CHAPTER II

BACKGROUND

As described before, phospholipids are the major building units of cell membrane and are considered as the structurally determining constituents of biological membranes. In fact, phospholipids in aqueous medium can form the structure consisting of lamellar bilayers separated by aqueous layers. Thus, phospholipids can be used in pure form or mixtures of different chain length phospholipids to form lipid bilayers. Other substances such as cholesterols, proteins, spin - probes and fluorescent probes can be used and incorporated into these bilayers for particular purposes. In these bilayers, the lipid hydrocarbon chains are interacting hydrophobically while the lipid polar head groups are facing the water.

Nature of each lipid molecule in bilayer membranes depends mainly on water content associated to this molecule. In nearly anhydrous forms, the lipid crystal structure can be obtained. Hydrocarbon chains in such structure are governed by steric and van der Waals forces, while the headgroup regions are controlled by mutual and water mediated hydrogen bonds. Nevertheless, when lipid molecules are in dry form, bulky lipid headgroups may interdigitate partly in order to

retain close chain packing and to establish the intermolecular hydrogen bond networks while their chains are largely parallel and sometimes tilt at an angle less than 40° ralative to the plane of the lipid layer.

Alteration of water content relates to the capability of lipid - water systems to undergo reversible structural changes. This alteration affects functional and structural changes in biological membranes as reported by Sackmann, E . and Trauble, H . (1972) .

The gel to liquid crystalline phase transition in phospholipid bilayer may be described as a highly cooperative order - disorder transition in which the bilayers undergo a lateral expansion together with decreasing in thickness and density. These mean that, more disorder and less localization of methyl group in the center of the bilayer in the liquid crystalline state has been established. In microscopic, hydrocarbon chain packing changes from all trans below the phase transition temperature (T_C) to a state in which the chains probably have from 7 to 12 gauche rotational isomers above the T_C. The state of membrane packing in phospholipid can be affected by the lipid component, the temperature and ionic composition of aqueous environment as previously reported by Sackmann, E. and Trauble, H.(1972). The sensitivity of a given bilayer structure to the composition of the aqueous solution is particularly

interesting and is illustrated that strictly on the chemical nature of headgroups, [Trauble and Eibl, 1974; Michaelson et al, 1974; Ohki and Papahadjoponlos, 1969]. It is seen from the fact that addition of water molecules to dry lipids causes a shift of the transition temperature of dipalmitoylphosphatidylcholine down to -58 $^{\circ}$C.The corresponding shift observed for phosphatidylethanolamine is by approximately a factor of two times smaller, -36 $^{\circ}$C, owing to the lower affinity of this latter lipid for water.

Although cholesterol has a marked effect on hydrocarbon chain organization, it was found that the phosphatidylcholine headgroup conformation is unchanged by addition of cholesterol to the bilayer. That is to say, the headgroup is oriented parallel to the plane of the bilayer for phosphatidyl - choline in both gel and liquid crystalline states.

Addition of cholesterol results in an increasing of density in the hydrocarbon chain region next to the headgroup through the center of the bilayer by about 10 $^{\circ}$A, concomitant with enlargement the width of bilayer caused by elimination of chain tilt, however, a slightly effect is achieved on the terminal methyl trough as additionally supported by McIntosh, T.J. (1978) .

Regardless of the chain length of the phospholipids, from 12 to 18 carbons, the cholesterol ring group, cholestene nucleus, is located in the same region of the bilayer. Considerably, the conformationally flexible cholesterol hydrocarbon tail extends from the ring toward the center of the bilayer between approximately 4 and 7 $^\circ$A depending on C 17 side chain length. Thus, the cholesterol tail reaches the center of the bilayer in the case of DLPC and comes within 0 - 3 $^\circ$A of the center of the bilayer of DPPC. However, there is a 3 - 6 $^\circ$A free region of pure DSPC chains between the end of the cholesterol tails. In DSPC / cholesterol systems, it is possible to conclude that mismatch of the hydrophobic length between phospholipid and cholesterol leads the occurrence of lateral phase separation by forming of hexagonal phase in hydrocarbon core. This is because terminal methyl trough of DSPC kicks cholesterol end tail .

It is known from several studies that biological membranes have been shown to contain lipids with various types of hydrocarbon chains and with difference in length; thus lateral phase separation in these membranes frequently occurs and displays the significant changes in biological membranes, [Phillip, et al., 1970 ; Linden, et al., 1973 ; Wu and McIntosh, 1975; Chapman, et al., 1974; Shimshick and McIntosh, 1973].

Membrane fluidity is one of the important factors that governs various functions in biological membrane. Although the concept of membrane fluidity still lacks an explicit definition, it has recently been proposed that changes in the fluidity are manifested in abnormal lipid composition of cell membrane or body fluid. In general, pathology causes membranes more fluid as already described for erythrocyte membranes of patients with Düchenne muscular dystrophy. For other examples, malignant lymphoma cells are found to be of a more fluid membrane than normal lymphocytes, moreover, a respiratory distress syndrome in newborns also relates to an improper lipid composition of the pulmonary surface lung surfactant. In two latter cases, DPH is used by measuring of fluorescence polarization for diagnosis and following up such diseases.

Besides pathology that produces membranes more fluid, lipid fluidity of biological membranes has been shown to influence a variety of membrane functions, such as the activity of membrane - associated enzymes, transport activities and the expression of receptors and cell surface antigens that responsible for lysis of cell. It was found that fluidity of liposomal membrane is a prerequisite and important factor for antigen - antibody reaction [Glagasigij, U., et al., 1988] .

For monitoring physiological changes occurring in biological and artificial model membranes, various kinds of membrane probes for example 1,6-diphenyl-1,3,5-hexatriene(DPH), merocyanine 540 (MC 540), etc. were already introduced to investigate such phenomena.

DPH is one of the most useful probes for monitoring acyl chain movement or fluidity in membrane interior, hydrocarbon region. From its chemical structure (Figure 4), it belongs to linear class. All of probes included in this class have long rigid molecules of roughly in shape and size that fully extent along phospholipid acyl chains. These fluorophores have excitation and emission dipoles (fluorescence dipoles) that are roughly colinear with their long molecular axes. For this reason, rotations about the long molecular axis make essentially no contribution to the depolarization of polarized fluorescence, while rotations about the two remaining rotational axes, orthogonal and orthogonal to the long axis, are equally capable of depolarizing fluorescence. From several reports, steady - state fluorescence depolarization theory can be used to characterize depolarization of this probe in membranes, although this theory corresponds rigorously to motional randomization of the orientation of only one axis (the axis of fluorescence polarization) during the lifetime of the corresponding excited electronic state, it is generally assumed that this loss of initial orientation is correlated with overall reorientations of

the probe molecules which in turn will reflect the fluidity of membranes.

On steady - state theory, the ground and excited state electronic distributions of any fluorophores define directions within the molecule in which the probabilities of absorption or emission of a photon are greatest. These are the directions of the excitation and emission dipole moments, the magnitudes of which determine the maximal probabilities of absorption or emission. When polarized exciting light (Figure 1) is directed toward a chromophore molecule whose excitation dipole moment is aligned with the electric vector of the exciting light, the chromophore will preferentially absorb this light. Since the absorption process is so much faster than molecular rotation, the use of oriented exciting light creates a population of preferentially oriented excited fluorophore. This is refered to as *photoselective*. Since the emission of a photon by the excited fluorophore requires a much longer time (the excited state lifetime, τ,) than does absorption, the fluorophore can often reorient before emission occurs as illustrated in Figure 1. If such a situation occurs (i.e., the rotational correlation time of the excited molecule is less than or on the order of the excited state lifetime), the emitted photon will no longer be polarized parallel to the exciting photon, even if the molecular excitation and emission dipoles are parallel within the fluorophore. The resulting polarization of fluorophore is often defined in

terms of the steady-state fluorescence polarization and is derived from the equation as shown below.

$$\text{Fluorescence polarization}(P) = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + GI_{\perp}} \quad (1)$$

Where $I_{\parallel}$ and $I_{\perp}$ are the intensities measured in directions parallel and perpendicular to the electric vector of the exciting light. The grating transmission factor (G) as measured by the method of Azumi and McGlynn (1962) must be included to account for the fact that emitted light polarized in the vertical and horizontal directions may not be passed with equal efficiency by the emission monochromater. In addition to fluorescence polarization, fluorescence anisotropy (r) can be used in the compromizing way and obtained by the following equation.

$$\text{Fluorescence anisotropy}(r) = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2GI_{\perp}} \quad (2)$$

All terms in equation (2) are defined as for fluorescence polarization.
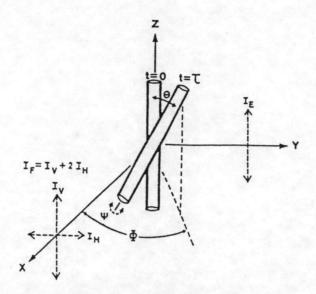
Figure 1. Fluorescence anisotropy of a cylindrically symmetric fluorophore, [Lentz, R.B., 1993 p 102].

Figure 1. shows fluorescence anisotropy of a cylindrically symmetric fluorophore whose excitation and emission dipoles are parallel to the symmetric axis. Vertically polarized light (I_E) from the Y - axis vertical polarizer (not shown) selectively excites (photoselective) a population of fluorophores whose excitation dipoles are assumed to be symmetrically distributed about the Z -axis polarizer (not shown). Rotation of the probe during the excited state lifetime, τ, diminishes the vertical component (I_V) and enhances the horizontal component (I_H) of analyzed light in a way described by time - dependence of fluorescence anisotropy [$r(t)$].

In aqueous media, DPH can form microaggregation or micelle which are practically void of fluorescence, however, when such a dispersion is mixed with lipid containing systems the probe incorporates very efficiency into hydrophobic domain and displays a sharp increase approximately 1000 fold in fluorescence signal, [Shinitzky, M. and Barenholz, Y., 1978]. The absorption and emission spectra of DPH in lipid below and above the phase transition are very similar to those in benzene or high paraffin solvents, [Cooper, R.A. and coworker; 1976]. These mean that DPH is localized almost exclusively in the hydrocarbon core of the lipid.

As a pure hydrocarbon, DPH probably reacts strongly with the lipid chains and becomes an integral part of the system, regardless of its phase or composition. Hence, in a heterogeneous system like a biological membrane, DPH can be considered as evenly distributed in the lipid layer and the derived fluidity or rigidity is therefore a weight average of all lipid domains.

In membrane, the exact vertical location of DPH is still a matter of controversy. This controversy has been addressed using fluorescence resonance energy transfer between DPH and a fluorophore located at the membrane surface. The conclusion is that transverse distribution of DPH is governed by a tendency to partition preferentially into more disordered region

of the bilayer. Thus, in egg phosphatidylcholine vesicles, DPH is found primarily close to the center of the bilayer, while in the dipalmitoylphosphatidylcholine vesicles, a fairly broad spread about the center is indicated, [Lentz, R.B., 1993] .

Although most fluorescent probes have certain advantages relative to other methods for characterizing the dynamic properties of membrane bilayers, foremost among these are the sensitivity of the fluorescence measurement, a relatively straightforward theoretical framework for interpreting data and the common availability of inexpensive equipment needed for the measurements, there are several drawbacks that should be aware. Firstly, probe molecules need not distribute equally between lateral domains or for that matter, even in a vertical direction in a membrane. Thus, the portion of the membrane where is occupied by probe will not be similar to the absent one. Secondly, probe may be labile under the experimental conditions due to oxidation which is activated by light.

From Shinitzky and Barenholz experiment (1974), when DPH was used as a membrane probe, prolonged exposure (about 1 min) to exciting light led to reversible bleaching of DPH fluorescence intensity and this phenomenon occurred more rapidly in less ordered membranes as a result of photo-induced *trans - cis* isomerization. Thus, experiments involving DPH should be performed in a short period of time .

Besides DPH, numerous reporter molecules as probes have been used to evaluate structural properties of lipid model membranes and biological membranes as well as transmembrane potential, [Lelkes, I.P., Bach, B. and Miller, R.I., 1980]. The versatility of the dye as an optical membrane probe explain the importance of a precise analysis of its spectroscopic properties. This is particularly valid for the evaluation of the still poorly understood monomer / dimer equilibrium. An estimation of the nature of the equilibrium is possible when the dye is dissolved in an aqueous medium and exhibits a double peak. In a less polar environment, however, dimers appear only as a shoulder on the monomer band.

Cyanine dyes have been frequently used as optical probes in membrane studies. In particular, the anionic dye merocyanine 540 (MC 540) has found widespread applications. Due to its amphipathic nature, MC 540 is soluble in solvents of a wide polarity range, including water and to a limited extent chloroform. The dye can easily incorporate into the bilayer of phospholipid model membranes and partitions into the lipid phase when added externally to an aqueous liposome suspension. MC 540 exhibits an equilibrium between fluorescent monomers and non - fluorescent dimers which depends on the dispersion medium. In phospholipid bilayers, the monomer chromophore is thought to be located near the membrane / water interface, while, dimer is suggested to be located in

hydrophobic region deep in membranes, [Verkman,S.A., 1987]. In addition, the λ_{max} of the dye's visible spectrum correlates very well with the dielectric constant of the solvent . Polarity changes in the microenvironment of the chromophore are monitored as λ_{max} shift.

Moreover, Williamson, P., et al., (1983) reported that MC 540 was able to sense the degree of lipid packing of bilayers and inserted preferentially into bilayers whose lipids were more widely spaced.

In addition to MC 540, bromcresol purple (BCP, 5,5-dibromo-o-cresolsulfonphthalein), an acid dye which has been widely used in the analysis of amine drugs, has been reported and used as a membrane probe to investigate structural features of fragmented sarcoplasmic reticulum (SR) of skeletal muscle, [Nakamaru,Y. and Sugii,Y.,1974; Nakamaru,Y.,1977; Nakamaru, Y. and Nomura, K., 1977]. Because spectral and intensity changes were obtained when divalent anion BCP was used to study conformational change of SR, therefore, it is intriguiting to investigate whether BCP can be used as a detecting probe to monitor structural changes, particularly fluidity change in model membranes. This research is now proposed to investigate for such a phenomenon and the experiments are performed in the following sequences.

1. The experimental parameters such as concentration of BCP used, incubation time and also lipid concentration were examined for obtaining the appropriate condition.

2. Large unilamellar vesicles were prepared from various types of phospholipid by the reverse - phase evaporation method that was minor modified by Glagasigij et al (1988). Afterthat such vesicles were incubated with BCP at $25^{\circ}C$ and absorption spectra were recorded. Furthermore, mixtures of phospholipids differing in acyl chain length were prepared and tested for the purpose of clarifying the state of fluidity and rigidity on liposomal membranes including domain formation created by non - ideal mixing of unequal acyl chain length.

3. Cholesterol was incorporated to both gel and liquid crystalline phase lipids in order to investigate effects of cholesterol below and above T_C of lipid used on binding of BCP.

4. To clarify effect of lipid packing on binding of BCP, both negatively and positively charged amphiphiles were also incorporated to liposomal membranes, additionally, effect of medium ionic strength on such binding was investigated as well.

5. Cholesterol analogues having various head group side chain lengths at 3- position, charges and polarities were added to DMPC or cholesterol containing DMPC. Consequently, the surface properties including fluidity of liposomes were modified and binding of BCP to such liposomes was investigated.

6. Fluorescence polarization of DPH was the parameter used for measuring fluidity of membrane lipid core. Eventhough, location of DPH in membrane is not similar to that of BCP it could be used and report fluidity in the average value of all lipid bilayers.

7. Finally, the relationship between the state of liposome membrane and binding of BCP was evaluated and discussed.