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PHOTOINDUCED FUSION OF LIPOSOMES

by

Doyle Edward Bennett

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF CHEMISTRY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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DEDICATION

To my mother, Jacqueline Kay Bennett, to my late father, John E. "Jack" Bennett, and to my two brothers, John M. Bennett and Daniel J. Bennett.

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ABBREVIATIONS

AIBN	azobis-(isobutyronitrile)
ANTS	1-amino-3,6,8-naphthalenetrisulfonic acid disodium salt
bis-SorbPC	1,2-bis-[10-(2',4'-hexadienoyloxy)decanoyl]-sn-glycero-3-phosphatidylcholine
CMC	critical micellar concentration
d	doublet (NMR)
DAG	diacylglycerol
DCC	dicyclohexylcarbodiimide
DMAP	4-(N,N-dimethylamino)pyridine
DOPC	dioleoylphosphatidylcholine
DOPE	dioleoylphosphatidylethanolamine
DOPE-Me	N-methyl-dioleoylphosphatidylethanolamine
DOPE-Me ₂	N,N-dimethyl-dioleoylphosphatidylethanolamine
DPX	N,N'-p-xylylene-bis(pyridinium bromide)
DSC	differential scanning calorimetry
EDTA	ethylenediaminetetraacetic acid tetrasodium salt
H _{II}	inverted hexagonal phase
ILA	interlamellar attachment
IMI	inverted micellar intermediate
L _α	liquid-crystalline lamellar phase
L _β	gel lamellar phase
LUV	large unilamellar vesicles
m	multiplet (NMR)
MLV	large multilamellar vesicles
mono-SorbPC	1-palmitoyl-2-[10-(2',4'-hexadienoyloxy)decanoyl]-sn-glycero-3-phosphatidylcholine
NBD-PE	N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine
NMR	nuclear magnetic resonance
OD	optical density
PA	sn-glycero-3-phosphatidic acid
P _β	ripple lamellar phase
PC	sn-glycero-3-phosphatidylcholine
PDC	pyridinium dichromate
PE	sn-glycero-3-phosphatidylethanolamine
PEG	poly(ethylene glycol)
PG	phosphatidylglycerol
PS	sn-glycero-3-phosphatidylserine
Q _{II}	inverted cubic phase
QELS	quasi-elastic light scattering
RES	reticuloendothelial system
RET	resonance energy transfer
Rh-PE	N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine
rt	room temperature

ABBREVIATIONS - *continued*

s	singlet (NMR)
SUV	small unilamellar vesicles
t	triplet (NMR)
T _c	critical fusion temperature
T _H	lamellar liquid-crystalline (L _α) to inverted hexagonal (H _{II}) phase transition temperature
T _I	lamellar liquid-crystalline (L _α) to inverted cubic (Q _{II}) phase transition temperature
TLC	thin-layer chromatography
T _m	gel to liquid-crystalline phase transition temperature
Tris	tris-(hydroxymethyl)amino methane
TX-100	triton X-100 detergent
X _n	degree of polymerization

ABSTRACT

The photopolymerization of two-component large unilamellar liposomes (LUV) composed of 3/1 dioleoylphosphatidylethanolamine (DOPE) and either 1,2-bis-[10-(2',4'-hexadienoyloxy)decanoyl]-sn-glycero-3-phosphatidylcholine (bis-SorbPC) or 1-palmitoyl-2-[10-(2',4'-hexadienoyloxy)decanoyl]-sn-glycero-3-phosphatidylcholine (mono-SorbPC) facilitated liposome fusion. Fusion was characterized by fluorescent assays for lipid mixing, aqueous contents mixing, and aqueous contents leakage. The rate and extent of the photoinduced fusion was dependent on the extent of polymerization, temperature, and the fusion initiation conditions, including the pH and the presence of Mg^{2+} ions. Examination of the temperature dependence of fusion for unpolymerized and polymerized liposomes showed that an enhancement of the rate of fusion occurred in the temperature range ΔT_I , which previous NMR studies have identified as the initial appearance of precursors to the formation of the inverted cubic phase. The phase behavior and fusion characteristics of the DOPE/bis-SorbPC (3:1) membranes provide unequivocal evidence that liposome fusion is mediated via intermediates associated with the lamellar to Q_{II} phase transition rather than the H_{II} phase. Photopolymerization of SorbPC-containing liposomes forms poly-SorbPC which enhances the lateral separation of the liposome components. The formation of enriched domains of polymorphic lipids, e.g. DOPE, causes isothermal induction of fusion by lowering the critical fusion temperature of the membranes.

CHAPTER I

INTRODUCTION

I.A. Supramolecular Lipid Assemblies

The development of biological membranes was critical to the origin of life (Sackmann, 1990). The most fundamental role of a biological membrane is maintaining a barrier between the living system and outer non-living environment. In higher organisms, this function is served by the plasma, or cytoplasmic, membrane. Cells of higher organisms also contain intracellular membranes that define such compartments as the nucleus and the mitochondria. Bacteria contain no intracellular membrane bounded compartments. In addition to defining compartments, membranes control communication between compartments by regulating the selective exchange of ions and molecules, and transmitting information through the conformational changes of membrane proteins. Membranes facilitate reactions that occur in two dimensions between enzymes anchored to the surface or the interior of the membrane. Cellular recognition also takes place via components on the membrane surface, including proteins and carbohydrates.

That membranes are composed of a thin layer of lipids and proteins has long been understood. But the basis of the presently accepted membrane structure was not articulated until 1972 by Singer and Nicolson in their well known fluid mosaic model (Singer & Nicolson, 1972; Singer, 1974). The authors' proposals were largely based on freeze-fracture electron micrographs that indicated the presence of globular proteins embedded in the

membrane. The model describes the membrane as a fluid-like phospholipid bilayer having proteins embedded and associated with the surface. The model allows for free lateral diffusion of both lipids and proteins in the membrane. Refinements of membrane structure have demonstrated that laterally differentiated domains and constraints on protein diffusion exist, and that boundary lipids surrounding proteins are important for their normal function (Jain, 1983; Jacobson, 1987).

Membranes are composed primarily of lipids and proteins, but also contain an appreciable amount of carbohydrates. The carbohydrate content, which may be as much as 10%, is typically a mixture of glycolipids and glycoproteins.

I.A.1. Lipid Self-Assembly

Membrane lipids are extremely diverse, but are structurally similar in their amphiphilic nature. Amphiphiles are dualistic in that they contain both polar (hydrophilic) and non-polar (hydrophobic) components. The polar or ionic group is usually called the head, and the hydrophobic portion, which is often composed of one or more aliphatic hydrocarbon chains, is usually called the tail. Figure I-1 shows the molecular structure of a phosphatidylcholine (PC), dipalmitoylphosphatidylcholine (DPPC), which is one of a class of lipids that compose a large part of biological membranes. The term amphiphile is applied to both biological and synthetic compounds.

As the concentration of a monomeric amphiphilic solution is increased above the critical micelle concentration (CMC), the amphiphiles spontaneously aggregate into micelles (Figure I-2). The driving force for the aggregation is the hydrophobic effect (Tanford, 1980).

Figure I-1: Molecular structure and schematic representation of the synthetic phosphatidylcholine (PC) dipalmitoyl-phosphatidylcholine (DPPC). The hydrophilic headgroup is represented by an oval, and the hydrophobic hydrocarbon tails are represented by two wavy lines. The dashed line indicates the water/hydrocarbon interface separating the hydrated headgroup region and the dehydrated tail region.

Figure I-2: Aggregation of single-chain amphiphiles (e.g. detergents) and double-chain amphiphiles (e.g. phospholipids) at concentrations exceeding their respective critical micellar concentrations (CMCs) to form supramolecular assemblies.

The hydrophobic effect arises not due to the attraction of the hydrophobic acyl-chains for one another, but due to the attractive forces between water molecules. The entropy of the water molecules is increased whenever any solute is introduced. Whereas polar or ionic compounds can form strong bonds with water molecules, which offsets the increase in entropy, hydrophobic molecules cannot provide such bond energy, and the

overall change in free energy for solvation is unfavorable. The aggregation of amphiphiles is associated with a decrease in entropy, but this is offset by the larger entropic gain of water molecules freed from solvating the tails.

Without any other constraints, the hydrophobic effect would favor complete separation of amphiphilic molecules from the water phase. For example, long chain hydrocarbons such as dodecane separate into different phases when they are mixed with water. But geometrical packing constraints prevent this. The requirement for the head group of the amphiphile to be in contact with water at the interface, and the condition that all parts of the amphiphile be within the extended length of the molecule from the interface, force the system to form aggregates (Israelachvili et al., 1980). Micelles are formed from amphiphiles having hydrocarbon chains which consist of a hydrophobic core, and a surface defined by the polar heads in contact with water. The hydrocarbon of the core is invariably a disordered fluid. Micelles can be spherical, ellipsoidal, cylindrical, or disk shaped.

The CMC, which was originally defined as the limiting concentration for monomeric single tail amphiphiles (surfactants), is also applied to double-chain amphiphiles that aggregate to form structures other than micelles, including bilayer vesicles (liposomes). Formation of closed aggregates minimizes the extent of chain-water contact and reduces the chemical potential of the chains, which is directly proportional to the area of the hydrocarbon-water contact. The solubility of amphiphiles depends on contributions from both the head and the tail(s). As a single tail increases in length, or when a second tail is attached, its contribution dominates the solvent interaction, and the amphiphile becomes progressively less soluble in aqueous solutions. For single-chain surfactants the CMC = 10^{-4} - 10^{-2} M, for

single-chain lysophospholipids $\text{CMC} = 10^{-7} - 10^{-6} \text{ M}$, and for double-chain zwitterionic phospholipids $\text{CMC} = 10^{-10} \text{ M}$.

Micelles are typically small, having aggregation numbers of 100 or less. A minimum size is required by the hydrophobic effect since a closed structure must be achieved in which no hydrocarbon/water interface exists. A maximum size is imposed by the repulsive force between the head groups and the geometrical requirement that the head groups approach closer to each other as the aggregation number increases. For ionic amphiphiles, the repulsion force is electrostatic; for uncharged amphiphiles, the force arises from the preference of the head groups for hydration over self-association. The head group surface area of single- and double-chain amphiphiles is similar, $60 - 70 \text{ \AA}^2$. But in the double-chain case, the area of the tail is twice that of the single-chain case. Since spherical micelles cannot accommodate the large hydrophobic volumes of such amphiphile, bilayer sheets or closed vesicles composed of bilayer vesicles must be formed. The aggregation numbers in these structures are in the tens of thousands. Hence, vesicles are much larger structures than micelles.

I.A.2. Biological Membrane Lipids

Amphiphiles can be divided into two categories: water-soluble, micelle forming amphiphiles, and water-insoluble, swelling amphiphiles (Lindblom & Rilfors, 1989). The double-chain phospholipids, 1,2-diacylphosphoglycerides, are the predominant lipids in most biological membranes. Figure I-3 illustrates the structures of some commonly found biological lipids.

Figure I-3: Common biological lipids: phosphoglyceride (phospholipid), plasmalogen, lysophosphoglyceride (lysophospholipid), phosphosphingolipid, glyceroglycolipid, and sterol.

Figure I-4: Biological Glycerophospholipids.

R = Alkyl chain

Phospholipid

Phosphatidic Acid (PA)

Phosphatidylcholine (PC)

Phosphatidylethanolamine (PE)

Phosphatidylserine (PS)

Phosphatidylglycerol (PG)

Phosphatidylinositol (PI)

Alcohol (-X)

—OH

—O—CH₂—CH₂—N⁺Me₃—O—CH₂—CH₂—N⁺H₃

$$\begin{array}{c} \text{—O—CH}_2\text{—CH—N}^+\text{H}_3 \\ | \\ \text{CO}_2^- \end{array}$$

$$\begin{array}{c} \text{—O—CH}_2\text{—CH—CH}_2\text{—OH} \\ | \\ \text{OH} \end{array}$$

The phosphoglycerides contain two aliphatic hydrocarbon chains (designated by R₁ and R₂ in Figure I-4) attached to a glycerol backbone by either ester or ether linkages. The head group contains phosphate and may also contain a variety of phosphoester linked alcohols. The configuration of the glyceride backbone is usually described by the stereospecific numbering (sn) system (Hauser et al., 1981). In this system, natural phosphoglycerides usually have the phosphate at the sn-3 position of the glycerol. The hydrocarbon tail at the sn-1 position is referred to as the α chain, and that at the sn-2 position as the β chain. Biologically derived phospholipids normally have the R (or D) configuration at the sn-2 glycerol carbon.

Since the phosphate is negatively charged, the head group may be either negative or zwitterionic, depending on the charge of the X group. No cationic lipids are found in nature.

The acyl-chains of biological lipids are invariably long and nearly always have an even number of carbons. Very few lipids contain tails of less than 16 carbons, and chain lengths of up to 24 carbons are common. Table I-1 lists a number of common fatty acids found in membrane lipids.

The two tails of glycerophospholipids are most often different in length and unsaturation. In animal cells, the sn-1 chain is usually saturated and the sn-2 chain is unsaturated, although bis-saturated and bis-unsaturated species also exist (Deemen, 1971). Unsaturated fatty acids can contain up to six double bonds. The double bonds are not usually conjugated and they are normally in the cis-configuration. The cis-double bonds create kinks in the hydrophobic tails that tend to disrupt the ordered packing of the tails by diminishing the van der Waals interactions between adjacent tails. Bacterial membranes have

been found to contain branched chains, cyclopropane-containing chains, and β -hydroxy substituted chains (Russell, 1984).

A biological lipid frequently used for model membrane studies is phosphatidylcholine extracted from egg yolk. EggPC is a mixture of lipids whose acyl-chains are about 50% saturated and 50% unsaturated (Huang et al., 1964). The fatty acyl chains have been identified to include palmitic, stearic, oleic, and linoleic acids. The physical properties of eggPC resemble those of the synthetic phospholipid, dioleoylphosphatidylcholine (DOPC), in several regards (DeGier et al., 1968).

Plasmalogens are phosphoglyceride variants in which the sn-1 of the ester linkages is replaced by an ether linkage. Cardiolipin, which is a phosphoglyceride dimer, is a predominant component of the mitochondrial inner membrane, the chloroplast membrane, and some bacterial membranes. It contains four acyl-chains attached to a single head group which contains two phosphates, linked by glycerol, and carries a double negative charge.

Phosphosphingolipids are another class of phospholipids whose hydrophobic groups contain ceramide rather than acyl-tails connected to a glycerol backbone. Head group structures can be the same as some glycerophospholipids, including choline, ethanolamine, inositol, and glycerol. These lipids are common in animal cells but not in plants or bacteria. A phosphosphingolipid commonly found in animal cell plasma membranes is sphingomyelin (ceramide 1-phosphorylcholine).

Glycoglycerolipids are similar to glycerophospholipids; the sn-3 position on glycerol is glycosidically linked to a carbohydrate rather than to a phosphate. These lipids are common in plants and bacteria but not in animal cells. The chloroplast thylakoid membrane is composed predominantly of one

Table I-1: Fatty acids commonly found in nature.

<u>Trivial Name</u>	<u>Chain length: Unsaturation^a</u>
Lauric	12:0
Myristic	14:0
Palmitic	16:0
Palmitoleic	16:1 cis Δ^9
Stearic	18:0
Oleic	18:1 cis Δ^9
Vaccenic	18:1 cis Δ^{11}
Linoleic	18:2 cis $\Delta^{9,12}$
γ -Linolenic	18:3 cis $\Delta^{6,9,12}$
α -Linolenic	18:3 cis $\Delta^{9,12,15}$
Arachidic	20:0
Behenic	22:0
Arachidonic	20:4 cis $\Delta^{5,8,11,14}$

^a Δ gives the position of the double bond(s).

type of this class of lipids, monogalactosyldiacylglycerol (Figure I-3) (Grounaris & Barber, 1983).

Glycosphingolipids are composed of carbohydrate headgroups glycosidically linked to the terminal hydroxyl of ceramide. The carbohydrate head group may be a single sugar or a complex oligosaccharide. Cerebrosides are monoglycosyl ceramides and have single neutral hexose sugars as head groups. Gangliosides are an important class of anionic glycolipids that have oligosaccharide head groups that contain one or more attached sialic acid (N-

acetylneuraminic acid) groups. Gangliosides function in the regulation of cell growth, and in cellular recognition by binding to specific receptors on other plasma membranes (Spiegel & Fishman, 1985). Globosides are neutral glycosphingolipids whose oligosaccharide head groups do not contain sialic acid. Glycosphingolipids are present as minor components in the outer surface of animal cell plasma membranes.

Single-chain lipids are present only in small quantities in biomembranes. Included in this category are lysophospholipids, free fatty acids, and various sterols, including cholesterol and its ester derivatives. Cholesterol is the predominant sterol in animal cells and can constitute up to 30% of the membrane lipids of some plasma membranes.

The reason for the vast diversity of membrane lipids is yet to be completely understood. Many lipids have been recognized as regulatory agents, such as gangliosides and phosphatidylinositol in animal cell plasma membranes. Other specific lipids are thought to be important for optimal enzyme activity. And certain lipids actively participate in membrane processes by stabilizing regions of high curvature, stabilizing junctions between membranes, and promoting membrane fusion (Gennis, 1989).

Since studying the whole lipid extract of cells is complicated, most studies of lipid systems are done with synthetic lipids, such as DOPC. The lipids can be easily prepared in high purity, and the acyl-chain and head group structures can be systematically varied.

The lipids extracted from biological membranes will spontaneously assemble to form lamellar liquid crystalline phases (bilayers) when mixed with water. And when the different types of biological lipids are isolated, many will individually form bilayers when hydrated. But a large fraction of

the isolated lipids (10 - 50%) will instead form nonlamellar (non-bilayer) phases (Cullis et al., 1985). The most common of these nonlamellar phases is the inverted hexagonal (H_{II}) phase, but various inverted cubic (Q_{II}) phases can also be formed. The presence of nonlamellar lipids has been postulated to be important both for transient formation of non-bilayer structures during membrane fusion (Siegel, 1986b; Ellens et al., 1989), and to endow the membranes with other physical properties crucial to the survival of the cell (Cullis et al., 1985; Gruner, 1985; Gruner, 1989). Nonlamellar lipid phases have been reviewed by a number of authors (Luzzati, 1968; Cullis et al., 1985; Gruner et al., 1985; Mariani et al., 1988; Larsson, 1989; Lindblom & Rilfors, 1989; Seddon, 1990; Tate et al., 1991).

I.A.3. Nomenclature of Lipid Phases

Mesophases are states of matter intermediate between crystalline and liquid phases. Such phases exhibit properties characteristic of both solids and liquids. Phospholipids exhibit both thermotropic and lyotropic mesomorphism. The stability of a particular lipid phase depends upon both the temperature and the lipid concentration. In binary systems, transitions between phases can only be induced by changes in temperature and lipid concentration (water content). But other isothermal transitions can be induced, depending on the specific lipid composition of the system, by introducing additional components, such as specific ions and peptides, or by changing such general environmental conditions as pH and ionic strength.

The various phases are usually designated by the nomenclature of Luzzati (Luzzati, 1968). Phases are classified according to three characteristics: 1.) long-range organization (i.e. lattice type), 2.) short range order of the

hydrocarbon chains (i.e. fluid or ordered chains), and 3.) the geometrical orientation, if the phase is nonlamellar (i.e. normal or inverted). The type of long range order is designated by an upper-case letter: L, one-dimensional lamellar; H, two-dimensional hexagonal; P, two-dimensional oblique; Q, three-dimensional cubic; and M, micellar. The conformational order of the chains is designated by a lower-case, Greek subscript: α , disordered (fluid); β , ordered, untilted (gel); β' , ordered, tilted (gel). The topological curvature of nonlamellar phases is designated by subscripted Roman numerals: type I, normal; type II, inverted. By convention, phases are designated normal if the head group surface curves toward the hydrocarbon, and inverted if the head group surface curves away from the hydrocarbon.

I.A.4. Lyotropic Phases

Lyotropic transitions can occur in systems that are below limiting hydration (excess water). Nonlamellar lyotropic mesophases exist as quasi-symmetric pairs. Normal (type I) oil-in-water phases consist of lipid aggregates in a matrix of excess water. Inverted (type II) water-in-oil phases consist of isolated water regions (e.g. cylinders or spheres) surrounded by a matrix of fluid hydrocarbon. Type I and II phases are not correctly envisioned as having the same topology with only the lipid and water regions reversed. The lipid region is of fixed and limited thickness, whereas the water region is not. This fundamental difference underlies the limited hydrations observed for inverted phases (Luzzati, 1968).

The sequence of lyotropic transitions at high temperature can be generalized for each of two lipid types, single-chain, and double-chain phospholipids. For single-chain ionic amphiphiles, the water concentration

is, in general, the more important variable for inducing phase transitions. For double-chain lipids, conversely, temperature is usually the more important variable. In the single chain case, the sequence of phases with decreasing hydration is the following: M_I , Q_I' , H_I , Q_I , L_α . At low lipid concentration, micelles exist in excess water. With increasing lipid concentration, a normal cubic phase, then a normal hexagonal phase, another normal cubic phase, and finally a lamellar phase are formed. In the double-chain case, the sequence with decreasing hydration is this : L_α , Q_{II} , H_{II} . The first phase in the sequence is the disordered lamellar phase, followed by an inverted cubic phase, and finally an inverted hexagonal phase. The nonlamellar phases are inverted, and with decreasing hydration the Q_{II} phase is formed first, followed by the H_{II} phase.

I.A.5. Thermotropic Phases

Thermotropic transitions are typically measured in excess water. Under such conditions, they are sharp and occur at characteristic temperatures. Pure lipid systems exhibit especially sharp, discontinuous (first order) transitions.

For double-chain phospholipids, the normal sequence of thermotropic phase transitions with increasing temperature is the following: L_β , P_β' , L_α , Q_{II} , H_{II} . Thermotropic transitions can occur between different lamellar phases and between the L_α phase and nonlamellar phases. The lamellar transition from the gel phase (L_β) to the rippled gel phase (P_β') is termed the pretransition (Figure I-5). This transition is not always observed since the rippled gel phase is not exhibited by all phospholipids. The main, or chain-

melting transition occurs from a gel phase to the fluid, or disordered lamellar phase (L_α) at the characteristic chain-melting transition temperature, T_m .

At still higher temperatures, transitions can occur from the L_α phase to inverted, nonlamellar phases. Just as lyotropic transitions for double chain amphiphiles occur from the L_α , to the Q_{II} , and then to the H_{II} phases with decreasing hydration, thermotropic transitions from the L_α to the Q_{II} and H_{II} phases result in successive dehydration of the lipids. Thermotropically formed H_{II} phases are less well hydrated and as a consequence, less dense, than thermotropically formed Q_{II} phases.

Pure lipids that can undergo these transitions are termed polymorphic. Unsaturated PEs are examples of polymorphic lipids, and their phase properties have been intensively studied. For many polymorphic lipids, including unsaturated PEs, transitions from the L_α phase to the H_{II} phases occur without intermediate formation of the Q_{II} phase. In some such systems thermodynamically stable Q_{II} phases have been formed through protracted manipulations of the samples (Gruner et al., 1988; Shyamsunder et al., 1988). When both inverted phases are observed, the sequence of thermotropic transitions of double-chain lipids is the same as for lyotropic transitions: the Q_{II} phase forms first at T_I , then the H_{II} phase forms at T_H .

I.A.6. Lamellar Phase

The lamellar phase consists of bilayer sheets of lipid stacked in a two-dimensional lattice separated by layers of water. This is the phase adopted by biological membranes during the overwhelmingly predominant period of their lives. Model biological membranes consisting only of lipids can be readily formed by dispersing the lipids in aqueous suspensions.

Biomembranes are modeled with a number of different systems, including monolayers, planar bilayers, and liposomes or vesicles. Liposomes are particularly suitable as model biological membranes since they are closed spherical assemblies consisting of an aqueous core and a bilayer membrane separating the internal and external aqueous milieu. Many phospholipids will spontaneously assemble into a heterogeneous mixture of liposomes when they are dispersed in water. Liposomes may either be composed of multiple bilayers arranged as concentric shells (multilamellar), or may be single-walled (unilamellar). A number of techniques have been developed that allow for the preparation of liposomes in specific ranges of size and lamellarity. Liposomes are loosely classified according to size; small unilamellar vesicles (SUV) are those with diameters of 200 to 500 Å, and large unilamellar vesicles (LUV) those with diameters of 500 to 5000 Å. Liposomes having even larger diameters are termed giant liposomes.

SUVs are typically unstable due to the high curvature (small radius of curvature) of their bilayers. When PC SUVs are incubated at their T_m , they will slowly grow in size over days or weeks until they reach diameters of about 100 nm (Lentz et al., 1987).

I.A.7. Inverted Hexagonal (H_{II}) Phase

The inverted hexagonal phase consists of cylindrical water cores arranged in a hexagonal lattice each of which is coated with a layer of lipids. The lipid head groups are positioned at the water surface and the acyl-chains fill the interstitial spaces between the water cylinders. Unlike the lamellar phase, the inverted phases exhibit highly curved water/hydrocarbon interfaces. The H_{II} phase for phospholipids was first observed in the X-ray

diffraction studies of Luzzati and coworkers (Luzzati & Reiss-Hudson, 1962; Luzzati & Reiss-Hudson, 1966; Luzzati et al., 1968).

I.A.8. Inverted Cubic (Q_{II}) Phases

Three inverted cubic phases are known to be formed by diacyl phospholipids: the primitive type, or "plumber's nightmare" (P or Q_{II}^{229}) (space group $Im3m$), the "double diamond" type (D or Q_{II}^{224}) (space group $Pn3m$); and the "gyroid" type (G or Q_{II}^{230}) (space group $Ia3d$) (Longley & McIntosh, 1983; Hyde et al., 1984; Mariani et al., 1988). The water content of these phases decreases in the order P, D, G. The G type phase is the most commonly observed of these phases for lipid systems.

The surfaces describing the middle of the lipid bilayers of these phases are infinite periodic minimal surfaces (IPMS) or Schwartz minimal surfaces. At all points on such surfaces, the mean curvature is zero. But at the polar head group surface the mean curvature is negative, as is the convention for inverted phases. In each of these phases, the minimal surfaces divide the water into two unconnected, interpenetrating cylinders. Although the phases are widely termed "bicontinuous", they are in fact quadricontinuous in that the two lipid monolayers making up the two leaflets of the minimal surface, and the two water phases are distinct (Gruner, 1989).

In phase diagrams, they can appear either in restricted regions (limiting hydration) or in the presence of excess water. In excess water, they are observed between the lamellar and inverted hexagonal phases. This position in the phase diagram is consistent with the curvature sequence of the phases: curvature of the inverted cubic phases is intermediate between the zero mean

curvature of lamellar phases and the large negative mean curvature of the inverted hexagonal phases.

Lysophosphatidylcholines (lysoPCs) form normal cubic phases. For example, palmitoyl lysophosphatidylcholine forms a cubic phase with space group $Pn3m$ (Q_I^{223}) which is thought to be composed of normal micelles (Arvidson et al., 1985).

I.B. Transitions between Lipid Phases

In general, biological membranes do not exhibit transitions between different lamellar phases, no less transitions between lamellar and nonlamellar phases. But model biological membranes composed of the purified components of natural membranes, or of synthetically derived lipids, do exhibit polymorphic phase behavior. And the behavior of model systems certainly has relevance to the interaction of components in biomembranes.

I.B.1. Lamellar (Chain-Melting) Phase Transition

The driving force for lamellar transitions is due to the increase in the chain rotational entropy, the increase in the intermolecular entropy, and the increased head group hydration. Figure I-5 shows representations for lipid bilayers undergoing transitions from the $L_{\beta'}$ phase to the $P_{\beta'}$ and the L_{α} phases. The chain-melting transition is opposed by decreased van der Waals attraction between the chains, the increased energy of the chains due to the increased rotational isomerism, and the increased hydrophobic exposure at the water/hydrocarbon interface due to the lateral expansion of the chains. The chain-melting transition temperature, T_m , is chain length dependent. With increasing chain length, the T_m increases due to increased van der Waals

Figure I-5: Schematic representation of lipids undergoing thermotropic transitions from the gel to the liquid-crystalline phases via an intermediate ripple phase.

attraction between tails, and the increased barrier to the formation of more numerous rotational isomers.

The mean molecular length of the acyl-chains at a given temperature is determined by the number of gauche rotamers that form about the saturated carbon-carbon bonds. This in turn is determined by a competition between the energy of introducing gauche rotamers (0.5 kcal/mol for each gauche rotamer) and the resultant increase in the entropy of the chains.

The bilayer thickness decreases by about 15% at the main phase transition due to the change from the all trans chain rotational isomers and large increase in the population of gauche rotamers. The increased number of gauche rotamers induces a large expansion of the chain area of about 20%.

The effect of these changes on the bilayer volume is an increase of about 5% (Marsh, 1991).

I.B.2. Lateral Phase Separation

In simple lipid mixtures, lipids having different miscibility can be separated into different domains. This lateral phase separation results in localized areas having different physical and chemical properties. Based on such model membrane studies, phase separation of the lipid mixtures of biomembranes has been proposed to occur.

Dissimilar lipids do not usually mix ideally. In particular, formation of the lamellar gel phase requires efficient packing of lipids. This is usually only possible for lipids having complementary molecular structures. And even if two lipids are miscible in the lamellar liquid crystalline phase, they may behave nonideally, as characterized by their experimentally determined phase diagram.

Phase diagrams for binary or multicomponent systems describe the fraction of lipid in each state during the phase transition. Mixtures exhibit broad melting transitions, in contrast to the sharp transitions of single-component systems. The transitions of complex biomembranes can be so broad that they are undetectable.

Similar lipids exhibit more ideal mixing behavior than dissimilar lipids. For example, dimyristoylphosphatidylcholine (DMPC) and DPPC, whose saturated tails differ only by two methylenes, behave nonideally in the gel phase, but ideally in the liquid crystalline phase. But they are completely miscible in both the gel and liquid crystalline phases. Mixtures of DLPC and DSPC, which differ by four methylenes, mix nonideally in both the gel and

liquid crystalline phases, and are nearly immiscible in the gel phase (Wilkinson & Nagle, 1979).

Phase separation of mixtures containing anionic lipids can be induced isothermally by addition of divalent cations, such as Ca^{2+} (Tokutomi et al., 1981; Haverstick & Glaser, 1987). Divalent cations can apparently form intermolecular bridges that result in clustering and lateral phase separation of the anionic lipids from the neutral lipids.

I.B.3. Nonlamellar Phase Transitions

The transition between the lamellar and nonlamellar phases requires a change in the geometry of the lipid interface. The enthalpy of the transition is dominated by curvature energy and chain packing restrictions. The entropy of the transition is dominated by changes in rotational isomerism of the chains, the ordering of water molecules of hydration, and by intermolecular contributions (Seddon, 1990).

These thermotropic transitions usually occur between the disordered lamellar (L_α) phase and a disordered inverted nonlamellar phase (either Q_{II} or H_{II}) at some characteristic temperature above the chain-melting transition temperature. Since the transition is between two disordered phases, the transition enthalpies and entropies are small compared to chain-melting transitions. The small transition enthalpy imparts on the transition a greater sensitivity to perturbations than is observed for the chain-melting transition. Hence, isothermal nonlamellar transitions can be induced by small changes in the physicochemical conditions such as hydration, salt concentration, and pH (Seddon, 1990).

Lateral stress imbalance can arise under conditions where either the effective head group size is reduced or the splay of the acyl-chains is increased. A number of factors that favor formation of the inverted hexagonal phase have been identified: decreasing the effective head group size (Seddon et al., 1983a; Gagne et al., 1985; Brown et al., 1986; Silvius et al., 1986; Gruner et al., 1988); deionizing the head groups (Hope & Cullis, 1980; Seddon et al., 1983b); adding Ca^{2+} to charged lipid systems (Tilcock, 1986); increasing the temperature (Seddon et al., 1984; Cullis et al., 1985; Kirk & Gruner, 1985); increasing the unsaturation of the acyl-chains (Cullis et al., 1985; Gruner et al., 1985; Lewis et al., 1989); increasing the length of the acyl-chains (Seddon et al., 1984; Brown et al., 1986; Epand et al., 1989; Lewis et al., 1989); and dehydrating the system (Luzzati et al., 1968; Seddon et al., 1983b; Cvec, 1987). Formation of the H_{II} phase can also be induced by addition of small amounts of long chain hydrocarbon, such as dodecane or squalene, to certain lipid-water systems (Kirk & Gruner, 1985; Sjolund et al., 1987; Siegel et al., 1989; Sjolund et al., 1989).

The inverted nature of the H_{II} phase is reflected in the difference in the chain length dependence of the transition temperature: in contrast to the chain-melting transition, the inverted hexagonal transition decreases in temperature with increasing chain length of PE (Lewis et al., 1989).

Recently, Lewis and coworkers (Lewis et al., 1994) reported the preparation of a new class of PCs that formed inverted nonlamellar phases. These lipids were analogs of DPPC having the normal PC headgroup and bis-substituted at the 2-position of both acyl tails with a variety of modestly sized hydrophobic groups. The hydrophobic substituents ranged in size from methyl to cyclohexylmethyl. Acyl substitution inhibited formation of the L_{C}

phase, and destabilized the L_β phase as evidenced by the decreased T_m of the analogs with respect to the parent compound, DPPC. And the L_α phase was destabilized with respect to the Q_{II} and H_{II} phases. The absolute temperatures at which the lamellar phases became unstable with respect to inverted phases increased with increasing length of the main acyl chains. This is the opposite dependence of that observed for most other nonlamellar phase forming lipids, including PE.

In the case of the smaller substituents, thermal induction of the Q_{II} phase but not the H_{II} phase was possible. In the case of larger substituents, the Q_{II} and H_{II} phases were formed sequentially with increasing temperature. The effect of the substituents on the phase behavior of the lipids was attributed to changes in the lateral stress profiles within the lipid monolayers. The stress profile is a theoretical construct suggested to capture a more exact description of a lipid system than either Israelachvili's single-valued shape parameters or Gruner's spontaneous radius of curvature (Israelachvili et al., 1977; Israelachvili et al., 1980; Kirk et al., 1984; Gruner, 1985). The stress profile arises from lateral interactions of lipids in three regions of a lipid monolayer: the headgroup (H) region, in which repulsive interactions occur for PCs; the interfacial, or hydrophilic/hydrophobic contact region (I) in which attractive interfacial tension exists; and the hydrophobic chain region in which repulsive interactions predominate. Currently, no experimental means of measuring the stress profile are known. However, the authors speculate that the primary effect of the 2-acyl substituents is to change the forces in the interfacial (I) region. This would also correspond to a change in the value of the spontaneous radius of curvature. Additionally, the authors suggested that the larger substituents may have induced a change in the conformation of the

glycerol backbone from its normal alignment perpendicular to the membrane (Figure I-1) to a conformation in which it is parallel to the bilayer surface. Such a conformational change might result due to the high energetic cost of placing the C2 acyl-substituents in a polar environment while maintaining the close van der Waals attractions between the main acyl chains.

I.B.4. Detection of Lipid Phase Transitions

Nonlamellar lipid structures are difficult to observe in biological membranes under physiologically relevant conditions. And the large scale formation of such structures is unlikely in cell membranes since the long lived formation of nonlamellar structures would compromise the barrier properties served by the membranes. In model membranes, phase transitions can be experimentally detected by a number of techniques including differential scanning calorimetry (DSC), ^{31}P -NMR, ^2H -NMR, X-ray diffraction, freeze-fracture electron microscopy, and cryo-transmission electron microscopy.

Determination of the long-range order of lipid-water phases (e.g. distinguishing between lamellar, inverted hexagonal, and inverted cubic phases) can only be accomplished by analysis of low angle X-ray diffraction patterns or by ^{31}P NMR. The H_{II} phase was first observed in the X-ray diffraction studies of Luzzati and coworkers (Luzzati & Reiss-Hudson, 1962; Luzzati & Reiss-Hudson, 1966; Luzzati et al., 1968; Luzzati & Tardieu, 1974) and the technique has since been employed by many others (Rand et al., 1971; Harlos & Eible, 1981; Gruner et al., 1988). The H_{II} phase has also be detected by ^{31}P -NMR (Cullis & de Kruijff, 1978; Cullis & de Kruijff, 1979); ^2H -NMR (Tilcock

et al., 1982); freeze-fracture electron microscopy (Deamer et al., 1970; de Kruijff et al., 1979; Van Venetie & Verkleij, 1981); fluorescence (Ellens et al., 1986a; Bentz et al., 1987; Hong et al., 1988); IR spectroscopy (Mantsch et al., 1981); ESR spectroscopy (Hardman, 1982); and differential scanning calorimetry (Harlos & Eible, 1981; Seddon et al., 1983a; Epand, 1985; Ellens et al., 1986a; Ellens et al., 1986b). H_{II} phases have been directly visualized by both freeze fracture electron microscopy (Deamer et al., 1970; Hui & Stewart, 1981) and cryo-transmission electron microscopy (Siegel et al., 1989; Talmon et al., 1990). The curvature of a phase -- type I or II -- cannot be directly determined from the X-ray diffraction data. But this information can usually be deduced from knowledge of the composition of the system and the approximate position in the system's phase diagram under the conditions where the X-ray data were collected. Figure I-6 gives the ³¹P NMR signals that typically correspond to the lamellar phase, the H_{II} phase, and the Q_{II} phase.

Short range interactions can be determined by differential scanning calorimetry (DSC). The P_β-L_β', L_β-L_α and the L_α-H_{II} phase transitions are routinely measured by DSC, although X-ray diffraction must be used to absolutely verify the phase structures. T_m and T_H values usually refer to the peak temperatures of the respective transitions on a first DSC heating scan. DSC cannot usually measure the inverted cubic phase transition temperature, T_I, since the transition occurs with a very small change in enthalpy. Instead, the emergence of isotropic resonances in the ³¹P-NMR is used to identify the transition.

Figure I-6: Typical ^{31}P NMR signals corresponding to the L_α , H_{II} , and Q_{II} phases.

I.B.5. Molecular Shape Model

Israelachvili and coworkers (Israelachvili et al., 1976; Israelachvili et al., 1977; Israelachvili et al., 1980) have formulated a model to attempt to predict the phase behavior of lipids based on their molecular shapes and other contributions such as head group interactions, hydration, and chain entropy. The model defines a critical packing parameter as v/a_0l_c , where v is the volume of the lipid molecule, l_c its critical, or extended fluid chain length, and a_0 the optimal surface area per molecule at the hydrocarbon/water interface. Here, optimal implies that the area corresponds rigorously to the minimum free energy of an idealized bilayer that is free of packing stresses. The model predicts that lipids having $v/a_0l_c < 0.5$ are cone shaped and form normal (type I) nonlamellar phases (micelles). Lysophospholipids and detergents are examples of this type. Those lipids having $v/a_0l_c = 0.5 - 1$ have cylindrical shapes and form lamellar phases. Such lipids include PC, PS, PG, sphingomyelin, and some saturated PEs. Lipids having $v/a_0l_c > 1$ have an inverted cone shape and form inverted (type II) nonlamellar phases. These include unsaturated PE, cardiolipin, and phosphatidic acid.

In this model, phase transitions occur due to packing stresses. Increasing temperature induces greater acyl-chain rotational (trans-gauche) isomerism, which in turn, reduces the critical chain length, and increases the critical packing parameter. A decrease in the water content would have a similar effect, due to a decrease of the optimal surface area at the hydrocarbon/water interface, and a resulting increase in the critical packing parameter. A great enough stress of either a thermotropic or lyotropic nature would induce lamellar to nonlamellar transitions.

I.B.6. Spontaneous Curvature Model

Gruner and coworkers (Kirk et al., 1984; Gruner, 1985; Gruner et al., 1985; Kirk & Gruner, 1985; Tate & Gruner, 1987; Anderson et al., 1988; Gruner, 1989; Tate et al., 1991) developed a model that explains nonlamellar phase transitions in terms of the structural and energetic factors that give rise to the tendency to curvature. In the model, the phase of a lipid assembly is determined by two opposing forces: curvature energy, and geometry-dependent energy terms. Since the opposed forces cannot be simultaneously minimized, lipid systems exist in a state of frustration. As the condition of the system changes, the sum of the forces changes. When the sum of the contributions can be reduced by a change of the morphology (e.g. from L_α to H_{II}), a phase transition occurs.

The geometry-dependent energy terms include hydrocarbon packing energy, van der Waals attraction energy, electrostatic repulsion energy, and hydration repulsion energy. But for a system composed only of neutral phospholipids in excess water the electrostatic and hydration energy terms can be ignored. Then, curvature energy is opposed only by hydrocarbon packing energy.

The elastic curvature free energy per molecule of a fluid lipid monolayer is given by the following expression:

$$E = k/2 (C_1 + C_2 - C_0)^2$$

where k is the elastic bending modulus of the monolayer, C_1 and C_2 are its principal curvatures, and C_0 is its spontaneous curvature (Helfrich, 1973). The principal curvatures are defined simply as the inverse of the principal radii of curvature, R_1 and R_2 : $C_1 = 1/R_1$, and $C_2 = 1/R_2$. The spontaneous curvature is the inverse of the spontaneous radius of curvature, R_0 : $C_0 = 1/R_0$.

The elastic free curvature energy is minimal when $C_1 + C_2 - C_0 = 0$. When the net curvature is equal to the spontaneous curvature the surface is relaxed. In flat bilayers, both principal curvatures are zero. But nonlamellar phases are characterized by non-zero (positive or negative) mean curvature. The mean curvature of a surface is arbitrarily assigned to be positive if the head group surface curves toward the hydrocarbon. Normal (type I) nonlamellar phases (e.g. micelles) are assigned positive mean curvature; inverted (type II) nonlamellar phases are assigned negative mean curvature.

The tendency of a lipid layer to curl arises from stresses in the layer due to an imbalance of the lateral interactions within the head groups and the acyl chains. The curvature energy of a phase depends on the spontaneous curvature, C_0 , of the monolayers that comprise the phase. C_0 depends on both temperature and lipid composition and it is also a colligative property of the system. So long as the different components of a system are intimately mixed, the system will exhibit an intermediate C_0 value. At the same temperature, lipids with smaller effective headgroups will have smaller values of C_0 . The value of C_0 is calculated from the value of the spontaneous radius of curvature, R_0 , which is experimentally determined from X-ray diffraction measurements (Rand et al., 1990). R_0 is taken to be approximately the value of the radius of water core of an H_{II} phase tube (Figure I-7). For systems composed of H_{II} competent lipids, R_0 decreases with increasing temperature, as evidenced by the decreased diameter of the H_{II} tubes as temperature is increased above T_H (Gruner et al., 1988; Tate et al., 1991).

Figure I-7: Spontaneous radius of curvature (R_0) and interstitial void volume as defined with respect to the inverted hexagonal (H_{II}) phase.

The hydrocarbon packing energy of the lipids in a given phase can be assessed by geometrical considerations. In the lamellar phase, all the component lipids exist in equivalent energy states. But in the H_{II} phase a fraction of the lipids must exist in a higher energy state than the bulk. The acyl tails of these lipids must assume a fully extended conformation in order to fill the interstices between adjoining H_{II} tubes. These lipids exist in an unfavorable entropic state compared to the bulk lipids.

The H_{II}/L_{α} phase transition can be understood by considering how both C_0 and hydrocarbon packing energy change as a consequence of decreasing temperature. Since C_0 increases with decreasing temperature, the dimensions of the H_{II} phase increase as the temperature is decreased from $T > T_H$ toward T_H . As the radii of the H_{II} tubes increase so do the volumes of the interstitial voids between them. In turn, this increases the hydrocarbon packing energy. At the transition, where the H_{II} tubes are at their maximum diameter, it is energetically favorable to pay the price of increased curvature energy to offset the growing price of hydrocarbon packing energy.

The formation of Q_{II} phases is also governed by the competition between curvature energy and the stretching energy of lipid chains (Anderson et al., 1988). As is the case in the hexagonal phase, the two terms are frustrated and cannot be simultaneously minimized. But the frustration of the Q_{II} phase is smaller than that in either the L_{α} or the H_{II} phases. The Q_{II} phase can appear between the L_{α} and H_{II} phases because in the Q_{II} phase the curvature energy can be smaller than for the lamellar phase, and the chain-packing energy can be lower than in the H_{II} phase.

The Q_{II} phases have constant but non-zero mean curvature. Gruner and coworkers (Anderson et al., 1988) calculated that for Q_{II} phases based on the

double diamond ($Pn3m$), the primitive ($Im3m$), or the gyroid ($Ia3d$) space groups, the conditions of constant bilayer thickness and constant curvature of the monolayers cannot be simultaneously minimized. Q_{II} phases satisfy the criteria of constant monolayer thickness and constant curvature at the expense of fluctuations in either the surface curvature or the thickness of the monolayers.

The most serious deficiency of the molecular shape model is its inability to explain the phase transitions of polymorphic lipids. The mean curvature of a monolayer undergoes an abrupt, discontinuous change at the L_{α}/H_{II} transition. But the critical packing parameter of the molecular shape model undergoes no such abrupt change due to changes in either temperature or hydration. In Gruner's spontaneous curvature model the frustration of opposing terms explains the lamellar to nonlamellar transition. The mean curvature changes abruptly at the phase transition. But the spontaneous curvature, or the curvature the system prefers to adopt, decreases continuously with increasing temperature until the curvature energy exceeds the hydrocarbon packing energy that results from H_{II} phase formation.

This model for the mesomorphic behavior of lipids allows lipids to be characterized on a continuum of spontaneous radius of curvature from highly lamellar-prone to highly non-lamellar prone. Nonlamellar-prone lipids readily form Q_{II} or H_{II} phases, but they will usually assume lamellar phases at sufficiently low temperatures. The equilibrium phase of unsaturated PEs at physiological pH and temperature is the H_{II} phase. Accordingly, these lipids are assigned small values of R_0 . Their L_{α} phases are characterized by high curvature energy but low hydrocarbon packing energy, whereas

their H_{II} phases have low curvature energy but high hydrocarbon packing energy. They are distinguished from lamellar-prone lipids, such as PCs, which do not easily undergo the L_{α}/H_{II} phase transition and exhibit large R_0 values. Representative examples of these two types of lipids are DOPE and DOPC. Below 6°C, both lipids are in the lamellar phase, but DOPE undergoes the L_{α}/H_{II} phase transition when heated above that temperature. DOPC remains in the L_{α} phase to temperatures above 85°C.

The largest factor contributing to their different phase behavior is the ability of PE to form hydrogen bonds between adjacent lipid head groups in the same membrane (Brown et al., 1986). The zwitterionic PC head group cannot form hydrogen bonds with its neighbors. Also, the effective head group size of DOPE is smaller than DOPC, since the ethanolamine head group is associated with fewer water molecules than the choline head group (Yeagle & Sen, 1986). DOPC exists in the L_{α} phase up to high temperatures since its high R_0 value corresponds to such large central water cores that the hydrocarbon packing price of forming the H_{II} phase is overwhelmingly prohibitive.

Since R_0 is a colligative property, mixtures of DOPC and DOPE exhibit values intermediate between the pure systems. Mixtures of DOPE and DOPC in mole ratios of 4:1, 3:1, and 2:1 exhibit T_H values determined by DSC at pH 4.5 of 40-48, 61, and 78-81°C, respectively (Ellens et al., 1987). In addition to undergoing the transition at higher temperatures with increasing content of DOPC, the mixtures exhibit larger water cores at their T_H values (Gruner, 1989).

In general, the mesomorphic behavior of lipids or of lipid mixtures can be predicted based on their absolute R_0 values. Those with small absolute R_0 values should undergo rapid L_{α}/H_{II} transitions, whereas those with large

absolute R_0 values should form stable L_α phases even at high temperatures. But those with certain intermediate absolute R_0 values should exhibit complex behavior at the L_α/H_{II} transition and are likely to form Q_{II} phases (Gruner et al., 1988). Lamellar prone lipids like DOPC have R_0 values of about -10 nm (Sjolund et al., 1989; Keller et al., 1993), whereas lipid compositions that form the H_{II} phase have R_0 values of about -3.3 nm (Rand et al., 1990). Systems having such intermediate R_0 values include certain PE/PC mixtures and DOPE-Me which has an R_0 value of -3.3 to -5.0 nm (Gruner et al., 1988; Siegel & Banschbach, 1990).

Despite its small negative R_0 value and facile L_α/H_{II} transition, a pure DOPE system has been induced to form a Q_{II} phase (Shyamsunder et al., 1988). But induction of the Q_{II} phase required cycling of the sample hundreds of times between temperatures just above the T_H and just below the T_m . Once formed, however, the phase was stable for weeks at 4°C, which is just below the T_H . The phase would probably be thermodynamically stable for even longer times, but the lipid degradation products apparently lead to its destabilization. By X-ray diffraction, the Q_{II} lattice was determined to be consistent with the either the $Pm3n$ (double diamond) or the $Pn3$ space group. This observation is suggestive that although the Q_{II} phases may exist as thermodynamic minima between L_α and H_{II} phases, their formation may be kinetically hindered, and may require the thermal generation of physical defects. Further, all lipids that undergo the L_α/H_{II} transition may be able to form intermediate Q_{II} phases under the appropriate conditions. The rate of formation of the Q_{II} phase from DOPE was found to decrease with increasing water content. Veiro and Rowe (Veiro & Rowe, 1988) observed the formation of an isotropic phase by ^{31}P -NMR in a thermally cycled sample of dielaidoylphosphatidylethanolamine (DEPE).

Chemical modifications of the head group or tails of lipids can change their spontaneous radii of curvature. Possible head group modifications include methylation, glycosylation, and ionization. N-Methylation of DOPE to form DOPE-Me, DOPE-Me₂, and DOPC can be used to successively increase the spontaneous radius of curvature (Figure I-8). Increased headgroup methylation changes the T_H values from 5 - 10°C for DOPE, to 65 - 75°C for DOPE-Me, and to > 85°C for both DOPE-Me₂ and DOPC (Gruner et al., 1988). Gruner and coworkers characterized the R₀ values associated with the lipids as small for DOPE, intermediate for DOPE-Me, and large for both DOPE-Me₂ and DOPC (Gruner et al., 1988).

DOPE-Me exhibits a slow L_α/H_{II} transition, compared to DOPE. At temperatures just below T_H, the lamellar lattice becomes unstable. And on incubation at 55°C, a Q_{II} phase slowly forms without the requirement of thermal cycling. The ³¹P-NMR spectrum exhibits an isotropic resonance, and the X-ray diffraction pattern is consistent with the Pn3m, or double diamond morphology (Gagne et al., 1985; Gruner et al., 1988). At room temperature, a DOPE-Me sample formed the cubic phase over the course of 1.5 years. The formation of cubic phases was facilitated by the slow room temperature formation of DOPE-Me degradation products. Once formed, the Q_{II} phase was thermodynamically stable, but the L_α phase could be reset by deep cooling of the system to temperatures below the chain melting transition temperature, T_m.

Figure I-8: Effect of N-methylation of the ethanolamine headgroup of DOPE on the T_H values of its methylated analogs, DOPE-Me, DOPE-Me₂, and DOPC.

	T_H (°C)	X
DOPE	5 - 10	
DOPE-Me	65 - 75	
DOPE-Me ₂	> 85	
DOPC	> 85	

The DOPE/DOPC (3:1, mol/mol) system exhibits similar polymorphic behavior to that of DOPE-Me. The T_H value of the mixture is similar to that of DOPE-Me, and the number of methyl groups per molecule on the quaternary nitrogen (0.75) is close to that in DOPE-Me (1.0).

The other geometry dependent contributions to the free energy have so far been neglected, since the systems under consideration have been composed of neutral lipids. When systems include charged lipids, the L_α phase is stabilized relative to the H_{II} phase due to electrostatic repulsion energy. For example, liposomes of pure unsaturated PE cannot be formed at physiological

temperature and pH, but they are easily formed above pH 9.0 where PE is negatively charged (Stollery & Vail, 1977). Stable PE-containing liposomes can also be formed at physiological pH if an acidic phospholipid component is added. Even the presence of small fractions of such negatively charged species is sufficient to stabilize PE into bilayers. Figure I-9 illustrates the effect of ionizing the PE headgroups to cleave intermolecular hydrogen bonds that exist at neutral pH.

Modification of the acyl tails may be accomplished by unsaturation, or insertion of various chemical moieties along any part of the chain. Lewis et al. (1989) prepared a series of synthetic diacyl PEs containing linear saturated chains, linear unsaturated chains, branched chains, and alicyclic chains of different lengths. The T_m and T_H values were measured by DSC and the identity of the phases was established by low-angle X-ray diffraction analysis. For a given homologous series of PE containing the same chemical moieties in the acyl chains, T_m was increased and T_H was decreased with increased effective chain length. For a nonhomologous series of PEs having the same length, both T_m and T_H were decreased by any modification of the basic linear saturated fatty acyl chain. And irrespective of the particular modification, for a given effective chain length, T_H occurred at a relatively constant reduced temperature above that of the T_m . The various structural modifications decreased T_m and T_H in the following order: linear saturated > methyl isobranched > ω -cyclohexyl > methyl anteisobranched > dimethyl isobranched > trans-monounsaturated > cis-monounsaturated > cis-diunsaturated. Structural variations in the acyl chains, and cis-unsaturation in particular, produced

Figure I-9: Effect of pH and the ionization state of the ethanolamine headgroup on the ability of PE to form intermolecular hydrogen bonds.

much larger changes in T_H values than variations in the chain length. Figure I-10 gives the structures and corresponding T_m and T_H values of linear saturated and unsaturated diacyl PEs.

Based on Gruner's model, one would predict that long-chain hydrocarbon added to a H_{II} phase lipid system would preferentially fill the interstitial voids between the H_{II} tubes, decrease the hydrocarbon packing energy, and thereby decrease T_H . In fact this has been observed, and systems with large R_0 values have been induced to form the H_{II} phase by the addition of small amounts of such hydrocarbons as dodecane or squalene (Sjolund et al., 1987; Gruner et al., 1988; Siegel et al., 1989; Sjolund et al., 1989; Turner & Gruner, 1992; Turner et al., 1992). Tate and Gruner (Tate & Gruner, 1987) also demonstrated that

Figure I-10: Effect of chain length and unsaturation on the T_m and T_H values of linear diacylPEs (Lewis et al., 1989).

<u>Linear Saturated PE</u>	<u>T_m (°C)</u>	<u>T_H (°C)</u>
16:0 (DPPE)	64.4	118.5
17:0	70.5	107.6
18:0	74.2	98
19:0	79.2	98
20:0	83.1	97.2
<u>Linear Unsaturated PE</u>		
18:1 $t\Delta^9$ (DEPE)	38.3	63.5
16:1 $c\Delta^9$	-30	42.5
18:1 $c\Delta^9$ (DOPE)	-16	10
18:2 $c,c\Delta^{9,12}$	-40	-15

addition of a small amounts of a PC having extra-long chains to a mixed PE/PC system decreased T_H . Addition of dodecane to DOPE-Me samples induced a low temperature H_{II} phase as well.

I.B.7. Molecular Mechanism of the L_α/H_{II} Transition

Molecular mechanisms of the L_α/H_{II} phase transition involving different intermediate structures have been developed by a number of workers. Siegel developed a detailed kinetic model for the transition by analyzing the stability of different intermediates, their lifetimes, and the energies required for their transformations during the transition (Siegel, 1984; Siegel, 1986a; Siegel,

1986c; Siegel, 1986b; Siegel, 1993c). The model was constructed to satisfy the observed effects of a system's lipid composition and other thermodynamic variables on the *rate* of the L_{α}/H_{II} transition.

The L_{α}/H_{II} transition is an interlamellar process that requires close apposition of L_{α} phase bilayers. Dilute suspensions of unilamellar vesicles will not undergo the transition, even at temperatures above T_H (Allen et al., 1990). They must first be aggregated so that interbilayer contact can occur and intermediates can form to facilitate the reorganization of the lipids. In Siegel's original model, inverted micellar intermediates (IMI) were invoked as the first intermediates in the phase transition. IMI are composed of a spherical inverted micelle sandwiched between two semi-toroidal (hour glass-shaped) monolayers (Figure I-11). The structures of IMI and their involvement in the L_{α}/H_{II} transition was first proposed by Verkleij and coworkers (Verkleij et al., 1980; Verkleij, 1984). Siegel refined his mechanism and eliminated IMIs as possible intermediates in favor of a more stable semi-toroidal structure termed a stalk (Siegel, 1993c). Siegel's stalk structure is a modified version of the stalk that was originally proposed by Hui et al. (Hui et al., 1982). Later Markin, Chernomordik and coworkers developed a molecular mechanism for the involvement of stalks in the L_{α}/H_{II} transition (Markin et al., 1984; Chernomordik et al., 1985; Chernomordik et al., 1987; Leikin et al., 1987; Kozlov et al., 1989).

Siegel argues that a modified stalk structure is a more probable component of the phase transition since it requires the least activation energy of any of the proposed intermediates (Figure I-12). Proposed intermediates are

Figure I-11: Schematic structure of an inverted micellar intermediate (IMI).

Adapted from Siegel (1984, 1986a).

required to have continuous lipid/water interfaces with no exposed monolayer or bilayer edges. The energy of the structures of the different possible intermediates is assumed to depend on the contributions of only two parameters: the curvature energy of the monolayers composing the structure, and the energy of any hydrophobic interstices. The curvature energy, defined in Section I.C.2, depends on the elastic bending modulus, k , of the monolayers of the intermediate, the principal curvatures of the monolayers, C_1 and C_2 , and the spontaneous curvature of the monolayers, C_0 . The value of k is composition-dependent, but its values are similar for a wide range of lipid compositions in both lamellar and inverted phases. The spontaneous curvature is a function of both the system composition and temperature.

Figure I-12: Kinetic model for the L_α/H_{II} phase transition. Adapted from Siegel (1993).

The phase transition from the L_α to the H_{II} phase creates interstitial voids between the lipid monolayers. The energy of an interstice is determined by the decrease in the configurational entropy of the acyl-chains that must stretch to their fully extended length to fill it. The transition is driven by a reduction in curvature energy, and it occurs at the temperature, T_H , where the decrease in curvature energy can be exactly offset by the increased energy associated with the creation of interstitial voids. The reduction of curvature energy can be calculated from the dimensions of the H_{II} phase at T_H , which comes from X-ray diffraction experiments. The energy of a given length of the interstitial void in the H_{II} phase can be calculated based on its cross sectional area. Similar voids must be formed in the first intermediate structure when the cis monolayers curl away from the trans monolayers to become joined. The interstice energy of various intermediates can be estimated by assuming that their energy per unit volume is the same as that of a given length of the interstitial void in the H_{II} phase.

Stalks can be formed when only a very small area of the two cis monolayers are apposed. The energy of formation of stalks is minimized since rupture of the cis monolayers must only occur over a small area. In contrast, formation of IMIs would require rupture of the apposed cis monolayers in a circular locus, rather than at just a contact point.

Stalks form rapidly when the two L_α phase bilayers are apposed at temperatures near T_H (Figure I-12). They can rapidly elongate into H_{II} phase precursors termed line defects (LD) within the planes of the cis monolayers. LDs are regions that resemble the walls of adjoining H_{II} tubes. This process should be very rapid for systems of unsaturated PE like eggPE or DOPE, but

stalks can also form other intermediate structures called interlamellar attachments (ILAs). ILAs are bilayer interconnections between apposed L_{α} phase lamellae.

Stalks can form ILAs via second intermediate, trans-monolayer contacts (TMCs). Stalks form TMCs by radially expanding within the cis-monolayers and allowing the trans-monolayers to dimple together. TMC formation is spontaneous for lipid compositions having certain C_0 values, where the energy of the TMC is lower than that of the stalk. Lipid compositions of this type form inverted cubic phases or inverted cubic phase precursors between the L_{α} and H_{II} phases. Formation of the TMC is opposed by the curvature energy of the trans-monolayers, which must be deformed into the dimple structure. Once formed, TMCs spontaneously form ILAs (fusion pores) by rupture of the monolayers of the dimple. Rupture of the dimple requires energy which is supplied by the interstice energy and the curvature energy associated with the dimple.

Two processes, therefore, compete to consume stalks: LD formation and ILA formation. The consequences of the two processes are completely different. Whereas LDs mediate formation of the H_{II} phase, ILAs cannot readily assemble into either the L_{α} or the H_{II} phase. Rather, ILAs kinetically trap stalks from facilitating the L_{α}/H_{II} transition. When they are initially formed, ILAs have dimensions similar to the TMC from which they arose. Both monolayers have high curvature, and the energy of the ILA is greater than that of a flat bilayer. The ILA can relieve the unfavorable curvature energy by expanding in the plane perpendicular to the original apposed lamellae.

Once this expansion occurs, reformation of either a TMC or a stalk is virtually impossible.

If they are formed in large enough numbers, ILAs can form arrays having similar morphology to inverted cubic phases. In fact, these arrays give rise to the some isotropic ^{31}P -NMR signals that are characteristic of Q_{II} phases. And under appropriate conditions, arrays of ILAs can form whole inverted cubic phases. Q_{II} formation can occur in systems of limiting hydration or in those having excess water. But just as H_{II} phase formation cannot occur in dilute suspensions of liposomes, neither can Q_{II} formation. Formation of Q_{II} phases are favored in stacks of lamellae where the L_{α} phase lamellae have long range order, and stalks can form between any bilayer and the ones above and below. At $T < T_{\text{H}}$, consumption of stalks by ILA formation can occur at steady state, without competitive formation of LDs. When a system is heated from temperatures well below T_{H} and maintained at temperatures just below T_{H} , ILAs can slowly accumulate. This restricts lipids from assuming either the L_{α} or the H_{II} phase, and result in hysteresis of the $\text{L}_{\alpha}/\text{H}_{\text{II}}$ phase transition. Upon heating to above T_{H} , the $\text{L}_{\alpha}/\text{H}_{\text{II}}$ transition will be slower than it would be for a sample rapidly heated to the same temperature but beginning at a temperature far below T_{H} . In certain systems, the absence of H_{II} phase formation has been observed at $T > T_{\text{H}}$ after long incubations at $T < T_{\text{H}}$ (Barry et al., 1992). Hysteresis is also observed in the cooling transition from a fully formed Q_{II} phase to the L_{α} phase. In PE/PC and other systems, the $\text{Q}_{\text{II}}/\text{L}_{\alpha}$ transition can either require hours to complete, or require deep freezing to temperatures below the $\text{L}_{\alpha}/\text{L}_{\beta}'$ transition (Gruner et al., 1988).

The polymorphic behavior of PE has been extensively studied. Studies of PE analogs have demonstrated that increasing its head group bulk, imparted

either by N-methylation of the PE ammonium group, or by addition of methylene groups to increase the phosphate-ammonium separation distance, leads to an increase in the nonlamellar transition temperature (Seddon et al., 1983a).

Stalks form the connection between the juxtaposed, or cis, monolayers of membranes. Formation of stalks requires bilayer contact and formation of these structures with an isolated bilayer cannot occur. If they form interlamellar attachments (ILAs), which are bilayer interconnections between apposed L_{α} phase lamellae that make both the membranes and the aqueous compartments of the lamellae continuous, they can mediate fusion between unilamellar vesicles.

Fusion should be more facile in systems with small C_0 values since both the energy of stalk formation and the energy of TMC formation decrease with decreasing C_0 . Hence, fusion should be rare in pure DOPC system, but should increase in probability as DOPE is added up to successively larger fractions, and as the temperature is increased towards T_H , since C_0 decreases with temperature.

The ILAs contain aqueous channels which can expand to allow intermixing of internal contents and formation of the vesicle fusion product. Elongation of stalks into LDs and formation of the H_{II} phase would lead to membrane rupture and leakage of the liposome contents.

I.C. Membrane Fusion

Biomembranes function as stable bilayers most of the time, but must also be capable of transient deformation. Certain essential cellular processes require transient, local disruption of the bilayer structure. These include cell division, fertilization, endocytosis, exocytosis, lipid exchange between and across bilayers, and infection of cells by enveloped viruses. From the simplest consideration of membrane fusion, it is evident that a molecular rearrangement of the juxtaposed bilayers must occur. Nonlamellar (non-bilayer) phase intermediates must form, however transiently, in this process. In fact, biological membranes and lipid extracts from biological membranes have been found to exist very near to the temperature associated with the L_{α}/H_{II} phase transition. This may be necessary for the cell to take advantage of stable bilayer membranes the majority of its lifetime, and transient nonlamellar phases during processes requiring bilayer transformations.

Membrane fusion is facile in biological systems; nonetheless it is not a spontaneous process. In biological membranes, which are often 50% or more protein by weight, fusion may be mediated by specific proteins. But the role of lipids is also important, and interactions between membranes is likely a cooperative process involving both proteins and lipids. Before two membranes can fuse, they must come into molecular contact. The strong forces that oppose fusion between biological membranes also act on liposomal membranes.

I.C.1. Forces Opposing Membrane Fusion

Interactions between membranes are controlled by interaction forces. These include attractive van der Waals forces, and three repulsive forces:

electrostatic (double-layer), solvation (hydration), and steric (Rand et al., 1990). For either neutral membranes (composed of nonionic or zwitterionic lipids) or negatively charged membranes in the presence of high salt, no strong electrostatic repulsion exists. In the absence of any other repulsive force, membranes under these conditions would be expected to come into strong, irreversible adhesion contact with no water layer between them. The strong short range force that controls the interactions between membranes that have closely approached one another and prevents spontaneous adhesion is the hydration force (LeNeveu et al., 1976; LeNeveu et al., 1977; Marra & Israelachvili, 1986). These forces arise from the oriented layers of water molecules that exist on both membrane surfaces. For pure PC membranes, each lipid molecule has two tightly bound water molecules and six or seven that are less tightly bound (Jenrasiak & Hasty, 1974). Dehydration of the membranes must be accomplished to allow the apposed bilayers to come into molecular contact. In fact this has been proposed to be the initial step in the fusion process (Rand & Parsegian, 1988). Membrane dehydration is highly energetically costly for membranes composed of well hydrated lipids such as PC, but much easier with membranes composed of less well hydrated lipids such as PE. Indeed, nature almost certainly chose as the components of biological membranes lipids whose head groups are well hydrated to ensure that spontaneous adhesion and fusion of membranes cannot occur.

I.C.2. Mass Action Kinetic Model for Membrane Fusion

Although the mechanism of liposome fusion remains uncertain, many factors that affect the fusion of liposomes have been determined. The following structural events have been proposed to contribute to fusion:

bilayer dehydration (Wilschut et al., 1985; Rand & Parsegian, 1988); local alterations in bilayer curvature (Nir et al., 1982; Lentz et al., 1987); changes in membrane fluidity (Wilschut et al., 1985; Düzgünes et al., 1987); bilayer-phase separation (Hoekstra, 1982); and locally induced non-bilayer phases (Ellens et al., 1989).

The overall process of fusion, for both liposomes and biological systems, involves the following sequence as illustrated in:

1. Adhesion (biological membranes) or
Aggregation (liposomes)
2. Close approach (apposition) of the membranes
3. Destabilization of the membranes in the region of contact
4. Intermixing of membrane components, and
5. Communion of the aqueous contents

The process is illustrated schematically in Figure I-13. After two membranes are in apposition, membrane destabilization is critical for fusion to occur. However, destabilization alone is not sufficient to produce fusion. After destabilization, membrane fusion intermediates must be formed, and these must then mediate the development of fusion, rather than other processes that lead to leakage or membrane rupture. The molecular mechanism of liposome fusion need not be the same as that for biological fusion processes.

Figure I-13: Schematic model for the fusion of two liposomes. The liposomes aggregate and their membranes come into adhesion with the consequence that the membranes are destabilized. When the membranes rupture in the region of adhesion, fusion results. When membrane rupture occurs outside of the region of membrane adhesion, membrane lysis results. Adapted from Bentz et al. (1988).

The initial fusion reaction is given by the following equation:

where V_1 denotes the liposomes, V_2 the aggregated dimer, and F_2 the fused doublet (Nir et al., 1980; Bentz et al., 1983). The aggregation rate constant for dimerization is denoted by C_{11} , and the deaggregation rate constant by D_{11} . The fusion rate constant is given by f_{11} .

The first step in membrane fusion is the same as for the L_α/H_{II} phase transition: molecular contact between the two apposed membranes. In order for such contact to occur, the repulsive forces between the membranes must be overcome. And following membrane contact, destabilization of the membranes must ensue. Aggregation and destabilization of liposomes can lead to a number of consequences only one of which is fusion, as defined in the biological sense. The *first* possible consequence is lipid mixing between the liposomes without concomitant fusion or leakage. This event is described as hemifusion. It can occur by formation of stalk structures between the liposomes which allows lipid mixing between the outer lipid monolayers. Reversion from the stalk to apposed bilayers could occur without causing either leakage from the liposomes or mixing of their aqueous contents. A *second* possible consequence is lipid mixing with both fusion and leakage. This is the usual occurrence for liposome systems that do undergo fusion. Since fusion is driven by destabilization of the liposomes, leakage occurs when such destabilization allows competitive formation of both ILAs and precursors

to the H_{II} phase. A *third* possible consequence is lipid mixing and leakage but no fusion. This occurs for systems that can form stable bilayers under conditions where no membrane contact can occur. Application of some stimulus that allows membrane contact may destabilize the membranes to the extent that formation of a stable fusion product is impossible. This is the situation encountered when pure PE liposomes are formed at pH 9.5, where they are negatively charged, and then membrane contact is initiated by neutralizing the membranes with either H⁺ or divalent cations (Allen et al., 1990). Contact between the PE membranes under these conditions results in H_{II} phase formation. A *fourth* possible consequence is lipid mixing and fusion without leakage. This is the situation in all cellular fusion events. But it has been reported in only one liposome fusion system, anionic PS liposomes in the presence of La³⁺ ions (Bentz et al., 1988).

Due to the variety of consequences that can result from aggregation and destabilization of liposomes, to fully comprehend different liposome fusion systems, one should utilize assays for all possible processes: lipid mixing, aqueous contents mixing, and aqueous contents leakage.

I.C.3. Fluorescence Assays for Membrane Fusion

A variety of physical methods have been employed to determine the effectiveness of different environmental conditions in facilitating fusion of liposomes, including DSC, NMR, electron microscopy, dynamic light scattering, and gel filtration. These methods can in many cases measure differences in the size or number of liposomes before and after the fusion event has been induced. But they cannot yield any kinetic information about the fusion

process or reveal the existence of transiently formed fusion products which may have limited lifetimes. Nor can they make a determination of the fundamental event that defines true fusion: mixing of the both the lipids and aqueous contents between two liposomes with no leakage of aqueous contents. Fluorescence assays have been developed to monitor lipid mixing, contents mixing, and contents leakage that may result from interaction between different liposome populations. These assays have recently been reviewed (Burgess & Lentz, 1993; Düzgünes & Wilschut, 1993; Hoekstra & Düzgünes, 1993; Leventis & Silviu, 1993).

I.C.3.a. Lipid Mixing Assays

A number of different lipid mixing assays have been developed that rely on resonance energy transfer (RET). These assays employ fluorescently labeled pairs of lipids that act either as donors or acceptors. The fluorophores are chosen such that the emission spectrum of the donor overlaps the excitation spectrum of the acceptor. Direct excitation of the donor can result in predominant emission from the acceptor when the probes are present in the same membrane in sufficiently close proximity. In most of the RET pairs, the fluorophores are present at the lipid/water interface. Vanderwerf and Ullman (Vanderwerf & Ullman, 1980) developed an assay using two headgroup labeled lipids, Dansyl-PE ($Ex_{max} = 340$, $Em_{max} = 578$) and Rhodamine-PE (Rh-PE) ($Ex_{max} = 550$, $Em_{max} = 590$), as the donor/acceptor pair. Uster and Deamer (Uster & Deamer, 1981) employed an uncharged acylated cholesterol derivative, cholesteryl anthracene-9-carboxylate (CA9C) as the donor ($Ex_{max} = 370$ nm, $Em_{max} = 450$), and a negatively charged dialkylammonium-NBD conjugate (DH-NBD) as the acceptor ($Ex_{max} = 450$, $Em_{max} = 530$). Pagano and

coworkers (Struck et al., 1981) introduced an RET assay utilizing the donor NBD-PE ($\text{Ex}_{\text{max}} = 460$, $\text{Em}_{\text{max}} = 534$) and the acceptor Rh-PE ($\text{Ex}_{\text{max}} = 550$, $\text{Em}_{\text{max}} = 590$). Presently the NBD-PE/Rh-PE is the most widely employed means of determining lipid mixing between different liposome populations. As originally developed, the assay was performed by preparing one liposome population labeled with a small percentage of NBD-PE (e.g. 1 mol%) and a second population with a similar fraction of Rh-PE (e.g. 2 mol%) in the so-called *probe mixing method*. At the beginning of the assay when the liposome populations were distinct, the fluorescence from the NBD-PE is intense since it is neither self quenched nor quenched by Rh-PE. Lipid mixing results in an increased concentration of Rh-PE with respect to NBD-PE with an attendant decreased fluorescence from NDB-PE. Subsequently, Papahadjopoulos and coworkers discovered that fluorescence quenching in the probe mixing method could result merely from aggregation of the differently labeled liposomes even when lipid mixing did not ensue (Düzgünes et al., 1987). The probe mixing method reports the sum interaction between liposome populations that is manifested as either lipid mixing or only as aggregation. Papahadjopoulos and coworkers developed a modified assay that was insensitive to aggregation between liposomes that did not result in authentic lipid mixing. In this version of the NBD-PE/Rh-PE lipid mixing assay, which they termed the *probe dilution method*, one liposome population is doubly labeled with both NBD-PE and Rh-PE, and a second liposome population is unlabeled. In this system, the fluorescence from NBD-PE is severely quenched at the beginning of the assay due to the high relative concentration of Rh-PE also present in the membrane as is illustrated in Figure I-14. Lipid mixing between the different liposome populations dilutes the relative concentrations

Figure I-14: Schematic illustration of the NBD-PE/Rh-PE probe dilution method to measure lipid mixing between different liposome populations.

of the two probes and results in relieved quenching of NBD-PE by Rh-PE. Aggregation of labeled and unlabeled liposomes that does not lead to genuine lipid mixing cannot affect the fluorescence from NBD-PE. For the probe dilution method, labeled liposomes are prepared containing 1 mol % each of NBD-PE and Rh-PE. By judicious use of both the probe mixing and probe dilution variations of this assay, one can compare the rates and extents of overall aggregation between liposomes under given conditions with the corresponding degree of lipid mixing that occurs as a direct consequence of the aggregation (Düzgünes et al., 1987).

A lipid mixing assay that relies on fluorescence self quenching of the probe molecules when they are above a critical concentration utilizes an 18-carbon, rhodamine headgroup labeled single-chain amphiphile (R18) (Hoekstra et al., 1984). In this assay, lipid mixing is reported as a consequence of increased fluorescence from R18 that results as a consequence of its dilution from the labeled liposomes into unlabeled ones. The assay is particularly useful for measuring lipid mixing between liposomal and viral membranes and between enveloped animal viral membranes and cellular membranes (Hoekstra & Klappe, 1993). In those assays, the liposomes are labeled with R18. Fusion of the liposomes with viruses results in large dilution of the probes from the liposomal membranes into the much larger viral structures.

I.C.3.b. Fusion Assays

In order to derive meaningful kinetic data from fusion assays monitoring the mixing of aqueous contents between different liposomes, the assays must meet stringent criteria (Düzgünes & Bentz, 1988). First, none of the fluorophores or their binding or dissociation products should bind to or

perturb the membranes. Second, the rate of the fluorescence reactions must be fast compared to the rate of the fusion event (i.e. the formation or dissociation of the fluorescence complex must not be rate limiting). Third, the leakage of the fluorophores or their products from intact liposomes before fusion is initiated must not occur, or must at least occur very slowly (e.g. occur with a half life of several days). Fourth, leakage of the fluorophores or their products during or after the course of fusion must reflect leakage of the encapsulated volume out of the liposomes. Fifth, the fluorophore and its adjuvant must not be allowed to react in the external medium upon leakage from their respective liposomes. Finally, the extent of the change in the fluorescence signal should reflect the extent of liposomes that have fused at a given time. Of course, since leakage that accompanies fusion affects the assay, the extent of fusion at any given time will not usually reflect the total extent of liposome fusion but only the percentage of liposomes fused at a given time.

A number of fluorescence assays have been developed to monitor the mixing of aqueous contents between separate liposome populations (fusion) or to measure the leakage of the internal liposome contents into the extra-liposomal medium (Düzgünes & Bentz, 1988). Two assays have been thoroughly examined for their reliability in a variety of liposome systems, the terbium/dipicolinic acid (Tb/DPA) assay, and the aminonaphthalene-trisulfonic acid/p-xylylene bis(pyridinium) bromide (ANTS/DPX) assay. The Tb/DPA and ANTS/DPX assays have also been compared to each other in identical liposome systems and under the same conditions (Ellens et al., 1985). Both these assays satisfy stringent criteria required for their use in determining fast kinetics for liposome fusion processes under biologically relevant conditions.

In the Tb/DPA assay, separate liposome populations are prepared containing either Tb^{3+} or DPA^{2-} (Wilschut & Papahadjopoulos, 1979; Wilschut et al., 1980). Both species exhibit high solubilities in aqueous buffers due to their charges. Fusion results in the formation of the fluorescent $[\text{Tb}(\text{DPA})_3]^{3-}$ chelation complex. The complex is excited at a wavelength near the absorption maximum of DPA (276 nm) and its emission spectrum is characteristic of Tb^{3+} . Intense fluorescence results from the excitation of DPA and internal energy transfer from DPA to Tb. Formation of the chelation complex results in an ca. 10,000-fold increase in the emission intensity from Tb. In order for the assay to report fusion and also subsequent leakage, the Tb/DPA chelation complex must be dissociated upon leakage into the extraliposomal medium. This is accomplished by including 0.1 mM EDTA in the external buffer since EDTA binds Tb more strongly than does DPA and consequently disrupts the fluorescent Tb/DPA chelation complex. When Tb^{3+} is encapsulated in anionic liposomes, citrate is usually included to prevent interaction between the trivalent cation and the negatively charged lipids. Citrate weakly chelates Tb and is rapidly and efficiently displaced by DPA after a fusion event. The fluorescence scale for fusion assays is set by taking the fluorescence intensity of the Tb-containing vesicles as 0% fluorescence (0% fusion). The 100% fluorescence (100% fusion) value is determined by preparing a population of mock fused vesicles containing coencapsulated Tb and DPA at the concentrations that would be expected if the Tb- and DPA-liposome populations employed in the fusion assay would have undergone random and complete fusion with one another.

The Tb/DPA assay can also be used to determine leakage occurring from liposomes under the conditions employed to aggregate and destabilize the

liposomes in fusion assays. For a leakage assay, a single liposome population may be prepared in which Tb and DPA are coencapsulated. Prior to leakage, the fluorescence from the liposomes is intense due to efficient internal energy transfer between DPA and Tb in their chelation complex. Leakage results in the dilution of the Tb/DPA complex into the external medium, which contains 0.1 mM EDTA, and rapid and complete disruption of the Tb/DPA complex and quenching of Tb fluorescence. The leakage scale is calibrated by taking the initial intensity of the unperturbed liposomes as 100% fluorescence (0% leakage). The value for 0% fluorescence, and 100% leakage, is determined by disrupting the liposome bilayers by addition of cholate or deoxycholate detergent. The nonionic detergent Triton X-100 should be avoided since it severely quenches Tb fluorescence.

The ANTS/DPX fluorescence assay is performed in much the same manner as the Tb/DPA assay. Separate liposome populations are prepared encapsulating either ANTS or DPX (Ellens et al., 1984; Ellens et al., 1985). The assay is based on the collisional quenching of ANTS by DPX (Smolarsky et al., 1977). Fusion results in the interaction of ANTS and DPX within the confines of the fused liposome and in the quenching of ANTS fluorescence as illustrated in Figure I-15. Notice that the assay differs from the Tb/DPA assay in that fusion is reported by a decreased fluorescence signal rather than by an increased one. The ANTS/DPX assay is superior to the Tb/DPA assay for fusion studies at mildly acidic pH since the protonation of DPA at ca. pH 5 interferes with formation of the Tb/DPA complex (Wilschut & Papahadjopoulos, 1979; Wilschut et al., 1980).

Figure I-15: Schematic representation of the ANTS/DPX assay for measuring aqueous contents mixing (fusion) between different liposome populations.

I.C.3.c. Leakage Assays

Assays for leakage of the encapsulated contents of liposomes into the extraliposomal medium must meet, in general, the same criteria applied to fusion assays: the fluorophores must not bind to the membranes, the fluorophores must not leak at appreciable rates through the intact liposomal membranes prior the induction of the assay, and the extent of fluorescence increase should mimic the extent of leakage from the destabilized liposomes.

The self quenching fluorophore calcein has been widely employed to measure dilution of the aqueous contents of liposomes resulting from poration or rupture of the membrane (Allen & Cleland, 1980). Calcein is superior to another self-quenched fluorophore, carboxyfluorescein (CF), since it does not become membrane permeable at low pH values where CF exhibits rapid leakage. Fluorophores covalently attached to macromolecules have been used to measure leakage that results from membrane rupture rather than from formation of small pores (Massenburg & Lentz, 1993).

The Tb/DPA and ANTS/DPX assays can also be used to measure contents leakage. For measuring leakage, either Tb/DPA or ANTS/DPX are coencapsulated in liposomes. Leakage of Tb/DPA out of liposomes, or the leakage of divalent cations or EDTA into the liposomes, results in the disruption of the Tb/DPA chelation complex and a decrease in the fluorescence. Leakage of ANTS/DPX from liposomes results in the relief of quenching of ANTS by DPX and an increased fluorescence from ANTS. Leakage assays can be performed using a single population of liposomes, since leakage from any of the liposomes gives rise to a detectable signal. These assays can also be performed by combining probe-containing liposomes with "empty" liposomes whose contents are the same as the bulk medium. In this situation, the "empty"

liposomes do not act as reporters of membrane destabilization, but they can present a different membrane surface on which the reported liposomes can be destabilized. By choosing the appropriate stoichiometries of reporter and "empty" liposomes, the probability of collisions between like and unlike membranes can be influenced (Ellens et al., 1987). Figure I-16 illustrates the use of the ANTS/DPX assay to measure leakage that results from interaction of ANTS/DPX-containing liposomes and "empty" liposomes.

Figure I-16: Schematic representation of the ANTS/DPX assay for measuring aqueous contents leakage resulting from the interaction of different liposome populations.

I.C.4. Liposome Fusion

Genuine fusion between two liposomes requires mixing of both their lipids and their aqueous contents. Aggregation of liposomes can result in mixing of the lipids in the outer leaflets without mixing of contents. This process is termed hemi-fusion, and is distinguished from true fusion. Leakage of liposome contents can occur as a result of aggregation. It can occur at the site of liposome contact, in which case fusion is said to be leaky, or it can occur due to rupture of the bilayer subsequent to non-leaky fusion. Biologically relevant fusion events are restricted to those in which fusion is non-leaky, and the fusion product is stable.

Insight into the fusion of biological membranes has resulted in part from the study of a variety of methods devised to induce the fusion of liposomes: anionic liposomes in the presence of multivalent ions (Bentz & Nir, 1981; Düzgünes et al., 1981; Ohki et al., 1982; Bentz & Düzgünes, 1985; Carmona-Ribeiro et al., 1985; Bentz et al., 1988); liposomes containing polymorphic lipids, such as phosphatidylethanolamine (PE)¹, at temperatures near T_H (Ellens et al., 1985; Ellens et al., 1986a; Ellens et al., 1986b; Ellens et al., 1987; Ellens et al., 1989; Siegel et al., 1989); neutral liposomes in the presence of dehydrating agents such as PEG (Parente & Lentz, 1986; Lentz et al., 1992; Massenburg & Lentz, 1993); so-called pH-sensitive liposomes at low pH or in the presence of multivalent cations (Connor et al., 1984; Ellens et al., 1984; Düzgünes et al., 1985; Nayar & Schroit, 1985; Straubinger et al., 1985; Leventis et al., 1987; Collins et al., 1989); formation of diacylglycerol (DAG) by reaction of phospholipase C with PC-containing liposomes (Nieva et al., 1989; Luk et al., 1993); and cationic liposomes in the presence of anionic liposomes (Felgner et al., 1987; Stamatatos et al., 1988; Leventis & Silviu, 1990).

Fusion of liposomes can be divided into general categories:

1.) anionic liposomes and multivalent cations, 2.) anionic and cationic liposomes, 3.) polymorphic liposomes, and 4.) neutral liposomes and dehydrating agents. The *first* category includes systems where multivalent cations (principally Ca^{2+}) are added to anionic phospholipid membranes having lamellar equilibrium phases. The cations do not induce a phase transition, or even the formation of nonlamellar phase precursors. Their role is multifold and includes aggregating the membranes, dehydrating the surfaces to allow the membranes to come into molecular contact, and destabilizing the membrane structure in the regions of contact. The *second* category is for fusion between liposome populations having complementary surface charges, i.e., fusion between liposomes possessing positive charge due to inclusion of a cationic component, and the classical anionic liposomes. The *third* category includes systems in which fusion is induced by the formation of nonlamellar phase precursor in membranes containing polymorphic lipids, such as PE, at temperatures below the H_{II} phase transition. It is restricted to ionotropically induced fusion events where the principal role of the protons or cations is to destabilize the membrane and facilitate the $\text{L}_{\alpha}/\text{H}_{\text{II}}$ phase transition or intermediates in the transition rather than to induce membrane contact. Destabilization occurs due to neutralization of charged groups or lateral separation of neutral and charged lipids and formation of destabilized phases. And the *fourth* category includes fusion of PC liposomes induced by high concentrations of dehydrating agents such as polyethylene glycol (PEG).

In all cases, membrane destabilization most likely occurs only after interbilayer contact. The prerequisite of membrane contact to the destabilization of anionic membranes in the presence of divalent cations was

demonstrated by a number of workers (Liao & Pestegard, 1980b; Liao & Pestegard, 1980a; Wilschut et al., 1980; Bentz et al., 1983; Nir et al., 1983a). Later, Ellens et al. (Ellens et al., 1984) showed that membrane contact was also necessary for destabilization of PE-containing liposomes. In their study, liposomes were composed of PE and a charged cholesteryl ester, cholesteryl hemisuccinate (CHEMS).

Liposome fusion has been studied with both LUV and SUV. Their fusion behavior is often very different under given conditions due to the instability of the SUV membranes. Due to the small radius of curvature, or alternatively the large curvature, of SUV membranes, the structures exist in a high energy state. They are often observed to spontaneously aggregate and fuse to produce larger structures free of such strain (Lentz et al., 1987). Here, the fusion behavior of LUV will be emphasized.

I.C.4.a. Anionic Liposomes and Multivalent Ions

Most studies of liposome fusion focus on the interaction of like charged lipid membranes that can be induced to aggregate, and in some cases fuse, when the electrostatic repulsions between the liposomes are reduced through the addition of counterions. The earliest liposome fusion systems developed involved liposomes composed wholly or in part of anionic lipids, such as PA, PG, cardiolipin, and PS in the presence of multivalent inorganic cations such as Ca^{2+} , Mg^{2+} , Ba^{2+} , and La^{3+} . The Ca/PS liposome system was principally used to develop the lipid mixing and aqueous contents mixing assays that were afterwards applied to other systems. At physiological pH, PA, PG, and cardiolipin liposomes are negatively charged due to the ionization of the phosphate group; PS liposomes are negatively charged due to the ionized and

negatively charged carboxylate and phosphate groups. For like charged vesicles to establish intermembrane contact, the repulsive electrostatic and hydration forces must be surmounted. The induction of fusion of identically charged liposomes by multivalent cations occurs as a result of both neutralization of the membrane surface charge and formation of ionic bridges by the cations via binding to anionic lipids in the cis-monolayers of separate liposomes. Following membrane adhesion, the intermembrane molecular contact may or may not result in fusion depending on the liposome composition, and the cation identity and concentration.

The aggregation rate constant for fusion has been found to increase with decreased surface charge density, as is expected due to diminished electrostatic repulsion between the liposomes (Nir et al., 1980; Bentz et al., 1983; Nir et al., 1983b; Bentz et al., 1985; Wilschut et al., 1985; Wilschut et al., 1985). The fusion rate constant, which is measured under aggregation rate limiting conditions, has been found to depend, in addition to the amount of bound cation, on the identity of the particular cation utilized (Düzgünes et al., 1981; Wilschut et al., 1981; Bentz et al., 1983; Bentz & Düzgünes, 1985; Bentz et al., 1985). Initial binding of the cations decreases the surface charge and facilitates approach of membranes. As the membranes come into closer apposition, the amount of bound cations increases due to double layer equilibration.

In the model for Ca^{2+} induced fusion of PS LUV, adhesion of the membranes promotes surface tension on the membranes, while overcoming the hydration forces, and resulting rupture of the membranes in the region of contact (Kachar et al., 1986; Bentz et al., 1988). An intermembrane intermediate may mediate the fusion event, but their short lifetimes have in most cases precluded their experimental observation. Since the initial fusion

event is nonleaky, lysis is believed to occur subsequently to fusion as a result of collapse of the fusion product and the formation of cochelate structures, rather than as a result of competitive rupture of the membranes outside the region of contact (Wilschut et al., 1980; Bentz et al., 1983; Wilschut et al., 1983; Bentz & Düzgünes, 1985; Bentz et al., 1985; Wilschut et al., 1985).

The fusion rate constants for PS LUV were found to depend in general on the stoichiometry of bound divalent cation, and for equal amounts of cations bound to isolated PS LUV, the fusion rate constants were determined to decrease in the order $\text{Ca} > \text{Ba} > \text{Sr} \gg \text{Mg}$ (Bentz & Düzgünes, 1985). The fusogenic ability of different cations is typically quantified by determining the threshold concentration required to induce fusion. The threshold concentration can be expressed as the concentration of the ion in the bulk medium or as the ratio of cations bound to anionic lipids. It is generally assumed that the amount of cation bound to the isolated membranes is a good measure of the amount bound at the fusion site during the actual fusion event.

For the Ca^{2+} induced fusion of PS LUV, increasing temperature led to increased lipid mixing relative to aqueous contents mixing. This was attributed to the increased reversibility of the aggregation step with increased temperature (Bentz et al., 1985). Following aggregation at the higher temperatures, lipid mixing occurs between the liposome cis-monolayers, but in most cases dissociation of the liposomes occurs prior to mixing of the lipids composing the trans-monolayers and mixing of the liposome aqueous contents.

Since both Ca^{2+} and Mg^{2+} ions are present *in vivo*, most fusion studies of PS liposomes have focused on the effects of these two ions. Their effects on PS LUV were found to be very different. Although both ions cause massive

aggregation of the anionic PS liposomes, Mg^{2+} results only in lipid mixing and does not promote fusion, while Ca^{2+} induces both lipid mixing and fusion which processes are followed by leakage (Düzgünes & Papahadjopoulos, 1983). For this reason, the processes of liposome aggregation and destabilization of the apposed bilayers leading to the merging of their bilayers and the mixing of their aqueous contents could be shown to be independent for PS LUV.

Binding of divalent cations to PS and PA membranes raises the main transition temperature. Addition of Ba^{2+} to bovine brain PS ($T_m \sim 10^\circ C$) raises the transition to $35^\circ C$, and addition of Ca^{2+} raises it to $>100^\circ C$ (Düzgünes et al., 1984). Isothermal phase transitions from the L_α to the L_β phase can therefore be induced in PS LUV at $20^\circ C$ by addition of either Ca^{2+} or Ba^{2+} . Nonetheless, the L_β/L_α phase transition has not been correlated with fusion of anionic liposomes in the presence of multivalent cations. And divalent ion binding to well hydrated PS liposomes at neutral pH does not induce H_{II} phase formation.

In the PS/ La^{3+} fusion system, fusion occurs without leakage and is unique in this regard with respect to all other liposome fusion systems (Bentz et al., 1988). The existence of a long lived intermembrane intermediate is inferred due to the rupture of the membranes exclusively in the region of membrane contact. Additionally, fusion can be disrupted without causing lysis by addition of excess EDTA to chelate the La^{3+} . The putative intermembrane intermediate has been proposed to be composed of a La/PS chelation complex in which the La^{3+} is bound at both the carboxyl and amino groups (Bentz et al., 1988). The binding of La^{3+} to the carboxylate, rather than the phosphate, group of PS is indicated by both the weak binding of La^{3+} to PC and the known structures of complexes of serine analogs with transition metals. Binding of

La^{3+} at the nitrogen of the ammonium group requires the release of a proton from the ammonium group. Evidence for the formation of this complex was derived from the pH dependence of microelectrophoresis during La^{3+} binding to PS liposomes. Although fusion is promoted at very low La^{3+} concentrations, $\geq 2 \mu\text{M}$, it is abolished at higher concentrations, $\geq 1 \text{ mM}$ at pH 7.4. This behavior is due to the strong La^{3+} -PS binding and the charge reversal of the PS membranes from anionic to cationic in the presence of excess La^{3+} .

I.C.4.b. Anionic and Cationic Liposomes

The combination of liposomes having oppositely charged lipid surfaces avoids the requirement for addition of chemical agents to induce fusion of like-charged liposomes. And while fusion of anionic liposomes at the plasma membrane of animal cells has not been achieved, cationic liposomes have been successfully used to transform cells in suspension (Felgner et al., 1987; Felgner, 1990; Legendre & Szoka, 1992). Cationic liposomes strongly adhere to the negatively charged surface of animal cells and are believed to deliver encapsulated DNA both by direct fusion at the plasma membrane and via fusion with the endosomal membrane following endocytosis (Figure I-17) (Legendre & Szoka, 1992). As a consequence of their strong tendency to adhere to anionic membranes, cationic liposomes are not capable of circulating in the blood.

Since cationic lipids are not known in nature, preparation of cationic liposomes requires synthesis of amphiphiles bearing positively charged groups. Synthetic double-chain cationic dimethyldialkylammonium halides

Figure I-17: Fusion of cationic liposomes with animal cells at either the plasma membrane or the endosomal membrane following endocytosis (Legendre & Szoka, 1992).

were utilized to prepare the first bilayers composed entirely of synthetic lipids (Kunitake & Okahata, 1977; Kunitake et al., 1977). Since these amphiphiles are toxic to cells, other cationic lipids have since been prepared and investigated.

Cationic liposomes formed from a 1:1 mol/mol mixture of DOPE and N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), which mixture has been termed lipofectin, have been reported to spontaneously interact with DNA to form lipid-DNA complexes in which the DNA is coated with lipid (Felgner et al., 1987). Lipofectin is a commercial reagent composed of equimolar amounts of DOTMA and DOPE. Lipofectin-DNA preparations have been demonstrated to be capable of efficient transfection of cells in suspension for a variety of cell lines.

Fusion of cationic and anionic liposomes has been studied as a means of evaluating the ability of different liposome preparations to undergo fusion with cellular membranes. Silvius and coworkers (Stamatatos et al., 1988) prepared an analogue of DOTMA, 1,2-bis(oleoyloxy)-3-(trimethylammonio)-propane (DOTAP), in a simple two-step synthesis. DOTAP differs from DOTMA in the attachment of the dioleoyl to the 2,3-dihydroxy-N,N,N-trimethylamino propane backbone via ester linkages rather than ether bonds, and should therefore be more efficiently biodegraded in the body. Inclusion of a minimum of 15 mol% DOTAP in liposomes composed otherwise of TPE was sufficient to stabilize the liposomes at physiological pH and temperature. Stable eggPC (EPC)/DOTAP could be prepared with any ratio of the lipids. Fusion of TPE/DOTAP and eggPC (EPC)/DOTAP liposomes with PS liposomes was compared using lipid mixing, contents mixing, and contents leakage assays.

The initial rates of lipid mixing between PE/PC (1:1) liposomes at pH 4.5 or at 9.5 in the presence of 10 mM Mg^{2+} or 10 mM Ca^{2+} were compared to the rates of mixing between PE/PC/DOTAP and PE/PC/PS liposomes in low ionic strength buffer at the same temperature (37°C) and total lipid concentration (30 μ M). The lipid mixing rate of the PC/PE liposomes (1.6% min^{-1} at pH 4.5, 5.1% min^{-1}

at pH 9.5/10 mM Mg^{2+} , and 6.8% min^{-1} at pH 9.5/10 mM Ca^{2+}) were substantially slower than the mixing rate for the PE/PC/DOTAP and PE/PC/PS liposomes (49% min^{-1}).

Gao and Huang (Gao & Huang, 1991) reported the preparation of a cationic cholesterol derivative. The lipid, 3 β [N-(N',N'-dimethylaminoethane)-carbamoyl]-cholesterol (DC-Chol), contains a dimethylamino moiety linked to the cholesterol hydroxyl group via a carbamoyl linkage. The authors suggest that this linkage should provide better stability to the lipid on storage than the ester linkages in DOTAP, and should allow more efficient biodegradation of the lipid in the body than DOTMA, since its acyl tails contain very stable ether linkages.

I.C.4.c. Polymorphic Liposomes

Transitions between lamellar and nonlamellar phases require a change in geometry. The first intermediates that participate in the structural reorganization of the membrane during the L_{α}/H_{II} transition have been proposed to be related to the structures that mediate fusion (Verkleij, 1984; Siegel, 1986a; Siegel, 1993a). However, completion of the H_{II} transition is antithetical to fusion. Lipid mixing and contact-mediated contents leakage were observed to be greatly enhanced at temperatures near to, but below the inverted hexagonal phase transition temperature, T_H (Ellens et al., 1986a). Liposome fusion was correlated with the onset of the isotropic ^{31}P -NMR resonances which are known to indicate the formation of inverted cubic phases or inverted cubic phase precursors in a variety of lipid systems (Ellens et al., 1986b; Ellens et al., 1989).

Isothermal transitions are possible whenever the transition temperature can be shifted. For example, a change in the composition of the aqueous phase can in turn change the surface electrostatics and hydration. Membranes that contain large amounts of nonlamellar type lipids are inherently stressed under conditions where the L_α phase is adopted. Even when the system is far from the phase transition, the forces that ultimately induce the lamellar to nonlamellar phase transition are present.

Lipids that exhibit phase transitions from lamellar to nonlamellar structures include unsaturated PEs, mono-galactosyldiacylglycerol, cardiolipin, and phosphatidic acid. The significance of non-bilayer phases in cellular processes, including fusion, has recently been postulated. This has led to interest in those non-bilayer lipids present in biological membranes, such as PE (Hui & Sen, 1989; Lindblom & Rilfors, 1989; Siegel et al., 1989; Tate & Gruner, 1989; Siegel & Banschbach, 1990).

Ellens et al. (1986a) studied the fusion of PE liposomes composed of DOPE, eggPE, or TPE (PE prepared by transphosphatidylation of eggPC). The liposomes were prepared at pH 9.5 and their fusion and phase behavior was studied under two different conditions: pH 4.5, and pH 9.5/20 mM Ca^{2+} . The different PE systems exhibited nearly the same T_H values at low pH and at high pH in the presence of calcium ions: DOPE, $T_H = 12^\circ\text{C}$, eggPE, $T_H = 45^\circ\text{C}$, and TPE, $T_H = 57^\circ\text{C}$. At temperatures far below their respective T_H values, the liposomes aggregated and underwent lipid mixing, limited contents mixing, and contents leakage. At all temperatures below T_H , both H^+ - and Ca^{2+} - induced fusion occurred only transiently. Fusion was more rapid and extensive at high pH in the presence of Ca^{2+} than at low pH. The rates and extents of fusion increased with increasing temperature up to T_H where both the rates and extents were

diminished. At temperatures $> T_H$, extensive lipid mixing and leakage occurred, but no fusion was observed.

Papahadjopoulos and coworkers (Allen et al., 1990) extended the study of fusion of PE liposomes by examining the effects of temperature, pH, ionic strength, liposome lamellarity (geometry), and the different concentrations of either Mg^{2+} or Ca^{2+} . In the studies, eggPE and TPE whose T_m and T_H values, as determined by DSC at pH 7.4 in isotonic saline buffer (100 mM NaCl), were 9 and 46°C, respectively for EPE, and 17 and 58°C, respectively for TPE. The fusion behavior of LUV prepared from either of the lipids was assessed by performing assays for aggregation, lipid mixing, contents mixing, and contents leakage. Stable liposomes, characterized as nonaggregating and impermeable, could be formed either at high pH (>8) in the presence of isotonic saline, or at physiological pH (7.4) and low ionic strength (< 50 mOsm $\sim$ 25 mM NaCl).

The liposomes were stable under either of two non-aggregating conditions: a) pH 7.4, 5 mM Na^+ , or b) pH 9.3, 100 mM Na^+ . Under both conditions, addition of either 7 mM Ca^{2+} or 7 mM Mg^{2+} resulted in the appearance of the L_α/H_{II} transition at a temperature similar to that observed at pH 7.4, and under the second condition, addition of 50 mM Na^+ was sufficient to induce the L_α/H_{II} transition. A summary of their observations is given in Figure I-18.

Addition of the divalent cations was proposed to have two effects: neutralizing the net negative charge on the PE LUV, and promoting the aggregation of the membranes by forming bridges between negatively charged ethanolamine headgroups on apposed membranes. These experiments

Figure I-18: Effect of the ethanolamine headgroup ionization state and temperature on the phase behavior of PE liposomes. Adapted from Allen et al., 1990.

confirmed the important rule that L_{α} phase lipids cannot undergo the L_{α}/H_{II} transition without first experiencing intermembrane contact.

At pH 7.4/5 mM Na^+ or at pH 9.3/100 mM Na^+ , increasing the temperature of LUV suspensions of either EPE or TPE to, or near to but below, their respective T_H values resulted in marked increases in aggregation (as evidenced by light scattering), lipid mixing (as evidenced by the probe dilution assay), and contents leakage (as evidenced by the calcein assay). Contents mixing, measured by the ANTS/DPX assay, occurred at temperatures near to, but ca. 20 °C below, the T_H . At or above the T_H , no contents mixing occurred due to competitive formation of the H_{II} phase which resulted only in leakage. At 20 °C below the T_H value, no evidence for the H_{II} phase or for H_{II} phase precursors was observed by DSC. Allen et al. (1990) inferred that formation of the H_{II} phase was not involved in the fusion event. Instead, they suggested that in that temperature range, other nonlamellar structures could have mediated the fusion event. Fusion was extremely transient whether induced by the addition of 100 mM Na^+ to LUV at pH 8.3, or by addition of Ca^{2+} or Mg^{2+} at pH 9.3, 8.3, or 7.4.

The effects of addition of Ca^{2+} or Mg^{2+} to the PE vesicles were almost indistinguishable. Both ions induced aggregation and destabilization of the PE LUV at approximately the same threshold concentrations. And under the appropriate conditions, both ions could induce lipid mixing, contents mixing, and contents leakage. In the case of PS liposomes, the effects of the two ions are different: Mg^{2+} can effect only aggregation and lipid mixing but not contents mixing or contents leakage, while Ca^{2+} can effect all three processes. Hence, the processes of aggregation and bilayer destabilization are known to

be separable for PS liposomes, but may not be separable in the case of PE liposomes.

Ellens et al. (1986b; 1989) studied the phase behavior and fusion of liposomes composed of DOPE-Me and of different mixtures of DOPE/DOPC (2:1, 3:1, and 4:1, mol/mol). These systems were chosen since they had been found by other workers to exhibit isotropic ^{31}P NMR signals at temperatures intermediate between the T_m and T_H values. In all cases, liposomes were prepared at pH 9.5 and their interactions were studied under four conditions: pH 4.5; pH 7.4; pH 7.4/5 mM Mg^{2+} ; and pH 9.5/20 mM Mg^{2+} . DSC was used to measure the T_m and T_H values for systems under the different conditions. The systems were assigned ΔT_I values based on ^{31}P NMR measurements of the temperature at which narrow isotropic resonances first appeared and the temperature at which they disappeared due to the onset of the H_{II} phase (T_H). Isotropic intermediates were observed in these systems at temperatures tens of degrees below their T_H values. The rates of fusion and leakage were measured by both the ANTS/DPX and Tb/DPA assays. These rates were then correlated with the corresponding phase behavior of the different systems to reveal the existence of distinct liposome behavior in three temperature ranges: below ΔT_I , little fusion or leakage occurred; within ΔT_I , fusion and leakage occurred with increasing rates as the temperature was increased toward T_H ; and $\geq T_H$ fusion was abolished due to the occurrence of aggregation-dependent lysis and H_{II} phase formation. Two observations distinguished these systems from pure PE systems: fusion did not occur at temperatures far below T_H where pure PE liposomes did undergo fusion; and fusion occurred without the completion of the transition to either the Q_{II} or H_{II} phase under conditions where pure PE

liposomes completed the L_{α}/H_{II} phase transition (e.g. high lipid concentration). The authors argued that the correlation of the increased rate of fusion and the onset of isotropic ^{31}P NMR resonances indicated that fusion was mediated by the same interbilayer intermediates that mediated the L_{α}/Q_{II} transition according to Siegel's kinetic model of the $L_{\alpha}/Q_{II}/H_{II}$ phase transition.

The polymorphic nature of PE has been exploited to develop liposomes that form stable, sealed structures at neutral pH but become destabilized at weakly acidic pH. So-called pH sensitive liposomes are prepared from two or more components, including a polymorphic lipid, such as PE, and a stabilizing component which possesses a titratable acidic group. For the liposomes to be useful under physiological conditions, the T_H of the mixture at neutral pH must be well above 37°C , and following acidification, the T_H should be near physiological temperature.

The development of these synthetic liposome systems was inspired by the strategies utilized by enveloped animal viruses to deliver their contents to the cytoplasm. Figure I-19 illustrates the strategy by which pH-sensitive immunoliposomes have been used to deliver liposomal contents to the cytoplasm of specific target cells *in vivo*.

In the endocytic pathway, biochemically distinct organelles are formed over time from the first formed early endosome. The aqueous compartment of these organelles have successively lower pH values. The late endosome fuses with a primary lysosome to form a secondary lysosome. The primary lysosome is itself acidic and it contains degradative enzymes which are capable of digesting most foreign matter taken up by the process of endocytosis.

Figure I-19: Schematic illustration of cytoplasmic delivery of the contents of pH-sensitive immunoliposomes to target cells *in vivo*.

Despite its large negative C_0 value, PE is stabilized in a bilayer morphology when the stabilizing component is charged. The curvature energy of PE, which drives the transition from the bilayer to the inverted hexagonal phase, is opposed by three geometric dependent terms: hydrocarbon packing energy, hydration energy, and electrostatic repulsion. The hydrocarbon packing energy can be overcome at physiological temperature if the appropriate PE is chosen, but bilayer contact can occur only if the hydration energy and electrostatic repulsion barriers can be overcome. Neutralization of the stabilizer, by protonation, largely eliminates both the electrostatic repulsion and the hydration energy from the outer leaflet of the liposomes. The destabilized membrane can then undergo either fusion or H_{II} phase transition upon contact with another membrane.

Stabilizers having different pK values can be used so that the liposomes are sensitive to different pH ranges. A stabilizer that has been used in much of the experimental work of Huang and coworkers is oleic acid (OA). Upon protonation, DOPE/OA liposomes exhibit a limited and transient amount of contents mixing that is accompanied by a substantial degree of leakage. This result is predicted by Siegel's model since no lamellar prone component (such as PC) is present in the system following the protonation step (Siegel, 1986c; Siegel, 1993b; Siegel et al., 1994). A serious drawback to the use of OA and other single chain acidic amphiphiles to stabilize pH-sensitive liposomes in the blood is that they can be rapidly removed by other membranes and serum components, including proteins such as serum albumin (Hamilton & Cistola, 1986; Storch & Kleinfeld, 1986). In the absence of stabilizing components, the pH-sensitive liposomes would rapidly leak their contents, since PE by itself does not form stable vesicles at neutral pH. Silvius and coworkers (Leventis et al., 1987) prepared a series of protonatable double-chain amphiphiles. They demonstrated that the amphiphiles could be combined 1:3 (mol/mol) with either TPE or dielaidoylphosphatidylcholine (DEPE, 18:1 trans) to form pH-sensitive liposomes that exhibited sensitivity over a range of different pH values depending on the specific composition of the amphiphile headgroup. The amphiphiles were all acylated derivatives of 2-substituted palmitic acid with the exception of 1,2-dioleoyl-3-succinylglycerol (succinyldiolein). The exchange rates of the double-chain amphiphiles vs. fatty acids were estimated by comparing the rates at which fluorescently-labeled fatty acids and a fluorescently-labeled analogue of a 2-acylated palmitic acid were extracted from pH-sensitive liposomes in the presence of either POPC/DOPG liposomes, serum albumin, or 25% fetal bovine serum. The single chain fatty acid, N-

NBD-12-(methyamino)stearic acid, was removed from pH-sensitive liposomes very rapidly (e.g. >50% within the first few seconds) in the presence of other liposomes, albumin, or the whole serum. By contrast, the double chain amphiphile, N-[N-NBD-12-(methyamino)stearoyl]-2-aminopalmitic acid, showed no detectable desorption from pH-sensitive liposomes over 15 min in the presence of other vesicles or albumin, and in the presence of whole serum, very slow desorption was observed (0.39%/min). Contents leakage was also compared for pH-sensitive liposomes prepared from TPE and either 30 mol% OA or 25 mol% of the double-chain amphiphile N-oleoyl-2-aminopalmitic acid in the presence of either POPC/DOPG liposomes, albumin, or whole serum. Substantial leakage was observed from the OA-containing vesicles in the presence of all of the fatty acid receptors, and the rate of leakage increased linearly with increasing amounts of the fatty acid receptors. Leakage was much slower from the double-chain amphiphile-stabilized liposomes than from the single-chain analogues in the presence of either POPC/DOPG liposomes or albumin. But the double-chain-containing pH-sensitive liposomes did undergo leakage at rates comparable to those for the TPE/OA liposomes in the presence of whole serum. However, the rate of leakage of the double-chain-stabilized liposomes was considerably lower than from liposomes prepared from POPC/DOPG (3:1), a system containing a comparable amount of acidic lipids and no PE.

Liposomes that have been formulated to be sensitive to protons are also destabilized by divalent cations, including Ca^{2+} and Mg^{2+} . As is the case for pure PE liposomes, the distinctive effects of these two cations observed with PS LUV do not occur with polymorphic LUV composed of mixtures of PE and other lipids. Whereas only Ca^{2+} promotes fusion of PS LUV, and Mg^{2+} induces

merely aggregation and lipid mixing, both ions are capable of promoting fusion of PE-containing LUV.

I.C.4.d. Enzymatic Formation of Diacylglycerol

Diacylglycerols (DAGs) are second messengers produced at low levels (ca. 1 mol% with respect to other membrane lipids) in cellular membranes by the phosphatidylinositol (PI) cycle (Priess et al., 1986). Phospholipase C cleaves the phosphoester bond of glycerophospholipids to generate diacylglycerol and a water-soluble phosphorylated alcohol. In the PI cycle, a PI-specific phospholipase C activated by a G-protein hydrolyzes PI to produce two second messengers: DAG and phosphorylated inositol. Other glycerophospholipids can be hydrolyzed by different phospholipase C species. Although one function of diacylglycerols is the activation of protein kinase C, it has also been suggested that their production makes membranes more fusogenic to facilitate processes such as exocytosis (Pickard & Hawthorn, 1978). In liposomes, addition of low levels of DAG have been demonstrated to decrease the L_{α}/H_{II} phase transition temperature (Dawson et al., 1984; Das & Rand, 1986; Coorsen & Rand, 1987; Siegel et al., 1989). Siegel et al. (1989) demonstrated that the addition of as little as 2 mol% DAG to DOPE-Me liposomes decreased both the T_H , and the onset of the appearance of isotropic resonances in ^{31}P NMR, by 15 to 20 $^{\circ}\text{C}$ depending on the identity of the DAG added. Siegel et al. further demonstrated that addition of 2 mol% 1-oleoyl-2-arachidonoylglycerol (OArG) resulted in a 15 $^{\circ}\text{C}$ shift in the fusion and leakage threshold temperatures for DOPE-Me liposomes. The strong correlation between the decreased temperature for the appearance of isotropic intermediates and the increase in the fusion rate was taken as strong evidence that the two processes are

mediated by the same mechanism. The authors proposed that the addition of DAG to polymorphic systems facilitates membrane fusion in two ways: increased hydrophobicity of the lipid/water interface, and decreased R_0 value of the system. The former change facilitates liposome aggregation by reducing the hydration repulsion between approaching membranes. The latter change facilitates formation of fusion intermediates after membrane aggregation at decreased temperatures relative to pure DOPE-Me systems. With respect to the mechanism by which DAG reduces the R_0 value, the effect of the DAG headgroup in decreasing the hydration of the membrane surface, rather than the stabilization of hydrophobic interstices between H_{II} tubes by the DAG tails, is probably responsible. NMR experiments confirmed that DAGs do not pack into the H_{II} interstices (Siegel et al., 1988).

Alonso and coworkers (Nieva et al., 1989; Burger et al., 1991; Nieva et al., 1993) demonstrated fusion of PC liposomes enzymatically catalyzed by phospholipase C. The workers employed a bacterial phospholipase C species that hydrolyzed PC and PE at comparable rates to generate DAG. Liposomes were prepared from pure PC or from (50:25:25 mol/mol/mol) PC/PE/cholesterol (CH) mixtures in the presence of 10 mM Ca^{2+} . In the absence of added phospholipase C, the liposomes were reported to be stable to both leakage and fusion up to 80°C. On addition of the enzyme, the liposomes underwent leakage-free fusion. The liposomes containing PE and CH exhibited much faster fusion kinetics than the pure PC liposomes as measured by the ANTS/DPX assay. This was due in part to the lower threshold concentration of DAG required to induce liposome fusion. The pure PC liposomes required 40 to 60 mol% DAG to undergo fusion, while the PC/PE/CH liposomes began to fuse in the presence of 4 mol% DAG. Remarkably, substitution of part of the

phospholipid components with 5 or 10 mol% DAG during their preparation did not apparently alter their stability against fusion or leakage. Although incorporation of DAG in the liposomes did induce liposome aggregation, the hydrolase activity of the enzyme was required to destabilize the membranes and induce fusion. Although the presence of Ca^{2+} was not necessary for enzymatic activity, reducing its concentration from 10 to 2 mM reduced the rate of fusion. The authors suggested that localized enzyme activity in the outer monolayers of aggregated liposomes was crucial to inducing liposome fusion.

Conspicuously absent from these reports are control experiments in which PE-containing liposomes are prepared under conditions where no interaction between liposomes of either the same or different populations can occur until such interaction is initiated in a controlled process that allows all subsequent liposome interactions to be monitored. For example, the liposomes could have been prepared at high pH (e.g. > pH 9.0) and in the absence of Ca^{2+} where the ca. 25 mol% PE component would carry sufficient negative charge to prevent liposome aggregation and fusion. The ability of DAG to enhance the fusion of PC/PE/CH liposomes under the conditions used by Alonso and coworkers could then be rigorously tested by rapid addition of either 2 or 10 mM Ca^{2+} and simultaneous reduction of the pH to 7.0 as was done in the experiments of Siegel et al. (1989).

Gomez-Fernandez and coworkers (Gomez-Fernandez et al., 1989) investigated the effect of addition of 10 mol% of three different DAGs on Ca^{2+} -induced fusion of PC/PS (1:1) liposomes. Fusion and leakage were measured at 50 μM lipid in the presence of 8 mM Ca^{2+} using the Tb/DPA assay. Addition of 1,2-dioleoylglycerol (1,2-DOG) increased the rate and extent of fusion and

leakage compared to the control liposomes composed of PC/PC (1:1). Addition of 1,2-dipalmitoylglycerol (1,2-DPG) or 1,3-dioleoylglycerol (1,3-DOG) did not strongly affect fusion or leakage as compared to the control. The phase behavior of the systems was investigated by ^{31}P NMR. Hydrated dispersion of PC/PS (1:1) exhibited characteristic lamellar NMR signals in the absence and presence of 20 mM Ca^{2+} . In the absence of Ca^{2+} , lamellar signals were observed after addition of 10 mol% of all three of the DAGs. Upon addition of Ca^{2+} to the DAG-containing systems, the appearance of isotropic resonances was observed. With 1,2-DOG, which was most effective in promoting fusion, the isotropic signal was most predominant. With 1,2-DPG and 1,3-DOG, which were less effective in promoting either leakage or fusion, isotropic components did exist, but the major component was due to the bilayer signal. The possibility that the enhancement of Ca^{2+} induced fusion by 1,2-DOG was due to the induction of phase separation of PC and PS was tested using a fluorescence assay. Fluorescence spectra measured for PC/PS/1,2-DOG (45/45/10) liposomes containing C6-NBD-PC were measured in the absence and presence of 8 mM Ca^{2+} . Self quenching of the fluorophore that would result from calcium-induced phase separation of PS from PC was not observed. Hence, the effect of addition of 1,2-DOG in promoting fusion of PC/PS liposomes is not likely due to phase separation, but to some other effect of DAG, such as the stabilization of fusion intermediates having isotropic symmetry.

Walter and coworkers (Walter et al., 1994) extended the examination of the effect of DAG on liposome fusion to a system that does not form inverted phases, pure bovine brain PS liposomes. PS can form H_{II} phases but does so only at extreme conditions: at low hydration (< 65% H_2O by weight) and in the presence of ≥ 20 mol % DAG (Das & Rand, 1986); or at very low pH (de Kroon et

al., 1990). In excess water and neutral pH, PS remains lamellar even in the presence of 50 mol% DAG and Ca^{2+} . The workers examined the effect of adding 3 mol% 1,2-DOG on the rate of PS LUV lipid mixing in the presence of either 3 mM Ca^{2+} or Ba^{2+} . On addition of DAG the lipid mixing rates were doubled while the aggregation rates were unaffected. The authors suggest that the effect of small amounts of DAG on the rates of membrane fusion may be general since the PS liposomes were far (thermodynamically) from a nonlamellar phase boundary.

I.C.4.e. Neutral Liposomes and Dehydration Agents

Poly(ethylene glycol) (PEG) is used to induce cell-cell fusion for such processes as production of monoclonal antibodies from immortal hybridoma cells and for injection of macromolecules into cultured cells from erythrocytes (Davidson & Gerald, 1977) or liposomes (Szoka et al., 1981). Lentz and coworkers have reported PEG (MW 8,000) induced aggregation and lipid mixing between liposomes composed of a single synthetic PC (e.g. DPPC or DOPC) but not fusion unless amphiphilic perturbants such as L- α -lysopalmitoylphosphatidylcholine (LysoPC), palmitic acid (PA), platelet activating factor (PAF), or 1,2-dilauroyl-3-sn-phosphatidylethanolamine (DLPE) were present (Burgess et al., 1991; Lentz et al., 1992). Although DPPC liposomes did not undergo fusion even after extreme dehydration of their surfaces in the presence of high concentrations of PEG, very low surface concentrations (1 - 5 mol %) of LysoPC, PA, and PAF were sufficient to facilitate fusion of DPPC liposomes in the presence of 20 - 30 wt % PEG. Since these perturbants exhibit positive intrinsic curvatures and increase the T_H of polymorphic membranes, their effect on fusion of DPPC liposomes could not be

ascribed to the introduction of isotropic intermediates. Further, no lateral phase separation is expected due to their introduction into the DPPC membranes. The effect of the perturbants was therefore attributed to the introduction of packing incompatibilities. Interestingly, similar amounts of some diacylglycerols, 1,2-dipalmitoyl-sn-glycerol, and 1,2-dioleoyl-sn-glycerol, did not promote PEG-mediated fusion of DPPC liposomes. DOPC liposomes were found to undergo fusion in the presence of PEG when large amounts (65 - 85 mol %) of DLPE were included in the membrane. With increasing DLPE content, the liposomes underwent fusion at decreasing PEG concentrations. Since DLPE does not form the H_{II} phase, its effect on inducing fusion of DOPE can not be attributed to its facilitating the formation of fusion intermediates having inverted structures. The authors suggest that the dehydration of the vesicle surface by DLPE reduced the concentrations of PEG required to induce fusion. And they propose that the effect of DLPE in promoting fusion of DOPE liposomes is the same as with the other perturbants: disrupted lipid packing in the interface of the headgroup regions of the bilayer.

Lentz and coworkers (Burgess et al., 1992; Massenburg & Lentz, 1993) developed a structural model for PEG-induced vesicle fusion based on lipid mixing, contents mixing, and contents leakage assays and on X-ray diffraction examination of the effects of high PEG concentrations on liposome morphologies. Their model proposes that liposomes in low PEG concentrations aggregate but are not deformed. With increasing PEG concentrations large osmotic pressures are exerted on the liposomes and both the interior and exterior bilayer surfaces are dehydrated. The vesicle morphology is changed from a sphere to a flattened disk. At the PEG concentrations where fusion will

occur, X-ray diffraction data suggest that the dimensions of the fluid spaces between adjacent liposomes and within liposomes are the same. The edges of the flattened disks are highly curved and additional osmotic stress results exclusively in membrane rupture in these regions. Even when the PEG concentration is restricted to the lowest value sufficient to induce fusion, contents mixing is inevitably accompanied by contents leakage. After comparing the leakage of a series of different high molecular weight fluorescently labeled dextrans and low molecular weight fluorophores, the workers proposed that the small and large markers leaked by the same mechanism. On this basis, they proposed that fusion and leakage occur by different mechanisms rather than as sequential events. Fusion is proposed to occur via the joining of bilayers in the closely apposed regions of the stressed bilayers at the highly curved ends, and leakage is proposed to occur due to membrane contact independent rupture at the highly curved ends.

I.C.5. Liposomes for *in vivo* Drug Delivery

Delivery of pharmaceuticals to specific tissues or cells *in vivo* is a goal that currently stimulates research across several disciplines. Targeting drugs to their therapeutic site of action reduces required dosages and minimizes side effects of drugs on healthy tissues. This technology is especially useful for cytotoxic drugs directed against cancers and other tumor cells. A useful drug delivery system must meet stringent requirements: it must deliver the drug to target cells with high specificity, release the drug only after delivery and in active form, avoid uptake by other cells -- especially those of the reticuloendothelial system (RES) -- and allow for subsequent *in vivo* clearance of the carrier. Also, the carrier should possess reasonable storage stability and

should not be too difficult to produce (Maruyama et al., 1990). The major limitation of vesicles as drug delivery systems is their rapid clearance from the circulatory system by the RES (Gabizon & Papahadjopoulos, 1988). The RES comprises one part of the cell-mediated immune response and includes the mononuclear phagocytes (Allen, 1989; Ostro, 1989). These cells emanate from the bone marrow as monocytes in the blood stream and differentiate into macrophages in such organs as the liver, spleen, and lungs (Alberts et al., 1983). By the process of phagocytosis, macrophages ingest and digest both antigenic and nonantigenic particulate material (Gennis, 1989). Attachment of macrophages to particles is mediated by surface receptors of two types: those that recognize specific ligands, and those that exhibit nonspecific binding. Antigen that is coated with antibody is said to be opsonized. Opsonization of antigen greatly increases the efficiency of antigen uptake by macrophages since specific receptors on the macrophage surface bind to the attached antibody.

The diameter, surface charge, and bilayer rigidity of vesicles influences their rate of uptake by the RES (Papahadjopoulos et al., 1989). Most vesicle preparations are readily recognized by the RES and rapidly cleared from the blood stream, but two types of vesicles have been developed that exhibit greatly increased circulation times in the blood stream. These have been termed stealth liposomes (Gabizon & Papahadjopoulos, 1988; Allen, 1989; Papahadjopoulos et al., 1989), and sterically stabilized vesicles (Blume & Cevc, 1990). These two preparations possess different compositions and properties and each utilizes a unique mechanism for evading the RES.

Stealth vesicles are composed of rigid bilayers in which a particular glycolipid, ganglioside GM₁, is incorporated. The bilayer must exhibit a gel-to-

liquid crystalline phase transition temperature, T_C , that is above physiological temperature so that it exists in the gel phase in vivo. Typically, the bilayers are prepared from PC and either cholesterol or sphingomyelin (SM). Stealth vesicles are taken up by the RES more rapidly if their bilayers are significantly larger than 100 nm, but the more rigid the bilayer, the less dependent the rate of uptake on vesicle size. In fact, an increase in RES uptake of only 1.4-fold results from increasing the size of SM-containing vesicles from 100 to 200 nm (Allen, 1989). Sterically stabilized vesicles consist of PC bilayers in which a small amount (ca. 10 mol %) of phosphatidylethanolamine having a covalently attached poly(ethylene glycol) head group (PE-PEG) is incorporated (Blume & Cevc, 1990). The PEG is a linear, water soluble polymer of about 100 atoms in length. The polymer coats the vesicles to an estimated thickness of about 2 nm. The PEG, which is non-antigenic and non-toxic, presumably prevents antibody recognition of the vesicle surface by steric interactions. Unlike stealth vesicles, these need not possess rigid, gel phase bilayers under physiological conditions to avoid uptake by the RES. However, rigid bilayers do reduce the rate of leakage of entrapped solutes. Sterically stabilized vesicles having diameters of about 100 nm successfully evade the RES.

One strategy to target vesicles to organs other than those of the RES is to attach antibodies to the vesicle surface. Antibodies are produced against antigens which are unique to the target cell surface. These antibodies are each covalently coupled to the head group of a lipid. The lipid portion of the antibody-lipid complex anchors it in the vesicle membrane. These immunoliposomes, on encountering target cells, become attached to the target cell surface via binding of the antibody to the target cell antigen. This exact

strategy has been successfully used with stealth immunoliposomes (Maruyama et al., 1990).

Drugs whose site of therapeutic action is in the cytoplasm must cross the formidable barrier of the plasma membrane to affect the cell. One process by which vesicles might gain access to the target cell cytoplasm is phagocytosis. Investigations by Papahadjopoulos and coworkers (Dalke et al., 1990) on the cellular fate of liposomal contents after phagocytosis revealed that much of the lipid component of the liposomes was retained by the phagocytes. These investigators hypothesize that cytoplasmic drug delivery via phagocytosis should be more efficient for lipophilic compounds than for water soluble ones. Delivery of lipophilic molecules is possible when the drug molecules can be entrapped in the bilayer walls of the vesicles. This strategy was tested by the use of an analog of the chemotherapeutic drug 5-fluorouracil (5FU) as the bis-palmitoylated nucleoside (Elorza et al., 1993).

I.D. Photosensitive Liposomes

Photosensitive liposomes have been described in recent years (reviewed by O'Brien & Tirrell, 1993). A variety of strategies have been employed, including the photochemical isomerization of chromophores in the acyl chains (Kano et al., 1981; Pidgeon & Hunt, 1983; Pidgeon & Hunt, 1987), photocleavage of lipid chains (Kusumi et al., 1989; Anderson & Thompson, 1992b), photoinduced change in the association of polyelectrolytes with liposomes (You & Tirrell, 1991), and the photopolymerization of lipids (Frankel et al., 1989; Lamparski et al., 1992). These methods were generally designed for liposome lysis. Only in the case of photopolymerization of liposomes has

evidence for liposome fusion been reported (Bennett & O'Brien, 1993; Bennett et al., 1994).

Photosensitive liposomes can be designed such that the lipids themselves possess a chromophore which absorbs light and undergoes a reaction, or a sensitizer can be included as the photosensitive component of the liposomes. In the former case, the characteristics of the lipid chromophores determine both the wavelength of the light that can be absorbed and the efficiency with which it is absorbed. Lipids exhibiting high absorptivities must usually contain bulky chromophores having extensive conjugation. This requirement can pose problems both for their accommodation in bilayers and also their synthetic preparation. In the latter case, the absorption characteristics of the sensitizer, rather than the lipid, determines the required excitation wavelength and the efficiency of the absorption of light. Since a variety of sensitizers are available, and no requirement exists for the accommodation of bulky chromophores in the reactive lipids, this strategy is a potentially superior one.

The potential utility of photosensitive liposomes depends on the number of photons per liposome (N_p) required to modify the bilayer properties. This value is the quotient of the number of lipids (N_L) per liposome that must react to produce the required change in the liposome properties and the quantum efficiency (Φ) of the photoreaction of the individual lipids. In turn, Φ is defined as the quotient of the number of molecules that undergo the reaction of interest and the number of photons that are absorbed. Since Φ is defined according to the number of absorbed photons, the inclusion of a sensitizer as only a minor component in a liposome does not reduce the photoefficiency of the liposome system compared, for example, to one composed entirely of

photosensitive lipids. If every absorbed photon results in the reaction of one molecule, Φ is unity. For stepwise reactions in which each reacting lipid must absorb a photon, Φ is usually < 1 , and therefore N_P is usually $> N_L$. But in a chain reaction in which a single absorbed photon causes the reaction of more than one molecule, Φ may be $\gg 1$. When a single excited state lipid causes the reaction of many ground state lipids, the quantum efficiency can be extremely large and N_P may be $\leq N_L$. In the extreme case, where only one photon per liposome must be absorbed in order to initiate a cascade of reactions which in turn cause the requisite modification of the liposome properties, N_P would be unity.

These arguments hold whether an excited state lipid is produced by direct absorption of light, by the transfer of energy from an excited state sensitizer, or from reaction with the product formed from the excited state sensitizer. In these different cases, the quantum yield is defined according to the ratio of the number of absorbed photons and the number of lipids that undergo the final reaction of interest, rather than the number of intermediates produced for example from the excited state sensitizer.

I.D.1. Photopolymerization of Liposomes

Polymerization represents a potential chain reaction that can be effected with lipids in bilayers. Polymerization of bilayers can result in either their stabilization or destabilization. Covalently linking the component lipids of a bilayer reduces their lateral mobility, eliminates the possibility of lipid exchange and desorption, can inhibit insertion of molecules such as proteins into the bilayer, and has even been reported to decrease the permeability of the bilayer to small molecular solutes. In fact, early work to realize

polymerized liposomes was motivated by the recognition that liposomes would leak their aqueous contents when exposed to biological fluids, including serum. Polymerization of liposome bilayers was pursued as one strategy to stabilize liposomes and increase their circulation times in vivo in the blood so that liposomes could be used as depots for either sustained release or targeted delivery of drugs. Since then, strategies have been developed by which polymerization of liposomes can destabilize them and induce either lysis of the membranes or fusion with other membrane bounded structures.

Polymerizable groups have been incorporated into a variety of amphiphiles capable of forming monolayers, micelles, and bilayers. Polymerization is generally successful with single- or multi-chain amphiphiles in monolayers at appropriate surface pressures, or with bilayer-forming amphiphiles, but polymerization of amphiphiles in micelles is generally unsuccessful with the exception of one recent report (Yasuda et al., 1993). Polymerization of supramolecular assemblies is governed by the comparative rates of polymerization and of exchange of the amphiphile between the assembly and the bulk phase (most often an aqueous phase). Under given conditions, the rate of exchange for amphiphiles depends on their monomeric solubility in the bulk phase. Micelle-forming amphiphiles are typically characterized as possessed of single hydrophobic chains and exhibiting relatively high CMC values (10^{-2} to 10^{-4} M). Bilayer-forming amphiphiles typically have at least two hydrophobic chains and exhibit CMC values (10^{-8} to 10^{-10} M) that are several orders of magnitude lower than those expressed by micelle-forming amphiphiles.

A variety of polymerizable groups have been incorporated into lipids that can undergo either stepwise or chain polymerization and have recently

been reviewed (O'Brien & Ramaswami, 1989; Singh & Schnur, 1993; O'Brien, 1994). Polymerization of bilayers has been initiated by thermal decomposition of radical initiators, by redox chemistry, and by both direct photolysis of lipids and by photolysis of sensitizers that generate various initiating species. Photopolymerization of lipids has been successfully demonstrated with acryloyl, methacryloyl, diacetylene, stryryl, dienoyl, muconoyl, and sorbyl groups (Figure I-20). Other polymerizable groups that have been incorporated into amphiphiles include thiol, lipoyl, and isocyanide.

Many different placements of polymerizable groups within various amphiphiles have been achieved and are illustrated in Figure I-21. At a given extent of polymerization (i.e. conversion of monomeric lipid to polylipid) the physical properties of the polymerized membrane (e.g. fluidity, permeability, stability) are strongly influenced by the size of the polymer domains. The length of the polymers depends on many factors including the choice of reactive group, its placement in the amphiphile, the initiation conditions, the phase of the assembly, temperature, etc.

Under given conditions, larger domains are usually expected if the polymers are cross-linked rather than linear. Formation of either linear or cross-linked polymers can usually be controlled by the choice of mono- vs. bis-substituted lipids and the use of appropriate initiation conditions (Figure I-22).

Polymerization of reactive groups in the acyl tails of lipids (Types A and B in Figure I-21) results in the formation of a polymer network in the membrane and can thereby significantly change the bilayer properties. Polymerization of surface associated reactive groups (Types C and D in Figure I-21) to form surface associated polymers does not necessarily lead to a

significant alteration of the hydrophobic region of the bilayer. In two component systems composed of polymerizable and nonpolymerizable lipids, polymerization at either the bilayer interior or the bilayer surface can potentially change the properties of both the bilayer interior and the bilayer surface. Such changes can occur if polymerization effects a lateral phase separation resulting in the transformation of an initially ideally mixed bilayer into a bilayer composed of discrete domains of either unreactive monomeric lipid or polylipid.

An alternative method to preparing polymerized vesicles is to polymerize the component monomeric lipids in isotropic solution, rather than in the liposome assembly, and to afterwards prepare assemblies from the resultant polylipids. This strategy has in fact been achieved with lipid having reactive groups attached to the headgroups via hydrophilic spacers (Type D in Figure I-20) (Kim et al., 1985). Of course, with respect to using a polymerization reaction to induce a change in the liposome membrane (e.g. inducing liposome fusion), this strategy is irrelevant.

Of the various polymerizable groups that have been incorporated into amphiphiles, diacetylene groups have been the most widely studied (Hub et al., 1980; Johnston et al., 1980; O'Brien et al., 1981; Lopez et al., 1982a; Lopez et al., 1982b; O'Brien et al., 1985; Kuo & O'Brien, 1990; Frankel & O'Brien, 1991; Kuo & O'Brien, 1991; Lee & O'Brien, 1992; Markowitz et al., 1992; Schoen et al., 1992). Diacetylene moieties have been incorporated into dialkylammonium lipids, phosphatidylcholines, double-chain glutamate lipids, and a variety of single chain amphiphiles. The diacetylene chromophore exhibits an absorption maximum of 256 nm and can be photochemically polymerized by exposure to

Figure I-20: Photopolymerizable groups that have been incorporated into amphiphiles.

Figure I-21: Polymerizable phospholipids with polymerizable moieties (denoted by X) placed in different positions: at the end of an acyl tail (Type A); in the middle of an acyl tail (Type B); attached to the headgroup via a hydrophilic spacer (Type C); and electrostatically associated with the headgroup (Type D).

Figure I-22: Polymerization of mono- and bis-substituted phospholipids resulting in either linear or cross-linked polymers, respectively.

Mono-Polymerizable Lipid

Linear Polymers

Bis-Polymerizable Lipid

Crosslinked Polymers

ultraviolet light from a low-pressure Hg lamp (254 nm). Diacetylene moieties undergo polymerization in a topotactic fashion which requires that the polymerizable groups be well ordered. For example, diacetylene groups placed too near the lipid backbone in dialkylammonium lipids were unreactive in polymerizations since the chromophores were unable to assume the necessary conformations required for reaction. The groups are usually placed in the center of the chain. In bilayers, diacetylene-containing lipids are typically unreactive in the L_α phase whereas they undergo facile polymerization in the L_β phase. In addition to forming bilayers, diacetylene-containing

amphiphiles form a variety of unusual supramolecular structures including tubules and helices which have been observed in many cases to retain their structures following polymerization. Following polymerization, polydiacetylenes are highly colored, ranging from red or orange to blue with increasing length of the polymer and degree of order in the conjugated π -orbitals.

The styrene moiety has been incorporated at the tails of both dialkylammonium lipids (Ishiwatari & Fendler, 1984; Reed et al., 1984; Fendler, 1985) and phosphatidylcholines (Matsushita et al., 1987). The styrene chromophore exhibits weak absorbance maxima at 273, 282, and 290 nm and can be photochemically polymerized by exposure to ultraviolet light from a high-pressure Hg lamp (360 nm) or a quadrupled ND-YAG laser. Styrene monomers are capable of producing very long polymers and degrees of polymerization (X_n) of up to 400 have been measured for a styrene-PC assembly (Matsushita et al., 1987).

Disulfide or lipoic acid groups within amphiphiles can be polymerized by redox chemistry. With these groups, polymerization proceeds in a stepwise fashion. And even to form linear polymers, two groups per lipid must undergo reaction. Liposomes stabilized by such polymers are attractive for medical applications since the polymer backbone is comprised of biodegradable groups.

Acryloyl and methacryloyl groups have been incorporated at a variety of positions within amphiphiles: at the acyl chain terminus, adjacent to the glycerol backbone, attached via hydrophilic spacers at the headgroup, and electrostatically associated to the headgroup. The groups have been incorporated into both dialkylammonium and PCs. The chromophores exhibit

weak absorption at a maximum of 210 nm (ϵ 3,000) with the result that photopolymerization via direct photolysis of the amphiphiles is difficult. These groups are usually polymerized by thermal or photochemical decomposition of radical initiators such as peroxides, azobis-isobutyronitrile (AIBN), and azocyanovaleic acid (ACVA). The groups are capable of producing high molecular weight polymers, having been reported to produce polymers with $X_n \sim 500$ in dialkylammonium amphiphiles (Dorn et al., 1983; Bolikal & Regen, 1984).

The dienoate amphiphiles comprise a number of different polymerizable groups including dienoyl, muconoyl, and sorbyl. Depending on their structures, the groups can be placed at the acyl chain terminus, at the top of the acyl chain near the lipid backbone, or in the center of the acyl chain. Dienoates have been incorporated into dialkylammonium amphiphiles, PCs, and double-chain glutamate amphiphiles (Ihara et al., 1992). The sorbyl moiety is employed in the work that is presented here. The dienoate moiety exhibits an absorbance maximum of 256 - 260 nm (ϵ 23,000 - 27,000) in both organic and aqueous solutions. When incorporated into amphiphiles, the groups can be photopolymerized by exposure to ultraviolet light from a low-pressure Hg lamp or by γ -irradiation, or chemically polymerized by thermal decomposition of radical initiators such as AIBN or peroxides, or by redox initiators (Tsuchida et al., 1992). Recently, the photosensitized polymerization of dienoate-PC was demonstrated using a visible light sensitive membrane-ballasted indocarbocyanine dye (Armitage et al., 1994). Dienoate groups can be polymerized in both liquid-crystalline and gel phase bilayers.

Dienoyl-PCs contain the polymerizable dienoyl groups near the glycerol backbone at the top of the acyl chains. Initiation by either UV-photolysis or γ -

irradiation facilitated complete conversion of the monomers to polymers, and polymerization of mono- and bis-substituted dienoyl-PCs was reported to produce linear and cross-linked polymers, respectively (Tsuchida et al., 1992). Polymerization of bis-dienoyl PC (bis-DenPC) by the radicals formed from the thermal decomposition of either the lipid soluble AIBN or the water soluble azobis-(2-amidinopropane) dihydrochloride (AAPD) proceeded to only ca. 50% conversion, and the polymerization produced only linear polymers. In the presence of both initiators, complete conversion of the monomers was achieved and a cross-linked polymer gel was produced. On this basis, the authors suggested that the dienoyl groups in the sn-1 and sn-2 chains of bis-DenPC are present in environments of sufficiently different polarity that they can be selectively polymerized with appropriate initiators. The hydrophobic radicals generated from the decomposition of AIBN react selectively with the sn-1 dienoyl groups that are buried in the hydrophobic region of the bilayer, while the water soluble radicals formed by AAPD react selectively with the sn-2 dienoyl groups that are situated in the aqueous/hydrocarbon interfacial region.

Sorbyl-PCs contain sorbyl groups at the acyl chain termini. Since the sorbyl groups are situated in the middle of the bilayer, selective polymerization of the sn-1 or sn-2 chains of bis-SorbPC is not possible.

Polymerization of mono- and bis-SorbPC (Figure I-23) has been demonstrated to result in the formation of linear and cross-linked polymers whether the reaction is initiated photochemically or by thermal decomposition of AIBN (Lamparski & O'Brien, 1994).

Figure I-23: Polymerizable sorbyl-PC lipids: bis-SorbPC_{17,17} and mono-SorbPC_{16,17}.

As mentioned above, polymerization of liposome bilayers can result in either their stabilization or destabilization. Polymerization of bi- or multi-component bilayers was shown to induce a lateral separation of the reactive and nonreactive bilayer components (Gaub et al., 1985; Tyminski et al., 1985;

Tyminski et al., 1987; Armitage et al., 1993). Tsuchida and coworkers (Takeoda et al., 1989; Takeoka et al., 1991) demonstrated the polymerization induced lateral phase separation of two component liposomes prepared from polymerizable bis-DenPC and either PC or saturated acyl-tail PE.

Polymerization was initiated either by thermal decomposition of radical initiators or by direct exposure of the polymerizable lipids with γ -irradiation and produced cross-linked polymeric domains. The resulting polymers could be "skeletonized" by removing the monomeric regions of the mixed liposomes either by drying the liposome suspensions and washing with chloroform, or by treating the liposome suspensions with detergents (Ohno et al., 1985). The polymeric lipid skeleton that remained after such treatments was imaged by electron microscopy to obtain dramatic evidence for the ability of the cross-linking of polymerizable lipids in two-component liposomes to effect efficient lateral separation of the different lipids.

The photopolymerization induced destabilization of multilamellar liposomes (MLV) has been demonstrated with two component systems containing the polymerizable bis-SorbPC and an unsaturated polymorphic PE, DOPE (Figure I-24). MLV destabilization was proposed to occur through intraliposomal membrane contact following photoinduced domain formation (Lamparski et al., 1992).

Figure I-24: Photoinduced leakage of DOPE/bis-SorbPC (3:1) MLV via intraliposomal membrane formation of a fusion pore (Lamparski et al., 1992).

I.D.2. Photomodification of Lipid Headgroups

The photoconversion of liposome lipid head groups represents a potential method by which to induce liposome fusion with light. Several strategies for the use of light to change the electrostatic and hydration characteristics of membranes can be readily envisioned: 1.) photocleavage of hydrophilic moieties in lipid headgroups to decrease the hydration barrier and facilitate membrane adhesion; 2.) photoconversion of negatively charged lipid headgroups to neutral ones to reduce electrostatic repulsion between like charged liposomes; and 3.) photoconversion of neutral or negatively charged lipid headgroups to positively charged ones to produce attractive electrostatic forces between unlike charged liposomes. If the lipids themselves are designed to absorb light, the chromophores must be hydrophilic to be accommodated in the head group region of the lipids. If sensitized photoconversion of lipids is to be used, the lipid head groups should be designed with a labile, reactive functional group which can be accommodated in the headgroup.

With respect to induction of membrane fusion by changing the polarity of the membrane surface, photoconversion of bilayer competent amphiphiles to species incapable of forming membranes is a poor strategy if the transformation compromises the membrane integrity. For example, conversion of a hydrophilic headgroup to one that is uncharged and hydrophobic may result in the loss of its bilayer competence. The high energy that would result from exposure of the hydrophobic head group at the water interface would probably force the molecule into the bilayer interior. Thus the desired change in the electrostatics or hydration of the membrane is not achieved, and an undesirable change in the bilayer interior instead results. Effecting more subtle changes in the polarity of the headgroup is a superior strategy. An example of such a change is the biologically important enzymatic conversion of PI to DAG. The inositol headgroup, with its numerous hydroxyls, is well hydrated and thereby very hydrophilic and bilayer competent. Cleavage of the inositol by phospholipase C results in the formation of DAG whose headgroup contains a single hydroxyl. Although DAG is less polar than PI, its headgroup is sufficiently polar to be aligned at the water/hydrocarbon interface.

Fuhrhop and coworkers (Fuhrhop et al., 1981; Fuhrhop & Mathieu, 1984) prepared amphiphiles containing benzenediazonium headgroups and acyl-linked aliphatic tails. The double-chain amphiphiles formed stable vesicles at pH 1 to 2, but were destabilized at higher pH values due to hydrolysis of the acyl chain linkages. Photosensitized decomposition of the vesicles in the presence of 0.1 mol % of the sensitizer 5, 10, 15, 20-porphyrinetetrakis(9-decene sulfonic acid) at the low pH values could be effected by irradiation with visible irradiation from a 60 W tungsten lamp (Figure I-25). Although the

photoproduct was not characterized, the photolysis presumably effected photoconversion of the hydrophilic and cationic headgroup to an uncharged and completely hydrophobic one via loss of the diazonium group as molecular nitrogen. This was the first report of *visible* light modification of synthetic lipid vesicles.

Figure I-25: Photosensitized decomposition of a benzenediazonium amphiphile.

Haubs and Ringsdorf (1987) developed photosensitive, bilayer-forming amphiphiles containing quaternary benzylammonium halide headgroups and ether-linked aliphatic tails. The double-chain amphiphiles formed vesicles when they were hydrated above their main phase transition temperature

($T_m = 36^\circ\text{C}$). The aliphatic-chain ether linkage presumably obviated the hydrophobic chain hydrolysis that Fuhrhop et al. observed in their system (Fuhrhop et al., 1981; Fuhrhop & Mathieu, 1984). The bilayers were sufficiently well packed that water-soluble dyes could be trapped in the aqueous interior of the vesicles. Photolysis with 254 nm light destroyed the vesicles as a result of the conversion of the cationic, quaternary ammonium headgroup to an uncharged and completely hydrophobic species (Figure I-26). By optical microscopy, the authors observed that during ultraviolet exposure, giant vesicles (diameter $> 10\ \mu\text{m}$) shrank in diameter while the thickness of the bilayer apparently increased. The authors proposed that the increased bilayer thickness was likely due the solubilization of the ballasted toluene photoproduct in the remaining intact bilayer. According to this hypothesis, the collapse of the bilayer occurred when the amount of bilayer-competent material in each vesicle became too low to accommodate the increasing amount of the photoproduct.

Figure I-26: Photocleavage of the benzylammonium halide headgroup of a quaternary benzylammonium double-chain amphiphile.

Haubs and Ringsdorf (Haubs & Ringsdorf, 1985; Haubs & Ringsdorf, 1987) prepared and characterized the photochemistry of another type of amphiphile whose polarity is changed on exposure to short wavelength visible light. Single- and double-chain amphiphiles were prepared whose headgroups were composed of derivatives of N-(1-pyridinio)amidates. On exposure to 366 nm light from high pressure Hg lamp, the headgroup pyridinium ylide functionality was converted to a 1,2-diazepine moiety (Figure I-27). Although the single chain derivatives did not form bilayers, vesicles could be formed from the double-chain N-(1-pyridinio)amidates under conditions where a critical fraction of the head groups was protonated and thereby rendered cationic. The zwitterionic form of the ylide was insufficiently hydrophilic to stabilize bilayer structures, but even a small

Figure I-27: Photoinduced conversion of polar, zwitterionic N-(1-pyridinio)amidate amphiphiles to nonpolar, uncharged 1,2-diazepine amphiphiles.

fraction of the cationic form of the ylide was a potent stabilizer of the lamellar phase. At pH 3 vesicles could be formed due to the presence of just 15% protonation of the pyridinium ylide headgroups. Protonation of the ylide headgroups renders them insensitive to 366 nm photolysis, but the unprotonated amphiphiles, comprising the major fraction of the bilayer, undergo a photoconversion to the uncharged 1,2-diazapine on absorption of 366 nm light. Since the diazapine headgroup is less polar and therefore less hydrophilic than the ylide headgroups, the hydration of the vesicle surface should occur which could facilitate vesicle aggregation and contact-dependent destabilization. However, no vesicle aggregation was observed. This could likely be explained by the existence of sufficient residual charge on the vesicles to prevent close approach of their membranes.

More recently, Morgan and coworkers (Chowdhary et al., 1993) reported that mixed liposomes composed of DPPC and N-stearoyl-L-histidine (NSH) and the sensitizer dihematoporphyrin (DHE) underwent photosensitive lysis and release of their contents on exposure to blue light. Sodium chloride could be trapped within liposomes containing up to 35 mol % NSH and were stable in the dark for several days. On exposure of DHE-containing liposomes to light from a 300 W "halight" slide projector equipped with a blue tungsten correction filter (400 - 450 nm), the liposomes underwent photolysis-dependent leakage. The leakage mechanism is attributed to the photosensitized oxidation and cleavage of the histidine headgroup of NSH to produce an aspartate headgroup. The photoreaction is dependent on the presence of oxygen and is thought to occur as a result of reaction of histidine with singlet oxygen. However, characterization of the photoproduct was not reported. Such a photocleavage

reaction would convert the single tail NSH amphiphile from a single negatively charged species to a doubly charged one.

The observation that NSH can be incorporated up to 35 mol % in liposomes composed primarily of DPPC indicates that NSH is a poor detergent having a relatively high CMC value. Photoconversion of NSH to a doubly charged species would be expected to dramatically decrease its CMC value and to thereby improve its solubilization properties. The % leakage of the liposomes that could be achieved under different conditions was not reported. And unfortunately, the efficiency of this system can not be gauged since neither the quantum yield for the photoreaction nor the percentage reaction of NSH per liposome required to induce leakage was given.

The authors suggested that liposome fusion occurred based on electron microscopy evidence that the liposomes increased in size as a result of photolysis. However, contents mixing between different liposome populations was not demonstrated. Because of the purported photoproduction of an amphiphile with surfactant qualities and whose presence would increase the liposome surface charge, the probability of liposome leakage occurring as a result of membrane-contact dependent fusion seems small. A greater likelihood should exist for membrane-contact independent lysis resulting from membrane disruption by the NSH photoproduct and formation of lipid/detergent mixed micelles.

I.D.3. Photocleavage of Lipid Tails

The conversion of bilayer-competent amphiphiles to bilayer-incompetent molecules is a good strategy for inducing liposome lysis and leakage of their aqueous contents. But as was stated above, formation of

photoproducts that compromise the membrane integrity is usually a poor strategy to induce membrane fusion. Photoconversion of double-chain, bilayer-competent amphiphiles to single-chain, bilayer-incompetent ones has been accomplished by Anderson and Thompson (1992). Production of single-chain amphiphiles in subsolubilizing concentrations may be a useful fusion strategy if the presence of such amphiphiles makes the bilayer fusogenic. But when the single chain amphiphiles are formed in sufficient number to actually cause solubilization of the membrane via mixed micelle formation, fusion cannot result since the bilayer structure is lost. Most single-chain amphiphiles, including as lyso PC, exhibit positive intrinsic curvatures and their presence in membranes inhibits the formation of fusion intermediates (Yeagle et al., 1994).

Kusumi et al. (Kusumi et al., 1989) prepared bis-substituted phosphatidylcholines containing 2-nitrobenzyl groups at both chain termini. Upon UV-light irradiation, the mononitrobenzyl esters were apparently cleaved to generate carboxylate moieties at the PC chain termini and the center of the hydrophobic region of the bilayer (Figure I-28). This putative photoreaction resulted in the catastrophic loss of bilayer integrity, as evidenced by the release of entrapped macromolecules of 45 kilodaltons, and disintegration of the liposomes as observed by phase contrast microscopy.

Anderson and Thompson (Anderson & Thompson, 1992b) have developed a photosensitive liposome system based on the peroxidation of lipids by photodynamic sensitization. The liposomes are composed principally of plasmenylcholines (plasmalogens). Plasmalogens are abundant in heart tissue and are a subcategory of phosphatidylcholines that share the identical ethanolamine headgroup and glycerol backbone but whose

Figure I-28: Photoinduced cleavage of 2-nitrobenzylate moieties at the acyl-chain termini of synthetic phospholipids (Kusumi et al., 1989).

sn-1 chains are attached to the glycerol backbone via a vinyl ether rather than an acyl linkage. Photooxidation of the sn-1 vinyl ether linkage results in the cleavage of the sn-1 chain via formation of lyso-PC and a fatty aldehyde (Figure I-29). Incorporation of lyso-lipids into synthetic bilayers or biomembranes typically leads to increased membrane fluidity, lipid flip-flop, and solute permeability.

Figure I-29: Photocleavage of plasmalogen sn-1 acyl-tail via photosensitized oxidation of the sn-1 vinyl ether linkage.

Vesicles were prepared from a semisynthetic plasmalogen whose sn-2 chain was palmitic, and whose sn-1 chain was a mixture of chain lengths with the 16 carbon length predominating. Vesicles were prepared from mixtures of plasmalogen and DPPS (9:1, mol/mol) by freeze/thaw - extrusion through 100 and 80 nm Nuclepore filters at a temperature above the main phase transition (38.9°C). Either of two photosensitizers was incorporated into the liposome

systems. A hydrophobic zinc phthalocyanine (ZnPc) was incorporated into the lipid bilayer (Anderson & Thompson, 1992b). Or a hydrophilic aluminum chlorophthalocyanine tetrasulfonate (AlClPcS₄) was trapped in the interior liposome aqueous compartments (Anderson & Thompson, 1992a). The ZnPc system was sensitive to exposure with >640 nm (red) light, as evidenced by increased permeability of entrapped glucose following photolysis. The AlClPcS₄ system exhibited an absorption maxima at 672 nm and was also sensitive to exposure with red light. In this case, the photooxidation of the plasmalogen was ascribed to the formation of singlet molecular oxygen.

I.D.4. Photoisomerization of Acyl Tails

The photoisomerization of cis-retinoyl in rhodopsin stimulated the incorporation of a variety of photoisomerizable moieties within the acyl chains of membrane-forming lipids. Both mono- and bis-substituted lipids have been formed and the effects of photoisomerization of a variety of moieties on the bilayer properties have been assessed.

Morgan and coworkers (Morgan et al., 1987) prepared both mono- and bis-substituted PCs whose acyl chains contained a photoisomerizable azobenzene chromophore. The lipids, 1-palmitoyl-2-(4-n-butylphenylzo-4'-phenylbutyroyl)-L- α -phosphatidylcholine (mono-AzoPC), and bis-(4-n-butylphenylzo-4'-phenylbutyroyl)-L- α -phosphatidylcholine (bis-AzoPC), underwent trans-to-cis isomerization on irradiation by 360 nm light from a high-pressure mercury lamp, and the reverse reaction on photolysis by visible light of >400 nm from a tungsten lamp (Figure I-30).

Figure I-30: Trans- to cis-photoisomerization of the azobenzene chromophores in bis-AzoPC.

Photoisomerization of the azobenzene chromophore from the cis- to the trans-form increases the effective molecular area of the acyl chain.

Irradiation of liposomes composed entirely of bis-AzoPC, or of DPPC and 6 mol % bis-AzoPC, did not result in the loss of the bilayer structure, but did result in bilayer destabilization and the leakage of aqueous contents. Liposomes formed in whole or part from mono-AzoPC did not exhibit photoisomerization-induced destabilization.

In addition to photoinduced leakage, the bis-AzoPC liposomes were also observed to undergo photoinduced aggregation and the formation of larger structures as evidenced by transmission electron micrographs recorded before and after liposome photolysis. The authors suggested that the increase in

liposome size observed in the electron micrographs was due to liposome fusion. Unfortunately, neither lipid mixing nor aqueous contents mixing was demonstrated, and the issue of whether genuine fusion did occur is unresolved. Nonetheless, the possibility that photoisomerization of the bis-AzoPC acyl chains leads to bilayer destabilization and fusion, perhaps via reduction of the membrane hydration and formation of nonlamellar intermediates, is an intriguing idea. And despite the fact that the bis-AzoPC possesses a well hydrated PC headgroup, the altered lateral interactions of the lipids that result upon photoisomerization might be similar to those of the nonlamellar competent 2-acyl substituted PCs reported by Lewis et al. (1994).

I.D.5. Photomodulation of Membrane Binding to Polymers

Polymers have been associated with bilayers by three strategies: electrostatic association via oppositely charged groups on the polymer and the lipid headgroups (Aliev et al., 1984; Regen et al., 1984; Fukuda et al., 1986; Ringsdorf et al., 1988; Higashi et al., 1990); anchoring of an otherwise hydrophilic polymer into the bilayer by attached hydrophobic pendant groups or lipids (Takada et al., 1984; Moellerfeld et al., 1986; Maeda et al., 1988; Sunamoto et al., 1992; Woodle & Lasic, 1992; Kono et al., 1994); and specific recognition of polymers and bilayers via formation of hydrogen bonds between complementary nucleotide bases (Pinilla et al., 1994). Tirrell and coworkers (Maeda et al., 1988) coated the exterior of PC vesicles with poly(2-ethylacrylic acid) (PEAA) by Michael addition of thiol groups on the polymer with a minor membrane component composed of maleimide-derivatized PE. PEAA undergoes a conformation change from an expanded, negatively charged coil at basic pH, to a uncharged, compact globular structure upon

protonation. Thus the character of the polymer can be transformed from hydrophilic to hydrophobic on proton stimulus. Vesicle suspensions in the presence of free PEAA or with covalently anchored PEAA are stable above pH 7.0, but are converted to polymer/lipid mixed micelles upon protonation to decrease the pH to 6.0 - 6.5. Disruption of vesicles in the presence of free PEAA was achieved photochemically by 254 nm irradiation of a water-soluble proton source, 3,3'-dicarboxydiphenyliodonium disulfate (You & Tirrell, 1991).

A similar strategy was recently employed to produce pH-sensitive PC liposomes coated with succinylated poly(ethylene glycol) derivatives (Kono et al., 1994). The work is an extension of the investigations of the ability of poly(ethylene glycol) (PEG) to induce fusion between liposomes by dehydrating the membrane surface and exerting osmotic stress on the lipid bilayers (Section I.C.4.e.). Poly(glycidol) has the same main chain structure as PEG and hydroxyl side chains via which the polymer can be derivatized. The poly(glycidol) pendant hydroxyl groups were partially succinylated to introduce carboxylic acid residues on the polymer chain. In turn, a fraction of the carboxyl groups were reacted with n-decylamine in order to attach hydrophobic groups on the polymer by which the polymer was anchored in the PC vesicles. The unreacted carboxyl groups possessed an apparent pK value of 5.4 and imparted pH-sensitivity to the polymer. At and above neutral pH, the polymers are negatively charged and sufficiently hydrated so that they do not strongly associate with the lipid bilayers. On decreasing the pH to 4.0, the carboxylate residues were neutralized and the polymers became strongly associated with the membrane surface. This resulted in membrane destabilization as evidenced by leakage of calcein and mixing of lipids between different liposome populations. Although the authors claim that such

destabilization resulted in fusion of different liposome populations, they did not demonstrate pH-induced mixing of aqueous contents between different liposome populations.

If this liposome system were combined with a photosensitive proton source, such as the 3,3'-dicarboxydiphenyliodonium disulfate employed by You and Tirrell (1989), these liposomes would be potentially capable of undergoing photoinduced fusion.

I.E. Research Goal

The goal of this research was the demonstration of the use of ultraviolet light to isothermally photodestabilize unilamellar liposomes and induce membrane fusion under physiologically relevant conditions. A larger goal related to this research is the development of a liposome system that can undergo fusion with cellular membranes following stimulus by visible or infrared light. A membrane system that could be controllably induced to undergo fusion with other membranes, by the use of light, would be potentially useful as an *in vitro* or *in vivo* drug delivery system. We envisioned these liposomes to be useful as *in vivo* drug delivery vehicles if their exposure to light could be used to control whether a toxic drug contained within their aqueous interior were allowed access to the cytoplasm of a given set of cells in the body. Access of the toxin to cells *in vivo* would rely on the photolysis of the liposomes after they were injected in to the body and were localized at the target cells. Liposomes not localized at the target cells and therefore not photolyzed would remain stable and would not deliver their toxic contents to any healthy cells.

This research is an extension of the work of Lamparski et al. (1992) in which photodestabilization of multilamellar liposomes was shown to induce leakage of the liposomal aqueous contents. The same lipid system was used in this research as was employed by Lamparski et al (1992). We chose a liposome system that can be photodestabilized as a result of a chain polymerization reaction since the destabilization should be made efficient, with respect to the number of photons required per liposome to induce membrane fusion, by chain reaction of many lipids following the activation of a single lipid. In the liposome systems used here, ultraviolet light induced polymerization is known to proceed by a step-wise, rather than a chain process. Since the liposomes can be destabilized as the result of a polymerization reaction initiated by a chain reaction, other means of destabilizing this liposome system will be investigated in the future.

CHAPTER II

EXPERIMENTAL

II.A. Materials

Di-oleoylphosphatidylethanolamine (DOPE), di-oleoylphosphatidylcholine (DOPC), N-(7-nitro-di-oleoylphosphatidylethanolamine (NBD-PE), and N-(lissamine Rhodamine B sulfonyl)-phosphatidylethanolamine (Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL) and used without purification (one spot TLC, 65:25:4, CHCl₃/CH₃OH/H₂O). 1,2-Bis-[10-(2',4'-hexadienoyloxy)decanoyl]-sn-glycero-3-phosphatidylcholine (bis-SorbPC) and 1-palmitoyl-2-[10-(2',4'-hexadienoyloxy)decanoyl]-sn-glycero-3-phosphatidylcholine (mono-SorbPC) were prepared as described previously (Lamparski et al., 1992). 1-Aminonaphthalene-3,6,8-trisulfonic acid (disodium salt) (ANTS) and p-xylylenebis(pyridinium bromide) (DPX) were obtained from Molecular Probes, Inc. (Junction City, OR). Water was distilled, then purified by a Milli Q filtration system (Millipore Corp.).

Oxalyl chloride, pyridinium dichromate (PDC), 4-(N,N-dimethylamino)pyridine (DMAP), and 1,3-dicyclohexylcarbodiimide (DCC) were obtained from Aldrich Chemical Co. (Milwaukee, WI) and were used as received. L-glycerophosphatidylcholine-cadmium chloride (GPC-CdCl₂) was purchased from Sigma Chemical Company (St. Louis, MO). Ion exchange resin AG 501-X8 was obtained from Bio-Rad (Richmond, CA). Silica gel for flash chromatography (200 - 425 mesh) was obtained from Fisher Scientific. Silicic acid for lipid chromatography (Biosil-A, 200 - 400 mesh) was obtained from Bio-Rad. Tetrahydrofuran (THF) and benzene were dried by distillation in the

presence of sodium. Chloroform was dried by distillation in the presence of phosphorus pentoxide. N,N-Dimethylformamide (DMF) was vacuum distilled and stored over molecular sieves (5Å).

II.B. Synthesis of Polymerizable Sorbyl-Lipids

1,2-Bis-[10-(2',4'-hexadienoyloxy)decanoyl]-sn-glycero-3-phosphatidylcholine (bis-SorbPC) and 1-palmitoyl-2-[10-(2',4'-hexadienoyloxy)decanoyl]-sn-glycero-3-phosphatidylcholine (mono-SorbPC) were prepared as described previously (Lamparski et al., 1992). All reactions were performed under yellow safe lights or in the dark to discourage photopolymerization of the sorbyl-containing compounds. The products were characterized by ^1H NMR as shown in the Appendix.

Sorbyl chloride was prepared from oxalyl chloride and 2,4-hexadienoic acid. Oxalyl chloride (100 g, 0.79 moles) was added dropwise to 2,4-hexadienoic acid (71.6 g, 0.64 moles) over ca. 1 hr at rt with magnetic stirring and the reaction was allowed to proceed overnight. The resulting pale yellow liquid was distilled under vacuum (1 Torr) at 50°C to produce a colorless liquid that could be stored several weeks at -20°C. Yield 78 g (93%). ^1H NMR (CDCl_3) 1.80 - 1.90 ppm (d, 3H, CH_3), 5.70 - 5.80 ppm (d, 1H, $\text{CH}=\text{CHCO}_2$), 6.10 - 6.40 ppm (m, 2H, $=\text{CHCH}=\text{CH}$), 7.15 - 7.30 ppm (m, 1H, $=\text{CHCH}_3$).

10-(Sorbyloxy)decan-1-ol was prepared by acylation of 1,10-decanediol with sorbyl chloride. Freshly distilled sorbyl chloride (31.0 g, 0.24 moles) was taken up in dry THF (100 mL) and added dropwise over ca. 2 hrs. to a solution of 1,10-decanediol (41.82 g, 0.24 moles) and pyridine (80 g, 1.01 moles) in THF (1.5 L) at rt and the reaction was allowed to proceed with magnetic stirring overnight. The solution was filtered by gravity to remove the white pyridine

hydrochloride precipitate and the resulting yellow filtrate was concentrated by rotary evaporation to an oil. TLC (hexanes/ethyl acetate, 9:1) revealed that the oil consisted of a mixture of the diol starting material and two principal products, presumably the mono- and bis-sorbyl esters of 1,10-decandiol. The product of interest was well resolved by TLC having an R_f (0.3) intermediate between the diol and the symmetric diester. The product was isolated by flash silica gel chromatography using a mixture of hexane and ethyl acetate (9:1) as eluent. Yield 35%; mp 33 - 34°C. TLC R_f = 0.3 (hexanes/ethyl acetate, 9:1). ^1H NMR (CDCl_3) 1.20 - 1.40 ppm (broad s, 12 H, CH_2), 1.45 - 1.65 ppm (m, 4H, $\text{CH}_2\text{CH}_2\text{R}$), 1.70 ppm (s, 1H, OH), 1.76 - 1.85 ppm (d, 3H, $\text{C}=\text{CHCH}_3$), 3.52 - 3.65 ppm (t, 2H, CH_2OH), 4.04 - 4.13 ppm (t, 2H, CO_2CH_2), 5.68 - 5.79 ppm (d, 1H, $\text{C}=\text{CHCO}_2$), 6.05 - 6.22 ppm (m, 2H, $=\text{CHCH}_2$), 7.12 - 7.27 ppm (m, 1H, $\text{CH}=\text{CHCH}_3$).

10-(Sorbyloxy)decanoic acid was prepared by oxidation of the corresponding alcohol in the presence of pyridinium dichromate (PDC). 10-(Sorbyloxy)decan-1-ol (20.1 g, 0.075 moles) in DMF (75 mL) was added dropwise to PDC (80.4 g, 0.21 moles) in DMF (75 mL) over ca. 2 hr at 4°C. Following addition, the reaction mixture was allowed to slowly warm to rt and was magnetically stirred overnight. The resulting purple suspension was diluted with an equivalent volume (150 mL) of ethyl acetate. The insoluble PDC was removed by vacuum filtration through ca. 200 g of flash silica gel suspended on filter paper in a Büchner funnel. The silica gel was washed with ethyl acetate (2 x 500 mL). The filtrate was concentrated to an oil by rotary evaporation. The product was isolated by flash silica gel chromatography using hexane/ethyl acetate (8:2) as eluent. Yield 68%; mp 54 - 55°C. TLC R_f = 0.15 - 0.35 (hexanes/ethyl acetate, 8:2). ^1H NMR (CDCl_3) 1.20 - 1.40 ppm (br s, 1OH, CH_2), 1.52 - 1.70 ppm (m, 4H, $\text{CH}_2\text{CH}_2\text{R}$), 1.78 - 1.86 ppm (d, 3H, $\text{C}=\text{CHCH}_3$), 2.28 -

2.37 ppm (t, 2H, CH₂CO₂H), 4.05 - 4.15 ppm (t, 2H, CO₂CH₂), 5.70 - 5.79 ppm (d, 1H, C=CHCO₂), 6.05 - 6.22 ppm (m, 2H, =CHCH=), 7.15 - 7.27 ppm (m, 1H, CH=CHCH₃).

1,2-Bis[10-(sorbyloxy)decanoyl]-sn-glycero-3-phosphatidylcholine (bis-SorbPC_{17,17}) was prepared by acylation of L-glycerophosphorylcholine-cadmium chloride complex (GPC-CdCl₂) at both hydroxyl positions with 10-(sorbyloxy)decanoic acid. GPC-CdCl₂ (460 mg, 1.04 mmoles) was dried by repeated (3 x) evaporation from a benzene suspension (5 mL) followed by evacuation under high vacuum overnight. To the dried GPC-CdCl₂, 10-(sorbyloxy)decanoic acid (1.1.2g, 3.90 mmoles), DMAP (265 mg, 2.17 mmoles), and DCC (700 mg, 3.39 mmoles) were added. The mixture was evacuated under high vacuum overnight and then was suspended in 10 mL of dry chloroform. The reaction was allowed to proceed in the dark, under argon, and at room temperature for five days. After this time, no change in composition of the reaction mixture was evident by TLC (65/25/4, CHCl₃, CH₃OH, H₂O). The white precipitate composed of the by-product dicyclohexylurea was removed by gravity filtration and washed with chloroform (2 x 20 mL). The filtrate was concentrated to ca. 15 mL by rotary evaporation. The filtrate was diluted with an equal volume of methanol, filtered by gravity, and the precipitate was washed with methanol (2 x 15 mL). The filtrate was stirred for 2 hr in the presence of Bio-Rad AG 501-X8 ion exchange resin (7 g). The resin was separated by gravity filtration and washed with a 1:1 mixture of methanol and chloroform (2 x 20 mL). The filtrate was concentrated to ca. 5 mL by rotary evaporation and the product was isolated by flash silica gel chromatography (2.5 x 35 cm column bed; Biosil A, 200 - 400 mesh) using 65/25/4, CHCl₃, CH₃OH, H₂O as eluent. The fractions containing the product were combined and concentrated to ca. 25 mL by rotary evaporation. Care was taken not to

concentrate the lipid to dryness. Benzene (50 mL) was repeatedly (3 x) added to the mixture which was afterwards concentrated to ca. 25 mL in order to azeotropically remove the water introduced by the chromatography eluent. Finally, the product was concentrated to dryness and immediately taken up in benzene. The product was stored as an amorphous ice (-30°C) in benzene after bubbling with argon. Yield 10 % (based on GPC). TLC R_f = 0.48 - 0.40 (65/25/4, CHCl_3 , CH_3OH , H_2O). ^1H NMR δ : 1.20 - 1.40 ppm (br s, 20H, CH_2), 1.48 - 1.70 ppm (m, 8H, $\text{CH}_2\text{CH}_2\text{R}$), 1.80 - 1.88 ppm (d, 6H, $=\text{CHCH}_3$), 2.20 - 2.32 ppm (m, 4H, $\text{CH}_2\text{CO}_2\text{R}$), 3.30 - 3.40 ppm (s, 9H, NCH_3), 3.75 - 3.83 ppm (br s, 2H, NCH_2CH_2), 3.88 - 4.00 ppm (br s, 2H, NCH_2CH_2), 4.05 - 4.12 ppm (t, 4H, $=\text{CHCO}_2\text{CH}_2$), 4.14 - 4.42 ppm (br m, 4H, POCH_2CH and CHCH_2CO_2), 5.14 - 5.25 ppm (br s, 1H, POCH_2CH), 5.72 - 5.80 ppm (d, 2H, $\text{O}_2\text{CCH}=\text{}$), 6.05 - 6.24 ppm (m, 4H, $=\text{CHCH}=\text{}$), 7.16 - 7.28 ppm (m, 2H, $=\text{CHCH}_3$). UV (λ_{max} = 258 nm, ϵ = 47,100, CH_3OH).

II.C. Liposome Preparation

Large unilamellar vesicles (LUV) were prepared by freeze/thaw -- extrusion techniques (Hope et al., 1985; Mayer et al., 1986). Lipid stock solutions were mixed in benzene, then lipid films (7 - 21 μ mole) were prepared in 10 mL flasks by evaporating the solvent under a stream of nitrogen gas and drying under vacuum for several hours. The resulting lipid film was hydrated by addition of the appropriate buffer followed by vortexing and brief irradiation in a bath-type sonicator. The lipid suspensions were freeze-thawed five times, employing alternating cycles between dry ice/isopropanol and warm (30°C) water baths. The freeze-thawed lipid suspensions were stored overnight at -40°C or were immediately extruded ten times through two stacked 0.1 μm pore size Nuclepore polycarbonate filters at room temperature

with nitrogen pressures of 300-500 psi using a stainless steel extrusion device (Lipex Biomembranes, Vancouver, B. C., Canada). The resulting populations of LUV were determined by quasi-elastic light scattering to be of monodisperse size with an average diameter of 120 ± 10 nm (Kölchens et al., 1993).

For lipid mixing assays, pairs of fluorescently labeled liposomes were prepared containing equivalent amounts of NBD-PE and Rh-PE. The fluorophores were added to weighed lipid films from an equimolar chloroform stock solution of NBD-PE and Rh-PE. For the first lipid mixing protocol, the LUV contained either 0.6 mol % or 0.3 mol % of each of the fluorophores. The mixed lipid films were hydrated with Tris buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4) to 13 mM before formation of LUV by extrusion. For the second lipid mixing protocol, the LUV contained 1.0 mol % or 0.1 mol % of each of the probes. In this case, the mixed lipid films were hydrated with glycine buffer (115 mM NaCl, 10 mM glycine, pH 9.5) to 13 mM before formation of LUV by extrusion.

LUV for the ANTS/DPX fusion assays were prepared according to methods described previously (Ellens et al., 1985; Ellens et al., 1989) with some modifications. LUV contained either (i) 25 mM ANTS and 60 mM NaCl, or (ii) 90 mM DPX. Both solutions were buffered with 10 mM glycine at pH 9.5. The ANTS/DPX leakage assays were also performed according to established methods (Ellens et al., 1987). LUV contained either (i) 12.5 mM ANTS, 45 mM DPX, and 30 mM NaCl buffered with 10 mM glycine at pH 9.5, or (ii) glycine buffer (10 mM glycine, 115 mM NaCl, and 0.1 mM EDTA, pH 9.5). Encapsulated material was separated from unencapsulated material on Sephacryl S-300 (Pharmacia) gel filtration columns (1.6 x 20 cm) using glycine buffer as the eluent. In all cases, the encapsulated solutions were isoosmotic to the buffers

used for gel filtration chromatography and for the subsequent fusion and leakage assays. In these experiments, the osmolarities of all buffers were determined to be 220 mosmol/Kg (Osmette S Osmometer, Precision Instruments). Vesicle concentration was determined spectroscopically from the bis-SorbPC chromophore ($\epsilon = 47,100$, $\lambda_{\text{max}} = 258$ nm, methanol) or from the mono-SorbPC chromophore ($\epsilon = 23,550$, $\lambda_{\text{max}} = 258$ nm, methanol) by diluting 25 μL aliquots of the chromatographed LUV suspensions into 1 mL volumes of methanol.

II.D. Liposome Photolysis

LUV samples (3.0 mL) were placed 1 cm from a low-pressure mercury vapor pen lamp (254 nm) in a stirred 3.5 mL fluorescence quartz cuvette which was thermostatted at 37°C. A Corning CS-9-54 filter (>230 nm) was used to attenuate the intensity of the 254 nm light incident on the sample. Photopolymerization was carried out continuously for each new sample, and photolysis times ranged from 0 to 3 min. The loss of monomeric mono-SorbPC or bis-SorbPC was monitored by the decrease in the UV absorption of the aqueous LUV suspensions at 258 nm. The extent of conversion of monomer (% polymerization) was determined using the following equation: % polymerization = $[(A_0 - A_t)/(A_0 - A_\infty)] \times 100$, where A_0 is the initial absorbance, A_t the absorbance after t min irradiation, and A_∞ the absorbance after complete conversion of monomer. For both the DOPE/bis-SorbPC and DOPE/mono-SorbPC systems, 100% polymerization was achieved by a 3.0 min exposure, and 50% polymerization was achieved by 0.5 min exposure of the bis-SorbPC system or 0.66 min exposure of the mono-SorbPC system.

II.E. Fluorescence Measurements

Fluorescence was measured with a Spex Fluorolog 2 fluorimeter (Spex Industries, Inc., Edison, NJ). The cuvette holder was thermostatted at various temperatures by a water circulator, and samples were thermally equilibrated for at least 5 min before the assays were initiated. For lipid mixing studies, excitation was 450 nm and emission was 530 nm; band slits were 2 mm for excitation and 4 mm for emission. For ANTS/DPX fusion and leakage studies, the excitation was 360 nm and emission was 520 nm; band slits were 8 mm for both excitation and emission.

II.F. Lipid Mixing Studies

Lipid mixing between different LUV populations was measured by the NBD-PE/Rh-PE assay (Struck et al., 1981) following the modifications of Düzgünes et al. (1987). Lipid mixing was measured using two different protocols. In the first protocol, LUV labeled with 0.6 mol % of NBD-PE and Rh-PE were combined 1:1 (mol/mol) with unlabeled LUV. The residual fluorescence at 530 nm of the labeled LUV containing 0.6 mol % each of NBD-PE and Rh-PE was taken as 0% of maximum fluorescence. Lipid mixing results in an increase in the fluorescence of NBD-PE due to decreased energy transfer efficiency to its quencher, Rh-PE, as the fluorescent probes are diluted from labeled LUV into unlabeled LUV. Multiple rounds of random fusion between the LUV populations would result in membranes containing 0.3 mol % each of the fluorescent phospholipids. The value for the theoretical maximum fluorescence was set by subjecting a mixture of the labeled and unlabeled LUV populations to five cycles of freeze/thawing (dry ice/isopropanol bath, 30°C water bath) (MacDonald & MacDonald, 1983). Randomization of the lipids by

this procedure was verified by preparing a separate LUV population containing 0.3 mol % of each probe, which would be the result of multiple rounds of fusion between labeled and unlabeled LUV. The total lipid concentration was usually 300 μ M. The labeled and unlabeled LUV stock solutions were combined by diluting aliquots from each to 3 mL. The mixed liposome suspension was either maintained in the dark or was exposed to the emission of a low pressure Hg pen lamp (254 nm) placed ca. 4 cm from the front face of the cuvette. Aliquots of 25 μ L were removed from the suspension at 5 min intervals and were diluted into 1 mL of Tris-buffer at 25°C. Emission spectra were measured for each 1 mL diluted aliquot suspension. The samples were thermally equilibrated at least 5 min at 25°C in the fluorometer since the resonance energy transfer efficiency between NBD-PE and Rh-PE was observed to be extremely sensitive to temperature fluctuations. The emission spectra were corrected for dilution errors by zeroing at 680 nm and normalizing at the isoemissive point (569 nm). The location of the isoemissive point was determined by measuring emission spectra of an undiluted mixed liposome suspension before and after subjecting it to repeated freeze/thaw and sonication cycles.

In the second lipid mixing protocol, LUV labeled with 1.0 mol % NBD-PE and Rh-PE were combined 1:9 (mol/mol) with unlabeled LUV. The lipid mixing scale was calibrated in a manner similar to that used in the first lipid mixing protocol. The total lipid concentrations was usually 300 μ M, although a limited number of experiments were performed at 50 μ M. The unlabeled LUV stock solution (0.81 μ mol) was diluted to ca. 2.9 mL and then photolyzed for the requisite time. The unirradiated stock solution of labeled LUV (0.09 μ mol) was then added to produce a 1:9 molar ratio of labeled to unlabeled liposomes of ca.

300 μ M. Lipid mixing was initiated in the fluorometer by injection of 75 μ L of various stock solutions into the magnetically stirred liposome suspension resulting in a final volume of 3.0 ml. Dark assays were performed in the same manner in the absence of photolysis of the unlabeled LUV. A 75 μ L aliquot of 2.0 M acetic acid/sodium acetate, pH 4.5 buffer was added to achieve pH 4.5. A 75 μ L aliquot of 0.5 M TES, pH 7.0 buffer was used to achieve pH 7.5. A 75 μ L aliquot of 0.5 M TES, 0.2 M MgCl_2 , pH 7.0 was added to achieve pH 7.5 and 5 mM Mg^{2+} . A 75 μ L aliquot of 0.8 M MgCl_2 , pH 6.8 was added to achieve pH 9.5 and 20 mM Mg^{2+} .

II.G. Fusion and Leakage Studies

The ANTS/DPX fusion assay reports mixing of aqueous contents between liposome populations containing either ANTS or DPX by decreased ANTS fluorescence due to DPX quenching (Ellens et al., 1985). The ANTS- and DPX-containing liposomes were combined 1:9 at a lipid concentration of 300 μ M. In order to avoid photobleaching of ANTS, only the DPX liposomes were exposed to the filtered 254 nm light. Following the exposure, the ANTS liposomes were added, and the mixture was thermally equilibrated at the temperature of interest for at least 5 min in the fluorimeter. The fluorescence scale was calibrated using the emission intensity of a 1:9 (mol/mol) mixture of ANTS and DPX liposomes in glycine buffer as 100% fluorescence (0% fusion), and the intensity of liposomes containing the coencapsulated ANTS/DPX as 0% fluorescence (100% fusion). Since ANTS is severely quenched by DPX, the latter value was close to zero.

The ANTS/DPX leakage assay employed LUV with coencapsulated ANTS and DPX. Leakage of aqueous contents, resulting in ca. 10^4 -fold dilution of the

probes into the extraliposomal medium, is reported as an increase in fluorescence from ANTS due to the relief of its quenching by DPX (Ellens et al., 1984; Ellens et al., 1987). The assay was calibrated using the initial intensity of the ANTS/DPX liposomes in the pH 9.5 glycine buffer as 0% fluorescence (0% leakage), and the intensity of the same solution following lysis of the LUV by the addition of Triton X-100 to a final concentration of 0.5 % (v/v) as 100% fluorescence (100% leakage). The 100% fluorescence value for each sample was determined since 254 nm exposure of the samples resulted in bleaching of ANTS to a limited, but significant extent. The fusion and leakage assays were initiated in exactly the same manner as the lipid mixing assays (described above) by injection of 75 μ L aliquots of various stock solutions into the stirred LUV suspensions. The initial rates of fusion and leakage were calculated from the slopes of the fluorescence curves during the period (5 s) immediately following the initiation of different events.

II.H. Light Scattering

Liposome size distributions were measured using dynamic laser light scattering (Brookhaven BI-8000AT Correlator with a 5-mW He-Ne laser light source; Brookhaven Instruments Corp.). LUV were examined at a total lipid concentration of 100 μ M at an angle of 90°. Two fitting methods, nonnegative least squares and CONTIN, were used to extract the set of exponential functions which made up the autocorrelation functions.

II.I. Solution Actinometry

The light flux from the low-pressure Hg pen lamp was determined by ferrioxalate actinometry according to the method of Hatchard and Parker (Hatchard & Parker, 1956). Solution A consists of 6 mM ferric oxalate in 0.1 M sulfuric acid; solution B is 0.1% (wt/vol) 1,10-phenanthroline; and solution C is 1 M sodium acetate in 0.36 M sulfuric acid. Solution A was prepared by dissolving 1.63 g (0.6 mmole) iron(III) chloride and 3.32 g (0.2 mmole) potassium oxalate in ca. 50 mL Milli Q water, adding 180 μ L of concentrated (18 M) sulfuric acid, and adjusting the volume to 100 mL with Milli Q water. Solution B was prepared by dissolving 0.1 g 1,10-phenanthroline in ca. 50 mL of hot Milli Q water and adjusting the volume to 100 mL with Milli Q water. Solution C was prepared by dissolving 8.2 g (0.1 mol) sodium acetate in ca. 50 mL Milli Q water, adding 6.48 mL concentrated (18 M) sulfuric acid, and adjusting the volume to 100 mL with Milli Q water.

Using an Eppendorf pipette, 2 mL of solution A was measured into a 3.5 mL quartz cuvette (inside dimensions = 1 x 1 cm). The solution was photolyzed 30 sec by a low-pressure Hg pen lamp. The lamp was positioned 1 cm from the front face of the cuvette which was magnetically stirred and thermostatted at 25°C by a jacketed brass cell holder through which water was circulated. The light incident on the front face of the cuvette was attenuated at 254 nm by a Corning CS 9-54 cut off filter (> 230 nm). The lamp was positioned in exactly the same position as used for the short time irradiations in the lipid mixing, contents mixing, and contents leakage experiments. A second identical solution was prepared but not photolyzed. To both solutions, 400 μ L aliquots of solution B and 1 mL aliquots of solution C were added. The solutions were allowed to develop for 1 hr with stirring at room temperature in the dark.

Finally, the spectrum of the photolyzed sample was measured versus the dark adapted sample using a Hewlet-Packard single-beam absorption spectrophotometer and the optical density was measured at the absorbance maximum.

The number of ferrous ions produced by the photooxidation of ferric oxalate ($N_{Fe^{2+}}$) was determined by the following equation:

$$N_{Fe^{2+}} = V_1 V_3 OD_{max} A_0 / V_2 b \epsilon$$

where V_1 is the volume of solution A that was irradiated (2 mL), V_2 is the volume of the photolyzed aliquot used for analysis (2 mL), V_3 is the final volume to which the photolyzed aliquot was diluted (3.4 mL), b is the path length (1 cm), ϵ is the experimental value of the molar extinction coefficient of the Fe^{2+} complex as determined from the slope of the calibration plot (approximately equal to $1.11 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$), and A_0 is Avogadro's number expressed in units of mM (6.023×10^{20} per mmol).

The light intensity incident on the front face of the cuvette (I_0) was calculated from the following equation:

$$I_0 = N_{Fe^{2+}} / [\Phi_{Fe^{2+}} t (1 - 10^{-OD_{max}})]$$

where t is the exposure time (30 sec), OD_{max} is the measured value of the optical density at the absorbance maximum for the photolyzed solution, $N_{Fe^{2+}}$ comes from the former equation, and $\Phi_{Fe^{2+}}$ is the quantum efficiency of $Fe^{2+}/1,10\text{-phenanthroline}$ complex at the irradiation wavelength (254 nm) employed ($\Phi_{Fe^{2+}}$ is approximately 1.25) (Hatchard & Parker, 1956).

CHAPTER III

RESULTS

III.A. Synthesis of bis-SorbPC

The synthesis of bis-SorbPC_{17,17} and a number of other bis-SorbPC lipids of various chain lengths has been reported (Lamparski et al., 1992; Lamparski et al., 1993; Lamparski & O'Brien, 1994). Bis-SorbPC_{17,17} was chosen for the focus of this work since its behavior in mixed DOPE systems -- before and after polymerization -- has been characterized (Barry et al., 1992). The degrees of polymerization of the mono-substituted analog of bis-SorbPC_{17,17}, i.e. mono-SorbPC_{16,17}, have also been determined (Lamparski & O'Brien, 1994).

Synthesis of bis-SorbPC_{17,17} was based on the methods of Khorana and coworkers (Gupta et al., 1977; Radhakrishnan et al., 1981). The general methods employed in lipid synthesis have been recently reviewed (Bittman, 1993). The synthesis of the symmetrical bis-SorbPC is performed using the biologically derived GPC headgroup which is enantiomerically pure. The products of GPC acylations are also enantiomerically pure and exhibit the same stereochemistry as do naturally occurring phospholipids. Synthesis of lipids that are asymmetric with respect to their sn-1 and sn-2 constituent acyl chain composition, including mono-SorbPC_{16,17}, can be accomplished by acylation of the sn-2 hydroxyl of any number of commercially available lysoPCs. The synthesis of mono-SorbPC_{16,17} was carried out by Dr. Henry Lamparski (Lamparski et al., 1992) and is not detailed here.

The synthesis of 10-(sorbyloxy)decanoic acid is outlined in Figure III-1.

Figure III-1: Synthesis of the polymerizable 10-(sorbyloxy)-decanoic acid.

Crystalline 2,4-hexadienoic acid (sorbic acid) was converted to the corresponding liquid acid chloride (sorbyl chloride) in nearly quantitative yield (> 90%). Sorbyl chloride was added in stoichiometric equivalence to 1,10-decanediol in a dropwise fashion and stirred overnight at room temperature to form a mixture of the mono- and bis-substituted sorbyl esters of 1,10-decanediol. Unreacted diol and the two esterified products were well resolved by TLC and were easily separated by flash chromatography. An alternative protocol employing 3 or 4 equivalents of diol to 1 equivalent of sorbyl chloride was avoided since both starting materials are inexpensive and removal of the excess diol is cumbersome and time consuming. The maximum theoretical yield of the monoester is statistically limited to ca. 50% due to the concomitant formation of the symmetric diester. Taking into account the formation of the symmetric diester, the mono-ester was recovered in good yield.

The 10-(sorbyloxy)decan-1-ol was converted to the corresponding fatty acid, 10-(sorbyloxy)decanoic acid, by oxidation in the presence of PDC in moderate yields. The yield was dependent upon the efficiency of the extraction of the product from the PDC precipitate during vacuum filtration through a bed of silica gel. The reaction mixture was purified by flash silica gel chromatography with a mixture of hexane and ethyl acetate as eluent. Since the reaction mixture was an oil, it could be loaded on the silica gel column without first being diluted with the elution solvent. After partial purification, the product formed a white precipitate. For subsequent column chromatography, the product was dissolved in the hexane/ethyl acetate elution solvent. Since the fatty acid had limited solubility in the solvent mixture, smaller loadings were necessary in these later columns.

Bis-SorbPC_{17,17} was synthesized by the reaction shown in Figure III-2. The lipid was prepared by acylating GPC-CdCl₂ in the presence of 3.9 equivalents of 10-(sorbyloxy)decanoic acid. During the reaction, the fatty acid is converted *in situ* to a symmetric anhydride by the dehydrating agent DCC. The anhydride is formed via an intermediate O-acyl urea formed by reaction of one equivalent each of fatty acid and DCC. The acylation reaction between GPC-CdCl₂ and the symmetric anhydride is catalyzed by the hindered nitrogen base DMAP. The acylation reaction is severely hindered by the presence of moisture which converts the anhydride to the constituent fatty acids. For that reason, GPC was dried by repeated evaporation from benzene immediately prior to the reaction and only fresh preparations of DMAP and DCC were used. Acylation of the primary hydroxyl at the sn-1 position is facile but subsequent reaction at the secondary hydroxyl at the sn-2 position is slow due to the decreased reactivity of the sn-2 hydroxyl and the steric hindrance imparted by the attached sn-1 fatty acyl chain. The reaction was allowed to proceed for five days after which time no change could be observed by TLC. The dicyclohexylurea by-product was removed by gravity filtration and the mixture was stirred in the presence of Bio-Rad ion exchange resin AG 501-X8. The ion exchange resin converts the product from the cadmium chloride complex to the free species as indicated by a color change of the resin from blue to yellow. Filtration of the resin in a methanolic solution also removed much of the DMAP acylation catalyst as evidenced by TLC. Unfortunately, the acylation reaction gave the lowest yields of all the steps in the synthesis and required a two-fold excess of the fatty acid which was not

Figure III-2: Synthesis of bis-SorbPC_{17,17}.

+

DMAP (2 equiv.)
 DCC (3.2 equiv.)
 CHCl_3
 5 days, RT

(bis-SorbPC_{17,17})

recovered during the lipid work-up since it traveled at the solvent front during the initial chromatographic resolution of the lipid. The low yields may have been due to insufficiently dry reaction conditions or to the difficulty of isolating the lipid by flash silicic acid gel chromatography. A problem associated with the chromatography of diacylPCs is 2,3-diacyl migration (Lammers et al., 1978; Ali & Bittman, 1989; Lamparski, 1993). To minimize the occurrence of this reaction, flash chromatography rather than gravity chromatography was employed to reduce the duration of lipid contact with the silicic acid. In this work, no by-products were observed by TLC indicating the formation of 2,3-diacyl migration products, but hydrolysis/methanolysis did occur to a limited extent. Repeated chromatography of the lipid was required to remove all traces of the fatty acid contaminants.

After purification, the product may be susceptible to subsequent reactions: acyl-chain cleavage by hydrolysis or methanolysis, and spontaneous polymerization. Hydrolysis or methanolysis can occur due to the presence of water or methanol, respectively, in the elution solvent. These reactions can be retarded by diluting the chromatographed lipid solution with benzene prior to concentrating the product by rotary evaporation. The water and methanol are removed by azeotropic distillation via repetitive additions of benzene followed by rotary evaporation. Polymerization was never observed with any of the sorbyl-containing intermediates but it did occur with the lipid during the first workup attempt when it was dried to a film under high vacuum. The occurrence of lipid polymerization was evidenced by the film's insolubility in benzene. Since the film was maintained under yellow safe lights or in the dark, polymerization was probably not photochemically activated but more likely occurred due to the presence of an unknown

impurity capable of initiating the polymerization. In subsequent workups, the lipid was never dried to a thin film but was always maintained as a benzene solution. After mixing bis-SorbPC_{17,17} with DOPE, thin films could be safely prepared and maintained overnight under vacuum without polymerization.

III.B. Photopolymerization of SorbPC Membranes

Liposomes of DOPE/bis-SorbPC and DOPE/mono-SorbPC in glycine buffer at 37°C exhibit absorbance maxima at 258 nm due to the sorbyl chromophore. Photolysis of the liposomes with UV light (254 nm) diminishes this absorbance band. The rate of the photopolymerization of the liposomes could be controlled by varying the light intensity incident on the liposome suspensions which may be controlled by changing the distance of the liposomes from the lamp or by the use of neutral density filters. In the initial lipid mixing assays, photopolymerization of DOPE/bis-SorbPC (3:1) LUV in 3 mL stirred suspensions was effectively complete after 30 min exposure to the filtered emission of a low-pressure Hg pen-lamp (predominantly 254 nm) placed 4 cm from the front face of a quartz cuvette. In the later lipid mixing, fusion, and leakage assays, LUV composed of either DOPE/bis-SorbPC (3:1) or DOPE/mono-SorbPC (3:1) at ca. 300 μ M in 3 mL stirred suspensions were completely polymerized by 3.0 min exposures under the same conditions except that the pen-lamp was placed 1 cm from the front face of a quartz cuvette. Polymerization of 50% of the bis-SorbPC or mono-SorbPC component was accomplished after 0.5 min or 0.66 min exposures, respectively, under those same conditions. The reaction rate is attenuated at high conversion of monomer due to the decreased sorbyl absorbance (De Schryver et al., 1975). For the same conditions used in the fusion and leakage assays, the extents of conversion of bis-SorbPC to poly(bis-

SorbPC) in DOPE/bis-SorbPC (3:1) LUV at different photolysis times are given in Table III-1.

Table III-1: Extents of conversion of bis-SorbPC_{17,17} to poly(bis-SorbPC) in DOPE/bisSorbPC (3:1) LUV in 37°C glycine buffer at different photolysis times.

Photolysis Time (sec)	OD ₂₅₈	% Polymerization
0	2.68	0
30	1.89	35
60	1.23	64
90	0.84	82
120	0.64	91
150	0.54	95
180	0.48	98
210	0.45	99
240	0.43	100

The shoulder of the broad absorption band of the isolated double bond in the 1,4-polymer was responsible for the non-zero OD₂₅₈ values at very long irradiation times. The polymerization data were extremely reproducible from sample to sample and from experiment to experiment as long as the lipid concentrations were adjusted carefully and the position of the lamp with respect to the sample cuvette was not changed. The rate of photobleaching did not change with temperature from 5 to 37°C.

Figure III-3: Effect of 254 nm photolysis of bis-SorbPC LUV on the sorbyl absorbance. A 10 μ M lipid sample in unbuffered Milli Q water was exposed at rt for 30 s intervals up to 60 s and was afterwards exposed continuously for 240 s. Isosbestic behavior is evident up to 60 s indicating the formation of principally one photoproduct. After long time exposure, the isosbestic behavior ceases, indicating the possible formation of another photoproduct.

After 3 min photolysis, virtually all (98%) of the monomeric bis-SorbPC_{17,17} was converted to polylipid. For all experiments under these conditions, 3 min photolysis was considered to result in complete LUV polymerization.

The photolysis of LUV in optically transparent media, i.e. water, reveals that the absorbance decrease at 258 nm is accompanied by an increase in the absorbance at 195 nm with a well defined isosbestic region at 222 nm (Figure III-3). The existence of the isosbestic region suggests that the photolysis yields principally one product (Lamparski et al., 1992). ¹H NMR studies of the polymer products of photolysis of bis-SorbPC and mono-SorbPC LUV identified the principal photoproduct as the 1,4-polymer (Lamparski & O'Brien, 1994). Figure III-4 illustrates the SorbPC 1,4-polymerization reaction.

Figure III-4: 1,4-polymerization of the sorbyl moiety contained at the acyl-chain terminus of mono-SorbPC or bis-SorbPC.

The insensitivity of the photoreaction to the presence of oxygen, and the direct proportionality of the rate of the reaction to light intensity, indicate that the reaction proceeds by photoactivated addition (Lamparski & O'Brien, 1994). Continued photolysis of bis-SorbPC LUV (30 min) at 254 nm results in the eventual disappearance of the band at 195 nm (Figure III-3). This was inferred to occur due to further reaction of the residual double bond. Low pressure mercury arc lamps emit primarily two bands of radiation centered at 185 and 254 nm (Parker, 1968). The line at 185 nm is more intense than the 254 nm line inside the lamp but it is less intense outside the lamp due to absorption by the quartz envelope. Because of the possibility that a second photoreaction could lead to uncontrolled cross-linking of the sorbyl lipids due to the 185 nm photolysis, precautions were taken against the occurrence of the reaction by both the use of short photolysis times (≤ 3.0 min) and the attenuation of the shorter wavelength light incident on the samples with a 230 nm high pass filter. Recently, the photoactivated polymerization of LUV composed of mono-SorbPC was shown to produce primarily short oligomers having repeat units of 3 - 7 (Lamparski & O'Brien, 1994). The size of the bis-SorbPC polymers is not known, but their greater insolubility in organic solvents than the polymers from mono-SorbPC implies that considerable cross-linking occurs with the bis-SorbPC.

The quantum yield for the SorbPC polymerizations was determined from knowledge of the light intensity (I_0) incident on the front face of the cuvette in which the liposome photopolymerizations were carried out (Section II.H.), and the fraction (I_a/I_0) of the light that was absorbed by the SorbPC molecules

under the conditions employed in the different assays. The quantum yield for a product B (e.g. poly(SorbPC)) is given by the following equation:

$$\Phi_B = n_B / I_0 (I_a/I_0) t = n_B / I_a t$$

where I_a is the number of photons absorbed by the solution, which is equivalent to the product of the light intensity incident on the front face of the cuvette (I_0) and the fraction of light absorbed. From knowledge of the transmittance (T) of the solution B at the wavelength of interest, the fraction of the light absorbed $[(100 - T) / 100]$ can be derived.

Prior to photolysis, LUV samples (270 μ M in ca. 3 mL) composed of DOPE and either bis-SorbPC or mono-SorbPC exhibited optical densities of ca. 2.7 or 1.7, respectively. Therefore, at the beginning of the photolysis, $\geq 98\%$ of the light was absorbed in both cases. The incident flux (I_0) at the front face of the cuvette was determined to be $\sim 8 \times 10^{15}$ photons s^{-1} (ca. 6 mW) by potassium ferrioxalate solution actinometry (Hatchard & Parker, 1956). Under these conditions, 50% conversion of the bis-SorbPC to poly(bis-SorbPC) required 30 sec, and 50% conversion of mono-SorbPC required 40 sec. Hence, the quantum yields for the reactions are 0.25 and 0.20, respectively.

III.B. Phase Behavior of Mixed Lipid Systems

Bilayers of PE are generally unstable at physiological pH unless they contain an L_α -competent colipid, e.g. PA or PC (Stollery & Vail, 1977). Colloidally stable 0.1 μ m LUV suspensions of 2:1, 3:1, and 4:1 mixtures of either DOPE/bis-SorbPC or DOPE/mono-SorbPC LUV can be prepared by extrusion (0.1 μ m pore size Nuclepore membranes) at physiological pH in aqueous buffers

(100 mM NaCl, 20 mM Tris, pH 7.4) at room temperature. However, under these same conditions, hydration and extrusion of 0.1 μ m LUV of 2:1, 3:1, and 4:1 mixtures of DOPE and DOPC results in lipid suspensions that rapidly aggregate and precipitate. The presence of the sorbyl group in the middle of the mixed lipid bilayers apparently has a profound effect on the equilibrium phase behavior of LUV composed principally of DOPE. Table III-2 shows the lamellar to nonlamellar phase transition temperatures for DOPE and its mixtures in various molar ratios with either DOPC or bis-SorbPC. Note that although DOPE/DOPC (3:1) systems readily adopt the H_{II} phase, neither DOPE/bis-SorbPC (3:1) nor DOPE/mono-SorbPC (3:1) systems have been observed by DSC to adopt the H_{II} phase at experimentally accessible temperatures.

Table III-2: Lamellar to Nonlamellar Phase Transition Temperatures.

Lipid System	Conditions	T _I (onset)	T _H (peak)
DOPE/DOPC (4:1)	pH 4.5		47±2 ^a
	pH 7.0	< 35 ^c	
DOPE/DOPC (3:1)	pH 4.5		60±4 ^a
	5 mM Mg ²⁺ /pH 7.4	45-50 ^a	
DOPE/DOPC (2:1)	pH 4.5		≥ 80 ^a
	pH 7.4	65-70 ^b	
DOPE/bis-SorbPC (3:1)	pH 7.4	49±0.5 ^d	> 85 ^d
DOPE/mono-SorbPC (3:1)	pH 7.4		> 85 ^d

^a Ellens et al. 1989^b van Duijn et al. 1986^c Tilcock et al. 1982^d Barry et al. 1992

The phase behavior of multilamellar dispersions of DOPE/bis-SorbPC (3:1) at relatively high concentrations (ca. 100 mM) was studied by ^{31}P NMR as a function of temperature at different extents of polymerization of the SorbPC component (Barry et al., 1992). ^{31}P NMR spectra were acquired at increasing temperatures with lipid mixtures that were either 0, 33, 51, or 90% polymerized by 254 nm photolysis. In all cases, the lipid systems were initially in the L_α phase at 25°C. At ca. 62°C, the 0%, 33, and 51% polymerized samples exhibited a completely isotropic ^{31}P NMR signal. Analysis of the 0 and 33% polymerized samples by X-ray diffraction confirmed that the isotropic NMR signals indicated the formation of precursors to a bicontinuous Q_{II} phase. With increasing extent of polymerization, the appearance of isotropic resonances occurred at successively lower temperatures. This indicated that increasing extents of polymerization of the multilamellar systems decreased the L_α/Q_{II} phase transition temperatures (T_I) to successively lower values. The 90% polymerized system did not exhibit a completely isotropic NMR signal even at high temperatures. This was inferred to indicate that the polymerized PC domains were sufficiently large that they were prevented from adopting the high curvature required by the Q_{II} phase. The temperatures at which 10, 50, and 100% of the lipids contributed to the isotropic ^{31}P NMR signal were quantified as T_I (10%), T_I (50%), and T_I (100%), respectively. Table III-3 gives the T_I values for dark-adapted and photopolymerized DOPE/bis-SorbPC (3:1) multilamellar assemblies as a function of both the extent of polymerization, and the ratio of DOPE to monomeric-PC in the system.

Table III-3: Effect of bis-SorbPC photopolymerization on the temperature of the L_{α}/Q_{II} phase transition of hydrated DOPE/bis-SorbPC (3:1). The lipid ratios are calculated for the total lipid present in the system. Data are from Barry et al. (1992).

% Loss of Monomer	DOPE/monomeric-PC	Temperature (°C) ^a		
		T _I (10%)	T _I (50%)	T _I (end)
0	3.1 : 1	49.1	54.0	58.0
33	4.3 : 1	44.8	47.8	55.0
51	6.1 : 1	42.7	45.6	54.0
90	31 : 1	38.1		

^a Temperatures where 10%, 50%, and 100% of the lipids contribute to the isotropic ³¹P NMR signal, termed T_I(10%), T_I(50%), and T_I(end), respectively.

III.C. Lipid Mixing Studies

Assays for contents mixing and contents leakage are fundamentally different from those for lipid mixing because of the possibility of leakage of the aqueous markers out of the liposomes after they are prepared and before the fusion and leakage assays can be performed. Fusion assays are distinguished from the other assays since the signal that is measured is almost always a transient one, while lipid mixing and leakage assays give rise to persistent signals. All three types of assays can be performed by combining two different liposome populations and immediately measuring a change that occurs over time. This protocol does not eliminate the possibility of leakage

when the liposomes within one of the populations interact with one another. Liposome interactions can sometimes be suppressed at low temperatures. When this is the case, separate liposome preparations can be maintained at low temperatures, combined immediately before the assay is performed, and then rapidly heated to allow liposome interactions and bilayer destabilization to occur. Since temperature gradients can affect the fluorescent assays that are used to measure mixing of lipids or contents, the signals for such assays are difficult to calibrate. Resonance energy transfer between NBD-PE/Rh-PE is strongly affected by temperature changes as small as several degrees (data not shown).

Wherever it is possible, liposomes should be prepared under conditions where no interaction between liposomes of either the same or different populations can occur until such interaction is initiated in a controlled process that allows all subsequent liposome interactions to be monitored. In the PS/Ca²⁺ liposome system, the negatively charged PS liposomes do not interact with one another until Ca²⁺ is injected into the medium. The titratable amino group of the ethanolamine headgroup of PE can be profitably employed to stabilize liposomes containing PE. The liposomes are prepared at pH 9.0 or above where PE is negatively charged. Liposome interactions are retarded by their negative charge until charge neutralization is achieved by the addition of protons or divalent cations to neutralize the charge contributed by PE. With respect to polymerization of lipids by photolysis or by other means, the advantage of preparing liposomes at high pH is that the polymerization reaction and the subsequent fusion event can be temporally separated. However, the requirement of temporally separating liposome

photopolymerization and fusion prevents the direct observation of the effect of photolysis on fusion.

The NBD-PE/Rh-PE lipid mixing assay was employed using two different protocols. In the first protocol, lipid mixing was monitored at the same time that the liposomes were photopolymerized. In the second protocol, the liposomes were photopolymerized at high pH where no liposome interaction could occur, and then lipid mixing was measured by neutralizing the surface charge by addition of protons, magnesium ions, or both.

The excitation and emission spectra of the NBD-PE and Rh-PE resonance energy transfer pair are shown in Figure III-5. Large spectral overlap exists for the emission spectrum of NBD-PE and the excitation spectrum of Rh-PE. Hence, when the two probes are in sufficiently close proximity, or alternatively, when they are in sufficiently high concentration within the same membrane, direct excitation of NBD-PE results almost exclusively in emission from Rh-PE due to efficient RET between the fluorophores. When the probes are diluted, as occurs when doubly labeled LUV fuse with unlabeled LUV, the direct emission from NBD is enhanced (Figure III-6).

Figure III-5: Structures and fluorescence spectra of the resonance energy transfer (RET) probes NBD-PE and Rh-PE. The spectra were measured in ethanol and are plotted on the same wavelength scale. In each panel the excitation spectrum is at the left and the emission spectrum is at the right.

Figure III-6: Emission spectra of NBD-PE and Rh-PE (1:1, mol/mol) at different combined concentrations with respect to the total lipid in DOPE/bis-SorbPC (3:1) LUV. The emission spectrum in the upper panel is for LUV containing 0.6 mol % of each fluorophore and corresponds to 0% lipid mixing in the NBD-PE/Rh-PE assay. The emission spectrum in the lower panel is for LUV containing 0.3 mol % of each fluorophore and corresponds to 100% lipid mixing. The effect of lipid mixing on RET efficiency is schematically illustrated beside the spectra.

Lipid mixing between labeled and unlabeled LUV populations was carried out under two protocols. In the first protocol, labeled LUV were prepared containing 0.6 mol % of each NBD-PE and Rh-PE and lipid mixing was measured after combining the two LUV populations 1:1, mol/mol. The LUV were prepared in Tris buffer (100 mM NaCl, 20 mM Tris) at pH 7.4 and were combined in a stirred and thermostatted cell with or without UV photolysis. Aliquots of 50 μ L were removed at 5 min intervals and were diluted 20-fold into 1 mL of Tris-buffer at 25°C to a final concentration of 15 μ M. Lipid mixing was quantified by immediately measuring the fluorescence emission spectra of the diluted aliquots. The spectra were corrected for dilution errors by normalization at the isoemissive point (569 nm). Figure III-7 illustrates the isoemissive behavior of the emission spectra for NBD-PE and Rh-PE as they are diluted from one liposome population into another.

Using this protocol, the effects of liposome composition, the extent of liposome photopolymerization, temperature, and presence of divalent cations on lipid mixing between labeled and unlabeled LUV were investigated (Bennett & O'Brien, 1993; Bennett et al., 1994). Wherever the effects of photopolymerization were investigated, both the labeled and unlabeled LUV populations were simultaneously irradiated.

Figure III-7: Isoemissive behavior of NBD-PE and Rh-PE emission spectra resulting from lipid mixing between labeled and unlabeled liposomes. The spectrum for 0% lipid mixing (a) was measured at 37°C using only labeled DOPE/bis-SorbPC (3:1) liposomes (150 μ M) containing 0.6 mol % of each fluorophore. Lipid mixing was initiated by combining labeled and unlabeled LUV 1:1 (mol/mol) at pH 7.4, 300 μ M, and 37°C with concurrent 254 nm photolysis. At different times emission spectra of the liposome mixture were measured at 37°C. Spectrum b is for 10 min irradiation (50% polymerization) and spectrum c is for 15 min irradiation (99% polymerization). Finally, the liposome mixture was subjected to repeated freeze-thaw and sonication cycles to equilibrate the fluorophores between the two liposome populations and to establish the spectrum corresponding to 100% lipid mixing (d). The spectra intersect in an isoemissive point at 569 nm.

To investigate the dependence of the presence of PE in the bilayers on liposome fusion, lipid mixing between LUV composed of either DOPE/bis-SorbPC (3:1) or DOPC/bis-SorbPC (3:1) was compared. A 1:1 mixture of labeled and unlabeled liposomes was incubated in the dark or was continuously photolyzed at 37°C and 300 μ M for 60 min. A controlled rate of photopolymerization was achieved by placing the low-pressure Hg pen lamp at a relatively large distance (4 cm) from the front face of the cuvette that contained the stirred liposome suspension. Photopolymerization was effectively complete (99%) after 30 min irradiation. The photolyzed DOPE/bis-SorbPC (3:1) LUV underwent significantly greater lipid mixing than the dark-adapted LUV (Figure III-8). For both the photolyzed and dark-adapted DOPE-containing LUV, lipid mixing increased throughout the assay, but it was very slow after 30 min. Control liposomes composed of DOPC/bis-SorbPC (3:1) exhibited virtually no lipid mixing even when continuously photolyzed (Figure III-8). The results of the control experiments are consistent with the observations of Lamparski et al. (1992) who found that photopolymerization of bis-SorbPC resulted in its phase separation from DOPC in two-component liposomes, but that the two-component MLV bilayers were not sufficiently destabilized to induce leakage of aqueous solutes.

Figure III-8: Effect of liposome composition on lipid mixing vs. irradiation time DOPE/bis-SorbPC (3:1) LUV (circles) or DOPC/bis-SorbPC (3:1) LUV (diamonds). Lipid mixing between NBD-PE/Rh-PE-labeled LUV and unlabeled LUV was initiated by combining the LUV 1:1 at 300 μ M lipid, pH 7.4 and 25°C and then heating the stirred suspension to 37°C in the dark (open symbols) or with continuous 254 nm irradiation (closed symbols). The data represent the average of at least three measurements and the error bars give the standard deviations.

Figure III-9: Effect of different extents of photopolymerization on lipid mixing between DOPE/bisSorbPC (3:1) LUV at 300 μ M lipid, 37°C, and pH 7.4. Lipid mixing was initiated as in Figure III-8 except that liposomes were thermostatted at 37°C in the dark (diamonds, 0% polymerization), or with irradiation for 5 min (triangles, 45% polymerization), 10 min (circles, 76% polymerization), or 30 min (squares, 99% polymerization) after the liposomes were combined.

The effect of different extents of photopolymerization on the extents of lipid mixing of DOPE/bis-SorbPC (3:1) LUV was measured by photolyzing the LUV for different durations following initiation of the assay. The LUV were photolyzed for 0, 5, 10, or 30 min to achieve 0, 45, 76, and 99% polymerization, respectively, of the bis-SorbPC component of the lipid bilayers. Lipid mixing was measured at 5 min intervals for 30 min total in all cases. All of the photolyzed LUV exhibited similar initial rates of lipid mixing that were significantly larger than that for the dark-adapted LUV. For the LUV photolyzed 5 or 10 min, the rate of lipid mixing plateaued after about 10 min, and the LUV underwent similar extents of lipid mixing over the remainder of the assay. The rate of lipid mixing for the continuously photolyzed LUV did not plateau even after 30 min (as was also observed in Figure III-9).

The effect of increasing the temperature from 25 to 30°C and to 37°C was examined under the conditions used in Figure III-8. Lipid mixing increased in rate and extent with increased temperature (Figure III-10). A more significant enhancement in lipid mixing occurred as a result of increasing the temperature from 30 to 37°C than from 25 to 30°C. The rate of lipid mixing was sustained for longer periods before plateauing at 37°C than at the lower temperatures.

The effect of increasing extents of photopolymerization on lipid mixing at 25, 30, and 37°C is shown in Figure III-11. The data are identical to Figure III-10, but are plotted in a manner that removes the time element. Significant differences in the extent of lipid mixing at the different temperatures are apparent only after ca. 70% polymerization. Thereafter, the differences increase with increasing monomer conversion.

Figure III-10: Effect of temperature on lipid mixing vs. irradiation time for DOPE/bis-SorbPC (3:1) LUV. Lipid mixing was initiated as in Figure III-8 except that LUV were continuously photolyzed at different temperatures: 25°C (diamonds), 30°C (triangles), or 37°C (squares). The data represent the average of at least three measurements and the error bars give the standard deviation.

Figure III-11: Effect of temperature on lipid mixing vs. extent of photopolymerization of DOPE/bis-SorbPC (3:1) LUV. The lipid mixing data are identical to those presented in Figure III-10, but lipid mixing is plotted vs. extent of polymerization rather than irradiation time. LUV were continuously photolyzed at 25°C (diamonds), 30°C (triangles), or 37°C (squares). The data represent the average of at least three measurements and the error bars give the standard deviation.

The effect of increasing the temperature from 25 to 37°C on lipid mixing was further examined by incubating labeled and unlabeled DOPE/bis-SorbPC (3:1) LUV for 30 min at 25°C in the dark or with continuous photolysis. Lipid mixing that occurred at 25°C was monitored at 5 min intervals as in the other assays. The mixed liposome suspensions were then rapidly heated to 37°C and lipid mixing was allowed to proceed for another 60 min. Lipid mixing was monitored as before at 5 min intervals for 60 min after the assay was initiated and a final sampling was made 30 min after that. As shown in Figure III-12, lipid mixing was partially, though not completely suppressed in both the photolyzed and the dark-adapted samples at 25°C. The effect on lipid mixing of increasing the temperature to 37°C after the 25°C incubation period was dramatic for both the photolyzed and the dark-adapted LUV. The increase in the rate and extent of lipid mixing was significantly greater for the photolyzed samples than the dark-adapted ones. Both the extents of lipid mixing and the difference between the photolyzed and dark-adapted samples after 60 min in Figure III-12 were similar to the values measured in Figure III-8 after 60 min incubations at 37°C where the liposomes were not preincubated at 25°C.

Figure III-12: Effect of increased temperature on lipid mixing between photopolymerized and dark-adapted DOPE/bis-SorbPC (3:1) LUV. Lipid mixing was initiated by combining NBD-PE/Rh-PE-labeled and unlabeled LUV (1:1) at 300 μ M and 25°C with 254 nm photolysis (filled circles) or in the dark (filled squares). After 30 min, the samples were rapidly heated to 37°C and maintained in the dark over the remainder of the assay. Lipid mixing for the photolyzed (99% photopolymerized) LUV is given by the open circles, and for unphotolyzed (unpolymerized) by open squares.

Figure III-13: Effect of concentration on lipid mixing between photopolymerized DOPE/bis-SorbPC (3:1) LUV at 37°C. Lipid mixing was initiated by mixing labeled and unlabeled LUV (1:1, mol/mol) at and pH 7.4 with continuous 254 nm photolysis. Lipid concentrations were 60 μ M (diamonds), 300 μ M (triangles), or 600 μ M (squares).

The effect of lipid concentration on the rate and extent of lipid mixing was determined for 60, 300, and 600 μ M lipid suspensions of DOPE/bis-SorbPC (3:1) LUV in the presence of continuous irradiation at 37°C (Figure III-13). No significant dependence of the lipid mixing kinetics was observed in this concentration range at 37°C. Thereafter, the lipid concentration was maintained at 300 μ M since the photopolymerizations could be conveniently and reproducibly carried out at that concentration.

Figure III-14: Effect of addition of Mg^{2+} on lipid mixing between photopolymerized and dark-adapted DOPE/bis-SorbPC (3:1). Lipid mixing was initiated by combining labeled and unlabeled LUV (1:1, mol/mol) at $300 \mu M$ in the presence (squares) or absence (circles) of $5 mM Mg^{2+}$. The LUV were maintained at $37^{\circ}C$ in the dark (open symbols) or in the presence of continuous $254 nm$ irradiation (filled symbols).

The effect of the presence of divalent cations on the rate and extent of lipid mixing between dark-adapted or photolyzed LUV was examined by addition of $5 mM Mg^{2+}$ to the extraliposomal medium ($150 mM NaCl$, $10 mM Tris$, $pH 7.4$). Lipid mixing for both dark-adapted and photolyzed LUV was significantly increased by the addition of $5 mM Mg^{2+}$ at $pH 7.4$ as shown in Figure III-14. The increased lipid mixing that occurred due to the addition of 5

mM Mg^{2+} was probably due to increased adhesion of the liposomes as a result of "bridging" by the Mg^{2+} ions between negatively charged ethanolamine headgroups in the cis-monolayers of aggregated liposomes. At pH 7.4, only ca. 0.5 mol % of the ethanolamine headgroups should be negatively charged as a result of the deprotonation of the amino group whose pK value is ca. 9.5. The Mg^{2+} ions might also interact with the negatively charged headgroups of either of the fluorophores present in the labeled liposomes.

The simultaneous photopolymerization and initiation of liposome interactions was suitable for determination of photoinduced lipid mixing which is evidenced by a persistent change in fluorescence, but the protocol could not be applied to the measurement of aqueous contents mixing due to the transient nature of the fusion event and the attendant changes in fluorescence. Fusion assays were attempted by combining liposomes at pH 7.4 and 25°C, photolyzing the mixtures, and then rapidly heating the suspensions to 37°C in the fluorimeter where the fluorescence was measured over time. Fluorescence changes indicating fusion events could not be consistently observed (data not shown). Leakage of the liposomal contents was apparently occurring prior to the fusion assays. Following preparation of the liposomes by extrusion, the fluorescent markers are uniformly distributed between the inside and outside of the liposome membranes. As the liposomes (ca. 10 mM) are separated from the unencapsulated dyes by gel filtration chromatography at rt, a concentration gradient for the fluorescent markers is formed. If liposome interaction occurs during or after the chromatography with the result of destabilizing the liposomal membranes, the concentration gradient experienced by the fluorophores should tend to stimulate their

leakage into the extraliposomal medium. Hence, even if photolysis of the liposomes at 25°C does induce destabilization of the membranes and subsequent liposome fusion, the fusion event cannot be observed since the fluorescent makers have already leaked out of the liposomes.

A second protocol, which allowed the temporal separation of the polymerization and fusion events, was required for determination of the effect of different extents of polymerization on fusion. In the second lipid mixing protocol, labeled and unlabeled liposomes were prepared in glycine buffer at pH 9.5 and combined (1:9). Lipid mixing was initiated by using either H^+ , or Mg^{2+} , or both ions to neutralize the initially negative bilayer surfaces of the LUV. With both the DOPE/bis-SorbPC (3:1) and DOPE/mono-SorbPC (3:1) systems, the rates and extents of lipid mixing increased in the following order: pH 7.5 < pH 7.5/5 mM Mg < pH 4.5 < pH 9.5/20 mM Mg. Large differences in lipid mixing were observed between the experiments performed at pH 4.5 and 7.5 as shown in Figure III-15. This was probably due to residual negative surface charge on the LUV at the higher pH due to incomplete protonation of the PE amino group. Papahadjopoulos and coworkers (Allen et al., 1990) reported the inhibition of aggregation of eggPE liposomes in the region of pH 7.4 - 8.0 and attributed the observation to a small residual surface charge contributed by a small percentage of ionized PE headgroups.

Figure III-16 shows the effect of lipid concentration on lipid mixing between photopolymerized and dark-adapted DOPE/bis-SorbPC (3:1) LUV at 37°C and pH 4.5. Lipid mixing was compared at 50 μM (panel A) and 300 μM (panel B). No significant difference in either the rates or extents of lipid mixing

Figure III-15: Effect of pH on lipid mixing of photopolymerized or dark-adapted DOPE/bis-SorbPC (3:1) LUV at 300 μ M and 37°C. Lipid mixing was initiated by combining labeled and unlabeled LUV 1:9 (mol/mol) and acidifying the suspensions from pH 9.5 to pH 7.5 (panel A) or to pH 4.5 (panel B). The unlabeled LUV were photopolymerized to the extents shown in the figure and the labeled LUV were unphotolyzed.

Figure III-16. Effect of lipid concentration on lipid mixing between photopolymerized and dark-adapted DOPE/bis-SorbPC (3:1) LUV at 37°C and pH 4.5. Lipid mixing was measured at 50 μ M (panel A) and at 300 μ M (panel B).

were observed for either the photopolymerized or the dark-adapted samples. This observation agrees with the results for lipid mixing at 60, 300, and 600 μ M obtained in the initial lipid mixing protocol (Figure III-13). These results suggest that lipid mixing is not aggregation rate limited at lipid concentrations as low as 50 μ M (Ellens et al., 1984; Ellens et al., 1986a). Due to experimental limitations, lipid mixing assays could not be performed at concentrations below 50 μ M. The remainder of the experiments were performed at 300 μ M lipid since at this concentration the assays were well behaved and the extent of polymerization of the liposomes could be reproducibly controlled.

Figure III-17 shows the effect of polymerization of DOPE/bis-SorbPC LUV on the time course of lipid mixing at 37°C after initiation in the presence of 5 mM Mg^{2+} at pH 7.5 (panel A), pH 4.5 (panel B), or in the presence of 20 mM Mg^{2+} at pH 9.5 (panel C). Time scans are shown for 0, 50, and 100% polymerization, as indicated in Figure III-17. In all cases, lipid mixing increases in both rate and extent with increasing conversion of bis-SorbPC to poly(bis-SorbPC).

Lipid mixing was also examined in this system at 20°C where the process was observed to be significantly retarded under all initiation conditions. Figure III-18 illustrates the temperature dependence of lipid mixing of DOPE/bis-SorbPC (3:1) LUV at pH 4.5.

In Figure III-19, the time course of lipid mixing is shown for DOPE/mono-SorbPC (3:1) under the same conditions as in Figure III-17, except that the studies were performed at 50°C. Just as in the bis-SorbPC system, lipid mixing increased with increased photopolymerization. In the mono-SorbPC system, lipid mixing was also strongly temperature dependent at all of the

Figure III-17: Effect of different initiation conditions on lipid mixing of photopolymerized and dark-adapted DOPE/bis-SorbPC (3:1) LUV. The time course of lipid mixing was measured at 300 μ M and 37°C at pH 7.5 (panel A), in the presence of 5 mM Mg^{2+} at pH 7.5 (panel B), at pH 4.5 (panel C), or in the presence of 20 mM Mg^{2+} at pH 9.5 (panel D).

Figure III-18: Temperature dependence of lipid mixing of DOPE/bis-SorbPC (3:1) LUV at pH 4.5. Lipid mixing was measured at 20°C (panel A) and at 37°C (panel B).

Figure III-19: Effect of different initiation conditions on lipid mixing of photopolymerized and dark-adapted DOPE/mono-SorbPC (3:1) LUV at 50°C. The time course of lipid mixing was measured under the same conditions as in Figure III-17 except at a higher temperature.

initiation conditions. Figure III-20 shows lipid mixing for photopolymerized and dark-adapted DOPE/mono-SorbPC (3:1) measured at pH 4.5 and three temperatures: 20, 37, and 50°C.

Comparison of Figures III-17 and III-19 reveals that with complete photopolymerization of the bis-SorbPC and mono-SorbPC systems, similar extents of lipid mixing are achieved at 37 and 50°C, respectively. The T_m values for bis-SorbPC and mono-SorbPC are 28.8 and 36.1°C, respectively (Lamparski et al., 1993). When the photopolymerizable lipids are combined 1:3 with DOPE, whose T_m value is ca. -20°C, the T_m values of the resulting mixed lipid systems are both less than 0°C. Nonetheless, the ca. 7 C° difference in T_m values for the two polymerizable lipids results in an apparent shift of ca. 15 C° to higher temperature of the fusion threshold for the DOPE/mono-SorbPC (3:1) LUV. Substitution of the sn-1 fatty acyl tail of bis-SorbPC system with the saturated 16-carbon tail of mono-SorbPC remarkably attenuates the lipid mixing of the LUV at equivalent temperatures (e.g. 37°C) where both systems are well above their T_m values as shown in Figure III-21. Interestingly, the difference between the dark-adapted and photopolymerized lipid mixing curves in the mono-SorbPC system at 50°C (Figure III-19) was markedly smaller than the corresponding difference between the curves measured for the bis-SorbPC system at 37°C (Figure III-17). This suggests that the cross-linking that results from extensive photopolymerization of the bis-SorbPC liposomes has a profound effect on the membrane properties compared to photopolymerization of the mono-SorbPC system which can only result in the formation of linear polymers.

Figure III-20: Temperature dependence of lipid mixing of DOPE/mono-SorbPC (3:1) LUV at pH 4.5. Lipid mixing was measured at 20°C (panel A), 37°C (panel B), and 50°C (panel C).

Figure III-21: Comparison of lipid mixing of photopolymerized and dark-adapted DOPE/mono-SorbPC (3:1) and DOPE/bis-SorbPC (3:1) LUV at pH 4.5 and 37°C. Panel A shows lipid mixing measured for the mono-SorbPC system and panel B for the bis-SorbPC system.

III.D. Fusion and Leakage Studies

The experimental design, in which only one population of LUV was photolyzed, was motivated by a reluctance to photobleach the ANTS fluorophore during the photopolymerization of the bis-SorbPC or mono-SorbPC component of the liposomes. An alternative fluorescence assay for aqueous contents mixing that employs the Tb^{3+} -dipicolinic acid (DPA) complex ($\text{Tb}(\text{DPA})_3^{3-}$) is inconvenient since the excitation band of encapsulated DPA ($\text{Ex}_{\text{max}} = 278 \text{ nm}$) is severely screened by the intense absorption band of the sorbyl chromophore ($\lambda_{\text{max}} = 258 \text{ nm}$).

Using the ANTS/DPX assay, the fusion behavior of ANTS- and DPX-containing LUV were compared under the same initiation conditions employed in the lipid mixing studies. Consistent with the latter lipid mixing assays, the ANTS- and DPX-LUV were combined 1:9 (mol/mol) in these assays. The 1:9 ratio was chosen instead of a 1:1 ratio for these assays since the sensitivity of the fusion assays are enhanced by the use of an excess number of DPX-LUV compared to ANTS-LUV. Aqueous contents mixing will only occur on contact of an ANTS-LUV with a DPX-LUV. A single round of ideal fusion is defined as the random production of liposome dimers. In a 1:1 system, after an ideal round of fusion, only 50% of the ANTS-LUV would undergo productive fusion by forming heterodimers with DPX-LUV whereas the other 50% of the ANTS-LUV fuse with themselves. The persistent fluorescence from the homo-dimers of ANTS is the source of the decreased sensitivity in this system. After an ideal round of fusion in the 1:9 system, 95% of the ANTS-LUV undergo productive fusion and only 5% of the ANTS-LUV form homo-dimers. Hence, the background fluorescence in this system is much less than in the 1:1 system, and the assay is therefore more sensitive to detecting fusion events.

For consistency with the fusion assays, leakage was studied with ANTS/DPX-containing LUV in the presence of "empty"-LUV (containing the same glycine buffer as composed the extraliposomal medium). In the fusion assays, only the nine-fold excess DPX-containing LUV were photolyzed; in the leakage assays, only the nine-fold excess "empty" LUV were photolyzed. Figure III-22 shows fusion and leakage for DOPE/bis-SorbPC (3:1) LUV at 40°C in the presence of 5 mM Mg^{2+} at pH 7.5 (panels A and D, respectively), at pH 4.5 (panels B and E, respectively), and in the presence of 20 mM Mg^{2+} at pH 9.5 (panels C and F, respectively). The shapes of the fusion curves are readily understandable in light of the respective leakage traces. In some cases, fusion is initially rapid but with time decreases in rate due to simultaneous and competitive leakage. On this time scale, when the DPX-LUV are completely polymerized and are afterwards combined with dark-adapted ANTS-LUV in the presence of 20 mM Mg^{2+} at pH 9.5 (panel C in Figure III-22), the rate of leakage initially lags the rate of fusion and later overcomes the fusion rate.

Figure III-22 shows fluorescence emission spectra measured for DOPE/bis-SorbPC (3:1) LUV prepared for use in ANTS/DPX fusion and leakage studies. Spectrum A is for ANTS-containing LUV at 30 μ M lipid. Spectrum B is for the same sample after dissolution of the liposomes by addition of 0.5 % (wt/vol) TX-100 detergent; the small decrease in the fluorescence intensity is due to dilution of the ANTS fluorophore. Spectrum D is for ANTS/DPX-containing LUV at 30 μ M lipid. Comparison of spectra A and D confirms that ANTS is efficiently quenched by DPX in the ANTS/DPX liposomes. Spectrum D is for the same sample after addition of 0.5 % TX-100. Dissolution of the LUV by the detergent releases the ANTS and DPX into the extraliposomal medium and results in essentially complete relief of quenching of ANTS by DPX.

Figure III-22. Fluorescence emission spectra measured for DOPE/bis-SorbPC (3:1) LUV containing ANTS or ANTS/DPX in the absence or presence of 0.5 % (wt/vol) TX-100 detergent at pH9.5, 30 μ M, and 37°C. Spectra A and B are for ANT-containing LUV in the absence and presence of TX-100, respectively. Spectra D and C are for ANTS/DPX-containing LUV in the absence and presence of TX-100, respectively. The ordinate is fluorescence intensity in arbitrary units.

The fluorescence intensity of spectrum C is about half that of spectrum A since ANTS is present at about half the concentration in the the ANTS/DPX-LUV as it is in the ANTS-LUV.

Figure III-23 shows raw data for ANTS/DPX leakage assays performed with DOPE/bis-SorbPC (3:1) LUV at 37°C and pH4.5. The scale on the ordinate is arbitrary fluorescence intensity. The three time scans at the bottom of the figure are leakage traces for samples photopolymerized to 0%, 50%, or 100% conversion of monomer to polymer as indicated in the figure. The scans at the top of the figure are the corresponding traces (as indicated in the figure) measured after addition of 0.5 % (wt/vol) TX-100 detergent to the LUV suspensions to effect complete dissolution of the membranes and complete release of the aqueous contents.

The uncorrected leakage spectra were normalized by the following procedure. First, the leakage traces were shifted left on the time axis so that the point at which the H^+ or Mg^{2+} buffer was injected corresponded to $t = 0$ sec. Second, the time scan following addition of TX-100 was corrected for dilution by multiplication by the appropriate factor (e.g. 1.1). Third, the leakage time scan was adjusted to a fluorescence intensity of zero at $t = 0$ sec by subtraction of the requisite factor; the same value was subtracted from the corresponding TX-100 scan. Fourth, the time scan was calibrated to a 100% leakage scale by division by the $t = 0$ sec value of the corresponding corrected TX-100 scan and then multiplication by 100%. All leakage scans were corrected by this procedure using their uniquely associated detergent scan since photolysis of the ANTS/DPX-LUV resulted in a limited but significant leaching of the ANTS fluorophore.

Figure III-23. Uncorrected ANTS/DPX leakage assay time scans for DOPE/bis-SorbPC (3:1) LUV at pH 4.5, 300 μ M, and 37°C. Leakage time scans were measured for 0, 50, and 100% photopolymerized LUV as indicated at the bottom of the figure. Time scans for each of the samples measured after addition of 0.5 % (wt/vol) TX-100 are shown at the top of the figure.

The uncorrected fusion spectra were normalized using a different procedure. The 100% fusion value was assumed to correspond to a zero fluorescence intensity as measured by the fluorimeter. The fusion scans were shifted along the time axis so that the point of injection of the H^+ or Mg^{2+} solutions corresponded to $t = 0$ sec. The scans were calibrated to a 100% fusion scale by dividing them by the fluorescence value at $t = 0$ sec and multiplying them by 100%. The scans were inverted, so that increasing fusion was represented by an increasing curve, by multiplication by -1 and addition of 100.

The extents of fusion and leakage under the different conditions are inversely related: at pH 4.5, both fusion and leakage are intermediate compared to the other initiation conditions; at pH 9.5/20 mM Mg, fusion is greatest and leakage is lowest; and at pH 7.5/5 mM Mg, fusion is lowest and leakage is greatest (Figure III-24).

Figure III-24: Effect of different initiation conditions on fusion (aqueous contents mixing) and leakage of photopolymerized and dark-adapted DOPE/bis-SorbPC (3:1) LUV at 40°C. Panels A and D show fusion and leakage, respectively, in the presence of 5 mM Mg^{2+} at pH 7.5. Panels B and E show fusion and leakage, respectively, at pH 4.5. And Panels C and F show fusion and leakage, respectively, in the presence of 20 mM Mg^{2+} at pH 9.5. Unpolymerized ANTS-LUV were combined in a 1:9 ratio at 300 μ M with DPX-LUV whose bis-SorbPC component was either unpolymerized, 50% polymerized, or 100% polymerized, as indicated in the figure.

The similarity in the behavior of the curves for 50 and 100% polymerization under the different initiation conditions is striking: under all conditions, the extent of fusion at 50% polymerization surpasses that at 100% polymerization after times of ca. 3 to 4 min, and the extent of leakage at 50% polymerization is lower than for 100% polymerization. Fusion measured at pH 4.5 and 50 μM was similar to that observed at 300 μM . Due to the excessive noise in the fluorescence time scans at the lower concentration, our studies focused on the higher concentration regime.

Although the absolute rates and extents of fusion varied when measured for different liposome preparations, repetitive time scans were very well reproducible for any given liposome preparation. Further, the trends observed on changing the fusion initiation conditions or the temperature were the same in different liposome preparations as shown in Figure III-25. The figure shows fusion data for DOPE/bis-SorbPC (3:1) LUV measured for two different liposome preparations and under two different initiation conditions, pH 9.5/20 mM Mg^{2+} , and pH 4.5. The data in panels A and C were collected for one liposome preparation while that in panels B and D were obtained for a separate preparation. The rates and extents of fusion for the different liposome preparations are shown at pH 9.5/20 mM Mg^{2+} in panels A and B, and at pH 4.5 in panels C and D. The DPX liposomes are photopolymerized to 0, 50, or 100% conversion of monomer to polymer in each case, as indicated in the figure.

Figure III-25. Reproducibility of fusion time scans for DOPE/bis-SorbPC (3:1) at 37°C and 300 μ M measured for different liposome preparations. Fusion was initiated at pH 9.5/20 mM Mg²⁺ (panels A and B), or at pH 4.5 (panels C and D). Different liposome preparations were used for determination of fusion for panels A and C than for panels B and D.

The effect of photopolymerization on the time course of fusion of DOPE/mono-SorbPC (3:1) LUV at 50°C and three different initiation conditions is shown in Figure III-26. Fusion is shown under the same conditions as those employed for the lipid mixing assays presented in Figure III-19. Strong correlation between the lipid mixing and fusion assays exists for the DOPE/mono-SorbPC (3:1) system. In both studies, LUV interaction increased in the same order: pH 7.5/5 mM Mg < pH 4.5 < pH 9.5/20 mM Mg. The difference in the rates and extents of fusion of photopolymerized or dark-adapted DOPE/mono-SorbPC LUV at 50°C are significantly smaller than those observed in the DOPE/bis-SorbPC (3:1) system at 37°C. The comparative effects of photopolymerization on fusion of the bis-SorbPC and mono-SorbPC systems are similar to those for lipid mixing. Taken together, the observations suggest that the greater changes that occur as a result of bis-SorbPC photopolymerization are due to a significantly larger change in the membrane properties than with the mono-SorbPC system. This greater membrane perturbation is likely due to more significant lateral phase separation in the bis-SorbPC system due to the formation of cross-linked polymers than in the mono-SorbPC system where only linear polymers can be formed.

Figure III-26: Effect of initiation conditions on fusion of photopolymerized and dark-adapted DOPE/mono-SorbPC (3:1) LUV at 300 μ M and 50°C. Panel A shows fusion in the presence of 5 mM Mg^{2+} at pH 7.5. Panel B shows fusion at pH 4.5. And Panel C shows fusion in the presence of 20 mM Mg^{2+} at pH 9.5. Conditions are otherwise identical to those in Figure III-24.

Figure III-27 (A-C) shows the effect of temperature on the fusion of DOPE/bis-SorbPC (3:1) LUV at pH 4.5 and at different extents of polymerization: 0% (panel A), 50% (panel B), and 100% (panel C). Figure III-27 (D-F) shows the corresponding effect of temperature on leakage of DOPE/bis-SorbPC (3:1) LUV under the same conditions. Evidently, fusion can proceed in the relative absence of leakage at low temperatures for both unpolymerized and polymerized LUV. As the temperature is raised, leakage progressively increases. While the initial rates of fusion increase as temperature is raised, the extents are diminished. In Figure III-28, the initial rates of fusion and leakage versus temperature are shown for the same systems as in Figure III-27. The rates shown in Figure III-28 were extracted from the initial slopes of the fusion and leakage curves up to ca. 6 s.

In Figure III-28, both the fusion and leakage curves are shifted to lower temperature with increasing photopolymerization of bis-SorbPC. The temperature threshold for the onset of rapid fusion, which is hereafter termed the critical fusion temperature (T_c), is defined arbitrarily under these conditions as the temperature at which the initial rate of fusion is ca. 15 % min^{-1} . The T_c value was nearly 65°C for the unphotolyzed LUV, but after 50% polymerization, the critical fusion temperature was reduced to 45°C, and on complete photopolymerization, to 40°C. Therefore, at temperatures between 40 and 65°C, photopolymerization of bis-SorbPC represents a mechanism to isothermally induce liposome fusion by decreasing the critical fusion temperature by ca. 25 C°.

Figure III-27: Temperature dependence of fusion and leakage of photopolymerized and dark-adapted DOPE/bis-SorbPC (3:1) LUV at 300 μ M and pH 4.5. Panels A-C are for fusion of unpolymerized ANTS-containing LUV combined 1:9 (mol/mol) with DPX-LUV whose bis-SorbPC component is either (A) unpolymerized, (B) 50% polymerized, or (C) 100% polymerized. Panels D-F are for leakage of unpolymerized ANTS/DPX-containing LUV combined 1:9 at 300 μ M with "empty" LUV whose bis-SorbPC component is either (D) unpolymerized, (E) 50% polymerized, or (F) 100% polymerized.

Figure III-28: Initial rates of fusion and leakage of photopolymerized and dark-adapted DOPE/bis-SorbPC (3:1) LUV at pH 4.5 and 300 μ M as a function of temperature. The initial rates of fusion are shown for unpolymerized ANTS-LUV with a nine-fold excess of DPX-LUV that are either unpolymerized (closed diamonds), 50% polymerized (closed triangles), or 100% polymerized (closed squares). The initial rates of leakage are shown for unpolymerized ANTS/DPX-containing LUV in the presence of a nine-fold excess of "empty" LUV that are either unpolymerized (open diamonds), 50% polymerized (open triangles), or 100% polymerized.

The effect of lipid composition on the critical fusion temperature of the DOPE/bis-SorbPC system was investigated by changing the relative amounts of the reactive and unreactive lipids. Mrs. Christina M. Miller performed fusion experiments with liposomes composed of DOPE/bis-SorbPC in either 4:1 or 2:1 ratios. Compared to the 3:1 DOPE/bis-SorbPC system, the proportion of PE was increased and that of bis-SorbPC decreased in the 4:1 system. Conversely, the proportion of PE was decreased and that of bis-SorbPC increased in the 2:1 system. Although the fusion data for these systems was collected in single trials, we would not suspect that the reproducibility of the fusion measurements for these systems should be different than for the DOPE/bis-SorbPC system, which was demonstrated to be very good within individual trials and between different data sets.

Figure III-29 shows the temperature dependence of fusion of the 4:1 DOPE/bis-SorbPC system at pH 4.5. The initial rates of fusion extracted from the photopolymerized and dark-adapted curves in Figure III-29 are plotted versus temperature in Figure III-30. The temperature dependence of fusion of the 4:1 DOPE/bis-SorbPC system is similar to that of the 3:1 DOPE/bis-SorbPC system in that the T_C values of the photopolymerized LUV are shifted to lower temperatures. The T_C value of the dark-adapted system is ca. 45°C. With 50 and 100% polymerization the T_C values are shifted to ca. 30 and 25°C, respectively. Unlike the 3:1 system, the critical fusion temperatures for the 50% and 100% photopolymerized systems are nearly identical.

Figure III-31 shows the temperature dependence of fusion of the 2:1 DOPE/bis-SorbPC system at pH 4.5. The initial rates of fusion extracted from the curves in Figure III-31 are plotted versus temperature in Figure III-32. The temperature dependence of fusion of the 2:1 system is similar to those of both

Figure III-29: Temperature dependence of fusion of DOPE/bis-SorbPC (4:1) LUV at 300 μ M and pH 4.5. Panels A - C are for fusion of unpolymerized ANTS-containing LUV combined 1:9 (mol/mol) with DPX-LUV whose bis-SorbPC component is either (A) unpolymerized, (B) 50% polymerized, or (C) 100% polymerized.

Figure III-30: Initial rates of fusion of photopolymerized and dark-adapted DOPE/bis-SorbPC (4:1) LUV at pH 4.5 and 300 μ M as a function of temperature. The curve at the extreme right shows the initial rate of fusion (closed diamonds) for unpolymerized liposomes. The middle curve shows the initial rate of fusion (closed triangles) for unpolymerized ANTS-containing LUV in the presence of a 9-fold excess of 50% polymerized DPX-containing LUV. The extreme left curve shows the initial rate of fusion (closed squares) for unpolymerized ANTS-containing LUV in the presence of a 9-fold excess of 100% polymerized DPX-containing LUV.

Figure III-31: Temperature dependence of fusion of DOPE/bis-SorbPC (2:1) LUV at 300 μ M and pH 4.5. Panels A - C are for fusion of unpolymerized ANTS-containing LUV combined 1:9 (mol/mol) with DPX-LUV whose bis-SorbPC component is either (A) unpolymerized, (B) 50% polymerized, or (C) 100% polymerized.

Figure III-32: Initial rates of fusion of photopolymerized and dark-adapted DOPE/bis-SorbPC (2:1) LUV at pH 4.5 and 300 μ M as a function of temperature. The curve at the extreme right shows the initial rate of fusion (closed diamonds) for unpolymerized liposomes. The middle curve shows the initial rate of fusion (closed triangles) for unpolymerized ANTS-containing LUV in the presence of a 9-fold excess of 50% polymerized DPX-containing LUV. The extreme left curve shows the initial rate of fusion (closed squares) for unpolymerized ANTS-containing LUV in the presence of a 9-fold excess of 100% polymerized DPX-containing LUV.

the 3:1 and 4:1 DOPE/bis-SorbPC systems with regard to decreased T_C values with increased photopolymerization. The differences between the 50 and 100% polymerization curves in the 2:1 system are greater than the corresponding differences in the 3:1 and 4:1 systems.

The ratios of DOPE to monomeric-PC after different extents of photopolymerization for two liposomes undergoing productive fusion (i.e. dark-adapted ANTS-containing LUV with photopolymerized or dark-adapted DPX-containing LUV) for the 4:1, 3:1, and 2:1 DOPE/bis-SorbPC systems are shown in Table III-4.

Table III-4: Effect of different extents of photopolymerization on the molar ratio of DOPE to monomeric-PC (PE/PC) in a hetero-dimer of two liposomes undergoing productive fusion and on their corresponding critical fusion temperatures (T_C). In all cases, only the DPX-LUV were photopolymerized.

Extent of Polymerization	Original DOPE/bis-SorbPC Ratio					
	4:1		3:1		2:1	
	PE/PC	T_C (°C)	PE/PC	T_C (°C)	PE/PC	T_C (°C)
0%	4:1	45	3:1	65	2:1	75
50%	5.3:1	30	4:1	45	2.6:1	60
100%	8:1	25	6:1	40	4:1	50

The data in the table indicate that the critical fusion temperature for two liposomes is controlled by the combined ratio of DOPE and monomeric-PC composing the liposomes of an aggregated dimer. In the 4:1, 3:1, and 2:1 DOPE/bis-SorbPC systems, when the DOPE to monomeric-PC ratio is ca. 4:1 (0% polymerization of the 4:1 system, 50% polymerization of the 3:1 system, and 100% polymerization of the 2:1 system, respectively), the T_C value is ca. 45 - 50°C. In the 3:1 and 2:1 systems, when the DOPE to monomeric-PC ratio is ca. 3:1 (0% polymerization of the 3:1 system, or 50% polymerization of the 2:1 system (PE/PC = 2.6), respectively), the T_C value is ca. 60 - 65°C. And in the 4:1 system, when the PE/PC ratio is 5.3:1 (50% polymerization), the T_C value is 30°C; whereas in the 3:1 system, when the PE/PC ratio is 6:1 (100% polymerization), the T_C value is 40°C. Finally, in the 4:1 system, when the PE/PC ratio is 8:1, the T_C value is ca. 25°C. The DOPE to monomeric-PC ratios for the different systems are plotted versus their corresponding T_C values in Figure III-33. Apparently, the effect of reducing the DOPE to monomeric-PC ratio above 5.3:1 is minimal. This is indicated in Figure III-33 by the flattened temperature dependence of the curve for T_C versus DOPE/monomeric-PC ratio as the ratio is increased above ca. 5.

The ANTS/DPX fusion assay measures only those fusion events occurring between ANTS-LUV, which are unpolymerized in all cases, and DPX-LUV, which are either unpolymerized or 50 or 100% polymerized. The critical fusion temperatures are defined based on the rates of the initial fusion events (up to ca. 6 s), and therefore depend primarily on fusion events occurring between aggregated heterodimers composed of an ANTS-LUV and a DPX-LUV. The putative first formed fusion intermediates (stalks) are necessarily composed of a mixture of the lipids contributed by the cis-

Figure III-33. Effect of photopolymerization on the DOPE to monomeric-lipid ratio in 4:1, 3:1, and 2:1 DOPE/bis-SorbPC LUV systems and on the corresponding critical fusion temperatures (T_C) determined by the ANTS/DPX fusion assay.

monolayers of both liposomes of an aggregated dimer. Monomeric lipid, rather than poly lipid, is more likely to participate in stalks formation since the lipids in the stalk structures must assume high curvatures. Lipids constrained by polymerization have been shown to be unable to adopt such high curvatures at high degrees of polymerization (Barry et al., 1992). The correlation of T_C values with the mole ratio of DOPE to monomeric-lipid calculated for two liposomes of a heterodimer, rather than for the entire

liposome sample, is therefore justifiable. Interestingly, when the data in Figure III-33 is extrapolated to pure DOPE in the area of contact (e.g. DOPE/monomeric-lipid mole ratio = 1), the best fit linear regression intersects the T_c axis at ca. 9°C which is within the range of T_H values accepted for pure DOPE systems (8 - 10 °C).

The relationship between the T_c values and the corresponding DOPE/monomeric-PC ratios calculated based on the lipid composition of the heterodimers during the initial fusion events is similar to that of the DOPE/monomeric-PC ratios and their associated $T_I(10\%)$ values as determined by ^{31}P NMR for photopolymerized multilamellar systems (Table III-3). At a limited number of DOPE/monomeric-PC ratios, calculated appropriately in the LUV or multilamellar systems based on the ability of interactions between the differently oriented membranes to give rise to a response in the different analysis methods applied to the systems (e.g. fluorescence time scans or ^{31}P NMR spectra), the T_c values agree more closely to the $T_I(10\%)$ values than to the $T_I(50\%)$ or $T_I(\text{end})$ values. In the ^{31}P NMR studies, the $T_I(10\%)$ values at DOPE/monomeric-PC ratios of 3:1, 4.3:1, and 6:1 were 49, 45, and 43°C, respectively. These agree well with DOPE/monomeric-PC ratios of 3:1, 4:1, and 6:1, whose associated T_c values are 65, 45 - 50, and 40°C. The correlation of T_c values with $T_I(10\%)$ values rather than $T_I(50\%)$ or $T_I(\text{end})$ values, at the same given PE/PC ratios, is understandable since in any isotropic liposome system, including the DOPE/bis-SorbPC system, fusion of apposed liposomes requires only the formation of Q_{II} phase precursors (i.e. stalks and ILAs) -- which are probably indicated by the first appearance of isotropic ^{31}P NMR resonances -- rather than the formation of the Q_{II} phase itself.

Next, the behavior of mono-SorbPC-containing liposomes was investigated in liposome fusion assays. Figure III-34 (A-C) shows the effect of temperature on the fusion of DOPE/mono-SorbPC (3:1) LUV under the same conditions as used in Figure III-27 (A-C). The corresponding leakage assays were not performed with the mono-SorbPC system. The initial rates of fusion versus temperature are shown in Figure III-35. All the curves for the mono-SorbPC systems are shifted to higher temperature than observed for the bis-SorbPC system as shown in Figure III-35. In addition, the effect of polymerization on the curves is relatively small in comparison to those for the bis-SorbPC system.

In the last fusion experiments, we returned to the DOPE/bis-SorbPC (3:1) system and compared the rates and extents of homo-fusion, where both liposome systems are either dark-adapted or photopolymerized, and heterofusion, where one liposome population is dark-adapted and the other is photopolymerized. The sensitivity of the fusion assay relies on quenching of ANTS by DPX, which is enhanced by use of an excess of the quencher. If both LUV populations were otherwise identical, then fusion would occur randomly and the numbers of asymmetric and symmetric fusion products would be governed by statistics. However, since only the DPX-LUV were photolyzed, hetero- and homo-fusion could not be expected to occur randomly. If homo-fusion of DPX-LUV occurred preferentially, it could indirectly reduce the extent of hetero-fusion that is observed. To test this possibility, fusion of DOPE/bis-SorbPC (3:1) liposomes where the ANTS-LUV were photopolymerized and the DPX-LUV were dark-adapted was compared with fusion of dark-adapted ANTS-LUV with photopolymerized DPX-LUV at pH 4.5 and 40°C (Figure III-36, curves A and B, respectively). Also shown in the figure is fusion ANTS- and

Figure III-34: Temperature dependence of fusion of photopolymerized and dark-adapted DOPE/mono-SorbPC (3:1) LUV at pH 4.5. Panels A-C are for fusion of unpolymerized ANTS-containing LUV combined 1:9 at 300 μ M with DPX-LUV whose bis-SorbPC component is either (A) unpolymerized, (B) 50% polymerized, or (C) 100% polymerized. Leakage data were not measured for this liposome system.

Figure III-35: Initial rates of fusion of photopolymerized and dark-adapted DOPE/mono-SorbPC (3:1) LUV at pH 4.5 and 300 μM as a function of temperature. The curve at the extreme right shows the initial rate of fusion (closed diamonds) for unpolymerized liposomes. The middle curve shows the initial rate of fusion (closed triangles) for unpolymerized ANTS-containing LUV in the presence of a 9-fold excess of 50% polymerized DPX-containing LUV. The extreme left curve shows the initial rate of fusion (closed squares) for unpolymerized ANTS-containing LUV in the presence of a 9-fold excess of 100% polymerized DPX-containing LUV. Leakage data were not measured for this system.

DPX-LUV when both are dark (C) and when both are photolyzed (D). Homofusion of two dark-adapted liposome populations occurred at a relatively slow rate under these conditions, as was also observed in the other assays. In the hetero-fusion assays, the initial rates of fusion up to 1 min were identical whether ANTS- or DPX-LUV were photopolymerized (A and B). Thereafter, the kinetics diverged. The rate of hetero-fusion for photopolymerized ANTS- and dark-adapted DPX-LUV continued unabated, but hetero-fusion of dark-adapted ANTS- and photopolymerized DPX-LUV fusion fell off precipitously. Where both populations were photopolymerized, essentially no fusion occurred. Apparently, if both membranes were destabilized by photolysis, stable fusion products could not form at this temperature, and interaction between the liposomes resulted only in leakage.

Figure III-36: Comparison of fusion of DOPE/bis-SorbPC (3:1) LUV resulting from symmetric and asymmetric lipid membrane interactions. ANTS- and DPX-containing LUV were combined 1:9 (mol/mol) at 300 μ M and 40°C and fusion was initiated at pH 4.5. Curve A is for hetero-fusion between photopolymerized ANTS-LUV and dark-adapted DPX-LUV. Curve B is for hetero-fusion between completely photopolymerized DPX-LUV and dark-adapted ANTS-LUV. Curve C is for homo-fusion between ANTS- and DPX-LUV that are both dark adapted, and curve D is for homo-fusion between ANTS- and DPX-LUV that are both photopolymerized. Complementary leakage assays were not performed.

CHAPTER IV

DISCUSSION

The goal of this research was the development of liposomes capable of containing aqueous solutes under physiological conditions and having a latent ability to aggregate and fuse upon exposure to light of the appropriate wavelength and intensity. This project was undertaken to explore the feasibility of using light to control cytoplasmic delivery of the aqueous contents of photosensitive liposomes across cellular membranes *in vitro* or *in vivo*. The liposome system described here is the first one in which photoinduced fusion has been unequivocally demonstrated. Many other types of liposome systems have been demonstrated to be capable of fusion. In these other systems, addition of chemical stimulants (such as multivalent cations or enzymes) is necessary to transform the liposomes from stable systems to fusogenic ones. The use of light for controlling cytoplasmic delivery *in vivo* is potentially advantageous since the simultaneous delivery of the liposomes and chemical agents is not required.

The fusion kinetics of certain liposome systems, composed of a subpopulation of those lipids capable of undergoing L_{α} to H_{II} phase transitions that can adopt an intermediate Q_{II} phase on the first heating scan, are strongly enhanced in the temperature range near, but below, T_H (Ellens et al., 1986b; Ellens et al., 1989). Precursors to the Q_{II} phase can be detected by ^{31}P NMR as narrow isotropic resonances (Gagne et al., 1985; Ellens et al., 1989), and are believed to correspond to the "lipidic particles" that have been visualized in electron micrographs during membrane fusion events (Verkleij et al., 1980;

Hui & Stewart, 1981; Siegel et al., 1989; Siegel et al., 1994). Several intermembrane structures have been proposed that could account for the resonances, including inverted micellar intermediates (IMI) (Siegel, 1984; Siegel, 1986a; Siegel, 1986b), stalks (Markin et al., 1984; Chernomordik et al., 1987; Siegel, 1993a), and interlamellar attachments (ILA) (Siegel, 1986c; Siegel et al., 1988). IMI and stalks have each been proposed as the first-formed fusion intermediates that evolve into ILA. Siegel has calculated that both IMI and stalks should have lifetimes too short to allow their observation even by fast-freezing electron microscopy techniques and that ILAs are therefore more likely to be responsible for isotropic ^{31}P NMR signals (Siegel et al., 1994).

Processes that trigger the phase separation of PEs from other lipids yield enriched domains of PE and modify its local phase behavior. Since PEs are significantly less hydrated than PCs (Parsegian et al., 1979; Rand, 1981), the formation of domains of PE in bilayer membranes facilitates the close approach of these regions of the bilayer surfaces. Contact between bilayers is a prerequisite for bilayer destabilization and liposome fusion (Ellens et al., 1984).

The polymerization of bi- or multi-component liposomes has been usefully employed to form domains of enriched lipids for the insertion of transmembrane proteins into partially polymerized liposomes (Tyminski et al., 1985; Tyminski et al., 1988), for the efficient photoinduced lysis of oligolamellar liposomes (Frankel et al., 1989; Lamparski et al., 1992), and for the enhancement of energy transfer between membrane surface bound dyes (Armitage et al., 1993). In each of these cases the covalent linking of the polymerizable lipids separates the lipids into polymeric and monomeric domains (Gaub et al., 1984).

Previously, 254 nm exposure of DOPE/bis-SorbPC (3:1) multilamellar vesicles (MLV) at pH 7.4 and 25°C resulting in leakage of the aqueous marker calcein was demonstrated (Lamparski et al., 1992). The leakage of calcein was attributed to photopolymerization of bis-SorbPC resulting in enriched phases of DOPE which facilitated formation of fusion pores by intraliposomal fusion of the bilayers comprising the MLV. Photoinduced leakage of the MLV was shown to increase with increasing extents of polymerization of bis-SorbPC. In a ^{31}P NMR temperature study of the effect of photopolymerization on the phase behavior of DOPE/bis-SorbPC (3:1) membranes, an isotropic signal appeared in the first heating scan of unpolymerized and photopolymerized systems (Barry et al., 1992). With increasing photopolymerization, the temperature of the appearance of the isotropic signal (T_I) decreased. However, at high conversion of monomer (90%), the system did not complete the transition to the Q_{II} phase. This behavior was attributed to trapping of DOPE in cross-linked polymer domains of poly(bis-SorbPC) that were apparently too large to form nonlamellar structures and therefore remained in the L_α phase.

In the present study, photopolymerization of LUV composed of either DOPE/bis-SorbPC (3:1) or DOPE/mono-SorbPC (3:1) was employed to induce fusion of the bilayers of distinct LUV populations. The phase behavior of such two-component membranes prior to polymerization depends on the identity of polymorphic and stabilizing components, their molar ratio, and their miscibility at the given molar ratio. PC and PE are typically miscible over wide ranges of mole ratios and this is also true of DOPE combined with either bis-SorbPC or mono-SorbPC. DOPE was chosen as the polymorphic component for its commercial availability, its well-defined chemical structure (e.g. as opposed

to the naturally derived eggPE), and its T_H value (10°C) that is well below physiological temperature. DOPE has been widely used to prepare fusogenic liposomes and is a principal component in the commercially available cationic liposomes sold as Lipofectin (Felgner et al., 1987). The low T_H of DOPE necessitates the addition of considerable amounts of stabilizing lipids in order to raise the T_H of the mixture to above 37°C. Other PEs having well-defined chemical structures and having T_H values below that of DOPE are also commercially available: PE 18:2 cis- $\Delta^{9,12}$ has a T_H value of -15°C, and PE 18:3 cis- $\Delta^{9,12,15}$ has a T_H value of -30°C. Liposomes formulated from these PEs would require greater proportions of a given stabilizing lipid, compared to the amounts required by DOPE systems, in order to exist in the lamellar phase at physiological temperature.

We chose to use PC to stabilize DOPE into liposomes rather than one of a variety of other well-hydrated lipids capable of stabilizing polymorphic lipids into lamellar phases, including fatty acids and negatively charged lipids such as PA, PG, and cardiolipin. The polymerizable PCs were designed with the polymerizable groups incorporated into the PC acyl tails, rather than as a part of the headgroups, in order to minimize the phase behavior in DOPE mixtures as compared to unreactive PCs such as DOPC. Preparing photosensitive liposomes with negatively charged surfaces would be disadvantageous for *in vivo* targeted delivery since anionic liposomes are more rapidly cleared from the blood stream than are neutral liposomes (Allen et al., 1989). In addition to the problems associated with their negatively charged headgroups, negatively charged single-chain amphiphiles are poor stabilizers for liposomes in the presence of other membranes since they are rapidly extracted from the liposomal membranes with the result that the liposomal membrane integrity is

compromised (Leventis et al., 1987). PG, PA, and cardiolipin are bilayer competent lipids which are negatively charged at neutral pH and are each capable of stabilizing DOPE into lamellar phases. Polymerizable PG or PA could be synthetically prepared using strategies similar to those used to prepare the polymerizable SorbPCs. At neutral pH, fusion and leakage would probably be strongly retarded between liposome systems in which the SorbPC component was substituted with polymerizable PA or PG. Polymerization of liposomes containing negatively charged stabilizing components would likely result in the formation of strongly hydrated anionic phases and dehydrated, neutral PE phases. The ability of such liposomes to undergo fusion might depend on whether the dehydrated phases of such liposomes were sufficiently large to enable two such liposomes to adhere and then fuse despite the negative charges expressed on some regions of the liposome surfaces.

Having chosen DOPE and bis-SorbPC as the constituents of the two-component polymerizable liposomes, their ratio was set to 3:1 for the initial investigations based on previous ^{31}P NMR experiments indicating that the L_{α}/Q_{II} phase transition temperature of the mixture was at least 5 to 10 C° above physiological temperature. The lipid composition following polymerization depends on the percent conversion of monomer to polymer and on the size of the domains that are formed. The conversion of monomer to polymer can be controlled in most polymerization systems and is easily controlled in a photoactivated polymerization by controlling the photolysis time. This determines the ratio of DOPE to monomeric-PC in the polymerized liposomes.

The size of the polymer-rich and monomer rich-domains depends on the extent of cross-linking of the polymers and on the degree of polymerization (X_n) of the polymers. The degree of crosslinking can be controlled by the

choice of monomer. The bis-SorbPC presumably undergoes extensive cross-linking since polymerization of pure bis-SorbPC systems results in formation of insoluble polymers. Conversely, polymerization of mono-SorbPC likely only forms linear polymers. Intermediate amounts of crosslinking could be achieved by polymerizing liposomes composed of DOPE and both bis-SorbPC and mono-SorbPC. The X_n for the polymerization strongly influences the size of the polymer domains that are formed. Presumably, the larger the polymer rich domains, the larger are the DOPE rich domains, and the greater is the effect of a given amount of polymerization on destabilizing the membrane. In general X_n may depend on the initiator concentration, the monomer concentration, and the reactivity of the monomer. The sorbyl group was chosen as the polymerizable moiety due to its facile polymerization on exposure to the relatively low intensity UV light readily available from a low-pressure Hg pen lamp. In both direct photopolymerization and photosensitized polymerization, X_n depends inversely on the light intensity when the effect of the photolysis is to generate a radical species that initiates a chain polymerization mechanism. However, with photoactivated polymerization, which proceeds by a stepwise mechanism, X_n is not likely to be influenced by light intensity.

Bulk phase parameters that may affect fusion include the ionic strength, pH, the presence of divalent cations, and the osmolarity relative to the aqueous interior of the liposomes. In all experiments reported here, the ionic strength was adjusted to mimic physiological conditions and was never intentionally varied. The NaCl concentrations of the bulk media were adjusted to osmotically balance the solutions with those composing the interior volume of the liposomes. The pH was varied from 9.5, to 7.5, and to 4.5. At pH 9.5, ca.

half of the PE should carry negative charge, at pH 7.5 ca. 0.5 mol % of the PE should carry negative charge, and at pH 4.5, the PE should be completely uncharged. At either pH 9.5 or pH 7.5, the opportunity exists for interaction of divalent cations with the negatively charged ethanolamine headgroups. The effect of addition of Mg^{2+} is physiologically relevant. No attempt was made to determine the threshold concentration of Mg^{2+} required to induce fusion of dark-adapted versus photopolymerized liposomes at different pH. Neither was the effect of addition of Ca^{2+} alone or in the presence of Mg^{2+} investigated.

The environmental parameters affecting liposome fusion include temperature, liposome concentration, and the geometry of the liposome interactions (e.g. LUV vs. MLV and homo-fusion vs. hetero-fusion). Temperature is of critical importance since it affects the physical state of the membranes and their ability to form the inverted phase intermediates critical to fusion. Liposome fusion was investigated at temperatures above and below physiological temperature to determine where the critical fusion threshold temperature was located at different membrane compositions (e.g. dark-adapted versus photopolymerized), and bulk phase compositions (e.g. pH 9.5/20 mM Mg^{2+} versus pH 4.5). The effect of liposome concentration on fusion was investigated over a limited range of lipid concentrations from 50 μM to 600 μM . Experimental limitations precluded the investigation of fusion below 50 μM . The absence of any dependence of fusion kinetics in this concentration range suggests that fusion is not aggregation rate limiting at concentrations as low as 50 μM . Thus the effect of polymerization on increasing the fusion rates cannot be unambiguously attributed to either increased rates of aggregation or increased rates of formation of fusion intermediates (Bentz et al., 1983). The use of MLV was avoided since in previous liposome fusion experiments leakage

from such liposomes has been attributed to intraliposomal membrane interactions (Ellens et al., 1984; Lamparski et al., 1992).

In the initial lipid mixing experiments, LUV were prepared at physiologically relevant conditions (150 mM NaCl, 10 mM Tris, pH 7.4) where enhanced lipid mixing due to photopolymerization of the DOPE/bis-SorbPC (3:1) liposomes was demonstrated. Lipid mixing was shown to be dependent on inclusion of PE in the bilayers, the extent of photopolymerization, the presence of divalent cations, and temperature (Bennett & O'Brien, 1994; Bennett et al., 1994). Fusion and leakage could not be profitably studied with LUV prepared under these conditions due to slow, but persistent leakage from the LUV that occurred as soon as the LUV were separated from the unencapsulated fluorescent dyes by gel filtration chromatography. In latter experiments, LUV were prepared at pH 9.5 where the PE component is negatively charged due to deprotonation of the amino group of the ethanolamine headgroup (pK 9.5) (Stollery & Vail, 1977). Electrostatic repulsion between LUV at pH 9.5 prevents their aggregation and allows the experimental separation of bilayer polymerization and LUV fusion.

Aggregation and membrane contact between liposomes was initiated by neutralizing the LUV with either H^+ or Mg^{2+} . These experiments compared the extent of interaction between LUV populations, as evidenced by lipid mixing, aqueous contents mixing, and aqueous contents leakage, where neither population was photopolymerized, or where just one population was photopolymerized to varying extents. In the latter lipid mixing experiments, the effect of photopolymerization of the membranes was much more dramatic than in the earlier experiments. This resulted in part from the capacity of the latter experimental protocol to measure the short time kinetics of the lipid

mixing events. In the assays performed with the latter protocol, the difference between lipid mixing of dark-adapted and photolyzed liposomes was affected by the identity of the polymerizable lipid, the temperature, and the composition of the bulk medium. The ability of liposomes to undergo lipid mixing is correlated with the appearance of isotropic resonances in the ^{31}P NMR spectra. Since these resonances appear over a temperature range, lipid mixing can apparently occur slowly at the bottom of the range and can occur rapidly at temperatures at or above the top of the range. Photopolymerization of the bis-SorbPC system under appropriate conditions can apparently shift the nonlamellar phase transition temperature of the system from a value below the temperature range to one at the top end of the range. With the bis-SorbPC system at 20°C, which is far below the nonlamellar phase transition temperature of the dark-adapted membrane, the difference in lipid mixing between the dark-adapted and photolyzed liposomes was small since the nonlamellar transition temperature of both the dark-adapted and photopolymerized systems was below the temperature range in which lipid mixing is promoted (Figure III-19). At 37°C, which is just below the nonlamellar phase transition temperature of the dark-adapted liposomes, the effect of photolysis was significant (Figure III-19). Apparently, photopolymerization of the membranes effected a shift of the nonlamellar transition temperature range of the membrane from above to below the environmental temperature. The difference between the lipid mixing of the dark-adapted and photolyzed DOPE/bis-SorbPC liposomes at 37°C was also significantly affected by the composition of the bulk medium. The difference between lipid mixing of the dark-adapted and photolyzed membranes

increased as the composition of the bulk medium was changed from pH 7.5, to pH 7.5/5 mM Mg, to pH 4.5, and finally to pH 9.5/20 mM Mg (Figure III-17).

The behavior of LUV containing either bis-SorbPC or mono-SorbPC was studied to evaluate the importance of either a cross-linked or linear polymer matrix, respectively, on the extent of lipid phase separation and subsequent bilayer interaction. In the lipid mixing assays, the difference between the dark-adapted and photopolymerized liposomes in the mono-SorbPC system was smaller than that of the bis-SorbPC system under conditions where the systems were situated at comparable distances from their nonlamellar phase transition temperatures (e.g. 37°C for the bis-SorbPC system and 50°C for the mono-SorbPC system). Photopolymerization of mono-SorbPC to a given extent has a more subtle effect on the phase transition temperature of its constituent membranes than does reaction of equivalent numbers of bis-SorbPC. Apparently, photopolymerization of mono-SorbPC does not sufficiently change the nonlamellar transition temperature so that the dark-adapted membranes can be situated on one side of the temperature range that defines the transition while the photopolymerized membranes can be situated on the other side.

Fusion of liposomes has been described by a mass action kinetic model in which the first step is aggregation of two stable vesicles to form a dimer, and the second step is the actual fusion process which produces the fusion product (Bentz et al., 1983). The latter step includes destabilization of the two vesicles and their communion via the fusion process. The kinetics of the overall fusion process depends on the rates of both aggregation and the fusion event. Polymerization of DOPE/bis-SorbPC membranes is proposed to induce fusion by facilitating both aggregation and subsequent fusion. A molecular

model for homo-fusion between DOPE/bis-SorbPC (3:1) liposomes following photopolymerization is shown in Figure IV-1. In the figure the DOPE lipid headgroups are unfilled and the bis-SorbPC headgroups are filled. The liposomes are photopolymerized at pH 9.5 where ca. half of the PE component is negatively charged and where the SorbPC lipids are zwitterionic (charges not shown). Following polymerization the lipid membranes are neutralized by addition of protons or magnesium ions. Adhesion of the neutralized membranes leads to sequential formation of a stalk, a TMC, and finally a fusion pore (ILA) as shown in the figure. Polymerization induces lipid phase separation with consequent decreased hydration of PE-rich domains, and decreased R_0 of the lipids composing the PE-rich domains. Since the monomeric domains contain only small fractions of PC compared to the polymeric PC domains, the surface hydration of these regions is diminished. The decreased R_0 of the PE domains is a consequence of both the increased ratio of DOPE to monomeric-PC (see Table III-2) and the decreased membrane hydration (Yeagle & Sen, 1986). Attractive hydrophobic forces promote adhesion of the membranes at these sites. After adhesion, formation of fusion intermediates is facilitated by the decreased R_0 of the lipids composing the apposed leaflets of the membranes.

Figure IV-1 shows homo-fusion between two completely polymerized DOPE/poly(bis-SorbPC) (3:1) liposomes. In this case, only the monomeric DOPE lipids would be likely to participate in formation of the stalk and TMC intermediates and the fusion pore product. Due to its very small R_0 value, DOPE by itself would not likely form very stable versions of these intermediates. This is supported by the fusion and leakage results in Figure III-36 comparing

Figure IV-1: Molecular model for homo-fusion of DOPE/bis-SorbPC (3:1) liposomes following photopolymerization.

homo-fusion between two photopolymerized liposome populations with hetero-fusion between dark-adapted and photopolymerized liposome populations. More persistent quenching of ANTS by DPX was observed in the hetero-fusion case than in the homo-fusion case. In the former case, both DOPE and monomeric-PC would likely participate in formation of the intermediate structures and the fusion pore. Mixtures of PE and PC exhibit medium R_0 values and are therefore more likely to form longer lived and more stable fusion intermediates (e.g. stalks).

The crucial question in this research was how different extents of polymerization, at a given temperature, affect the stability of aggregated LUV at the site of bilayer contact and whether the extent of destabilization could be used to control whether fusion or leakage ensues. The critical fusion temperature (T_C) for DOPE/bis-SorbPC (3:1) LUV was shifted from ca. 65°C for the case of homo-fusion between two dark-adapted LUV populations to ca. 45 and 40°C for hetero-fusion between dark-adapted LUV and either 50% polymerized or 100% polymerized LUV, respectively. The change in the temperature threshold for leakage was similar. The effectiveness of polymerization of only one of the liposome populations in shifting the fusion threshold of these LUV by 20 to 25 C° represents a potentially useful strategy for triggering fusion of liposomes with cellular membranes at physiological pH. Consider for example the fusion of liposomes with endosomal membranes which have a relatively high content of PC. These circumstances would probably shift the fusion threshold to higher temperatures. The composition of the photosensitive liposomes could be changed in a variety of ways to lower the critical fusion temperature with PC-rich membranes to 37°C. These include a reduction in the proportion of the polymerizable PC, bis-SorbPC, or a

change in the chain length of the SorbPC, which is known to alter its main phase transition temperature, T_m (Lamparski et al., 1993). The polymorphic component (e.g. DOPE) could be replaced by a lipid exhibiting a lower T_H (e.g. plasmenylethanolamine). Recently, Glaser and Gross (1994) reported that substitution of plasmenylethanolamine for PE promoted calcium induced fusion between liposomes also containing PC and PS. Finally, the critical temperature for the onset of fusion could be modified by the addition of other lipids, e.g. diacylglycerol (DAG), to the photosensitive LUV.

The photoactivated polymerization of mono-SorbPC produces only short oligomers of 3 - 10 repeat units (Lamparski & O'Brien, 1994), and in this case the photoreaction does not necessarily produce a dramatic phase separation of the membrane components. Although the size of the polymers produced by photopolymerization of bis-SorbPC is unknown, the polymers are known to be crosslinked. The number of monomers comprising the polymer domains may be as large as the square of the X_n value for the linear oligomers formed from mono-SorbPC. Regardless of the polymer domain size, subtle changes in the composition of membranes have previously been shown to exert dramatic influences -- both inductive and inhibitive -- on membrane fusion. Siegel and coworkers (Siegel et al., 1989; Siegel et al., 1989) showed that just 2 mol % of DAG was sufficient to decrease the T_H value of DOPE-Me membranes by ca. 15 C°. The temperature of the onset of the appearance of isotropic resonances was lowered by a similar extent, and a sharp increase in the fusion rate accompanied addition of DAG. The rate of divalent cation-induced fusion of PS liposomes was increased by the addition of either DAG or hexadecane to the liposomal membranes (Walter et al., 1994). Chernomordik et al. (1993) reported that lysoPC at subsolubilizing concentrations inhibited Ca^{2+} , GTP-, and pH-

dependent biological fusion processes. And Yeagle and coworkers (Yeagle et al., 1994) demonstrated that very low concentrations of lysoPC (1 - 9 mol %) inhibited fusion and leakage of liposomes composed of DOPE-Me. In these studies, the authors attribute the effect of the low level perturbants on the rates of fusion to their abilities to either stabilize or destabilize formation of stalk structures (Siegel, 1993c; Siegel et al., 1994).

The potential utility of photosensitive liposomes depends on the number of photons per liposome required to modify the bilayer properties. This value is the product of the quantum efficiency of the photoreaction of the individual lipids and the number of lipids per liposome required to modify the bilayer properties. The photoactivated polymerization of bis-SorbPC has a quantum yield of 0.25. Hence, reaction of 50% of the polymerizable lipids in 130 nm diameter DOPE/bis-SorbPC (3:1) LUV (composed of ca. 1.3×10^5 lipids) requires ca. 8.1×10^4 photons per liposome. Although this is a relatively large value, it is readily achieved in a minute or less under the experimental exposure conditions used here, which are well within the range used in photodynamic therapy.

Much higher efficiencies for photopolymerization of liposomes are possible by the use of a chain polymerization rather than the stepwise UV-photoactivated polymerization used in these studies. Radically initiated polymerization of mono-SorbPC and bis-SorbPC liposomes proceeds via a chain mechanism and produces high molecular weight polymer having repeat units numbering from 50 to 600 (Lamparski & O'Brien, 1994). Hence, radical polymerization of either DOPE/bis-SorbPC or DOPE/mono-SorbPC LUV should effect more dramatic changes in the membrane properties than photopolymerization. Consequently, large shifts of the initial rates of fusion

vs. temperature would be expected at low conversion of bis-SorbPC to polymer. This is particularly important because the photosensitized polymerization of bis-SorbPC has been achieved using membrane-bound cyanine dyes and visible light (Armitage et al., 1994). Photosensitized polymerization of bis-SorbPC, which is believed to occur via a chain reaction following radical initiation, has been achieved using the membrane-bound indocarbocyanine dye, N,N'-dioctadecyldimethyl-indocarbocyanine (DiIC18(3)), which is anchored in the bilayer with two 18-carbon fatty acyl tails. Using > 440 nm light, polymerization of bis-SorbPC LUV has been accomplished (Armitage et al., 1994). The polymerization is believed to proceed by a radical mechanism following photoproduction of hydroxyl radicals. Thus, *visible* light-induced fusion of DOPE/bis-SorbPC liposomes under physiologically relevant conditions is potentially possible using photosensitized polymerization (Bennett et al., 1994).

Other strategies by which photopolymerization of liposomes could be used to induce cytoplasmic delivery of reagents *in vivo* can be readily imagined. For example, the three attributes of steric stabilization, targetability, and polymerizability could be combined by synthesis of a polymerizable PEG-PE and attachment of an antibody or antibody fragment to the PEG terminus (see section I-C.6.). Small to moderate amounts of such lipids (e.g. 1 to 10 mol %) could be incorporated into bi- or multi-component liposomes. Monomeric PEG-PE lipids would endow the liposomes with stealth properties required for evasion of the RES and binding at the target site. Attachment of antibodies to some of the PEG-PE lipids would endow the liposomes with targetability to those cells expressing unique antigens for which antibodies can be prepared. Following binding at the target site, the

PEG coat could be unzipped by polymerization of the PEG-PE. By covalently linking the acyl tails of the PEG-PE component, the steric stabilization of the liposome would be restricted to only one or a few regions of the liposome. The exposed monomeric regions of the liposome could then undergo fusion at the plasma or endosomal membrane of the target cell. The unpolymerized region of the liposome could be composed of a polymorphic lipid mixture or of cationic lipids. Such a liposome system could therefore potentially exploit the unique properties of cationic lipids for *in vivo* use. While bare cationic liposomes cannot circulate in the blood, sterically stabilized liposomes with buried and latent cationic lipid interiors may exhibit long circulation times. Such a scheme is depicted in Figure IV-2 where the PEG-PE lipids have unfilled headgroups and the cationic lipids have filled headgroups. Some of the polymerizable PEG-PE lipids are conjugated to antibodies that mediate targeting of the liposomes to unique antigens expressed on the target cell surface.

These studies into the photoactivated fusion of liposomes demonstrate a significant enhancement in the fusion of LUV. The results complement the previous demonstration of photoinduced destabilization of oligolamellar liposomes. Both methods rely on the temporal and spatial characteristics of light to deliver reagents from liposomes to other bilayer bounded structures in the case of LUV or release of reagents to the aqueous media surrounding MLV.

Figure IV-2: Photoinduced fusion of PEG-PE liposomes following *in vivo* targeting to specific antigens on the target cell plasma membrane.

APPENDIX

¹H NMR SPECTRA

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Figure A-1. ^1H NMR spectrum of 10-(sorbyloxy)decan-1-ol in CDCl_3 .

Figure A-2. ^1H NMR spectrum of 10-(sorbyloxy)decanoic acid in CDCl_3 .

Figure A-3. ^1H NMR spectrum of 1,2-bis[10-sorbyloxy)decanoyl]-sn-glycero-3-phosphatidylcholine (bis-SorbPC_{17,17}).

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