Photochemical Applications to the Study of Complexity
Phospholipid Bilayer Environments

Christopher John Wohl Jr.
Virginia Commonwealth University

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PHOTOCHEMICAL APPLICATIONS TO THE STUDY OF COMPLEXITY
PHOSPHOLIPID BILAYER ENVIRONMENTS

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry at Virginia Commonwealth University.

by

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support of his own research group is sure to be a bright spot in the future of the VCU chemistry department.

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Abstract

PHOTOCHEMICAL APPLICATIONS TO THE STUDY OF COMPLEXITY
PHOSPHOLIPID BILAYER ENVIRONMENTS

By Christopher John Wohl Jr, Ph.D.

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry at Virginia Commonwealth University.

Virginia Commonwealth University, 2006

Major Director: Dr. Darius Kuciauskas
Assistant Professor, Department of Chemistry

The physical and biophysical properties of a biological membrane model, phosphatidylcholine bilayers, were investigated using novel spiropyran/merocyanine molecular probes. The femtosecond to second dynamics of this system’s photochemistry enabled bilayer viscosity and free volume to be studied over a broad time scale. Spiropyrans/merocyanines with different polarity were synthesized by changing the substitution of the indole moiety enabling determination of the trans-membrane properties.
of the bilayer. In addition, transient grating spectroscopy was used to study thermal energy transfer in phospholipid bilayers on a picosecond time scale.

Femtosecond transient absorption spectroscopy was used to study the photo-induced spiropyran ring-opening and isomerization reactions that produce the highly polar merocyanine species. The hindered rotation of the merocyanine bridge results in several metastable merocyanine isomers. The merocyanine ground state was determined to be populated predominantly by two isomers (TTC and TTT). Selective photoexcitation of these isomers results in excited state isomerization producing a third isomer (τ = 60 ps). Merocyanine thermal ring-closing was observed on a seconds time scale. Reaction kinetics, and solvatochromic and photochromic properties of merocyanines and spiropyrans were used to determine the bilayer physical properties. Bilayer viscosity was determined from merocyanine isomerization kinetics. Phospholipid bilayer free volume (the unoccupied volume enclosed in the bilayer) was determined from a modified Kramers’ analysis. The greatest free volume was found in the extreme interior of the bilayer, while the head-group region exhibited the least free volume in qualitative agreement with molecular dynamics simulations of these bilayer systems. Free volumes determined via ps experiments were lower than those determined on a seconds time scale due to reduced acyl chain dynamics on the ps time scale.

Femtosecond transient grating spectroscopy was used to study the rate of thermal energy transfer from photo-excited porphyrin molecules to the surrounding solvent. Thermal energy transfer was observed as photo-acoustic waves propelled through the
system upon relaxation of photo-excited porphyrin molecules in aqueous solution and embedded in bilayers. For liposome solutions, a bimodal energy transfer model was developed. The determined rate constants suggest that energy transfer occurs predominantly via thermal diffusion and vibrational energy transfer, while lipid dynamics (isomerizations) are not involved.

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Chapter 1: Introduction

1.1 Physical Properties of Phospholipid Membranes

Biological membranes represent a unique system for molecular separation and discrimination. Composed of lipids, cholesterol and membrane-bound proteins, these active barriers prevent the loss of intracellular components, enable concentration gradient formation of various biologically relevant ions and enable biological functions to occur. The phospholipid bilayer environment is highly anisotropic and physical characteristics transform dramatically upon translation through the bilayer (for example the dielectric constant, \( \varepsilon \), changes from \( \varepsilon = 2 \) in the nonpolar aliphatic interior to \( \varepsilon = 81 \) for bulk water). Other physical properties of phospholipid membranes depend on composition; for example, the packing density, microviscosity, and water permeability are dramatically affected by the presence of cholesterol. In order to study the variation of these physical properties, the complexity of the system must be reduced.

Researchers often turn to phospholipid bilayers made of one or several biologically relevant components. Studies have been conducted on phospholipid monolayers, bilayers, multi-bilayers, Langmuir-Blodgett films, single unilamellar vesicles (SUV’s), giant unilamellar vesicles (GUV’s), and multi-lamellar vesicles. In order to probe these environments spectroscopically, molecular sensitizers must be incorporated into these
structures. Two approaches have yielded success: 1) incorporation of molecular sensitizers into the bilayer itself; and 2) synthetically modified phospholipids possessing a pendant probe molecule.

Several reviews have been dedicated to the determination and control of the physical properties of biomaterials by the incorporation of spectroscopically active probe molecules.\textsuperscript{1-4} Spiropyran molecules (discussed in detail in section 1.2) have been shown to alter the rate of ion transport,\textsuperscript{5,6} affect trans-membrane potential,\textsuperscript{7} detect and augment enzyme activity,\textsuperscript{8,9} and modify protein structure.\textsuperscript{10} Functionalized spiropyrans were also coupled directly to protein molecules\textsuperscript{8,11} enabling investigation of lipid - protein interactions.\textsuperscript{12} In this work, spiropyran molecular switches are employed to determine the depth and temperature dependent viscosity variation in phospholipid bilayers without significant perturbation to the bilayer structure.

Two aspects of phospholipid bilayer complexity are addressed, variation of the physical properties of the interface and lateral heterogeneities in mixed lipid bilayer systems. Lateral and vertical variations in phospholipid bilayer physical properties, relative to the bilayer plane, depend upon the geometry and composition of a phospholipid bilayer. To understand this dependence, the structure of phospholipid membranes is briefly discussed. Microscopic heterogeneities as a result of the bilayer architecture are described with an emphasis on microviscosity and bilayer free volume. Thermotropic phase transition of single component phospholipid bilayers and self-sorting in multi-component lipid systems are related to changes in viscosity, free volume and water
penetration depth. The diffusion of small molecules and lipids in a phospholipid bilayer
are correlated with bilayer composition and phase.

**Phospholipid Membrane Structure**

Phospholipids self assemble into micellar and membrane architectures to minimize
unfavorable interactions between the alkyl chains and the surrounding aqueous solution.\(^{13}\) Formation of structures with the polar head-groups located on the exterior and the interior
comprised solely of alkyl chains reduces these unfavorable interactions by “shielding” the
alkyl chains from the surrounding solvent.
Scheme 1: A) Architecture of a phospholipid vesicle. B) The structure of dimyristoylphosphatidylcholine (DMPC), a commonly studied lipid, consists of a zwitterionic head-group and two aliphatic chains.
The phospholipid bilayer can be divided into four distinct regions (Figure 1A):\textsuperscript{12,14,15} 1) The perturbed water region. Water orients in response to the presence of lipid head groups which protrude into this layer. 2) The interface. This region has the highest density and all of the water present here is strongly hydrogen bonded. Interactions in this region are believed to be responsible for the phase behavior of phospholipid aggregates. 3) The soft polymer region. This region has a high chain density and low free volume. 4) The decane region. This region, consisting of the most internal portions of the alkyl chains, demonstrates low density and high free volume characteristics similar to a long-chain saturated alkane. Molecular density distributions for the various bilayer components are shown in Figure 1B. The total molecular density distribution reaches a maximum in the headgroup region (region 2 in Figure 1A) and a minimum at the bilayer center (region 4 in Figure 1A).
Figure 1: A) Four region model of the phospholipid bilayer as described by Marrink et al.\textsuperscript{15} B) Molecular density distribution of individual bilayer components (a) and the total molecular density of the phospholipid bilayer (b).\textsuperscript{16} Note the water penetration depth, up to $\sim$ 10 Å from the bilayer center.
Microscopic Heterogeneity

The amphiphilic nature of the phospholipid molecules results in chemical complexity in the self-assembled vesicular structure. The variation in chemical and physical properties of a phospholipid bilayer affects a myriad of different biophysical events (diffusion, lipid clustering, bilayer permeability, etc.). This complexity will be exploited in the studies described here to derive several important physical parameters of a phospholipid embedding environment.

Polarity. The dielectric constant, \( \varepsilon \), of a phospholipid bilayer is depth dependent. The interior of the bilayer, comprised of hydrophobic alkyl chains, is nonpolar with a dielectric constant \( (\varepsilon \approx 2 \text{ D})^{17} \) similar to that of nonpolar organic solvents (toluene \( \varepsilon = 2.4 \text{ D}, \) benzene \( \varepsilon = 1 \text{ D}, \) decane \( \varepsilon = 1.8 \text{ D} \)). The head-group region of the bilayer consists of functionalities that exhibit favorable interactions with water and are highly polar. Recent molecular dynamics simulations have predicted local polarity values in the head-group region in excess of water \( (\varepsilon > 81 \text{ D})^{18} \).

Viscosity. Viscosity measures the retardation of flow of components within a biological membrane.\(^{19} \) Alteration of the microenvironment surrounding membranous components has been implicated in several biologically relevant processes: conformation changes of trans-membrane portions of proteins,\(^{20} \) alteration of lipid composition,\(^{21} \) membrane bound zinc concentrations,\(^{22} \) etc. Several drugs also play an important role in the alteration of phospholipid membrane viscosity. The efficacy of a series of nonsteroidal
anti-inflammatory drugs was recently evaluated based upon their ability to perturb the viscosity of splenocyte membranes.\textsuperscript{23} The strong interactions between phospholipids and propofol (a popular anesthetic), which were attributed to its unique structure, was confirmed by measurement of changes in bilayer microviscosity upon incorporation of propofol into lipid membranes.\textsuperscript{24} Extended studies concerning modulation of membrane viscosity could inspire the creation of more effective medications.

The viscosity in the bilayer interior varies according to bilayer depth and temperature. Near the head-groups, viscosity values become very large ($\sim 1000$ cP) due to the high density of this area. In the aliphatic interior, "micro" viscosity values become strongly temperature dependent due to the dramatic changes that occur in this region upon phase transition.

Literature values for viscosity vary widely due to differences in experimental technique (a summary of the determined microviscosity values up to 1986 can be found in the work of Kung and Reed).\textsuperscript{25} Phospholipid membrane viscosity values are substantially higher than the viscosity determined for saturated alkane solutions of similar chain length.\textsuperscript{26} Viscosity values for a large range of naturally occurring lipid membranes were determined ($\eta = 3009 - 360$ cP) from fluorescence depolarization values of several dyes.\textsuperscript{27} Monitoring excimer formation of pyrene, a dye known to reside in the bilayer interior, resulted in viscosity values ranging from $\eta = 125$ cP for the L\textsubscript{B'} phase to $\eta = 13$ cP at 60$^\circ$C for the L\textsubscript{A} phase in DMPC membranes.\textsuperscript{28,29} The fluorescence quantum yield of 9-(dicyanovinyl)julolidine has been determined to be viscosity dependent and incorporation of this molecule into DPPC membranes resulted in viscosity variation from 120 – 70 cP
over the temperature range of 10 - 60°C.\textsuperscript{25} Shafirovich et. al. studied the rate of radical ion pair recombination for a series of porphyrin-viologen dyads embedded in DPPC membranes.\textsuperscript{30} The viologen (located at the head-group region) and porphyrin (located in the bilayer interior) moieties were separated by varying length linker groups (C\textsubscript{6} and C\textsubscript{10} methylene bridges). They determined that the “micro” viscosity values depend upon the distance from the head-group region, with the C\textsubscript{6} linker dyad exhibiting higher viscosity values than the C\textsubscript{10} dyad over a temperature range of 42 - 70°C (liquid phase).\textsuperscript{30}

Clearly, the variation in determined viscosity values in these relatively simple systems is prohibitive to application of these methods to more complex phospholipid membrane environments such as membrane “rafts.” In spite of this, the qualitative dependence of bilayer viscosity on lipid composition has been investigated. The addition of cholesterol reduces the free volume in the bilayer interior resulting in higher viscosities.\textsuperscript{31}

**Free Volume and “Micro” viscosity.** Viscosity, diffusion, and permeability variation in phospholipid membranes can be related to bilayer depth-dependent changes in free volume. The free volume is different in each bilayer region and therefore the “micro” viscosity is different. In DPPC, for example, the free volume in the decane region is \(~54\%\textsuperscript{32}\) with a viscosity similar to a saturated alkane of similar length.\textsuperscript{33} Cholesterol reduces this free volume.\textsuperscript{31} Free volume also depends on the lipid structure. For example, Shinoda et. al. conducted simulations on dipalmitoyl phosphatidylcholine (DPPC) and diphytanoyl phosphatidylcholine (DPhPC) that focused on the effects of chain branching at
the interior of phospholipid membranes.$^{34-36}$ They determined that branching in region 4 results in a dramatic reduction of the free volume (Figure 2) due to the interposing of neighboring alkyl chains. This resulting in slower chain motions, increased water penetration depth, reduced bilayer thickness, and a greater number of gauche conformations.$^{36}$
Figure 2: Shinoda et. al. conducted molecular dynamics simulations exploring the effects of chain branching on phospholipid membranes.\textsuperscript{34} They determined that the chain branching present in the aliphatic interior of bilayers comprised of DPhPC lipids resulted in the presence of fewer voids in the bilayer interior (B) compared to DPPC lipids (A). The phospholipid membrane voids shown here are time-averaged probability distribution isosurfaces after 1.5 ns MD trajectories.
The shape of void spaces in bilayers has also been considered. Jedlovszky et. al. determined that voids are generally cylindrical and align roughly parallel to the bilayer normal.\textsuperscript{37} The addition of cholesterol was shown to enhance the number of large voids at the expense of smaller open spaces. The smaller spaces have been determined to be occupied by the cholesterol molecules themselves.\textsuperscript{31}

**Phase Behavior: Single Lipid Component.**

Phospholipid bilayers exhibit polymorphism: the ability to reversibly transition between structures upon environmental changes. The phases, consisting of a highly-ordered gel phase, L\textsubscript{c}, a sub-gel phase, L\textsubscript{\beta} or L\textsubscript{\beta\prime}, a rippled phase, P\textsubscript{\beta\prime}, and a liquid phase, L\textsubscript{\alpha}, are termed thermotropic phases (Figure 3). Thermotropic phases can be attained simply by changing temperature. Transition temperatures are affected by chain length and degree of unsaturation and head group size, polarity, and hydrophilicity. Recent Monte-Carlo simulations studied the structural changes that occur upon transition from the L\textsubscript{c} to L\textsubscript{\alpha} phases.\textsuperscript{38,39} The L\textsubscript{c} phase is not biologically relevant; therefore, the following discussion begins with the L\textsubscript{\beta\prime} phase. Thermotropic phase transition results in dramatic changes to the bilayer physical properties (water penetration depth, viscosity, density, membrane thickness, free volume, etc.) and dynamic properties (lipid diffusion, permeability, etc.).
Figure 3: The thermotropic structures of phospholipid membranes show variation in the packing density of the lipid chains. Indicated here are cross-sectional views of the bilayer.
**L⁰⁻to P⁰ Phase Transition.** The L⁰ phase is characterized by the tilt angle of the lipid chains \((0° \leq \phi \leq 30°)\). The hydrocarbon chains in this phase are in the fully extended, all *trans*, configuration, and densely packed. The transition temperature, \(T_p\), for DPPC is 35°C. At this temperature, the P⁰, "rippled" phase forms. As the temperature approaches \(T_p\), rotations in the head group about the P-O bond become more frequent as the head group begins to "melt."\(^{41}\) The area of the lipid molecules increases resulting in a decrease in the thickness of the bilayer. Increased rotation of the polar head groups and alkyl chains results in undulation along the bilayer plane.\(^{42}\) These undulations make this phase microscopically heterogeneous. The tilt angles for the lipid chains range from 0 to 19° and are normal to the local bilayer plane.\(^{43}\)

**P⁰⁻to L⁰ Phase Transition.** This transition, main chain melting, is the most widely studied transition involving phospholipids and the resulting phase, L⁰, is the phase in which most lipids, in biological systems, exist. The corresponding transition temperature, \(T_m\), for DPPC is 41°C. The enthalpy of this phase transition (39.2 kJ/mol)\(^{44}\) is approximately ten times that of the other two transitions. Several other parameters for this phase transition can be found in the literature.\(^{41,42,44}\)

The primary change during this phase transition is the number of *gauche* interactions in the alkyl chain.\(^{42}\) As the head group rotational frequency increases, the bilayer expands (≈20%) to minimize repulsive interactions between head groups. This lateral expansion also minimizes the interactions between lipid chains and can be seen as
an “ironing-out”\textsuperscript{41} of the rippled phase undulations. This greater surface area results in decreased bilayer thickness (≈15%). This surface area increase also results in a greater degree of hydration as now, the head groups have more space and can hydrate maximally. This lateral expansion and decrease in bilayer thickness also decreases the cohesive forces within the bilayer.

**Multiple Component Lipid Bilayers: “Rafts” and Transient Domains**

Biological membranes are not comprised of one component, but rather exist as heterogeneous mixtures of phospholipids, cholesterol, and integral membrane proteins. Research on these multi-component systems reveals several distinct features not found in single component membranes. The distinct phase transitions vanish in mixed lipid bilayers and are replaced with the formation of phase domains, or lipid “rafts.”\textsuperscript{45} Lipid “rafts” have been extensively studied due to evidence that these domains are important for several biological functions (signal transduction, lipid trafficking etc.)\textsuperscript{46-49}. Similarly, lipid rafts have recently been implicated in the onset of neurodegenerative diseases (Alzheimer disease specifically). Formation, metabolism and aggregation of amyloid-β (Aβ) protein (believed to be the major contributor to amyloidal plaque formation) is thought to occur in cholesterol-rich lipid “rafts.”\textsuperscript{50}
Figure 4: Schematic representation of domain, “raft,” formation in multi-component membranes. The dark regions represent “ordered” domains. Small domains exhibit a transient nature (lifetimes $\approx$ ps to ns); while large domains persist for minutes.
Thermodynamically, the formation of phase segregation in phospholipid bilayers resulting in domain formation can be attributed to differences in interaction energies (chemical potential) between unlike lipids (hetero-interactions) and like lipids (homo-interactions).\textsuperscript{51,52} For a recent review, see Almeida et al.\textsuperscript{53} One of the most studied systems, monolayer DMPC:DSPC films, was investigated using Brewster angle microscopy\textsuperscript{54} and analyzed using classical nucleation theory. This theory describes the formation of these domains as being dependent upon line tension and chemical potential:

\[
\Delta G = -n \Delta \mu + 2\pi r \lambda
\]

(1.1)

where \( n \) is the number of molecules in the seed, \( \Delta \mu \) is the chemical potential difference between the two phases, \( r \) is the radius of the seed, and \( \lambda \) is the line tension (a one-dimensional analogue of surface tension) between the two phases. Monte-Carlo simulations were employed to study the state separation of this system (DMPC:DSPC) and gain insight into the interaction between lipids.\textsuperscript{55} Interchain interactions were determined to be important based upon the presence of a coexistence region in the phase diagram in qualitative agreement with experimental results from previous EPR experiments.\textsuperscript{56} The coexistence region is comprised of inhomogeneous domains surrounded by a homogeneous system. The appearance of this phase separation requires temperatures higher than the percolation threshold temperature determined to be 303 K (30\(^\circ\) C) for the DMPC:DSPC system.\textsuperscript{55} The formation of fluid phase domains enriched in DLPC were recently observed using coarse-grained simulations of a DLPC: DSPC system.\textsuperscript{57}
Figure 5: Hypothetical phase diagram for a two component phospholipid membrane comprised of lipids differing only in alkyl-chain length. At temperatures below the transition temperature for lipid A, a heterogeneous gel phase exists. As temperature increases, a homogeneous gel-phase surrounds an inhomogeneous liquid phase. Approaching the upper solid line (the line of gel separation), the system consists of a homogeneous fluid phase with inhomogeneous gel phase domains. Above this line, only a homogeneous fluid phase exists.
Visualization of these domains, spectroscopically, requires the incorporation of probe molecules that exhibit preferential partitioning into one phase of the bilayer (for a recent review of domain partitioning properties of fluorescent probes, see Vaz and Melo).\(^{58}\) Observation of domains in DPPC:DOPC systems was achieved using confocal fluorescence microscopy with the fluorescent BODIPY probe molecule.\(^{59}\) BODIPY labels attached at the head group of the phospholipid DPPE exhibit preferential partitioning into the fluid DOPC domains. Fluorescent images of the domains, confirmed with atomic force microscopy (AFM), suggest that the ordered domains span both leaflets of the bilayer. Fluorescence correlation spectroscopy (FCS) has been used to observe domain formation in giant unilamellar vesicles of DOPC:SM:Cholesterol.\(^{60}\) In another study, phosphatidylinerine (PS) lipids labeled with pyrene sensitizers were mixed with phosphatidylcholines (PC) and the variation in the \(I_{\text{excimer}}/I_{\text{monomer}}\) ratio was measured. An increase in this ratio as the PC chain length was reduced indicated that self-sorting was more substantial when the hydrophobic mismatch (difference in the alkyl chain length) was greatest.\(^{52}\)

Time-resolved AFM experiments have demonstrated the formation of domain structures and branched networks of gel-phase lipids on nanometer length scales and seconds time scales.\(^{61}\) These branched networks are believed to be biologically relevant for bilayer-active species that exhibit different activities based upon the length of the interface between two bilayer phases (phospholipase A\(_2\) has been demonstrated to exhibit enhanced activity at bilayer domain interfaces).\(^{62}\) Monte Carlo simulations, however, have attributed the enhanced activity of phospholipase A\(_2\) at lipid transition temperatures
to clustering of these peripheral proteins at the bilayer surface due to preferential interactions with a particular lipid phase. Preferential interactions with a specific lipid-enriched domain have been demonstrated by several proteins. The specificity demonstrated by these protein is often ascribed to more favorable hydrophobic interactions.

Orientational order can be induced in phospholipid bilayers through the introduction of molecules, lipids, or proteins and is important in biological processes. Cholesterol has been shown both experimentally and with simulations to induce ordering effects in phospholipid systems. Spontaneous segregation of bilayer components forming ordered and disordered domains, “rafts,” induced by the presence of cholesterol has been observed. The degree of hydrophobicity and the hydroxyl functionality present at C3 in cholesterol have been determined to be the major structural requirements for sterols to induce local ordering effects. Raft formation induced by the presence of proteins have also been observed in simulations and induced “raft” formation by GPI-anchored proteins in the influenza virus envelope protein has been observed experimentally. These large stable rafts are believed to be formed by the coalescence of smaller, unstable domains and have been linked to the onset of downstream signaling processes. Vesicle aggregation and other bilayer structural rearrangements have also been observed upon incorporation of proteins into mixed lipid bilayers.
Diffusion in a Phospholipid Bilayer

Free volume and viscosity variation in a phospholipid bilayer result in complex diffusion dynamics exhibited by permeating molecules. To better understand the diffusion of bilayer embedded species the thermodynamics controlling bilayer diffusion need to be understood. To this end, several researchers have addressed the variation of both lateral diffusion (diffusion parallel to the bilayer plane) and vertical diffusion (through the bilayer) of several different entities (proteins, lipid, small molecules, etc.). Some of the conclusions relevant to the work described here are discussed below.

Filippov et. al. studied the lateral diffusion of lipid molecules in oriented bilayers using pulsed field gradient 1H NMR. Variation in the water content of DMPC bilayers resulted in a reduction in the relative diffusion rate. Likewise, the addition of cholesterol resulted in retardation of the lateral diffusion rate. The measured lateral diffusion rate at a cholesterol content of 33%, I, domain formation occurs at this composition, was reduced 70% relative to the diffusion rate measured in a pure DMPC bilayer. Filippov also determined an activation energy for the lateral diffusion process of approximately 30 and 50 kJ mol\(^{-1}\) at a water concentration of 55% and cholesterol concentrations of 0 and 33% respectively. Fluorescence recovery after photobleaching (FRAP) experiments conducted on bilayers containing NBD-PE molecules have yielded similar results. FRAP experiments have also concluded that increased cholesterol content resulted in increased (reduced) lateral diffusion coefficients below (above) the main-chain melting transition temperature of DMPC (\(T_M = 24 \degree C\)). Papahadjopoulos et. al. determined that
amphiphilic molecules (relevant to the results given in this dissertation) of intermediate molecular weight (300 – 3000 g mol$^{-1}$) should exhibit lateral diffusion coefficients of $D \sim 10^{-8} – 10^{-7}$ cm$^2$ s$^{-1}$ based on their results obtained with an alkylated cyanine dye.$^{91}$ Lateral diffusion rates were determined to depend on the packing density and free volume properties of bilayers with diffusion coefficients determined for DOPC > DPPC ≈ Sphingomyelin due to differences in packing densities.$^{92}$ Simulations have also predicted a reduction in the lateral diffusion rate upon increasing cholesterol content.$^{93}$ Reduced lateral diffusion rates were related to reductions in the free area (a two-dimensional equivalent to free volume) with increasing cholesterol content. Ubiquinone (UQ, a small molecule involved in photosynthesis) was found to exhibit different diffusion rates depending on where it was initially located in simulations.$^{94}$ UQ initially placed in the DPPC bilayer midplane diffused three times faster than the surrounding lipids due to bilayer reduced viscosity and density, while UQ initialized in the bilayer head-groups exhibited diffusion characteristics similar to the surrounding lipid molecules. Translation between the lipid head-groups and the aliphatic interior of UQ was not observed.

The diffusion of a small molecule across a phospholipid bilayer can occur either actively (through an appropriate channel or using a chaperone) or passively (basal transport). For the purposes of the work described here, only passive ion transport will be discussed. Passive ion transport comprises three steps,$^{95}$ 1) Loss of the waters of solvation as the molecule partitions from the aqueous phase into the bilayer. 2) Diffusion of molecule through the bilayer. 3) Partitioning of the molecule from the bilayer to the
aqueous phase on the other side of the bilayer. Particle induced X-ray and γ-ray emission experiments studied the rate of transport of alkali metal ions through a phosphatidylcholine bilayer. Heavier ions were found to diffuse faster and as hydrated species, not bare ions or ion pairs. Likewise the rate of ion transport increased concomitantly with a decrease in alkyl chain length.

The composition and phase of the lipids in the bilayer has been found to dramatically alter the rate of ion transport. Second harmonic generation (SHG) experiments have shown that malachite green will not diffuse across a gel-phase bilayer. Erythrocytes enriched in cholesterol (in vitro) exhibited dramatically reduced ion transport activity, both active and basal transport. ESR experiments have found that bilayer permeability increases upon addition of cholesterol up to region 3 of a phospholipid bilayer coupled with a dramatic reduction of transport across the bilayer midplane. This is in agreement with FCS results which observed a moderate increase in diffusion coefficients upon increased cholesterol content. The rate of passive and active trans-bilayer ion flux was studied in different phospholipid subclasses. Gross et. al. demonstrated that an increasing degree of alkyl chain unsaturation resulted in enhanced passive ion transport and entropic differences were the predominant factor in determining the rate of passive ion transport for bilayers with similar surface charge density.

Simulations have also predicted the rate of passive ion transport. Xiang observed a decrease in the diffusion rate of noble gas atoms of increasing size. Local isomerizations were determined to be responsible for lateral diffusion while overall chain rotation would result in the formation of elongated free volume regions necessary for trans-
bilayer transport.\textsuperscript{100} Essex et. al. studied the rate of transport of several small organic molecules through a DPPC bilayer.\textsuperscript{101} They determined that the diffusion coefficient decreased dramatically upon incorporation into the bilayer. The permeation barrier of hydrophilic compounds (water, methanol, acetic acid, etc.) was the aliphatic interior, while the polar head-groups present the greatest permeation barrier for hydrophobic compounds (benzene, ethane, methyacetate).\textsuperscript{101}
1.2 Spiropyran: Physical Properties and Photochemistry

Spiropyran, first studied in the early 1960’s,\textsuperscript{102-104} consists of two weakly interacting moieties, substituted indole and benzopyran, connected orthogonally through a spiro-carbon. Upon exposure to UV light, photochemical bond cleavage of the $C_{\text{spiro}}$-O bond, and subsequent isomerization results in formation of a planar merocyanine (MC) form allowing conjugation to extend across the molecule. Increasing the conjugation length in this system is readily observed as a dramatic color change. Spiropyran, with no visible absorption, is converted to merocyanine, which has an intense $\pi\pi^*$ transition observable in the visible region of the spectrum. This property of SP molecules enables utilization of this class of compound to an array of applications. This photochemical reaction results in dramatic changes to the physical properties of the molecule. The dipole moment increases dramatically from 4.3 D to 17.7 D.\textsuperscript{105} As can be seen in Scheme 2, the spiro form has a chiral center at the spiro carbon atom; the barrier to conversion between the two enantiomers has been determined to be $\Delta G^\ddagger = 85.9$ kJ mol$^{-1}$.\textsuperscript{106} For the work described here, the spiro form is considered to be a racemic mixture of the two enantiomeric species.
Scheme 2: Spiropyran exists in equilibrium with the ring-opened merocyanine form

\[
\text{SP} \quad \overset{k_1}{\rightleftharpoons} \quad \text{MC}
\]
A number of reviews discussing the photochemistry and photophysics of spiropyran have been published.\textsuperscript{107-110} In spite of the intense scrutiny, there are several aspects of spiropyran photophysics that still require elucidation (the structure of the reactive intermediate formed in the SP $\rightarrow$ MC reaction, the ground state MC isomeric distribution, the molar absorptivity of the MC form).

The thermal transition between SP and MC forms has been studied in an effort to understand the ground-state potential energy surface connecting SP and MC forms.\textsuperscript{111} The ground state ring-opening reaction has been studied in several simulations.\textsuperscript{112-115} Experimentally, Görner has determined the activation energy for thermal conversion of SP to MC to be $E_a = 80 - 110 \text{ kJ/mol.}\textsuperscript{116}$ In agreement with this, DFT calculations have determined an activation energy of 130 kJ/mol.\textsuperscript{114}

The merocyanine structure possesses a central methine bridge consisting of three C-C bonds (labeled $\alpha$, $\beta$, and $\gamma$, see section 2.2 scheme 4) that possess both single and double bond nature. Therefore, rotation about these bonds requires more energy than rotation about a single bond. This results in the possible formation of 8 different isomers.\textsuperscript{117} MC isomers possessing a "cis" conformation at the $\beta$ position have been determined to be unstable; therefore, only four possible conformations of ground state MC isomers (TTC, TTT, CTC, CTT) are stable.\textsuperscript{115} $C_{\text{spiro}}$-O bond cleavage results in two possible isomerization pathways both leading to TTC isomer formation (This isomer has been determined to be the most stable, see Chapter 2). The first pathway involves formation of the highly unstable CCC isomer followed by the CTC form and ultimately TTC formation.\textsuperscript{114} The second pathway involves formation of TCC which isomerizes to
the TTC form directly.\textsuperscript{115} There is no general agreement concerning which thermal isomerization pathway is more likely. The thermal conversion of SP to MC was determined to be rate-limited by the isomerization about the central bond $\beta$ on the methine bridge,\textsuperscript{117} which suggests that the first pathway may be preferred.

The thermal reversion mechanism, MC $\rightarrow$ SP, has also been studied. The decoloration kinetics were determined to be mono-exponential in nonpolar and polar-aprotic solvents with an activation energy of $E_a = 70$ and 100 kJ mol$^{-1}$ respectively.\textsuperscript{118} In polar-protic solvents, the thermal decay of MC was observed to be bi-exponential with activation energies of 70 and 100 kJ mol$^{-1}$ for the slow and fast component respectively.\textsuperscript{118} A three species reaction scheme (with two MC species, a “colored” and “uncolored” form) with solvent-dependent transition state energies was employed to explain these observations (Figure 6A). The reduced activation energy for the ring-closing reaction of MC in nonpolar solvents has been attributed to greater stability of the quiniodal resonance form in comparison to the zwitterionic form which is likely to be more stable in polar solvents (Figure 6B).\textsuperscript{119} In the quiniodal form, the central $\beta$ bond has essentially single-bond character allowing rotations to occur with reduced energy requirements.
Figure 6: A) Potential energy surface determined for the thermal decoloration of MC indicating the presence of multiple MC forms. B) Resonance forms of MC. The quinoidal form is believed to be stabilized in nonpolar environments. The zwitterionic form, with greater charge separation is likely to be more stabilized in polar environments.
Hartree-Fock calculations concerned with the change in energy resultant from changes in the dihedral angles of the methine bridge bonds determined that isomerization about the α or γ bonds is most likely the first step towards ring closure.\textsuperscript{119} Subsequent isomerization about the α or γ bond, depending on which bond was rotated in the first step, followed by the final isomerization about the β bond results in formation of the highly-strained CCC form which invariably leads to SP formation.

As indicated above, an equilibrium exists between ring-closed SP and ring-opened MC forms. SP is thermodynamically more stable; however, excitation of SP results in ultrafast $C_{\text{spiro}}$ bond cleavage and formation of the MC product. For the spiropyran discussed here, possessing a nitro substituent at the 6 position of the benzopyran moiety, formation of the MC photoproduct is believed to arise from inter-system crossing ISC from the excited SP singlet state forming the triplet state, $T_1$.\textsuperscript{120} Removal of this substituent results in evolution along the excited singlet potential energy surface.\textsuperscript{115}

The photophysical pathway connecting the $S_1$ state of SP with the $S_0$ state of MC has been intensely studied. Picosecond,\textsuperscript{117,121-126} nanosecond,\textsuperscript{120,127-131} and microsecond\textsuperscript{116} time scales have been studied using various spectroscopic techniques. Few computational studies have been dedicated to this process due to difficulties in simulating excited state processes.\textsuperscript{115} No undisputable description of the photophysics involved in the ring-opening reaction has been published. Several reviews have been written concerning this topic.\textsuperscript{107-110} The photochemical ring-opening reaction has also been studied in the gas phase.\textsuperscript{132} A popular SP derivative, spiroxazine, has also received a great deal of attention, due to greater fatigue-resistance,\textsuperscript{133,134} and detailed studies can be found elsewhere.\textsuperscript{135-138}
The reactivity of intermediate species in the photochemical ring-opening reaction has been attributed to the reduced fatigue-resistance of spiropyran molecules.\textsuperscript{139}

Excitation of SP results in efficient population of the $S_1 (n\rightarrow\pi^*)$ state followed by intersystem crossing (ISC) to the triplet state. Ring opening, $C_{\text{spiro-O}}$ bond scission, occurs as fast as 200 fs after excitation\textsuperscript{122} due to the presence of a conical intersection of the ground and excited state potential energy surfaces (Figure 7).\textsuperscript{140} This conical intersection was determined to continue through the isomerization process of the MC form resulting in efficient formation of the trans-MC form from the cis-MC intermediate.\textsuperscript{141} This has been assigned to excited state crossing of the energetically proximal $n\rightarrow\pi^*$ and $\pi\rightarrow\pi^*$ transitions.\textsuperscript{142} For merocyanines that possess a 6-nitro substituent, the photochemical evolution producing the merocyanine species is believed to occur predominantly along a triplet manifold.\textsuperscript{115} The photochemical ring-closing reaction for merocyanines that do not possess a 6-nitro substituent was also determined to proceed through conical intersections explaining the rapid ring-closing observed upon excitation of these molecules.\textsuperscript{143} Gas phase dynamics of the spiropyran ring-opening reaction have suggested that a high degree of charge separation is present during the isomerization reaction.\textsuperscript{132}
Figure 7: The conical intersection of the potential energy surfaces was determined to be the reason that ring-opening occurs so quickly in spiropyran.\textsuperscript{140}
Recently, Fidder et. al. determined that the internal conversion (IC) process from the SP S\text{1} excited state has a lifetime of several ps and a quantum yield as high as 0.8.\textsuperscript{144} According to Kasha's rule, IC processes are generally inefficient and occur on a µs to ms time scale, much slower than the lifetimes determined by Fidder. Application of the standard energy-gap law, a relation of the IC rate to the electronic excitation energy gap, did not accurately explain the behavior of the SP S\text{1} state. Flexible portions of the molecule resulting in large conformational changes accounted for the efficient IC process.\textsuperscript{144}

Even fewer studies have focused on direct excitation of the MC form. Hobley et. al. investigated the photochemical reaction dynamics of a MC molecule possessing two nitro functionalities.\textsuperscript{145} Incorporation of a second nitro substituent at the 8 position resulted in a semi-permanent MC molecule allowing direct MC excitation. Excitation of MC resulted in efficient population of an excited state (Figure 8). This excited state, S\text{\textsubscript{n}}, quickly relaxes (τ ~ 10 ps) resulting in formation of a twisted "cis" form. This species then decays bi-exponentially (τ\textsubscript{1} ~ 75 ps and τ\textsubscript{2} ~ 400 ps) resulting in MC-S\textsubscript{0} population and formation of SP. Hobley determined a quantum yield for ring-closure in acetonitrile of $\Phi_{MC\rightarrow SP} \sim 0.44$.\textsuperscript{145} We conducted ultra-fast studies on MC in toluene and concluded that the ring-closure reaction was unfavorable in nonpolar environments.\textsuperscript{146} Instead, internal conversion ($\Phi_{IC} \sim 0.8$) and excited state isomerization processes resulting in formation of other MC isomers were observed with little evidence of SP formation up to 3 ns.
Figure 8: The reaction mechanism determined by Hobley et. al. for the direct excitation of MC isomers. Excitation of merocyanine was determined to populate a singlet excited state, which quickly relaxes to the first singlet excited state followed by an equilibration process with the excited “cis” isomer. Further reaction of the “cis” isomer results in SP formation and isomerization forming the “trans” conformation which is also populated directly from internal conversion.145
SP molecules have been incorporated in a myriad of applications from solid state lasers\textsuperscript{147} to anti-tumor treatments.\textsuperscript{148} Spiro compounds have been used to attenuate fluorescent emission\textsuperscript{149} and selectively bind with metal ions resulting in novel materials.\textsuperscript{150-154} Monitoring the photochemistry of the SP/MC system is particularly relevant in highly viscous or solid state environments. The photochemical conversion between SP and MC forms was determined to have a molecular volume change of \(\sim 78 \, \text{Å}^3\) molecule\(^{-1}\) (in cycloalkane solvents).\textsuperscript{131} The reaction kinetics have been demonstrated to be sensitive to the available free volume in a polymer matrix.\textsuperscript{155} Studies of SP molecules embedded in polymeric systems have demonstrated the response of the thermal ring-closing reaction to changes in viscosity\textsuperscript{156,157} and local polarity.\textsuperscript{158} Incorporation of spiropyrans in liquid crystalline systems have shown the response of MC molecules to phase changes\textsuperscript{159} with phases possessing reduced density and viscosity exhibiting enhanced ring-closing rates.\textsuperscript{160} The spiropyran ring-opening reaction was used to test different models for reaction kinetics in amorphous polymeric environments.\textsuperscript{161} Surface effects (differences in polarity, hydrophobicity and intermolecular forces such as hydrogen bonding) were examined using the solvatochromic nature of merocyanine molecules.\textsuperscript{162} Diffusion from nonpolar to polar environments have been observed for ring-opening reactions of spirooxazines (resulting in dramatic changes in the dipole moment) embedded in phospholipid membranes.\textsuperscript{17}
Figure 9: Model of the diffusion of the ring-opened form of spirotaxazine to a more polar environment observed in phospholipid bilayers as a blue-shift in the MC absorption spectrum.\textsuperscript{17}
1.3 This Work

In this work, a series of novel photochemical spiropyran / merocyanine sensitizers were synthesized to study the physical properties of phospholipid bilayers. The structure of all the spiropyrans can be found in Scheme 3. One of the spiropyran molecules described in this work is a novel compound and the synthetic details can be found in section 4.3. Porphyrin molecular sensitizers were also used to study the rate of thermal energy relaxation in phospholipid bilayers and the structures of these species can be found in section 5.2 (Scheme 9). Several different architectures of phospholipid bilayer were explored through the course of the work described here. The preparation of supported planar phospholipid bilayers are described in Appendix C. Frequently, aqueous dispersions of unilamellar vesicles comprised of a single lipid were employed and details concerning the preparation and exact composition of the vesicles pertinent to each chapter will be found in the corresponding experimental sections.
Scheme 3: Merocyanine and spiropyran molecular sensitizers

Merocyanine (MC)

Spiropyran (SP)

MC1: $R = \text{CH}_3$
MC2: $R = \text{CH}_2\text{CH}_2\text{OH}$
MC3: $R = \text{CH}_2\text{CH}_2\text{OCOCH}_2(\text{CH}_2)_{13}\text{CH}_3$
Predominantly, spectroscopic techniques were used to explore various phospholipid bilayer phenomena. Two different types of ultra-fast spectroscopy were employed, pump-probe spectroscopy and transient grating spectroscopy. The home-built femtosecond / picosecond spectrometer is described in detail in section 2.2. Likewise, the experimental set-up used to collect the transient grating photo-acoustic data is described in section 5.4. Linear dichroism spectroscopy was used to determine the orientation of membrane embedded sensitizers in phospholipid bilayers and a brief description of the experimental techniques and mathematical treatment of the obtained data can be found in Appendix C.

The work described here addresses several questions pertinent to the understanding of the variation of phospholipid bilayer physical properties. Free volume variation within a phospholipid bilayer (as outline in section 1.1) has been theoretically explored. However, no experimental studies have addressed this problem. Using our merocyanine sensitizers, we were able to study the free volume variation within a phospholipid bilayer on a picosecond (Chapter 3) and second (Chapter 4) time scale. The free volume of the bilayer interior was determined according to a modified Kramers’ analysis of the viscosity dependence of the isomerization rate constants. Our results were consistent with simulations which predict the free volume present in the bilayer midplane to be substantial. With merocyanines embedded at different bilayer depths, we developed a free volume profile perpendicular to the bilayer plane. Different detected free volumes on a ps and second time scale can be correlated with relative rates of phospholipid dynamics (acyl chain reorientation times).
The rate at which thermal energy translates through a phospholipid bilayer is also difficult to determine. We employed transient grating photo-acoustic spectroscopy to study this process (Chapter 5). Membrane-embedded porphyrin molecules were photoexcited and allowed to relax to the ground state. Due to the choice of porphyrin molecule (porphyrins with an iron atom chelated in the center of the porphyrin macrocycle) the predominant relaxation pathway was a very fast inter-system crossing pathway producing a triplet state which also quickly undergoes internal conversion. This process releases energy into the surrounding phospholipid environment. In turn, the phospholipid bilayer expands slightly launching an acoustic compression wave into the surrounding solvent. Subsequently, thermal energy transfer occurs between the bilayer and the surrounding solvent resulting in shrinking of the phospholipid bilayer and the release of an additional acoustic wave. Detection of these processes was achieved by measuring the change in diffraction intensity at an angle that satisfies the Bragg conditions particular to the experimental set-up. We developed a model to explain these experimental observations (found in section 5.6) and determined that thermal diffusion occurs predominantly by Brownian motion rather than by a concerted mechanism in which the thermal energy is transferred along the alkyl chain vibronically. However, a more cooperative vibrational energy transfer method could not be excluded. Lipid isomerizations and local melting effects were determined to not play a role in the thermal energy transfer.
Chapter 2: Excited State Dynamics of Spiropyran-Derived Merocyanine Isomers


2.1 Overview

Merocyanine (MC1) isomers that are formed after absorption of a UV photon by 6-nitroBIPS spiropyran (1′,3′-dihydro-1′,3′-3′-trimethyl-6-nitrospiro[2H-1-benzopyran-2′,2′-(2H)-indole]) were studied. Several, predominantly TTC and TTT, merocyanine isomers are present in toluene solution ("T" and "C" indicate trans- and cis- conformations of the C-C bonds in the methine bridge). Excitation in the MC1 visible absorption band (at 490 nm, 550 nm, and 630 nm) with 100 fs laser pulses was used to study MC1 excited state dynamics. Internal conversion on the ps time scale was found to be the dominant relaxation pathway. Excited state isomerization reactions were also observed. Excitation at 630 nm (assigned to TTC isomer excitation) leads to formation of a third isomer (either CTC or CTT). Excitation at 490 nm (assigned to TTT isomer excitation) leads to more complex excited state relaxation, including formation of two isomers: TTC (absorption at 600 nm) and CTC or CTT (absorption at 650 nm).
2.2 Merocyanine Isomers Background

Photochromic molecules have been used in a broad array of both applied and fundamental studies. This is a consequence of their unique optical properties. Investigations of photochromic reactions have contributed to an improved understanding of twisted internal charge transfer (TICT), conical intersections of potential energy surfaces, and other effects. New technologies such as optical switching, optical data storage, and molecular logic gates have benefited from the incorporation of photochromic materials. Biological applications are also pursued due to the often dramatic changes in molecular structure, polarity, and volume occurring in photochromic reactions.

Perhaps the most extensively studied photochromic compound is 6-nitroBIPS spiropyran, SP1 (Scheme 4). As mentioned in section 1.2, it consists of weakly interacting substituted indole and benzopyran moieties connected orthogonally at a spiro carbon atom. Spiropyran converts to a planar form, merocyanine (MC1), upon absorption of a UV photon. This reaction results in an increased dipole moment (from 4.3 D to 17.7 D), larger molecular volume, and the onset of absorbance in the visible region. This new absorption band is indicative of increased conjugation in the MC1 form. The reaction is both thermally and photochemically reversible.

Merocyanine contains a methine bridge consisting of three conjugated C-C bonds. Three dihedral angles (labeled $\alpha$, $\beta$, and $\gamma$ for the TTC isomer in Scheme 4) can be altered to form several isomers. It has been suggested that the TTC form is the most stable
ground state isomer due to hydrogen bonding interactions;\textsuperscript{167,168} however, the relative energies of the remaining isomers are still unclear. \textsuperscript{1}H and \textsuperscript{13}C NMR studies\textsuperscript{168-170} and density-functional theory (DFT) calculations\textsuperscript{114,115} suggest that the TTT isomer is the second most stable isomer. The relative MC1 isomer energies determined from quantum chemical calculations and NMR line broadening studies are given in Scheme 4.\textsuperscript{114,115,170} The activation barrier for isomerization in the ground state is \(\approx 40 \text{ kJ/mol}.\textsuperscript{170}\)
Scheme 4: Spiropyran-derived merocyanine isomers

\[ \text{6-nitroBIPS} \xrightarrow{hv_1} \begin{cases} \text{TTC, } E = 0 \text{ kJ mol}^{-1} \\ \text{TTT, } E = 4.6 \text{ kJ mol}^{-1} \\ \text{CTC, } E = 7.8 \text{ kJ mol}^{-1} \\ \text{CTT, } E = 9.1 \text{ kJ mol}^{-1} \end{cases} \]

\[ \text{hv}_2, \Delta \]
The presence of multiple merocyanine isomers have been implicated in photo-initiated ring-opening\textsuperscript{123,124} and thermal decoloration reactions.\textsuperscript{118,152} Few studies, however, have been conducted with direct excitation of specific merocyanine isomers.\textsuperscript{126,145} Hobley et al. studied the excited state dynamics for a merocyanine possessing nitro substituents at the 6 and 8 positions of the benzopyran moiety (6,8-dinitroBIPS).\textsuperscript{145} They found that excitation at 390 nm did not result in selective MC isomer excitation.\textsuperscript{145}

In this chapter, we show that transient absorption spectra and kinetics measured using several excitation wavelengths in the visible absorption band region indicate the presence of multiple MC1 isomers. Using ultrafast pump-probe experiments, we selectively excited these isomers to determine their excited state relaxation pathways.

2.3 Pump-Probe Spectroscopy

\textbf{Ultrafast transient absorption spectroscopy.} The optical scheme of the pump-probe transient absorption spectrometer is shown in Scheme 5. A regeneratively amplified Ti:Sapphire laser system (Spectra Physics) provided 100 fs, 0.7 mJ pulses at 780 nm and 1 kHz repetition rate. Excitation pulses were obtained using an optical parametric amplifier (Topas, Quantronix/Light Conversion). A white-light continuum was generated from focused probe pulses incident on a sapphire window. A stable continuum was generated by controlling probe light intensity with a \(\lambda/2\) waveplate and a Glan-Taylor polarizer and by translating a 2 mm thick sapphire plate in the beam waist region using a micrometer stage. The relative polarizations of pump and probe beams were adjusted to 54° (the
"magic angle"). Kinetics measurements from 100 fs to 3 ns were made by varying the probe beam time delay with a computer-controlled linear translational stage. Electrical signals from Si photodiodes were gated with a boxcar. To improve the signal/noise ratio, an optical chopper blocked every other pump pulse, and computer software (developed using LabView, National Instruments) was used to take the difference of signals measured with and without excitation.
Scheme 5: Optical scheme of a pump-probe spectrometer

![Optical Scheme Diagram]

OPA - optical parametric amplifier, BS1, BS2 - beamsplitters (90% T), M - mirrors, WP - λ/2 waveplate, P - polarizer, L - plano-convex lenses (L1 f=100 mm, L2 f=50 mm, L3 f=250 mm, L4 f=350 mm, L5 f=75 mm), SW - sapphire window, F1 - low-pass filter, F2 - interference filter (10 nm bandwidth), B - beam block, S - sample, PD1, PD2 - photodiodes, CH - chopper. The angle between the pump and probe beams Θ = 4°.
SP1 solutions in toluene (2.5 mM) were measured in a 1-mm optical path length flow cell connected to a peristaltic pump equipped with Viton tubing. The temperature was 25°C for all experiments. Preparation of samples containing a substantial merocyanine concentration was achieved using an UV-lamp (364 nm) to illuminate the sample reservoir and the volume of the flow cell immediately preceding the pump and probe beams. The peristaltic pumping rate was approximately 1.7 mL/min. Variation of the repetition rate of the laser indicated no change in the transient absorption characteristics. Absorption spectra were obtained using a HP8452A diode-array spectrometer. Fluorescence measurements were conducted with a Varian Cary-Eclipse fluorimeter. Merocyanine formation was induced using a low power Entela UV-lamp (central irradiation wavelength of 364 nm).

2.4 Resolution of MC Isomers

Merocyanine steady state absorption and fluorescence properties. In toluene, the ring-closed form of SP1 predominates. Upon UV-excitation, ring-opening and subsequent isomerization results in the appearance of a new absorption band with the maximum of absorption at 579 nm (Figure 10A). The emission spectrum of the MC1 form (maximum at 625 nm) is shown in Figure 10B.
Figure 10: A: SP1 absorption spectra in toluene before (-----) and after UV irradiation (---). The inset shows the expansion of the MC1 visible absorption band region. The wavelengths used for excitation in ultrafast experiments are indicated with arrows. B: Fluorescence emission spectrum measured in toluene with excitation at 490 nm. C: Fluorescence excitation spectra measured in toluene at 620 nm (-----) and 700 nm (---). D: Shift of the maximum of the fluorescence excitation spectrum with variation of the emission detection wavelength.
It is well established that multiple MC isomers can be formed in the SP→MC reaction, but absorption (and emission) properties of these various isomers have not been identified. The presence of multiple MC1 isomers in toluene solution is suggested by the fluorescence excitation spectra shown in Figure 10C. Depending on the wavelength at which fluorescence emission is measured (620 nm or 700 nm), the maximum of the excitation spectrum shifts from 567 nm to 600 nm. Several fluorescence excitation spectra were obtained when the detection wavelength was between 600-700 nm. At wavelengths below 610 nm, the maximum of the excitation spectrum was at 562 nm. As the detection wavelength is increased from 620-650 nm, the maximum of the excitation spectrum shifted from 569 nm to 593 nm. The maximum was at 598 nm for experiments with the emission measured above 660 nm.

This data could be interpreted by assuming the presence of at least two MC1 isomers in toluene solution, where emission at 600-610 nm is due to the first isomer, and emission between 660-700 nm is due to a second isomer. (At 610-660 nm, the overlapping emission of both isomers leads to a 36 nm shift of the excitation spectrum maximum depending on the detection wavelength.) Further evidence for this model was obtained in the time-resolved studies.

**Merocyanine transient absorption difference spectra.** To further characterize the MC1 isomers, we used 100 fs laser pulses to excite the MC1 absorption band at 490 nm, 550 nm, and 630 nm (see inset in Figure 10A). Figure 11A shows the transient absorption difference spectrum measured at 1 ps time delay after 550 nm excitation. The
negative signal with a minimum at \( \approx 575 \) nm is assigned to MC1 ground state bleaching, as it overlaps with the normalized MC1 steady-state absorption spectrum. The positive signal at 460-500 nm is assigned to the MC1 excited state absorption.\textsuperscript{145} The negative signals at 650-690 nm could have contributions from stimulated emission, as MC1 steady-state emission is observed at these wavelengths (Figure 10B).
Figure 11: Transient absorption difference spectra in toluene solution measured 1 ps after excitation with 100 fs laser pulses at 550 nm (■, A), 490 nm (○, B) and 630 nm (△, B). In A, the normalized and inverted steady-state absorption spectrum of MC1 in toluene is also shown (---). Error bars are indicated in the transient absorption spectra.
Different results are obtained when 1 ps transient absorption spectra are measured with 490 nm and 630 nm excitation (Figure 11B). While the major features (bleaching, excited state absorption, and stimulated emission) are also evident in these spectra, the bleaching minima shift from ≈ 575 nm for 550 nm excitation to ≈ 568 nm for 490 nm excitation and ≈ 600 nm for 630 nm excitation. The dependence of the bleaching minimum on the excitation wavelength supports the presence of multiple MC1 isomers in toluene solution.

Since excitation of the “red” (630 nm) and “blue” (490 nm) sides of the MC1 absorption band are most likely to excite different isomers, we proceed to an analysis of ΔA spectra measured using these excitation wavelengths (Figure 12 A and B). The decrease of the bleach and excited state absorption signal amplitudes at longer time delays indicates that excited state reactions are occurring on a ps time scale. Interestingly, the differences in the 2 ns time delay spectra shown in Figure 12 (A and B) and Figure 13 suggest that MC1 isomers do not follow the same excited state relaxation pathways.
Figure 12: Transient absorption spectra in toluene solution collected with $\lambda_{\text{exc}}=630$ nm (A) and $\lambda_{\text{exc}}=490$ nm (B). Time delays: 0 ps (—□—), 1 ps (—○—), 50 ps (—○—), 200 ps (—○—), and 2000 ps (—○—). Error bars are indicated for each measurement and 2000 ps spectra are multiplied 10 times for clarity.
Figure 13 compares the long-time (2 ns) difference spectra. The minima at 568 nm and 600 nm (measured with $\lambda_{\text{exc}}=490$ nm and $\lambda_{\text{exc}}=630$ nm, respectively) correspond to ground state bleaching. The positive absorption at 650 nm seen in both spectra suggests that following either "red" (630 nm) or "blue" (490 nm) excitation, a new isomer was formed in an excited state reaction. In addition, the $\lambda_{\text{exc}}=490$ nm spectrum has positive amplitude at 600 nm. The simplest model that is consistent with this data is the presence of three MC1 isomers in toluene solution. Two isomers would correspond to bleaching minima at 568 nm and 600 nm, while the third isomer would have absorption at 650 nm. Because the 650 nm absorption signal amplitude is small, the quantum yield of the third isomer is low (see section 2.5).
Figure 13: 2 ns time delay transient absorption difference spectra for $\lambda_{exc}=490$ nm (---○---) and $\lambda_{exc}=630$ nm (---△---). The spectra have been normalized at the maxima. Absorption at 650 nm in both spectra indicates formation of a third isomer.
This model is consistent with the identification of TTC and TTT isomers in acetonitrile solutions of merocyanine formed from 6,8-dinitroBIPS (NMR lineshape analysis was used in these studies).\textsuperscript{169} In experiments with ns time resolution, a positive absorption signal with a maximum at 635 nm was also attributed to a new MC1 isomer.\textsuperscript{120} The amplitudes of the bleaching and positive 650 nm signals in Figure 13 are similar to the results obtained by Göner et al.\textsuperscript{120} One important new finding in the current experiments is the unambiguous demonstration that the 650 nm signal amplitude is small in comparison to the initial bleaching amplitude (amplitude at 2 ns time delay is about 10\% of the initial amplitude, see Figure 12 and Figure 14 below). Therefore, the isomerization yield is low, and the major MC1 excited state relaxation pathway is internal conversion. The same conclusion was recently reached for the “closed-form” SP1 spiropyran.\textsuperscript{124}

**Excited state dynamics.** Figure 14 shows the transient absorption kinetics measured at 480 nm, at the MC1 ground state bleaching minimum, and at 650 nm. 490 nm and 630 nm were the excitation wavelengths.
Figure 14: Transient absorption kinetics measured at 480 nm (A), 568 nm/600 nm (B), and 650 nm (C). In all graphs □ shows data measured with \( \lambda_{\text{exc}}=490 \) nm and ○ shows data measured with \( \lambda_{\text{exc}}=630 \) nm. Note the breaks in both axes in B and C. Solid lines are fitting results. Kinetics were measured at equal MC1 concentrations in toluene solution; 490 nm excitation kinetics have been multiplied 5 times for clarity.
**Excited state absorption.** The positive signal at 480 nm is assigned to excited state absorption in agreement with earlier studies. For $\lambda_{\text{exc}} = 630$ nm, signal rise is faster than 100 fs and is followed by a 1.5±0.5 ps lifetime decay. In marked contrast, $\lambda_{\text{exc}} = 490$ nm leads to bi-exponential rise, where the smaller amplitude second rise component has a 2±0.5 ps lifetime. These 1.5-2 ps lifetime components could reflect vibrational relaxation or electronic relaxation from $S_n$ to $S_1$ states. The difference in amplitudes (positive or negative) could reflect different excited state absorption properties of MC1 isomers. On the longer time scale (data not shown in Figure 14A), 480 nm excited state absorption kinetics decay with the same lifetimes as the bleach recovery signals described below.

**Bleaching.** Figure 14B shows the bleaching kinetics at 568 nm ($\lambda_{\text{exc}} = 490$ nm) and at 600 nm ($\lambda_{\text{exc}} = 630$ nm). These wavelengths represent measured bleaching minima for the respective excitation wavelengths. Excitation at $\lambda_{\text{exc}} = 630$ nm results in faster bleach recovery than excitation at $\lambda_{\text{exc}} = 490$ nm. At $t>10$ ps, all kinetics were fit to exponential functions and the results are summarized in Table 1. The ground state recovery measured with $\lambda_{\text{exc}} = 630$ nm can be described by a 67±5 ps lifetime single exponential decay. The $\lambda_{\text{exc}} = 490$ nm ground state recovery yielded more complex kinetics which required a bi-exponential fit with $\tau_1 = 36±4$ ps and $\tau_2 = 270±25$ ps. Bleach recovery is incomplete for both kinetics in Figure 14B; the persistent bleach amplitudes are 10-12 % (Table 1).
Table 1: Excited state lifetimes and MC1 isomer spectral assignments

<table>
<thead>
<tr>
<th>$\lambda_{\text{exc}}, \text{nm}$</th>
<th>$\tau_1$, ps</th>
<th>$\tau_2$, ps</th>
<th>Bleach amplitude at $t &gt; 2000$ ps</th>
<th>Bleaching minimum, nm</th>
<th>MC1 isomer$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>630</td>
<td>$67 \pm 5$</td>
<td></td>
<td>$10 \pm 3%$</td>
<td>600</td>
<td>TTC</td>
</tr>
<tr>
<td>490</td>
<td>$36 \pm 4$ (60 %)$^a$</td>
<td>$270 \pm 25$ (28 %)$^a$</td>
<td>$12 \pm 4%$</td>
<td>568</td>
<td>TTT</td>
</tr>
</tbody>
</table>

$^a$ amplitudes of $\tau_1$ and $\tau_2$ components

$^b$ assignments based on isomer relative energies determined in Ref. 114, 115, and 170
**Stimulated Emission / Isomerization.** At t<10 ps, signals at 650 nm (Figure 14C) exhibit similar characteristics to the excited state absorption signals in Figure 14A. Excitation at \(\lambda_{\text{exc}} = 630\) nm results in the appearance of a negative amplitude signal within 100 fs followed by decay with a 1.5±0.5 ps lifetime. The kinetics measured with \(\lambda_{\text{exc}} = 490\) nm required bi-exponential fitting with \(\tau_1 = 100\) fs and \(\tau_2 = 2±0.5\) ps. At intermediate time delays (10 ps < t < 500 ps), the 650 nm kinetics behave in a manner similar to the bleaching signals. Excitation with \(\lambda_{\text{exc}} = 630\) nm resulted in a single exponential decay \((\tau_1 = 67±5\) ps) whereas excitation at \(\lambda_{\text{exc}} = 490\) nm yielded biexponential decay kinetics with \(\tau_1 = 36±4\) ps and \(\tau_2 = 270±25\) ps. At t > 500 ps, positive transient absorption signals were observed at this wavelength; this absorption band (Figure 13) was assigned to formation of the third MC1 isomer.

Thus, measurements at the excited state absorption, bleaching, and stimulated emission/new isomer absorption wavelengths can be consistently explained using one rate constant for \(\lambda_{\text{exc}} = 630\) nm and two rate constants for \(\lambda_{\text{exc}} = 490\) nm.

### 2.5 Analysis of Selective MC Isomer Excitation Data

**MC1 isomers and excited state relaxation pathways.** The excited state lifetime dependence on the excitation wavelength (Table 1) and shifts of the bleaching minima (Figure 11) suggest the presence of multiple MC1 isomers in toluene solution. Transient absorption difference spectra in Figure 13 (albeit measured with low resolution) 2 ns after excitation indicate the presence of at least three MC1 isomers with absorption at 568 nm,
600 nm, and 650 nm, respectively. The bleaching signals at 568 nm and 600 nm indicate that the corresponding MC1 isomers are present in the ground state. The two predominant ground state isomers are likely to be TTC and TTT, as they have the lowest ground state energies (Scheme 4). The third isomer could be either CTC or CTT. An NMR lineshape analysis also indicated the presence of TTT and TTC isomers in solutions of a related merocyanine.\textsuperscript{169}

The positive 650 nm signal (Figures 13 and 14C) suggests that this isomer is formed in excited state isomerization reactions but is not significantly populated in the ground state. This assignment is consistent with results obtained by Görner and coworkers, who, in experiments with ns time resolution, have observed positive signals with maxima at 635 nm which have been attributed to formation of “cis” isomers.\textsuperscript{120}

The difference spectra in Figure 13 also suggest that TTC and TTT isomers follow different excited state relaxation pathways. Excitation of the isomer with absorption (bleaching) at 600 nm leads to absorption at 650 nm, while excitation of the isomer with absorption at 568 nm leads to formation of both 600 nm and 650 nm absorbing isomers.

It is possible to assign TTC/TTT isomers to the 600/568 nm absorption bands if we assume that MC1 photochemical reactions take place on the singlet excited state potential energy surface and estimate excited state energies from the absorption and emission spectra. A singlet excited state isomerization pathway was proposed by Sheng et al, who theoretically analyzed merocyanine isomerization and decoloration reactions.\textsuperscript{115} They found that the isomerization energy barrier in the excited singlet state is much lower than barriers determined in the triplet and ground states (2.8 kJmol\textsuperscript{-1} in the S\textsubscript{1} state, 12.0
kJmol$^{-1}$ in the T$_1$ state, and 25.1 kJmol$^{-1}$ in the ground state).\textsuperscript{115} Another possibility would be fast internal conversion to hot vibrational energy levels of the ground state, and isomerization due to this excess vibrational energy in the ground state. (In equilibrium, isomerization between TTC/TTT isomers would be slow, as the energy barrier for ground state isomerization is $\approx$40 kJ/mol for 6-nitro-8-bromo-BIPS derived merocyanine.\textsuperscript{170})

Our results are consistent with excited state isomerization. Figure 15 shows that excited state absorption kinetics at 480 nm decay with a 67 ps lifetime. (Transient absorption signals in this spectral region were assigned to excited state absorption by Hobley et al.\textsuperscript{145}) Formation of the CTC/CTT isomer (kinetics at 650 nm at longer time delay), can also be described by the same lifetime. Decay of the excited states with the same lifetime as product (CTC/CTT isomer) formation reflects an excited state isomerization reaction.
Figure 15: Comparison of 480 nm (—■—) and 650 nm (—○—) kinetics measured with 630 nm excitation in toluene solution. The solid lines are fitting results. The same lifetime ($\tau=67$ ps) describes the 0.5-2000 ps data at both probe wavelengths.
Scheme 6 shows a state diagram constructed to explain the transient absorption results. After excitation of the TTC (600 nm) isomer, isomerization is possible to only CTC/CTT conformers (absorption at 650 nm, rate constant $k_{iso1}$). In contrast, excitation of the TTT isomer (568 nm), leads to TTC and CTC/CTT formation (absorption at 600 nm and 650 nm, rate constants $k_{iso3}$ and $k_{iso2}$, respectively). Experiments with ns time resolution are required to determine further dynamics of the CTC/CTT (650 nm) isomers, and in particular to estimate the quantum yield of the "closed ring" SP1 spiropyran (Scheme 4). While SP1 is perhaps the most extensively investigated photochromic compound, to our knowledge no studies have addressed complete fs to μs dynamics for the same system (same temperature, solvent, excitation wavelength). Because of the complex potential energy surface of this reaction (conical intersections of the ground and excited state potential energy surfaces,\textsuperscript{140} excited singlet and triplet state reactions,\textsuperscript{120} and unusual intersystem crossing properties\textsuperscript{144} were reported for spiropyrans) such studies are required to obtain a full description of SP1 spiropyran $\leftrightarrow$ merocyanine photophysical and photochemical reaction pathways.
Scheme 6: Reaction pathways upon selective MC1 isomer excitation
**Rate constants and quantum yields of the excited state reactions.** Scheme 6 indicates that excited states of the TTC isomer decay due to internal conversion (rate constant \( k_{\text{IC1}} \)) and isomerization (rate constant \( k_{\text{iso}} \)) to CTC/CTT forms. Internal conversion is the dominant relaxation pathway (the 650 nm absorption amplitude is only \( \approx 10\% \) of the initial 600 nm bleach amplitude, see Table 1 and Figure 13). Internal conversion was also found to be the dominant excited state relaxation pathway for "closed form" SP1 spiropyran in tetrachloroethylene and deuterated acetonitrile solutions.\(^ {124,144} \)

MC1 isomerization can be followed by a photochemical decoloration (or "ring-closing") reaction. In experiments on 6,8-dinitroBIPS derived merocyanines, an estimate for the ring closing reaction yield was obtained by comparing the initial and final bleach amplitudes.\(^ {145} \) Using a similar method, we can estimate the quantum yields of isomerization, \( \Phi_{\text{iso}} \), and ring closing, \( \Phi_{\text{RC}} \). Such an estimate yields \( \Phi_{\text{iso}} + \Phi_{\text{RC}} \approx 10\% \). Assuming that the extinction coefficients are similar for all isomers, \( \Phi_{\text{iso}} \) is higher than \( \Phi_{\text{RC}} \). (Persistent bleaching and positive absorption at 650 nm have approximately equal amplitudes.) Therefore, we see no evidence for ring closing on a \(< 2.5 \text{ ns} \) time scale. The difference between our results and those obtained on 6,8-dinitroBIPS could be related to solvent polarity. Hobley et al studied MC1 dynamics in acetonitrile, while our experiments were carried out in toluene. Spiropyran photochemical reactions are generally faster in polar solvents.\(^ {116,171,172} \) In addition, spiropyran photochemical reaction yields depend on solvent polarity.\(^ {116,171,172} \) This sensitivity could be related to the unusual internal conversion properties of spiropyran compounds.\(^ {144} \)
From the data in Table 1, $k_{IC1} + k_{isol} = (67 \text{ ps})^{-1} = 1.49 \times 10^{10} \text{ s}^{-1}$. Because the yield of the new isomer is low, $k_{IC1} > k_{isol}$ and $k_{IC1} \approx 1.49 \times 10^{10} \text{ s}^{-1}$. Assuming $\Phi_{isol} = 10\%$ yields $k_{IC1} = 1.34 \times 10^{10} \text{ s}^{-1}$. Kasha’s rule describes internal conversion as being relatively inefficient and occurring on a ns-μs time scale for the $S_1$ state of many organic compounds.\textsuperscript{173} Fast internal conversion from the spiropyran $S_1$ state was recently found and attributed to large conformational changes upon excitation.\textsuperscript{123,124,144} The same mechanism could be important for spiropyran-derived merocyanines, and lead to picosecond internal conversion for the MC1 isomers.

Excited state relaxation processes observed with 490 nm excitation are more complex (Scheme 6). In addition to internal conversion (rate constant $k_{IC2}$), isomerization to the TTC form (rate constant $k_{isot2}$) and to the third isomer (CTC/CTT, rate constant $k_{isot3}$) are observed. The origin of the biexponential TTT isomer excited state relaxation is ambiguous. Biexponential kinetics could be observed if the excited states of several isomers were in equilibrium. Biexponential kinetics could also reflect excitation of multiple isomers at 490 nm. In this case, $\tau_1^{-1}$ and $\tau_2^{-1}$ components in Table 1 would represent internal conversion and isomerization rate constants, $k_{IC} + k_{isot}$, for these isomers. It has been suggested that differences in solvent stabilization for MC1 isomers could lead to different excited state lifetimes for these isomers.\textsuperscript{120} This argument supports the presence of two isomers with absorption at 490 nm, but more detailed studies are required to assign the kinetic components observed with 490 nm excitation. The average excited state relaxation rate constant obtained with 490 nm excitation is $k_{av} = (a_1\tau_1^{-1} + a_2\tau_2^{-1})/(a_1 + a_2)$.
\(=2\times10^{10} \text{ s}^{-1}\), which is somewhat faster than the TTC isomer excited state decay rate constant of \(1.5\times10^{10} \text{ s}^{-1}\). When using 490 nm excitation, 650 nm absorption signal amplitude is about 10% of the initial bleaching amplitude; therefore internal conversion is more efficient than isomerization, \(k_{\text{IC2}}>k_{\text{iso2}}, k_{\text{iso3}}\), for all MC1 isomers.

2.6 Summary

Several merocyanine isomers are formed after UV excitation of SP1; these isomers include the TTC and TTT forms. Variable excitation wavelength ultrafast pump-probe spectroscopy was used to determine MC1 isomer excited state relaxation pathways. We find that internal conversion is the dominant relaxation pathway, and it occurs on the picosecond time scale. In addition, isomerization reactions in excited singlet states were studied. Following TTC isomer excitation at 630 nm, a third isomer (with absorption at 650 nm, either CTC or CTT) is formed. In contrast, following excitation at 490 nm, isomerization to both TTC and CTC/CTT forms is observed. Determination of the isomeric distribution, excited state photochemical reaction pathways, and relaxation dynamics for photochromic spiropyran/merocyanine molecules may contribute to the development of novel molecular materials and photonic devices. In particular, the properties of multiple merocyanine isomers should be considered when designing molecular switches that incorporate spiropyran compounds.
Chapter 3: Isomerization Dynamics of Spiropyran Molecular Switches in Phospholipid Bilayers


3.1 Overview

Transient absorption spectroscopy was used to investigate the dynamics of the photochromic indolinobenzospiropyran reaction in toluene solution and in phosphatidylcholine bilayers (DMPC, DPPC, and DOPC). After excitation with UV light, colorless (R/S)-2-(3′,3′-dimethyl-6-nitro-3′H-spiro[chromene-2,2′-indol]-1′-yl)ethanol (SP2) derivatives are converted to colored merocyanine products in high yield; Φ=0.45 in dimyristoylphosphatidylcholine (DMPC) liposomes. We find that the reaction occurs in the bilayer aliphatic region in gel (Pβ) and liquid (Lα) phases. The Arrhenius activation energy for the isomerization in DMPC bilayers was ~3.5 times larger in liquid phase (Lα, $E_a=26.0\pm1.0$ kJmol$^{-1}$) than in gel phase (Pβ, $E_a=7.3\pm1.6$ kJmol$^{-1}$). Analysis of the isomerization rate constant temperature dependence allows an estimation of the bilayer viscosity and free volume properties in Lα phase.
3.2 Introduction

Phospholipid bilayer aggregates, such as liposomes, are commonly used as cell membrane models. The physical properties of phospholipid bilayers are often derived from spectroscopic studies conducted on membrane-embedded molecular sensitizers.\textsuperscript{174} Fluorescent membrane probes with large solvatochromic shifts are often used; however, different molecular probes and experimental approaches are needed to study a wider range of bilayer properties. One such approach is the study of excited state reactions in phospholipid bilayers.\textsuperscript{175}
Scheme 7: SP2 photochemical reaction in phospholipid bilayers.
We investigated the spiropyran/merocyanine photochemical reaction in phospholipid bilayers (Scheme 7). (R/S)-2-(3',3'-Dimethyl-6-nitro-3'H-spiro[chromene-2,2'-indol]-1'-yl)ethanol molecular sensitizers (SP2) consist of two orthogonal moieties, substituted indole and benzopyran, weakly interacting in the ground state.\textsuperscript{176} The SP2 molecular structure is similar to that of well-characterized 6-nitroBIPS spiropyran,\textsuperscript{177,178} except that the dimethylindole moiety of SP2 is substituted with a (CH\textsubscript{2})\textsubscript{2}OH group. Ring-opening at the conical intersection of the excited state and the ground state potential energy surfaces results in ultrafast (several hundred femtoseconds) spiropyran C\textsubscript{spiro}-O bond cleavage.\textsuperscript{107,177,178} Subsequent isomerization leads to merocyanine (MC2) formation. Experimental and theoretical studies were applied to spiropyran photochemical reaction dynamics,\textsuperscript{144,177-180} excited state potential energy surfaces,\textsuperscript{140} photochemical reaction pathways,\textsuperscript{181} and the structural identity of reaction intermediates and products.\textsuperscript{114,167} The two merocyanine isomers shown in Scheme 7 have been reported as having the lowest ground state energies.\textsuperscript{114,115} Our earlier analysis of MC1 excited state dynamics showed that at least 2 isomers are present in toluene solution.\textsuperscript{182} One of the unusual features of spiropyran/merocyanine photochemistry is the sensitivity of this reaction to the medium's polarity, rigidity, and free volume; this supports the use of SP/MC reactions in various applications.\textsuperscript{107} Interaction of spiropyran/merocyanine compounds with lipid and surfactant membranes has been studied.\textsuperscript{6,183,184} In particular, the spiropyran/merocyanine photochemical reaction has been used to reversibly control ion transport across the bilayer.\textsuperscript{6}
By utilizing ultrafast SP→MC photochromic reaction and femtosecond pump-probe spectroscopic experiments, we study dynamics in phospholipid bilayers on a picosecond time scale. In particular, we consider phase transitions in phosphatidylcholine bilayers. Phospholipid bilayers exhibit rich polymorphism. In single component phosphatidylcholine bilayers, the gel ($L_{\beta'}$ and $P_{\beta'}$) and liquid ($L_{\alpha}$) phases are most significant, and the $P_{\beta'}$↔$L_{\alpha}$ phase transition is considered the most important.\(^{185}\) Several physical properties are altered during this transition. In particular, the bilayer thickness in $P_{\beta'}$ phase is greater by \(~25\%) than in $L_{\alpha}$ phase.\(^{185}\) The lipid leaflets are clearly separated in $P_{\beta'}$ phase.\(^{185}\) The conformations of the lipid chains change from a highly ordered all-trans geometry in the $P_{\beta'}$ phase to a more disordered, liquid-like structure in the $L_{\alpha}$ phase.\(^{185}\) The $L_{\alpha}$ phase best corresponds to the biological membrane state. Theoretical models developed to study reactions in solution have been applied to reactions in phospholipid bilayers.\(^{186}\)

Below we show that SP→MC isomerization dynamics are sensitive to the structural changes that occur during the $P_{\beta'}$↔$L_{\alpha}$ phase transition in phosphatidylcholine bilayers. In the $L_{\alpha}$ phase, isomerization dynamics are similar to those observed in liquids, except that higher viscosities present in phospholipid bilayers reduce the reaction rate. The isomerization rate constants in bilayer $L_{\alpha}$ phase are analyzed using Kramers theory and the results are compared to bilayer free volume properties predicted in simulations.\(^{14,31,34,37,87}\)
3.3 Transient Absorption Spectroscopy

The pump-probe transient absorption spectrometer was described in the previous chapter (section 2.3). 325 nm laser pulses were used for selective spiropyran excitation (spiropyran absorbance at this wavelength was ~0.3, merocyanine absorption was <0.1). Excitation pulses (100 fs, 1 kHz) were obtained by frequency-doubling 650 nm OPA pulses in a 0.5 mm thick BBO crystal. The energies of the pump and probe pulses were ~2 and <1 µJ, respectively. To eliminate polarization effects, the relative polarization of the pump and probe beams was set at a “magic angle” (54°). 10 nm bandwidth interference filters were used to select the probe wavelength from a white light continuum generated in a 2 mm thick sapphire plate. Sample temperature was adjusted with a thermostat to ±1°C. A 1 mm optical path flow cell attached to a peristaltic pump was used to remove reaction products from the measurement volume. The flow rate was 1.7 mL/min. Changing the laser repetition rate or sample flow rate did not influence the transient absorption signals.

3.4 SP2 Ring-opening / Isomerization Sensitivity to Bilayer Viscosity

SP2/MC2 transient absorption difference spectra and kinetics in DMPC bilayers and in toluene solution. Transient absorption difference spectra of dimyristoylphosphatidylcholine (DMPC) bilayers embedded with SP2 sensitizers measured after excitation with 325 nm, 100 fs laser pulses are shown in Figure 16. At 0-20
ps time delay, a positive signal at 480-540 nm and a negative signal at 600-650 nm are observed. In earlier ultrafast studies of the related 6-nitroBIPS spiropyran (SP1), the positive 480-540 nm signal has been assigned to the cisoidal intermediate X formed after breaking the $C_{spiro}$-O bond (Scheme 7).$^{177,179}$ Recently it was suggested that this signal is observed due to spiropyran internal conversion.$^{178}$ The negative signal at 600-650 nm is attributed to intermediate X and merocyanine MC2 stimulated emission.$^{145}$ In this wavelength region, MC2 steady-state fluorescence emission is observed.$^{182}$ To investigate this assignment further, we measured ps difference spectra with direct excitation of the MC2 form (a 380 nm excitation wavelength was used). The negative signal amplitude at 600-650 nm was enhanced with 380 nm excitation, thus confirming the negative signal assignment to stimulated emission.

A strong transient absorption signal with the maximum at $\approx 580$ nm grows on a ps time scale. This photochromic absorption band is assigned to merocyanine.$^{177,179}$ At 800 ps (and longer) time delay, the transient absorption spectrum in DMPC bilayers is essentially identical to the MC2 absorption spectrum in toluene solution (see dashed line in Figure 16A). As merocyanines have large solvatochromic shifts (MC2 absorption maximum shifts from 585 nm to 525 nm depending on solvent polarity), similarities in the absorption spectra indicate that the polarity of the MC2 molecular environment in the bilayer is essentially the same as that in the toluene solution. Therefore, the sensitizers are embedded in the aliphatic interior of the phospholipid bilayer where the effective dielectric constant is $\approx 2.$$^{17,18}$ (The polarity of the phospholipid bilayer varies from $\varepsilon \approx 2$ in the aliphatic interior to $\varepsilon = 81$ for the free water; $\varepsilon$ is the dielectric permittivity).$^{17,18}$
Figure 16: A: SP2/DMPC transient absorption difference spectra. Time delays: -1 ps (prior to excitation, ■), 0 ps (●), 20 ps (▲), 100 ps (▲) and 800 ps (▲). Error bars are indicated in the spectra. The dotted line (-----) shows the MC2 absorption spectrum in toluene solution. B: 568 nm transient absorption kinetics measured for SP2/DMPC liposomes (■) and for SP2 in toluene solution ( ○). Fits to the data at t>10 ps are also shown; rate constants are $k=(3.2\pm0.2)\times10^9$ s$^{-1}$ for SP2/DMPC and $k=(7.6\pm0.4)\times10^9$ s$^{-1}$ for SP2 in toluene. Excitation was at 325 nm with 100 fs pulses, temperature 25°C.
The MC2 $\lambda_{\text{max}}$=580 nm absorption peak does not shift on the 3 ns time scale investigated in our transient absorption experiments. On a much slower time scale ($\gg$3 ns) the MC2 absorption maximum in DMPC bilayers shifts to $\lambda_{\text{max}}$=520 nm. Merocyanine has a much larger dipole moment (17.7 D was determined for a related 6-nitroBIPS spiropyran) than the closed-form SP (4.3 D for SP1).\textsuperscript{105} Thus, this solvatochromic shift is assigned to MC2 diffusion from the aliphatic bilayer interior to the more polar phosphate headgroup region. Spirooxazine-derived merocyanine diffusion in phospholipid bilayers has been investigated.\textsuperscript{17}

Transient absorption kinetics measured in DMPC bilayers and in toluene solution using 568 nm probe wavelength are shown in Figure 16B. (Within signal-to-noise for the experiment, 580 and 568 nm kinetics were determined to have identical rise times. A probe wavelength of 568 nm was used in temperature dependence studies to avoid stimulated emission contributions to the measured signal). In bilayers at $>10$ ps time delay, the kinetics can be described with a single exponential rise term $B (1-\exp(-kt))$, where $B$ is amplitude, $t$ is time, and $k$ is a rate constant. Nonlinear least squares fitting of the data to this expression yields $k=(315\pm10$ ps)$^{-1}$ = (3.2$\pm$0.2)$\times10^9$ s$^{-1}$. Rate constant $k$ is assigned to isomerization leading to product MC2 formation. Earlier studies of several spiropyrans in organic solvents\textsuperscript{144,177-180} also found single exponential isomerization dynamics. These relatively simple isomerization kinetics enable analysis of the reaction temperature and viscosity dependence and determination of phospholipid bilayer effects on isomerization rates. Although the polarity of the bilayer interior is similar to that of
toluene (solvatochromic $\lambda_{\text{max}}$ values are the same), the more viscous environment present in phospholipid bilayers leads to slower isomerization. Isomerization kinetics in toluene solution shown in Figure 16B are single exponential (at $t>10$ ps) with $k=(131\pm7$ ps)$^{-1}$ $(7.6\pm0.4)\times10^9$ s$^{-1}$.

**Isomerization in liquid and gel phase phosphatidylcholine bilayers.**

SP2→MC2 reaction dynamics were investigated in the gel and liquid phases of three common phosphatidylcholines. Table 2 lists lipid phase transition temperatures and MC2 absorption maxima 800 ps after the excitation. The phospholipids in Table 2 have identical choline headgroups (-CH$_2$CH$_2$N(CH$_3$)$_3^+$, Scheme 7) but differ in the length of the fatty acid chains: 14 carbon atoms for DMPC, 16 for DPPC, and 18 for DOPC (DOPC lipids also have an unsaturated C=C bonds at carbon-9 which lowers the phase transition temperature, $T_{PT}$, to 11°C due to packing defects as a result of the cis geometry about the point of unsaturation). For all phosphatidylcholine bilayers, 800 ps transient absorption difference spectra are similar to the steady-state spectrum in toluene. In both gel (8°C) and liquid (53°C) phases, isomerization kinetics (and experimental rate constants $k$) did not differ significantly between DOPC, DMPC, and DPPC lipids (Table 2). For example, in liquid phase bilayers at 53°C, isomerization rate constants ranged between $(9.4-9.7)\times10^9$ s$^{-1}$. In contrast, differences between the gel and liquid phase data are important.
Table 2: Phase transition temperatures, merocyanine absorption maxima, and experimental rate constants in gel and liquid phase phosphatidylcholine bilayers and in toluene solution.

<table>
<thead>
<tr>
<th></th>
<th>$T_{PT}$, °C</th>
<th>$\lambda_{\text{max}}$ nm$^a$</th>
<th>$k_r \times 10^9$ s$^{-1}$$^b$</th>
<th>$k_r \times 10^9$ s$^{-1}$$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>800 ps</td>
<td>8°C - gel phase</td>
<td>53°C - liquid phase</td>
</tr>
<tr>
<td>DMPC 14:0</td>
<td>23</td>
<td>580</td>
<td>3.2±0.2</td>
<td>9.7±0.7</td>
</tr>
<tr>
<td>DPPC 16:0</td>
<td>42</td>
<td>580</td>
<td>1.9±0.3</td>
<td>9.5±0.9</td>
</tr>
<tr>
<td>DOPC 18:1</td>
<td>11</td>
<td>580</td>
<td>3.8±0.2</td>
<td>9.4±0.3</td>
</tr>
<tr>
<td>toluene</td>
<td>-</td>
<td>580</td>
<td>7.6±0.4</td>
<td>11.4±0.7</td>
</tr>
</tbody>
</table>

$^a$ Merocyanine $\lambda_{\text{max}}$ 800 ps after the excitation with 100 fs pulses at 325 nm

$^b$ Obtained from single exponential fits to 568 nm kinetics at 10-2000 ps time delay
**Isomerization rate constant temperature dependence.** Temperature dependence studies were carried out on SP2/DMPC bilayers between 8-53°C. This temperature range spans the \( P_\beta \leftrightarrow L_\alpha \) phase transition for DMPC lipids. (Because of the results shown in Table 2, we expect that the conclusions are also applicable to other phosphatidylcholines.) The isomerization kinetics at all temperatures were single exponential, but the rate constant \( k \) increased from \((3.2\pm0.2)\times10^9 \text{ s}^{-1}\) at 8°C to \((9.7\pm0.7)\times10^9 \text{ s}^{-1}\) at 53°C. The transient absorption signal amplitudes at 1500-3000 ps time delay were similar at all temperatures, suggesting that the quantum yield for the reaction, \( \Phi \), did not change in this temperature range.

The model of Scheme 7 was used to determine isomerization rate constants, \( k_{iso} \), from the experimental data. To describe the photochemical processes in Scheme 7, a system of four first order differential equations is required.\(^\text{188}\) Such a system could only be solved numerically.\(^\text{188}\) The analysis can be simplified because \( k_i \gg k_{iso} \) for spiropyran compounds.\(^\text{144,177-180}\) Therefore, very fast formation of intermediate \( X \) is followed by slower isomerization (\( k_{iso} \)) and ring-closing (\( k_2 \)) processes. Differential equations describing the time-dependent concentrations of intermediate \([X](t)\) and product \([MC](t)\) are:

\[
\frac{d[X](t)}{dt} = -(k_{iso} + k_2)[X](t) \tag{3.1}
\]

\[
\frac{d[MC](t)}{dt} = k_{iso}[X](t) = k_{iso}[X_0] \exp(-(k_{iso} + k_2)t) \tag{3.2}
\]
(Formation of $[X](t)$ is not included in the model, but is described using $[X_0]$ - the concentration of the intermediate formed after excitation.) The solution of Eq.(3.2) is:

$$[MC](t) = \frac{k_{iso}}{k_{iso} + k_2} \left(1 - \exp\left(-\frac{k_{iso} + k_2}{k_{iso}} t\right)\right) = \Phi[X_0] \left(1 - \exp\left(-\frac{k_{iso}}{\Phi} t\right)\right) \quad (3.3)$$

where $\Phi$ is the quantum yield of the reaction, $\Phi = \frac{k_{iso}}{k_{iso} + k_2}$. Eq.(3.3) represents experimentally measured $[MC](t)$ concentration time dependence. Therefore, the experimentally measured rate constant, $k$, is equal to $k=k_{iso}/\Phi$ and the isomerization rate constant, $k_{iso}$, is:

$$k_{iso} = k \Phi \quad (3.4)$$

The quantum yield for the reaction, $\Phi$, was determined from comparison of MC2 absorption amplitudes in toluene and in DMPC bilayers ($\Phi=0.64$ in toluene solution was reported for a related 6-nitroBIPS spiropyran which was used as a reference). In DMPC liposomes, we found $\Phi=0.44$. The $k_{iso}$ temperature dependence calculated according to Eq.(3.4) is shown in Figure 17.
Figure 17: Temperature dependence of isomerization rate constants in DMPC bilayers
(-■-) and in toluene solution (-○-). $P_{\gamma} \leftrightarrow L_{\alpha}$ phase transition temperature is indicated in the graph.
As the polarity of the SP2 membrane environment is similar to that of toluene (solvatochromic $\lambda_{max}=580$ nm, Table 2 and Figure 16A), isomerization dynamics in toluene solution were studied as well. Similar to the results in phospholipid bilayers, 568 nm kinetics in toluene solution could be described by a single-exponential rise with $k=(7.6\pm0.4)\times10^9 \text{ s}^{-1}$ at $8^\circ\text{C}$. At $53^\circ\text{C}$, the experimental rate constant increased to $(11.4\pm0.7)\times10^9 \text{ s}^{-1}$. Isomerization rate constants in toluene solution (also calculated using Eq.(3.4)) are contrasted with the results obtained in DMPC liposomes in Figure 17.

As shown in Figure 17, $k_{iso}$ data in toluene solution could be described using an Arrhenius expression, $k=A\cdot \exp(-E_a/RT)$, with an activation energy of $E_a=7.1\pm0.9 \text{ kJmol}^{-1}$ and a preexponential factor $A=(9.8\pm0.2)\times10^{10} \text{ s}^{-1}$. It is apparent from Figure 17 that the $k_{iso}$ temperature dependence in DMPC bilayers differs from that in toluene solution. In particular, a single Arrhenius expression cannot be used to fit SP2/DMPC data over the entire temperature range. Instead, $P_{\beta}$ and $L_\alpha$ phase data was analyzed independently using two Arrhenius expressions. Arrhenius preexponential factor, $A$, and activation energy, $E_a$, changed at $\sim20^\circ\text{C}$, which is attributed to the $P_{\beta} \leftrightarrow L_\alpha$ phase transition ($T_{PT}=23^\circ\text{C}$ for DMPC lipids, Table 2). In $P_{\beta}$ phase, $E_a=7.3\pm1.6 \text{ kJmol}^{-1}$ and $A=(3.3\pm0.3)\times10^{10} \text{ s}^{-1}$. In $L_\alpha$ phase, $E_a=26.0\pm1.0 \text{ kJmol}^{-1}$ and $A=(6.9\pm0.6)\times10^{13} \text{ s}^{-1}$. The origin of reduced activation energy and smaller preexponential factor in $P_{\beta}$ phase is discussed below.

**Intrinsic barrier for the isomerization reaction.** Two effects contribute to the temperature dependence of rate constant $k_{iso}$: the intrinsic barrier for the reaction and
molecular friction of the environment.\textsuperscript{190-192} These contributions can sometimes be separated using the activated barrier crossing formula, where $E_{iso}$ is the intrinsic barrier height, $R$ is the gas constant, and the reduced rate, $F(\zeta)$, describes frictional effects.\textsuperscript{190-192}

$$k_{iso} = F(\zeta) \exp(-E_{iso} / RT)$$

(3.5)

Molecular activation energy, $E_{iso}$, can be estimated if rate constants are measured in solutions of equal viscosity.\textsuperscript{190-192} This is commonly achieved using a homologous series of solvents and adjusting the temperature to obtain isoviscosity conditions.\textsuperscript{190-192} We performed such an analysis in $n$-alcohol solutions (C$_4$-C$_9$) and obtained $E_{iso} = 14.2 \pm 0.7$ kJmol$^{-1}$ (see Supporting Information; alcohol solvents were used to obtain sufficient viscosity variations in the temperature range accessible with a flow cell). $E_{iso}$ values found for SP2/MC2 compounds are similar to the isomerization barriers for cyanine dyes and polyenes.\textsuperscript{192}

Specific intermolecular interactions could be considered as a potential third contribution to the activation energy for the spiropyran ring-opening/isomerization reaction (for example, hydrogen bonding interactions between lipid carbonyl groups and the terminal alcohol of the SP2 species). These effects have been evaluated according to the activation energies determined in hydrogen bonding solvents ($n$-alcohols) for spiropyran molecules with and without the terminal alcohol functionality. The determined activation energies (data not shown) were very similar for both species indicating that specific solvation-type interactions occurring on the nitrogen functionality of the indole portion of
the spiropyran molecule do not play a significant role in the transition-state between spiropyran and merocyanine forms.

3.5 Modified Kramers Theory Applied to SP2 Isomerization Rate Constants

Isomerization in the gel phase of the bilayer. SP2→MC2 isomerization in the gel P\text{g} phase is unexpected, as this phase closely resembles a solid state system.\textsuperscript{185} Photochromic reactions have been investigated in the solid state.\textsuperscript{193,194} Using spiropyran and spirooxazine microcrystals and amorphous solids, it was shown that the first step of the reaction, breaking of the C\textsubscript{spiro}-O bond, is followed by the reverse reaction without formation of a merocyanine product.\textsuperscript{193,194} This was attributed to the limited free volume available in the solid state.\textsuperscript{193,194} Results are different in phospholipid bilayers, where even in the solid-like P\text{g} phase the SP2→MC2 reaction occurs with a high quantum yield and the isomerization reaction has lower activation energy than in the L\textsubscript{\alpha} phase (Figure 17).

Formation of the MC2 product in gel phase bilayers could be attributed to the structural heterogeneity of this system. In the P\text{g} phase, the bilayer thickness is about 25% greater than in L\textsubscript{\alpha} phase.\textsuperscript{44} This is largely due to the all-trans alkyl chain configuration of the lipids and some separation of the lipid leaflets. In the center of a P\text{g} phase bilayer (the region of the slip plane between the lipid leaflets), molecular packing is lower than in the fatty acid chain region.\textsuperscript{14,31,34,37,187} Spiropyran localization in the middle of the bilayer (between the lipid leaflets) could explain the observed efficient isomerization and reduced
activation energy in the \( P_\beta \) phase. Such a localization would not result in solvatochromic shifts, as the polarity of the central region of the bilayer (∼20 Å thickness for DMPC lipids) is approximately constant.\textsuperscript{17,18} Localization in the bilayer slip plane may also explain the reduced viscosity dependence (manifesting as a smaller pre-exponential factor compared to the \( L_\alpha \) phase data). Therefore, detailed analysis of isomerization rate constants in a gel phase requires determination of the molecular geometry (structure) of the spiropyran/bilayer system, which is not presently known.

Observations of facile (high yield and low activation energy) SP2/MC2 isomerization in the gel phase bilayers support the use of such photochromic reactions to modify phospholipid bilayer properties, including various light-sensitive liposome-based membrane transport\textsuperscript{6} and drug delivery\textsuperscript{195} systems.

**Analysis of DMPC bilayer viscosity based on MC isomerization rates in \( L_\alpha \) phase bilayers.** First we consider the possibility of local heating of the bilayer in the course of the photochemical reaction. A liposome with a diameter of 75 nm (determined by dynamic light scattering) contains ∼57,000 lipid and ∼1,200 SP2 molecules (an average DMPC headgroup surface area is 58.2 Å\(^2\);\textsuperscript{73} the phospholipid/SP2 molar ratio is 50/1). Using the excitation energy as an upper limit (325 nm, \( Q=368 \text{ kJmol}^{-1} \)), a sample absorbance of ∼0.3 at 325 nm, and a DMPC lipid heat capacity of \( C_p=1.65 \text{ kJmol}^{-1}\text{K}^{-1} \),\textsuperscript{44} the expected temperature change would be \( \Delta T<2 \text{ K} \). Since the MC2 ground state has been calculated to be higher in energy than the SP2 ground state\textsuperscript{181} and some SP2 excited state energy is used in the photochemical reaction, the energy released to the liposome from
excited SP2 molecules will be less than the excitation energy. Therefore, liposome heating is not expected to be significant.

As described in the previous section, the isomerization rate constant, \( k_{iso} \), depends on the molecular activation energy, \( E_{iso} \), and the viscosity of the medium (see Eq.(3.5)). If we assume that \( E_{iso} \) value determined in organic solvents can be used to analyze the data obtained in phospholipid bilayers, the viscosity-dependent factor \( F(\xi) \) can be determined from Eq.(3.5) and the data in Figure 17.

Classical Kramers theory of unimolecular reactions predicts that, subject to strong friction, factor \( F(\xi) \) of Eq.(3.5) will vary as \( F(\xi) \propto \xi^{-1} \), where \( \xi \) is a friction coefficient.\(^{191,192}\) If the friction in the isomerization reaction arises only from the viscosity of the solvent, \( \eta \), and Stokes law (\( \xi = 6\pi r \eta \) for the diffusion of a particle with a radius \( r \)) applies, then \( F(\eta) \) scales with:\(^{191,192}\)

\[
F(\eta) \propto \eta^{-1} \tag{3.6}
\]

Several isomerization reactions show this inverse dependence on the solution viscosity, which is often called the Smoluchowski limit.\(^{191,192,196}\) If Eq.(3.6) applies, determination of the phospholipid membrane viscosity based on calculated \( F(\eta) \) values is possible. A normalization factor required to use Eqs.(3.5)-(3.6) to estimate bilayer viscosity could be obtained from comparison of isomerization rates in solutions and in bilayers. Similar \( k_{iso} \) values in DMPC bilayers (\( k_{iso} = 4.3 \times 10^9 \text{ s}^{-1} \) at 53°C) and in nonanol
solution \(k_{iso}=4.0 \times 10^9 \text{ s}^{-1}\) at 42°C and 5 cP) suggest that \(\eta \approx 5\) cP for DMPC at 53°C. Using this approximation, data in Figure 17, and Eqs.(3.5)-(3.6), the temperature dependence of L\(_\alpha\) phase bilayer viscosity shown in Figure 18 was estimated.
Figure 18: DMPC bilayer viscosity temperature dependence determined from $k_{iso}$ data in Figure 17, Eqs.(3.5)-(3.6), and assuming $\eta = 5$ cP at 53°C. Viscosity activation energy is $E_\eta = 11.3 \pm 1.1$ kJmol$^{-1}$. 
The DMPC bilayer viscosity in Figure 18 appears to have activated temperature
dependence. Such an assumption is common in liquid studies and was used for
phospholipid bilayers.\textsuperscript{192,197-199}

\[ \eta = \eta_0 \exp\left(\frac{E_\eta}{RT}\right) \]  \hspace{1cm} (3.7)

Application of Eq.(3.7) to the data in Figure 18 yields $E_\eta = 11.3 \pm 1.1$ kJmol$^{-1}$ for L$\alpha$
phase DMPC bilayers. The change in $\eta$ is modest, $\eta = 7.4-5$ cP at $T = 23-53^\circ$C. In other
studies, much higher $E_\eta$ values have been found in L$\alpha$ phase bilayers.\textsuperscript{197-199} For example,
$E_\eta = 36.0$ kJmol$^{-1}$ have been found in dipyrenylpropane excimer formation experiments in
DMPC bilayers at $T = 30-60^\circ$C ($\eta = 22-13$ cP).\textsuperscript{197,198} Likewise, measurements of triplet
radical recombination rates in zinc porphyrin-viologen dyad linked by (CH$_2$)$_6$ bridge
yielded $E_\eta = 35$ kJmol$^{-1}$ ($\eta = 80-27$ cP at 45-70$^\circ$C) in L$\alpha$ phase DPPC liposomes.\textsuperscript{199}

Possible explanations for the modest viscosity variation and low $E_\eta$ value in Figure
18 include failure of the Stokes law and “internal friction” effects (or both). These
questions are carefully analyzed for reactions in solution,\textsuperscript{191,192} but more work is required
to establish the limits of validity of Stokes law and Kramers theory approximations in
phospholipid bilayers. In biophysical cell membrane and phospholipid bilayer studies,
Stokes law is commonly used.\textsuperscript{200} For merocyanine 540 excited state isomerization,
rotational diffusion times have been shown to vary linearly with solvent viscosity
indicating that these processes can be described by the Stokes law.\textsuperscript{201} Therefore, we
assume that the model of Eq.(3.6) predicts inaccurate $E_\eta$ values primarily because of the internal friction effects in our system. In further analysis, we use a modified Kramers model that approximates internal friction with an empirical parameter "$a$."\textsuperscript{191,192}

$$F(\xi) \propto \eta^a, \quad 0<a<1$$\hspace{1cm} (3.8)

The model of Eq.(3.8) has been applied to reactions ranging from photochemical ring closure,\textsuperscript{202} isomerization of organic molecules,\textsuperscript{192} protein folding,\textsuperscript{191} and merocyanine decoloration.\textsuperscript{156,203} Fractional viscosity dependence has also been studied theoretically. For example, a recent calculation of frequency-dependent friction using a mode coupling theory gives a behavior similar to the power law form.\textsuperscript{196}
Figure 19: Reduced rate $F(\eta)$ temperature dependence in L$_\alpha$ phase DMPC bilayers. Upper axis shows DMPC bilayer viscosity. $F(\eta)$ values were obtained using Eq.(3.5) and $k_{iso}$ data in Figure 17. Solid lines show $F(\eta)$ viscosity temperature dependence estimated according to Eq.(3.8) with $a=0.33$ and $a=1$. 
Molecular free volume in $L_\alpha$ phase DMPC bilayers. To apply model of Eq.(3.8) to isomerization in phospholipid bilayers and to determine an exponent $a$ value, bilayer viscosity should be determined independently. We use Eq.(3.7) and the literature value $E_\eta=36$ kJmol$^{-1}$ for $L_\alpha$ phase phosphatidylcholines.$^{197-199}$ In this case, bilayer $\eta$ values range between 40-10 cP at 20-50°C (Figure 19, upper axis). We find that $a=0.33\pm0.03$ should be used to describe experimental $F(\eta)$ data in Figure 19 (obtained from Figure 17 using Eq.(3.5)).

A simple model that accounts for fractional viscosity dependence, $a<1$, is based on consideration of the available free volume compared with the free volume needed for isomerization.$^{204}$ The free volume required for isomerization consists of two parts: the van der Waals volume occupied by the molecule and additional volume provided by the reaction medium. Therefore, determination of exponent $a$ in different systems can lead to determination of their free volume properties. Free volume in phospholipid bilayers primarily depends on lipid rotation and alkyl chain isomerization. Because of the ultrafast (picosecond time scale) dynamics considered here, lipid isomerization and rotational motions are essentially frozen. Such conditions are essential for comparison with bilayer simulations carried out on similar time scales.$^{14,31,34,37,187}$

Clustering of the “cavities” between some lipid alkyl chains has been predicted in simulations.$^{31,34,37}$ Such clustering might be necessary for the large scale isomerization considered here. In the SP→MC isomerization, the substituted benzene moiety moves $\sim0.5$ nm, while, for a sphere of 0.5 nm radius in the aliphatic bilayer region, the available free volume is $<1\%$. $^{14}$ Computational studies also predicted that the distribution of
“cavities” in the bilayer depends on the lipid structure\textsuperscript{34} and on the presence of other membrane components, such as cholesterol.\textsuperscript{31,37} Because of the complex depth profile of the bilayer free volume,\textsuperscript{14,31,34,187} isomerization rates for sensitizers embedded at different depths in the phospholipid bilayers are expected to have different exponent “α” values. Different exponent “α” values could also be predicted for bilayers composed of several lipids, especially if cholesterol is present\textsuperscript{31,37} or lipids have branching fatty acid chains.\textsuperscript{34} These predictions remain to be verified experimentally and underscore the importance of studies on well-defined systems. More detailed studies of isomerization reactions in bilayers composed of different lipids that have different free volume distributions\textsuperscript{14,31,34,37,187} are required to determine if isomerization rate sensitivity to the bilayer free volume is a general result.

3.6 Summary

Isomerization reactions of photochromic spiropyran/merocyanine compounds were studied in organic solvents and in phosphatidylcholine bilayers. Spiropyran/merocyanine photochromic molecular probes could be used in biophysical membrane studies and provide an interesting system to examine reaction dynamics in a complex environment. We find that isomerization reactions occur in gel and liquid bilayer phases. Efficient isomerization in the gel phase is unexpected and suggests that spiropyran sensitizers are localized between the lipid leaflets in the gel phase. When the temperature dependence of the isomerization rates in the liquid phase bilayers are analyzed using a modified Kramers
theory, results could be related to the free volume properties of phospholipid bilayers.

This suggestion should be tested in further studies.

The determination of the intrinsic barrier for spiropyran isomerization reaction can be found in Appendix A.
Chapter 4: Phospholipid Bilayer Free Volume Analysis Employing the Thermal Ring-Closing Reaction of Merocyanine Molecular Switches


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4.1 Overview

An assay incorporating photochromic and solvatochromic merocyanines has been developed to determine the free volume properties of phospholipid bilayers. Substitution on the merocyanine indole moiety is used to control the orientation and embedding depth of the merocyanines in bilayers. Analysis of the rate constants for the merocyanine ring-closing reaction is used to determine the free volume changes in three regions of the phosphatidylcholine bilayer: the bilayer aqueous interface (region 1), the phospholipid headgroup region (region 2), and the bilayer aliphatic interior (region 3). Free volume variations during the $P_b$ (gel) $\leftrightarrow$ $L_\alpha$ (liquid) phase transition are observed in region 1, in accordance with large structural rearrangements between the gel and liquid phases in this region. In agreement with the computational studies of the free volume in bilayers, the highest free volume is found in region 3, the lowest – in region 2. Comparison between the free volume in region 2 of two saturated phosphatidylcholines, DPPC and DMPC, shows the sensitivity of this method to small structural differences between lipids. The
free volume is found to be ~2 times larger in DPPC bilayers, which could be related to different merocyanine interaction with the two phosphatidylcholines. Free volume properties determined on picosecond and second time scales are compared based on the rate constant analysis for merocyanine formation and decoloration reactions respectively.

4.2 Bilayer Free Volume Effects and Selective Merocyanine Localization

Permeability, viscosity, and diffusion in biological membranes depend on the free volume properties of these complex systems. For example, a greater degree of acyl chain unsaturation results in a dramatic increase in the rate of passive trans-bilayer ion flux.\textsuperscript{99} This can be directly correlated with an increase in free volume.\textsuperscript{205} Simulations have predicted greater bilayer density\textsuperscript{73} and reduced free volume\textsuperscript{31} as a result of the addition of small amounts of cholesterol to a phospholipid bilayer. Experimentally, this has been observed as reduced diffusion rates of NBD-labeled lipids in phospholipid/cholesterol liposomes.\textsuperscript{206} Similarly, the presence of proteins in a phospholipid membrane can have a dramatic impact on the distribution of membrane free volume.\textsuperscript{32} Embedding of anesthetics into the aqueous interface of phospholipid bilayers is believed to perturb the free volume distribution.\textsuperscript{207} This changes the lateral pressure profile which shifts the ion-channel conformational energy to favor the closed form.\textsuperscript{207} Likewise, the incorporation of several small molecules results in dramatic changes in membrane viscosity that may be explained by changes in membrane free volume.\textsuperscript{23,24}
The complexity of a phospholipid bilayer requires compartmentalization of the membrane to describe the variation of physical properties associated with different sections of the bilayer (i.e. the head-groups, the glycerol backbone, the alkyl chains, etc.). In computational studies, a minimum in the free volume was found at the phospholipid head-groups and a maximum in the ordered aqueous phase and between the lipid leaflets.\textsuperscript{14,31,36,37,205} The current study appears to be the first to experimentally analyze the bilayer free volume profile. We report here the free volume distribution in saturated phosphatidylcholine bilayers using a series of merocyanine/spiropyran molecular sensitizers (Scheme 3) embedded in well-defined regions of the membrane (Scheme 8).
Scheme 8: Merocyanine localization in different regions of a phosphatidylcholine bilayer and the structure of DPPC and DMPC lipids
Merocyanines are planar conjugated compounds with a large dipole moment (17.7 D for MC1).\textsuperscript{105} The merocyanines shown in Scheme 3 undergo thermal ring-closing reactions described by first order kinetics.\textsuperscript{189} The products of such reaction, spiropyrans, consist of two orthogonal moieties, substituted indole and benzopyran, connected by a spiro carbon atom. The photochromic and solvatochromic properties of merocyanines have been applied to characterize complex systems, such as glasses.\textsuperscript{156,161,208} The photochemical reactions of membrane-embedded merocyanines, similar to the ones used in this study, have been used to manipulate bilayer permeability to ions and small molecules.\textsuperscript{5,6}

Substitution of the indole moiety (R in Scheme 3) was used to control the localization and orientation of merocyanines in the bilayers which allowed studies of well-defined membrane regions. Three-\textsuperscript{37,205} and four-region\textsuperscript{14,15,31,209} models for the bilayer have been proposed (see section 1.1). As illustrated in Scheme 8, MC1 is localized at the surface of the bilayer (region 1, which includes the bilayer/aqueous interface and phosphatidylcholine head-groups), MC2 is found near the phospholipid glycerol backbones and the ordered region of the lipid chains (region 2, which has the highest density), and MC3 is embedded in the aliphatic interior of the bilayer (region 3). We show that the kinetics of the ring-closing reaction for the three merocyanines reflect variations in the phospholipid bilayer free volume.
4.3 SP3/MC3 Synthesis

**Materials and methods.** All reagents and solvents were obtained from Aldrich. SP3/MC3, (R/S)-hexadecanoic acid-2-(3′,3′-dimethyl-6-nitro-3′H-spiro[chromene-2,2′-indol]-1′-yl)-ethyl ester was synthesized as described below. $^1$H NMR spectra were recorded on a Varian Mercury instrument operating at 299.865 MHz. $^{13}$C NMR spectra were recorded on the same instrument operating at 75.400 MHz. Chemical shifts are reported in ppm relative to the residual protonated solvent peak. High resolution mass spectra were recorded on a Micromass Q-TOF-2 instrument (Manchester, U.K.) using electrospray ionization (positive mode). The samples were introduced into the mass spectrometer using a flow rate of 1 μL/min and a nanospray source, the needle voltage was set to 2000 V with an ion source at 120 oC, and a cone voltage of 35 V. Polyaniline was used for internal calibration. Electronic absorption spectra were measured with a HP8452A diode-array spectrometer and corrected for scattering from the liposomes. Dynamic light scattering measurements were performed with a Zeta Nanosizer ZS (Malvern Instruments).

(R/S)-hexadecanoic acid-2-(3′,3′-dimethyl-6-nitro-3′H-spiro[chromene-2,2′-indol]-1′-yl)-ethyl ester, MC3. (R/S)-2-(3′,3′-dimethyl-6-nitro-3′H-spiro[chromene-2,2′-indol]-1′-yl)ethanol, MC2 (0.71 g, 2.02 mmol) was dissolved in 125 mL of acetonitrile. Pyridine (0.80 g, 10.10 mmol, 5 mol eq.) and palmitoyl chloride (1.67 g, 6.05 mmol, 3 mol eq.) were added to the solution and stirred for 5 h at room temperature until the purple solution became green and a white precipitate formed. The solution was filtered and
concentrated under reduced pressure. The crude yellow-brown oil was purified by extraction with ethyl acetate (150 mL) and water (150 mL). The organic layer was washed with water (2 × 150 mL) and dried over MgSO₄. The solvent was removed under reduced pressure to afford a crude product as a purple oil (1.80 g). Further purification by column chromatography (silica gel, hexanes/ethyl acetate 4:1) afforded pure 3 (0.66 g, 1.12 mmol) in a 59% yield as a pink solid. ¹H NMR (300 MHz, CD₃CN, δ): 8.09 (d, 1H, J = 3 Hz), 8.01 (dd, 1H, J = 9 and 3 Hz), 7.18 (t, 1H, J = 8 Hz), 7.12 (d, 1H, J = 7 Hz), 7.05 (d, 1H, J = 11 Hz), 6.87 (t, 1H, J = 7 Hz), 6.73 (d, 1H, J = 9 Hz), 6.70 (d, 1H, J = 8 Hz), 6.00 (d, 1H, J = 11 Hz), 4.20 (dt, 2H, J = 5 and 3 Hz), 3.55 – 3.35 (m, 2H), 2.21 (t, 2H, J = 7 Hz), 1.50 (m, 2H), 1.27 (m, 24H), 1.24 (s, 3H), 1.14 (s, 3H), 0.90 (t, 3H, J = 7 Hz). ¹³C NMR (CD₃CN) δ: 173.7, 159.9, 147.4, 141.7, 136.4, 128.6, 128.2, 126.1, 123.2, 122.4, 122.3, 120.1, 119.5, 115.8, 107.3, 107.2, 62.6, 53.1, 42.7, 34.3, 34.2, 32.2, 29.9 – 29.2, 25.7, 25.1, 22.9, 19.5, 13.9. MS (ESI, high resolution) [M+H]⁺ rel intensity 100.00: 591.3773, calcd. for C₃₆H₅₁N₂O₅ 591.3798.

4.4 Merocyanine Thermal Ring-Closing Kinetics Measurements

Kinetic measurements were conducted using an HP8452A diode-array spectrometer equipped with a temperature controlled cell holder. For all measurements, the UV (deuterium) lamp of the spectrometer was turned off to prevent merocyanine formation induced by the spectrometer. Samples were placed in 1 cm optical path length quartz cuvettes and were allowed to equilibrate until an external temperature probe displayed a stable temperature for about 10 minutes. The sample cell was exposed to UV illumination
(325 nm, Entela hand-held lamp) for 150 s to induce merocyanine formation and allow for equilibration with the bilayer. Sample stability was monitored by measuring its absorption spectrum. Merocyanine aggregation (which could result in absorption band narrowing)\textsuperscript{210} was not observed in any of the experiments.

4.5 Merocyanine Solvatochromism and Arrhenius Analysis

Merocyanine solvatochromism and embedding depth in phospholipid bilayers.

The electronic absorption spectra of merocyanines MC2 and MC3 in several organic solvents are shown in Figure 20A. The 450-600 nm absorption band is not present for spiropyans. Therefore, the MC $\rightarrow$ SP ring-closing kinetics can be studied by following the disappearance of the photochromic absorption band.
Figure 20: A: MC2 and MC3 (inset) absorption spectra in methanol (—, \( \lambda_{\text{max}} = 528 \) nm for MC2, 540 nm for MC3), 1-pentanol (—, \( \lambda_{\text{max}} = 542 \) nm for MC2, 555 nm for MC3), and toluene (—, \( \lambda_{\text{max}} = 580 \) nm for MC2, 587 nm for MC3). B: Solvatochromic shifts for MC2 in organic solvents. Blue lines indicate the maximal deviation of the solvatochromic data from the linear fit.
The solvatochromic properties of MC1 - MC3 are similar to those of other merocyanines.\textsuperscript{162} As shown in Figure 20, the maximum of the photochromic absorption band, $\lambda_{\text{max}}$, shifts to shorter wavelengths in more polar solvents (for example, $\lambda_{\text{max}}$ for MC2 is at 576 nm in benzene and at 528 nm in ethanol). In agreement with other studies,\textsuperscript{119} substitution on the indole moiety does not influence $\lambda_{\text{max}}$ significantly (for example, in toluene solution, $\lambda_{\text{max}} = 580$ nm for MC1 and MC2 and $\lambda_{\text{max}} = 587$ nm for MC3). A modified Reichhardt's scale, $E_T^N(30)$, used in the analysis of the solvatochromic shifts in Figure 20B, describes solvent polarity and specific solvent effects, such as hydrogen bonding.\textsuperscript{162} An apparent linear correlation between $\lambda_{\text{max}}$ and $E_T^N(30)$ suggests that the polarity of the merocyanine molecular environment can be determined from the maxima of the solvatochromic absorption band.\textsuperscript{5,6}
Figure 21: Merocyanine absorption spectra in DPPC bilayers: MC1 (—, $\lambda_{\text{max}} = 505$ nm), MC2 (—, $\lambda_{\text{max}} = 520$ nm), and MC3 (—, $\lambda_{\text{max}} = 555$ nm).
The width of the merocyanine solvatochromic absorption band in phosphatidylcholine bilayers is similar to that in organic solvents (70 – 80 nm, Figure 21), but $\lambda_{\text{max}}$ shifts from 505 nm for MC1 to 520 nm for MC2 and 555 nm for MC3. The merocyanine absorption spectra did not change significantly in the 20 - 60°C temperature range (see Figure 33 in Appendix B). As several merocyanine isomers could be present in solution (we have reported the isomeric distribution and excited state dynamics of MC1), the constant width of the solvatochromic absorption band suggests that the same isomers are present in solvents and bilayers. The most stable MC1 - MC3 isomers are shown in Scheme 3.

The solvatochromic shifts in Figure 21 indicate that merocyanines are localized in bilayer regions of different polarity, with $E_T^N(30) \approx 1.12$ for MC1, $\approx 0.97$ for MC2, and $\approx 0.42$ for MC3. The uncertainty is largest for MC3, for which $E_T^N(30)$ can range from 0.28 to 0.50 (values typical for cyclohexanone and acetonitrile respectively). In spite of this variability, MC3 is in a substantially less polar environment than MC1 or MC2. The polarity variations in the direction of the bilayer normal have been established. Therefore, merocyanine solvatochromism data indicate that MC1 is localized in region 1, MC2 is found in region 2, and MC3 is embedded deeply into the bilayer (region 3, Scheme 8). Notably, Stern and Feller predicted polarity values at the bilayer / aqueous interface to be higher than the polarity of the bulk water. Our results for MC1 ($E_T^N(30) > 1$ for MC1 in region 1, while $E_T^N(30) = 1$ for water) appear to be in agreement with this prediction.

Merocyanine orientation in phospholipid bilayers was determined from absorption and fluorescence linear dichroism. The angle between the bilayer normal and
merocyanine transition dipole moment varied from 16° to 45° (see Figure 34 in Appendix C).

**Merocyanine ring-closing kinetics in L_α phase DPPC bilayers.** Well-defined merocyanine localization in phospholipid bilayers allows characterization of three distinct regions of the membrane from the ring-closing reaction kinetics. Merocyanine reactions were studied in solvent mixtures\textsuperscript{118} and in polymers.\textsuperscript{156,161,208} In polymers, however, the kinetics are nonexponential and more complex models are needed to analyze reaction rates.\textsuperscript{156,161,208} In phospholipid bilayers, single exponential thermal ring-closing kinetics allow solution-phase models to be used in the analysis.
Figure 22: MC1 (A) and MC3 (B) ring-closing kinetics in toluene solution (■) and in L_α phase DPPC bilayers (○) at 50°C. Insets on the log-linear scale show that the reactions can be described by single exponential kinetics. Solid lines show single exponential fits to the data. Rate constants for MC1 are 0.572 ± 0.003 s\(^{-1}\) in toluene and \((7.86 ± 0.01) \times 10^{-3}\) s\(^{-1}\) in bilayers; rate constants for MC3 are 0.584 ± 0.005 s\(^{-1}\) in toluene and \((4.44 ± 0.01) \times 10^{-2}\) s\(^{-1}\) in bilayers.
Merocyanine ring-closing kinetics in DPPC bilayers and in toluene solutions are compared in Figure 22 (temperature $T = 50^\circ$C, bilayers are in the liquid-like L$_\alpha$ phase). The kinetics of MC1 and MC3 are similar in toluene solutions (the rate constants are $0.572 \pm 0.003$ s$^{-1}$ and $0.584 \pm 0.005$ s$^{-1}$, respectively). In DPPC bilayers, the ring-closing rate constant decreases $\sim$70 times for MC1 $(7.86 \pm 0.01) \times 10^{-3}$ s$^{-1}$) and $\sim$10 times for MC3 $(4.44 \pm 0.01) \times 10^{-2}$ s$^{-1}$). Similar results were obtained for the MC2/DPPC system: the ring-closing rate in the DPPC bilayers is $\sim$87 times slower than in toluene solution (respective rate constants are $(3.08 \pm 0.02) \times 10^{-3}$ s$^{-1}$ and $0.269 \pm 0.005$ s$^{-1}$).

In general, the merocyanine ring-closing rates are sensitive to the polarity and viscosity of the reaction medium.$^{189}$ The polarity of the aliphatic interior of the bilayer, where MC3 is localized, is similar to that of the toluene solution.$^{18}$ Therefore, the slower rate for MC3 in Figure 22B is attributed to the higher viscosity of the DPPC bilayers. For MC1 and MC2, polarity differences could also contribute to the change in rates. We analyzed the temperature dependence of the MC1 - MC3 ring-closing reactions to determine the relative contributions of viscosity and polarity effects.

**Evidence for the $P_b \leftrightarrow L_\alpha$ phase transition in the MC1 rate constant data.**

Figure 23A shows the ring closing kinetics of MC1/DPPC at several temperatures. All of the kinetics were analyzed as single exponential decays; the rate constants are summarized in Figure 23B. For comparison, the rate constants determined in toluene solution are also shown. The Arrhenius parameters (the pre-exponential factor, $A$, and the activation
energy, \( E_a \) were determined by fitting the rate constant temperature dependence data to the expression

\[
k = A \exp(-E_a/RT)
\]  

(4.1)

where \( R \) is the gas constant. \( E_a = 71 \pm 1 \text{ kJ mol}^{-1} \) determined in toluene solution (Figure 23B and Table 3) is in excellent agreement with the literature value of 70.1 kJ mol\(^{-1}\).\textsuperscript{118,217}
Figure 23: A: MC1/DPPC ring-closing kinetics at 28°C (□, $(2.80 \pm 0.09) \times 10^{-4}$ s$^{-1}$), 35°C (○, $(7.90 \pm 0.0) \times 10^{-4}$ s$^{-1}$), 39°C (△, $(2.20 \pm 0.01) \times 10^{-3}$ s$^{-1}$), 42°C (▽, $(4.47 \pm 0.02) \times 10^{-3}$ s$^{-1}$), and 53°C (◇, $(1.25 \pm 0.01) \times 10^{-2}$ s$^{-1}$). Single-exponential fits are shown as solid lines. B: Temperature dependence of the MC1 ring-closing rate constant, $k$, in DPPC (●) and DMPC (□) bilayers and in toluene solution (■). Solid lines are fits according to the Arrhenius expression. The change in activation energy for MC1/DMPC (at $\sim 23$°C) and MC1/DPPC (at $\sim 40$°C) is attributed to the $P_{\alpha} \leftrightarrow L_\alpha$ phase transition.
For the MC1/DPPC system, the slope of the Arrhenius graph decreases from $E_a = 191 \pm 22$ kJ mol$^{-1}$ at $<40^\circ$C to $E_a = 81 \pm 9$ kJ mol$^{-1}$ at $>40^\circ$C. For MC1/DMPC, $E_a$ decreases from $128 \pm 25$ kJ mol$^{-1}$ at $<23^\circ$C to $78 \pm 3$ kJ mol$^{-1}$ at $>23^\circ$C (Table 3). This change in activation energy can be attributed to the gel-liquid ($P_{\beta'} \leftrightarrow L_\alpha$) phase transition, which occurs at $\sim 41^\circ$C for DPPC and $\sim 23^\circ$C for DMPC bilayers. During the $P_{\beta'} \leftrightarrow L_\alpha$ phase transition, the structure of the bilayer changes from the more-ordered $P_{\beta'}$ gel phase to the liquid-like $L_\alpha$ phase. The changes in the bilayer structure are the most significant in the phospholipid headgroup region and at the aqueous interface (region 1). As MC1 is found at the interface, ring closing kinetics reflect bilayer structural rearrangements due to the $P_{\beta'} \leftrightarrow L_\alpha$ phase transition. Activation energies in both phases of the bilayer are higher than $E_a = 71$ kJ mol$^{-1}$ in toluene solution, which is attributed to the higher viscosity and polarity of the bilayer region 1 (Scheme 8) relative to toluene.
Table 3: Merocyanine MC1 - MC3 photophysical properties in toluene solution and in phosphatidylcholine bilayers

<table>
<thead>
<tr>
<th></th>
<th>(\lambda_{\text{max}}, \text{nm}^a)</th>
<th>(E_a (\text{kJ mol}^{-1}), \ln(A))</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>DMPC Bilayer(^b)</td>
<td>DPPC Bilayer(^c)</td>
<td>Toluene</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>(\text{L}_\alpha)</td>
<td>(P_{\beta'})</td>
<td>(\text{L}_\alpha)</td>
<td>(P_{\beta'})</td>
</tr>
<tr>
<td>MC1</td>
<td>501 - 507</td>
<td>78 ± 3,</td>
<td>128 ± 25,</td>
<td>81 ± 9,</td>
<td>191 ± 22,</td>
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<tr>
<td></td>
<td></td>
<td>23.8 ±</td>
<td>44.0 ±</td>
<td>25.7 ±</td>
<td>67.4 ±</td>
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<td></td>
<td></td>
<td>1.1</td>
<td>10.2</td>
<td>3.3</td>
<td>8.7</td>
</tr>
<tr>
<td>MC2</td>
<td>512 - 520</td>
<td>83 ± 5,</td>
<td>63 ± 2,</td>
<td>100 ± 3,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>29.4 ±</td>
<td>20.6 ±</td>
<td>31.7 ± 1.0</td>
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<td></td>
<td></td>
<td>0.6</td>
<td>0.4</td>
<td></td>
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<tr>
<td>MC3</td>
<td>549 - 555</td>
<td>124 ± 3,</td>
<td>123 ± 6,</td>
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<tr>
<td></td>
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<td>43.4 ± 1.1</td>
<td>43.1 ± 2.5</td>
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</table>

\(^a\) Maximum of the solvatochromic and photochromic merocyanine absorption band in DMPC and DPPC bilayers determined over the temperature range 20 – 60 °C

\(^b\) \(P_{\beta'} \leftrightarrow \text{L}_\alpha\) phase transition temperature \(\sim 23^\circ\C\)

\(^c\) \(P_{\beta'} \leftrightarrow \text{L}_\alpha\) phase transition temperature \(\sim 41^\circ\C\)

\(^d\) MC2 kinetics in toluene were bi-exponential. The amplitude of the second component was \(< 30\%\) of the total amplitude, the lifetime was \(\sim 10\) times longer than that of the first, and the activation energy was \(25 \pm 9 \text{ kJ mol}^{-1}\).
MC2 and MC3 kinetics in DMPC and DPPC bilayers. The temperature dependence of the ring-closing rate constants for MC2 and MC3 in bilayers and in toluene are shown in Figure 24; the Arrhenius parameters are summarized in Table 3.

The MC3 kinetics in DMPC and DPPC bilayers are the same, within experimental uncertainty. This suggests that the molecular environment surrounding this merocyanine is similar in both lipids. The results for MC2 are different – the rate constants for MC2/DPPC are ~100 times lower than those for MC2/DMPC. (In addition, for MC2/DMPC, $E_a$ is higher by ~30% in $L_a$ phase, while no such change is evident for MC2/DPPC.) The contrasting data for MC2/DPPC and MC2/DMPC suggests that either the free volume of the glycerol backbone region of these two lipids is not identical (see Discussion) or MC2 perturbs the local structure of the bilayer differently for these two phosphatidylcholines. As DPPC and DMPC lipids have identical headgroups and only differ in the length of their acyl chains (Scheme 8), this sensitivity is surprising. The isomerizing molecular probes could be more sensitive to the bilayer structural variations in this region, because the molecular density is the highest in the glycerol backbone region (and the free volume is the smallest).
Figure 24: MC2 (A) and MC3 (B) ring-closing rate constant temperature dependence in DPPC (●) and DMPC (□) bilayers and in toluene solution (■). Solid lines are fits according to the Arrhenius expression.
4.6 Development of the Phospholipid Bilayer Free Volume Profile

In the previous section, we showed that merocyanine ring-closing kinetics, rate constants, and Arrhenius parameters that describe the rate constant temperature dependence vary: (i) in regions 1-3 of the bilayer (Scheme 8) and (ii) for phosphatidylcholines with different saturated acyl chain lengths. Now we apply models from condensed-phase reaction dynamics\textsuperscript{192} to analyze the free volume properties of these bilayer systems.

**Rate constant analysis using a Kramers' model.** Effects from the molecular activation energy, $E_0$, and the viscosity, $\eta$, on the rate constants can be determined from\textsuperscript{192}

$$k \propto \frac{1}{\eta^a} \exp\left[-\frac{E_0}{RT}\right]$$

(4.2)

Classical Kramers' theory predicts that the exponent $a$ is equal to one ($a = 1$).\textsuperscript{192} When the Kramers' model was applied to protein folding,\textsuperscript{219} ring closing reactions,\textsuperscript{202} and isomerization of cyanine dyes,\textsuperscript{220-222} it was found that in systems with relatively high viscosities, the power law dependence predicted by classical Kramers' theory fails, but $a < 1$ could adequately explain the data.\textsuperscript{192,196} The temperature dependence for the viscosity, $\eta$, is commonly written as:\textsuperscript{30}

$$\eta(T) = \eta_0 e^{E_\eta/RT}$$

(4.3)

where $\eta_0$ is the viscosity as temperature approaches infinity and $E_\eta$ is the viscosity activation energy. Dipyrenylpropane excimer studies yielded $E_\eta = 35$ kJ mol\textsuperscript{-1} for L\textsubscript{α} phase phosphatidylcholines.\textsuperscript{28,29} In more recent triplet radical ion pair recombination
studies, it was found that $E_\eta = 35.6$ kJ mol$^{-1}$ in the lipid head-group region but $E_\eta = 73.6$ kJ mol$^{-1}$ in the aliphatic interior of the bilayer.$^{30}$ Therefore, we assumed that $E_\eta = 35.6$ kJ mol$^{-1}$ in regions 1 and 2 of the bilayer$^{28-30}$ and that $E_\eta = 73.6$ kJ mol$^{-1}$ in region 3.$^{30}$ (Our analysis is limited to L$_\alpha$ phase bilayers, as the viscosity and viscosity activation energy in the gel P$_\beta$ phase is not established.)

Comparison of Eq. (4.1) and Eqs. (4.2) - (4.3) shows that the Arrhenius activation energy is related to the molecular activation energy, $E_0$, and the viscosity activation energy, $E_\eta$.$^{156}$

$$E_a = E_0 + aE_\eta$$ \hspace{1cm} (4.4)

To estimate $aE_\eta$, the energy $E_0$ should be determined independently. In principle, $E_0$ can be obtained from isoviscosity studies.$^{192}$ In the temperature range used in our studies, viscosity of toluene varies much less than the viscosity of the bilayer (toluene viscosity activation energy is $E_{\eta,\text{toluene}} = 9.2$ kJ mol$^{-1}$).$^{223}$ Therefore, we estimated molecular activation energies as $E_0 = E_{a,\text{toluene}} - E_{\eta,\text{toluene}}$. Results are summarized in Table 4.
Table 4: Analysis of the free volume properties of Lα phase phosphatidylcholine bilayers

<table>
<thead>
<tr>
<th></th>
<th>$E_a$, kJ mol$^{-1}$</th>
<th>$E_0$, kJ mol$^{-1}$</th>
<th>$aE_\eta$, kJ mol$^{-1}$</th>
<th>Free volume exponent $a$</th>
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<tbody>
<tr>
<td>DMPC</td>
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<tr>
<td>DPPC</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MC1</td>
<td>78 ± 3</td>
<td>81 ± 9</td>
<td>62 ± 1</td>
<td>16.0 ± 0.3</td>
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<td>19.0 ± 1.1</td>
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<td></td>
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<td>0.45 ± 0.03</td>
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<td>0.03$^a$</td>
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<td>0.05$^a$</td>
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<tr>
<td>MC2</td>
<td>83 ± 5</td>
<td>100 ± 3</td>
<td>68 ± 4</td>
<td>17.0 ± 0.6</td>
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<td>32.0 ± 1.4</td>
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<td>0.48 ± 0.04</td>
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<td></td>
<td>0.04$^a$</td>
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<td></td>
<td>0.07$^a$</td>
</tr>
<tr>
<td>MC3</td>
<td>124 ± 3</td>
<td>123 ± 6</td>
<td>67 ± 1</td>
<td>57.0 ± 1.3</td>
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<td>56.0 ± 2.4</td>
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<td>0.77 ± 0.06</td>
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<td>0.76 ± 0.09</td>
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$^a E_\eta = 35.6$ kJ mol$^{-1}$ was assumed$^{28-30}$

$^b E_\eta = 73.6$ kJ mol$^{-1}$ was assumed$^{30}$
Phosphatidylcholine bilayer free volume. The exponent $a$ in Table 4 is proportional to the available free volume, $V_f^{220,224}$

$$a \propto \frac{V_f}{V_0}$$

(4.5)

where $V_0$ is the molecular volume required for the reaction. Using laser-induced optoacoustic spectroscopy, the volume change due to the MC1 photochemical reaction was determined to be $\sim 78 \text{ Å}^3 \text{ molecule}^{-1}$ (in alkane solvents).\textsuperscript{131} Therefore, merocyanine photochromic molecular switches could detect free volume changes by a similar amount.

Phosphatidylcholine bilayer free volume has been investigated in computational studies.\textsuperscript{14,31,36,37,205,209} The total free volume in the bilayer mid-plane has been calculated to be as large as 54\% for a DPPC membrane,\textsuperscript{32} however, $V_f$ is less than the total free volume. The available free volume depends on the size of the solute and on the distribution of the free volume “voids” in the bilayers. Free volume voids have been predicted to be larger in the bilayer mid-plane than in the ordered aqueous phase.\textsuperscript{14} These voids often have the shape of elongated ellipsoids.\textsuperscript{37} The highest density (and smallest $V_0$) of a phospholipid bilayer is found in region 2.\textsuperscript{209} Because of the uncertainty in estimating $V_0$ for merocyanines and spiropyrans, we cannot determine absolute $V_f$ values. Rather, we analyze $V_f$ variation in the direction of the bilayer normal.

For MC3 (region 3 in Scheme 8), exponent $a$ values (and rate constants in Figure 24B) in DPPC and DMPC bilayers are identical, $a = 0.76 - 0.77$, respectively. Thus, the aliphatic region of the bilayer is similar for both phosphatidylcholines. In addition, the same Arrhenius activation energy describes the temperature dependence of rate constants
in L\(_\alpha\) and P\(_{\beta'}\) phases. This result is in agreement with smaller structural changes in this
region of the bilayer during the liquid - gel phase transition.

For MC1 (region 1), \(a = 0.45\) for DMPC and \(a = 0.53\) for DPPC bilayers. This result suggests that \(V_f\) is somewhat lower in the L\(_\alpha\) phase of DMPC bilayers; however, the
difference is not large relative to the uncertainty of the data. As shown in Figure 23B and
Table 3, MC1 ring-closing rate constants in DPPC and DMPC bilayers differ more
significantly in the P\(_{\beta'}\) phase. This result might be related to the different orientation of
MC1 in the gel phase of these bilayers (the angle between the bilayer normal and the
merocyanine dipole moment was 16° in DPPC and 25° in DMPC bilayers, see Figure 34 in
Appendix C).

The most interesting results are obtained from the MC2 data. This merocyanine is
localized in region 2, which includes the glycerol backbone and the ordered region of the
acyl chains. As shown in Figure 24A, the rate constants for MC2 in DPPC and DMPC
bilayers differ by almost 2 orders of magnitude, and the available free volume exponents
are \(a = 0.90\) for DPPC and \(a = 0.48\) for DMPC (Table 4). This suggests that in region 2
the available free volume is \(~2\times\) larger in DPPC bilayers. This outcome could reflect
intrinsic properties of the bilayers or could be induced by the membrane-embedded
merocyanines.

In computational studies, the free volume properties of DMPC and DPPC bilayers
were found to be very similar in all membrane regions.\(^{209}\) Thus, our results agree with
computational free volume analysis for regions 1 and 3, but not for region 2. This region
of the membrane is the most densely packed (total free volume is \(~30\%\) less than in region
3 and ~15% less than in region 1).\textsuperscript{14} When the available free volume is considered, the differences between the bilayer regions are even more pronounced.\textsuperscript{14} If merocyanines are perturbing the local structure of the bilayer, such perturbations are likely to have the largest effect in the most dense membrane region. The presence of cholesterol (mole fraction of 0.4) in DMPC bilayers led to a reduction in the free volume in the bilayer midplane and an increase in free volume in the head-group region.\textsuperscript{37}

In experimental studies, differences between DMPC and DPPC bilayers have been reported. For example, the antimicrobial peptide microcin J25 was shown to induce dramatic fluidity change in DPPC membranes but not in DMPC membranes.\textsuperscript{225} The selective membrane microviscosity variation demonstrated by this peptide may be related to the available free volume.

In summary, we found that the largest available free volume is in region 3, the smallest – at the interface (region 1). For DMPC bilayers, the largest change in $V_f$ (more than 150% increase) takes place between regions 2 and 3. For DPPC bilayers, a similar increase in $V_f$ is observed between the regions 1 and 2.

**Phospholipid bilayer dynamics and free volume.** Phospholipid dynamics range from picoseconds (trans/gauche isomerizations) to hours (lipid exchange between the bilayer leaflets),\textsuperscript{39} and therefore the bilayer free volume will depend on the time scale of the reaction that is used to probe this complex system. SP $\rightarrow$ MC and MC $\rightarrow$ SP reaction dynamics are uniquely suitable for such an analysis as the ultrafast ring-opening reaction, SP $\rightarrow$ MC, occurs on the picosecond time scale, while the thermal ring-closing reaction,
MC \rightarrow SP, is much slower. On the picosecond time scale, major bilayer reorganizational dynamics are essentially "frozen" and ultrafast reactions reveal the static structure of the bilayer. On the slower time scale studied in this work, the phospholipids can rearrange in response to the photochemical reaction.

Recently we studied the ultrafast ring opening and isomerization of spiropyran SP2 (SP2 \rightarrow MC2) in the aliphatic region 3 of DPPC, DMPC, and DOPC bilayers (see Chapter 3).\textsuperscript{226} On the picosecond time scale in L_{\alpha} phase bilayers, the free volume exponent was $a = 0.33 \pm 0.03$ - much smaller than $a = 0.76 - 0.77$ determined in the current study for region 3. The difference suggests that on a ps time scale $\nu_f$ is about two times smaller than on the time scale examined here.

Comparison of data measured at different time scales shows other differences. For the slower reactions studied here, $E_a$ was generally higher in the P_{\beta'} phase than the L_{\alpha} phase (Table 3), which is consistent with the presumed higher viscosity of gel phase bilayers. In contrast, on the ps time scale, $E_a$ in region 3 increased from 7.3 kJ mol$^{-1}$ in P_{\beta'} phase to 26 kJ mol$^{-1}$ in L_{\alpha} phase.\textsuperscript{226} Although this hypothesis requires further study, the smaller $E_a$ values for ultrafast reactions in P_{\beta'} phase could be related to free volume void aggregation around spiropyrans in bilayer, similar to the cholesterol effects found in simulations.\textsuperscript{31}

The time scale of many biological processes, such as diffusion in the membrane or transport across the bilayer, occurs on a millisecond and second time scale. Thus, the results of the current experiments could be more directly applicable to biological
membrane phenomena than the results of ultrafast studies\textsuperscript{226} and ps time scale simulations.\textsuperscript{14,15,31,37,205,209}

4.7 Summary

Photochromic and solvatochromic merocyanines appear to be sensitive molecular probes of phospholipid bilayer structure and dynamics: (i) merocyanines can be embedded into well-defined regions of the bilayer, (ii) ring closing rate constants are sensitive probes of the structural changes that occur during gel - liquid phase transitions, (iii) even small differences in bilayer properties (such as the contrasting free volume properties in region 2 of DMPC and DPPC bilayers) are reflected in the rate constant data, and (iv) bilayer properties on ultrafast (picosecond) and slow (second) time scales can be analyzed using spiropyran and merocyanine reactions.

We determined the free volume profile of saturated phosphatidylcholine bilayers and developed a new method to study this important physical property. This method, based on the rate constant analysis of the intramolecular ring-closing reactions, can be applied to other bilayer systems. Computational studies showed that alkyl chain unsaturation\textsuperscript{205} and gauche conformations\textsuperscript{227} result in enhanced free volume, while branching in the alkyl chains\textsuperscript{35} and the presence of cholesterol\textsuperscript{31} reduce the available free volume. These predictions remain to be tested experimentally. Our method could also be applied to the analysis of the free volume distribution in bilayers with components segregated into domains. The results described here suggest that molecular probes in
region 2 of the bilayer are the most sensitive to the variation of membrane properties, and
could be applied to analyze lipid self-segregation behavior. To study bilayer domains,
photochemical molecular probes with affinities to these membrane regions should be
developed.

Temperature dependent merocyanine absorption spectra in DMPC and DPPC for
MC1 – MC3 can be found in Appendix B. Linear dichroism experimental details and
analysis can be found in Appendix C.
Chapter 5: Nonlinear Optical Spectroscopic Studies Thermalization Dynamics of Phospholipid Bilayer Liposomes Embedded with Porphyrin Sensitizers


5.1 Overview

Transient grating kinetics were measured at different temperatures (1°C – 40°C) for DMPC phospholipid bilayer liposome aqueous dispersions. 5,10,15,20-tetrakis-(4-hydroxyphenyl)-21,23H-porphyrin sensitizers were embedded into the phospholipid bilayers. Fluorescence emission temperature dependence and quenching studies were used to determine that hydroxyphenyl porphyrins are localized in the aliphatic region of the phospholipid bilayer. Porphyrin localization does not change in gel (P_{β'}) and liquid (L_{α}) phases of the membrane. In the transient grating experiment, following excitation of aqueous liposome dispersions with 100 fs 410 nm pulses, temperature and density modulations were observed as acoustic grating signals. Grating signal amplitude and time when the acoustic peak reaches maximum were temperature-dependent. Negative time delay of the acoustic peak maximum was observed and explained by considering two thermal processes: 1) thermalization of the porphyrin in the phospholipid bilayer
(described by a rate constant $k_1$) and 2) energy transfer from the phospholipid bilayer to the surrounding solvent (described by a rate constant $k_2$). A model accounting for time-dependent thermal expansion of phospholipid bilayers and water was developed and used to determine rate constants $k_1$ and $k_2$. The rate constant $k_1$ equals to $7.14 \times 10^{10} \text{ s}^{-1}$ ($k_1^{-1} = 14 \text{ ps}$) and is temperature-independent at $10^\circ \text{C}-35^\circ \text{C}$. $k_2$ increases from $3.33 \times 10^{10} \text{ s}^{-1}$ ($k_2^{-1} = 30 \text{ ps}$) at $10^\circ \text{C}$ to $5.56 \times 10^{10} \text{ s}^{-1}$ ($k_2^{-1} = 18 \text{ ps}$) at $35^\circ \text{C}$. The value and temperature dependence of $k_2$ suggests that thermalization in DMPC membrane occurs by thermal diffusion and vibrational energy transfer, but other phospholipid degrees of freedom (such as isomerization) are not involved in this process.

5.2 Energy Transfer and Porphyrin Molecules

Phospholipid bilayer vesicles (liposomes) are good cell membrane models and are widely used to study physical properties of biological membranes. Spectroscopic investigations of liposomes usually rely on membrane embedded fluorescent or spin molecular probes; properties of such probes indirectly reflect properties of the phospholipid bilayer.\textsuperscript{228} Likewise, liposomes are self-organized nanoscale molecular assemblies and have become increasingly important in molecular electronics and artificial photosynthesis applications. The function of such structures (for example, energy storage or signal transduction) can be controlled by light. This is achieved by using membrane embedded molecular sensitizers.\textsuperscript{229} Due to their large extinction coefficients and favorable redox properties, porphyrins are often used as probes in various membrane-based artificial
assemblies. Additionally, understanding porphyrin interactions with membranes is also important in photodynamic cancer therapy.

All applications described above depend on energy transfer in liposome – sensitizer molecular assemblies. While thermal processes in solids have long been understood and molecular vibrational relaxation is also widely studied, thermal processes in nanoscale structures have not been investigated thoroughly. The well-defined structure and narrow size distribution of liposomes make them well suited for studies of thermal processes in nanoscale molecular assemblies. We examined thermal processes in 50 nm diameter liposomes. Our interest was primarily in energy transfer from porphyrin molecular sensitizers to phospholipid bilayers and energy transfer from the bilayer membrane to the aqueous solvent. The system is also interesting because phospholipid bilayers undergo a phase transition from an ordered solid (gel) phase to a liquid phase. This phenomenon is sometimes associated with membrane functions in biological systems. The study of thermal processes in different phospholipid bilayer phases allows the role of gel – liquid phase transition on phospholipid bilayer thermal relaxation and volume thermal expansion to be investigated.
Scheme 9: Porphyrin structures and energy transfer model in a phospholipid bilayer.

1: M = H₂,  
2: M = Fe(III)Cl₂, R = OH  
3: M = Fe(III), R = N⁻

\[ k_1 \] thermalization in the membrane

\[ k_2 \] energy transfer to aqueous solvent
We examined phospholipid bilayer liposomes with embedded 5,10,15,20-tetrakis-(4-hydroxyphenyl)-21,23H-porphyrins 1 and 2 (Scheme 9). Hydroxyphenyl porphyrins sensitize singlet oxygen\textsuperscript{232,233} and have been used in photodynamic cancer therapy.\textsuperscript{234} Previous work has shown that porphyrin localization in phospholipid bilayers critically depends on the macrocycle substitution.\textsuperscript{235-239} The central metal has no influence on the localization.\textsuperscript{240} Therefore, lipophilic (not soluble in water) free-base (H\textsubscript{2}) porphyrin 1, which shows strong fluorescence, was used to determine porphyrin localization in the phospholipid bilayers. Nonfluorescent Fe(III)Cl hydroxyphenyl porphyrins 2 were used in nonlinear optical experiments. These porphyrins were used because their shorter excited state lifetime afforded picosecond time resolution in transient grating dynamics studies. Energy transfer in phospholipid bilayers (excited porphyrin $\rightarrow$ phospholipid bilayer $\rightarrow$ water) was compared with direct energy transfer from porphyrins to water. Direct energy transfer from porphyrin to solvent was studied using the water-soluble Fe(III) tetra-(N-methyl-4-pyridinium) porphyrin 3.

Experimentally, energy transfer between molecules and the surrounding solvent has been examined by various spectroscopic techniques, including fluorescence lineshape analysis, transient absorption, and Raman scattering. In particular, transient grating spectroscopy permits the study of systems that are non-fluorescent. As such, this technique is well suited for experiments on phospholipid bilayers. Transient grating spectroscopy has been used to study thermal processes for molecules in solution, films, and processes in heme proteins.\textsuperscript{241-243} We applied transient grating spectroscopy to the study of porphyrin
excited state relaxation and vibrational energy transfer in phospholipid bilayer membranes and in aqueous solution.

5.3 Transient Grating Spectroscopy

Thermalization dynamics in membranes were investigated using the transient grating method (Scheme 10). In the transient grating experiment, two excitation pulses reach the sample at the same time. Because of the excitation beam interference, light intensity is modulated along the coordinate, \( x \). If the light intensity is sufficiently high, nonlinear interactions lead to absorbance and/or refractive index modulation along \( x \), thus creating a transient diffraction grating. In the case of resonant excitation, grating properties are largely determined by the changes in the absorption spectra and the corresponding modulation of the refractive index. After nonradiative relaxation of the electronic excited states, the grating is still present because of the sample heating, \( dT \), and density changes, \( d\rho \), due to the thermal expansion. The modulation of the refractive index along the coordinate \( x \) may be described by a sine function: \( \Delta n = \Delta n_{\text{max}} \sin(2\pi x / \lambda) \). The grating period, \( \lambda \), depends on the angle between excitation beams, \( \Theta \), and on the excitation wavelength, \( \lambda_e \): \( \lambda = \lambda_e /[2 \sin(\Theta/2)] \). The probe pulse (which reaches the sample at the variable delay after the excitation pulses) is diffracted from the grating. Intensity of the diffracted signal, \( I_{\text{grating}} \), under the weak diffraction conditions is given by:

\[
I_{\text{grating}} \propto [\Delta n(t)]^2 = \left[ \left( \frac{\partial n}{\partial T} \right)_\rho dT + \left( \frac{\partial n}{\partial \rho} \right)_T d\rho \right]^2
\]  

(5.1)
In this expression, the \((\partial n/\partial T)_p dT\) term represents heating, and the \((\partial n/\partial \rho)_T d\rho\) term represents density change due to the thermal expansion. (If the sample absorption at the probe wavelength changes, expression for \(I_{\text{grating}}\) will also have terms related to the absorption modulation).\(^{243}\) \(dT\) and \(d\rho\) are time-dependent, therefore, by measuring kinetics of the diffracted signal, dynamic properties of the transient grating can be investigated. Since the experiment relies on the \(\Delta n\) measurement, systems under study do not have to be fluorescent or have other "spectroscopic signatures" required for linear spectroscopy. An important advantage of the transient grating experiment is sensitivity — it is a zero background technique.

The \((\partial n/\partial T)_p dT\) and \((\partial n/\partial \rho)_T d\rho\) terms have different time dependences. The first term describes heat transfer from the solute to the medium and heat diffusion in the medium. In our experiments this term is much smaller (arguments for this statement are presented below), therefore we will neglect it. The second term describes thermal expansion of the medium. Such expansion occurs with the speed of sound and the system evolution may be described by a standing wave model. Experimentally, standing waves of density changes are observed as oscillations in the diffracted light intensity (Scheme 10). In the case of instantaneous solute excited state relaxation and instantaneous heat transfer from solute molecule to medium, a sinusoidal transient grating signal would be observed:

\[
I_{\text{grating}} \propto A[\sin(\omega t)]^2 = A \left( \sin \left( \frac{\pi t}{T} \right) \right)^2 \quad (A \text{ is amplitude, } \omega \text{ is frequency}, \ T = 2\pi/\omega \text{ is period, and } t \text{ is time})
\]

This case is illustrated as a dotted line in Scheme 10. If the excited state relaxation and energy transfer to the medium is not instantaneous but occurs with a finite
rate, the phase of the acoustic wave, $\phi$, will be different. The signal will then be described as: $I_{\text{grating}} \propto A[\sin(wt + \phi)]^2 = A\left(\sin\left(\frac{\pi(t - t_c)}{T}\right)\right)^2$ ($t_c$ is an acoustic peak delay). This case is illustrated with a solid line in Scheme 10. Therefore, the time when the first acoustic peak reaches its maximum, $t_{\text{max}} = T/2 + t_c$, depends on the energy transfer rate.
Scheme 10: Transient grating diagram and photoacoustic peak shift schematic.

[Diagram of transient grating setup with labels for excitation beams, probe beam, sample, and diffraction signal, along with a graph showing diffracted signal amplitude over time delay.]

\[ t_{\text{max}} \]

\[ t_c \]
Terazima and coworkers have studied several dyes in different organic solvents and have shown that $t_c$ is equal to the thermalization time.\textsuperscript{244,245} Therefore, by determining $t_c$ values, rate constants for energy transfer from the solutes to the surrounding solvent can be obtained. Acoustic peak delays have been analyzed in biological systems. Miyata and Terazima have recently observed negative $t_c$ values for deoxymyoglobin aqueous solution.\textsuperscript{246} By modeling transient grating data, these authors were able to determine that 17\% of the heme excess energy was transferred to water directly, while the major part of the heme energy was transferred to the protein.\textsuperscript{246} Miller and coworkers studied picosecond conformational changes in carboxymyoglobin following ligand photolysis by analyzing acoustic peak delay.\textsuperscript{247}

The amplitude of the transient grating signal, $A$, is also an important parameter due to its temperature dependence. The amplitude of the acoustic signal in aqueous solution would be negligible at 4\(^\circ\)C, when the water thermal expansion coefficient is zero, while acoustic signals would be larger at higher temperatures. Temperature dependence measurements were successfully applied in transient grating studies of heme proteins and dye molecules.\textsuperscript{241,248,249} As will be discussed below, the temperature dependence of the transient grating signal for liposome aqueous dispersion can be described by a simple model.

5.4 Experimental

Materials and Methods. Porphyrins 1 and 2 were obtained from Porphyrin Systems (Germany), porphyrin 3 was purchased from Sigma. All other reagents and
solvents were obtained from Aldrich. Nano-differential scanning calorimeter DSC II (Calorimetry Sciences Corporation) was used to obtain heat capacity profiles. Within experimental accuracy, heat capacity profiles were the same for 100:1 porphyrin sensitized concentration liposomes and for liposomes without porphyrins; both were similar to DSC data reported in the literature.\textsuperscript{44} Electronic absorption spectra were measured with a HP8452A spectrometer and corrected for scattering. Fluorescence spectra were acquired with a Varian Eclipse fluorimeter equipped with a temperature controller.

**Nonlinear Spectroscopy.** The spectrometer for ultrafast nonlinear experiments was described previously,\textsuperscript{250} it was modified for transient grating studies. A regeneratively amplified Ti:Sapphire laser system (Spectra Physics) provided 100 fs, 0.8 mJ pulses at 820 nm. The laser beam was split into two excitation beams (second harmonics generation in a 0.5 mm thick BBO crystal was used to obtain excitation pulses at 410 nm) and a probe beam (820 nm). The energies of the excitation and probe pulses were 2 $\mu$J and 1 $\mu$J respectively. All beams were p-polarized. The grating period was $\lambda = 1.73$ $\mu$m ($\Theta = 13.6^\circ$ and $\lambda = 410$ nm). The probe beam was aligned to satisfy the phase matching conditions (the angle between the grating normal and the probe beam was equal to 13.6$^\circ$, Scheme 10). Excitation beams were aligned in the horizontal plane, while the probe beam was offset from the horizontal plane by a small angle ($\sim 3^\circ$) to achieve better separation of the diffracted signal from the scattered light. Two iris diaphragms placed in the optical path of the signal beam reduced scattered probe light. A red filter was used to eliminate scattered excitation light. The diffracted signal was detected with a PMT (photosensor module
H6780-20, Hamamatsu). The signal was amplified with a 300 MHz bandwidth amplifier (SR445, Stanford Research Systems) and was gated by a box-car (SR250, Stanford Research Systems). A computer-controlled linear translation stage allowed adjustment of the probe beam time delay necessary to measure kinetics on the 100 fs to 3 ns time scale. Samples were placed in 2 mm pathlength cuvettes and stirred with a small magnet. Porphyrin absorption at 410 nm was approximately 0.65 for all experiments. Temperature was adjusted with a high-sensitivity thermostat (Flash 200, Quantum Northwest).

5.5 Porphyrin Bilayer Embedding and Photoacoustic Data

Fluorescence properties of liposomes with hydroxyphenyl porphyrins embedded into the phospholipid bilayer. Electronic absorption spectra of porphyrins 1 and 2 and fluorescence emission spectrum of porphyrin 1 are shown in Figure 25A. Porphyrin spectra are very similar in organic solvents and in phospholipid bilayers. For example, absorption band maxima for porphyrin 1 are at 419 (Soret band), 516, 554, 594, and 650 nm (Q bands) in acetone solution and at 423, 519, 557, 596, and 651 nm in liposomes. Porphyrin 1 is highly emissive in organic solvents (fluorescence emission quantum yield in acetone is 0.13\cite{232,233} emission maxima are at 657 and 723 nm). Emission maxima for porphyrin 1/DMPC liposomes are at 660 and 720 nm. Absorption maxima for porphyrin 2 in acetone solution are at 422 nm, 512 nm, 571 nm (shoulder), and 699 nm. For porphyrin 2/DMPC liposomes, Soret band maximum is at 423 nm and the other weaker absorption bands are broadened.
Depending on the temperature, phospholipid membranes have very different structural and thermodynamic properties. Three major phases of phosphatidylcholine bilayers are the gel phase \( (L_{\beta^{'}}) \), the rippled gel phase \( (P_{\beta^{'}}) \), and the liquid phase \( (L_{\alpha}) \).\textsuperscript{228} The enthalpy for the \( P_{\beta^{'}} \leftrightarrow L_{\alpha} \) phase transition has the highest value because of the major structural changes in the aliphatic interior of the bilayer.\textsuperscript{44,228} The \( P_{\beta^{'}} \leftrightarrow L_{\alpha} \) transition occurs at \( \sim 23^\circ C \) for DMPC lipids \( (\sim 42^\circ C \) for DPPC).\textsuperscript{44,228} Porphyrin 1 fluorescence emission intensity increases by about 12% when the phospholipid bilayer undergoes \( P_{\beta^{'}} \leftrightarrow L_{\alpha} \) phase transition (Figure 25B). A well-defined phase transition temperature \( (\sim 23^\circ C) \) observed in the fluorescence emission data indicates that porphyrins 1 are localized in the aliphatic interior of the membrane.\textsuperscript{236} (Porphyrins that localize close to the membrane surface have different temperature dependence with no discontinuity at the \( P_{\beta^{'}} \leftrightarrow L_{\alpha} \) phase transition temperature\textsuperscript{235-239} because structural changes at the membrane interface are much less important for this phase transition.) Within accuracy of the experiments, the same \( P_{\beta^{'}} \leftrightarrow L_{\alpha} \) phase transition temperature was obtained in differential scanning calorimetry experiments on liposomes with and without porphyrins. This result suggests that porphyrin sensitizers do not significantly perturb the phospholipid bilayer.
Figure 25: A. Electronic absorption spectra for porphyrins 1 (—) and 2 (---) in acetone solution. Inset a: Expansion of the porphyrin Q band region in the absorption spectrum. Inset b: Porphyrin 1 fluorescence emission spectra in acetone solution (—) and embedded in liposomes (-----). B. Fluorescence emission intensity temperature dependence for porphyrin 1/DMPC liposomes. Phospholipid/porphyrin ratio was 1000:1, excitation wavelength was 550 nm. The data was obtained by integrating fluorescence emission between 620 nm and 760 nm. Porphyrin 1/DMPC absorption and emission spectra do not change in this temperature range.
Additional information about porphyrin localization in the phospholipid bilayers can be obtained from the fluorescence emission quenching studies.\textsuperscript{235-239} For these experiments, different amounts of KI were added to liposome solutions while keeping the ionic strength of the solution constant with KCl (0.1 mM Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3} was also used to prevent oxidation of I\textsuperscript{-}). At higher iodide concentration, porphyrin fluorescence emission yield was lower due to excited state quenching by iodide. Analysis of the quenching data according to a Stern-Volmer model yields the excited state quenching rate.\textsuperscript{200} However, a modified Stern-Volmer model was used because a simple Stern-Volmer model was not consistent with the data. The modified Stern-Volmer model is based on the assumption that there are two-distinct groups of porphyrins, one group that is quenched efficiently and the other group that is quenched more slowly. The difference between the two groups could be their depth in the phospholipid bilayer. While several porphyrins embedded in phospholipid bilayers were extensively investigated (because of their potential as anti-cancer agents),\textsuperscript{235-239} hydroxyphenyl porphyrin embedding depth has not been determined conclusively. Therefore, a more complex distribution, such as the presence of more than two distinct populations or a continuous depth distribution, cannot be excluded. We are primarily interested in whether the porphyrin distribution in the liposome membrane (embedding depth) is the same in \( L_\beta' \) and \( L_\alpha \) phases of the membrane. To answer this question, Stern-Vormer analysis is sufficient.

A modified Stern-Volmer plot of porphyrin 1/DPPC fluorescence emission intensity versus the inverse of iodide concentration is shown in Figure 26. Two data sets
were collected, at 25°C (L_β' phase of the bilayer) and at 55°C (L_α phase). Fluorescence intensity, \( F \), was analyzed as:

\[
\frac{F_0}{\Delta F} = \frac{1}{f_a K_a [KI]} + \frac{1}{f_a}
\]  

where \( F_0 \) is fluorescence intensity without quencher, \( \Delta F = F_0 - F \), \( f_a \) is the fraction of porphyrin molecules which are accessible to the quencher, \( K_a \) is the Stern-Volmer quenching constant of the accessible fraction, and \([KI]\) is concentration of the quencher. Fitting of the data yields similar \( f_a \) values at both temperatures, \( f_a = 0.78 \) (25°C) and 0.81 (55°C); therefore, the fraction of the porphyrin molecules exposed to the quencher does not change significantly in gel and liquid crystal phases of the membrane. Based on this result, we assume that porphyrin localization does not change in different phases of the bilayer. The Stern-Volmer constant is somewhat larger at 55°C, \( K_a = 6.8 \, M^{-1} \), than at 25°C, \( K_a = 5.9 \, M^{-1} \). The Stern-Volmer constant for quenching with iodide has similar values as determined for other porphyrins, especially those that localize in the aliphatic region of the phospholipid.^{235}
Figure 26: Modified Stern-Volmer plot of porphyrin 1/DPPC fluorescence emission quenching with iodide. [KI] concentration is varied from 0 to 0.80 M. Phospholipid/porphyrin ratio was 1000:1. Excitation was at 550 nm, integrated emission was measured at 620 nm – 760 nm. Temperature was 25°C (■) and 55°C (●). Solid lines were obtained by fitting data to Eq. (5.2).
Photophysical characteristics of porphyrin 1 in phospholipid bilayers can be contrasted to those of carboxylic porphyrins (such as hematoporphyrin). In DMPC and DPPC liposomes, carboxylic porphyrin fluorescence emission decreases at higher temperatures; porphyrin distribution between various compartments of the membrane and aggregation behavior are also influenced by the temperature changes. This does not appear to be the case for porphyrin 1.

Transient grating kinetics for aqueous solutions of porphyrin 3. Transient grating kinetics for porphyrin 3 aqueous solution are shown in Figure 27. Temperatures at which data were collected are indicated on the graph. Within the accuracy of the experiment, dynamics at $t < 3$ ps are indistinguishable. The peak at $t = 0$ ps (when excitation and probe pulses overlap in time) is assigned to the Optical Kerr effect (related to the third-order nonlinear polarization). The width of this peak (FWHM = 0.40 ps) is somewhat larger than FWHM of the laser pulses used for excitation; this broadening is observed because of the relatively large angle between the excitation pulses ($\Theta = 13.6^\circ$, Scheme 10). Both resonant and nonresonant effects contribute to the femtosecond Optical Kerr effect signal. The resonant contribution arises due to the iron porphyrin excited state absorption at the probe wavelength of 820 nm, while nonresonant contributions are related to the nonlinear polarizability of the hydroxyphenyl porphyrin macrocycle.
Figure 27: Porphyrin 3 aqueous solution (phosphate buffer, pH 7) transient grating kinetics measured at 30°C, 17°C, 10°C, and 4°C (amplitudes from the highest to the lowest). Note different scale used for time axis. For clarity, all signals at 20 to 3000 ps time delay were multiplied ×10. Solid lines are fits according to Eq. (5.3).
A small amplitude picosecond decay component is also evident at 0.5-3 ps time delay in Figure 27. Assuming an exponential decay, the lifetime of this component is 1.5 ps. The amplitude is the same at all temperatures. Because the grating response is quadratic (see Eq. (5.1)), the apparent 1.5 ps decay corresponds to 3 ps relaxation of the porphyrin exited state. Similar ps relaxation components observed for iron porphyrins were attributed to electronic and vibrational relaxation.\textsuperscript{253-255} For example, anti-Stokes band $\nu_4$ (which corresponds to 1375 cm$^{-1}$ macrocycle skeletal vibrations) for porphyrin 3 had a 1.9 ps lifetime in water.\textsuperscript{255} Based on the analysis of the Optical Kerr effect signal, we conclude that the electronic excited states for porphyrin 3 relax within several picoseconds.

Next we analyze the oscillating signal at 20 to 3000 ps in Figure 27. At 4°C, when the water volume thermal expansion coefficient, $\beta_w$, is equal to zero, and the amplitude of the oscillating signal is negligible. Signal amplitude increases at higher temperatures concomitant with an increase in $\beta_w$. Therefore, the oscillating part of the kinetics is assigned to the acoustic signal (see methods section) in aqueous solution. A nonlinear least squares Levenberg-Marquardt algorithm was used to fit the acoustic data (measured at 20 to 3000 ps time delay) to the following function ($\alpha$ is the grating decay rate constant):

$$I_{\text{grating}} = A \left( \sin(\pi(t - t_e) / T) \right)^4 \exp(-\alpha t)$$  \hspace{1cm} (5.3)

Fitting results are shown as solid lines in Figure 27; the values of $A$, $T$, and $t_e$ at different temperatures are summarized in Figure 29 A-C. The exponential term in Eq. (5.3) describes the transient grating decay; $\alpha \approx 1 \times 10^8$ s$^{-1}$. Study of the rate constants $\alpha$ by
Transient grating provides detailed information about the microscopic viscosity. However, our experiments were limited to 3 ns time scale. More reliable $\alpha$ values would be obtained by analyzing longer time kinetics. Period $T$ data (Figure 29B) simply reflects the temperature dependence of the speed of sound, $c$, in aqueous solution ($c = \Lambda/T$; based on $T$ value at 25°C and $\Lambda = 1.73$ μm, $c = 1.55 \times 10^3$ m s$^{-1}$; literature value for $c$ in water at this temperature is $1.50 \times 10^3$ m s$^{-1}$).

The most important results were obtained from the amplitude, $A$, and the acoustic peak delay, $t_e$, temperature dependence data. Figure 29C shows that $t_e$ varies between 20 and 30 ps (small amplitude of TG signals precluded reliable $t_e$ determination at temperatures <10°C). Following the analysis of Terazima and coworkers, $t_e$ data is assigned to porphyrin thermalization with $\tau \approx 25$ ps. Because electronic excited state relaxation for porphyrin 3 is significantly faster than 25 ps, thermalization with $\tau \approx 25$ ps reflects vibrational energy transfer from the porphyrin electronic ground state to water.

**Transient grating kinetics for porphyrin 2/DMPC liposomes.** Transient grating kinetics measured for porphyrin 2/DMPC liposomes at several temperatures are shown in Figure 28. Assignment of the $t = 0$ ps peak to the Optical Kerr effect and ~1.5 ps relaxation component to the electronic and vibrational relaxation (with 3 ps lifetime) is the same as for porphyrin 3 aqueous solution.
Figure 28: Transient grating kinetics for 2/DMPC liposomes measured at 35°C, 20°C, 10°C, 4°C, and 1°C (amplitudes from the highest to the lowest). Note different scale used for time axis. For clarity, all signals at 20 to 3000 ps time delay were multiplied ×5. Solid lines represent fits according to Eq. (5.3). Inset: expansion of 17°C acoustic signals at 20 to 1100 ps for porphyrin 2/DMPC liposomes (■■■) and for porphyrin 3 aqueous solution (○○○). Kinetics differ in amplitude and in acoustic peak delay.
Oscillating (acoustic) signals at 20 to 3000 ps for porphyrin 2/DMPC liposomes were also fitted to the function of Eq. (5.3); parameters are summarized in Figure 29 A-C (grating decay rate constant $\alpha \approx 1 \times 10^8$ s$^{-1}$). Period $T$ values (B) appear to be the same as for porphyrin 3 aqueous solution, while amplitude, $A$, (A) and acoustic peak delay, $t_c$, (C) values are different. The main differences are: 1) acoustic signals for porphyrin 2/DMPC liposomes have larger amplitude, 2) porphyrin 2/DMPC liposome data amplitude is significant at 4°C, when the water thermal expansion coefficient is zero and porphyrin 3 aqueous solution signal amplitude is negligible, 3) the phase shift for porphyrin 2/DMPC acoustic signals is negative ($t_c < 0$) and strongly temperature-dependent.

The larger amplitude for porphyrin 2/DMPC liposome transient grating signals is attributed to the liposome thermal expansion. The volume thermal expansion coefficient for lipids, $\beta_L$, has similar values in both gel ($\beta_L = 0.00088$ K$^{-1}$) and liquid ($\beta_L = 0.001$ K$^{-1}$) phases of the bilayer. In contrast, volume thermal expansion coefficient for water, $\beta_W$, changes nonlinearly with temperature. The difference between $\beta_L$ and $\beta_W$ values is greatest at 4°C, when $\beta_W = 0$.

The negative phase shift ($t_c < 0$) is particularly interesting. As described in above, a finite relaxation rate in systems described by a single thermalization rate constant could only result in positive acoustic peak delays, $t_c > 0$. A positive acoustic peak delay was observed for porphyrin 3 aqueous solution at 12°C to 30°C (Figure 29C). The inset in Figure 28 contrasts 17°C kinetics for porphyrin 3 aqueous solution and for porphyrin 2/DMPC liposomes. Clearly, acoustic signals for porphyrin 2/DMPC liposomes reach maximal values at earlier times, which results in $t_c < 0$ (Figure 29C). In the following
sections we develop a model to explain the negative acoustic peak delays by accounting for two steps of porphyrin thermalization in phospholipid bilayers – energy transfer to the membrane followed by energy transfer to the surrounding solvent (Scheme 9). Interference of the acoustic waves created by the two processes causes the apparent negative acoustic peak delay.

Finally, Figure 29D shows the experimentally determined time of the first acoustic peak maximum for porphyrin 2/DMPC liposomes (—○—; also see Scheme 10). Surprisingly, $t_{\text{max}}$ does not vary with temperature when the acoustic period changes; the increase in $T/2$ at low temperatures is compensated by larger negative phase shift, and $t_{\text{max}} = T/2 + \tau_c \approx 545$ ps.
Figure 29: Amplitude \( A \) (A), period \( T \) (B) and acoustic peak delay \( t_c \) (C) temperature dependence determined by fitting data to Eq. (5.3). Parameters are shown for porphyrin 2/DMPC liposomes (\(--\bullet--)\) and for porphyrin 3 aqueous solution (\(--\square--)\). D - Experimentally determined time when the first acoustic peak reaches its maximum for porphyrin 2/DMPC liposomes (\(--\bullet--)\); this time is equals \( t_{\text{max}} = T/2 + t_c \). In the case of instantaneous thermal relaxation and volume expansion, \( t_{\text{max}} \) would be observed at \( T/2 \) (\(--\triangle--)\).
5.6 Modeling the Thermalization Behavior of Membrane-embedded Porphyrin Molecules

**Thermal relaxation dynamics and volume expansion.** Following excitation at 410 nm, 3.02 eV (or 291 kJ M\(^{-1}\)) excess energy is absorbed by the porphyrin. Porphyrin heating can exceed tens of degrees.\(^{261}\) Electronic excited state relaxation of Fe(III)Cl porphyrins contributes to the Optical Kerr effect signals in Figures 27 and 28 (kinetics at time delay < 3 ps). As explained above, electronic relaxation can be approximated with femtosecond and 3 ps lifetime components. For porphyrin 2/DMPC liposomes, porphyrin electronic excited state relaxation is followed by a vibrational energy transfer to the phospholipid bilayer. (Based on fluorescence temperature dependence, we assume that there is no direct energy transfer from porphyrin 2 to water). The upper limit for the increase in liposome temperature can be estimated as 1.4 K. (50 nm diameter liposomes consist of ~20000 phospholipids, heat capacity of DMPC phospholipid bilayer is 1600 J M\(^{-1}\) K\(^{-1}\),\(^{1,44}\) ~150 porphyrins are excited in one porphyrin 2/DMPC liposome based on sample absorbance of 0.65 and 100:1 lipid:porphyrin ratio.) An increase in the liposome temperature leads to the thermal expansion.

Porphyrins embedded in the lipid bilayers (porphyrin 2/DMPC) can perhaps be compared to the heme in deoxymyoglobin. Li and Champion modeled thermal processes in deoxymyoglobin by considering the thermal response of an instantaneously heated chromophore embedded in a protein and surrounded by water.\(^{262}\) The temperature for such a system (obtained by numerically solving a two-boundary thermal transport problem) was well-approximated by a two-exponential function (in a longer time limit solutions “rolled
over" to a $t^{3/2}$ decay of a very small amplitude).\textsuperscript{262} Similarly, cooling of deoxymyoglobin heme in transient mid-IR absorption experiments was modeled by an exponential decay with a time constant $\tau$ of 3 to 10 ps.$^{263}$ Thermalization of dye molecules in various organic solvents was also assumed to be exponential.$^{264}$ Therefore, an exponential function is a satisfactory first order approximation for thermal relaxation of photoexcited porphyrins (experimental data does not justify a more complex model, such as including a second exponent or a $\sim t^{3/2}$ component):

$$Q_p = Q \exp(-k_1 t)$$

(5.4)

where $k_1$ is a first order rate constant that describes porphyrin thermalization, $Q_p$ is porphyrin excesses energy (heat), and $Q$ is total excess energy. Similarly, excess heat in liposomes and in water is described as:

$$Q_L = \frac{Qk_1}{k_2 - k_1} \left\{ \exp(-k_1 t) - \exp(-k_2 t) \right\}$$

(5.5)

$$Q_W = Q - Q_p - Q_L = \left( 1 + \frac{k_1 \exp(-k_1 t) - k_2 \exp(-k_2 t)}{k_2 - k_1} \right) Q$$

(5.6)

where $k_2$ is the first order rate constant that describes liposome thermalization (Scheme 9). $Q_L$ and $Q_W$ are excess energies for liposomes and water respectively. Deposition of excess heat causes volume thermal expansion; such expansion can be separated into liposome expansion, $dV_L(t)$, and water expansion, $dV_W(t)$:

$$dV = dV_L(t) + dV_W(t) = \frac{\beta_L}{\rho_0 C_{PL}} \frac{dQ_L}{\rho_0 C_{PL}} + \frac{\beta_W}{\rho_0 C_{PW}} \frac{dQ_W}{\rho_0 C_{PW}}$$

(5.7)
where $\rho_0$ is density, $\beta_L$ and $\beta_W$ are the volume expansion coefficients for phospholipid bilayers and water ($\beta = V^{-1}(\partial V/\partial T)_p$), $C_{PL}$ and $C_{PW}$ are heat capacities for phospholipid bilayers and water respectively ($C_p = (\partial H/\partial T)_p \approx dQ/dT$).

**Model for transient grating data taking into account time dependence of phospholipid bilayer and solvent thermal expansion.** Chen and Diebolt derived an analytical expression for describing the temporal profile of the acoustic wave created by the thermal expansion of the medium (they also considered molecular volume change in a chemical reaction).\textsuperscript{265} Puchenkov and Malkin considered pulse-width effect on the rise time of the transient grating kinetics.\textsuperscript{266} Deåk et al. modified this model to include time-dependent structural relaxation.\textsuperscript{247} We also start with the models of Puchenkov and Malkin\textsuperscript{266} and Deåk et al.\textsuperscript{247} but consider time-dependent thermal expansion. The wave equation describing density modulation, $\delta(t,x) = \rho(t) - \rho_0$, due to liposome and solvent thermal expansion is:\textsuperscript{247,266}

$$\frac{\partial^2 \delta(t,x)}{\partial x^2} - \frac{1}{c^2} \frac{\partial^2 \delta(t,x)}{\partial t^2} = \frac{\rho_0}{c^2} \frac{\partial^2 V}{\partial t^2}$$

(5.8)

Substitution, $\delta(t,x) = D(t)\sin(2\pi x/\lambda)$, is used to separate temporal and spatial variables; $D(t)$ describes maximal density changes for the grating. The time dependence for $D(t)$ is represented by:

$$\frac{d^2 D(t)}{dt^2} + \omega^2 D(t) = -\rho_0 \frac{d^2 V}{dt^2}$$

(5.9)
The second derivative for \( V(t) \) is obtained from Eqs. (5.5) – (5.7). Substituting this result into Eq. (5.9) yields:

\[
\frac{d^2 D(t)}{dt^2} + \omega^2 D(t) = -\frac{Qk_1^2}{k_2 - k_1} \left( \frac{\beta_L}{C_{PL}} k_1 - \frac{\beta_w}{C_{PW}} k_2 \right) \exp(-k_1 t) - \frac{Qk_1 k_2^2}{k_2 - k_1} \left( \frac{\beta_L}{C_{PL}} - \frac{\beta_w}{C_{PW}} \right) \exp(-k_2 t)
\]

(5.10)

Eq. (5.10) can be solved analytically using the initial conditions \( D(0) = 0 \) and \( D'(0) = 0 \) (imposing no density perturbations at \( t = 0 \)). The solution is comprised of three terms – exponential relaxation, \( \sin(\omega t) \), and \( \cos(\omega t) \) acoustic waves:

\[
D(t) = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + A_3 \sin(\omega t) + A_4 \cos(\omega t)
\]

(5.11)

where coefficients are:

\[
A_1 = \frac{Qk_1^2 (\beta_w C_{PL} k_2 - \beta_L C_{PW} k_1)}{C_{PL} C_{PW} (k_2 - k_1)(k_1^2 + \omega^2)}, \quad A_2 = \frac{Qk_1 k_2^2 (\beta_L C_{PW} - \beta_w C_{PL})}{C_{PL} C_{PW} (k_1 - k_2)(k_2^2 + \omega^2)},
\]

\[
A_3 = \frac{Qk_1 \beta_L C_{PW} \left( k_1^3 k_2^2 + k_1^2 k_2^3 + k_1^3 k_2^3 + k_1^3 w^2 + k_2^3 w^2 - \beta_w C_{PL} k_2 \left( 2k_1^2 k_2^2 + k_1^2 w^2 + k_2^2 w^2 \right) \right)}{C_{PL} C_{PW} (k_1 - k_2)(k_1^2 + \omega^2)(k_2^2 + \omega^2)w}, \text{ and}
\]

\[
A_4 = \frac{Qk_1 - \beta_w C_{PW} \left( 2k_1^2 k_2^2 + k_1^2 w^2 + k_2^2 w^2 \right) + \beta_w C_{PL} k_2 (k_1 + k_2)(k_1 k_2 + w^2)}{C_{PL} C_{PW} (k_1 - k_2)(k_1^2 + \omega^2)(k_2^2 + \omega^2)}
\]

The physical significance of the two oscillating terms becomes clear from the analysis of the thermal expansion process of this complex system. The thermal expansion coefficient of liposomes is larger than that of water, particularly at low temperatures when \( \beta_w \) approaches zero. Therefore expansion of the total volume, \( V_L + V_w \), is maximal when the heat energy is deposited in the liposome. The \( \sin(\omega t) \) term is related to the total volume expansion due to the liposome heating. The \( \cos(\omega t) \) term is related to the decrease
in the total volume due to heat transfer to the aqueous solvent. Comparison with the data shows that the sin($\omega t$) amplitude is greater, but the cos($\omega t$) contribution determines acoustic peak shift $t_c$ values.

Finally, $[D(t)]^2$ can be compared to the experimental data, $I_{\text{grating}}$ (see Eq. (5.1)):

$$I_{\text{grating}} \propto \left( \frac{\partial n}{\partial \rho} \right)^2 \frac{8^2(t)}{T} = \left( \frac{\partial n}{\partial \rho} \right)^2 \left[ D(t) \sin \left( \frac{2\pi x}{\Lambda} \right) \right]^2$$

(5.12)

The term due to temperature change ($\frac{\partial n}{\partial T} e^{dT}$ of Eq. (5.1)) is not included in Eq. (5.12) because, as we previously mentioned, it is much smaller. In general, the phases of the thermal and acoustic terms are different – thermal contributions appear following electronic and vibrational relaxation, while density changes evolve with acoustic wave propagation.\textsuperscript{247} Negligible transient grating signal amplitudes at $t \approx 20$ ps, $t \approx 1100$ ps and $t \approx 2200$ ps suggest that only density changes are important and therefore only the acoustic grating is considered.

**Modeling results.** Eqs. (5.11) and (5.12) were used to model porphyrin $2$/DMPC transient grating kinetics measured at different temperatures. While Eq. (5.11) is complex, there are only two adjustable parameters – rate constants $k_1$ and $k_2$. Other parameters used for fitting were obtained from the literature: $C_{PL} = 1600$ J M$^{-1}$K$^{-1}$,\textsuperscript{44} $C_{PW} = 75.3$ J M$^{-1}$K$^{-1}$,\textsuperscript{257} $\beta_L = 8.8 \times 10^{-4}$ K$^{-1}$.\textsuperscript{44} The water volume thermal expansion coefficient, $\beta_W$, was calculated as $\beta_W = V_{w}^{-1} (\partial V_{w}/\partial T)_P$, and the cubic approximation for the water volume temperature dependence was used: $V_w = V_{0W}(1-0.06427 \times 10^{-3} T + 8.5053 \times 10^{-6} T^2 - 6.7900 \times 10^{-8} T^3)$, where $T$ is temperature expressed in °C.\textsuperscript{267} Temperature dependence of the frequency $w$ was
obtained from the grating period data (Figure 29B). Linear approximation yielded \( w = 0.00267 + 5.6 \times 10^{-6} \tilde{T} \).

Figure 30 shows experimental and simulated porphyrin 2/DMPC liposome acoustic signals measured at 10°C (A) and 24°C (B). At 10°C, two simulated transient grating kinetics are shown. Simulated kinetics with \( k_1 = 1.0 \times 10^{12} \text{ s}^{-1} \) and \( k_2 = 3.33 \times 10^{10} \text{ s}^{-1} \) (———, \( t_{\text{max}} = 576 \text{ ps} \)) does not fit the data, while simulated kinetics with \( k_1 = 7.14 \times 10^{10} \text{ s}^{-1} \) and \( k_2 = 3.33 \times 10^{10} \text{ s}^{-1} \) (——, \( t_{\text{max}} = 546 \text{ ps} \)) is in agreement with the experimental results. As evident from the two simulations shown, slower energy transfer from porphyrin to the phospholipid bilayer results in an earlier \( t_{\text{max}} \) time of the acoustic wave – and therefore negative \( t_c \) values. As explained above, this effect is caused by the interference of the two acoustic waves created due to liposome and aqueous solution thermal expansion. As shown in Figure 30B, 24°C data can be simulated using the same porphyrin thermalization rate constant, \( k_1 = 7.14 \times 10^{10} \text{ s}^{-1} \), but the rate constant \( k_2 \) is somewhat larger, \( k_2 = 4.35 \times 10^{10} \text{ s}^{-1} \) (———, \( t_{\text{max}} = 545 \text{ ps} \)). The model is sensitive to the variations in the rate constant values. For example, simulation with \( k_1 = 7.14 \times 10^{10} \text{ s}^{-1} \) and \( k_2 = 3.33 \times 10^{10} \text{ s}^{-1} \) does not fit the data in Figure 30B (———, \( t_{\text{max}} = 477 \text{ ps} \)).
Figure 30: Porphyrin 2/DMPC liposome transient grating data (●) measured at 10°C (A) and at 24°C (B). Following rate constants were used in simulations according to Eqs. (5.11) and (5.12): A) $k_1 = 1.0 \times 10^{12} \text{ s}^{-1}$, $k_2 = 3.33 \times 10^{10} \text{ s}^{-1}$ (-----) and $k_1 = 7.14 \times 10^{10} \text{ s}^{-1}$, $k_2 = 3.33 \times 10^{10} \text{ s}^{-1}$ (-----); B) $k_1 = 7.14 \times 10^{10} \text{ s}^{-1}$, $k_2 = 3.33 \times 10^{10} \text{ s}^{-1}$ (-----) and $k_1 = 7.14 \times 10^{10} \text{ s}^{-1}$, $k_2 = 4.35 \times 10^{10} \text{ s}^{-1}$ (—).
Figure 31 summarizes temperature dependence of rate constants $k_1$ and $k_2$ determined from simulations. At higher temperatures ($\geq 10^\circ$C) $k_1 = 7.14 \times 10^{10}$ s$^{-1}$ ($k_1^{-1} = 14$ ps), while at 4$^\circ$C and 8$^\circ$C $k_1$ values are lower. Rate constant $k_1$ describes vibrational energy transfer from iron porphyrins to the phospholipid bilayer. Below 13$^\circ$C, DMPC bilayers are in the highly-ordered $L_{\beta'}$ phase. Different interaction between the porphyrin and the phospholipid alkyl chains might explain lower $k_1$ values in the $L_{\beta'}$ phase. Rate constant $k_1$ values are in good agreement with the heme thermalization rate constants determined using computer simulations,$^{268,269}$ transient absorption,$^{261,263,270}$ time-resolved Raman,$^{255,271}$ and transient grating$^{272}$ experiments. Similar electronic and vibrational relaxation rate constants were also reported for iron porphyrins in solution.$^{253-255}$

Rate constant $k_2$ describes vibrational energy transfer in phospholipid bilayers. In contrast, $k_2$ is temperature-dependent and ranges between $k_2^{-1} = 50$ ps (at 4$^\circ$C) to $k_2^{-1} = 18$ ps (at 35$^\circ$C). In the next section $k_2$ values are compared to the results obtained in other membrane studies. Determination of $k_2$ is significant because this information cannot be obtained from experiments that only study linear spectroscopic properties of membrane embedded molecular probes. Extension of transient grating studies to other phospholipid bilayer membranes, which differ in thickness or phospholipid interactions, could provide new information about the physical characteristics of biological membranes.
Figure 31: Temperature dependence of rate constants $k_1$ (■) and $k_2$ (○) for porphyrin 2/DMPC liposomes. Rate constants were determined by simulating transient grating data (Figure 28) with Eqs. (5.11) and (5.12).
Analysis of thermalization in membranes and comparison with other studies.

The DMPC bilayer thickness is 4.8 nm in the gel phase (L\(\beta\)\(^-\)) and 3.9 nm in the liquid (L\(\alpha\)) phase,\(^{44,228}\) therefore the distance through which excess energy is transferred is about 2 nm (assuming that porphyrins are localized in the aliphatic region of the bilayer as suggested by fluorescence studies). Two estimates can be made for the rates of vibrational energy transfer from the membrane aliphatic interior to the surrounding water. First, the thermal diffusion coefficient in proteins (such as the photosynthetic reaction center and myoglobin) is \(D_T = 7 - 14 \ \text{Å}^2/\text{ps}^{273,274}\). The time required for energy transfer across the distance \(L = 2\) nm in a system with such diffusion coefficient is \(t_D = L^2/(2 \ D_T) = 28-56\) ps. Second, the rate of vibrational energy transfer along the alkyl backbone has been directly examined; the time for vibrational energy transfer increased by about 0.4 ps per CH\(_2\) group.\(^{275}\) DMPC fatty acid residues have 13 CH\(_2\) groups, so estimated energy transfer time through such fatty acid residue is 5.2 ps. The first vibrational energy transfer rate estimate is in better agreement with experimentally measured \(k_2\) values. This suggests that energy transfer through the phospholipid bilayer predominantly occurs by thermal diffusion. In the liquid phase of the membrane (L\(\alpha\)), phospholipid fatty acid chains adopt various conformations. While disordered, they remain in van der Waals contact. Such conformational disorder may explain diffusion-like vibrational energy transfer in liquid-phase liposome membranes. At lower temperatures (L\(\beta\)\(^-\) phase), membranes have highly-ordered structure, but the membrane thickness increases by about 25% (in the P\(\beta\)\(^-\) phase phospholipids are also ordered, but the bilayers have rippled structure). Decrease in \(k_2\) values in the ordered bilayer phases could be the result of an increase in bilayer thickness.
While rate constant $k_2$ increases from $2.0 \times 10^{10}$ s$^{-1}$ to $5.6 \times 10^{10}$ s$^{-1}$ when the temperature changes from 4°C to 35°C, the change appears monotonous and no discontinuity is observed at the phase transition temperatures (13°C and 23°C for DMPC lipids). This is unexpected, since many liposome parameters change at this temperature. Different results were obtained from acoustic spectroscopy. In these experiments, phospholipid bilayer relaxation times were obtained from modeling ultrasonic excess attenuation spectra measured at frequencies up to 2 GHz. Close to the main phase transition ($P_\beta' \leftrightarrow L_\alpha$) temperature, ns and µs relaxation times become significantly longer. These relaxation times were assigned to isomerization of the fatty acid residues and density fluctuations in the bilayer. In our time-domain experiments, the acoustic period varied between 1080 – 1180 ps (Figure 29B), which in frequency domain correspond to 0.85 - 0.93 GHz – but by analyzing acoustic peak shifts, we examined much higher frequency dynamics. Higher frequency (picosecond) dynamics observed in our experiments could explain the difference from broadband acoustic spectroscopy results.

Other experiments examined ultrasonic velocity at a single acoustic frequency (at < 10 MHz). Such ultrasonic velocity had discontinuity close to the main phase transition temperature. Unlike these results, our investigations showed that the speed of sound in 0.85 - 0.93 GHz frequency domain, which corresponds to the acoustic period of 1080 – 1180 ps, has no discontinuity at the $P_\beta' \leftrightarrow L_\alpha$ phase transition temperature. Much higher frequency dynamics examined in transient grating experiments could explain this difference as well.
Phospholipid dynamics in membranes consists of a wide range of motions that span many different time scales – from $10^{-14}$ s for vibrations to hours for lipid exchanges between the inner and outer surface of the bilayer. Only vibrations, torsional oscillations, and trans-gauche isomerizations occur on the subnanosecond time scale. Other motions are slower. While bilayer properties are very different in $L_\alpha$, $P_\beta^\prime$, and $L_\beta^\prime$ phases, correlation times for vibrations, torsional oscillations, and isomerization do not differ significantly. The insensitivity of $k_2$ to the phase of the bilayers suggests that energy relaxation in the phospholipid bilayer membrane following excitation with short laser pulses happens through vibrational energy transfer, but phospholipid isomerization and other large scale motions are not excited.

5.7 Summary

We have examined ultrafast thermal processes in phospholipid bilayer liposomes with embedded porphyrin sensitizers. Use of transient grating nonlinear optical spectroscopy allowed us to study energy transfer from molecular sensitizers to phospholipid bilayers as well as energy transfer from phospholipid bilayers to the aqueous solvent. Modeling of the transient grating acoustic peak delay data was used to obtain rate constants that describe these processes. We find that porphyrin electronic and vibrational relaxation in phospholipid bilayers occurs with similar rate constants as in organic solvents, $k_1 = 3.7 \times 10^{10}$ s$^{-1}$. Energy thermalization in liposomes happens by thermal diffusion and vibrational energy transfer but without exciting other phospholipid dynamic
modes. Picosecond thermal energy transfer from phospholipid bilayers to water occurs with similar rates in gel and liquid phases of the bilayer.
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APPENDIX A

Determination of the Intrinsic Barrier for the Spiropyran Isomerization Reaction in Alcohol Solutions.

Merocyanine isomerization kinetics were measured in n-alcohol (C₄ – C₅) solutions with shear viscosity of $\eta = 3$ cP, 3.6 cP, and 5 cP. Isomerization rate constants in alcohol solutions are summarized in Figure 32. Although the alcohol viscosity varies from 3 cP to 5 cP, isomerization rate constants can be described using the same Arrhenius activation energy of $14.4\pm0.5$ kJ mol⁻¹. Therefore, in alcohol solutions SP isomerization dynamics does not depend on the molecular nature of the solvent. The intrinsic barrier for the isomerization reaction, $E_{iso} = 14.2\pm0.7$ kJ mol⁻¹, was estimated from $k_{iso}$ temperature dependence at 3 cP solvent viscosity. Very similar results are obtained from other data sets: $E_{iso} = 14.3\pm1.2$ kJ mol⁻¹ based on 3.6 cP data, $E_{iso} = 13.7\pm0.8$ kJ mol⁻¹ based on 5 cP data.
Figure 32: Isomerization rate constants in n-alcohol solutions (butanol – black symbol, hexanol – red symbols, heptanol – green symbols, and nonanol – blue symbols).

Temperature for the measurements was adjusted to obtain 3 cP (■), 3.6 cP (●), and 5 cP (▲) solvent viscosity. Fit to Arrhenius expression (activation energy of 14.4 kJ mol⁻¹) is shown as a solid line.
APPENDIX B

Merocyanine Absorption Spectra in Gel and Liquid Phases of the Bilayer

The merocyanine absorption spectra at different temperatures are shown in Figure 33. As merocyanines have large solvatochromic shifts (see Figure 21) and the polarity varies significantly in different regions of the bilayer, the results suggest that merocyanine localization in the bilayers is the same in $\beta'$ and $L_\alpha$ phases. Small shifts of the photochromic absorption band maximum, $\lambda_{\text{max}}$, are observed only for MC1 in a DPPC bilayers (where $\lambda_{\text{max}} = 502$ nm at low temperatures and $\lambda_{\text{max}} = 507$ nm at $> 40^\circ\text{C}$). This merocyanine is localized in the head group region of the bilayer (region 1, Scheme 8) and changes in temperature dramatically affect the penetration depth and density of water in this region.\textsuperscript{281}
Figure 33: Merocyanine absorption spectra in phospholipid bilayers corrected for scattering. A) MC1 in DPPC measured at 21°C (---), 28°C (---), 35°C (---), 42°C (---), and 53°C (---). B) MC1 in DMPC measured at 20°C (---), 25°C (---), 35°C (---), 46°C (---), and 50°C (---). C) MC2 in DPPC measured at 30°C (---), 35°C (---), 40°C (---), 49°C (---), and 59°C (---). D) MC3 in DPPC measured at 21°C (---), 27°C (---), 35°C (---), 41°C (---), and 50°C (---).
APPENDIX C

Linear Dichroism Experimental Details and Analysis

Absorption and fluorescence emission linear dichroism experiments were used to determine merocyanine orientation (the angle between the bilayer normal and the merocyanine transition dipole moment).\textsuperscript{213,214,216,282} Supported membranes for linear dichroism measurements were prepared on silica slides using a literature method.\textsuperscript{213,214,216,282} The hydrophobicity of the silica slides was enhanced by first rinsing with dilute HCl (0.1 M) followed by deionized water and toluene. The slides were left to dry overnight. Approximately 100 µL of a chloroform solution containing phospholipids (~5 mM) and spiropyran (~200 µM) was placed on a microscope slide. A gentle N\textsubscript{2} stream was used to remove excess solvent. Deionized water was sparingly added to the film to rehydrate the lipids resulting in greater membrane integrity. The excess water was allowed to evaporate. Such preparation yielded optically uniform films of ~1 cm\textsuperscript{2} area. Fluorescence emission spectra were measured with a Varian Eclipse fluorescence spectrophotometer and were not corrected.

The theoretical framework used to determine the orientation of molecules within oriented matrices has been developed by Castanho et. al.\textsuperscript{213} Briefly, assuming that the molecules are cylindrical with a long molecular axis oriented parallel to the electric dipole moment, than the only necessary orientational parameter is the angle between the
molecule's electric dipole moment and the system director vector, $\psi$. Since the system is at equilibrium, a distribution of orientations will be present according to a Boltzmann potential. Therefore, the orientational distribution function will be:

$$ f(\psi) \sin(\psi) $$

(A.1)

where $f(\psi)$ is a single molecule term and $\sin(\psi)$ accounts for the orientational distribution about $\psi$. The functional form of $f(\psi)$ is generally that of a Legendre polynomial:

$$ f(\psi) = \sum_{L \text{ even}} \frac{1}{2} (2L+1) \langle P_L \rangle P_L \cos(\psi) $$

(A.2)

which satisfies the condition that:

$$ \int \sin(\psi) f(\psi) d\psi = 1 $$

(A.3)

Experimentally, only $\langle P_2 \rangle$ and $\langle P_4 \rangle$ can be determined. Therefore, once the $\langle P_2 \rangle$ and $\langle P_4 \rangle$ parameters are known, only the functional form of $f(\psi)\sin(\psi)$ needs to be determined. Generally, truncation of the $f(\psi)$ function at $\langle P_4 \rangle$ results in negative amplitude probabilities. To correct for this problem, Castanho recasts the formula accounting for the maximal possibility of distributions for the given $\langle P_2 \rangle$ and $\langle P_4 \rangle$ parameters. The resulting equation is:

$$ f(\psi) = A \exp \left( \sum_L \lambda_L P_L \cos(\psi) \right) $$

(A.4)

Experimental determination of $\langle P_2 \rangle$ and $\langle P_4 \rangle$ requires UV-Vis and fluorescence experiments. By measuring the change in absorption of the aligned multi-bilayer sample after changing the angle of incidence, the following relation can be constructed relating the
integrated absorbance band at a particular angle to the absorbance at a perpendicular angle of incidence:

$$\frac{\sin(\omega)A_{\omega}}{A_{\omega=\pi/2}} = 1 + \frac{3\langle P_2 \rangle}{(1-\langle P_2 \rangle)n^2} \cos^2(\omega)$$  \hspace{1cm} (A.5)$$

where $A_{\omega}$ is the absorbance at the angle $\omega$, $A_{\omega=\pi/2}$ is the absorbance at perpendicular incidence, $n$ is the refractive index, and the $\sin(\omega)$ term accounts for the increased illumination volume as the angle is increased. $\langle P_2 \rangle$ can be determined by simple linear regression. For fluorescence measurements the experimental data is collected by measuring the fluorescence emission intensity without polarization and in the four possible permutations of vertical (v) and horizontal (h) polarization settings on the entrance and exit slits of the instrument (hh, hv, vh, vv). The data is fit to the following equations:

$$\frac{G I_{sh}}{f'(\alpha) I_{rv}} = m \sin^2(\alpha) + b$$  \hspace{1cm} (A.6)$$

$$m = \frac{a}{n^2}$$  \hspace{1cm} (A.7)$$

$$a = \frac{\left[ \left( \frac{3}{7} \langle P_4 \rangle + \frac{4}{7} \langle P_2 \rangle - \langle P_2 \rangle^2 \right) w + \langle P_2 \rangle^2 \right]}{c}$$  \hspace{1cm} (A.8)$$

$$b = \frac{1}{3} \left[ 1 - \left( \frac{2}{5} + \frac{36}{35} \langle P_4 \rangle + \frac{4}{7} \langle P_2 \rangle - 2 \langle P_2 \rangle^2 \right) \omega + 2 \langle P_2 \rangle^2 + \langle P_2 \rangle \right]$$  \hspace{1cm} (A.9)$$

$$c = \frac{1}{3} \left[ 1 + \left( \frac{4}{5} - \frac{4}{7} \langle P_2 \rangle + \frac{22}{35} \langle P_4 \rangle - \langle P_2 \rangle^2 \right) \omega + \langle P_2 \rangle^2 - 2 \langle P_2 \rangle \right]$$  \hspace{1cm} (A.10)$$
\[ \omega = \frac{\tau_o}{\tau_o + \tau} \tag{A.11} \]

where \( n \) is the refractive index, \( \tau_o \) is the rotational relaxation time, \( G \) is an instrument correction function according to:

\[ G = \frac{I_{hv}}{I_{hh}} \tag{A.12} \]

and \( f'(\alpha) \) is a angle-dependant (\( \alpha \) in degrees) correction factor for reflection according to:

\[ f'(\alpha) = 1 + \left(1.14 \times 10^{-2} \alpha \right)^{4144} \tag{A.13} \]

We found that rotation of the supported merocyanine/phosphatidylcholine films led to changes in the absorption and fluorescence emission signals. Such characteristics show that merocyanines are not randomly distributed in the bilayers, but have a preferential orientation.

The MC transition dipole moment orientational distribution functions are shown in Figure 34. The maxima of the distribution functions are at 16-45°. Phosphatidylcholine fatty acid chain tilt in the bilayer gel phase is about 20°,\textsuperscript{218} this suggests that the phospholipid fatty acid chains may induce merocyanine orientation in bilayers. However, there are significant differences between DMPC and DPPC films. For DPPC, the maxima are at 16-25°, while for DMPC the angles are larger, 25-45°. This result could be attributed to some difference in the bilayer thickness (the distance between the phosphate headgroups of the two lipid leaflets about 4.7 nm for DPPC\textsuperscript{283} and about 4.4 nm for DMPC\textsuperscript{284}), or to the variation in the \( P_{b'} \leftrightarrow L_\alpha \) phase transition temperatures. In particular, the greatest
difference in the width of the distribution function is observed for MC1, which is localized at the bilayer/aqueous interface. During the $P_{β′} \leftrightarrow L_α$ phase transition this region undergoes the largest structural rearrangements.
Figure 34: The orientational distribution function, $\varphi$(angle), for MC1 (---), MC2 (--), and MC3 (-----) in DPPC (A) and DMPC (B) multibilayers determined from absorbance and fluorescence emission linear dichroism. In DPPC (DMPC), the maxima are at $16^\circ$ ($25^\circ$), $24^\circ$ ($37^\circ$), and $25^\circ$ ($45^\circ$) for MC1, MC2, and MC3 respectively. The inset in (A) depicts the approximate tilt angle ($20^\circ$) of the phospholipid acyl chains in a gel phase phospholipid bilayer.
APPENDIX D

Liposome Preparation and other Experimental Details

**Materials and methods.** 1',3'-dihydro-1',3'-3'-trimethyl-6-nitrospiro[2H-1-benzopyran-2',2'-(2H)-indole] (SP1) was purchased from Aldrich and used as received. Spiropyran molecular sensitizers [(R/S)-2-(3',3'-dimethyl-6-nitro-3'H-spiro[chromene-2,2'-indol]-1'-yl)ethanol, SP2] were synthesized using a literature method\(^{176}\) and characterized by NMR, EA, and UV-vis spectroscopy. 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) lipids were obtained from Avanti Polar Lipids. Absorption spectra were measured with a HP8452A diode array spectrometer and corrected for light scattering from the liposomes. All other reagents and solvents were obtained from Aldrich. Deionized distilled water was used to prepare the phosphate buffer solution.

**Liposome preparation.** To prepare liposomes, sensitizer and phospholipid solutions were mixed in a round-bottom flask (the phospholipid/spiropyran molar ratio was 50:1 for the ultrafast studies in Chapter 3 and 100:1 for the thermal ring-closing studies in Chapter 4 and the phospholipid/porphyrin molar ratio was varied from 100:1 to 1000:1 for fluorescence experiments; for transient grating experiments lipid to porphyrin ratio was 100:1) and the organic solvent was removed under an N\(_2\) stream. The flask was placed in a warm-water bath (with the temperature above the phospholipid main phase transition
temperature) and the phospholipids were hydrated with a phosphate buffer (50 mM, pH 7) to a final phospholipid concentration of 7.4 mM. This aqueous solution was then agitated with a low power (10 W) ultrasonic bath until homogeneous in appearance and extruded 11 times through a polycarbonate filter (50 nm pore diameter). This preparation yielded single lamellar liposomes with a narrow size distribution and an average liposome diameter of 75±2 nm. Dynamic light scattering with ZetaSizer from Malvern Instruments was used to determine liposome size.
VITA

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Publications:

Wohl, C.; Kuciauskas, D. “Phospholipid Self-Segregation Results in Greater Environmental Heterogeneity near the Membrane Surface” in preparation


Presentations:

Phospholipid Bilayers as Biological Membrane Models, NASA Langley Research Center, Hampton, VA, June 29, 2006.

Discerning Phospholipid Membrane Physical Properties via Merocyanine Photochemistry. Graduate School Graduate Student Research Symposium, Virginia Commonwealth University, Richmond, VA, April 12, 2006
