Nobel Prize in 1948 was awarded for his discovery of DDT's use as a poison against arthropods (including mosquitos) in the midst of the world battling mosquito-delivered malaria.³⁷ By the 1960s, biologists were linking the decline of bird species high in the food chain to eggshell thinning- a direct result of DDT biomagnification.^{38,39} The US Congress limited DDT use in 1972, and only five years later, the US banned PCB production due to their potential carcinogenicity and their propensity to bioaccumulate.^{36,40} Over the ~50 years that PCBs and DDT were used in the United States, they entered the environment via soil, water, and air, and *to this day* remain in the environment due to their resistance to degradation through photolytic, biologic, or chemical processes.^{36,40}

In 1976, the United States enacted the Toxic Substance Control Act (TSCA), which mandated risk assessment of chemicals used at the time, and enacted regulations on which chemicals may be produced and used in the future. The United Nations and Canada have since implemented similar strategies for regulating potentially hazardous chemicals.⁴¹ To date, approximately 100,000 chemicals are regulated, and 1,000+ new chemicals are assessed each year. Best hazard assessment of these chemicals would come from ecotoxicological data, however the cost associated with such tests has led to the development and application of models manipulating quantitative structure-activity relationships (QSARs), empirical models, and mass balance models.

Bioaccumulation is one of the hazard risks closely monitored through environmental protection agencies. Most commonly, bioaccumulation is reported as a bioconcentration factor (BCF):

$$BCF = \frac{C_B}{C_w} \quad (1.2)$$

where C_B is the concentration *in biota* and C_w is the concentration dissolved in water.⁴²

The bioaccumulation factor (BAF) accounts for more ecological factors than the BCF, and often is used to more thoroughly describe environmental hazards:

$$BAF = \frac{C_B}{C_S + C_W + C_F} \quad (1.3)$$

where concentration of the solute in sediment (C_S), water (C_W), and food (C_F) is

considered. The BCF of a solute is generally assumed to be proportional to the log P value, whereas the BAF is typically not.^{43,44} Although the BCF and BAF of a compound may provide valuable predictions of the impact of a chemical on the environment and are often used to develop regulatory policies for exposure estimates^{45,46}, these empirical correlations simplify many other factors known to affect bioaccumulation such as lipid content or environmental temperature.^{41,47}

1.3.1 Lipid Content

The influence of lipid content on the bioconcentration of 1,2,4-trichlorobenzene in fish was investigated in 1985, in which Geyer *et al.* noted a positive correlation between the lipid content of 8 different species of fish and their bioconcentration factor.⁴⁸ A study of the bioaccumulation of a series of PCBs in the Lake Ontario ecosystem was conducted 3 years later. Results from this study attributed the large bioaccumulation of PCBs in high food-web organisms to their higher body lipid content. Data shown in Figure 1.2 are derived from this study, where the lowest lipid percentages (of wet weight) belonged to plankton, and the highest lipid percentages were attributed to lake fish.⁴⁹

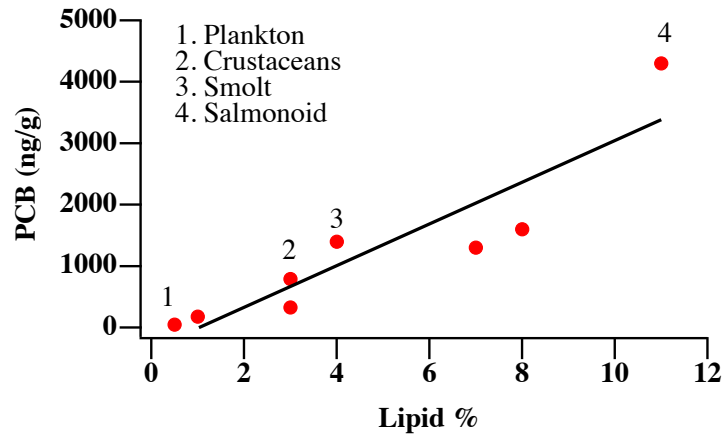


Figure 1.2. Concentrations of PCBs in 8 organisms compared to the organism body composition (wet weight lipid percent). Data adapted from Oliver *et al.*⁴⁹

The global effect of bioaccumulating pollutants and biomagnification is observed in the pristine Arctic, where concerning levels of persistent organic pollutants have been observed in high food-web organisms such as polar bears, gulls, and even indigenous human populations- despite the minimal use of the chemicals in the local environment. The increased concentration is attributed largely to the biomagnification of persistent organic pollutants (POPs) by organisms high in the food web, but is also attributed to the large lipid stores in Arctic organisms.⁵⁰

Evidence of the effects of lipid content on bioconcentration was shown in an experiment monitoring the bioconcentration of DDT in rainbow trout.⁵¹ Trout that were already exposed to DDT were starved and forced to consistently swim through a current. Starvation and rigorous exercise did not result in a net decrease in DDT concentration, however these activities decreased the body fat of the trout (and thus lipid content), increasing the DDT concentration in the brain (within 15 days) and blood and muscle (within 20 days).⁵¹

Studies outlined above (amongst numerous others not summarized here) have shown that both the lipid composition of organisms *and* the identity of the lipids composing the membranes can significantly alter the solvation and accumulation of a solute within a lipid membrane.

1.3.2 Temperature

Changes in temperature and membrane composition have been shown to alter the permeation rate and equilibrium concentrations of solutes in vesicles.^{52,53} In general, solutes will preferentially partition into a lipid membrane when the membrane is in the liquid-crystalline phase. Partitioning enhancement above the melting temperature may be due to a number factors, including the additional free volume present when a membrane changes from its gel to liquid crystalline state⁵⁴⁻⁵⁶, and general change in mechanical properties of vesicles.⁵⁷ At the melting temperature of the membrane, a large enhancement in partitioning is often observed.^{58,59} Two models have been proposed to explain the increase in permeability. The “leaky membrane” hypothesis suggests solutes will preferentially partition into the boundaries of coexisting gel and fluid domains.^{58,60} A second model suggests membrane permeability is enhanced due to the increase in area per lipid, membrane lateral compressibility, and maximum area fluctuations.⁶¹⁻⁶³

The effects of temperature on bioconcentration have been shown in numerous ecological studies. A 314% increase in the bioconcentration factor of dichlorodiphenyldichloroethane (DDE) was observed in water fleas when the local temperature was raised from 5 to 25 °C.⁶⁴ The BCF of a PCB in rainbow trout increased

from 7,400 to 10,000 over a temperature range of 5–15 °C, while the BCF of the same PCB in sunfish increased from 6,000 to 50,000 over the same temperature range.⁶⁵

Membrane partitioning and accumulation likely increases with temperature due purely to thermodynamic solvation and membrane mechanical properties. However, biological mechanisms may also play a role in bioaccumulation as a mechanism secondary to that of fundamental partitioning thermodynamics. Poikilotherms (such as fish) are organisms whose internal temperature varies drastically with ambient environmental temperature. To maintain structural and functional integrity of their cells, these organisms have temperature sensitive enzymes that are activated upon environmental temperature changes. These enzymes are able to quickly and reversibly compensate for the change in environmental temperature through altering the physical properties of their cell membranes.⁶⁶ Typically these enzymes turn saturated fatty acids into unsaturated fatty acids as the environmental temperature decreases, thus maintaining membrane integrity and increasing the fluidity of the membrane at lower temperatures.⁶⁷

In general, bioconcentration factors for PCBs in aquatic species are proportional to both the log P values as well as the lipid content of the species, and thus may be semi-accurately predicted. Although partition coefficients of PCBs can prove useful in predicting BCFs, simplistic models do not account for any variation such as the amount of lipid present in the organism or what types of lipids compose their cell membranes. These factors differ significantly different among species (and even among individuals of the same species).^{47,68} Moreover, partition coefficients often are measured at single temperatures and do not allow for partitioning's temperature dependence.

To advance current descriptions of membrane partitioning phenomena such as bioaccumulation and pharmaceutical activity, new models are needed not only to quantify solute uptake by biological membranes as a function of temperature but also to identify how solutes are distributed within the membranes themselves and how different solvation environments affect solute behavior. Meeting these needs requires quantitative data such as molecular specificity, quantitative accuracy, and the ability to discriminate changes in solvation forces that may occur over very short distances. Membranes themselves are only ~4 nm wide and provide local environments that range from highly charged to polar to hydrophobic. Studies described in this thesis were performed to develop methods capable of providing this information and to test these methods by examining solute partitioning into model biological membrane systems.

1.4 Previous Work

Studies described in this dissertation build upon a sizable body of work describing solute partitioning in membranes. Membrane mechanics and solute partitioning have been studied extensively both by experiment and computation.⁶⁹ Experimental materials include liposomes,^{57,59,70,71} lipid monolayers,^{72,73} and supported lipid bilayers (SLBs)⁷⁴, while experimental techniques include fluorescence,⁷⁵⁻⁷⁸ FTIR,⁷⁹ differential scanning calorimetry (DSC),⁸⁰⁻⁸³ X-ray and neutron diffraction,⁸⁴ second harmonic generation (SHG),⁸⁵ EPR,⁸⁶ NMR,⁸⁷ AFM,⁸⁸⁻⁹⁰ Langmuir trough,⁹¹⁻⁹⁴ and isothermal titration calorimetry.^{95,96}

Computational simulations frequently use several ns-long, all-atom MD simulations to determine parameters such as solute ingress and egress rates, depth-dependent partition coefficients, the number of non-covalent interactions between solutes and surrounding lipids and water, and preferred solute orientation.^{97,98} Although atomic-level molecular dynamic simulations have provided valuable insight into membrane partitioning, these simulations come at a large computational cost. Another common method of studying solute partitioning computationally is the use of course-grain modeling techniques. However, due to the sensitivity of membrane partitioning to solute structure, even minor changes in solute atomic structure can lead to differences (orders of magnitude) in the permeability coefficients.⁹⁹ Modern approaches, termed multiresolution models or concurrent schemes, combine the sensitivity of atomic-level simulations at the computational cost of course-grain techniques through modeling solutes atomistically and the surrounding membrane with course-grain techniques.⁹⁹⁻¹⁰² Analyses such as these, however, can overlook the sensitive interactions that occur due between solute, lipid, and surrounding water. Moreover, systems studied using the multiresolution models must be relatively small, as every increase is accompanied by computational cost. Studying small systems may create artificial scenarios or overlook long-range effects in membranes that may affect solute solvation.

Summarized below are results from experiments particularly relevant for the work described in this dissertation. Included in these sections are the analysis of membrane structure and solute solvation as determined with computational analysis, DSC, and SHG. These previous studies, although informative, do not quantify solute distributions within

bilayers nor characterize the immediate solvation environments sampled by solutes solvated in lipid membranes.

1.4.1 Computational Analysis

Computational simulations have proven useful for describing diffusion of specific molecules across lipid membranes. For example, water can diffuse freely across lipid membranes¹⁰³⁻¹⁰⁶ as can small alcohols^{105,107} and benzene.^{105,107} Such studies have noted permeation properties vary considerably for different solutes due to the anisotropic and inhomogeneous structure of the lipid bilayer.^{98,100,108-115}

Computational simulations have also provided detailed insight into the structure and fluid dynamics of lipid bilayers.¹¹⁶⁻¹¹⁸ A widely accepted structural framework for lipid bilayers is the “four-region model,” proposed first by Berendsen¹⁰³ in 1994 and adopted by numerous others.^{97,119,120} Since the four regions, defined below, have different polarities and viscosities, a solute may preferentially partition into a specific region. Solute structure and reactivity may depend sensitively on the local solvation environment within the membrane.

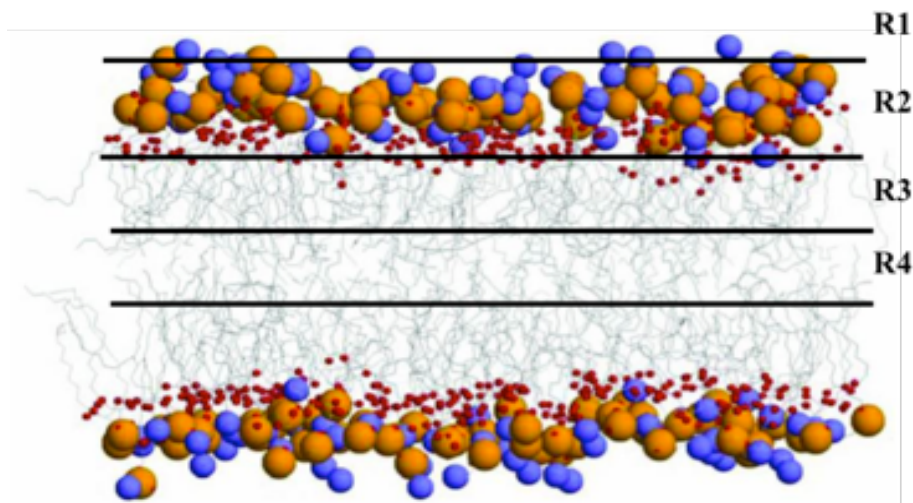


Figure 1.3. Snapshot of a DMPC bilayer taken from a MD simulation presented by Volkov *et al.*,¹²¹ adapted to show the boundaries of the four-region model as described in text. The red, blue, and orange spheres represent oxygen, nitrogen, and phosphorous atoms, respectively. Hydrophobic acyl chains are shown with sticks, and water molecules are not shown.

The four-layer model of a lipid bilayer includes regions having markedly different solvation forces:

Region 1 (R1) is the highly polar region of the phospholipid bilayer. This region includes the positively charged choline/ethanolamine groups that extend into the bulk water, causing water perturbation. Region 1 extends from the end of bulk water perturbation to about 0.8 nm into the bilayer.

Region 2 (R2) is also very polar, a description attributed to the high density of phosphate groups existing in this region. A small portion of the carbonyl density may also stretch into this region. In the membrane's fluid (physiological) state, water may provide hydrogen bridges between neighboring phosphate groups. In the gel state, virtually no water is present. This region extends 0.75 nm past Region 1.

Region 3 (R3) extends ~0.7 nm past Region 2 and contains the ester groups which associate with one other to form the lipid's 3-carbon glycerol backbone. This region also contains the bulk of the hydrocarbon chain density, providing no solvation environment for water. Region 3 is described as the interface between the hydrophobic and hydrophilic entities of the lipids.

Region 4 (R4) describes the middle of the bilayer where the methyl groups of opposing lipids meet. This region has very low alkane density and is thought to have properties resembling those of hexane. This region is about 1.1 nm thick, and extends to Region 3 of the opposite side.

In this dissertation, fluorescence decay traces of solutes in vesicle solutions are used to determine the local solvation environment. Due to the photophysical behavior of the chosen solutes, we adopt a "three-region" model for data analysis. Solute are either solvated in bulk buffer (no membrane association), in a polar protic environment (similar to region 1 in Figure 1.3), in a polar aprotic environment (region 2 in Figure 1.3), or in a nonpolar environment (regions 3-4 in Figure 1.3).

Sugii *et al.* investigated the effects of the hydrocarbon chain length of phospholipids on the permeability of H₂O.¹²² Computational simulations showed that the local resistance to the permeation of water was higher for phospholipids with longer carbon chains. Shinoda *et al.* analyzed the effect of chain branching of lipid molecules on the permeation of water by comparing permeation in lipid bilayers composed of DPPC and monounsaturated diphytanoylphosphatidylcholine (DPhPC). They concluded that the chain branching of DPhPC reduces permeability of water by restricting water

motility, but that the branching alone does not significantly change the partition coefficient.¹²³

Although computational simulations such as these can provide useful data (often complementing experimental data), models used in simulations are based on numerous assumptions. Accurately modeling the complexity of the heterogeneous membrane requires significant computational resources, and thus the complexity of the membrane is frequently simplified to reserve the computational power for accurate solute permeation and binding thermodynamics. These assumptions and simplifications may compromise the accuracy of information regarding sensitive molecular interactions within the lipid membrane-solute system.

1.4.2 Partitioning Insights from Thermotropic Behavior

Differential scanning calorimetry (DSC) has been used in several studies to assess drug-membrane interactions. Krill and coworkers investigated the change in phase behavior in DMPC vesicles in the presence of four β -blockers.¹²⁴ Modulations in thermotropic behavior showed decreasing partitioning from propranolol to metoprolol to oxprenolol to nadolol, which agreed favorably with simplistic partition coefficients. In a study performed by Lucio *et al.*, calorimetric curves were used to investigate how a series of nonsteroidal anti-inflammatory drugs (NSAIDs) interacted with DPPC membranes.¹²⁵ Membrane perturbation was observed upon introduction of all three NSAIDs investigated, with a lowering in melting temperature strongly correlated to the molar fraction of drug introduced into the system. At a mole fraction of 0.4, acetamin

decreased the melting temperature of DPPC vesicles by ~ 5 °C. The same mole fraction of indomethacin and nimesulide decreased the melting temperature by ~ 3.5 °C and ~ 1 °C, respectively. Although useful in determining *relative* extent of partitioning (e.g. of drugs with similar structures), data resolved from DSC studies are lacking the fundamental description of partitioning (*where?*) as well as a quantifiable description of partitioning (*how much?*).

1.4.3 Using SHG to Investigate Tetracaine Binding

Nguyen and Conboy have investigated interactions between tetracaine (a water insoluble local anesthetic) and lipid bilayers composed of DOPC, SOPC, DMPC, and DPPC using second harmonic generation (SHG).¹²⁶ Lipid phase was shown to influence tetracaine binding, as the binding affinity with liquid-crystalline phase lipids was higher than in solid-gel phase lipids. Furthermore, the amount of tetracaine associated with membranes composed of unsaturated lipids was larger than that of lipids composed of fully saturated lipids. The authors rationalized the high degree of order and structural rigidity between lipid molecules in the gel phase inhibits tetracaine permeation into the lipid membrane. These results further support earlier measurements by Zhang *et al.*¹²⁷ who measured tetracaine-membrane interactions using steady state fluorescence measurements.

1.5 Phospholipid Membranes

Biological cells contain a diverse mixture of lipids that will affect solute partitioning and can lead to preferential accumulation of solutes in the biomembranes of

one organ over others. For example, human erythrocyte membranes are diverse environments consisting of three major components: lipids (43.6%), proteins (49.2)%, and carbohydrates (7.2%).^{128 129} Phospholipids, amphiphilic molecules containing a hydrophilic phosphate headgroup and two acyl hydrocarbon chains, constitute 60.5% of the overall lipid content.⁶⁹ Phospholipids are diverse; headgroups can vary in charge and size, and the chains can be symmetric or differ in length and degree of unsaturation. Phospholipid chain length is primarily 14 to 22 carbons long, and are either saturated or *cis*-unsaturated.⁶⁹

In aqueous solution, lipids spontaneously form bilayers and multilamellar structures. Sonicating and extruding these structures through filters creates spherical vesicles that approximate the structure of biological cells. These vesicles provide solutes with distinct heterogeneous environments into which they can partition.¹³⁰ The clearest example of this heterogeneity is the contrast between the polar, aqueous environment on either side of the membrane and the hydrophobic interior of lipid bilayers where effective dielectric constants can assume alkane-like limits.^{103,131} Such a description is the basis for the four-layer model described above.¹⁰³ More subtle are changes in solvation that occur near the membrane surface and within a lipid bilayer's polar, glycerol-backbone region. Coulomb forces, local functional group dipoles and intercalated water all conspire to create regions that have been modeled using simplified approximations such as additive (nonpolarizable) force fields, where polarized charges are later added as constants to mimic polar (aqueous) and nonpolar (alkane) regions.^{113,132,133}

Lipid membranes exist in one of three phases: a ‘gel’ phase characterized by tight lipid packing (L_β), a ‘ripple’ phase characterized by tight lipid packing with long range structure (P_β), and a ‘liquid crystalline’ phase (L_α) characterized by increased fluidity and volume within the bilayer (Figure 1.4).¹³⁴ From a biological perspective, transitioning from fluid to gel phase can cause membrane proteins to cluster, reduce the activity of membrane-associated enzymes, reduce lateral protein diffusion rate (thus reducing diffusion coupled transfer) and may increase permeability to specific solutes.¹³⁵⁻¹³⁷

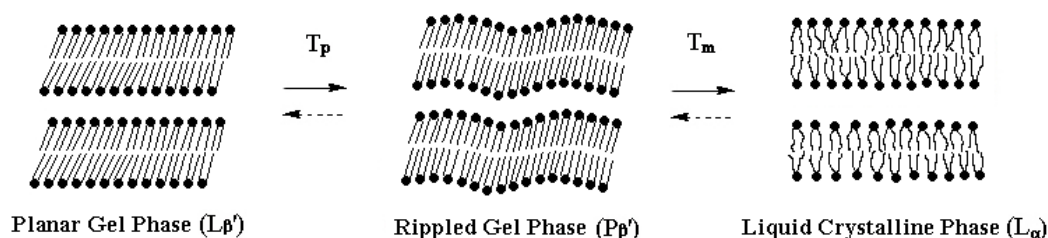


Figure 1.4. Phases of a phospholipid bilayer. Figure adapted from Smith *et al.*¹³⁴

A complete analysis of lipid bilayer phases as they relate to the experiments described in this dissertation is further described in Chapter 2.

1.6 Thesis Organization

Research described in this dissertation is organized as follows:

Chapter 2 describes the materials and techniques used throughout the experiments described in the later chapters. Included here are discussions validating our choice of materials used through the experiments and justification of the methods used for data interpretation.

Chapter 3 describes partitioning of coumarin 152 in a simple phosphocholine bilayer (DMPC) and the effect of temperature on membrane partitioning.

Chapter 4 describes the partitioning of coumarin 152 in phosphocholine bilayers (DLPC, DMPC, and DPPC), elucidating the factors that influence partitioning such as alkyl chain length and temperature.

Chapter 5 describes the partitioning of coumarin 152 and coumarin 461 in phosphocholine bilayers (DPPC) and present evidence justifying the pharmaceutical industry's use of halogenated moieties in drugs.

Chapter 6 describes the partitioning coumarin 152 in vesicles composed of phosphocholine, phosphoethanolamine, and binary mixtures of the two. These studies start to clarify the partitioning differences in membranes composed of lipids with differing headgroups.

Chapter 7 includes the summary of research described in Chapters 3-6 and includes a discussion on further experiments that should be performed to continue this research project.

Chapter 3 consists of a (modified) journal article published in 2016. Chapter 4 consists of an accepted manuscript, and Chapter 5 is prepared in manuscript format for future submission. Due to their publication (or anticipated publication) in literature, information in the introductions, materials, and methods sections of these chapters may appear more than once in this dissertation.

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CHAPTER TWO

MATERIALS AND METHODS

2.1 Phospholipids

The research described in this dissertation uses two families of phospholipids: phosphatidylcholines (PCs) and phosphatidylethanolamines (PEs). Dimyristoylphosphatidylcholine (a member of the PC family) and dimyristoylphosphatidylethanolamine (a member of the PE family) are shown in Figure 2.1.

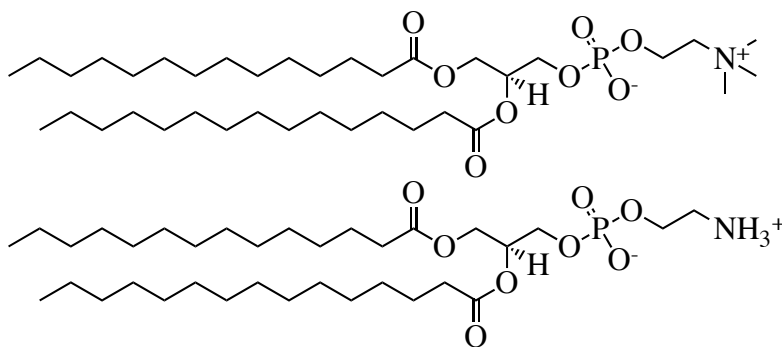


Figure 2.1. Molecular structure of dimyristoylphosphatidylcholine, DMPC (top) and molecular structure of dimyristoylphosphatidylethanolamine, DMPE (bottom). The only difference in structure is the quaternary and primary amine headgroups.

Three of the phospholipids discussed throughout this dissertation are phosphatidylcholines, which comprise 17.4% of the overall lipid content in human erythrocyte membranes. Two phospholipids discussed in detail in Chapter 6 are phosphatidylethanolamines, a category of lipids comprising 17.2% of the overall lipid content in human erythrocyte membranes.^{128,129}

PCs and PEs used in these experiments are structurally similar. Each lipid type contains a hydrophilic, negatively charged phosphate and a positively charged amine, that together with the ester group form the “headgroup.” The identity of the amine (primary or quaternary) dictates the last two letters of the four-letter acronym describing individual phospholipids (Table 2.1). The hydrophobic lipid tail consists of two acyl hydrocarbon chains. Although PCs and PEs may contain unsaturated alkane chains, all lipids used in these experiments are fully saturated. The number of carbons in the acyl chains depicts the first two letters of the four-letter acronyms. The lipids used here remain zwitterionic over a large range of pHs. Nomenclature and physical characteristics of the lipids used in the research described in this dissertation are summarized in Tables 2.1 and 2.2.

Common Name	IUPAC Name	Headgroup	Carbon Chain Length:Degree of Unsaturation^a
DLPC	1,2-didodecanoyl- <i>sn</i> -glycero-3-phosphocholine	PC	12:0
DMPC	1,2-ditetradecanoyl- <i>sn</i> -glycero-3-phosphocholine	PC	14:0
DPPC	1,2-dihexadecanoyl- <i>sn</i> -glycero-3-phosphocholine	PC	16:0
DLPE	1,2-didodecanoyl- <i>sn</i> -glycero-3-phosphoethanolamine	PE	12:0
DMPE	1,2-dimyristoyl- <i>sn</i> -glycero-3-phosphoethanolamine	PE	14:0

Table 2.1. Descriptions of the phospholipids used in this research.

^aCarbon chains of lipids used in this research are symmetric.

Lipid	Area/ Lipid ¹³⁸ (Å ²)		Bilayer Thickness ¹³⁸ (Å)		Pretransition Temp. (°C)	Transition Temp. (°C)
	30 °C	60 °C	30 °C	60 °C		
DLPC	63.1	66.0	31.6	30.4		-3
DMPC	60.2	65.0	36.0	34.9	14	23
DPPC		63.4		38.9	35	41
DLPE	53.8	58.4	35.1	33.1		31
DMPE	52.5 ^a	59.6	40.4 ^a	37.8		50

Table 2.2. Physical properties of the phospholipids used in this research. Transition and pretransition temperatures were determined using instrumentation described below. Data recorded agree with literature values.¹³⁹

^aArea per lipid and bilayer thickness measured at 40 °C.¹³⁸

As described in Chapter 1, lipid membranes can exist in one of three thermodynamic states at a given temperature. Hydrated medium-chain length phosphatidylcholines and phosphatidylethanolamines undergo transitions between different lamellar phases as the temperature of the system is increased (Figure 2.2).

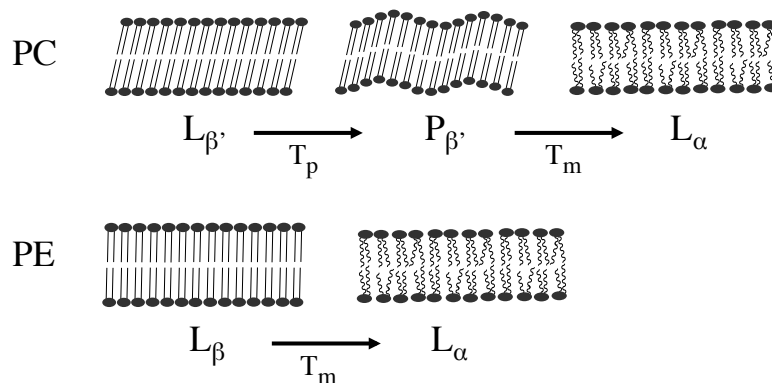


Figure 2.2. Phases and transitions associated with phosphatidylcholine lipid bilayers (top) and phosphatidylethanolamine lipid bilayers (bottom).

At low temperatures, phosphatidylcholines in a bilayer exist in a gel phase, characterized by tightly packed acyl chains that are tilted with respect to the bilayer

surface. The tilted acyl chains are thought to be due to the size mismatch between the bulky headgroup and the acyl chains as well as the propensity of PCs to associate with water in the gel phase.¹⁴⁰ Phosphatidylethanolamines are less strongly hydrated than phosphatidylcholine bilayers and stand normal to the bilayer surface when in the gel phase.

As the temperature of PC bilayers is increased to the pretransition temperature (T_p), a rippled gel phase ($P_{\beta'}$) forms. This transition occurs at ~ 13 °C for DMPC vesicles and at ~ 36 °C for DPPC vesicles.^{79,139} Electron density profiles determined from small angle X-ray scattering show an undulating pattern in ripples with a wavelength of 120-160 Å.¹⁴⁰ Because of the periodic ripples, the surface area of the $P_{\beta'}$ phase of DMPC bilayers is 6% less than the area of DMPC bilayers in their gel phase.⁷⁹ Phosphatidylethanolamine bilayers show no evidence of a rippled phase at any temperature.¹³⁹

As the temperature of phosphatidylcholine vesicles is increased to the (chain-melting) transition temperature (T_m), bilayers transition into their disordered liquid-crystalline (L_α) phase. Most biological membranes (upper epidermis excluded) exist in the liquid-crystalline phase. Other physical changes in membrane structure above the T_m include the decreasing in the length of each acyl hydrocarbon tail due to the formation of rotation isomers around the C-C bonds (gauche-defects),^{135,136} an overall decrease in the bilayer thickness (Table 2.2),¹³⁸ and an increase in surface area ($\sim 20\%$ for DMPC bilayers).⁷⁹ The result of these phase changes is an increase in the total free volume in

which solutes may partition, be it in the polar headgroup region or laterally between the hydrocarbon chains.¹⁴¹⁻¹⁴⁶

Of interest is the difference in melting temperature due to the identity of the phospholipid headgroup. Compared to PCs with the same acyl chain length, PEs have a higher melting temperature. This is thought to be due to a lack of a bulky headgroup, and thus the propensity to form strong intermolecular hydrogen bonds between N-H and O-P groups of neighboring lipids. The thermal energy required to break these intermolecular bonds is higher than the energy needed to break any water-lipid bonds that may form in PCs, causing PE lipid bilayers to have a larger transition temperature. The inter- and intra- molecular forces between lipids and water in lipid bilayers undoubtedly play a role in solute solvation in these heterogeneous environments. Data summarized in Chapter 6 elaborates upon the differences solute partitioning in membranes composed of PC, PE, and mixtures of the two.

2.2 Coumarins

Solutes used in the experiments described in this thesis are 7-aminocoumarins, highly fluorescent, photostable, and well-characterized dyes (Figure 2.3). Coumarins form excited states with large dipole moments, exemplified through fluorescence behavior dependent on local solvent polarity.¹⁴⁷ This solvent-dependent behavior allows us to determine the solvation environment(s) of the coumarins within a lipid membrane. Moreover, analysis of time-resolved fluorescence decays allows us to determine a relative percent of solute in each environment. Experiments outlined in Chapters 3-5 of this

thesis utilize 7-dimethylamino-4-trifluoromethyl-coumarin (coumarin 152, C152).

Experiments in Chapter 5 utilize both C152 and C461 (coumarin 461, 7-dimethylamino-4-methyl-coumarin). The solvent-dependent steady-state absorbance and emission traces are shown in Figures 2.4 and 2.5. Time-resolved fluorescence decay traces in various solvents are shown in Chapters 3-5.

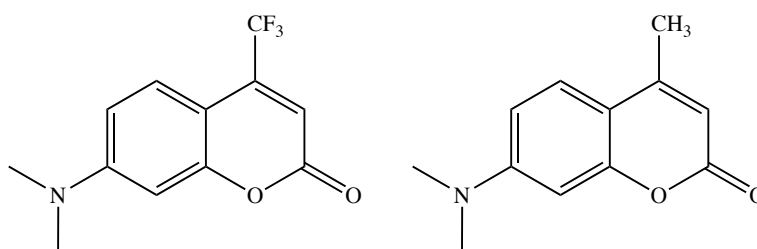


Figure 2.3. Molecular structures of coumarin 152 (left) and coumarin 461 (right).

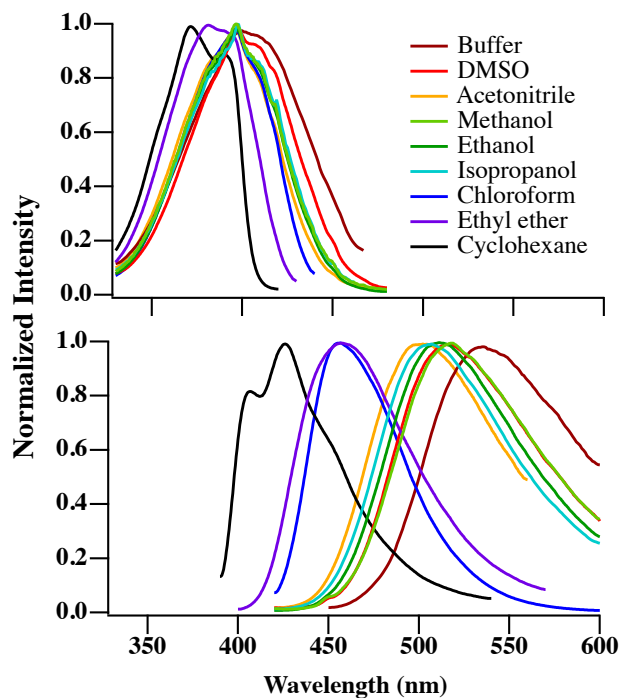


Figure 2.4. Fluorescence excitation (top) and emission (bottom) spectra of C152 in selected solvents.

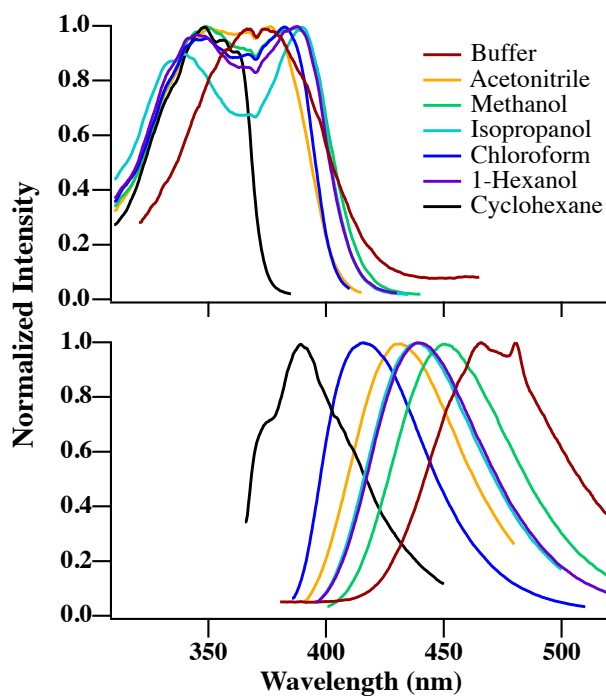


Figure 2.5. Fluorescence excitation (top) and emission (bottom) spectra of C461 in selected solvents.

In the ground state, the trisubstituted amine in C152 adopts sp^3 hybridization, forming a pyramidal structure (Figure 2.6). In nonpolar environments, the excited state structure of C152 is planar.¹⁴⁸ Excited state relaxation of C152 in nonpolar solvents is due to the change in amino group geometry and is characterized by high quantum yields and long (~ 3 ns) fluorescence lifetimes.

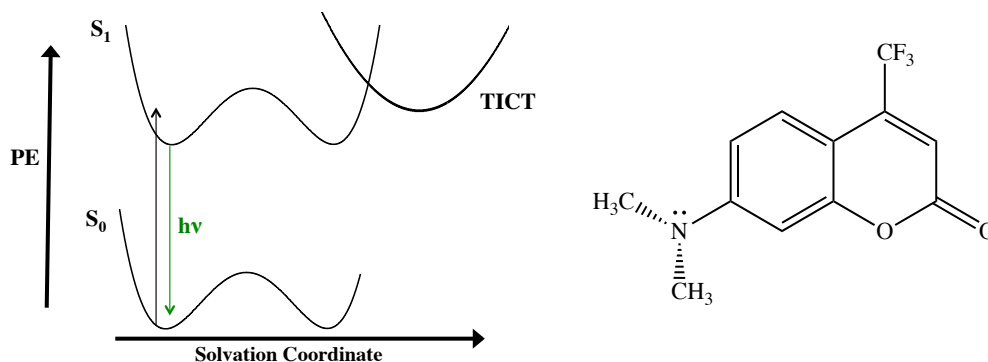


Figure 2.6. Energy level diagram and molecular structure of C152 in nonpolar solvents.

Upon photoexcitation in aqueous and polar environments, a nitrogen lone pair electron is promoted into an aromatic π^* orbital. The change in electron configuration accompanies a structural isomerization (“twist”) of the methyl groups on the nitrogen. In this twisted intramolecular charge transfer state (TICT), the methyl groups lie perpendicular to the benzene rings, thus increasing the charge separation (Figure 2.7).^{148,149}

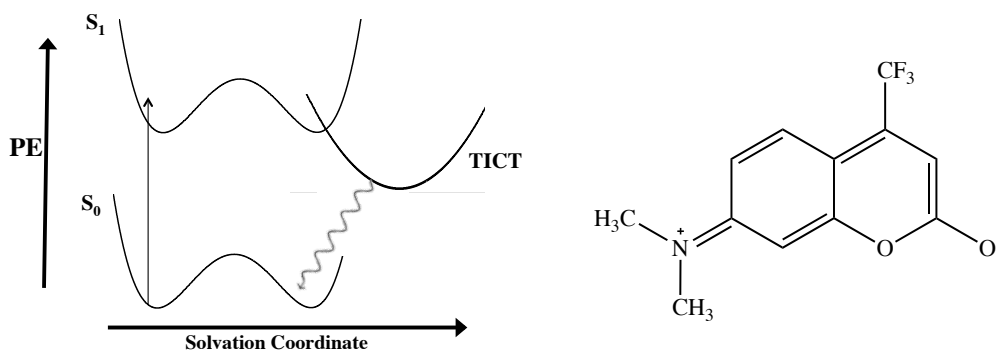


Figure 2.7. Energy level diagram and molecular structure of C152 in polar solvents.

De-excitation from the TICT state requires conformational change to return to the ground state geometry, resulting in a non-radiative de-excitation pathway. Any radiative

decay seen with deexcitation from C152 in polar or aqueous solvents is characterized with a short (sub ns) lifetime and a low quantum yield.^{147,148}

Although the photophysics of C461 have not been studied as extensively as those of C152, data presented in this dissertation (Figure 2.5 and Chapter 5) agree well with literature.^{147,150}

From the standpoint of membrane partitioning, C152 and C461 should behave similarly due to their similar structures. Both molecules are hydrophobic, with C152 having slightly more hydrophobic character ($\log P = 2.72$) than C461 ($\log P = 2.29$). (Log P values were calculated using ChemDrawPrime 16.0.) The ground state dipole moments of C152 and C461 are identical (~ 6.3 D), while the excited state dipole moment of C152 (12.8 D) is slightly higher than that of C461 (10.0 D).¹⁴⁷ Data shown and discussed in Chapter 5 in fact show substantial differences in membrane partitioning based on identity of the solute.

2.3 Large Unilamellar Vesicle Preparation

Large unilamellar vesicles (LUVs) were formed from solid lipids purchased from Avanti Polar Lipids (*Alabaster, AL*). Following standard protocols,¹⁵¹⁻¹⁵³ ~ 1 mg/mL lipid/chloroform was mixed thoroughly and the chloroform was removed via rotary evaporation (>50 °C) leaving behind a lipid film on the round-bottom flask. The sheets were rehydrated using phosphate buffered saline (PBS, 10 mM, pH = 7). Vesicles prepared for fluorescence experiments were prepared with a 1.5 mM concentration, while vesicles prepared for DSC experiments were prepared at 20 mM. Upon rehydration,

multilamellar vesicles spontaneously form, and the solution was then sonicated for 30 min to produce LUVs. Solutions prepared for DSC measurements were used as produced from this procedure.

For fluorescence experiments, the LUVs were then filtered through a 450 nm pore PTFE syringe filter. Vesicle solutions were spiked with PBS solutions containing coumarins. This final solution was filtered 12 times with a miniextruder (Avanti Polar Lipids) through a 200 nm pore membrane. Extrusions were performed above the melting temperature of the lipid bilayer.

2.4 Dynamic Light Scattering

DMPC vesicle size distributions were confirmed using a Brookhaven 90Plus Particle Size Analyzer equipped with a solid state 15 mW 633 nm laser. Scattered light was detected at 90° from the incident beam. Measurements were recorded at room temperature.

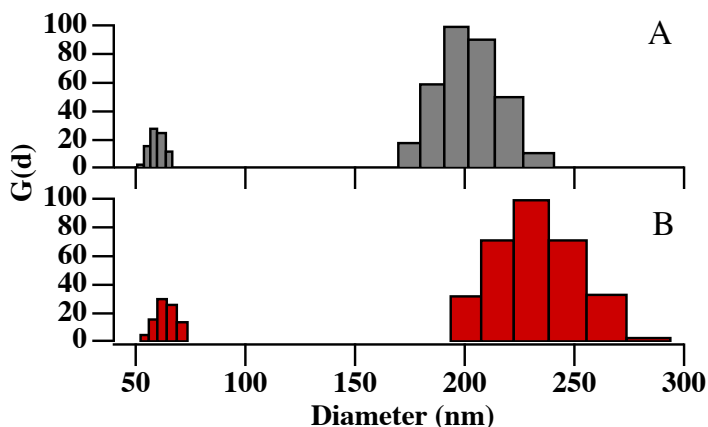


Figure 2.8. Dynamic light scattering data of two independent samples of DMPC vesicles. G(d) describes the relative percent contribution.

Two independent trials (Figure 2.8) confirm the average vesicle diameter of 1 mM DMPC vesicles (prepared using the technique described in Chapter 2.3) as ~210 nm. The bimodal distribution in Figure 2.8A shows ~20% of the vesicles have a diameter centered at ~55nm and the remainder with the average size of 195nm. ~15% of the DMPC vesicles in Figure 2.8B have an average diameter of ~75 nm and the remainder of vesicles have a diameter of ~245 nm. The minor populations of small diameter vesicles were likely created upon sonication. Due to their small size, the mini-extruder was not able to remove these vesicles from solution. No observable changes in solute partitioning were observed in experiments using DMPC vesicles of ~50 nm diameter (data not shown).

2.5 Steady-State Absorbance and Fluorescence

Steady state absorbance was recorded using a Hitachi U-3010 UV/Vis spectrophotometer. Bulk steady-state fluorescence was recorded using Jobin-Yvon Horiba FluoroLog 3 FL3-11 fluorometer. The FluoroLog produces molecular excitation through a 450 W xenon arc lamp. Sample temperature on the FluoroLog is controlled using a flow cell pumped by an external water circulator. Both instruments were used to determine fluorescence quantum yield.

2.6 Quantum Yield Measurements

The quantum yields of C152 in bulk solvation environments were determined following standard protocol, and summarized briefly here.^{154,155} The fluorescence quantum yield of C152 in bulk PBS buffer (10 mM, pH = 7) was determined relative to a

fluorescent standard, coumarin 153 in ethanol ($\phi_f=0.53$).¹⁵⁵ Quantum yields of C461 in buffer, acetonitrile, methanol, and cyclohexane were determined relative to two fluorescence standards: coumarin 153 in ethanol and coumarin 153 in cyclohexane. Absorbance and fluorescence emission spectra were measured at temperatures ranging from 10–70 °C. Integrated fluorescence intensity was graphed as a function of absorbance for a series of four dilutions and fit to lines with slopes m_s (standard) and m_x (sample). Fluorescence quantum yield of a standard (ϕ_s) and a sample (ϕ_x) is related through:

$$\phi_x = \phi_s \left(\frac{m_x}{m_s} \right) \left(\frac{n_x^2}{n_s^2} \right) \quad (2.1)$$

where n_x and n_s are the refractive indices of the sample and the standard. The quantum yield of C152 in buffer from 10–55 °C is shown in Figure 2.9, and the quantum yields of C461 in buffer, methanol, acetonitrile, and cyclohexane from 10–70 °C are shown in Figure 2.10.

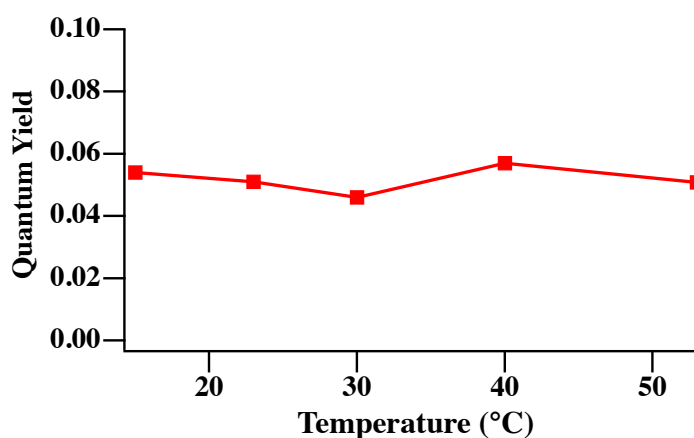


Figure 2.9. Quantum yields of C152 in 10 mM phosphate-buffered saline buffer as a function of temperature. Errors in quantum yield measurements are on the order of ± 0.03 .

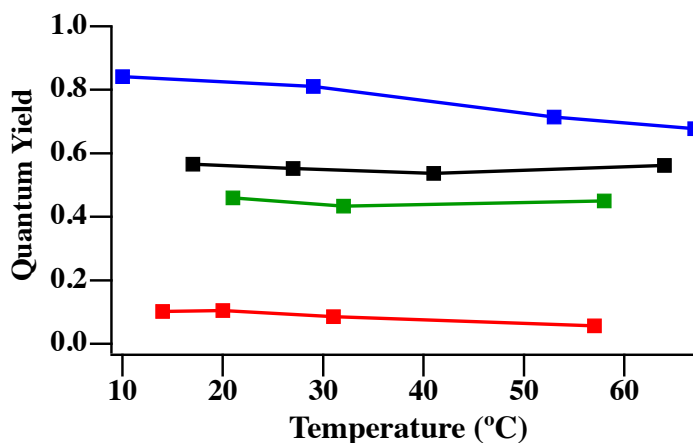


Figure 2.10. Quantum yields of C461 in 10 mM PBS buffer (red), methanol (green), acetonitrile (black), and cyclohexane (blue). Errors in quantum yield measurements are on the order of ± 0.03 .

Quantum yields determined using the method described above agree with literature values.^{147,148}

2.7 Differential Scanning Calorimetry

The melting temperatures of 20 mM lipid vesicles (in 10 mM PBS buffer, pH = 7) were determined using a TA Instruments Q2000 Differential Scanning Calorimeter (DSC). To investigate whether the addition of a coumarin solute affected the melting temperature of the vesicles, vesicle solutions (20 mM DMPC in 10 mM PBS buffer (pH=7, doped with 6 μ M coumarin) were also prepared. Pure buffer references were used for both pure and doped samples. The vesicles were equilibrated well below the melting temperature, after which the temperature was increased at a rate of 1 $^{\circ}\text{C min}^{-1}$. Temperature sensitivity of the Q2000 DSC is ± 0.1 $^{\circ}\text{C}$. Data were analyzed using TA Instruments Trios software. DSC results of pure lipid vesicles (Figure 2.11) agree with

literature.^{139,156,157} Solute doped vesicles were found to have the same melting temperature (within error) of pure vesicles (Figure 2.12).

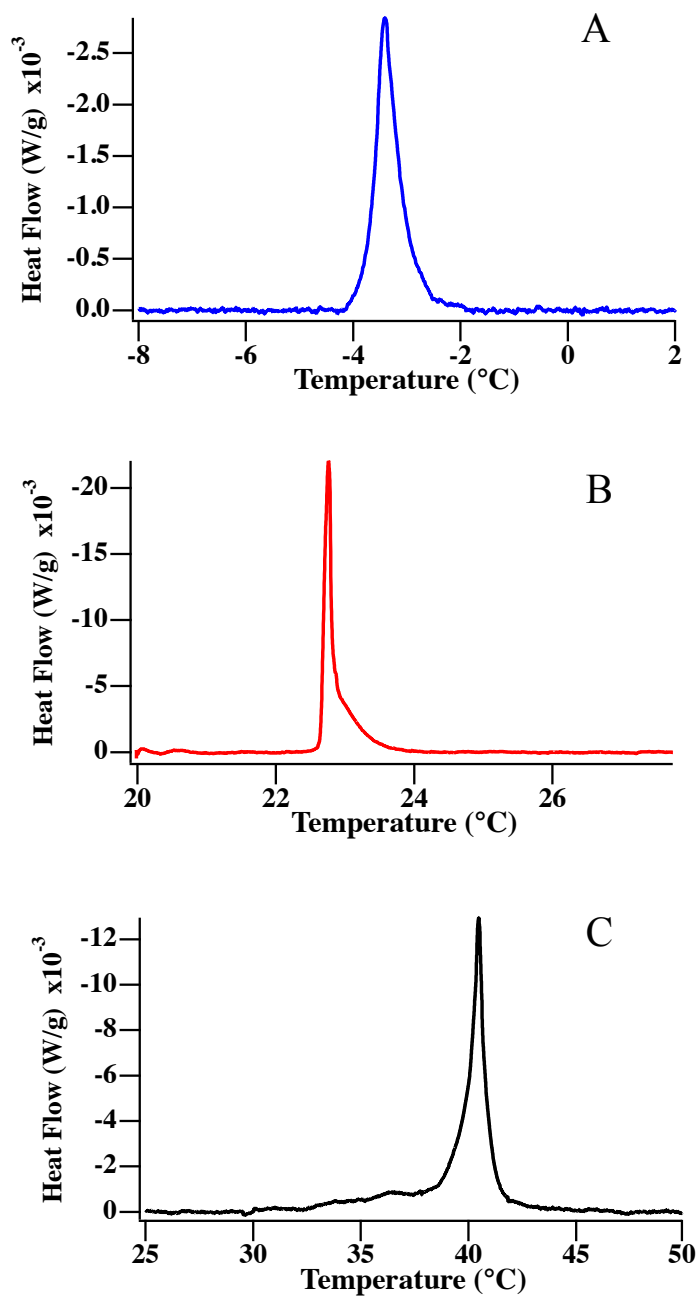


Figure 2.11. Differential scanning calorimetry traces of 20 mM solutions containing DLPC (top) DMPC (middle) and DPPC (bottom) vesicles.

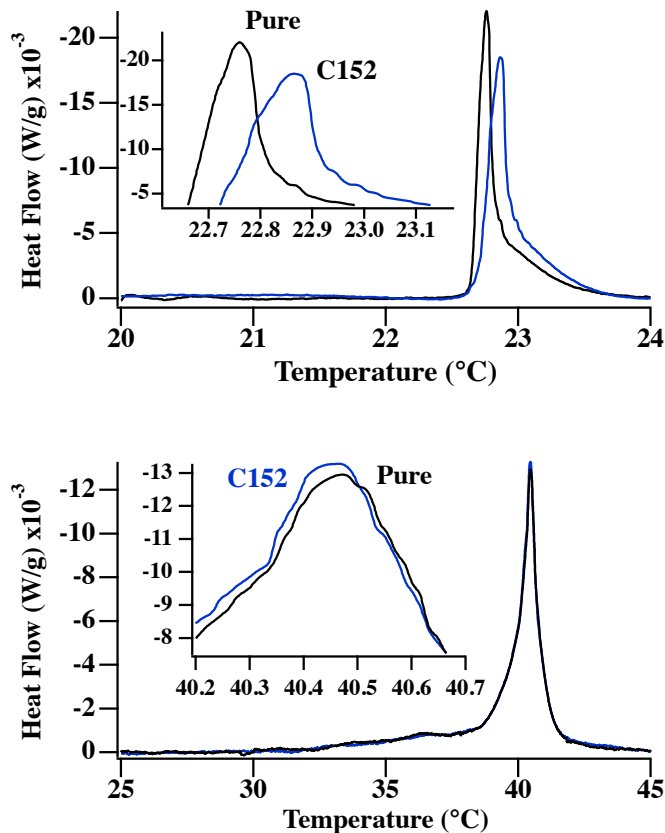


Figure 2.12. DSC traces of DMPC (top) and DPPC (bottom) vesicles with and without C152 present in solution.

Melting curves in Figure 2.12 indicate that C152 does not disrupt the order in DMPC or DPPC membranes. These results were unexpected, as DSC has been used numerous times to successfully identify solute-induced disorder in membranes. For example, Lucio *et al.* report a broadening of the melting temperature of DPPC vesicles upon introduction of three NSAIDs. Depending on the identity of the drug and the molar concentration of the drug, the melting temperature of DPPC membranes was observed to decrease up to $\sim 5^\circ\text{C}$.¹²⁵ Results in Figure 2.12 seemingly contradict partitioning data presented in Chapters 3-6. However, due to the low solubility of C152 in water, molar drug:lipid ratios as high as those used in Lucio's research (<0.4) could not be explored.

2.8 Time-Correlated Single Photon Counting

2.8.1 Experimental Design

The time-correlated single photon counting (TCSPC) setup at MSU (Figure 2.13) uses a Coherent Chameleon laser (80 MHz, 85 fs pulse length) with an APE autotracker containing a doubling crystal and tripling crystal to generate output wavelengths from 226–340 nm (tripling crystal), 340–510 nm (doubling crystal), and 680–1020 nm (fundamental). A Conoptics Model 350-10 modulator is used to reduce the repetition rate to 4 MHz. Samples are excited using vertically polarized light, while fluorescence emission is collected at the magic angle (54.7356°). Picoquant PicoHarp 300 and FluoTime 200 software are used for data collection, detecting individual photon arrival times after sample excitation.

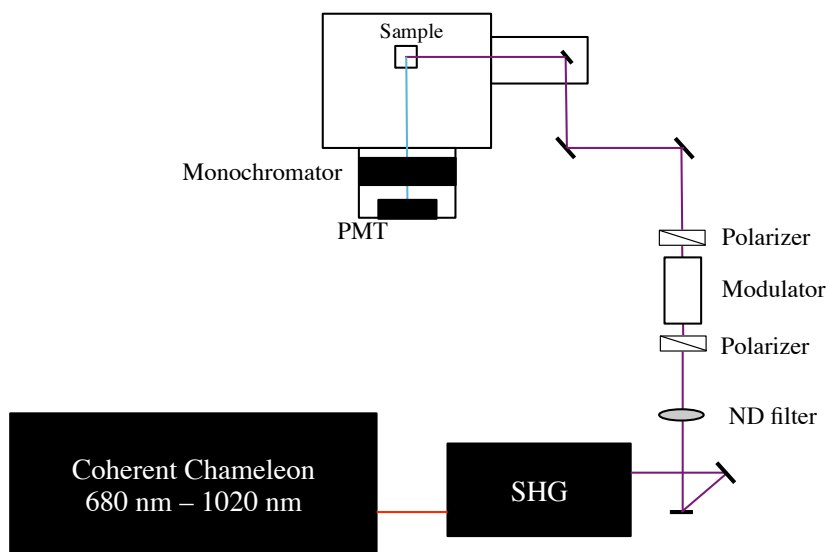


Figure 2.13. Experimental setup of the second-harmonic path of the TCSPC

The difference in time between the excitation and photon arrival at the detector is output as a histogram with time bins of a set time difference. FluoFit (Picoquant) is used to analyze the histogram by creating an exponential model with reconvolution to account for the instrument response function (IRF).

2.8.2 Data Analysis

To explain the techniques and assumptions we used to analyze fluorescence decay traces, the fluorescence decay of C152 in a representative vesicle solution is used as an example below. A similar method was used to analyze C461 fluorescence decays in vesicle containing solutions.

The procedure for calculating solute distribution in vesicle solutions is as follows: raw fluorescence data were collected until the fluorescence intensity at time $t=0$ reached 8,000 counts. Data were fit unconstrained to a sum of exponential functions:

$$I(t) = \int_0^t \text{IRF}(t') \sum_{i=1}^n A_i e^{-\frac{t-t'}{\tau_i}} dt' \quad (2.2)$$

where A_i is the amplitude of component i and τ_i is the lifetime of component i . The quality of fits was initially evaluated by assessing each fit's χ^2 value. The corrected Akaike Information Criterion (AIC), described elsewhere,¹⁵⁸ was used to statistically estimate the number of exponential terms (n) in Equation 2.2 required to fit the decay trace. Three exponential terms were suggested for C152 in vesicle solutions, while only two exponential terms were suggested for C461 in vesicle solutions. Typical χ^2 values ranged from 0.90 to 1.10 when the model accounted for three lifetimes and three associated amplitudes:

$$I(t) = \int_0^t \text{IRF}(t') \left[A_a e^{-\frac{t-t'}{\tau_a}} + A_b e^{-\frac{t-t'}{\tau_b}} + A_c e^{-\frac{t-t'}{\tau_c}} \right] dt' \quad (2.3)$$

where a, b, and c refer to unique solvation environments in a vesicle solution. At any given instant, the fluorescence intensity $I(t)$ is also equal to the number of excited solutes in each environment ($C_i^*(t)$) multiplied by the appropriate, solute dependent radiative rate constants (k_r):

$$I(t) = C_a^*(t)k_{r,a} + C_b^*(t)k_{r,b} + C_c^*(t)k_{r,c} \quad (2.4)$$

where $I(t)$ and $C_i^*(t)$ are implicitly referenced to a standard state (e.g. 1 M). Assuming the three populations are independent of each other and do not interconvert, solutes decay via first order kinetics:

$$C_i^*(t) = C_i^*(0)e^{-(k_{r,i}+k_{nr,i})t} \quad (2.5)$$

where $C_i^*(0)$ is the population of excited C152 in environment i at the time of excitation. Equation 2.5 may be substituted into the expression for the fluorescence intensity at time t (Equation 2.4) to arrive at the expanded expression:

$$I(t) = C_a^*(0)k_{r,a}e^{-(k_{r,a}+k_{nr,a})t} + C_b^*(0)k_{r,b}e^{-(k_{r,b}+k_{nr,b})t} + C_c^*(0)k_{r,c}e^{-(k_{r,c}+k_{nr,c})t} \quad (2.6)$$

At time $t=0$, Equation 2.6 simplifies to equal to the sum of the excited state populations multiplied by the relevant radiative rate constants:

$$I(0) = C_a^*(0)k_{r,a} + C_b^*(0)k_{r,b} + C_c^*(0)k_{r,c} \quad (2.7)$$

Equation 2.2 may also be related to Equation 2.7 to show the relationship between the amplitude associated with component i (A_i) and the excited state concentration of the species in environment i :

$$A_i = C_i^*(0)k_{r,i} \quad (2.8)$$

To calculate the distribution of solutes in each environment (i), Equation 2.8 clarifies that the amplitudes associated with the solute in environment i (as provided from Equation

2.2) must be divided by the radiative decay rate of the solute in bulk environment i .

These rate constants have either been calculated in this work using experimentally measured quantum yields (Section 2.6) or taken from literature.

Finally, the relative percent of solute in environment i at the moment of excitation (X_i) may be calculated through a simple normalization:

$$X_i = \frac{C_i^*(0)}{C_a^*(0) + C_b^*(0) + C_c^*(0)} = \frac{\frac{A_i}{k_{r,i}}}{\left(\frac{A_a}{k_{r,a}} + \frac{A_b}{k_{r,b}} + \frac{A_c}{k_{r,c}}\right)} \quad (2.9)$$

where numerical values for A_i are produced using Equation 2.2 (FluoFit software), and radiative rates are calculated using the fluorescence lifetimes and quantum yields as determined earlier in Section 2.6.

An important point to note is that the analysis of C152 decay traces in vesicle containing solutions required several assumptions. First, we assumed that the lifetimes that resulted from fitting the data that corresponds to C152 sampling solvation environments corresponding to different bulk solution limits. Such action seemed reasonable given that unconstrained fitting of each decay consistently resulted in three lifetimes: one short, sub-ns lifetime, one intermediate lifetime that shortened from ~ 2.4 ns at low temperatures to ~ 1.0 ns at higher temperatures and one long lifetime of ~ 4.5 ns. The short lifetime matched exactly the lifetime of C152 in bulk aqueous buffer and was assigned to solutes that remained unassociated with lipid vesicles. The intermediate lifetime at low temperatures corresponded to C152 in polar aprotic solvents (such as acetonitrile) and at higher temperatures (when lipid bilayers were in their fluid, liquid

crystalline state), the ~ 1.0 ns lifetime corresponded to C152 in polar protic solvents (such as methanol).

Correspondingly, this intermediate lifetime was assigned to C152 solutes solvated in the polar, headgroup and glycerol backbone regions of the lipid bilayer, and the shift from polar aprotic to polar protic solvation was attributed to water permeation into this region at temperatures above the gel-liquid crystalline transition temperature. The long lifetime (~ 4.5 ns) is markedly longer than C152's lifetime in alkanes (~ 3.5 ns), but given that the nonpolar environment in lipid membranes is only ~ 3 nm wide and solvation is constrained by long, ordered acyl chains, one might reasonably expect that C152's lifetime will be lengthened in such confinement.^{159,160} Consequently, this third lifetime is assigned to C152 sampling the nonpolar, alkane like environment lipid bilayer. We also assume that the radiative rates of C152 in vesicle containing solutions correspond to the radiative rates of C152 in their assigned bulk solvation environment.

The method of analysis described in Equations 2.2–2.9 also requires the assumption that the equilibrium distribution of C152 solutes prior to excitation does not change during an experiment (i.e. solutes do not move from one environment to the other following excitation). Finally, we assumed that the bulk solution solvation mechanisms (e.g. TICT state formation) remained accurate for the heterogeneous solvation environments sampled by solutes in vesicle containing solutions.

Initially fluorescence data were analyzed incorrectly by dividing A_i by the fluorescence quantum yield (ϕ_i). Data reported in Chapter 3 of this dissertation were analyzed with this incorrect approach, but for comparative purposes, the data are also

included in Chapter 4 where they are corrected using the radiative-rate approach outlined above. The method of data analysis described in Equations 2.2–2.9 is also used in the remainder of the thesis (Chapters 5 and 6).

Data correction following the incorrect quantum-yield approach artificially suppressed C152 population in nonpolar environments of vesicle solutions. Although the population of C152 in nonpolar environments (and consequently the populations of C152 in bulk buffer and polar regions) was erroneously reported in Chapter 3, the driving thermodynamics controlling C152 partitioning in DMPC vesicles remain unchanged within experimental error. For example, using the incorrect, quantum yield treated data, Chapter 3 reports C152 partitioning into gel phase DMPC membranes as endothermic ($\Delta H = 27 \pm 9$ kJ/mol) and entropically favorable ($\Delta S = 88 \pm 32$ J/(mol K)). Using the correct method of data analysis in Chapter 4, C152 partitioning into gel phase DMPC membranes is also reported endothermic ($\Delta H = 34 \pm 3$ kJ/mol) and entropically favorable ($\Delta S = 127 \pm 12$ J/(mol K)).

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CHAPTER THREE

TEMPERATURE DEPENDENT SOLVATION AND PARTITIONING OF
COUMARIN 152 IN PHOSPHOLIPID MEMBRANES

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

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Contributions: Collected and analyzed all time-resolved fluorescence data. Assisted in collection of differential scanning calorimetry data. Assisted in writing and editing manuscript.

Co-Author: Victoria A. Kong

Contributions: Collected and analyzed differential scanning calorimetry data.

Co-Author: Robert A. Walker

Contributions: Assisted with analysis and edited manuscript.

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CHAPTER THREE

TEMPERATURE DEPENDENT SOLVATION AND PARTITIONING OF
COUMARIN 152 IN PHOSPHOLIPID MEMBRANES3.1 Introduction

Biological membranes are nature's means of separating the machinery necessary for cellular function from extracellular conditions. While membranes are typically considered to serve as barriers, they are, in fact, permeable to a wide variety of polar solutes.^{116,161,162} Water, for example, can diffuse freely across biological membranes^{77,103,163} as can polar organic species such as urea,^{77,164} small alcohols,¹⁶⁵ and DMSO.^{166,167} Larger organic solutes that are more lipophilic tend to become solubilized within the lipid bilayer.¹⁶⁸⁻¹⁷⁰ Solute partitioning into biological membranes constitutes the principle mechanism of bioaccumulation and many nonspecific anesthetics function by penetrating into a cell's plasma membrane and disrupting biochemical signaling.^{45,165} Exposure estimates used to develop regulatory policies are based, in large part, on solute solubility in cell membranes.^{45,46}

Once within a membrane's interior, a dissolved solute can minimize its solvation energy by taking advantage of asymmetric and distinctly heterogeneous interactions. Lipids, the primary component of most plasma membranes, consist of charged headgroups, polar glycerol backbones and long acyl chains.^{161,171} Consequently, solute partitioning into a lipid bilayer can sample Coulomb and dipolar interactions as well as hydrophobic forces and excluded volume effects.^{87,172,173} Solute partitioning into

membranes comes at a cost, however, as inclusion requires structural changes within the membrane itself, potentially changing properties such as compressibility and permeability as well as the membrane's ability to support the functions of embedded proteins.^{127,174-177}

Not surprisingly, solute partitioning into lipid membranes has attracted considerable interest from many communities.^{167,178-182} The simplest approach to treating solute partitioning in biological systems requires measuring an equilibrium constant based on how much of a solute remains in solution and how much has been taken up by the cell membrane. These studies and related log P water-octanol partitioning coefficient experiments provide data that are used to develop empirical models correlating membrane permeability with a solute's functional group composition.^{161,183-188} This approach, however, does not address directly how solutes are solvated within the membrane interior. Similarly, differential scanning calorimetry experiments have shown that membrane soluble solutes will change membrane phase behavior, but the mechanism(s) responsible for these effects are often unclear.^{80-83,189,190}

Optical studies, including fluorescence emission and second harmonic generation (SHG), have used changes in spectroscopic signatures to illustrate how certain coumarin-based solutes prefer to associate with a membrane's polar headgroup region while closely related but more hydrophobic coumarin species accumulate within the membrane interior.^{75,78,146,191-193} Relative amplitude contributions to fluorescence lifetimes have been used to measure partitioning of solutes in phospholipid bilayers. Datta *et al.* probed the microenvironments of DMPC vesicles using C480 at 26 °C and observed two distinct fluorescent lifetimes. These lifetimes were different than the lifetimes of C480 in bulk

water and hydrocarbons, leading the authors to suggest that C480 partitioned into the vesicle water pool.⁷⁵ Fluorescence lifetimes of several organic dyes including Nile Red and Rhodamine 6G in phosphatidylcholine membranes indicated the dyes experienced distinct solvation environments, which could be related to similar lifetimes in pure organic solvents.⁷⁶ SHG imaging studies have shown how the anesthetic tetracaine will penetrate a phospholipid bilayer when the bilayer is in its more fluid, liquid crystalline phase but is forced out of the bilayer when the bilayer freezes into its gel state.¹²⁶ These measurements provide direct support for solute partitioning models that emerged from earlier measurements using steady state fluorescence data and solutions containing unilamellar vesicles.¹²⁷

Similarly, electron spin resonance studies also show that a phospholipid bilayer will expel small nitroxide-based solutes when solutions are cooled below the membrane phase transition temperature.⁸⁶ NMR spectra of small lipophilic molecules in membrane solutions show an 8-fold increase in partitioning into the lipid bilayer as the solution was heated above phase transition.⁸⁷ The common denominator to all of these reports is that lipid membranes are more susceptible to solute permeation when in their fluid or liquid-crystalline state. Whether this effect is simply mechanical (the lipids films are more compressible and can accommodate solutes more easily) or if specific, noncovalent chemical interactions bear some responsibility for observed changes in partitioning remains a topic of debate.

Studies described below use time-resolved fluorescence emission spectroscopy to measure the partitioning of coumarin 152 (C152) into vesicle bilayers where the bilayers

consist of saturated, symmetric 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (14:0 DMPC). Structures of a model lipid bilayer, C152, and a DMPC are shown in Figure 3.1.

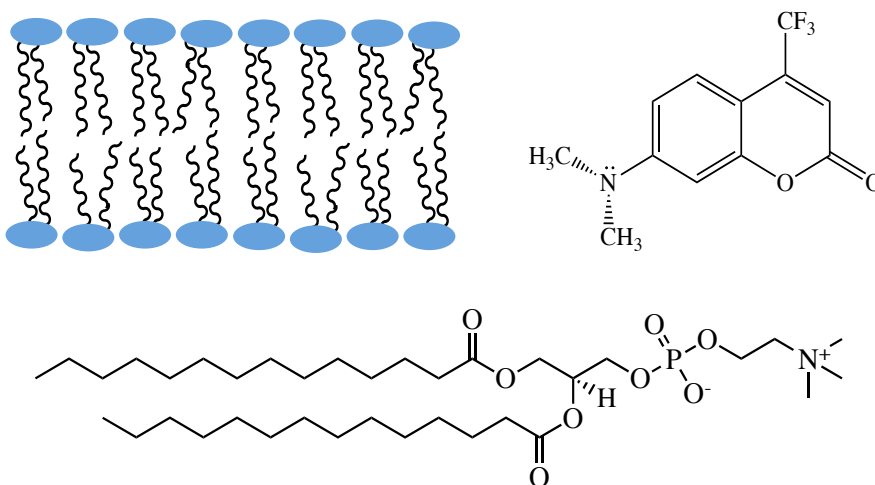


Figure 3.1. Schematic diagram of a lipid bilayer (left), and the chemical structures of coumarin 152 (right), and DMPC (bottom).

Changes in solute partitioning are observed as a function of temperature, and more C152 appears to migrate into the lipid bilayer when the bilayer transitions from its gel to liquid crystalline state. On the basis of fluorescence lifetime measurements, most C152 associated with the lipid bilayer samples a polar solvation environment implying that C152 was solvated by the 3-carbon glycerol backbone. These same fluorescence lifetime data also indicated that very little C152 samples the hydrophobic bilayer interior. Interestingly, at higher temperatures C152 affinity for the lipid membrane diminishes and solute population redistributes itself back into the bulk buffer. Such behavior implies that solute partitioning into phosphocholine membranes reflects a sensitive balance between enthalpic and entropic contributions.

3.2 Materials and Methods

3.2.1 Chemicals

The phosphocholine 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (14:0 DMPC) was purchased as powder from Avanti Polar Lipids (*Alabaster, AL*). Laser grade coumarin 152 (C152) was purchased from Exciton (*Dayton, OH*). Both materials were used as received without further purification. Solvents were HPLC grade and received from Sigma. Phosphate buffered saline solutions for vesicles were prepared using Millipore water (18.2 M Ω).

3.2.2 Large Unilamellar Vesicle Preparation

Large unilamellar vesicles (LUVs) were formed from pure DMPC following standard protocols (Chapter 2). Vesicle solutions were spiked with PBS solutions containing C152 (6 μ M) to produce a 1 mM vesicle/ 2 μ M C152 solution of 200 nm diameter vesicles.

3.2.3 Vesicle Characterization

The melting temperatures of 20 mM lipid vesicles (in 10 mM PBS, pH = 7) were determined using a Texas Instruments Q2000 DSC. Data were analyzed using TA Instruments Trios software. Data collection and analysis is described in Chapter 1, and summarized briefly here. The vesicles were equilibrated at 20 °C, after which the temperature was increased at a rate of 1 °C min⁻¹ from 20 to 28 °C. A second vesicle solution (20 mM DMPC in 10 mM PBS buffer, pH=7, doped with 6 μ M C152) was used

to verify the addition of C152 does not change the melting temperature of DMPC vesicle solutions (Figure 3.2). DSC results reported here agree with literature.^{139,156,157}

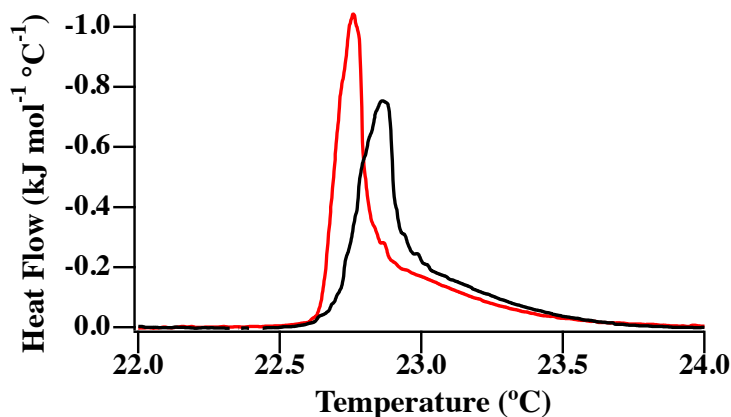


Figure 3.2. Differential scanning calorimetry traces of (red) 20 mM DMPC vesicles and (black) 20 mM DMPC vesicles doped with 6 μ M C152.

Vesicle size was verified using a Brookhaven 90Plus dynamic light scattering (DLS) apparatus (details in Chapter 2).

3.2.4 Time-Resolved Fluorescence

The time correlated single photon counting assembly used in these experiments has been described in Chapter 2. Briefly, time-resolved fluorescence emission data were collected using Picoquant PicoHarp 300 and FluoTime 200 software. An APE autotracker containing a SHG crystal and pumped by a Chameleon Ti:sapphire oscillator (Coherent, 80 MHz, 680-1080 nm) produced excitation pulses (85 fs pulsewidth) at a wavelength of 400 nm. A Conoptics model 350-105 modulator reduced the repetition rate to 4 MHz. Temperature of the solution was monitored by a Quantum Northwest TC152 control. A long-pass filter (80% transmission at >455 nm) was placed after the sample to reduce

scattering from the vesicles. Photon emission (500 nm) was collected until the maximum intensity at early detection times reached a threshold of 8,000 counts.

Emission decays were fit to a multi exponential model (Equation 2.2). For C152/DMPC vesicle solutions, the best model consistently included three lifetimes.

3.3 Results and Discussion

3.3.1 C152 in Bulk Solvents

Emission decays of C152 in selected solvents and a DMPC vesicle solution are shown in Figure 3.3. Solvents were chosen to represent the different solvation environments that C152 might experience inside the lipid bilayer. These environments include nonpolar (represented by cyclohexane), polar aprotic (acetonitrile), polar protic (methanol) and the buffered aqueous solution. One lifetime was used to fit the emission decays of C152 in cyclohexane, acetonitrile, and methanol. The decay for C152 in PBS buffer was fit to two lifetimes, and C152 in a DMPC vesicle solution was fit to three lifetimes. A summary of fluorescence lifetimes and relative amplitudes appears in Tables 3.1 and 3.2. These results compare favorably with values previously reported by Pal *et al.*¹⁴⁸

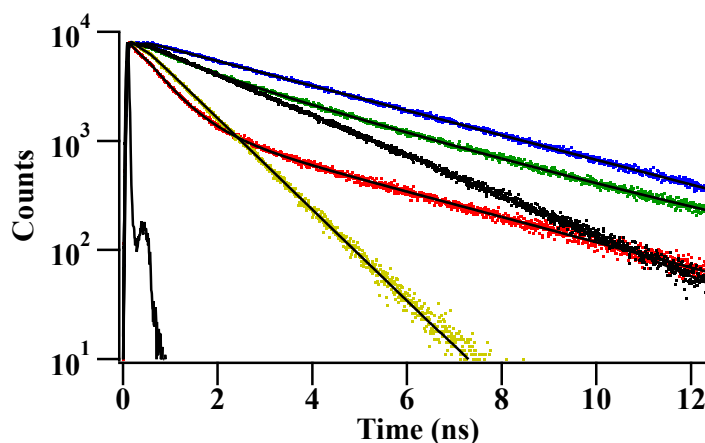


Figure 3.3. Time resolved fluorescence emission from C152 in cyclohexane (blue), acetonitrile (black), methanol (yellow), PBS buffer (red), and a DMPC vesicle solution at 20 °C (green). Solid black lines are fits from analyzing the traces according to Equation 2.2. Also included is a representative instrument response function from a scattering solution. Typical IRF traces measured ~ 100 ps.

Solvent	$\lambda_{\text{abs, max}}$ (nm)	$\lambda_{\text{em, max}}$ (nm)	τ_f (ns)
Cyclohexane	372	426	3.86
Acetonitrile	396	502	2.34
Methanol	397	515	1.09
0.01M PBS buffer	404	527	0.62 (0.83), 3.75 (0.17)

Table 3.1. Fluorescence properties of C152 in varying solvents and solutions at 10 °C. Excitation and emission wavelengths of C152 in the solvents correspond to wavelengths at intensity maxima. For the DMPC solutions, wavelengths report experimental excitation and emission conditions used in experiments. Uncertainties in reported lifetimes are ± 0.10 ns. Uncertainties in reported amplitudes (for the PBS buffer solution) are ± 0.05 .

Several aspects of the C152 bulk solution data deserve attention. First, lifetimes are generally long (~ 4 ns) in nonpolar solvents and short (0.5 – 2 ns) in polar solvents.^{147,148,194,195} This behavior has been attributed to C152's ability to form a nonradiative, twisted intramolecular charge transfer (TICT) state that is stabilized in polar solvents. Efficient TICT formation makes for larger k_{nonrad} and correspondingly shorter

emission lifetimes. Second, the lifetimes themselves are temperature sensitive. Figure 3.4 shows how lifetimes change as solution temperature increases from 10 to 60 °C. The nonpolar lifetime is invariant to temperature, but lifetimes for acetonitrile, methanol and aqueous buffer all diminish linearly. (Figure 3.4 shows results only for the dominant, subnanosecond lifetime (τ_1) in aqueous solution. The longer lifetime (τ_2) also decreases from 3.8 to 3.4 ns, although its contribution to the overall decay remains constant with $A_2 = 0.17$.)

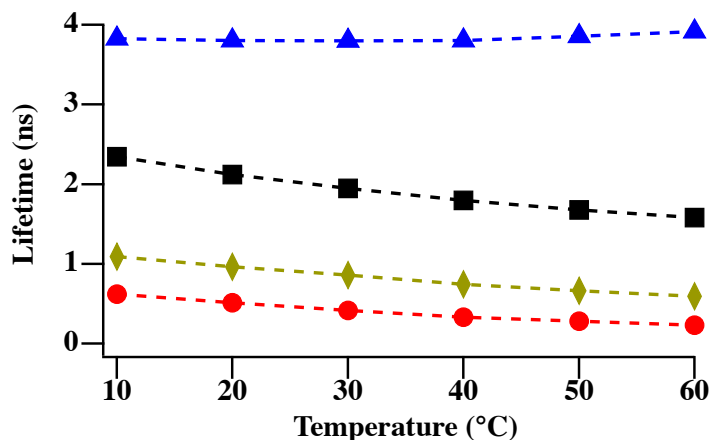


Figure 3.4. Emission lifetimes of C152 in cyclohexane (blue triangles), acetonitrile (black squares), methanol (green diamonds), and buffer (red circles) as a function of temperature. Uncertainties in lifetimes are ± 0.10 ns.

3.3.2 C152 in DMPC Vesicles

In solutions containing DMPC vesicles, C152 emission shows three characteristic lifetimes: one long (~ 4.5 ns), one intermediate (2.0 – 0.5 ns, depending on temperature), and one short (sub nanosecond). These lifetimes and their relative contributions to the emission decay traces vary with temperature.

Figure 3.5 shows time-resolved C152 emission from DMPC vesicle solutions at temperatures of 10, 30, and 60 °C. Table 3.2 reports the lifetimes and relative amplitudes for these three temperatures.

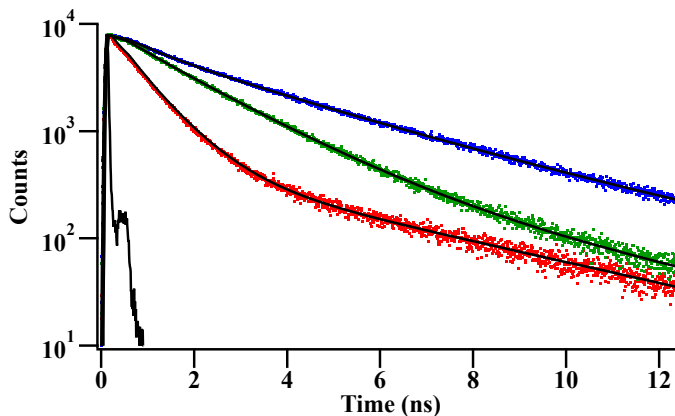


Figure 3.5. Fluorescence decay traces of C152 in DMPC vesicle solutions at 10 °C (blue), 30 °C (green), and 60 °C (red). Also included is a representative instrument response function (IRF). Black lines through data represent results of fitting decay traces to Equation 2.2 with three different decay pathways.

Temp (°C)	$\tau_1 (A_1)$	$\tau_2 (A_2)$	$\tau_3 (A_3)$
10	0.63 (0.22)	2.51 (0.47)	4.89 (0.31)
30	0.49 (0.09)	1.75 (0.79)	5.01 (0.12)
60	0.32 (0.26)	0.80 (0.63)	4.42 (0.11)

Table 3.2. Fluorescence lifetimes and associated amplitudes of C152 in DMPC vesicles at 10, 30, and 60 °C. The relative amplitudes are not corrected for quantum yield. Maximum lifetime uncertainties in DMPC vesicle solutions are typically ≤ 0.2 ns.

In Figure 3.5, two traces correspond to temperatures above DMPC's gel-liquid crystalline phase transition temperature of 23 °C. C152 fluorescence in the vesicle containing solutions shows a strong temperature dependence implying that C152's solvation environment is affected by the DMPC vesicle's bilayer structure.

In terms of assigning the C152 lifetimes measured in the vesicle solutions, the shortest lifetime (τ_1) matches the dominant short lifetime of C152 in the aqueous buffer (Figure 3.3, Figure 3.4, and Table 3.1) and is assigned to C152 monomers not associated with the DMPC vesicles. Because the DMPC vesicles used in these experiments are 200 nm in diameter and membrane perturbation to interfacial water structure extends only 1 nm past the headgroup,¹⁷¹ C152 trapped on the inside of the vesicle water pool would experience the same local environment as C152 between vesicles, making C152 within the water pool indistinguishable from solutes in the buffer outside of the vesicles. Confidence in this assignment is strengthened by short lifetime's temperature dependence; in vesicle solutions as the temperature rises, the short lifetime diminishes from 0.6 to 0.3 ns. This result is virtually identical to the short lifetime behavior of C152 in buffer solution without vesicles ($\tau_1 = 0.6$ ns at 10 °C and 0.2 ns at 60 °C).

The long lifetime (τ_3) in the emission decays reported in Figure 3.5 stays approximately constant at 4.8 ns from 10 to 40 °C and then diminishes slightly to 4.4 ns at 60 °C. This lifetime is assigned to C152 solutes that sample the hydrophobic, nonpolar interior of the DMPC vesicle bilayer. The most interesting result is the intermediate lifetime (τ_2). In vesicle solutions at 10 °C, τ_2 is 2.5 ns, the same result observed for C152 in a polar, aprotic environment (Figure 3.3, Table 3.1). At 60 °C, τ_2 has diminished to 0.8 ns, a quantity that matches the single lifetime measured for C152 in protic organic solvents such as methanol (Figure 3.3, Table 3.1). This temperature dependent behavior of τ_1 , τ_2 , and τ_3 is illustrated in Figure 3.6 with emission lifetime information from C152 in different bulk solvents (Figure 3.4) superimposed.

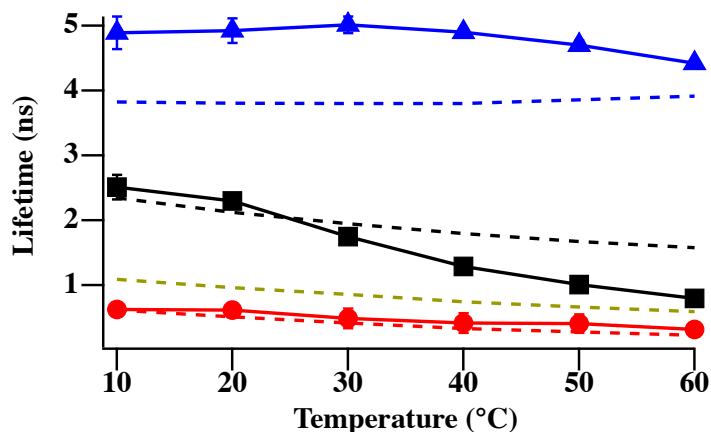


Figure 3.6. Lifetime data from vesicle solutions with bulk solution data (from Figure 3.4) represented only as dashed lines. The three lifetimes correspond C152 in buffer (red), nonpolar (blue), and polar (black) environments. Uncertainty in the reported C152 lifetimes in vesicle solutions is ≤ 0.2 ns.

Results shown in Figure 3.5, Figure 3.6, and Table 3.2 show that C152 in DMPC vesicle solutions samples several different solvation environments and that the distribution of C152 between these environments changes with temperature. To treat these data more quantitatively, the amplitudes must be corrected for quantum yield (QY). Quantum yields of C152 in the different solvents shown in Figure 3.3 (cyclohexane, acetonitrile, and methanol) have been reported previously¹⁴⁸ and/or measured in our own laboratory.

Following the approach of Wurth *et al.*, the QY of C152 in a buffer solution was found to be ~ 0.05 from 15 to 55 °C, as determined with absorption and integrated fluorescence (Figure 3.7). The quantum yield did not change over the temperature range studied.

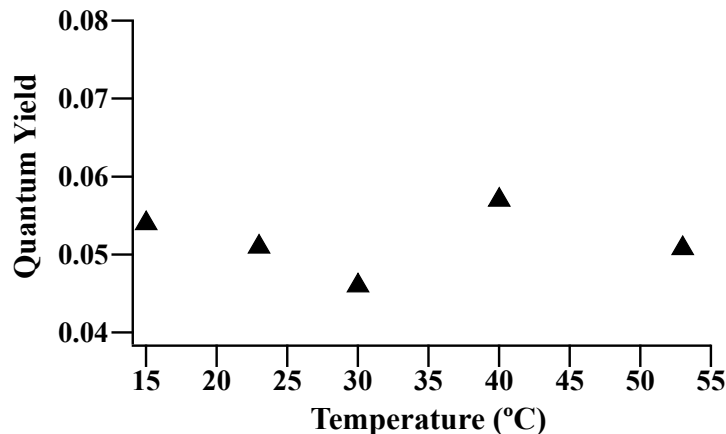


Figure 3.7. Quantum yields of C152 in bulk aqueous solution (10 mM PBS buffer) from 15–55 °C. Errors in quantum yield measurements are on the order of ± 0.03 .

In nonpolar solvents, the tertiary amine of C152 is sp^3 hybridized, making the lone pair nonresonant with the rings' pi-system. Upon excitation, C152 is promoted into an intramolecular charge transfer state, in which the amine has a similar geometry. C152 deexcitation in nonpolar solvents is characterized by a long fluorescence lifetime and large quantum yield. When C152 is excited in polar solvents, a charge separated twisted intramolecular charge transfer geometry is favored. Decay from this state is primarily nonradiative, and any radiative decay from C152 in polar solvents is through a short fluorescence lifetime (with a low quantum yield).

In alkanes and aqueous phases, C152 quantum yields were found to be independent of temperature. The quantum yield of C152 in acetonitrile decreased slightly with temperature.¹⁴⁸ If we assume that the bulk solution quantum yields also apply to the heterogeneous solvation environments sampled by C152 in vesicle solutions, then the amplitudes reported in Table 3.2 can be adjusted to reflect C152's relative

partitioning between bulk buffer, the polar region of the DMPC vesicle bilayer and the hydrophobic bilayer interior (shown in Table 3.3).

Results for C152 partitioning are shown in Figure 3.8. These quantum yield corrected data can, at first glance, appear misleading. For example, experimental data for the 10 °C vesicle solutions were fit to a three-term decay expression with amplitudes A_1 (aqueous) = 0.22, A_2 (polar headgroup) = 0.47, and A_3 (hydrophobic interior) = 0.31. However, given a QY for C152 in buffer of only 0.05 and assuming a QY of 1.0 for C152 in the hydrophobic bilayer interior, the effective concentration of C152 in buffer relative to the membrane interior is ~14:1. Including the C152 associated with the polar headgroups (giving rise to τ_2 and an assumed QY of 0.2), we calculate a relative C152 distribution in the 10 °C vesicle solution of 0.62 (buffer): 0.33 (headgroup): 0.04 (bilayer interior).

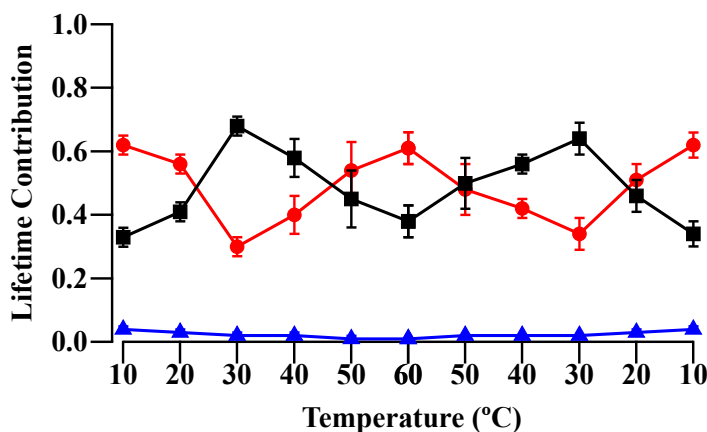


Figure 3.8. Quantum yield corrected amplitudes of C152 populations in DMPC vesicle solutions plotted as a function of temperature. Reported uncertainties reflect the standard deviation from at least three sets of independent measurements.

Temp (°C)	τ_1 (ns)	A ₁	τ_2 (ns)	A ₂	τ_3 (ns)	A ₃
10	0.63 ± 0.08	0.62 ± 0.03	2.51 ± 0.19	0.33 ± 0.03	4.89 ± 0.25	0.04 ± 0.01
20	0.62 ± 0.11	0.56 ± 0.03	2.30 ± 0.12	0.41 ± 0.03	4.92 ± 0.19	0.03 ± 0.01
30	0.49 ± 0.15	0.30 ± 0.03	1.75 ± 0.05	0.68 ± 0.03	5.01 ± 0.13	0.02 ± 0.01
40	0.42 ± 0.15	0.40 ± 0.06	1.29 ± 0.05	0.58 ± 0.06	4.90 ± 0.05	0.02 ± 0.01
50	0.41 ± 0.14	0.54 ± 0.09	1.01 ± 0.04	0.45 ± 0.09	4.70 ± 0.07	0.01 ± 0.01
60	0.32 ± 0.05	0.61 ± 0.05	0.80 ± 0.02	0.38 ± 0.05	4.42 ± 0.03	0.01 ± 0.00
50	0.32 ± 0.07	0.48 ± 0.08	0.94 ± 0.03	0.50 ± 0.08	4.49 ± 0.26	0.02 ± 0.00
40	0.44 ± 0.08	0.42 ± 0.03	1.23 ± 0.02	0.56 ± 0.03	4.85 ± 0.04	0.02 ± 0.00
30	0.50 ± 0.07	0.34 ± 0.05	1.65 ± 0.06	0.64 ± 0.05	5.08 ± 0.16	0.02 ± 0.00
20	0.57 ± 0.09	0.51 ± 0.05	2.18 ± 0.11	0.46 ± 0.05	4.74 ± 0.26	0.03 ± 0.01
10	0.63 ± 0.10	0.62 ± 0.04	2.57 ± 0.24	0.34 ± 0.04	5.04 ± 0.34	0.04 ± 0.01

Table 3.3. Fluorescence lifetimes and amplitudes of C152 in DMPC vesicles at 10, 20, 30, 40, 50, and 60 °C. Fluorescence lifetimes and amplitudes were then recorded at the same temperatures as the temperature of the C152/DMPC vesicle solution was decreased. The relative amplitudes are corrected for quantum yield.

We note that given the biexponential decay of C152 in buffer solution with the minority lifetime (3.75 ns) being similar to that of C152 in alkanes (3.86 ns, Figure 3.3 and Table 3.1), the possibility that the long lifetime measured from C152 in vesicle solutions might be assigned to the C152 population sampling a bulk buffer environment. However, the long lifetime contribution to these emission decays is significantly longer than the long buffer lifetime and is more consistent with what one expects from solutes that are conformationally hindered and unable to form a nonradiative twisted

intramolecular charge transfer (TICT) state (vide infra). Regardless of how one interprets the long lifetime contribution to the C152 emission from vesicle solutions, the conclusion remains the same: very little (if any) C152 partitions into the hydrophobic, nonpolar bilayer interior.

The temperature dependent partitioning reported in Figure 3.8 and Table 3.3 shows surprising behavior. At the lowest temperature sampled (10 °C), more than 60% of the C152 samples a bulk aqueous environment and is not associated with the DMPC membrane. The long-lifetime contribution to the fluorescence decay (τ_3) assigned to C152 solvated in the membrane's hydrophobic interior is also largest (4%) at 10 °C. The balance of C152 in solution appears to be associated with the polar region of the DMPC bilayer. As the sample temperature passes through the DMPC gel-liquid crystalline phase transition temperature at 23 °C, the relative populations of C152 in buffer and C152 solvated by the polar region of the bilayer flip so that at 30 °C, 68% of the C152 samples the polar headgroups while only 30% of available C152 remains in buffer. The relative amount of C152 in the hydrophobic interior has dropped almost to the limit of our detection of 2%.

This behavior is consistent with previous studies showing that solute partitioning into membranes increased once the membrane was in its fluid, liquid crystalline phase. For example, using resonance enhanced second harmonic imaging and an array of patterned lipid bilayers comprised of DOPC, SOPC, DMPC, and DPPC, Nguyen and Conboy measured the association of the water-soluble solute anaesthetic tetracaine with the bilayer.¹²⁶ Results showed stronger affinity between solute and bilayer when the

bilayer was in its more fluid, liquid crystalline state. Furthermore, the total amount of associated solute also increased with bilayers comprised of unsaturated lipids. Signal from the solute disappeared for lipid bilayers in the more tightly packed gel phase. The authors rationalized that structural rigidity and high degree of ordering between acyl chains inhibit tetracaine permeation into the membrane.

Data shown in Figure 3.8 and Table 3.3 illustrate that just above the DMPC phase transition temperature, a majority of the C152 in solution samples an environment consistent with solvation in the vesicle's polar headgroup region. Curiously, as the temperature was raised in the C152/DMPC samples, the partitioning behavior reversed itself a second time so that at temperatures of ≥ 50 °C, the majority of C152 was again solvated in the buffer solution and not associated with the lipid bilayer. As shown in Figure 3.8, these results were fully reversible. This additional change in partitioning behavior suggests that C152 solvation in the lipid bilayer depends sensitively on a host of noncovalent interactions that are carefully balanced to minimize the system's free energy. Once the DMPC bilayer is in its liquid crystalline state, more C152 can take advantage of the favorable enthalpic interactions with the lipid headgroups but at an entropic cost.

3.3.3 Thermodynamic Analysis

The balance between enthalpic and entropic contributions to the system's free energy can be visualized in a Van't Hoff plot. By treatment of amplitudes in Table 3.3 as the relative amounts of C152 in buffer (A_1) and associated with the vesicle's polar headgroup region (A_2), equilibrium constants were calculated for the different, temperature dependent distributions. This analysis neglected the small amount of C152

($\leq 4\%$) solvated in the vesicle's hydrophobic interior (A_3). Figure 3.9 plots the natural log of these equilibrium constants as a function of reciprocal temperature for data both above and below the DMPC phase transition temperature assuming that membrane associated C152 is the 'product' and C152 in buffer is the 'reactant'.

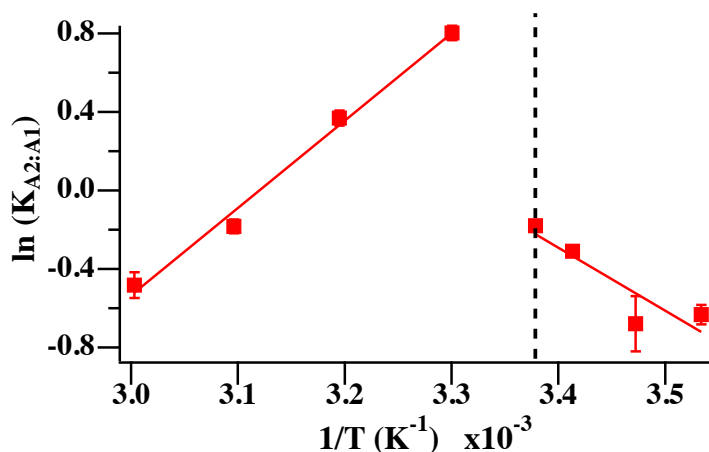


Figure 3.9. van't Hoff plot treating the ratio of C152 associated with the polar part of the bilayer relative to C152 in buffer as an effective equilibrium constant. The dashed vertical line corresponds to the phase transition temperature of DMPC bilayers. Data on either side of the transition temperature were fit to lines to calculate enthalpies and entropies of C152-membrane association.

As the temperature of the vesicle solution increases from 10 to 22 °C, $\Delta H = 27 \pm 9$ kJ/mol, and $\Delta S = 88 \pm 32$ J/(mol K). These data indicate that partitioning into the gel state bilayer is endothermic but entropically favored. At temperatures above 23 °C, DMPC is in its liquid crystalline state, meaning that the lipid chains are more fluid and the bilayer has more free volume.¹⁹⁶ X-ray diffraction experiments have shown that the area per lipid of DMPC increases from 47.2 Å² in the gel phase to 59.7 Å² in the liquid-crystalline phase.^{55,197} The corresponding change in bilayer volume means that solutes can be accommodated into the membrane more easily and helps explain results from

numerous studies that solute partitioning into a lipid bilayer increases significantly above the gel-liquid crystalline transition temperature.

The negative slope of the van't Hoff plot above the transition temperature demonstrates that C152 partitioning into the bilayer is exothermic ($\Delta H = -37 \pm 3$ kJ/mol), but the negative intercept indicates that partitioning is entropically unfavorable ($\Delta S = -115 \pm 8$ J/(mol K)). We propose that this enthalpically driven solvation of C152 in the bilayer is driven by the expansion of DMPC lipids and C152's correspondingly easier access to the polar functional groups of the lipid glycerol backbones. However, localizing C152 into this polar region of the lipid bilayer comes with an entropic cost. Between 40 and 50 °C, the entropic penalty of partitioning into the bilayer becomes too great, leading to the 'exsolvation' of the C152 out of the bilayer and back into the buffer.

The lifetime data for C152 in DMPC solutions (Table 3.3) can be analyzed in one additional way. Following Pal *et al.*,¹⁴⁸ the quantum yields for C152 in a bulk polar environment and C152 in a bulk aqueous buffer environment (0.2 and 0.05, respectively) and the fluorescence lifetime (τ_f) of C152 in polar and buffer regions of a DMPC vesicle solution were used in Equation 3.1 to find the temperature-independent radiative decay rate, k_f .

$$k_f = \frac{\phi_f}{\tau_f} \quad (3.1)$$

In aqueous and polar environments, C152 can quickly change geometry upon photoexcitation (further described in Chapter 2). The conformational changes accompanying deexcitation from an excited state promotes a non-radiative de-excitation

pathway. The major component (63%) of C152 fluorescence is attributed to the short (0.62 ns) fluorescence lifetime.

The Arrhenius equation (Equation 3.2) can be used to relate the temperature dependent, radiative lifetime to the activation energy of the nonradiative deactivation process¹⁴⁸:

$$\frac{1}{\tau_f} = k_f + k_{nr} \exp\left(\frac{-\Delta E_a}{RT}\right) \quad (3.2)$$

where k_{nr}^0 is the preexponential factor and ΔE_a is the activation energy for the nonradiative decay process. A linear trace of $[\ln(1/\tau_f) - k_f]$ vs. $(1/T)$ was used to determine the activation energy according to Equation 3.2, shown in Figure 3.10.

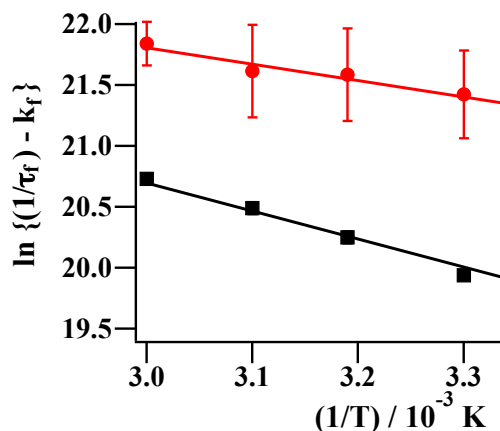


Figure 3.10. Arrhenius plots of C152 in the aqueous environment (red circles) and associated with DMPC vesicles near the lipid headgroups (black squares).

In the DMPC vesicle solutions, the activation energies of the non-radiative paths are 11 ± 1 kJ/mol and 19 ± 1 kJ/mol for C152 in the aqueous and polar regions, respectively. Pal *et al.* reports the non-radiative activation pathway to be 9 kJ/mol for C152 in acetonitrile and 10 kJ/mol for C152 in methanol.¹⁴⁸ Activation energies

calculated from data in Figure 3.10 show that when C152 is in the aqueous buffer, the energetics of the TICT state C152 match those of C152 in a bulk acetonitrile or bulk methanol, but for C152 associated with the DMPC bilayer the activation energy is a factor of 2 larger. This result may reflect a more hindered environment within the polar region of the bilayer that prevents excited C152 from forming the twisted conformer. This hypothesis remains to be tested, however.

3.4 Conclusions

Time-resolved fluorescence decays were used to determine C152 partitioning and local solvation in DMPC model membranes. When DMPC vesicles are in their gel state, more than 60% of C152 remains in the bulk aqueous environment and approximately 35% of C152 is associated with the polar region of the DMPC bilayers. The remainder C152 (<5%) is solvated in the membrane interior. Temperature dependent data below the gel-liquid crystalline transition temperature indicate that partitioning into the gel state DMPC bilayer is endothermic but entropically favored. As the solution temperature increases above the membrane's melting temperature, close to 70% of the available C152 solvates in the polar region of DMPC vesicles, 30% of C152 remains in the bulk aqueous environment, and the population of C152 solvated in the membrane interior decreases to $\leq 2\%$. With DMPC in its liquid crystalline state, C152 partitioning into the bilayer is enthalpically favored and entropically unfavorable. Above 40 °C the entropic contribution to C152 partitioning becomes dominant and a majority of C152 dissociates from the membrane to become solvated in the buffer again. These results were shown to be fully reversible as temperature decreased from 60 to 10 °C.

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CHAPTER FOUR

TEMPERATURE DEPENDENT PARTITIONING OF C152 IN
PHOSPHATIDYLCHOLINE LIPID BILAYERS

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CHAPTER FOUR

TEMPERATURE DEPENDENT PARTITIONING OF C152
IN PHOSPHATIDYLCHOLINE LIPID BILAYERS4.1 Introduction

Biological membranes create locally heterogeneous environments that can accommodate a wide variety of solutes.^{172,198,199} The clearest example of this heterogeneity is the contrast between the polar, aqueous environment on either side of the membrane and the hydrophobic interior of lipid bilayers where effective dielectric constants can assume alkane-like limits.^{103,131} More subtle are the changes in solvation that occur near the membrane surface and within a lipid bilayer's polar, glycerol backbone region. Coulomb forces, local functional group dipoles and intercalated water all conspire to create regions that have been modeled using simplified approximations such as additive (nonpolarizable) force fields, where polarized charges are later added as constants to mimic polar (aqueous) and nonpolar (alkane) regions.^{113,132,133} Experimental validation of these models, however, remains elusive. Complicating this picture is molecular organization within the membrane itself. Lipid bilayer phase diagrams show a wealth of phases and coexistence regions depending on temperature, bilayer composition and solution phase ionic strength.^{200,201} With so many variables to consider, predictive, molecularly specific mechanisms describing solute solvation in lipid membranes are difficult to formulate.

Such mechanisms, however, are essential for understanding phenomena including bioaccumulation,⁴⁴ anesthetic activity,²⁰² and active peptide-facilitated transport.¹⁰⁸ In drug delivery applications, for example, a solute's lipophilicity helps determine its ADMET (Absorption, Distribution, Metabolism, Elimination, and Toxicology) profile.^{15,203} Partitioning of a potential drug between two environments is sometimes tested rigorously using natural membranes,²⁰⁴⁻²⁰⁶ but more often, characterization uses less labor intensive empirical methods known as high-throughput screenings (HTS).^{6,207,208} The log P scale is one such HTS where a solute's ADMET profile is quantified by its tendency to partition between an aqueous solution and an immiscible, 1-octanol phase.²⁰⁹ While models characterizing partitioning behavior tend to rely on collections of constants (such as the log P value), empirical correlations such as Lipinski's Rule of 5¹⁵ or qualitative structure-permeability relationships (QSPRs),⁹ these models often lack a quantifiable description of solute-lipid interactions within the membrane bilayers themselves.

More rigorous methods have been developed to study solute-membrane binding, including techniques such as X-ray and neutron diffraction,^{84,170} differential scanning calorimetry,²¹⁰ FTIR, NMR,⁸⁷ EPR,⁸⁶ and SHG.¹²⁶ Most of these methods, however, do not probe directly the noncovalent interactions experienced by permeating solutes. Solute distributions and dynamics in model membranes have been studied extensively *in silico*, but detailed experimental evidence remains scarce.^{6,103,211,212} Moreover, HTS and other labor intensive methods rarely account for temperature dependent solute partitioning into membranes. Temperature dependent partitioning is of specific interest

from an environmental perspective, as organic pollutants may show differences in bioaccumulation depending on local thermal conditions.²¹³

In addition to changes in solute properties induced by partitioning into membranes, partitioning affects membranes themselves. Near the gel (L_β) to liquid crystalline (L_α) transition temperature, specific solutes have been shown to enhance or suppress the formation of large-scale (10-1000 Å) domains.⁶² For example, anesthetics^{61,202,214} and some pesticides and insecticides²¹⁵⁻²¹⁷ are known to disrupt order within lipid bilayers, while cholesterol^{165,218} and pyrene-labeled lipid analogs⁶² have been shown to increase superstructures within the fluid phase of lipid bilayers. Furthermore, lipid vesicle phase transition temperatures have been shown to decrease when chlorinated benzenes⁸⁰ and anesthetics^{61,202} accumulate above regulatory limits. Partition coefficients and diffusion rates of certain solutes were determined to be higher in the L_α phase than in the L_β phase, with the largest partition coefficients and diffusion rates observed at the main transition temperature.^{58,60,61} The increase in membrane permeability at the chain-melting temperature of lipids has been attributed to a maximum in lateral compressibility.^{60,63,219,220} These studies, however, fail to provide insight into the specific solute-membrane interactions responsible for observed changes in membrane structure and properties.

Experiments described in this work use time-resolved fluorescence spectroscopy to examine how a coumarin dye, coumarin 152 (C152), partitions into phosphatidylcholine vesicle bilayers. C152 is a hydrophobic solute with a log P value of 2.72 (calculated using ChemDrawPrime 16.0). Because C152's emission lifetime is

sensitive both to local polarity as well as hydrogen bonding opportunities, measurements are able to identify not only what fraction of C152 associates with the lipid bilayer but also where within the bilayer C152 localizes.^{148,221} Results show a large uptake of C152 by the bilayer as it passes from its L_{β} to L_{α} phase (23 °C for DMPC vesicles and 42 °C for DPPC vesicles), but as the temperature rises further, C152 is forced back out of the bilayer so that at temperatures above ~50 °C, C152 partitions preferentially back into the aqueous buffer. Thermodynamic analysis of the temperature dependent partitioning coefficient shows that for vesicle bilayers in their L_{α} crystalline state, C152 partitioning into the bilayer is exothermic but entropically unfavorable, whereas in lipid bilayers in their L_{β} state, partitioning is generally endothermic and entropically driven. These results, however, depend sensitively on the phosphocholine identity with vesicles prepared from three different saturated lipids – DLPC, DMPC and DPPC – all showing quantitative differences in their ability to accommodate C152. From these studies emerge a suggestive mechanism describing how C152 partitions into lipid membranes and how the thermodynamics of membrane partitioning change as a function of lipid identity and bilayer phase. Furthermore, data provide clear benchmarks for emerging computational models of membrane structure and dynamics.

4.2 Experimental

4.2.1 Materials

1,2-Dilauroyl-*sn*-glycero-3-phosphocholine (12:0 DLPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (14:0 DMPC), and 1,2-dipalmitoyl-*sn*-glycero-3-

phosphocholine (16:0 DPPC) were purchased in powder form from Avanti Polar Lipids (Alabaster, AL). Laser grade coumarin 152 was purchased from Exciton (Dayton, OH). Millipore water (18.2 M Ω) was used to make phosphate buffered saline. Solvents were purchased from Sigma Aldrich. All materials were used as received.

4.2.2 Large Unilamellar Vesicle Preparation

Large unilamellar vesicles (LUVs) were prepared according to the procedure described in Chapter 2. Briefly, DLPC, DMPC, or DPPC was dissolved in chloroform that was then removed via rotary evaporation, creating a thin lipid film. The film was rehydrated using 10 mM phosphate buffered saline (PBS, pH = 7) to form a 1.5 mM lipid vesicle suspension. The suspension was sonicated for 30 minutes at a temperature 10 °C above the respective lipid's melting temperature. The solution was then passed through a PTFE syringe filter (450 nm) to remove the giant unilamellar vesicles. C152 in PBS buffer (10mM, pH = 7, 6 μ M) was added to the vesicle suspensions producing a 1mM vesicle/ 2 μ M C152 solution. The solution was heated above the vesicle melting temperature and filtered through an Avanti Mini Extruder (membrane pore size of 200 nm) 10 times.

Differential scanning calorimetry and differential light scattering were used to characterize vesicles. Vesicle transition temperatures as found using differential scanning calorimetry agree with previously reported values.²⁰¹ Instrumentation descriptions and results are reported in the Chapter 2.

4.2.3 Time-Resolved Fluorescence

Time-resolved fluorescence emission data were collected using Picoquant PicoHarp 300 and FluoTime 200 software. An APE Autotracker containing a SHG crystal situated after a Chameleon laser (Coherent, 80 MHz, 680-1080nm) produced excitation pulses (85 fs pulsewidth) at a wavelength of 400 nm. A Conoptics Model 350-105 modulator reduced the repetition rate to 4 MHz. Samples were equilibrated at reported temperatures for approximately 5 minutes. Extending the equilibration time led to no observable changes in the measured lifetimes or relative amplitudes. The sample temperature was monitored by a Quantum Northwest TC152 control. A long-pass filter (80% transmission >455 nm) was placed after the sample to reduce scattering from the vesicles. Photon emission was collected at 500 nm.

In a given TCSPC experiment, signal was collected until the maximum intensity at early times reached a threshold of 8000 counts. Depending on the local solvation environment of C152 (and thus fluorescence quantum yield and radiative rate), the time required for each experiment varied with temperature and lipid identity. The instrument response function was accounted for by reconvolution of each experiment's raw data and fluorescence decays were fit to a sum of decaying exponentials terms corresponding to independent radiative lifetimes (Equation 2.2). Data were treated according to the technique described in Chapter 2.

4.3 Data and Results

Fluorescence decay traces of coumarin 152 in selected bulk solvents and a solution containing DPPC vesicles are shown in Figure 4.1. The solvents selected for bulk fluorescence measurements were chosen to model a lipid bilayer's distinctive solvation environments.^{103,168} Solvents investigated included cyclohexane (representative of the hydrophobic core of a lipid bilayer), acetonitrile and methanol (representative of the polar aprotic and polar protic regions near the phosphate headgroup), and buffer (the environment associated outside of the lipid membrane).

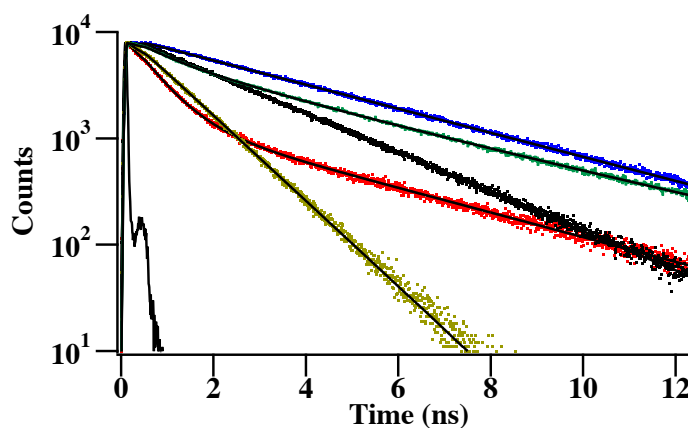


Figure 4.1. Fluorescence lifetime traces of C152 in bulk methanol (yellow), buffer (red), acetonitrile (black), and cyclohexane (blue). Fluorescence trace of C152 in DPPC vesicles is shown in green. An instrument response function was collected and is shown in black. All data were collected at 20 °C.

Steady-state and time-resolved fluorescence data for C152 in bulk solvents are briefly summarized in Table 4.1. Instrumentation used in absorption and fluorescence emission measurements and quantum yields are reported in Chapter 2. Time-resolved fluorescence traces in the bulk solvents were fit to one or two exponential decays.

Solvent	λ_{exc} (nm)	λ_{em} (nm)	k_f (10^7 s^{-1})	ϕ_f	τ_f (ns) ^c
Cyclohexane	372	426	25.1	0.97 ^a	3.86
Acetonitrile	396	502	9.8	0.23 ^b	2.34
Methanol	397	515	8.3	0.09 ^a	1.09
0.01M PBS buffer	404	527	8.1	0.05 ^b	0.62 (0.83), 3.75 (0.17)

Table 4.1. Photophysical properties of C152 in selected solvents at 10 °C

^a Quantum yields as reported by Pal *et. al.*¹⁴⁸

^b Quantum yields measured in this work. Experiments were conducted at room temperature, and remain virtually unchanged from 10 – 70 °C.

^c Uncertainties in reported lifetimes are ± 0.10 ns. Uncertainties in reported amplitudes (for the PBS buffer solution) are ± 0.05 .

Fluorescence properties of C152 in the four solvents shown in Table 4.1 and Figure 4.1 vary systematically with solvent polarity. Unusual behavior of C152 in high polarity solvents was observed, including a substantial reduction in quantum yield and shortened fluorescence lifetimes. The authors concluded that fluorescent states of C152 in nonpolar solvents were different than the fluorescence states of C152 in polar solvents, and subject to different relaxation mechanisms. Figure 4.1 shows the distinct difference in fluorescence lifetimes depending on the solvent polarity. In buffer, C152 exhibits two fluorescence lifetimes. The short lifetime contributes the majority (83%) of the overall fluorescence decay. Fluorescence decay in cyclohexane shows distinctly different fluorescence properties that are described with a single, long lifetime. Two different conformations have been assigned to C152's excited states. In nonpolar solvents, the tertiary amine retains its pyramidal geometry, while polar solvents stabilize a non-emissive twisted intramolecular charge transfer (TICT) state.⁷³

Fluorescence traces in Figure 4.1 indicate that C152 in lipid vesicle solutions do not have the same time dependent emission behavior as in bulk solvents, suggesting C152

is sampling more than one solvation environment. The separable and measurably different C152 emission lifetimes in different bulk solvents allows for assignment of individual lifetimes and associated amplitudes of C152 in vesicle containing solutions to discrete local environments.

To assign local solvation environments of C152 in lipid membranes, fluorescence traces were fit to a three-exponential decay. Each of the three lifetimes is similar to lifetimes of C152 in a different solvent (Figure 4.2). These data indicate C152 samples at least three distinct local solvation environments in vesicle solutions. The three lifetimes (triangles, squares, and circles) of C152 in DPPC vesicles match closely C152 fluorescence lifetimes of C152 in three bulk solvents: cyclohexane, methanol/acetonitrile, and buffer.

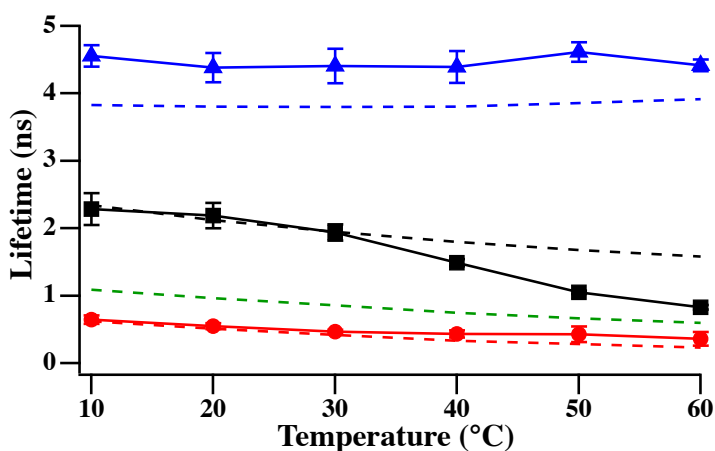


Figure 4.2. Fluorescence lifetimes of C152 in bulk solvents represented by dashed lines: (blue) cyclohexane, (black) acetonitrile, (green) methanol, (red) buffer. The long (minor) lifetime of C152 in buffer is omitted. The three lifetimes of C152 in DPPC vesicles are represented by blue triangles (τ_3 , nonpolar), black squares (τ_2 , polar), and red circles (τ_1 , buffer).

Results in Figure 4.2 show clear similarities between C152 fluorescence lifetimes in bulk solvents and in DPPC vesicle solutions. Similar correlations have been reported for C152 in DMPC vesicle containing solutions (Chapter 3). The long lifetime of C152 in DPPC vesicles (τ_3) remains constant over the temperature range investigated. C152 in alkanes has a similarly long lifetime that remains constant with temperature, thus we assigned this contribution to C152 decay to solutes in the hydrophobic core of the lipid membrane. The short lifetime of C152 in DPPC vesicles (τ_1) also remains approximately constant over the temperature range investigated. The fluorescence lifetime tracks the fluorescence lifetime of C152 in bulk buffer solution. Thus τ_1 , the short (sub ns) lifetime of C152 in DPPC vesicles, was attributed to solutes that did not interact with the vesicle. The intermediate fluorescence lifetime reported by C152 in DPPC vesicle solutions (τ_2) changed with temperature. At low temperatures, τ_2 matched C152's lifetime in polar aprotic solvents, whereas at higher temperatures, τ_2 converged to the polar, protic limit. We assign this lifetime to C152 solvated in the polar region of the lipid bilayer and discuss this result in greater detail below.

Figure 4.3 shows the temperature dependent fluorescence decay traces of C152 in DPPC vesicle solutions.

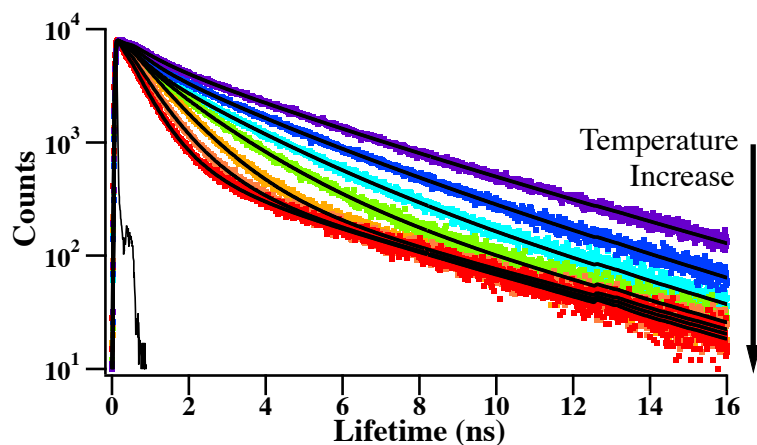


Figure 4.3. Raw fluorescence traces and exponential fits of C152 in DPPC vesicles at 10, 20, 30, 40, 50, 60, and 70 °C. Fluorescence traces were fit to an exponential decay with three distinct lifetimes. The instrument response (black trace) is typically ~ 100 ps.

Time dependent C152 fluorescence emission changes noticeably with the shorter lifetime, τ_3 , becoming more pronounced at higher temperatures. To investigate the partitioning changes within the vesicle solutions quantitatively, the raw amplitude of each of the three fluorescence lifetimes was corrected by the radiative rate of C152 in methanol/acetonitrile, buffer, or cyclohexane. The radiative rate of C152 in buffer was determined using the measured quantum yield and lifetime of C152 in buffer. The quantum yield remained ~ 0.05 from 15-55 °C, and thus we determined the radiative rate for C152 in buffer to also remain relatively constant ($8.1 \times 10^7 \text{ sec}^{-1}$). The quantum yield of C152 in acetonitrile changes from ~ 0.25 to ~ 0.16 from 10-70 °C.¹⁴⁸ For the time-resolved data analysis, we ascribed the radiative rate of τ_2 to be the average of the radiative rates of C152 in methanol and acetonitrile ($9.0 \times 10^7 \text{ sec}^{-1}$). Quantum yields for C152 in nonpolar solvents are temperature independent, and thus time-resolved data analysis used the radiative rate of C152 in cyclohexane ($25.1 \times 10^7 \text{ sec}^{-1}$). Amplitude data

from fluorescence decays shown in Figure 4.3 were corrected with the following radiative rates: $8.1 \times 10^7 \text{ sec}^{-1}$ (buffer), $9.0 \times 10^7 \text{ sec}^{-1}$ (methanol/acetonitrile), and $25.1 \times 10^7 \text{ sec}^{-1}$ (cyclohexane). The new amplitudes at each temperature were renormalized to unity. Lifetime contributions from C152 in the buffer (τ_1), polar (τ_2), and nonpolar (τ_3) regions at selected temperatures are summarized in Table 4.2 (DLPC), Table 4.3 (DMPC), and Table 4.4 (DPPC).

To visualize the relative population of C152 in three solvation environments (buffer, polar, and nonpolar environments), the lifetime contributions of τ_1 , τ_2 , and τ_3 are shown in Figure 4.4. The behavior of C152 in DPPC vesicle solutions is similar to results reported previously describing C152 partitioning into DMPC vesicles although the quantitative details have changed due to the correction for radiative rates as described above.²²¹ At the lowest temperature studied (5 °C), approximately 40% of C152 remains in the buffer, 20% of C152 partitions into the membrane interior, and the remainder partitions near the phospholipid headgroup. As the temperature increases through the pre-transition temperature (characterized by the formation of membrane ripples at 14 °C), more C152 partitions into the polar headgroup region. C152 partitioning into the membrane is maximized just above the phase transition temperature (25 °C). At high temperatures, some C152 moves out from the membrane back into the buffer solution, although most C152 remains associated with the membrane. This behavior is quantitatively reversible.

DPPC contains the same phosphocholine headgroup as DMPC, with the only difference in structure being the addition of two methylene groups to each acyl chain.

The increase in acyl length increases inter and intramolecular van der Waal forces, thus increasing the melting temperature of DPPC vesicle bilayers from 23 °C (DMPC) to 42 °C (DPPC). C152 partitioning behavior follows similar qualitative trends in DPPC and DMPC vesicles but with distinctive quantitative differences. Figure 4.4 shows only minor changes in partitioning in L_{β} DPPC vesicles (5–30 °C). At the lowest temperature studied (5 °C), 22% of C152 is embedded in the membrane interior, 37% is associated with the headgroups, and the remainder of C152 remains in the buffer solution. As the temperature increases to 20 °C, the amount of C152 solvated in the membrane interior diminishes to virtually nothing, while the fraction of C152 in buffer and membrane headgroups increases. When the sample reaches 30 °C, a sharp decrease in lifetime contribution from C152 in the buffer coincides with an increase in the contribution from the second lifetime (C152 in a polar environment) although the amount of C152 in the nonpolar region of the membrane remains largely unchanged. At the phase transition temperature, C152 partitioning into the membrane is maximized, similar to the behavior of C152 partitioning in DMPC membranes. At temperatures higher than the transition temperature, C152 again shows weaker affinity for the lipid bilayer and exsolvates from the membrane back into aqueous solution.

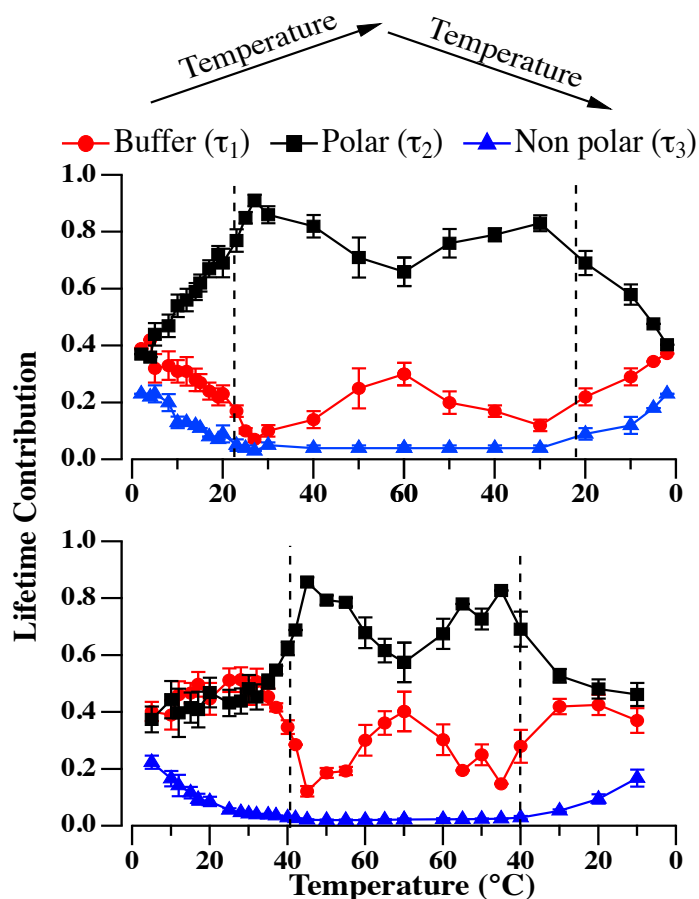


Figure 4.4. Quantum yield corrected fluorescence contributions of C152 populations in (top) DMPC vesicle solutions and (bottom) DPPC vesicle solutions plotted as a function of temperature. Each contribution reported corresponds to three lifetimes, associated with three local solvation environments for C152 in vesicle solutions, buffer (τ_1 , red circles), nonpolar (τ_3 , blue triangles), and polar (τ_2 , black squares) environments. The dashed lines indicate the melting temperature of DMPC and DPPC vesicles.

C152 membrane affinity increases with temperature reaching a maximum near the transition temperature. C152 partitioning into bilayers in their L_α phase appears exothermic with C152 concentration in the bilayer decreasing as the temperature continues to increase above the transition temperature. These effects are completely reversible and occur in less than 5 minutes. Experiments varying the time between

reaching a constant sample temperature and acquiring the fluorescence trace showed no difference in partitioning over two hours of equilibration (Appendix A).

To test the generality of these observations, similar experiments were performed for DLPC vesicles. DLPC's melting temperature is $-2\text{ }^{\circ}\text{C}$ so all experiments were carried out with DLPC vesicles in the L_{α} state. Radiative rate corrected lifetime contributions (normalized amplitudes) are shown in Figure 4.5, and the trends match those observed for both DMPC and DPPC. Uncertainties associated with fluorescence lifetimes and amplitudes are reported in Table 4.2 (DLPC), Table 4.3 (DMPC), and Table 4.4 (DPPC).

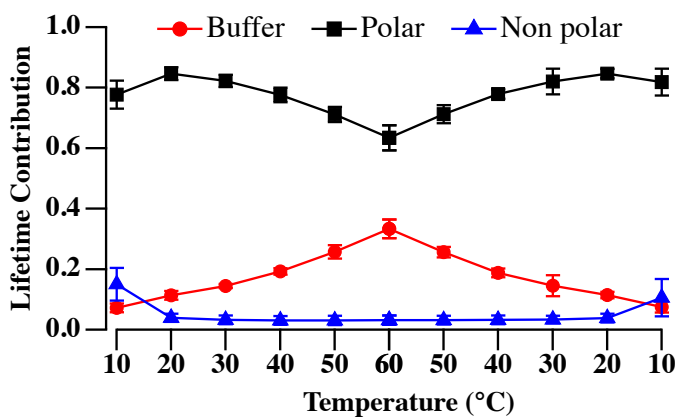


Figure 4.5. Radiative rate corrected fluorescence lifetime contributions of C152 in DLPC vesicle solution plotted as a function of sample temperature. The red circles indicate the short fluorescence lifetime of C152 (buffer), black squares are the contributions of the intermediate fluorescence lifetime (polar) and blue triangles are the contributions of the long fluorescence lifetime (non-polar).

Temp. (°C)	Buffer τ_1 (A ₁)	Polar τ_2 (A ₂)	Nonpolar τ_3 (A ₃)
10	0.69 ± 0.15 (0.07 ± 0.01)	2.35 ± 0.16 (0.78 ± 0.05)	4.18 ± 0.62 (0.15 ± 0.05)
20	0.48 ± 0.04 (0.11 ± 0.01)	1.93 ± 0.07 (0.85 ± 0.02)	4.80 ± 0.35 (0.04 ± 0.01)
30	0.42 ± 0.08 (0.14 ± 0.01)	1.49 ± 0.02 (0.82 ± 0.02)	4.84 ± 0.32 (0.03 ± 0.01)
40	0.41 ± 0.07 (0.19 ± 0.01)	1.17 ± 0.02 (0.78 ± 0.02)	4.74 ± 0.17 (0.03 ± 0.01)
50	0.34 ± 0.06 (0.26 ± 0.02)	0.90 ± 0.02 (0.71 ± 0.03)	4.56 ± 0.09 (0.03 ± 0.02)
60	0.29 ± 0.04 (0.33 ± 0.03)	0.74 ± 0.02 (0.63 ± 0.04)	4.37 ± 0.07 (0.03 ± 0.01)

Table 4.2. Fluorescence lifetimes and associated radiative rate corrected amplitudes of C152 in a DLPC vesicle solution. Lifetimes are reported in ns. Relative amplitudes are included in parenthesis. Each data point represents the average of four samples.

Temp. (°C)	Buffer τ_1 (A ₁)	Polar τ_2 (A ₂)	Nonpolar τ_3 (A ₃)
10 (n=7)	0.65 ± 0.07 (0.31 ± 0.04)	2.47 ± 0.17 (0.54 ± 0.04)	4.70 ± 0.30 (0.13 ± 0.02)
20 (n=7)	0.61 ± 0.11 (0.23 ± 0.03)	2.27 ± 0.11 (0.69 ± 0.05)	4.73 ± 0.30 (0.09 ± 0.03)
30 (n=7)	0.50 ± 0.15 (0.10 ± 0.02)	1.76 ± 0.05 (0.86 ± 0.03)	4.92 ± 0.22 (0.05 ± 0.01)
40 (n=7)	0.45 ± 0.15 (0.14 ± 0.03)	1.28 ± 0.05 (0.82 ± 0.04)	4.90 ± 0.21 (0.04 ± 0.01)
50 (n=7)	0.41 ± 0.13 (0.25 ± 0.08)	1.00 ± 0.04 (0.71 ± 0.08)	4.69 ± 0.14 (0.04 ± 0.01)
60 (n=6)	0.31 ± 0.07 (0.30 ± 0.04)	0.78 ± 0.02 (0.66 ± 0.05)	4.40 ± 0.09 (0.04 ± 0.01)

Table 4.3. Fluorescence lifetimes and associated radiative rate corrected amplitudes of C152 in a DMPC vesicle solution. Lifetimes are reported in ns. Relative amplitudes are included in parenthesis. Sample size is included next to each temperature.

Temp. (°C)	Buffer τ_1 (A_1)	Polar τ_2 (A_2)	Nonpolar τ_3 (A_3)
10 (n=8)	0.65 ± 0.06 (0.39 ± 0.05)	2.28 ± 0.24 (0.44 ± 0.06)	4.56 ± 0.16 (0.17 ± 0.03)
20 (n=8)	0.55 ± 0.04 (0.45 ± 0.06)	2.19 ± 0.19 (0.47 ± 0.05)	4.38 ± 0.22 (0.09 ± 0.02)
30 (n=8)	0.47 ± 0.03 (0.48 ± 0.05)	1.94 ± 0.12 (0.48 ± 0.05)	4.41 ± 0.26 (0.04 ± 0.01)
40 (n=8)	0.48 ± 0.05 (0.35 ± 0.02)	1.49 ± 0.06 (0.62 ± 0.02)	4.39 ± 0.23 (0.03 ± 0.01)
50 (n=9)	0.43 ± 0.11 (0.19 ± 0.02)	1.05 ± 0.03 (0.79 ± 0.02)	4.61 ± 0.14 (0.02 ± 0.01)
60 (n=9)	0.36 ± 0.10 (0.30 ± 0.05)	0.83 ± 0.03 (0.68 ± 0.05)	4.42 ± 0.09 (0.02 ± 0.01)
70 (n=9)	0.30 ± 0.07 (0.40 ± 0.07)	0.68 ± 0.03 (0.57 ± 0.07)	4.17 ± 0.05 (0.02 ± 0.01)

Table 4.4. Fluorescence lifetimes and associated radiative rate corrected amplitudes of C152 in a DPPC vesicle solution. Lifetimes are reported in ns. Relative amplitudes are included in parenthesis. Sample size is included next to each temperature.

4.4 Discussion

Studies described above provide information necessary for identifying mechanisms responsible for solute partitioning into lipid membranes. In this context, several observations require discussion. These observations include reversible, temperature dependent changes in τ_2 , similarities and differences in temperature dependent partitioning in DPPC, DMPC and DLPC, and thermodynamic behavior of C152 partitioning into different lipid phases.

4.4.1 Reversible, Temperature Dependent Changes in τ_2

The short (τ_1) and long lifetimes (τ_3) of C152 in vesicle solutions are attributed to C152 in buffer and a nonpolar environment, respectively. These two fluorescence

lifetimes change little with temperature, while the intermediate lifetime, τ_2 changes significantly. τ_2 data for DLPC, DMPC, and DPPC as a function of temperature are shown in Figure 4.6. Also shown in Figure 4.6 are the temperature dependent fluorescence lifetimes of C152 in acetonitrile and methanol. At low temperatures, τ_2 in vesicle solutions is ~ 2.4 ns and matches almost exactly the lifetime of C152 in acetonitrile (2.3 ns), a polar aprotic solvent. This result suggests that C152 in phosphocholine membranes at low temperatures (e.g. 10 °C) is sensitive to the high dipole density found along the lipid glycerol backbone but does not accept any hydrogen bonds. As the vesicle solution temperature rises, τ_2 converges to ~ 0.8 ns (at 60 °C), the same lifetime as C152 in bulk methanol.

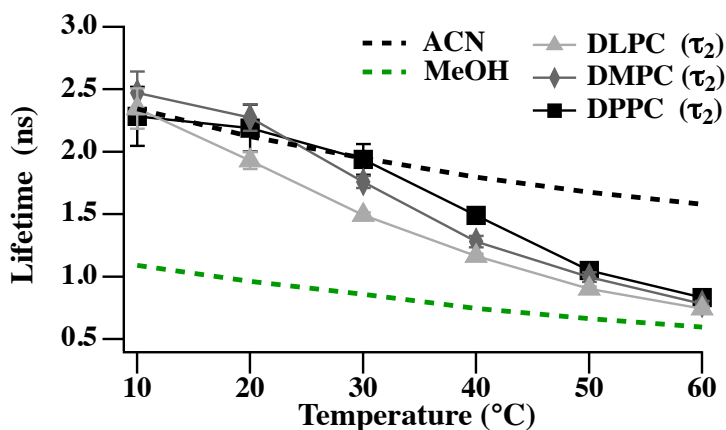


Figure 4.6. Fluorescence lifetime of C152 in bulk acetonitrile (dashed black line) and bulk methanol (dashed green line). The intermediate fluorescence lifetime of C152 in vesicles composed of DPPC (black squares), DMPC (dark grey diamonds) and DLPC (light grey triangles). The intermediate lifetime is attributed to C152 in a polar region of the membrane.

We propose that this change in τ_2 reflects a change of hydrogen bonding opportunities within the polar region of the membrane. If the convergence of τ_2 to the

protic polar limit at higher temperatures reflects the emergence of C152 being able to accept hydrogen bonds, then this result implies that as the temperature increases, water permeates into the polar part of the lipid bilayer and interacts directly with partitioned solutes. This result is supported by predictions from computational simulations that show a membrane in the L_α phase has almost double the number of waters bonded to each phosphocholine compared to a membrane in the L_β phase.²²²

4.4.2 Similarities and Differences in Temperature Dependent Partitioning in DPPC, DMPC and DLPC

Normalization of temperature with respect to each lipid's melting temperature allows for direct comparison of C152 partitioning into vesicles composed of different lipids. Figure 4.7 shows the lifetime contributions observed in DMPC and DPPC vesicle solutions at reduced temperatures, where temperature dependent data from DPPC and DMPC solutions are normalized to each lipid's respective phase transition temperature. (For clarity, DLPC is omitted from Figure 4.7 and appears in Appendix A.)

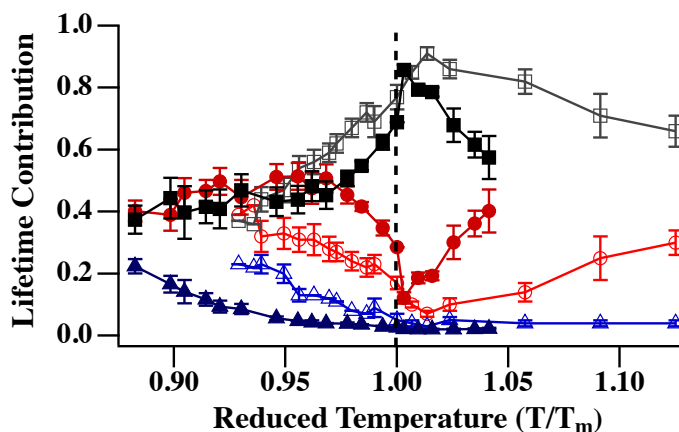


Figure 4.7. Lifetime contributions of C152 in DMPC and DPPC vesicles at a reduced temperature. The open points are lifetime contributions of C152 in a DMPC vesicle solution, and the filled points are C152 lifetime contributions in DPPC vesicle solution. Red markers indicate C152 in the buffer (τ_1), black markers indicate C152 in the polar region (τ_2), and blue triangles indicate C152 in the nonpolar region (τ_3). Solutions were allowed to fully equilibrate at each temperature before recording fluorescence decays.

Similarities between the different lipid systems are striking. Lipid bilayers show a strong uptake of C152 in the immediate vicinity of the transition temperature followed by a gradual return of C152 into bulk solution as the temperature continues to rise. Interestingly, even the minority species – C152 in the hydrophobic interior (with lifetime τ_3) behaves similarly in the two different lipid systems with small populations decreasing even further as temperature rises.

Despite these similarities, however, quantitative differences between DPPC and DMPC do exist. Specifically, C152 partitioning into DMPC vesicles shows more gradual temperature dependence than C152 partitioning into DPPC vesicles. Starting at a reduced temperature of 0.90, C152 shows a steady increase in its affinity for DMPC vesicles, while in the DPPC solution, C152 remains largely in the buffer. Furthermore, C152 shows an overall lower partitioning coefficient in DPPC solutions above the

transition temperature relative to DMPC solutions. Reported results illustrate that even though DPPC and DMPC share virtually equivalent structures, changes in acyl chain length have measurable effects on properties associated with the lipid bilayer headgroup region.

One possible explanation for this effect comes from Monte Carlo simulations. Findings reported by Ipsen *et al.* and others have shown that close to the phase transition temperature, as the membrane starts to melt, it forms transient L_{α} domains.^{220,223} These domains remain surrounded by lipids in their L_{β} phase. Clusters are created, destroyed, and vary in size. Monte Carlo simulations have shown the lateral density fluctuations are inversely related to chain length (shorter chains have larger fluctuations). Fluctuations will also affect the temperature range over which clusters can form, meaning clusters will form over a broader temperature range in DMPC vesicles compared to DPPC vesicles. Given that lipid bilayers in the L_{α} state are more receptive to C152 permeation, these simulation results predict that partitioning into DMPC vesicles should start at lower temperatures relative to the transition temperature, and show a greater overall affinity compared to DPPC vesicles.²²³ This prediction supports our finding that C152 partitions more into L_{α} DLPC vesicles compared to L_{α} DMPC vesicles (Figure 4.8 and Table 4.5).

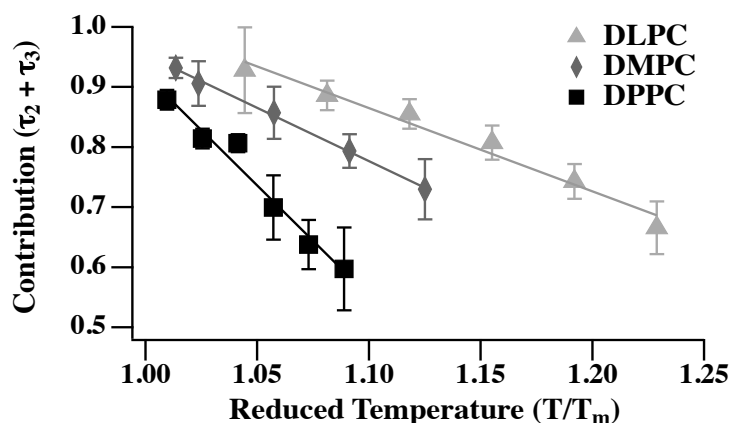


Figure 4.8. Contribution from intermediate and long lifetimes (τ_2 and τ_3) in the overall lifetime of C152 in DLPC (light grey triangles), DMPC (dark grey diamonds) and DPPC (black squares). The reduced temperature elucidates the differences in partitioning at the main transition temperature of each individual lipid.

Lipid	1.01 T/T_m	1.05 T/T_m	1.10 T/T_m	1.15 T/T_m
DLPC	(1 °C) 98% ^a	(12 °C) 93%	(25°C) 86%	(39°C) 80%
DMPC	(27 °C) 94%	(38°C) 87%	(53°C) 78%	(68°C) 69%
DPPC	(45 °C) 88%	(58°C) 74%	(74°C) 55% ^a	(89°C) 37% ^a

Table 4.5. Lifetime contribution of membrane associated C152 ($A_2 + A_3$) at reduced temperatures.

^aContributions are extrapolated from linear fits of data in Figure 4.8. (Note that reduced temperatures are calculated using absolute temperatures (K) but are reported in °C for the purposes of comparison to data presented in this work.)

These lipid dependent partitioning effects become more pronounced at higher temperatures. Just above the transition temperature ($T/T_m \sim 1.01$), the percentage of C152 associated with DMPC membranes is ~94%. This fraction diminishes with increasing temperature but the effect is less extreme with DLPC vesicles and most extreme with DPPC vesicles. This behavior is similar to previous findings showing that

chlorobenzenes partitioned into DMPC vesicles more so than DPPC vesicles by almost an order of magnitude in the vicinity of the transition temperature.⁸⁰

Solute permeation in lipid membranes near the phase transition has been studied extensively, with numerous experiments confirming the highest permeation of certain solutes to be at the melting temperature.^{58,59} Two models have been proposed as reasons for the increase in permeability. The “leaky interface” model suggests the increased permeation at the melting temperature results from solutes preferentially partitioning into boundaries between gel and fluid domains.^{58,60} The second model suggests the spike in membrane permeability near the transition temperature is caused by the increase in area per lipid, membrane lateral compressibility, and maximum area fluctuations.^{59,62,63} Computational simulations investigating solute partitioning at the main phase transition determined permeation was not enriched at domain boundaries, casting doubt on the leaky interface description. Yang *et. al* thus attributed increased solute/membrane permeation to the increased area per lipid in liquid-crystalline domains.²²⁰ X-ray and neutron scattering and computational simulations found the area per DMPC lipid increases by 22% as the sample temperature was raised from 10 °C (L_β) to 30 °C (L_α), and the area of a DPPC molecule increases by 25% over its transition temperature (25 °C to 50 °C).^{56,224,225} Permeation of water into membranes has been shown to increase with area per lipid, suggesting at $T \gg T_m$, C152 exsolvation from the membrane may be a result of more energetically favorable lipid headgroup hydration.^{56,225}

Studies conducted by Aliva *et al.* showed a similar trend in dissociation of benzocaine in aqueous DMPC vesicle solutions by measuring the partitioning coefficient

($K_{\text{DMPC/W}}$) above DMPC's melting temperature. $K_{\text{DMPC/W}}$ increased from 93 at 25 °C to 248 at 30 °C. Partitioning then decreased as the temperature was raised to 40 °C. The small partitioning coefficient within 1 °C of the melting temperature was omitted from their van't Hoff analysis as the value was viewed as anomalous.⁷⁰ However, data reported by Aliva *et al.* also show that immediately below the melting temperature, solute partitioning decreases, a result that is inconsistent with findings shown here and elsewhere.^{58,60,61,80}

4.4.3 Thermodynamic Behavior of C152 Partitioning into Different Lipid Phases

Temperature dependent partitioning allows the enthalpic and entropic contributions to membrane permeation to be calculated. Figure 4.9 shows a van't Hoff analysis of the partitioning coefficients calculated from data in Figure 4.4 and Figure 4.5. This analysis considers contributions from C152 solvated in buffer solution (from τ_1) and the sum of C152 associated with the polar and nonpolar parts of the lipid membrane (τ_2 and τ_3). Included in Figure 4.9 are data from DPPC, DMPC and DLPC vesicle solutions. Enthalpy and entropy results are summarized in Table 4.6.

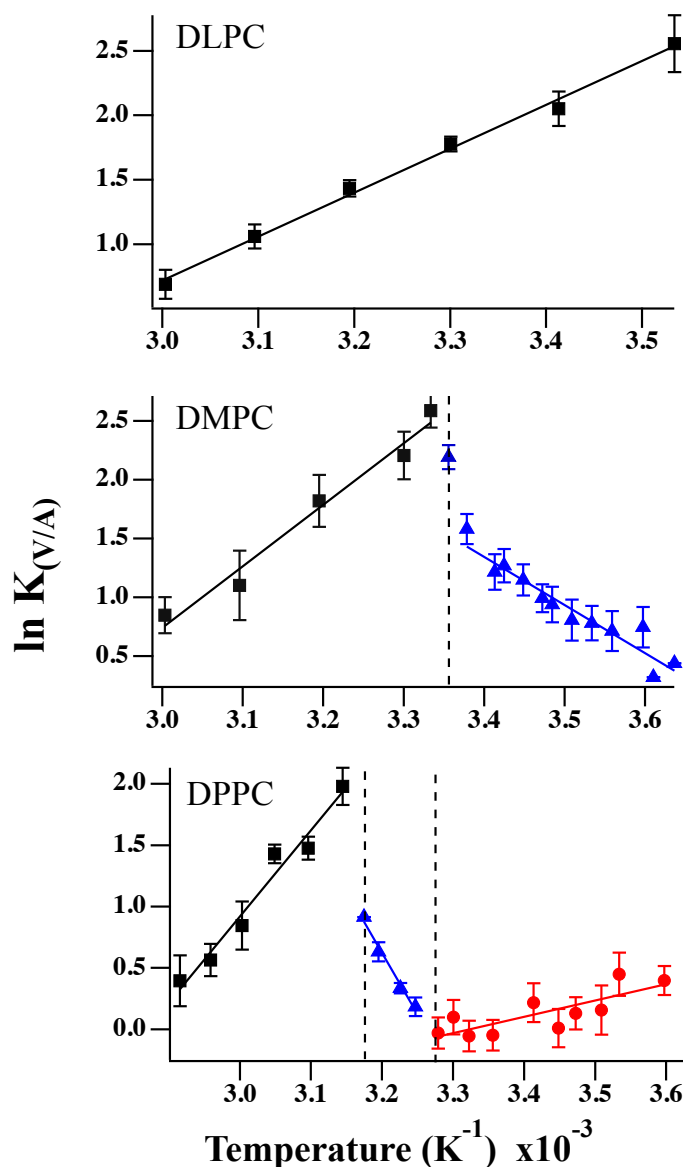


Figure 4.9. van't Hoff plots for C152 in (top) DLPC, (middle) DMPC, and (bottom) DPPC vesicle solutions. The ratio of C152 population associated with the lipid membrane to the C152 population in the buffer (unassociated with the vesicle) is plotted as a function of temperature. Red markers indicate ratios of populations well below the melting temperature (membranes in the gel phase), blue markers are associated with data recorded in the pre-transition temperature of vesicles, and black markers are ratios of C152 populations while the membranes are in their liquid-crystalline state. The transition temperature of DMPC vesicles (middle) is indicated with a dashed line, and both the pretransition and transition temperatures of DPPC vesicles are shown with dashed lines (bottom).

Vesicle	ΔH (kJ/mol)	ΔS (J/mol K)
$T < T_m$		
DPPC	-11 ± 3	-37 ± 10
$T \approx T_m$		
DMPC	34 ± 3	127 ± 12
DPPC	84 ± 7	272 ± 24
$T > T_m$		
DLPC	-28 ± 1	-79 ± 3
DMPC	-43 ± 4	-125 ± 12
DPPC	-58 ± 5	-166 ± 16

Table 4.6. Enthalpic and entropic contributions to the systems' free energies of partitioning.

4.4.3a $T \gg T_m$. C152 permeation into the lipid bilayer above the transition temperature is exothermic and entropically unfavorable. ΔH and ΔS both become increasingly negative with longer lipid chain length. The exothermicity associated with C152 being excluded from the membrane can be understood in terms of changes that occur in the lipid membrane upon melting. Specifically, after passing from the L_β to the L_α state, lipids form more headgroup-water interactions, in turn decreasing the number of headgroup-headgroup interactions and increasing the area per lipid.^{54,222,226} At high enough temperatures, we propose that headgroup-associated C152 solutes are less energetically favored than headgroup-water interactions thus promoting C152 exsolvation. Exsolvation into the buffer, however, will also be entropically unfavorable, given that C152 will force higher conformational order in the surrounding solvent.

What remains puzzling is the systematic changes in magnitude of ΔH and ΔS as a function of chain length. Although the general partitioning behavior is consistent between all three lipids above the melting temperature, we observe a constant increase in

exothermic behavior and a decrease in entropy as the carbon chains are lengthened. The increase in exothermicity is ~ 15 kJ/mol with the addition of two methylene groups per chains, while the entropy increment decreases by ~ 40 J/mol K.

The systematic thermodynamic changes must be attributed to the lipids' acyl chains, as the phosphocholine moiety remains identical for each lipid. The hydrophobic interior of a membrane has long-range effects on the water near the polar headgroups. However, the "second shell" of water (the solvation shell of choline-associated water) that is sensitive to the surface area per lipid (a factor determined by alkyl chain length). Regardless of phase, more waters can associate with longer chain lipids due to their increased area/lipid.^{56,226} At a given temperature (dependent on alkyl chain length), water association with the headgroups becomes more favorable, apparently at the expense of C152 solvation in the headgroup region.

4.4.3b $T \approx T_m$. Just below the transition temperature, C152's partitioning behavior changes qualitatively. ΔH and ΔS are both positive, meaning that C152 permeation into the membrane becomes *endothermic* and entropically favorable. As noted above, the temperature window where this behavior is observed is considerably broader for DMPC vesicles than for DPPC vesicles. This result may be a consequence of the more extensive domain formation in the DMPC bilayers predicted by Yang *et al.*²²⁰

More surprising is the magnitude of ΔH and ΔS for the DPPC vesicles in this intermediate temperature range. The magnitude of entropic and endothermic partitioning is more pronounced than for DMPC vesicles. Partition coefficients sampled at small temperature intervals directly below and above DMPC's transition temperature may help

clarify subtle changes in a membrane's ability to accommodate permeating solutes in a regime where membrane structural fluctuations become quite large.

4.4.3c $T < T_m$. The final observation from Figure 4.9 is that at temperatures well below the DPPC's transition temperature, the presence of C152 in the bilayer is weakly exothermic (-11 kJ/mol) and slightly entropically unfavorable. We interpret these results as follows: removing C152 from solution is net exothermic as the solvating water molecules are able to form stronger solvent-solvent hydrogen bonds. However, solvating the C152 in the membrane requires disrupting the tightly packed, highly ordered lipid membrane, a procedure that is net endothermic. Given the relatively small enthalpy of partitioning measured at temperatures below the transition temperature, we surmise that these two processes are similar in magnitude and opposite in sign. Similar arguments can be made to rationalize the small partitioning entropy.

Computational simulations have shown small molecules (argon and methane) can insert themselves near lipid headgroups without disrupting the local water networks, and thus no enthalpic cost.²²⁷ Similarly, we believe the small population of C152 observed as associated with the polar headgroups in the L_β phase does not significantly disrupt the water networks.

4.5 Conclusions

We have shown the similarities and differences in temperature-dependent partitioning of coumarin 152 in three phosphocholine lipid membranes. C152 partitioning in DLPC, DMPC, and DPPC show a similar general trend: in the L_β phase,

C152 favors the buffer surrounding the vesicles. Upon phase change to the L_α phase, C152 solvates near the polar headgroups of the phospholipid bilayer. Well above the phase transition, C152 is expelled from the membrane and once again remains in the buffer. Only at temperatures below the membrane melting temperatures is a significant population ($\sim 1520\%$) of C152 observed in the hydrophobic core of the membranes. The trend remains true regardless of heating or cooling the solution, indicating nearly perfect reversibility in partitioning.

Enthalpic and entropic contributions to C152 partitioning in vesicles also follow a trend: Below the melting temperature, the C152 association with DPPC membranes is largely temperature independent as evident by the small and opposing partitioning enthalpy and entropy. Near the melting temperature, partitioning into the membrane is endothermic and entropically favorable. The thermodynamic processes are $\sim 2.5\times$ larger in DPPC vesicle solutions than in DMPC vesicle solutions. Well above the melting temperature, partitioning into the membrane is exothermic and entropically unfavorable. A systematic increase in exothermic behavior and negative entropy is observed between all three phosphatidylcholines. Exothermic behavior increases by ~ 15 kJ/mol and entropy decreases by ~ 40 J/(mol K) as the lipids' alkyl chains increase in length.

We conclude C152 partitioning near the lipids' melting temperatures is the result of the larger area/lipid in the melting temperature region and a larger membrane lateral compressibility, while membrane expulsion at $T \gg T_m$ is the result of C152 displacement by more energetically favorable interactions forming between water and lipid headgroups.

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CHAPTER FIVE

QUANTIFYING SOLUTE PARTITIONING
IN PHOSPHATIDYLCHOLINE MEMBRANES

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Contributions: Assisted with data interpretation and edited manuscript.

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CHAPTER FIVE

QUANTIFYING SOLUTE PARTITIONING
IN PHOSPHATIDYLCHOLINE MEMBRANES5.1 Introduction

Modern drug design commonly utilizes selective halogenation to improve pharmacological profiles, as halogenated drugs are generally more lipophilic and more likely to be stable against metabolic degradation.²²⁸⁻²³⁰ A common practice to enhance passive diffusion of drugs into membranes is to replace hydrogens selectively with a chlorine or trifluoromethyl residue. Fluorinated drugs make up ~20% of all pharmaceuticals,²³¹ up to ~30% of agrochemicals, and a quarter of all patents and papers related to medicinal chemistry involve selective halogenation as a final step of drug synthesis.²³² Despite the importance of halogens in modern pharmacology, however, relatively few models can predict quantitative, molecular-level details about drug-membrane interactions. The enhanced lipophilicity shown by halogenated pharmaceuticals has been attributed to larger molecular volumes and increased cross sectional areas, but these effects fail to account for specific, noncovalent forces that drive membrane partitioning. Furthermore, once a pharmaceutical has permeated into a membrane, empirically developed descriptions of biological activity cannot anticipate solute distributions across the heterogeneous membrane environment.

Along with solute structure, membrane packing order strongly influences drug permeation and binding. As membrane packing changes drastically with temperature,

drug/membrane partition coefficients have similarly been shown to have strong temperature dependence. Below a membrane's melting temperature, phospholipid membranes exist in a highly ordered, rigid, gel state. Lower partition coefficients have been recorded when membranes are in this phase as compared to membranes in their fluid, liquid crystalline phase. Studies using a wide variety of solutes show that the partition coefficient reaches a maximum at the membrane melting temperature due to an increase in lateral density fluctuations.^{63,219} A reduction in partition coefficients have been observed with beta blockers, dopamine, and local anesthetics at temperatures well above the membrane melting temperature.¹²⁶ The decrease in partitioning may be attributed to the increase in water solubility of hydrophobic molecules at higher temperatures or the conformational changes of the lipid headgroups.^{126,233}

Many studies have investigated how halogen identity and position changes a solute's membrane permeability, however the techniques used in these reports do not *directly* measure partitioning into membranes at different temperatures. Common techniques used in pharmacological development include computational simulations^{211,234} and QSARs (quantitative structure-activity relationships).^{6,15,235} Air/water partitioning experiments are sometimes used to imitate partitioning at the membrane interface,²²⁹ but such experiments do not account for the small heterogeneous microenvironments present in membranes. Differences in lipid-water partitioning thermodynamics of promazine, perazine, and perphenazine (and their halogenated analogs) have been quantified using surface activity measurements. This study found that replacing a hydrogen by a chlorine and trifluoromethyl group increases the partitioning coefficient by factors of ~2 and ~9,

respectively, while the replacement increases the free energy of partitioning on average by -1.3 and -4.5 kJ/mol, respectively.²²⁹ These experiments reported that the increase in partitioning coefficient was dominated by the increase in solute hydrophobicity as opposed to the increase in molecular cross-sectional area.²²⁹ Missing from this study, however, was a description about where these solutes partitioned within a lipid membrane itself.

Several other techniques have also been used to detect drug-membrane interactions, including immobilized lipid chromatograph (ILC),²³⁶ SHG,¹²⁶ FTIR,²³⁷ NMR,²³⁸ fluorescence emission,²²¹ and DSC.²¹⁰ Partitioning of 12 anthracyclines, anticancer drugs, was studied through relating general fluorescence intensity to partitioning depth in the membrane. This study showed that the partition coefficient of anthracyclines in LUVs was generally related to the drug's hydrophobicity.²³⁹ Conboy and coworkers measured the binding affinity of tetracaine as a function of lipid physical state using SHG. The binding affinity of tetracaine to liquid crystalline state DMPC and DPPC (46 °C) was 5,500 M⁻¹ and 2,400 M⁻¹, respectively. Binding of tetracaine to gel state DMPC and DPPC bilayers could not be quantitatively assessed, however, as signal levels were below detection limits.¹²⁶ Temperature effects of membrane partitioning have been studied using ILC. Liu and coworkers found that of 15 drugs studied, membrane partitioning coefficients increased over a broad temperature range, while the partitioning coefficients of four drugs (including three beta blockers) decreased.²³⁶ While these techniques all provide a general framework for describing solute-membrane interactions,

the molecular mechanisms responsible for a solute's membrane affinity remain poorly defined.

In experiments described below, we investigate the partitioning of C152 and C461 in DPPC membranes. Both coumarins used in this work are *N,N*-dimethyl substituted tertiary amines. Coumarin 152 (C152) has an electron withdrawing $-\text{CF}_3$ group in the 4-position, while coumarin 461 (C461) has a weak electron donating $-\text{CH}_3$ group in the 4-position (Figure 5.1). Both molecules are hydrophobic, with C152 having slightly more hydrophobic character ($\log P = 2.72$) than C461 ($\log P = 2.29$) due to the trifluoromethyl group. (Log P values were calculated using ChemDrawPrime 16.0.) The ground state dipole moments of C152 and C461 are identical (~ 6.3 D), while the excited state dipole moment of C152 (12.8 D) is slightly higher than that of C461 (10.0 D).¹⁴⁷ Partitioning of C152 and C461 in DPPC membranes is quantified as a function of bulk C152 concentration as well as the ratio of concentration to DPPC vesicle in solution. Time-correlated single photon counting (TCSPC) fluorescence spectroscopy is used to determine the relative percent of solutes present in distinct lipid vesicle microenvironments. Fluorescence traces are fit to a multi-exponential model, with three distinct lifetimes representing the solute in bulk buffer, near the polar lipid headgroups, and in the nonpolar lipid bilayer core. Additional details of this analysis can be found in Chapter 2.

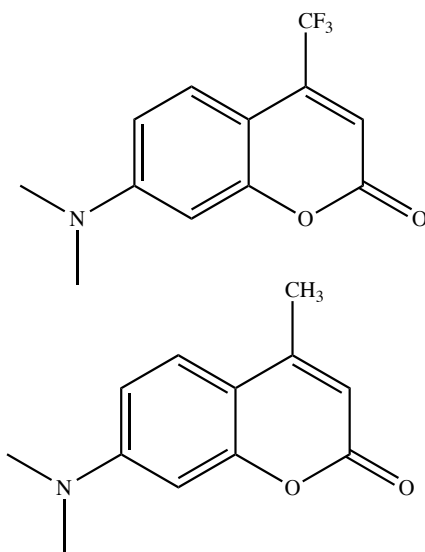


Figure 5.1. Molecular structures of (top) coumarin 152 (C152) and (bottom) coumarin 461 (C461).

Results presented here quantitatively measure C152 and C461 partitioning into phosphocholine membranes above and below each membrane's gel-liquid crystalline transition temperature. Furthermore, characteristic photophysical behaviors are used to clarify *where* solutes partition within a model membrane at different temperatures. Data show that the absolute number of C152 solutes associated to DPPC membranes is approximately an order of magnitude higher than the number of C461 solutes associated to DPPC membranes. Results presented here expand and quantify findings from previous studies investigating C152 partitioning in phosphocholine membranes.²²¹

5.2 Experimental

5.2.1 Materials

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (16:0 DPPC) was purchased in powder form from Avanti Polar Lipids (Alabaster, AL). Laser grade coumarin 152 and coumarin 461 were purchased from Exciton (Dayton, OH). Millipore water (18.2 M Ω) was used to make phosphate buffered saline (PBS buffer). Solvents were purchased from Sigma Aldrich. All materials were used as received.

5.2.2 Large Unilamellar Vesicle Preparation

Large unilamellar vesicles (LUVs) were prepared according to the procedure described in Chapter 2. Briefly, DPPC was dissolved in chloroform and subsequently removed via rotary evaporation, creating a thin lipid film. The film was rehydrated using 10 mM phosphate buffered saline (PBS, pH = 7) to form a 1.5 mM lipid vesicle suspension. The suspension was sonicated for 30 minutes at ~50 °C. The solution was then passed through a PTFE syringe filter (450 nm) to remove the giant unilamellar vesicles and subsequently heated (~50 °C) and passed through an Avanti Mini Extruder (membrane pore size of 200 nm) 10 times. This procedure resulted in a distribution of vesicles having diameters measuring approximately 210 nm in diameter with a low population having markedly smaller diameters (60 nm).

5.2.3 Time-Correlated Single Photon Counting

Time-resolved fluorescence emission data were collected using Picoquant PicoHarp 300 and FluoTime 200 software. An APE Autotracker containing a SHG

crystal situated after a Chameleon laser (Coherent, 80 MHz, 680-1080nm) produced excitation pulses (85 fs pulsewidth) at a wavelength of 400 nm. A Conoptics Model 350-105 modulator maintained a 4 MHz pulse repetition rate. Sample temperatures were maintained by a Quantum Northwest TC152 temperature control. A long-pass filter (80% transmission >455 nm) was placed after the sample to reduce scattering from vesicles in solution. Photon emission was collected at 500 nm. Additional details about this assembly and data analysis can be found in Chapter 2.

The local environments sampled by C152 and C461 in vesicle containing solutions were identified from each fluorescence lifetime and correlated with an equivalent bulk solvent having a quantitatively similar lifetime. The amplitudes associated with individual lifetimes were then corrected for the radiative rate of C152 or C461 in each assigned environment. The resulting amplitudes were normalized, producing the fractional lifetime contribution.

5.2.4 Titrations

To further quantify the partitioning behavior of C152 and C461 in DPPC vesicles, DPPC vesicles were slowly titrated into a coumarin/buffer solution. The fluorescence emission of the coumarin/buffer solutions were recorded from 10 – 70 °C. A DPPC vesicle solution (1.5 mM) was subsequently titrated stepwise into the bulk C152 or C461 solutions. With each step of the titration, fluorescence emission of the solution was recorded from 10 – 70 °C.

5.3 Data and Results

5.3.1 Bulk Fluorescence

Fluorescence absorbance and emission of C152 and C461 in bulk solvents are shown in Table 5.1 and Table 5.2, respectively. Four solvents were chosen to represent the different fluorescence environments C152 and C461 may encounter in a lipid vesicle solution: cyclohexane (hydrophobic membrane interior), acetonitrile and methanol (polar regions near the lipid headgroups), and buffer (unassociated with the vesicle or fully solvated within the water pool inside the vesicle).

Solvent	$\lambda_{\text{abs, max}}$ (nm) ^c	$\lambda_{\text{em, max}}$ (nm) ^c	k_f (10 ⁷ s ⁻¹)	ϕ_f	τ_f (ns) ^d
Cyclohexane	372	426	25.1	0.97 ^a	3.86
Acetonitrile	396	502	9.8	0.23 ^b	2.34
Methanol	397	515	9.0	0.09 ^a	1.09
0.01M PBS Buffer	404	527	8.1	0.05 ^b	0.62 (0.83), 3.75 (0.17)

Table 5.1. Photophysical properties of C152 in selected solvents.

^a Quantum yields as reported by Pal *et. al.*¹⁴⁸

^b Quantum yields measured in this work. Experiments were conducted at room temperature, and remain virtually unchanged from 10 – 70 °C.

^c Measured at room temperature.

^d Uncertainties in reported lifetimes are ± 0.10 ns. Uncertainties in reported amplitudes (for the PBS buffer solution) are ± 0.05 . Data were collected at 10 °C.

Solvent	$\lambda_{\text{abs, max}}$ (nm) ^a	$\lambda_{\text{em, max}}$ (nm) ^a	k_f (10 ⁷ s ⁻¹)	ϕ_f ^a	τ_f (ns) ^b
Cyclohexane	349	390	31.9	0.82	2.57
Acetonitrile	363	431	14.7	0.55	3.73
Methanol	368	451	13.6	0.46	3.38
0.01M PBS Buffer	372	473	5.4	0.08	1.48 (0.92), 5.13 (0.08)

Table 5.2. Photophysical properties of C461 in selected solvents.

^a Measured at room temperature.

^b Uncertainties in reported lifetimes are ± 0.10 ns. Uncertainties in reported amplitudes (for the PBS buffer solution) are ± 0.05 . Data were collected at 10 °C.

Fluorescence emission decay traces of C152 and C461 in bulk solvents are shown in Figure 5.2. Fluorescence lifetimes of C152 vary systematically with solvent polarity. C152 in buffer is fit to two exponential decays, with a large contribution due to a fast (~ 0.5 ns) decay. In polar solvents such as methanol and acetonitrile, the fluorescence lifetime is longer (~ 1 - 2 ns), while in nonpolar solvents, fluorescence decay is significantly longer (~ 4 ns). A correlation with fluorescence lifetime and solvent polarity is not observed for C461.

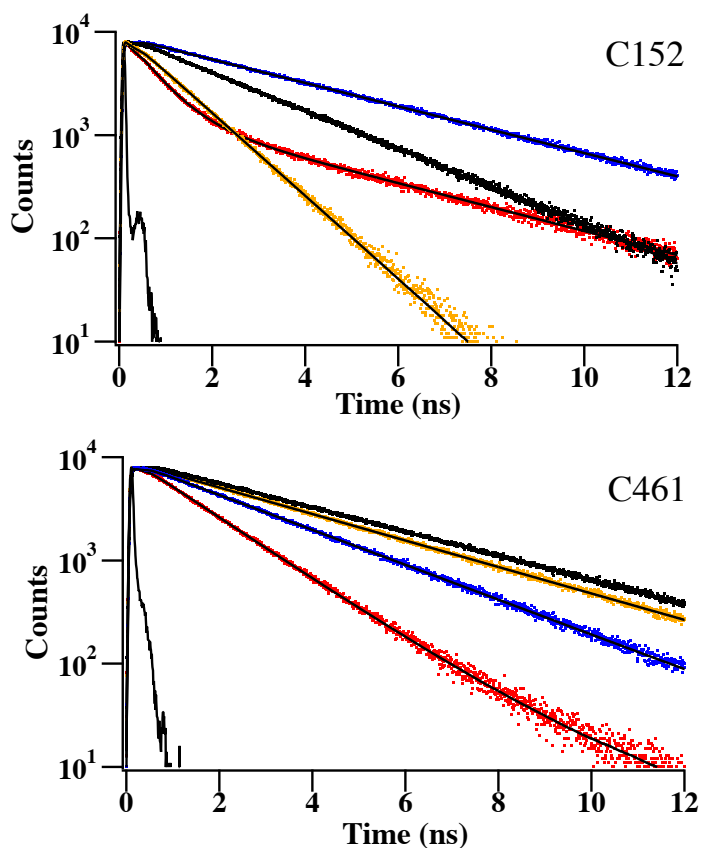


Figure 5.2. Fluorescence traces of C152 and C461 in buffer (red), acetonitrile (black), methanol (orange), cyclohexane (blue). All fluorescence traces were collected at 10 °C.

5.3.2 Fluorescence in DPPC Vesicles

When C152 and C461 are added individually to DPPC vesicle solutions, fluorescence lifetime behavior changes dramatically (Figure 5.3), indicating that vesicles are providing solutes with heterogeneous solvation environments. The fluorescence decay of C152 in DPPC solution is best fit to three lifetimes, while emission decay of C461 in DPPC vesicles is best fit to two lifetimes.

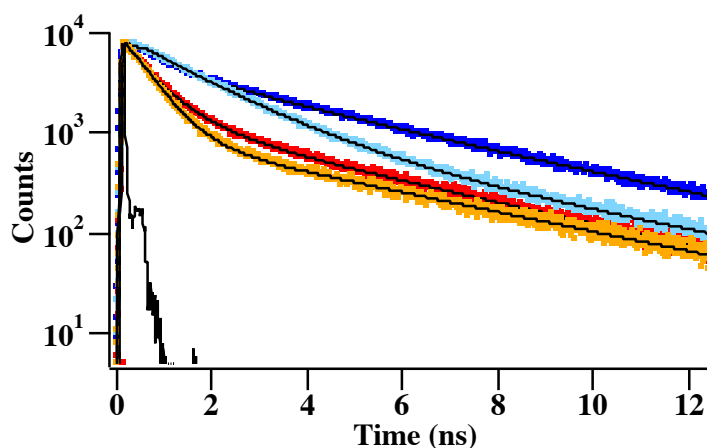


Figure 5.3. Fluorescence traces of C152 in DPPC solutions at 10 °C (dark blue) and 70 °C (red), and C461 in DPPC vesicle solutions at 10 °C (light blue) and 70 °C (orange).

At temperatures from 10 – 70 °C, fluorescence decays of C152 in DPPC vesicle solutions are fit to a short lifetime ($\tau_1 = \sim 0.5$ ns), an intermediate lifetime ($\tau_2 = \sim 1.5$ ns), and a long lifetime ($\tau_3 = \sim 4$ ns). Due to their similarities with lifetimes measured in bulk solvents over the range of temperatures sampled, τ_1 and τ_3 are assigned to C152 solvated in bulk aqueous solution and in the nonpolar, hydrophobic bilayer interior, respectively. Only the intermediate lifetime, τ_2 , changes significantly with temperature, shortening from 2.15 ns at 10 °C to 1.03 ns at 70 °C. The C152 population assigned to this lifetime

is assigned to the solvation environment created by the DPPC glycerol backbone region of the DPPC vesicles. As the lipid bilayer becomes more fluid and permeable to water, τ_2 moves from a value consistent with solvation in a polar aprotic environment to the polar protic limit. This change is believed to reflect increasing hydrogen bonding opportunities within the bilayer interior as water begins to permeate the headgroup region.²²⁶

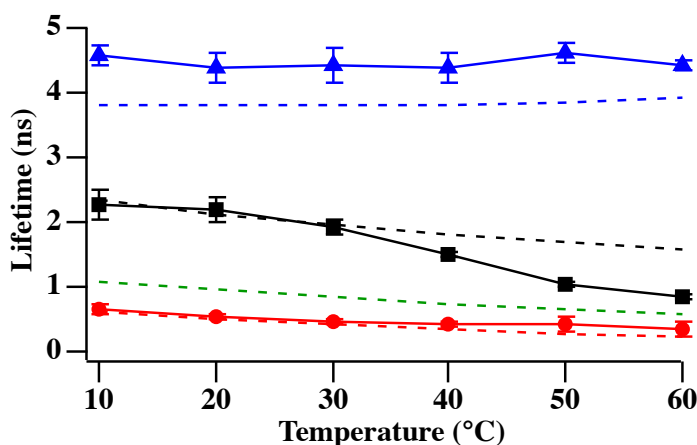


Figure 5.4. Fluorescence lifetimes of C152 in bulk solvents represented by dashed lines: (blue) cyclohexane, (black) acetonitrile, (green) methanol, (red) buffer. The long (minor) lifetime of C152 in buffer is omitted. The three lifetimes of C152 in DPPC vesicles are represented by blue triangles (τ_3 , nonpolar), black squares (τ_2 , polar), and red circles (τ_1 , buffer).

Fluorescence decay traces of C461 in DPPC vesicle solutions were fit to a short ($\tau_1 = \sim 1$ ns) and a long ($\tau_2 = \sim 4.8$ ns) lifetime. As shown in Figure 5.5, the short lifetime (τ_1) of C461 in DPPC vesicles tracks almost exactly with the dominant short lifetime of C461 in buffer. The long lifetime (τ_2) of C461 in the DPPC vesicle solutions is most similar to the long (minor amplitude contribution) lifetime of C461 in buffer solution. However, τ_2 of C461 in DPPC vesicles decreases abruptly between 40 – 50 °C, a trend that differs significantly from the second lifetime of C461 in buffer solution. Since the

melting temperature of DPPC vesicles is in this temperature range, we have assigned this C461 lifetime to solutes associated with the vesicle in some manner. This hypothesis is further confirmed by the trend in fluorescence traces of C461 occurring at lower molar ratios: as the amount of DPPC vesicles present in solution increases, we see τ_2 's amplitude decrease even more significantly.

Molecular dynamics simulations of fully hydrated DPPC bilayers in their liquid crystalline state have provided some insight into the electrostatic interactions occurring between lipid headgroups. When fully hydrated, the charge density of the choline and phosphate groups are completely compensated by the charge density of the dipoles of the surrounding waters (11-16 water molecules).²⁴⁰

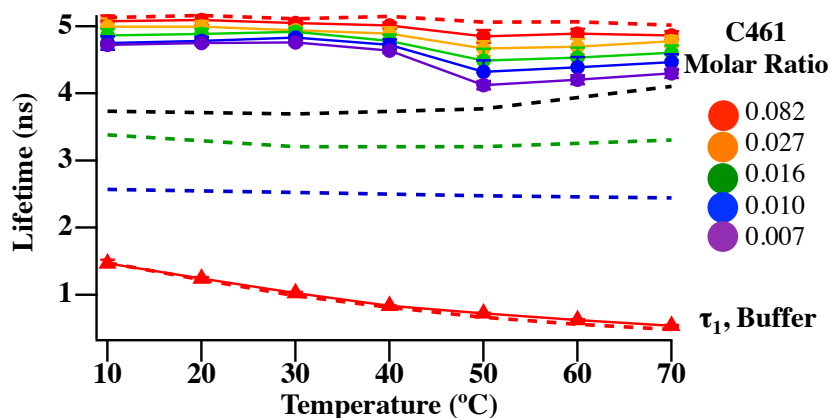


Figure 5.5. Fluorescence lifetimes of C461 in bulk solvents represented by dashed lines: (blue) cyclohexane, (black) acetonitrile, (green) methanol, and (red) buffer. The short lifetime (τ_1) of C461 in DPPC solutions is shown with solid red triangles. The longer lifetime of C461 in DPPC solutions is shown with circles from 4–5 ns.

5.3.3 DPPC Titrations

To quantify partitioning of C152 and C461 in DPPC vesicles, DPPC vesicles were slowly titrated into a coumarin/buffer solution. The fluorescence emission of 2.5 mL of C152 (3.73 μM) was recorded from 10 – 70 °C. A DPPC vesicle solution (1.5 mM) was titrated stepwise into the bulk C152. With each step of the titration, fluorescence emission of the solution was recorded from 10 – 70 °C. A DPPC vesicle solution was added in five steps, corresponding to C152:DPPC lipid ratios of 0.0009, 0.001, 0.002, 0.005, 0.008, 0.012, 0.021, and 0.062. The corresponding C152 bulk concentrations in these solutions were 1.07 μM , 1.24 μM , 1.74 μM , 2.52 μM , 2.83 μM , 3.11 μM , 3.33 μM , and 3.59 μM . However, in related partitioning studies,^{241,242} the concentrations are reported as solute:lipid ratios, and that convention is adopted in the discussion below.

To observe the change in C152 partitioning with temperature, the fluorescence decay was decomposed into three lifetimes and associated amplitudes using Equation 2.2. The raw amplitudes of the fluorescence decays were corrected by the radiative rates of C152 in buffer (τ_1), methanol/acetonitrile (τ_2), or cyclohexane (τ_3). The resulting radiative rate corrected amplitudes were then normalized, resulting in the percent of C152 molecules in each environment.

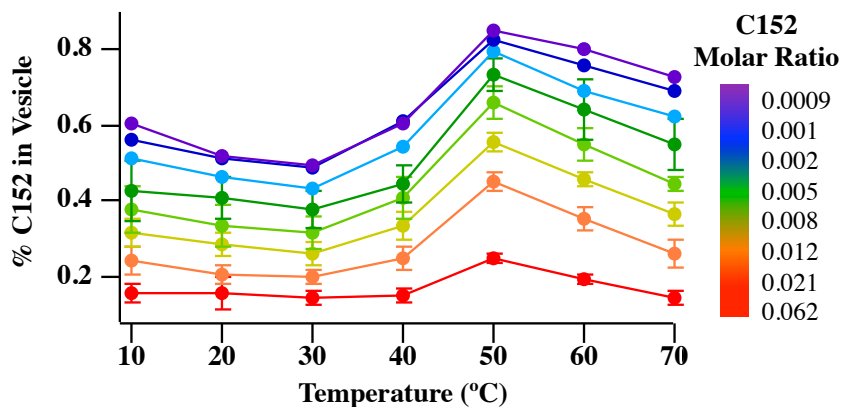


Figure 5.6. Membrane partitioning of C152 in DPPC vesicles as a function of solution temperature and C152:DPPC molar ratio. C152 molar concentration decreases from red to purple.

Figure 5.6 shows the partitioning of C152 into DPPC vesicles as a function of both C152:DPPC molar ratio and temperature. The percent of C152 in vesicles comprises the sum of the amplitudes associated with the medium (τ_2 , polar environment) and long lifetimes (τ_3 , nonpolar environment). At all temperatures, the percent of C152 in the vesicle is strongly dependent on the amount of C152 in the solution. As more DPPC is titrated into the solution, we observe an increase in C152 partitioning into the vesicles. We also observe an increase in partitioning near the melting temperature (42 °C). The percent of C152 in the vesicle reaches a maximum near 50 °C, and C152 exsolvation from the membrane is observed as the temperature is increased further to 70 °C.

C461 (4.11 μ M)/DPPC (1.5 mM) vesicle titrations were conducted similarly to the C152/DPPC titrations described above. The C461:DPPC molar ratios were 0.007, 0.010, 0.016, 0.027, and 0.082. These ratios correspond to bulk C461 concentrations of 2.94

μM , $3.24 \mu\text{M}$, $3.52 \mu\text{M}$, $3.74 \mu\text{M}$, and $3.98 \mu\text{M}$. C461/DPPC fluorescence traces were fit to two lifetimes. The short lifetime, τ_1 , remains identical (within error) to the short lifetime of C461 in a pure buffer solution (Figure 5.5) and therefore C461 with this fluorescence decay are assumed to remain unassociated with the DPPC membrane. The long lifetime of C461 in the DPPC membrane (τ_2) was attributed to C461 associated with the vesicle membrane. The amplitudes associated with τ_1 and τ_2 were corrected for the radiative rates of C461 in buffer and acetonitrile, respectively, to determine partitioning ratios.

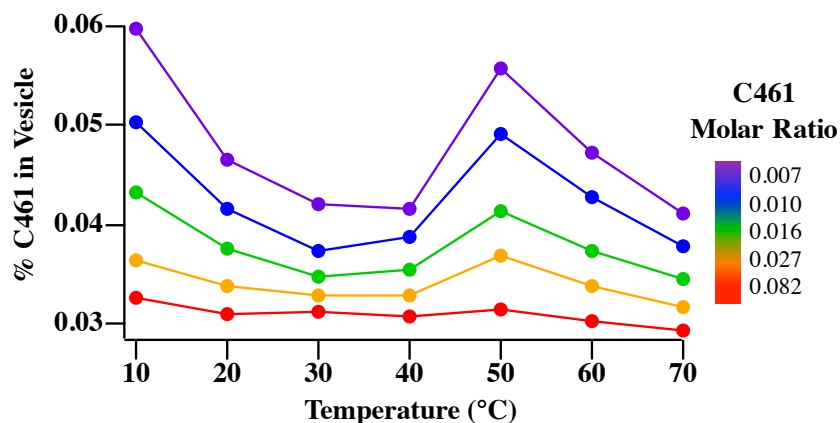


Figure 5.7. Membrane partitioning of C461 in DPPC vesicles as a function of solution temperature and C461:DPPC molar ratio.

The percent of C461 was also plotted as a function of molar ratio and temperature (Figure 5.7). Below the melting temperature of DPPC, the trend in partitioning differs from that of C152 in the same temperature range. At the lowest temperature (10 °C), C461 association with the membrane is relatively large (relative to the other temperatures investigated), although still much less than C152 partitioning at any temperature. As the

temperature increases towards the melting temperature, C461 dissociates from the membrane into solution. This trend is more pronounced at low C461:DPPC molar ratios. Similar to the behavior of C152 in DPPC vesicles, an increase in solute-membrane association is observed at DPPC's melting temperature. Above 50 °C, we once again observe C461 exsolvation from the membrane.

The most striking result of these coumarin/DPPC titration experiments is the difference in magnitude of membrane association. At all temperatures investigated, the average absolute number of C152 molecules associated with vesicles is an order of magnitude larger than the average number of C461 molecules associated with each vesicle. To visualize this comparative partitioning, data presented in Figures 5.6 and 5.7 can be used to determine the number of coumarins partitioning into vesicle membranes (as a function of temperature and mole concentration). Using several coarse approximations, the number of vesicles in solution was determined using the number of DPPC lipids in solution. When assuming a spherical vesicle with an average 200 nm diameter (based on DLS results), an average bilayer thickness of 4 nm²²⁵, and an average DPPC molecular area of 57 Å²,²⁴³ each DPPC vesicle contains ~400,000 DPPC molecules. With these assumptions and following the concentrations of coumarins and DPPC solutions for each titration, we calculated the average number of coumarins per vesicle at different coumarin molar concentrations (Figure 5.8).

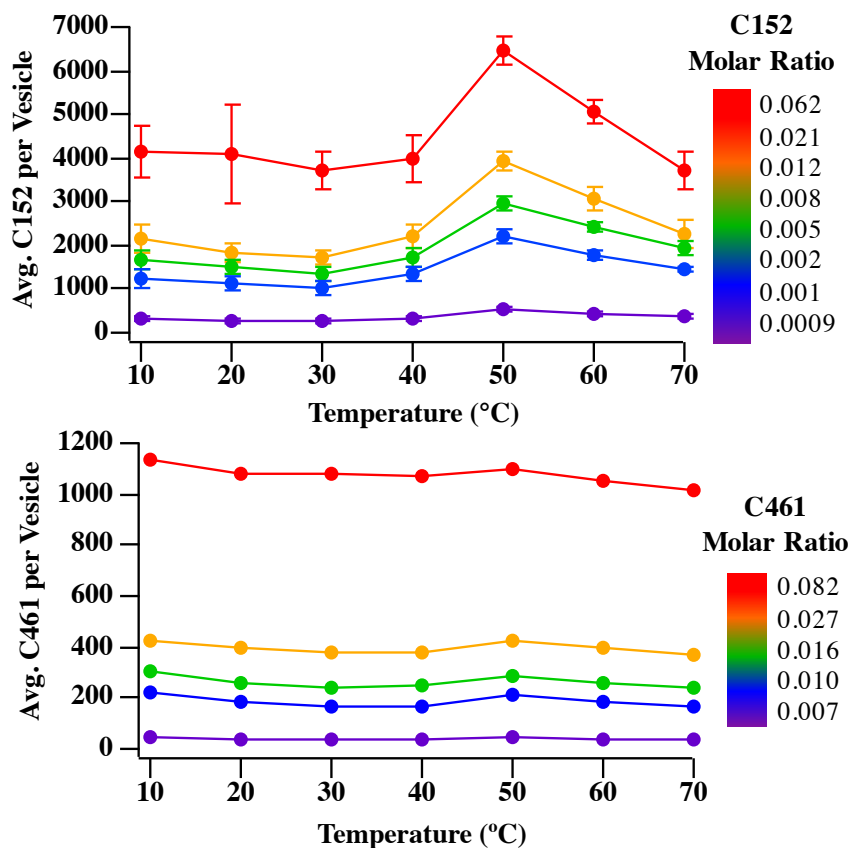


Figure 5.8. Average number of C152 (top) and C461 (bottom) molecules per vesicle as a function of temperature. Colored traces indicate the molar ratio of coumarins to DPPC lipids.

At first, Figures 5.6 and 5.7 appear to contradict Figure 5.8. Figures 5.6 and 5.7 show that at higher mole ratio of coumarins to DPPC lipids, a smaller percent of coumarin solutes associate with vesicles. Figure 5.8 and Tables 5.3 and 5.4 clarify this apparent contradiction by showing that as the number of DPPC vesicles in solution increases, the number of coumarins associated with each vesicle also increases.

Mole Ratio (C152:DPPC)	[C152]_{bulk} (μM)		10 °C	30 °C	50 °C	70 °C
0.062	3.59	# C152	4200	3700	6500	3700
		% C152	16%	14%	25%	14%
0.008	2.83	# C152	1200	1000	2200	1500
		% C152	38%	32%	66%	45%
0.002	1.74	# C152	500	400	700	600
		% C152	52%	43%	80%	62%
0.0009	1.07	# C152	300	200	400	300
		% C152	60%	50%	85%	73%

Table 5.3. Average number of C152 and percent of C152 per vesicle in select steps of C152:DPPC titration.

Mole Ratio (C461:DPPC)	[C461]_{bulk} (μM)		10 °C	30 °C	50 °C	70 °C
0.082	3.98	# C461	1140	1080	1100	1020
		% C461	3.3%	3.1%	3.1%	3.0%
0.027	3.74	# C461	420	380	430	370
		% C461	3.6%	3.3%	3.7%	3.2%
0.01	3.24	# C461	220	160	210	170
		% C461	5.0%	3.7%	5.0%	3.8%
0.007	2.94	# C461	50	40	50	30
		% C461	6.0%	4.2%	5.6%	4.1%

Table 5.4. Average number of C461 and percent of C461 per vesicle in select steps of the C461:DPPC titration.

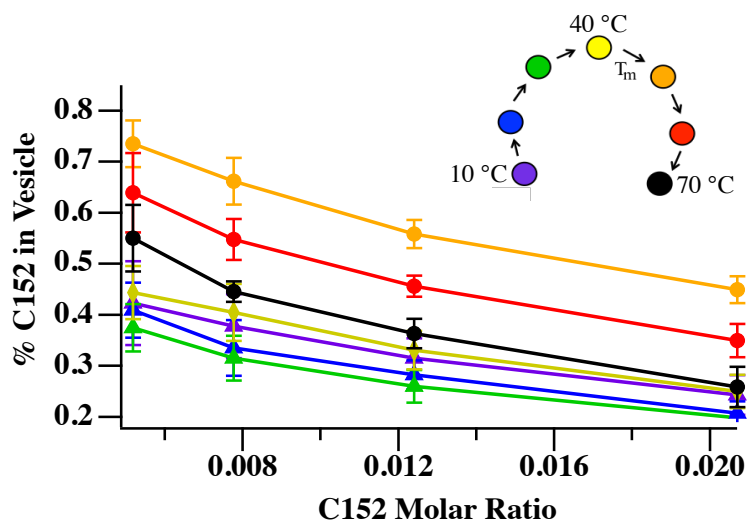


Figure 5.9. Percent of C152 population solvated in the DPPC bilayer are plotted as a function of C152 molar ratio. Colored traces indicate solvation at 10 °C (purple), 20 °C (blue), 30 °C (green), 40 °C (yellow), 50 °C (orange), 60 °C (red), and 70 °C (black).

At all C152 molar concentrations investigated, the percent of C152 that partitions into the vesicle is strongly temperature dependent (+30% increase in partitioning from gel to liquid crystalline). Figure 5.9 also shows that at a given mole fraction, C152 partitioning remains similar at all temperatures below the melting temperature. Based on the similarity between the traces at 30 and 40 °C, no evidence of any change in partitioning accompanies DPPC structural change at the “pre-transition” temperature (35 °C). This result implies that changes in bilayer structure and fluidity accompanying the pretransition are not significant enough to induce any changes in partitioning behavior.

5.3.4 Partitioning Coefficients

The partitioning of C152 into DPPC vesicles can also be cast as the equilibrium constant of C152 in the aqueous phase and the lipid phase (K_{part}) as a function of the C152 molar ratio (Figure 5.10) and bulk C152 concentration (inset).

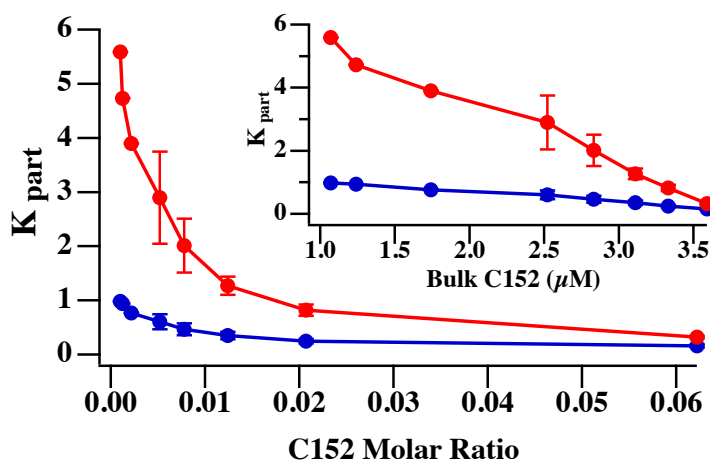


Figure 5.10. Partitioning coefficient (K_{part}) of C152 in DPPC vesicles as a function of C152:DPPC molar ratio and (inset) bulk C152 concentration. Red traces indicate partitioning coefficients of C152 into liquid crystalline phase DPPC membranes (50 °C), and blue traces indicate partitioning coefficients of C152 into gel phase DPPC membranes (30 °C).

Regardless of the mole ratio of C152:DPPC in Figure 5.10, the partitioning coefficient (K_{part}) of the solution at 50 °C remains larger than the K_{part} of the solution at 30 °C. Furthermore, data recorded below the melting temperature of the vesicles (42 °C) show little dependence on mole ratio of C152:DPPC. These results demonstrate clearly that when DPPC is in its gel phase, the ratio of C152 in the lipid bilayer to aqueous buffer remains constant, regardless of temperature. Most interesting is the drastic change in the partition coefficient of C152 in DPPC vesicles as a function of C152:DPPC molar ratio. At high molar ratios, the partitioning coefficient in both the gel and the liquid crystalline

phases decreases towards zero (as expected in the limit of excess C152 bulk solution concentrations). As the C152:DPPC molar ratio decreases, we observe a steep increase in the partition coefficient, indicating we are reaching the dilute limit. At infinitely small C152 mole ratios, we expect K_{part} to reach ~ 500 (as predicted by the log P of C152).

5.4 Thermodynamic Analysis

A van't Hoff plot analysis (Figure 5.11) was used to calculate the enthalpic and entropic contributions to the thermodynamics of C152 membrane permeation. Partitioning is analyzed as a function of the amount of C152 in the lipid membrane to the amount C152 in the aqueous phase (K_{part}).

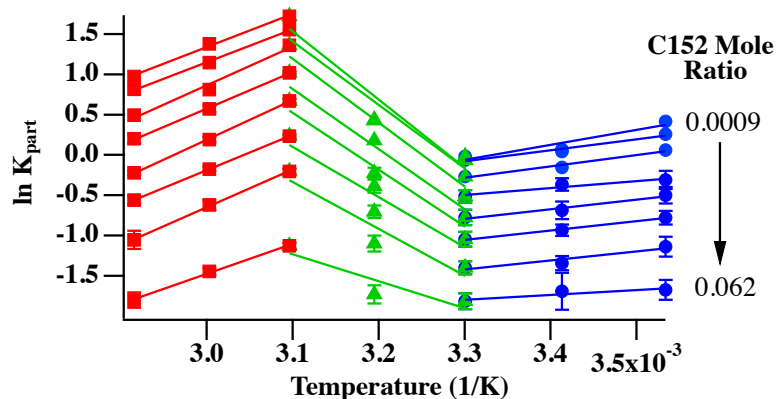


Figure 5.11. van't Hoff analysis of C152 partitioning into DPPC vesicles as a function of temperature. The color schemes indicate the temperature range (blue circles) below DPPC melting temperature, (green triangles) near the transition temperature of DPPC, and (red squares) above the melting temperature. C152:DPPC mole ratios decrease with increasing partition coefficients.

The partitioning is further analyzed in terms of the enthalpic and entropic contributions to the overall C152 partitioning thermodynamics (Figure 5.12 and Table 5.5).

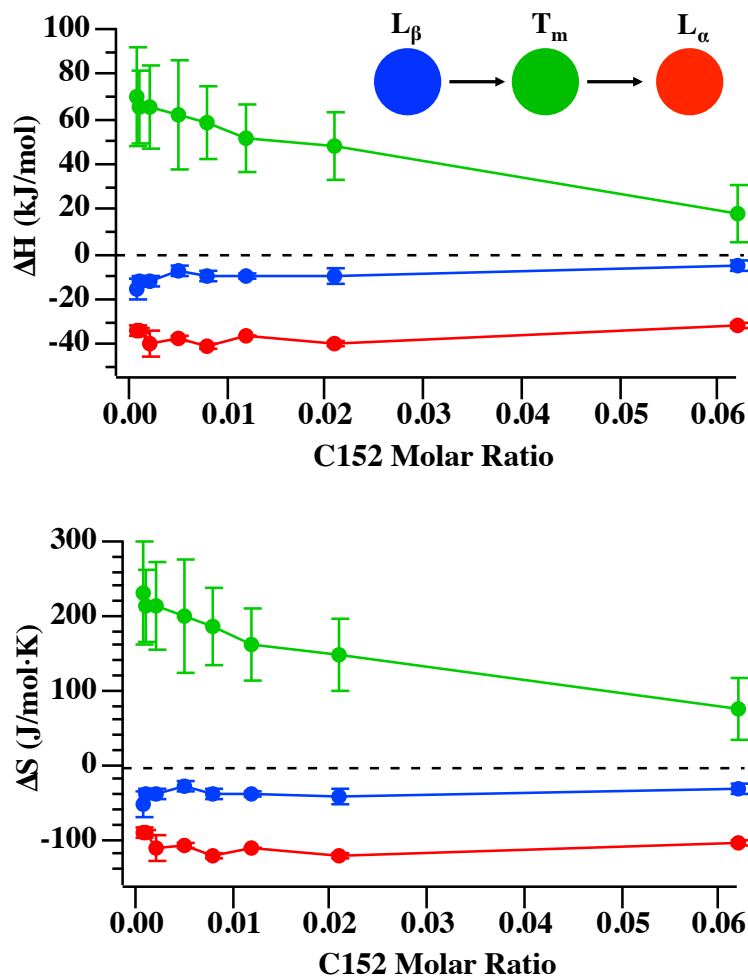


Figure 5.12. Enthalpy (top) and entropy (bottom) of C152 partitioning into DPPC vesicles as a function of C152:DPPC molar ratio. The three lines in each figure indicate the temperature range (blue) below DPPC melting temperature, (green) near the transition temperature of DPPC, and (red) well above the melting temperature.

Bulk C152 Conc. (μM)	Molar Ratio (C152: DPPC)	$T < T_m$		$T \sim T_m$		$T > T_m$	
		ΔH (kJ/mol)	ΔS (J/mol K)	ΔH (kJ/mol)	ΔS (J/mol K)	ΔH (kJ/mol)	ΔS (J/mol K)
3.59	0.062	-5 ± 2	-32 ± 8	19 ± 13	76 ± 41	-31 ± 2	-105 ± 5
3.33	0.021	-9 ± 3	-43 ± 10	48 ± 15	147 ± 48	-39 ± 1	-123 ± 3
3.11	0.012	-10 ± 1	-41 ± 2	52 ± 15	161 ± 48	-37 ± 1	-111 ± 1
2.83	0.008	-10 ± 2	-39 ± 6	59 ± 17	186 ± 54	-41 ± 1	-122 ± 4
2.52	0.005	-7 ± 2	-28 ± 7	62 ± 24	199 ± 76	-38 ± 1	-108 ± 4
1.74	0.002	-12 ± 2	-40 ± 6	66 ± 19	214 ± 60	-40 ± 5	-113 ± 16
1.24	0.001	-11 ± 2	-38 ± 7	65 ± 16	215 ± 50	-34 ± 1	-93 ± 4
1.07	0.0009	-15 ± 5	-52 ± 17	70 ± 21	231 ± 68	-34 ± 2	-92 ± 7

Table 5.5. Phase dependent enthalpy and entropy contributions to C152 partitioning in DPPC membranes.

The enthalpic and entropic forces governing C152 partitioning into lipid membranes are the sum of the enthalpies and entropies arising from two distinct but coupled processes: C152 solvation (or desolvation) in bulk buffer, and C152 desolvation (or solvation in the lipid membrane).

Entropic and enthalpic contributions to membrane partitioning differ significantly with the physical phase of the membrane. Below the melting temperature of DPPC, C152 partitioning is slightly exothermic and entropically favored. Small thermodynamic contributions to partitioning in gel-phase membranes have similarly been reported throughout literature. Kwon *et al.* investigated the partitioning of 16 endocrine disruptors in gel-phase DPPC membranes. 14 of the selected solutes showed entropic driven partitioning (~ 5 to 55 J/(mol K)) with a small endothermic contribution ($+2$ to 30 kJ/mol).²⁴⁴ Rogers and coworkers found the partitioning of four p-alkylphenols in gel-

phase DMPC membranes to be endothermic ($\sim +1$ to 9 kJ/mol) and entropically favorable ($+65$ to 75 J/(mol K)).²⁴⁵

Near DPPC's melting temperature we observe a large increase in partitioning, driven entropically with a large endothermic contribution. In this temperature range, small changes in temperature create large structural changes within the bilayer, including an increase in volume ($\sim 4\%$), area (15 - 25%),²⁴⁶ and enthalpy (20 - 40 kJ/mol).²⁴⁷ The structural changes in the membrane in this temperature region accompany large lateral lipid density fluctuations. We attribute the increase in membrane partitioning in this narrow temperature region to be a result of the increase in lipid density fluctuations.

Above the melting temperature, we observe enthalpy driven C152 partitioning as evidenced by a relatively large ΔH_{part} , a result similar to findings in numerous other studies. van Wezel *et al.* investigated partitioning of a series of chlorobenzenes partitioning into DPPC vesicles in the liquid crystalline phase. For all 7 analogues studied, partitioning was exothermic (~ -10 to -30 kJ/mol) and slightly entropically favored ($20 - 60$ J/(mol K)).⁸⁰ Partitioning of paclitaxel, an anti-cancer drug, into liquid-crystalline POPC vesicles was found to be exothermically driven (-105 kJ/mol).²⁴⁸ Partitioning of three indole derivatives into liquid-crystalline POPC membranes was similarly found to be exothermic (-21 to -30 kJ/mol) and slightly entropically favorable ($10 - 40$ J/mol K).¹⁹⁹

Although enthalpic and entropic behavior could tentatively be assigned to specific processes in the C152/DPPC lipid vesicle system, results from these experiments cannot negate or validate the proposed origin of these thermodynamic parameters. Molecular

dynamic simulations of C152 in lipid vesicles would be advantageous in both confirming the results presented in this work and elucidating the processes that provide the enthalpic and entropic contributions that drive solute partitioning.

Additionally, of interest is the apparent differences in thermodynamic parameters found for solutions with different C152:DPPC molar ratios. Below the melting temperature, the enthalpy remains small and negative for all C152:DPPC mole ratios. Above the phase transition temperature, partitioning is more strongly exothermic and entropically unfavorable. Similar to below the melting temperature, we see no systematic change in enthalpy or entropy of partitioning at different mole ratios. Near the phase transition temperature, however, the enthalpy of partitioning becomes endothermic and entropically favorable (Figures 5.11 and 5.12 and Table 5.4). Partitioning energetics within this small temperature range are derived from a line fit to three points (for each mole ratio). Moreover, lines fit to the data in this region consistently miss the data point at 40 °C. The origin of this behavior remains unresolved.

A similar van't Hoff analysis was used to treat C461 membrane partitioning (Figure 5.13). The nonlinearity of the slope of the C461 van't Hoff plot near and below the phase transition temperature of DPPC prevents a definitive analysis of these data. We attribute the nonlinearity of these data to multiple mechanisms of solute-membrane association.

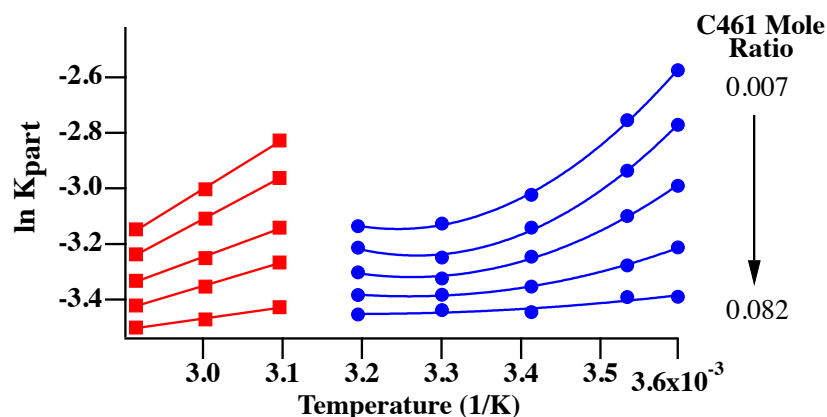


Figure 5.13. van't Hoff plot analysis of C461 partitioning in DPPC vesicle solutions. The color schemes indicate the temperature range (blue circles) below DPPC melting temperature and (red squares) above the melting temperature. C152:DPPC mole ratios decrease with increasing partition coefficients.

A similar analysis was performed with data recorded from C461/DPPC titrations (Figure 5.13). Partitioning thermodynamics could be determined for temperatures higher than the melting temperature (Table 5.6). However, near the melting temperature (and temperatures below the melting temperature), data show nonlinear behavior. Such behavior on a van't Hoff indicates the presence of different equilibrium states and a balance of complex partitioning behavior at these different temperatures.

Bulk C461 Conc. (μM)	Molar Ratio (C461:DPPC)	ΔH (kJ/mol)	ΔS (J/mol K)
3.98	0.0822	-3.4 ± 0.3	-39 ± 1
3.74	0.0274	-7.1 ± 0.4	-49 ± 1
3.52	0.0164	-8.8 ± 0.6	-53 ± 2
3.24	0.0103	-12.7 ± 0.2	-63 ± 1
2.94	0.0069	-14.7 ± 0.5	-69 ± 2

Table 5.6. Enthalpy and entropy contributions to C461 partitioning in DPPC membranes above the melting temperature (42°C).

C461 partitioning above the melting temperature is slightly exothermic and entropically unfavorable. The magnitude of exothermicity of partitioning above the melting temperature is significantly smaller for C461/DPPC membrane partitioning than for C152/DPPC partitioning. We attribute the smaller (magnitude) enthalpy in C461/DPPC partitioning studies to the smaller relative number of solutes interacting with the membrane at any given temperature (Table 5.4).

5.5 Conclusions

Data presented in this chapter have shown the similarities and differences in temperature-dependent partitioning of coumarin 152 and coumarin 461 in DPPC lipid vesicles. Results indicate that C152 has a ~10-fold greater affinity than C461 for lipid bilayers, despite both solutes having similar hydrophobicities as calculated from their log P values. The increase in membrane affinity is attributed to the presence of a -CF₃ group present in the 4-position of C152. Results presented here support several other empirical studies that link drug halogenation to increased membrane affinity.²²⁸⁻²³⁰ An important contribution from work described in this chapter is the quantitation of dissolved solute accommodated by vesicles as a function of solution temperature and solute concentration. We expect that similar information can come from related systems provided that the solute of interest has well defined properties that are distinctly sensitive to heterogeneous solvation environments.

Temperature dependent partitioning data were also used to calculate enthalpies and entropies of C152 partitioning as a function of concentration. These values were

used to extrapolate to the infinitely dilute limit. Above and below the lipid gel-liquid crystalline temperature, partitioning is exothermic with negative changes in entropy. Near the transition temperature, these quantities change sign with ΔH_{part} becoming endothermic (+40-70 kJ/mole) and $\Delta S_{\text{part}} = +100\text{-}200 \text{ J}/(\text{mole K})$. Enthalpic and entropic contributions represent a sensitive balance between solute solvation in the lipid membrane or bulk aqueous buffer. Also contributing to these thermodynamics are the energetic costs (or gains) due to membrane disruption (solute insertion) or membrane reordering (solute exsolvation).

5.6 References

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CHAPTER SIX

TEMPERATURE DEPENDENT PARTITIONING OF C152 IN BINARY
PHOSPHATIDYLCHOLINE MEMBRANES AND MIXED
PHOSPHATIDYLCHOLINE/PHOSPHATIDYLETHANOLAMINE
MEMBRANES

6.1 Introduction

Since the “fluid-mosaic model” of the cell membrane was proposed in 1972²⁴⁹, scientists have been investigating the influence of lipids on cellular membrane structure and function.²⁵⁰ Of particular interest is how lipids are distributed within the outer and inner leaflets of the membrane bilayer.^{251,252} Entropic considerations would lead to a homogeneous distribution of lipid mixtures with each leaflet having equivalent compositions. However, in mammalian cells, distinct heterogeneity is observed between inner and outer leaflets. For example, the inner leaflet of the cell membrane generally is enriched in phosphatidylserine and phosphatidylethanolamine, and the outer leaflet is enriched with phosphatidylcholine. Deviation in standard lipid distribution is often an indicator for other cellular processes. For example, an enrichment of phosphatidylserine in the outer leaflet is a susceptibility signal for phagocytosis and a propagation signal for blood coagulation.^{1,253,254} Transmembrane lipid arrangement is yet another example of local lipid ordering in lipid bilayers. Fluid phase binary mixtures of lipids differing by only 2 methylenes per tail have been shown to anti-correlate in positions across a bilayer in a phenomenon termed “transbilayer complementarity.”²⁵⁵

Furthermore, lipids (as well as other membrane components) are not homogeneously mixed within individual membrane leaflets. Lipid domains or “rafts” are formed in fluid membranes creating lateral heterogeneity.²⁵⁶ Lipid domain size varies from nanometer to micrometer diameter.^{250,257,258} Directly related to lipid domain size and composition is their cellular function. Lipid domains are instrumental in gene expression, enzyme binding, enzyme activation, and cell division.^{252,254,256,259,260}

Heterogeneity is even observed in lipid membranes composed of one type of lipid. Near main phase transition, clusters of 2° phases form in the bulk equilibrium phase, i.e. below the transition temperature, liquid-crystalline phase domains form in the bulk gel phase, and above the phase transition temperature, gel phases form in the bulk liquid-crystalline phase. The average domain size is a sensitive equilibrium that relies on the precise temperature of the system as well as the characteristics of the lipid (or lipids) comprising the membrane.

Phosphatidylcholines and phosphatidylethanolamines differ only by the headgroup (Chapter 2). Both lipids are zwitterionic (positive amine and negative phosphate group) at neutral pH. Elaborate networks of interconnected lipids and water molecules are formed in PC bilayers. Computational simulations of interfacial water at pure liquid-crystalline phase DMPC membranes show that water bridges link 76% of DMPC molecules, while coulombic associations are observed between 93% of the DMPC lipids.^{222,261} PE membranes include far fewer interactions with water. Instead, the stability of these membranes (and higher phase transition temperature) is attributed to the

interactions that occur between neighboring PEs, including hydrogen bond donating and accepting and strong Coulomb associations.^{197,262-267}

To identify structure-function relationships within complex biological membranes, lipid mixing has been studied in model membranes composed of binary or ternary phospholipid mixtures. Studies use a multitude of computational strategies²⁶⁸⁻²⁷⁰ and experimental methods including DSC,^{260,271} fluorescence,^{272,273} ESR,^{62,274} electron microscopy,²⁷⁵⁻²⁷⁸ and NMR.^{279,280}

Lipid domain formation and lipid mixing phenomena are of great interest to the pharmaceutical industry, as membrane lipids often control the ADMET (absorption, distribution, metabolism, excretion, and toxicology) of drugs. Overwhelming evidence has shown that the permeation of certain solutes is enhanced near the phase transition temperature of PC membranes. Enhanced permeation is thought to be a consequence of unique solvation environments resulting from interfacial boundaries of lipid domains that are present only near the melting temperature.^{59,281} The physical structures of lipid domains and their interfaces are dependent on many factors including lipid headgroup, acyl chain length, and the precise temperature of the system. Although enhancement of solute permeation near the phase transition temperature is well documented and generally accepted to be a consequence of lipid domain formation, reports in the literature generally lack molecularly specific descriptions of *how* solutes permeate into mixed lipid membranes near the phase transition temperature.

Miscibility in binary mixtures of phosphatidylcholines has been studied extensively. The lipid miscibility in DMPC/DPPC membranes was studied using ESR.

Results show solid and liquid-crystalline phase coexistence at 30 °C for DMPC membranes composed of 25-60 mol percent DPPC.²⁵⁶ DSC has also been used in numerous lipid miscibility studies.^{82,268,274,282,283} In general, binary mixtures of saturated phosphatidylcholines are miscible if the difference in acyl chain length is two carbons long. When the acyl chains of two PCs differ by four or more carbons, non-ideal mixing (lateral phase segregation) is observed.^{284,285} Time-resolved fluorescence experiments described in this chapter were used to determine if solute partitioning behavior depended on the ideal/non-ideal phase behavior observed in lipid bilayers consisting of two separate components.

In one of many published studies, Silvius *et al.* used DSC to determine lipid miscibility in the solid and liquid phases of binary phosphatidylcholine/phosphatidylethanolamine mixtures.²⁷¹ Results indicate that binary mixtures of DMPC and DMPE and binary mixtures of DPPC and DPPE are completely miscible in the liquid phase. Using ³¹P-NMR, Arnold *et al.*²⁷⁹ characterized lipid miscibility in the same system. As the temperature of the mixed membrane system was decreased, DPPE crystallized first due to its higher melting temperature. As the temperature was decreased even further, DPPC also crystallized, but lipids in clusters with similar lipids (complete immiscibility). Even above the melting temperatures of both lipids (completely fluid membrane), ³¹P-NMR results suggest a nonrandom distribution of DPPE and DPPC molecules. These findings indicate interactions between two DPPC molecules or two DPPE molecules are stronger than between a DPPC and DPPE molecule. Because the

acyl chains of DPPC and DPPE are identical, the non-ideal mixing behavior was attributed to the difference in the headgroup composition.^{271,275,279}

Fewer studies investigate the permeability of solutes in mixed lipid membrane systems. The permeability of ^{22}Na was measured as a function of temperature for a series of PCs (DMPC, DPPC, and DSPC) and PEs (DLPE, DMPE). Increased permeability was observed in the phase transition region for all three PCs, with the largest increase in permeability observed in DMPC vesicles ($> \text{DPPC} > \text{DSPC}$). No increase in ^{22}Na permeability was observed in vesicles composed of DLPE or DMPE near their phase transition temperatures.²⁸¹ Results indicate that permeability in PC bilayers is enhanced near the melting temperature due to lateral domain formation. Characteristics of lipid domains including size and structure are a function of PC acyl chain length, and thus permeability can be directly correlated to the identity of phosphocholine or phosphocholine mixture. In PE bilayers, membrane melting does not alter the permeability of ^{22}Na . Of interest for work presented below is whether the presence of PE in lipid vesicles composed of PC and PE will create a wider variety of interfaces in which C152 may partition (enhancing overall partitioning) or whether the intermolecular lipid interactions between PEs and neighboring PCs or PEs prevent solute partitioning.

In this chapter, binary mixtures of two phosphatidylcholines (DLPC/DMPC) and binary mixtures of a phosphatidylcholine and a phosphatidylethanolamine (DMPC/DMPE) are investigated using differential scanning calorimetry. Calorimetric traces provide insight into the mixing behavior of PCs and PCs/PEs in the gel and liquid

crystalline phases. Experimental data were compared to theoretical ideal mixing phase diagrams.

Of specific interest was how C152 partitioning membranes would be altered if the membrane were composed of two similar phosphatidylcholines (12:0/12:0 DLPC and 14:0/14:0 DMPC), or a phosphatidylcholine and a phosphatidylethanolamine (14:0/14:0 DMPC and 14:0/14:0 DMPE). Time-correlated single photon counting was used to determine the relative amount of C152 partitioning into model membranes and how the partitioning behavior is altered by temperature. Studies presented in this chapter show how C152 partitioning changes when a solute is introduced to membranes with specific features: miscible or immiscible gel phases, coexisting gel and liquid-crystalline phases, and miscible or immiscible liquid-crystalline phases. Results show nearly ideal partitioning of C152 into all mixtures investigated, regardless of membrane composition or deviation of lipid mixing from ideal behavior.

6.2 Differential Scanning Calorimetry

The melting temperatures of 20 mM lipid vesicles were determined using differential scanning calorimetry. Details of sample preparation and instrumentation are found in Chapter 2.7.

6.2.1 DLPC/DMPC Mixtures

The calorimetric traces of vesicles composed of pure DLPC, pure DMPC, and mixtures of DLPC and DMPC are shown in Figure 6.1. The melting temperatures are summarized in Table 6.1.

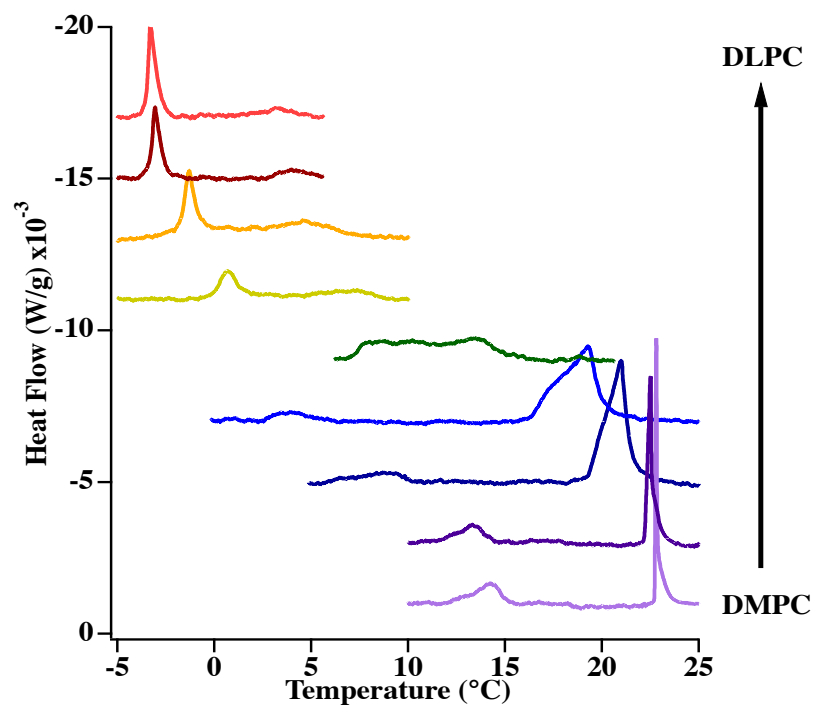


Figure 6.1. Calorimetric traces of DMPC vesicles (light purple), DLPC vesicles (red), and binary mixtures of DLPC and DMPC: 0.99 DMPC (dark purple), 0.90 DMPC (dark blue), 0.80 DMPC (royal blue), 0.50 DMPC (green), 0.20 DMPC (yellow), 0.10 DMPC (orange), 0.01 DMPC (dark red).

Mole Ratio DMPC:DLPC	T_m (°C)
0:100	-3.3
1:99	-3.1
5:95	-2.2
10:90	-1.3
20:80	0.7
50:50	7.3-14.9
80:20	16.4-20.5
90:10	19.4-22.1
95:5	22.0
99:1	22.5
100:0	22.8

Table 6.1. Melting temperatures of large unilamellar vesicles composed of binary mixtures of DLPC and DMPC.

Previous calorimetric studies of binary phosphocholine lipid mixtures have shown ideal mixing behavior when the two alkyl chains of the phosphatidylcholines differ by only two carbons (as is the case in with DLPC and DMPC). Evidence of miscibility in these vesicles is found by comparing phase diagrams derived from the experimental data with expectations based on ideal mixing behavior. In binary mixtures of lipids, the presence of one main calorimetric peak can indicate lipid miscibility. However, binary lipid mixtures more often exhibit broad phase transitions, indicating some degree of non-ideal behavior and domain formation. The broad calorimetric traces are thought to result from one species of lipid melting and expelling the higher melting-temperature lipid from the newly formed liquid-crystalline phase.²⁶⁰ Computational simulations were used to investigate the nature of the domains that formed in a mixture of DPPC (16:0) and 20:0 PC. As expected, gel domains contained acyl chains of 20:0 PC while the fluid region was composed mostly of 16:0 PC. However, simulations showed that the interface of the fluid 16:0 PC regions and the gel 20:0 PC regions was enriched by gel phase 16:0 PC.⁶² Regardless of membrane composition (single or multiple lipid species), domains form in the vicinity of the membrane melting temperature. Of interest is whether the formation of new varieties of domains in binary PC lipid mixtures near their melting temperatures affects solute/membrane interactions.

All DSC traces in Figure 6.1 (with the exception of equimolar DLPC/DMPC) show a singular (primary) transition temperature, indicating these membranes enjoy a large degree of lipid miscibility. To qualitatively investigate the degree of ideality in binary PC membrane mixtures, the theoretical ideal mixing curves were calculated

according to Sturtevant *et al.*⁸² (Figure 6.2). The dashed lines in Figure 6.2 are the ideal solidus (s) and liquidus curves (l), calculated using the following expressions:

$$X_{\text{DMPC}}^{(l)} = \frac{1-\alpha}{\beta-\alpha} \quad (6.1)$$

$$X_{\text{DMPC}}^{(s)} = \beta X_{\text{DMPC}}^{(l)} \quad (6.2)$$

where $X_{\text{DMPC}}^{(l)}$ and $X_{\text{DMPC}}^{(s)}$ are the mole fractions of DMPC in the liquidus and solidus phases, and α and β are described by:

$$\alpha = \exp \left[\frac{\Delta H_{\text{DLPC}}}{R} \left(\frac{1}{T} - \frac{1}{T_{\text{DLPC}}} \right) \right] \quad (6.3)$$

$$\beta = \exp \left[\frac{\Delta H_{\text{DMPC}}}{R} \left(\frac{1}{T} - \frac{1}{T_{\text{DMPC}}} \right) \right] \quad (6.4)$$

where ΔH_{DLPC} is the enthalpy of transition of pure DLPC (+7.1 kJ/mol), ΔH_{DMPC} is the enthalpy of transition of pure DMPC (+22.8 kJ/mol), T_{DLPC} is the melting temperature of pure DLPC (-3.3 °C), and T_{DMPC} is the melting temperature of pure DMPC (22.8 °C).

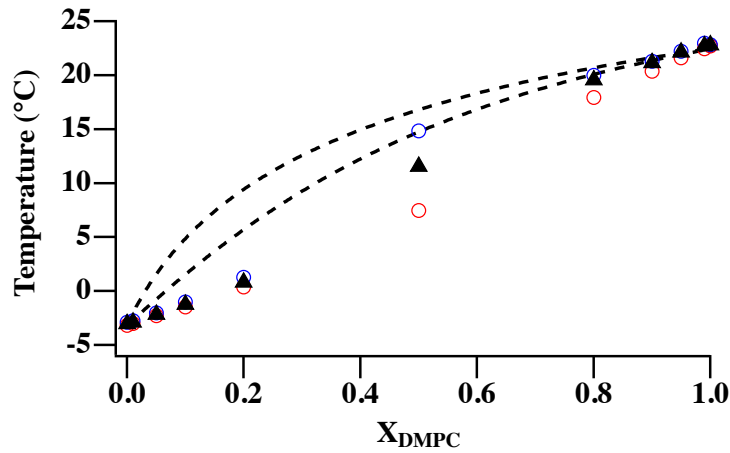


Figure 6.2. Calculated transition curves (---) using Equations 6.1– 6.4. Black triangles indicate the peak melting temperature in experimental data, red circles indicate the temperature of the start of the transition, and blue circles indicate the temperature where the transition is complete.

Figure 6.2 shows ideal mixing only at high mole ratios of DMPC. Moreover, the non-ideality observed here is more pronounced for the gel phase than for lipids in the liquid crystalline phase. Non-ideal mixing seen the gel phase of membranes is a direct result of domain formation, and because enhanced solute permeation near membrane melting temperatures has been attributed to domain formation, solute partitioning could also be enhanced in gel phase membranes.

6.2.2 DMPC/DMPE Mixtures

Calorimetric traces of vesicles composed of DMPC/DMPE are shown in Figure 6.3 and summarized in Table 6.2.

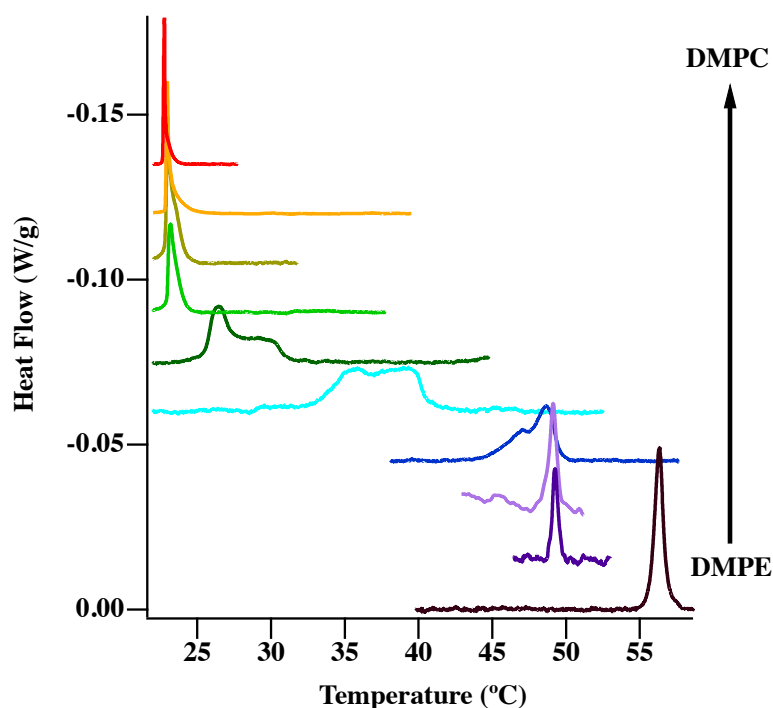


Figure 6.3. Calorimetric traces of DMPC vesicles (red), DMPE vesicles (black), and binary mixtures of DLPC and DMPC: 0.99 DMPC (orange), 0.95 DMPC (yellow), 0.90 DMPC (light green), 0.80 DMPC (dark green), 0.50 DMPC (light blue), 0.10 DMPC (dark blue), 0.05 DMPC (light purple), 0.01 DMPC (dark purple).

Mole Ratio DMPE: DMPC	T_m (°C)
0:100	22.8
1:99	23.0
5:95	23.0
10:90	23.2
20:80	25.5-30.8
50:50	33.0-40.6
80:20	44.8-47.9
90:10	46.3-49.8
95:5	49.1
99:1	49.3
100:0	56.3*

Table 6.2. Melting temperatures of large unilamellar vesicles composed of binary mixtures of DMPC and DMPE.

* Observed melting temperature. Literature melting temperature (gel to liquid-crystal) is 50.2 °C.²⁶⁷

Complicating the analysis of DMPC/DMPE mixtures are the two crystal phases of DMPE. The “stable” crystal phase directly melts into the liquid crystalline phase at ~56 °C, while the “less stable” crystal phase melts into the liquid-crystalline phase with an intermediate gel phase. The main transition temperature present in membranes with the intermediate gel phase exists at ~50 °C. For the gel phase of PEs to form, a PE membrane must be stored around -5 °C for >5 days prior to heating.²⁶⁷ In studies described here, we assume the melting temperature is 56.3 °C for pure DMPE vesicles (Figure 6.3). We propose that the addition of 1 mol % DMPC prevents the stable gel phase of DMPE from forming, and instead, the transition at 49.3 °C is a result of the melting of the gel phase DMPE bilayer.

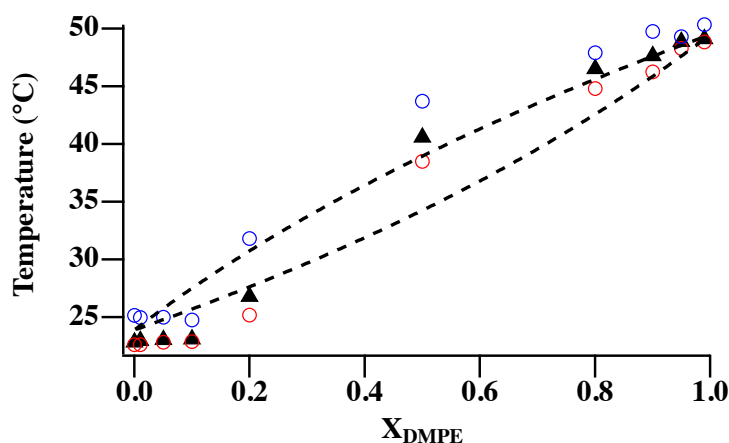


Figure 6.4. Calculated transition curves (---) of DMPC/DMPE mixtures using Equations 6.1–6.4. Black triangles indicate the peak melting temperature in experimental data, red circles indicate the temperature of the start of the transition, and blue circles indicate the temperature at which the transition is complete.

The calorimetric data for DMPC/DMPE mixtures deviate significantly from the calculated ideal mixing transition curves in Figure 6.4. Interestingly, at low mole ratios of DMPE, the transition temperature is lower than expected if ideal mixing were occurring, however, beyond 20 mol percent DMPE, the melting temperatures are higher than predicted were ideal mixing occurring. A nonlinear trend in melting temperature of DMPC/DMPE lipid mixtures has also been observed by Melchior *et al.*, who attributed the asymmetric and broad calorimetric traces to fractional crystallization.²⁸⁶

6.3 Time-Resolved Fluorescence

TCSPC was used to investigate the partitioning of C152 in lipid bilayers composed of DLPC/DMPC and DMPC/DMPE mixtures. The experimental assembly and process of data analysis are summarized in Chapter 2.8.

6.3.1 DLPC/DMPC Mixtures

The partitioning of C152 in pure DLPC and pure DMPC lipid vesicles are described in detail in Chapter 4. Summarized in Figure 6.5 are the fluorescence contributions of C152 in buffer, the polar headgroup region, or imbedded in the membrane interior. Dashed lines in Figures 6.5 and 6.6 represent ideal C152 partitioning. The theoretical lifetime contribution (A^i) of C152 in buffer, the polar headgroup, or nonpolar membrane interior was determined for mixture:

$$A_{X_{DMPC}}^i \text{ (theoretical)} = X_{DMPC} \cdot A_{DMPC}^i + X_{DLPC} \cdot A_{DLPC}^i \quad (6.5)$$

where X_{DMPC} is the mole fraction of DMPC and $X_{DMPC} + X_{DLPC} = 1$ and A^i is the lifetime contribution where i is the buffer, polar, or nonpolar regions.

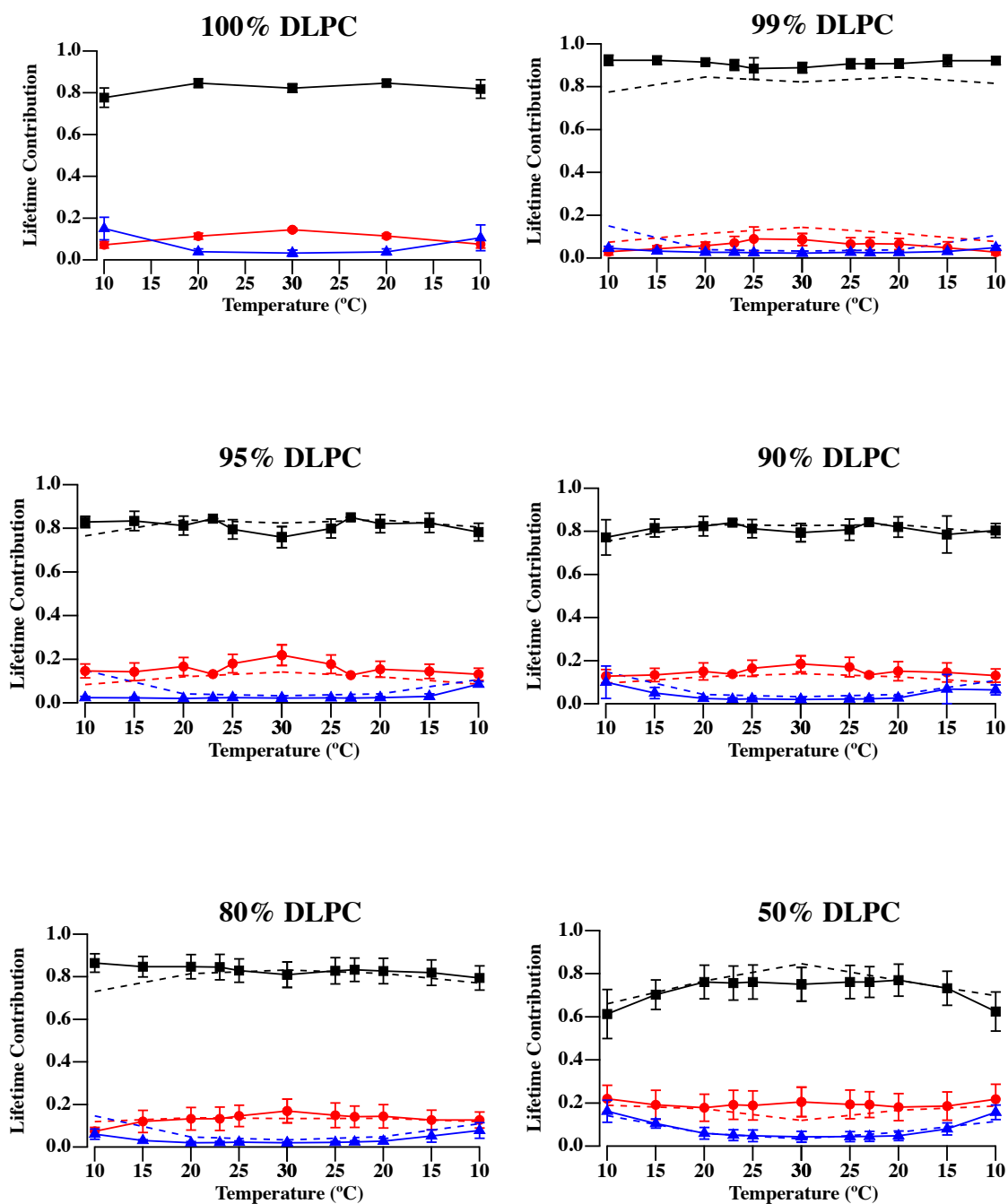


Figure 6.5. Lifetime contribution of C152 in buffer (red circles), polar (black squares), and nonpolar (blue triangles) environments in vesicles composed of pure DLPC (top left), 99% DLPC/ 1% DMPC (top right), 95% DLPC/ 5% DMPC (middle left), 90% DLPC/ 10% DMPC (middle right), 80% DLPC/ 20% DMPC (bottom left), and 50% DLPC/ 50% DMPC (bottom right).

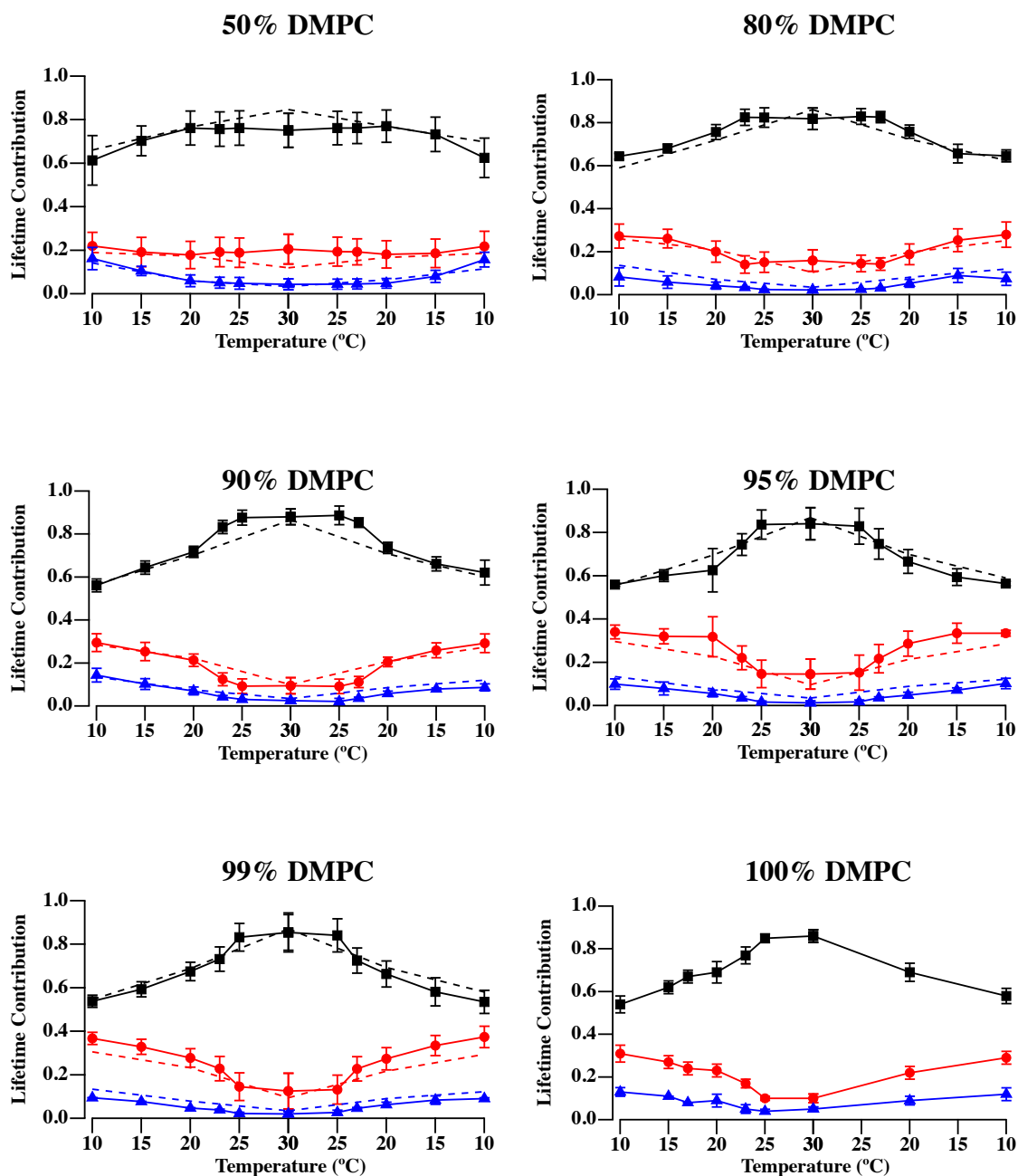


Figure 6.6. Lifetime contribution of C152 in buffer (red circles), polar (black squares), and nonpolar (blue triangles) environments in vesicles composed of DMPC enriched vesicles: 50% DMPC / 50% DLPC (top left; equimolar concentration is reshown here for comparison purposes), 80% DMPC, 20% DLPC (top right), 90% DMPC/ 10% DLPC (middle left), 95% DMPC/ 5% DLPC (middle right), 99% DMPC/ 1% DLPC (bottom left), and pure DMPC (bottom right).

DLPC:DMPC	Temperature (°C)	%C152: Buffer	% C152: Polar	%C152: Nonpolar
Pure DLPC (n=4)	10	7 ± 2	78 ± 5	15 ± 5
	20	11 ± 1	85 ± 2	4 ± 1
	30	14 ± 1	82 ± 2	3 ± 1
90 DLPC: 10 DMPC (n=4)	10	13 ± 3	77 ± 8	10 ± 7
	20	15 ± 4	82 ± 5	3 ± 1
	30	19 ± 4	79 ± 4	2 ± 1
80 DLPC: 20 DMPC (n=3)	10	7 ± 2	87 ± 4	6 ± 2
	20	13 ± 5	85 ± 5	2 ± 1
	30	17 ± 6	81 ± 6	2 ± 1
50 DLPC: 50 DMPC (n=4)	10	22 ± 6	61 ± 11	16 ± 5
	20	18 ± 6	76 ± 8	6 ± 3
	30	21 ± 7	75 ± 8	4 ± 3
20 DLPC: 80 DMPC (n=3)	10	27 ± 6	64 ± 1	8 ± 4
	20	20 ± 5	76 ± 3	4 ± 2
	30	16 ± 5	82 ± 5	2 ± 1
10 DLPC: 90 DMPC (n=3)	10	29 ± 4	56 ± 3	14 ± 3
	20	21 ± 3	72 ± 3	7 ± 2
	30	9 ± 4	88 ± 4	3 ± 1
Pure DMPC (n=7)	10	31 ± 4	54 ± 4	13 ± 2
	20	23 ± 3	69 ± 5	5 ± 2
	30	9 ± 2	87 ± 3	4 ± 2

Table 6.3. Percent of C152 in the buffer, polar region, and nonpolar region of vesicles at 10, 20, and 30 °C. Standard deviations are calculated from the number of trials displayed next to each vesicle solution.

Partitioning of C152 in mixed DLPC/DMPC vesicle shows only slight deviations from ideal behavior (as determined by Equation 6.5). DLPC exists in its liquid crystalline state over all the entire sampled temperature range. In pure DLPC vesicles at 10 °C, ~10-15% of C152 partitions into the nonpolar region of the lipid membrane, ~5-10% of C152 is solvated in the buffer, and the majority of C152 (~80%) partitions into the polar lipid headgroup region (~80%). At 20 °C, C152 is exsolvated from the membrane interior, and the population of C152 associated with the lipid headgroups and C152 in bulk aqueous solution increases accordingly.

This behavior changes little in vesicle mixtures of 99% DLPC and 1% DMPC. The largest deviations are seen at temperatures below 20 °C. We attribute the non-ideal partitioning behavior to the presence of DMPC molecules disrupting the native DMPE crystalline structure and promoting formation of a DMPE gel phase. Although the number and size of the gel phase DMPC domains may be small, the interfacial regions would provide favorable environments for C152 insertion. Moreover, the non-ideality is reduced above 20 °C, near the temperature at which any gel phase DMPC domains would transition into liquid-crystalline domains.

Ideal C152 partitioning behavior is observed in vesicles composed of 95% DLPC, 90% DLPC, and 50% DLPC. However, a significant change in partitioning is observed in vesicles composed of equimolar DLPC and DMPC. As determined by DSC, the phase transition in vesicles having equimolar composition occurs over a broad temperature range: 7.3–14.9 °C (Figure 6.1, Table 6.1). At the lowest temperature sampled (10 °C), the largest population of C152 is in the polar region of the lipid headgroups (~60%), while 20% of C152 is in the membrane interior and the remaining 20% of C152 is not associated with the membrane. At 20 °C, the population of C152 solvated in the membrane interior decreases to <5%, reflected by an increase in C152 population near the membrane headgroups. From 20–30 °C, the population of C152 in these regions does not change. The increase in C152 partitioning near the headgroups from 10–20 °C is attributed to lipid domain formation, similar to the non-ideality seen in the 99% DLPC/ 1% DMPC vesicles. However, the variety of domains that form in equimolar binary solutions provide C152 with unique interfacial regions not present in 99% DLPC/ 1%

DMPC mixtures. In theory, three lipid interfaces could exist in 50% DLPC/ 50% DMPC lipid vesicles from 10–30 °C: domains formed between liquid-crystalline DLPC and gel phase DMPC, domains formed between liquid-crystalline DLPC and liquid-crystalline DMPC (less probable as DLPC and DMPC are very miscible in liquid crystalline phases), and domains formed between liquid-crystalline DMPC and gel phase DMPC.

In mixtures of DLPC and DMPC composed of >80% DMPC, C152 partitioning is nearly ideal and follows the same trend observed with partitioning into DMPC and DPPC vesicles (discussed in Chapter 4). Most evident is the increase in C152 association with the polar lipid headgroups near the main phase transition temperature. In vesicles composed of 80% DMPC, the phase transition starts at ~16 °C and is completed by ~20 °C. The amount of C152 associated to the lipid headgroup gradually increases from 10–20 °C and reaches a maximum at ~23 °C. Similar behavior is observed with vesicles comprised of 90% DMPC ($T_m = 19\text{--}22$ °C). Vesicles composed of $\geq 95\%$ DMPC ($T_m = 22$ °C) show a maximum uptake of C152 at a slightly higher temperature (25 °C), following the trend with maximum uptake near the melting temperature.

Of interest is the relative amount of C152 associated with the membrane (K_{part}) as a function of the reduced temperature (T/T_m). (A similar analysis was used DLPC, DMPC, and DPPC vesicles in Chapter 4.) Although membranes with lower compositions <50% DMPC show no trend in the partitioning coefficient with reduced temperature, partitioning coefficients plotted as a function of reduced temperatures in DLPC/DMPC mixed vesicles with DMPC compositions $\geq 50\%$ (Figure 6.7) provide additional insight into behavior.

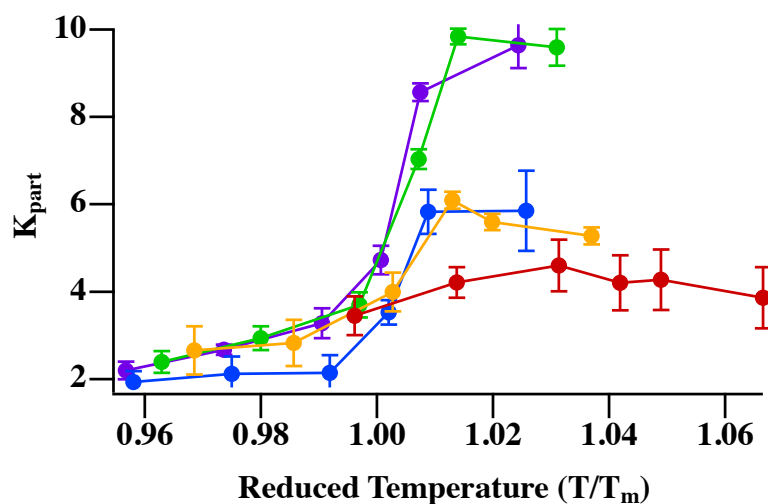


Figure 6.7. Partitioning coefficient ($(A_2+A_3)/A_1$) of C152 in selected binary mixtures as a function of reduced temperature: 100% DMPC (purple), 95% DMPC/ 5% DLPC (blue), 90% DMPC/ 10% DLPC (green), 80% DMPC/ 20% DLPC (orange), and 50% DMPC/ 50% DLPC (red)

Adopting the reduced temperature convention introduced in Chapter 4, the membrane: aqueous partitioning coefficient was calculated at reduced temperatures < 1.00 and found to be virtually unaffected by membrane composition. Partitioning coefficients remain < 4 at these temperatures. A sharp increase in C152 partition coefficient at reduced temperatures > 1.00 is seen for pure DMPC, 95% DMPC, and 90% DMPC vesicles. The increase in partition coefficient is more gradual for vesicles composed of 80% DMPC. Vesicles comprised of equimolar DLPC/DMPC mixtures showed almost no change in partitioning near the admittedly ill-defined transition temperature. Moreover, the partition coefficients of C152 in pure DMPC vesicles and 90% DMPC vesicles are almost double that of vesicles with DMPC compositions of 95%, 80%, and 50%.

6.3.2 DMPC/DMPE Mixtures

C152 partitioning in vesicles composed of mixtures of DMPC and DMPE was analyzed according to the methods described in the previous section.

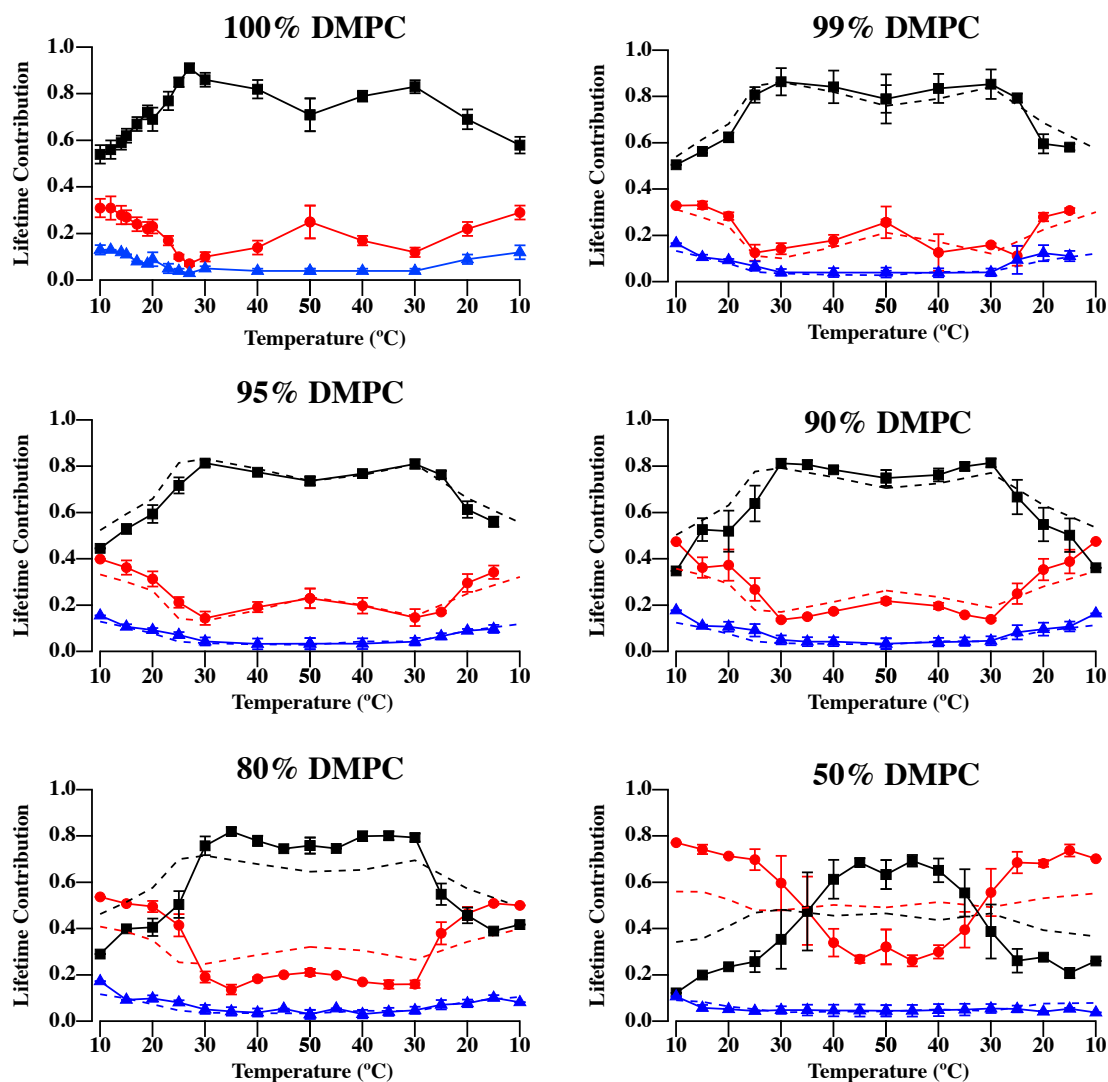


Figure 6.8. Lifetime contribution of C152 in buffer (red circles), polar (black squares), and nonpolar (blue triangles) environments in vesicles composed of pure DMPC (top left), 99% DMPC/ 1% DMPE (top right), 95% DMPC/ 5% DMPE (middle left), 90% DMPC/ 10% DMPE (middle right), 80% DMPC/ 20% DMPE (bottom left), and 50% DMPC/ 50% DMPE (bottom right).

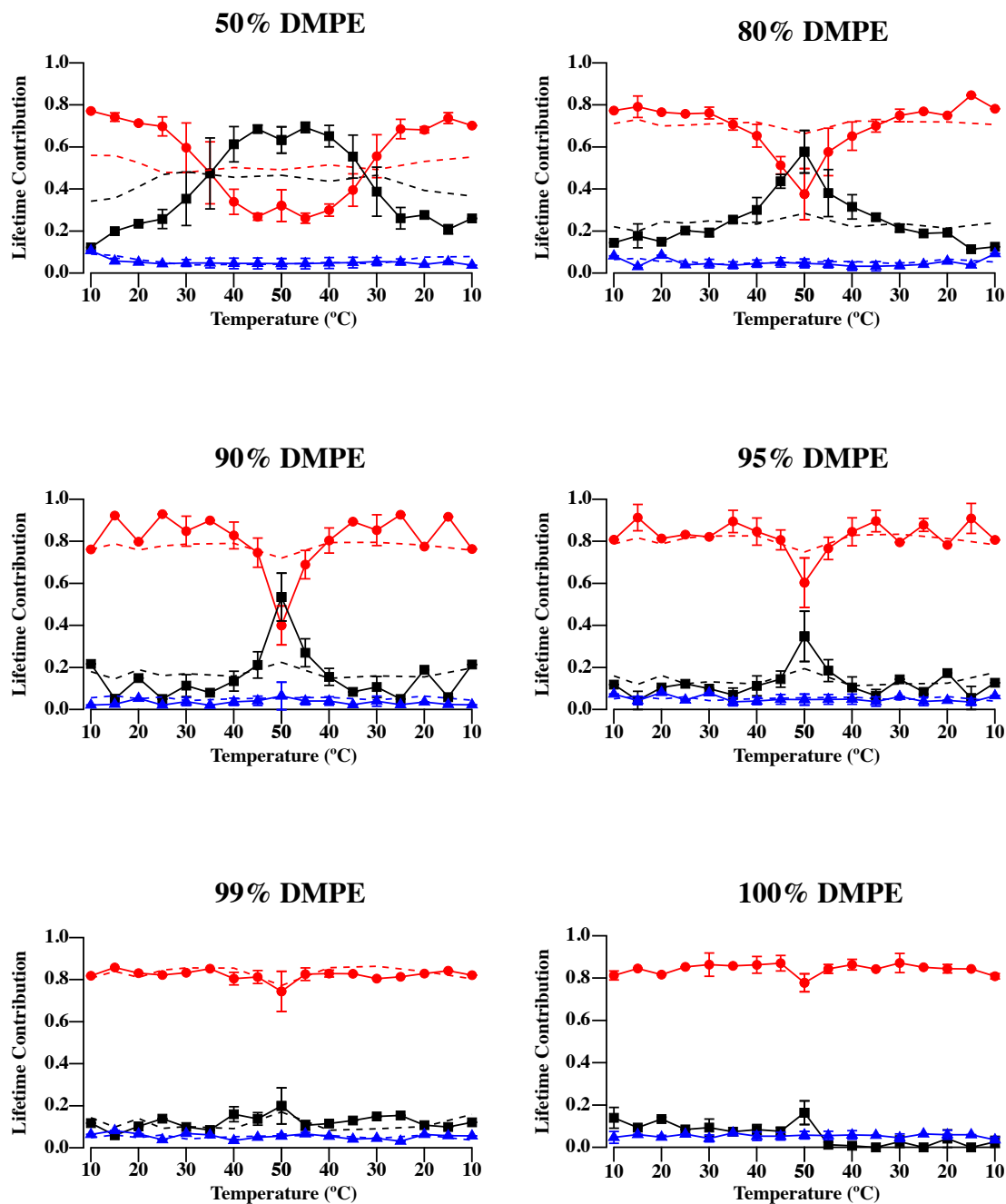


Figure 6.9. Lifetime contribution of C152 in buffer (red circles), polar (black squares), and nonpolar (blue triangles) environments in vesicles composed of 50% DMPE/ 50% DMPC (top left, equimolar concentration is reshown here for comparison purposes), 80% DMPE/ 20% DMPC (top right), 90% DMPE/ 10% DMPC (middle left), 95% DMPE/ 5% DMPC (middle right), 99% DMPE/ 1% DMPC (bottom left), and pure DMPE (bottom right).

DMPC:DMPE	Temperature (°C)	%C152: Buffer	% C152: Polar	%C152: Nonpolar
Pure DMPC (n=7)	10	31 ± 4	54 ± 4	13 ± 2
	20	23 ± 3	69 ± 5	5 ± 2
	30	9 ± 2	87 ± 3	4 ± 2
	50	25 ± 7	71 ± 7	4 ± 1
90 DMPC: 10 DMPE (n=3)	10	47 ± 1	35 ± 1	18 ± 0
	20	37 ± 7	52 ± 9	11 ± 2
	30	14 ± 1	81 ± 2	5 ± 2
	50	22 ± 1	75 ± 3	3 ± 2
80 DMPC: 20 DMPE (n=4)	10	53 ± 1	29 ± 1	17 ± 1
	20	50 ± 2	41 ± 4	10 ± 1
	30	20 ± 2	76 ± 4	5 ± 2
	50	21 ± 1	76 ± 3	3 ± 2
50 DMPC: 50 DMPE (n=3)	10	77 ± 2	12 ± 1	11 ± 1
	20	71 ± 1	23 ± 1	5 ± 1
	30	60 ± 12	35 ± 13	5 ± 2
	50	32 ± 8	66 ± 6	6 ± 2
20 DMPC: 80 DMPE (n=3)	10	77 ± 5	14 ± 6	8 ± 1
	20	77 ± 1	15 ± 1	8 ± 1
	30	76 ± 3	19 ± 2	4 ± 2
	50	38 ± 12	58 ± 10	5 ± 2
10 DMPC: 90 DMPE (n=4)	10	76 ± 1	22 ± 1	2 ± 1
	20	80 ± 1	15 ± 1	5 ± 1
	30	85 ± 7	11 ± 5	4 ± 2
	50	40 ± 10	54 ± 11	6 ± 6
Pure DMPE (n=7)	10	82 ± 2	14 ± 5	5 ± 3
	20	82 ± 1	13 ± 1	5 ± 1
	30	86 ± 5	10 ± 5	4 ± 2
	50	78 ± 4	16 ± 6	6 ± 2

Table 6.4. Percent of C152 in the buffer, polar region, and nonpolar region of vesicles at 10, 20, and 30 °C. Standard deviations are calculated from the number of trials displayed next to each vesicle solution.

Similarities in DSC traces (Figure 6.3) of DMPC rich vesicles indicate that relatively small amounts (10 mol %) of DMPE do not alter the physical properties of DMPC vesicles. Time-resolved fluorescence decays indicate that partitioning into vesicles with these compositions is ideal (Equation 6.4), as no abnormal partitioning is

observed in vesicles composed of $\leq 10\%$ DMPE (Figure 6.5). Partitioning behavior deviates from ideality in vesicles composed of 80% DMPC (20% DMPE). Ideal partitioning behavior predicts 46% (10 °C) to 70% (25 °C) of C152 molecules would be associated with the polar lipid headgroups. Experimentally, only 29% (10 °C) and 50% (25 °C) of the C152 population is associated with the lipid headgroups with the difference between ideal and measured amounts remaining in the aqueous buffer. Calorimetric traces indicate the melting temperature of these binary lipid vesicles occurs from $\sim 26\text{--}31$ °C). However, the characteristic maximum in membrane partitioning (observed for C152 in PC vesicles) is instead observed at a temperature ~ 10 °C higher than the melting temperature. These results are surprising and contradict conventional wisdom that predicts significant membrane uptake of solutes *at* the phase transition temperature.

The discrepancies in C152 population distribution and melting temperature (as determined by solute uptake by the membrane) are even more pronounced in equimolar DMPC/ DMPE vesicles. Singer studied the permeability of glucose and sodium in an equimolar DMPC/DMPE lipid mixture. DSC showed that phase separation occurred from 29 to 43 °C, and that permeability of ^{22}Na reached a maximum at 30 °C. Moreover, the permeation behavior in the equimolar mixture was more similar to the permeation in pure DMPC vesicles rather than the permeation behavior in pure DMPE vesicles. The author concluded that the defect regions formed upon melting are more populated by PCs than PEs. Data presented above cannot test this hypothesis directly, but results show clearly that in these heterogeneous bilayers, solute partitioning shows strongly non-ideal

behavior. These findings provide grist for the mill for future simulations intending to capture membrane-solute interactions.

Partitioning of C152 in vesicles composed of $\geq 80\%$ DMPE is ideal (within error) below 45 °C. From 45–50 °C, a slight increase in the population of C152 near the headgroups is observed. Since DMPE limits membrane permeation by forming strong interactions with neighboring lipids, the increase in membrane permeation at higher temperatures must be a result of the presence of small amounts of DMPC in the membrane. If C152 permeation were due *solely* to the phase transition of DMPC, we would expect enhancement of partitioning to occur at the melting temperature of DMPC (~23 °C) instead of ~50 °C. Therefore, partitioning at these higher temperatures must result from complex interactions that remain unresolvable using techniques described in this work.

6.4 Conclusions

We have shown the distinct differences in C152 partitioning into vesicles composed of binary mixtures of phosphatidylcholines and binary mixtures of phosphatidylcholines and phosphatidylethanolamines. Differential scanning calorimetry traces show high lipid miscibility in mixtures of 12:0 and 14:0 PCs. Partitioning of C152 in mixed PC membranes also follows ideal behavior. In lipid mixtures that exhibit a phase transition within the temperature range used in partitioning experiments, C152 association with the polar lipid headgroups is enhanced. These results agree with partitioning data discussed in Chapter 4. Distinctly non-ideal behavior is observed in

mixed PC/PE membranes and deserves closer scrutiny in future studies outlined in

Chapter 7.

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CHAPTER SEVEN

CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Summary

Experiments summarized in this thesis were designed to clarify the complex nature of solute interactions with lipid bilayer membranes. Investigations used a variety of techniques: time-resolved fluorescence, steady-state fluorescence, differential scanning calorimetry, and dynamic light scattering. Time-resolved fluorescence was used to measure the fluorescence lifetimes of two solutes (C152 and C461) in lipid vesicle solutions. Lipid vesicle compositions were varied systematically to investigate the effect of membrane structure on solute partitioning. Research presented in Chapters 3 and 4 reported how C152 samples heterogeneous solvation in aqueous vesicle solutions and how the distribution of solutes between different environments changes with temperature and with lipid identity. Phospholipid vesicles were comprised of DLPC (12:0 PC), DMPC (14:0 PC), and DPPC (16:0 PC). C152 in all three lipid systems showed partitioning behavior that was qualitatively similar but quantitatively different.

Partitioning into a gel phase membrane was slightly exothermic and slightly entropically unfavorable. Partitioning of C152 near the lipid membrane headgroups was entropically driven and endothermic. Well above the melting temperature, exsolvation of C152 from the membrane back into the aqueous buffer was enthalpically driven but entropically unfavorable. Regardless of solution temperature, relatively little (<10%) C152 partitioned into the hydrophobic core of the membrane. The magnitudes of the

forces driving C152 partitioning systematically increased with alkyl chain length (DLPC < DMPC < DPPC). Differences in partitioning between the three vesicle systems are attributed to differences in surface area per lipid as membrane phase changes from the gel to liquid-crystalline state.

In Chapter 5, solute partitioning into DPPC lipid vesicles was quantified as a function of solute identity (C152 and C461), solute concentration, and temperature. C152 and C461 differ solely in the 4-position where C152 has a trifluoro methyl group in place of C461's $-\text{CH}_3$ group. These studies are particularly relevant for understanding quantitatively the common practice of improving solute bioavailability by adjusting its hydrophobicity. In vesicle containing aqueous solutions, multi-exponential fluorescence decay shown by both solutes indicates distinctive solute populations in the aqueous buffer, in the vesicle headgroup region, in the acyl chain bilayer interior. Fluorescence amplitudes were used to calculate absolute partition coefficients and average number of solutes per vesicle. C152 shows a ~10-fold greater affinity than C461 for lipid bilayers, despite both solutes having similar log P values. The enthalpies and entropies of C152 partitioning as a function of concentration were determined from temperature dependent partitioning data. Above and below the lipid gel-liquid crystalline temperature, partitioning is exothermic with negative changes in entropy. Near the transition temperature, these quantities change sign with ΔH_{part} becoming endothermic (+70 kJ/mole) and $\Delta S_{\text{part}} = +200 \text{ J}/(\text{mole K})$.

Biological membranes are, themselves, comprised of lipid mixtures. Results presented in Chapter 6 build upon discoveries reported in Chapters 3 and 4 by examining

the temperature-dependent solvation of C152 in mixed lipid vesicle solutions. The effect of phosphatidylcholine alkyl chain length on lipid miscibility and C152 partitioning was investigated using vesicles composed of DLPC (12:0 PC), DMPC (14:0 PC), and mixtures of DLPC/DMPC. Differential scanning calorimetry traces show moderate miscibility between DLPC and DMPC. Time-resolved fluorescence decays show ideal C152 partitioning into mixed PC; that is, even if mixed PC vesicles *do* form single lipid domains, C152 partitioning behavior is largely unaffected. Similar experiments were performed with vesicles composed of DMPC (14:0 PC) and DMPE (14:0 PE) to investigate the role of the lipid headgroup in lipid miscibility and C152 solvation. DSC traces show low miscibility between PC and PE lipids in vesicle solutions, and time-resolved fluorescence decays show C152 into PC/PE membranes is distinctly non-ideal. The cause of the non-ideal behavior requires further studies.

7.2 Future Directions

Results summarized in Chapters 3-6 of this dissertation help to identify some of the sensitive interactions between solutes and lipid membranes. Research presented here also provokes additional questions that could be easily answered using the methods developed in this research. Additional experiments outlined below can answer questions related to the effects of solute and lipid structures on partitioning.

7.2.1 Solute Structure

In Chapter 5, the effect of solute structure on membrane partitioning was investigated. Results showed that partitioning decreased by an order of magnitude with

the replacement of the $-\text{CF}_3$ on 4-position of C152 with a $-\text{CH}_3$ (C461). Results also demonstrated a positive correlation between the empirical log P value and membrane partitioning: C461 (log P = 2.29) showed less membrane partitioning than C152 (log P = 2.72). However, this correlation was not quantitative. Based on their log P values, we would expect that C152 should show a $\sim 2.5\times$ greater affinity than C461 for lipid bilayers, but measured data show this difference to be closer to an order of magnitude. Given the methodology developed in this dissertation, we are now poised to examine correlations between log P values for multiple solutes and their corresponding membrane affinity.

To further investigate the relationship between solute structure and membrane partitioning behavior, similar studies as those outlined in Chapter 5 could be performed using coumarin 481 and coumarin 151 (Figure 7.1).

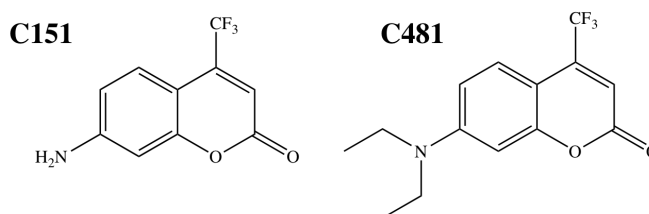


Figure 7.1. Molecular structures of coumarin 151 (C151, left) and coumarin 481 (C481, right).

C151 and C481 are viable candidates for future partitioning studies due to their photostability, large quantum yields, and sensitivity to local solvation environment. Moreover, they are extensively characterized both computationally and experimentally in the literature.¹⁴⁷⁻¹⁴⁹ Structural similarity to solutes already used in partitioning studies will allow for a direct comparison of partitioning based solely on solute structure.

Considering the simple “like dissolves like” argument and the increased hydrophobicity and log P of C481 (log P = 3.4), we expect membrane partitioning of this solute would be larger than that of C152 and C461. Based on similar arguments, we expect much less membrane partitioning for C151 (log P = 1.63) than observed for C152 and C461. However, partitioning behavior may not follow simple hydrophobicity and log P considerations given that membrane solubility will also depend sensitively on solute size. Although the log P of C481 is the largest of the four solutes discussed in this dissertation, the molecular diameter of C481 is also the largest. Considering the lipid-lipid interactions present in membranes, the energetic costs associated with membrane disruption may prevent larger solutes (such as C481) from partitioning into the membrane. Partitioning experiments using C151 and C481 as solutes will provide insight into whether the log P values can accurately predict membrane partitioning.

Experiments designed to investigate the role of solute structure on partitioning would proceed in the same way as those presented in Chapters 3-6. Absorption and emission behavior of C151 and C481 in bulk solvents is well reported in literature. Preliminary time-resolved fluorescence decays of these solutes in bulk solvents and DMPC vesicles have been recorded and analyzed (Tables 7.1 and 7.2, Figure 7.2).

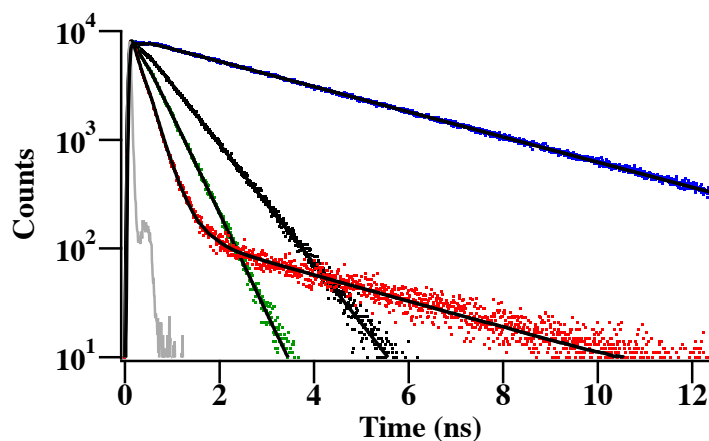


Figure 7.2. Time-resolved fluorescence decays of C481 in buffer (red), methanol (green), acetonitrile (black), and cyclohexane (blue). A typical IRF is shown in grey.

Solvent	τ_f (A)
Buffer	0.26 (0.99)
	3.51 (0.01)
Acetonitrile	0.80
Methanol	0.48
Cyclohexane	3.75
DMPC (10 °C)	0.62 (0.06)
	2.04 (0.49)
	4.04 (0.45)
DMPC (30 °C)	1.55 (0.91)
	2.99 (0.09)

Table 7.1. Time-resolved fluorescence of C481 in bulk solvents (10 °C) and DMPC vesicle solutions at 10 and 30 °C. Fluorescence lifetimes are reported in ns. Amplitudes reported in DMPC vesicle solutions are not corrected for radiative rate.

Similar to C152 and C461, fluorescence lifetimes of C481 change with environmental polarity. The emission decays of C481 in DMPC vesicle solutions are fit to 2-3 lifetimes (depending on temperature), indicating C481 experiences more than one local solvation environment in DMPC vesicle solutions. The quantum yields and radiative rates of C481 in bulk solvents were not determined, so the amplitudes reported in Table 7.1 cannot be directly correlated to the percent of C481 in specific solvation

environments in vesicle solutions. Moreover, fluorescence behavior of C481 in a variety of other solvents must be characterized to determine the environment in which C481 decays with a 2-3 ns fluorescence lifetime.

Solvent	τ_f (A)
Buffer	4.48
Acetonitrile	5.13 ¹⁴⁹
Methanol	5.30
Cyclohexane	1.06 (0.94) 1.92 (0.06)
DMPC (10 °C)	2.24 (0.11) 5.31 (0.89)
DMPC (30 °C)	2.00 (0.09) 5.46 (0.91)

Table 7.2. Time-resolved fluorescence of C151 in bulk solvents (10 °C) and DMPC vesicle solutions at 10 and 30 °C. Fluorescence lifetimes are reported in ns. Amplitudes reported in DMPC vesicle solutions are not corrected for radiative rates.

Assignment of C151 fluorescence lifetimes to local solvation environment has similarly proven difficult. Promising, however, are the two distinct fluorescence lifetimes of C151 in DMPC solutions. Clarifying fluorescence behavior of C151 in a wider variety of solvents may also elucidate membrane partitioning behavior.

7.2.2 Vesicle Structure

Experiments discussed in Chapters 4 and 6 were designed to investigate the role of lipid structure on solute partitioning. From these studies, we determined that C152/phosphatidylcholine membrane association reached a maximum just above the melting temperature of phosphatidylcholine membranes, regardless of the acyl chain length. However, acyl chain length *was* shown to influence the magnitude of C152 partitioning.

Membrane association of C152 also changed drastically with phospholipid headgroup. Partitioning into phosphatidylethanolamine membranes was limited at all temperatures investigated (Chapter 6). We attributed the lack of partitioning into PE membranes to the presence of intermolecular hydrogen bonds (or intermolecular water bridges), which create physical barriers blocking solute permeation. Studies in Chapter 6 prompt interest in the relationship between PE chain length and membrane permeability. If intermolecular interactions in PE membranes truly create a physical barrier for solutes, we should see little to no change in C152 partitioning behavior in vesicles composed of 12:0, 14:0, or 16:0 phosphatidylethanolamine. Experiments and analysis described in Chapter 4 could easily be repeated with the substitution of the PC lipids by their analogue PEs.

Also of interest is how solute partitioning into membranes may change in the presence of divalent cations. The effect of Ca^{2+} on the structure of phosphatidylcholine membranes has been studied extensively.^{287,288} NMR suggests Ca^{2+} creates conformational ordering in the headgroups and acyl chains of PC membranes.²⁸⁹ IR spectroscopy indicates the conformational ordering is created by Ca^{2+} induced displacement of water near lipid headgroups.²⁹⁰ Spectroscopic studies of C152 partitioning into membranes in the presence of Ca^{2+} could provide insight into the mechanisms driving solute and Ca^{2+} membrane permeation. If Ca^{2+} does displace water in the headgroups of PCs, we expect to observe a decrease in the amplitude associated with C152 fluorescence in a polar environment. Moreover, we anticipate partitioning into PC membranes in the presence of Ca^{2+} would not be sensitive to acyl chain length.

Studies should start with measuring the melting temperature of DLPC, DMPC, and DPPC vesicles in the presence of Ca^{2+} . Ca^{2+} concentrations should vary from very dilute (<1 mM) to concentrated (100 mM). Fluorescence decays of C152 in Ca^{2+} solutions should also be measured to determine the anticipated fluorescence lifetime of C152 in bulk aqueous (Ca^{2+}) environment. Finally, fluorescence decays of C152 in vesicle solutions should be measured at temperatures surrounding the melting temperature (as observed from DSC traces). Data analysis will follow the procedure summarized in Chapter 2.

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APPENDIX

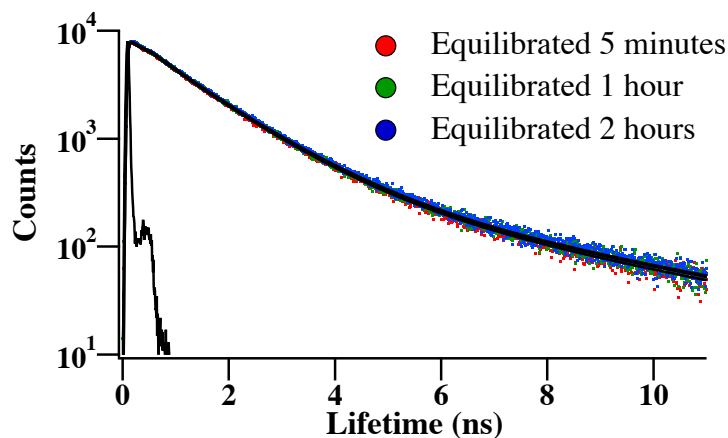


Figure A.1. Fluorescence lifetimes of C152 in DPPC vesicles at 44 °C. The black trace represents an instrument response function and is typically <100 ps.

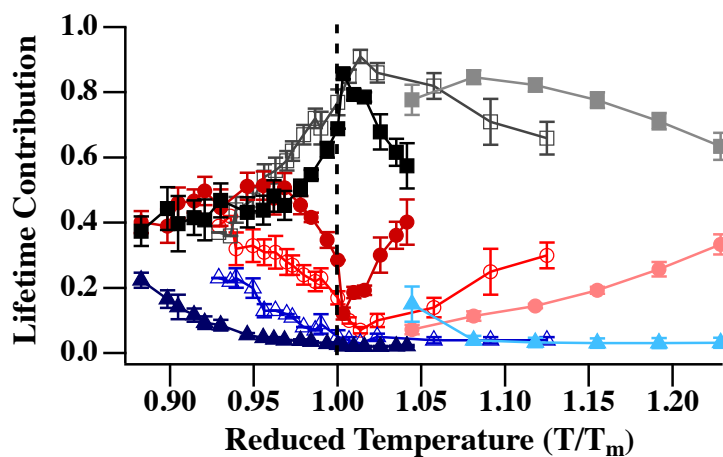


Figure A.2. Lifetime contributions of C152 in DLPC, DMPC, and DPPC vesicles at reduced temperatures. Filled (light) markers are C152 lifetime contributions in DLPC vesicle solution, open markers are lifetime contributions of C152 in a DMPC vesicle solution, and filled (dark) markers are C152 lifetime contributions in DPPC vesicle solution. Red markers indicate C152 in the buffer (τ_1), black markers indicate C152 in the polar region (τ_2), and blue triangles indicate C152 in the nonpolar region (τ_3). Solutions were allowed to fully equilibrate at each temperature before recording fluorescence decays.